MicroRNAs and their role within the teleost (*Oncorhynchus mykiss*) stress response

by

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Author's Declaration

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

I understand that my thesis may be made electronically available to the public.

Abstract

The stress response is a highly conserved signalling cascade that signals between tissues to increase energy availability. Changes in glucose regulation are studied extensively due to the glucose intolerant phenotype of rainbow trout (*Oncorhynchus mykiss*). Recent studies have started to investigate microRNAs (miRNAs) in this phenotype, and previous work in other models has linked microRNAs to stress as well. MiRNAs are small, non-coding RNAs that bind to the 3' UTR of mRNA and act to silence translation or degrade the transcript. These miRNAs can also be present in circulation by association with extracellular vesicles (EVs; such as exosomes), which have been shown to signal between tissues. Therefore, the primary aim of this thesis was to establish the role of miRNAs in the teleost stress response, both within and between tissues. The two studies used *in vivo* and *in vitro* techniques to determine how stress and the hormone cortisol impacts miRNA levels.

In the first chapter, rainbow trout were exposed to a 3-minute acute air exposure and allowed to recover for 1-, 3-, or 24-hours. MiRNA levels in plasma EVs, anterior kidney, and liver were measured, and changes linked to pathways that could be impacted using KEGG analysis. Overall, miRNAs increased in EVs in circulation. Tissue miRNA abundances either remained constant or decreased. KEGG revealed that predominantly metabolic pathways were the targets for these miRNAs. Therefore, the circulating miRNAs could be stored in the blood to increase the metabolic potential of tissues and then reabsorbed later during recovery. Tissue abundances were likely reflecting the increase in circulating glucose observed by allowing for increased glycolytic potential.

In the second chapter, rainbow trout hepatocytes were exposed to increasing concentrations of cortisol to determine how the hormone might influence the miRNA associated

with glucose metabolism. Hepatocytes were exposed for 2- or 4-hours to 0, 30, 300, or 3000 ng/mL of cortisol and both cell and supernatant fractions taken. MiRNA predicted to target key enzymes involved in gluconeogenesis (fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase) and glycolysis (glucokinase and pyruvate kinase) were measured to see how cortisol impacted their epigenetic regulation. However, supernatant levels of LDH were found to be different between fish, which may indicate necrosis of the cells during the exposure. Transcript abundances of glycolytic enzymes and the miRNAs predicted to target them were not impacted at any timepoint, but cortisol increased gluconeogenic transcripts and miRNAs. Enzyme activities were also not affected by cortisol exposure, but time-dependant decreases in glycolytic enzymes could be due to changes in glucose availability during the experiment. Although few changes were measured, the miRNA predicted to impact enzymes have been implicated in other glucose-intolerant phenotypes.

The results of these studies indicate that miRNAs can be impacted by stress and could be involved in regulating translation during recovery. Future studies should further investigate the role of miRNAs in the teleost stress response as the *in vivo* experiment clearly shows acute changes occurring that can influence physiology during recovery. The miRNAs predicted in chapter 2 should also be investigated further for their role in glucose metabolism within the context of stress.

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Chapter 1

General introduction

1.0 Introduction

The stress response is a highly conserved process that allows for organisms to respond to changes in their environment. Chronic stressors experienced by organisms often cause detrimental effects such as decreased growth rate and inhibition of reproduction, side effects that the aquaculture industry would like to reduce (Tort, 2013). However, acute stressors such as handling, can be adaptive, as trout are able to respond and recover faster after subsequent handling (Tort, 2013). Handling stress is often experienced in aquaculture and angling competitions, which accounted for $7.9 billion in revenue in 2015 (Department of Fisheries and Oceans, 2019; Twardek et al., 2018). Mitigating these effects is important to maintain healthy fish populations. Investigating how stress is communicated between cells and tissues can help with development of methods to reduce these effects. Hormone cascades in teleosts are well-studied and demonstrate how fish overcome a perceived stressor using endocrine signalling (Bonga, 1997; Mommsen et al., 1999). My work will add to our knowledge of how stress alters microRNA levels both within and between tissues to understand the role of non-coding RNAs within the well studied teleost stress response.

1.1 The Stress Response

Organisms respond and adapt to changes in their environment using endocrine signalling cascades to cause physiological changes in tissues throughout the body. Stress is integrated in the hypothalamus and communicated to the anterior pituitary using corticotropic releasing hormone (CRH; Bonga, 1997; Mommsen et al., 1999). This causes release of adrenocorticotropic hormone (ACTH) into circulation where it induces cortisol production from the interrenal cells of the anterior kidney (Fig 1; Bonga, 1997; Mommsen et al., 1999). After an acute stressor, interrenal cells respond to ACTH by upregulated expression of melanocortin receptor (MC2R),

steroidogenic acute regulatory protein (StAR), cytochrome P450 side chain cleavage (P450scc), and 11β-hydroxylase to aid in the production of cortisol (Fierro-Castro et al., 2015).

Cortisol acts on many tissues to induce major physiological changes associated with the stress response. The presence of elevated plasma glucose levels is used to confirm that a fish is in a stressed state (Bonga, 1997; Mommsen et al., 1999). Activation of the glucocorticoid receptor (GR) in hepatocytes upregulates genes associated with metabolism, as well as endocrine and stress related genes (Aluru & Vijayan, 2007). This results in increased gluconeogenesis in the liver, or production of glucose from non-carbohydrate-based sources that can then be used as fuel for energy demanding tissues (Bamberger et al., 1996; Mommsen et al., 1999; Sathiyaa & Vijayan, 2003; Walton & Cowey, 1979; Wiseman et al., 2007). GR activation stimulates upregulation of the key gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (Pepck), which is involved in catalyzing the production of phosphoenolpyruvate (PEP) from oxaloacetate (Aluru & Vijayan, 2007; Mommsen et al., 1999; Sathiyaa & Vijayan, 2003; Vijayan et al., 2003; Wiseman et al., 2007). However, glycolytic genes are also induced during stress responses as a way of metabolizing excess glucose in fish. Glucokinase, the enzyme responsible for catalyzing the production of glucose from glucose-6-phosphate and pyruvate kinase, the enzyme responsible for converting PEP into pyruvate, have both been shown to be upregulated during recovery from a stress response (Wiseman et al., 2007). Acute stress has also been shown to enhance certain immune functions (Dhabhar & McEwen, 1996; Dhabhar et al., 2010; Dhabhar & Viswanathan, 2005; Pickford et al., 1971). These benefits come at the repression of other pathways such as growth, reproduction, and digestion to conserve energy for escaping/avoiding the perceived stressor (Aluru & Vijayan, 2009; Bonga, 1997; Mommsen et al., 1999). Overall,

this shows the important role cortisol has in both modulating energy mobilization while inhibiting pathways that are not required when overcoming a stressor.

Cortisol uses glucocorticoid receptor signalling to activate transcription of stress responsive elements (Aluru & Vijayan, 2009; Charmandari et al., 2005). Liver levels of Pepck, a protein involved in gluconeogenesis, increases *in vitro* and *in vivo* in response to increased cortisol levels (Aluru & Vijayan, 2007; Sathiyaa & Vijayan, 2003; Vijayan et al., 2003). Transcripts for protein catabolism (*cathepsin D* and *gad65*), protein chaperones (*hsp90*), and enzymes involved in contaminant metabolism (*metallothionein, cytochrome P450*) have been shown to be upregulated, while transcripts important for reproduction (*estrogen receptor* and *vitellogenin*) are downregulated in the liver by cortisol (Aluru & Vijayan, 2007; Lethimonier et al., 2000). Previous work in fish has found that cortisol upregulates expression of *gr* and *pepck* mRNA while downregulating protein levels of both in the liver (Aluru & Vijayan, 2007; Sathiyaa & Vijayan, 2003). These results clearly show that transcript levels are regulated by hormone signalling, both at the transcriptional and translational level.

1.2 microRNAs

MicroRNAs (miRNA) are small, 22 nucleotide-long, non-coding RNA that bind to the 3' untranslated region (UTR) of mRNA transcripts to either target them for degradation or inhibit translation (Bartel, 2004; Lee et al., 1993; Wightman et al., 1993). MiRNAs are created through initial transcription in the nucleus into pri-miRNA, which often forms into hairpin loops that are further processed into shorter pre-miRNAs by Drosha, a type III RNase (Bizuayehu & Babiak, 2014; Lee et al., 2003). This pre-miRNA is then transported out of the nuclease where Dicer, another type III RNase, produces a 22-nucleotide long RNA duplex which can have one strand loaded into Argonaut 2 (Ago2; Krol et al., 2010; Lee et al., 2002; Lee et al., 2003). Ago2 and the

bound miRNA are part of the RNA-induced silencing complex (RISC) which can bind to the 3'UTR and target the transcript for degradation or suppress expression (Bartel, 2004; Brennecke et al., 2005; Wightman et al., 1993). Each miRNA can influence the expression of thousands of different mRNA transcripts due to the seed pairing only requiring 2-7 nucleotides (Lewis et al., 2005). In tissues, miRNA transcript abundance changes in response to environmental conditions as well as cell signalling events, such as hormones, making them ideal targets for understanding the impact of anthropogenic stressors in the environment (Cameron et al., 2015).

MiRNA expression can be regulated by targeting transcription of the miRNA itself, targeting enzymes involved in the production of miRNAs, or targeting the proteins involved in the action of the miRNAs (Cameron et al., 2015; Leung & Sharp, 2010). Furthermore, previous studies have demonstrated that miRNA levels have been altered by hormonal signalling. Estradiol in humans acts on RNA polymerase II to regulate miRNA transcription as well as inhibiting Drosha (Cameron et al., 2015; Gupta et al., 2012). Gonadotropins have been shown to increase key miRNA transcripts that target luteinizing hormone receptors in the ovaries of rats (Kitahara et al., 2013). Knockdown of Dicer in human adrenal cells has been shown to influence expression of cytochrome P450 side chain cleavage (*p450scc* or *cyp11a1*), 17α-monooxygenase (*cyp17a1*), and 11β-hydroxylase (*cyp11c1*), key transcripts in the cortisol production pathway (Robertson et al., 2013; Robertson et al., 2017). As previously stated, cortisol was shown to upregulate GR mRNA levels, but protein levels were found to be downregulated (Aluru & Vijayan, 2007; Sathiyaa & Vijayan, 2003). However, in mice, GR levels in adrenal glands have been shown to be influenced by miRNAs (Riester et al., 2012). These observations clearly show a link between hormones and miRNA, and these changes are likely passed on to the transcripts they target as another level of regulation.

1.3 Extracellular miRNAs

Another way that cells respond to stress is by secreting vesicles. Extracellular vesicles (EVs) are classified based on their size and release. Exosomes (30-100 nm) are EVs that are created by inward budding of multivesicular endosomes, while microvesicles (50-1000 nm) are produced by budding of the plasma membrane into extracellular space (Raposo & Stoorvogel, 2013; van Niel et al., 2018). Apoptotic bodies (1-5 μm) are another class of circulating vesicles that are released from cells undergoing programmed cell death (Sohel, 2016; Turiák et al., 2011). All contain proteins, mRNA, and miRNAs that can influence function in cells that absorb these contents (Pegtel et al., 2010; Raposo & Stoorvogel, 2013; van Niel et al., 2018). EVs have been found in biological fluids such as blood, urine, and saliva, making them easily accessible (Caby et al., 2005; Ogawa et al., 2011; Pisitkun et al., 2004). EVs provide protection for RNAs and proteins from catabolic enzymes in these biological fluids, making them enriched sources for information on cell signalling (Cheng et al., 2014).

MiRNAs are also quite stable in circulation when associated with other complexes such as proteins and lipids. As mentioned above, miRNAs associate with Ago2 to form the RISC for translational repression (Bartel, 2004; Brennecke et al., 2005; Wightman et al., 1993). Ago2 has been found to be stable in circulation and most of the miRNAs found in circulation are thought to be associated with such protein complexes (Arroyo et al., 2011; Turchinovich et al., 2011). Although currently no studies have found a functional role for these Ago2 associated miRNAs, their potential for paracrine action is high considering their importance in RNA silencing. Additionally, miRNAs associated with lipoproteins have been shown to be biologically active in circulation and can be transported to tissues at a distance (Babin & Gibbons, 2009; Kim et al., 2007; Lee et al., 2009). High-density-lipoprotein associated miRNAs have been shown to

influence expression in cultured hepatocytes as well as functionally impacting endothelial cell function (Tabet et al., 2014; Vickers et al., 2011). Together with EVs, these sources of miRNAs offer the potential for understanding signalling between tissues, epigenetically, due to their high stability.

1.4 Extracellular vesicles and stress

Previous work has shown that stress responsive pathways, particularly immune functions, are reinforced through exosomal signalling. For example, exosomes contain adhesion molecules that allow them to be recruited to immunologically compromised sites (Skokos et al., 2001). In mice, exposure to stress showed a decrease in miRNAs within exosomes that target cytokine signalling pathways indicating that exosomes are helping to prime the organism for potential injury by stimulating the innate immune system (Beninson et al., 2014). Stress-produced exosomes also had increased levels of danger associated molecular patterns (DAMPs), which can activate leukocytes to support the immune system (Beninson et al., 2014). Previous studies have also shown that EV formation is regulated by the cargoes they package (Buschow et al., 2009; van Niel et al., 2018). MHC class 2 is a cargo that promotes the formation of EVs and has been shown to be upregulated in livers of stressed rainbow trout, indicating that stress could increase EV production and aid in transducing this response (Buschow et al., 2009; Wiseman et al., 2007).

Few studies have investigated the role of EVs or circulating miRNAs in teleosts. Studies in fish immunology have found that anterior kidney leukocytes can be simulated to release exosomes enriched with MHC class 2 after being stimulated by DNA un-methylated CpG motifs (Iliev et al., 2010). The protein content of circulating EVs from Atlantic salmon was also found to be altered by infection to enhance immune function for overcoming the pathogen (Lagos et al.,

2017). In rainbow trout, circulating exosomes produced in response to heat stress were shown to be enriched in Hsp70, a protein important in the cellular stress response and influenced by cortisol (Faught et al., 2017). *In vitro*, cortisol reduced Hsp70 bound to exosomes produced by fish hepatocytes, showing that cortisol influenced exosome content (Faught et al., 2017). Although circulating miRNAs have been shown to be enriched within exosomes and provide a useful biomarker for cholesterol metabolism, no studies have currently investigated their role in communicating the stress response (Sun et al., 2017; Zhu et al., 2018).

1.5 Study organism

For this thesis, rainbow trout (*Oncorhynchus mykiss*) was used. Its' large size is beneficial in order to provide the blood volume required for the protocols in this study. Rainbow trout have been used as a model species for studying stress for the last three decades, making it well-documented in literature (Tort, 2013). Sequencing of both the genome and miRNA transcriptome in different tissues makes rainbow trout an excellent model organism for miRNA studies, especially in combination with stress physiology (Berthelot et al., 2014; Juanchich et al., 2016).

1.6 Study objectives

The primary aim of this thesis was to expand the current literature on miRNAs within teleosts through the stress response and investigate how stress alters miRNAs in tissues and circulation. The objectives for these *in vivo* and *in vitro* studies were:

1. Assess the changes to miRNA abundances in circulation and within tissues of rainbow trout recovering from an acute air stress to determine if stress is communicated both hormonally and epigenetically.

2. Determine the liver's role in contributing to circulating miRNA changes by focusing on one of its primary responses to the glucocorticoid cortisol to establish if miRNAs are important regulators of glucose production and metabolism during recovery from stress.
3. Compare the *in vivo* and *in vitro* effects to expand our knowledge on how stress in fish is transduced both at the tissue level and the entire organismal level.

1.7 Hypothesis

I hypothesize that during the recovery from a stressor, miRNA levels will be altered to transduce the stress response between and within tissues. Cortisol will be instrumental in influencing these changes as it has a central role in metabolic regulation during the return to homeostasis. The liver, in combination with cortisol, will undergo metabolic reprogramming during the recovery from stress due to its function as the primary site for gluconeogenesis.

1.8 General predictions

I predict that an acute air handling stressor will induce a stress response in rainbow trout and result in elevated plasma cortisol and glucose. This will also induce miRNA changes in the anterior kidney and liver due to their importance in hormonal signalling and repartitioning of metabolic resources during the recovery period. Stress will result in changes in circulating miRNA levels to transduce stress between tissues epigenetically. At the level of the liver, miRNA predicted to target key transcripts such as *pepck* and *gk* will be altered due to the liver's primary role in liberating glucose under stressful events. These miRNAs will be secreted by hepatocytes as a way of paracrine signalling to maintain the metabolic state of the liver during this recovery period.

Chapter 2

Impacts of acute air exposure on circulating, liver, and kidney microRNAs in rainbow trout (*Oncorhynchus mykiss*)

2.1 Introduction

In aquaculture facilities, regular handling is necessary to monitor and maintain fish health. However, this can cause a stress response similar to stress responses of fish in the wild and fish exposed to handling during angling (Barton & Iwama, 1991; Twardek et al., 2018). The HPI axis is the primary driver of the physiological change associated with stress in teleost species and uses the hormone cortisol to communicate between tissues. This response begins with the hypothalamus which stimulates the anterior pituitary to secrete adrenocorticotropic releasing hormone (ACTH) using corticotropin releasing hormone (CRH; Bonga, 1997; Mommsen et al., 1999). In teleosts, ACTH stimulates the interrenal cells in the anterior kidney to secrete cortisol, the primary stress hormone responsible for inducing cortisol (Bonga, 1997; Mommsen et al., 1999). Cortisol has many functions systemically, but one of its main action in the stress response is stimulating the release of glucose from the liver to provide energy in a stressed state (Bonga, 1997; Mommsen et al., 1999). Other actions of cortisol include influencing osmoregulation, suppression of growth and reproduction, and suppression of the immune system under chronic stress conditions (Berg et al., 2004; McCormick et al., 2008; Milla et al., 2009; Mommsen et al., 1999; Ramsay et al., 2009) Handling stressors have been shown to increase blood concentrations of cortisol and result in the physiological changes described above (Bonga, 1997; Ramsay et al., 2009; Ritola et al., 1999). Maintaining fish health is important for industries such as aquaculture and understanding how handling stressors is communicated can help mitigate deleterious effects.

MicroRNA (miRNA) are a small, non-coding type of RNA that have been shown to be altered by changes in the environment and are important for epigenetic regulation (Cameron et al., 2015). miRNA act to silence genes by binding to the 3’ untranslated region of mRNA

transcripts and either target them for degradation or prevent translation from occurring (Bartel, 2004; Lee et al., 1993; Wightman et al., 1993). Since these molecules are short (20-22 bp) and only require a seed region of 7-8 bp, they can bind to many different mRNA targets and can therefore have powerful repression and silencing effects in different tissues (Lewis et al., 2005). MiRNAs are also altered by hormonal signalling, either by having a direct impact on miRNA transcript abundance or by altering the proteins associated with miRNA action (Cameron et al., 2015; Gupta et al., 2012; Kitahara et al., 2013). Cortisol has been shown to modulate miRNAs in the same fashion by playing an important role in repression of the immune system in mammals (Chen et al., 2016; Clayton et al., 2018; Smith et al., 2010; Smith et al., 2013). In rainbow trout, miRNAs have been implicated in the metabolism associated with social hierarchies and the chronic stress associated with these hierarchies (Kostyniuk et al., 2018; Kostyniuk et al., 2019). Understanding the role that miRNAs play during stress responses will help to mitigate the detrimental effects associated with such physiological change.

MiRNA are also protected from degradation in circulation through association with vesicles, lipids, or proteins (Cheng et al., 2014). Extracellular vesicles (EVs) are released by both healthy and perturbed cells and have been found in biological fluids such as blood, urine, and saliva (Caby et al., 2005; Ogawa et al., 2011; Pisitkun et al., 2004; Raposo & Stoorvogel, 2013). Different EVs are classified based on their size and method of formation. Exosomes are produced via inward invagination of multivesicular endosomes, microvesicles are produced by outward budding of the plasma membrane, and apoptotic bodies are released by cells undergoing programmed cell death (Bartel, 2004; Raposo & Stoorvogel, 2013; Sohel, 2016; Turiák et al., 2011). The composition of EVs can be influenced *in vitro* by environmental factors such as heat,

hypoxia, irradiation, and the ambient media, showing that they could be used as a method to adapt to the environment (Beninson & Fleshner, 2014; Faught et al., 2017).

Teleost serum derived EVs have been shown to contain miRNA, making them a good vehicle for systemic miRNA transport (Sun et al., 2017). Other studies with fish have outlined the immunological role of EVs produced response to infection (Iliev et al., 2010; Lagos et al., 2017). Exosomes have been shown to be altered during a stress response, but changes in miRNA content have not been reported in teleosts (Faught et al., 2017). However, in mammals, the exosomal miRNA profile has been shown to alter as a way of supporting the stress response (Beninson et al., 2014). Although miRNA have been investigated in humans from a medical perspective, there has been little work on how miRNA expression in circulating exosomes is influenced by environmental conditions in teleosts.

For this study, the model species was rainbow trout (*Oncorhynchus mykiss*). Its size makes it an ideal model for circulating exosome studies due to its larger blood volume. The recent mapping of the rainbow trout genome as well as miRNA transcriptome make them an excellent model for genetic and epigenetic studies (Berthelot et al., 2014; Juanchich et al., 2016). Rainbow trout are important aquaculture and wild fishery species worldwide, making them economically relevant and important to study for handling stressor influence (Ellis et al., 2002). Rainbow trout are often used in studying the teleost stress response, making them well documented in literature (Alderman et al., 2008; Caldwell et al., 1991; Krasnov et al., 2005; Vijayan & Moon, 1992). Finally, rainbow trout serum has already been confirmed to contain exosomes, making them the ideal model for teleost exosome study (Faught et al., 2017).

Studying EV trafficking of miRNA has the potential to provide information on how stressors can cause epigenetic changes between tissues. EVs can be used as bioindicators of

environmental perturbations, which makes studying their production within fish systems highly important. It is hypothesized that the EV and tissue miRNA profile will be altered during a stress response as a way of epigenetic modification during recovery from stress. MiRNAs measured in this study (Table 2.1) were chosen based on their high relative abundance in the tissues essential to the stress response (Juanchich et al., 2016). These tissues include the anterior kidney, which produces cortisol in response to adrenocorticotropic hormone (ACTH), and the liver which is one of the primary targets for cortisol resulting in the liberation of glucose (Mommsen et al., 1999). This is the first study to examine the EV miRNA profile of a teleost species exposed to a handling stressor.

Table 2.1: Abundance of miRNAs in key rainbow trout tissues for responding to stress. Abundances of miRNAs were determined based on sequencing by Juanchich et al., (2016). MiRNAs in italics were not measured in the current study.

	miRNA Expression		
Organ	**Most Expressed**	**2nd Most Expressed**	**3rd Most Expressed**
Brain	miR-21	miR-143	*miR-146*
Pituitary	miR-21	let-7a	*let-7e*
Anterior Kidney	miR-21	miR-143	*miR-146*
Heart	*miR-1*	*miR-499*	miR-21
Liver	miR-21	*let-7e*	*miR-146*

2.2 Materials and Methods

2.2.1 Study Animals

Rainbow trout (*Oncorhynchus mykiss*) were purchased from Lyndon Fish Hatcheries in Petersburg, ON and donated for this study by Dr. Brian Dixon (University of Waterloo). Trout of mixed sex (average weight 660 ± 33 g) moved to 180-gallon flow through tanks at a density of 6 fish per tank and allowed to acclimate for 48 hours. During the study, a 12-hour light-dark cycle was maintained and temperature, dissolved oxygen, pH, and conductivity monitored. Water

quality parameters were constant over the course of the study and fish were fasted 24 hours prior to the start of the experiment. Experimental procedures and the use of animals in this study was approved by the University of Waterloo Animal Care Committee and within CCAC guidelines (AUPP 40315).

Table 2.2: Acute air exposure rainbow trout information. Rainbow trout were acutely exposed to an air stressor for 3 minutes in the case of treatment groups or immediately sampled in control. Fish sex was determined using presence of male or female gonads and weight determined prior to collecting any tissue.

Treatment	Sex	Weight (g)
Control	Male	552.50
	Female	587.60
	Male	715.60
	Male	627.11
	Male	557.90
	Male	580.40
1 hour	Male	483.70
	Female	794.30
	Female	973.90
	Female	742.42
	Male	402.80
	Female	742.77
3 hours	Male	559.50
	Female	939.20
	Male	748.70
	Female	752.70
	Male	650.21
	Male	534.10
24 hours	Female	488.36
	Female	621.14
	Male	507.79
	Female	668.02
	Male	540.29
	Female	1060.38

2.2.2 Acute Air Handling Stressor Experiment

Following 48 hours of acclimation and 24 hours of fasting, fish were either sampled (control) or exposed to a handling stressor. For this study the handling stressor was a 3 minute air exposure since this has been shown to induce a stress response and imitates a common source of stress in the aquaculture industry (Barton, 2000; Ramsay et al., 2009; Skrzynska et al., 2018). Fish exposed to the handling stress recovered for 1-, 3-, or 24-hours while control fish were immediately euthanized in 0.5 g/L bicarbonate buffered MS-222. Blood was collected via caudal puncture, aliquoted into two 2 mL tubes containing 5 µL of 0.5 M EDTA to prevent coagulation and kept on ice until further processing. Liver and anterior kidney tissues were collected following blood collection and flash frozen using tissue clamps in liquid nitrogen. One blood aliquot was centrifuged at 10 000 x *g* for 3 mins to collect plasma while the other fraction was differentially centrifuged to remove cellular material for exosome analysis based on a protocol from personal communication with FroggaBio and Kenigsberg (2016). Cellular components were removed by centrifuging twice at 4°C, 2500 x *g* for 15 mins. Plasma supernatant was then centrifuged at 300 x *g* for 10 mins, 2000 x *g* for 10 mins, and lastly 12 000 x *g* for 30 mins to remove unwanted blood components that could impact the results of downstream analysis. This final fraction of plasma was passed through a 0.22 µm filter to clear out larger vesicles and cellular debris that might have remained after centrifugation steps. All samples were subsequently stored at -80°C until analysis.

2.2.3 Transmission Electron Microscopy validation of extracellular vesicle extraction

One plasma sample from each timepoint was thawed on ice and EVs were extracted using a modified protocol for the SeraMir Exosome RNA Amplification kit (System Biosciences; Cat# RA800A-1). Plasma samples (300 µL) was incubated with 75 µL of ExoQuick (System

Biosciences) for 30 mins at 4°C. Samples were then centrifuged at 1500 x *g* for 30 mins to precipitate the EVs and the supernatant aspirated. Residual ExoQuick was then centrifuged again for 1500 x g for 5 mins and all traces removed. This pellet was resuspended in 30 µL of RNase free water and stored at -80°C. EVs were then visualized using transmission electron microscopy (TEM) by depositing undiluted and diluted (1:5) samples on Formvar/Carbon coated grids. These were air dried and stained with a saturated solution of uranyl acetate for 10 mins and images obtained using a Phillips CM10 TEM at 60 KV.

2.2.3 Plasma Glucose and Cortisol determination

Plasma samples were thawed on ice and glucose concentrations determined using a spectrophotometric assay. Plasma or glucose standard (10 µL) was added to a 96 well plate and combined with a 50 mM Hepes reaction buffer (pH 7.4). This buffer contained 10 mM $MgCl_2$, 100 mM KCl, 1 mM NADP, 1 mM ATP, 1 unit/mL of G6PDH (Sigma-Aldrich) and 1 unit/mL hexokinase (Sigma-Aldritch). The change in ratio of NADP to NADPH was measured over the course of 20 mins in a SpectraMax 190 Microplate Reader at 340 nm. Concentrations were determined by comparing absorbances to a 6-point standard curve.

Cortisol concentrations were determined by using a commercially available kit for measuring plasma cortisol (Cayman Chemical, Cat# 500360). The protocol was followed based on the manufacturer's guidelines. Plasma samples were diluted 1:100 in MilliQ Ultrapure Water to allow for readings to fall within the 8-point standard curve. Concentrations were determined after incubating for 90 mins with Ellman's reagent by using a SpectraMax 190 Microplate Reader at 412 nm and comparing to the 4-parameter logistic standard curve.

2.2.4 RNA Extractions

Exosomal miRNA was extracted using a commercially available SeraMir Exosome RNA Amplification kit (System Biosciences; Cat# RA800A-1) that specifically targeted exosomes through a proprietary extraction method. The extraction protocol was followed based on the manufacturer's guidelines without deviation. A SeraMir control RNA spike-in (Cat# RA805A-1) was added to provide a control for qPCR normalization that was unaffected by the treatment conditions. Samples were kept at -80°C until subsequent analysis.

Tissue RNA was extracted from liver and kidney tissues using the Qiagen miRNeasy Mini Kit (Cat# 217004). Flash frozen organs were ground up in liquid nitrogen using a motor and pestle and 25-50 mg of tissue used for RNA extraction. The extraction protocol was followed based on the manufacturer's guidelines without deviation. 30 µL of RNase-free water was used to elute RNA and all samples stored at -80°C until subsequent analysis.

2.2.5 RT-qPCR

Extracted samples were thawed on ice and RNA concentration determined using a SpectraDrop Micro-Volume Microplate (Molecular Devices). Either 50 ng of exosomal RNA or 1000 ng of tissue RNA was used for cDNA synthesis. Qiagen's miScript II RT kit (Cat# 218161) was used since it was able to generate miRNA specific cDNA. The manufacturer's protocol was followed without deviation. HiSpec buffer was used for exosomal samples while the HiFlex buffer was used for tissue samples since both can be used to make cDNA for mature miRNA but the HiFlex buffer is more ideal for reverse transcribing mRNA. A pooled sample was also reverse transcribed from each tissue in order to generate standard curves.

Standard curves were run for each target in the exosomal and tissue fractions. Dilutions within these curves were 4x, 16x, 64x, 256x, and 1024x and standard curves for each primer was

found to be linear after the 16x dilution. Therefore, all samples were diluted 20x to reduce qPCR inhibition. Each qPCR reaction contained 1 µL of diluted cDNA (0.125 ng of exosomal cDNA and 2.5 ng of tissue cDNA per reaction), 5 µL of Biorad SYBR Green Master Mix (Cat# 1725272), 0.5 µL of 5 µM forward primer for the target miRNA or mRNA (Sigma-Aldritch), 0.5 µL of 5 µM Universal Primer for miRNAs (Qiagen, Cat# 218073) or reverse primer for mRNA (5 µM) , and 3 µL of water. Samples were run on CFX96 Touch Real-Time PCR Detection System (Biorad). Each run consisted of 30 seconds at 95°C initially, followed by 40 repeated cycles of 10 seconds at 95°C and 15 seconds at 60°C. All runs finished off with a melt curve to make sure that only one qPCR product was generated during the run. Primer sequences can be found in Table 2.2. MiRNAs were chosen based on their high relative abundance in the tissues important to the stress response or their importance in epigenetic regulation (Juanchich et al., 2016; Kuc et al., 2017). Relative abundances were calculated by either normalizing to the spike-in for exosomal samples or to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the standard $2^{-\Delta\Delta Ct}$ equation (Livak & Schmittgen, 2001).

Table 2.3: Primer sequences for targets of acute air handling stress study. Forward sequences for miRNAs were acquired from sequencing data of the miRNA expression in rainbow trout tissues while reverse primers were proprietary Qiagen sequences (Juanchich et al., 2016). Amplicon length was unable to be determined for these sequences.

Target	Forward Primer Sequence	Reverse Primer sequence	Amplicon length
omy-miR-21-3p	GACAGCTTACAGACCGTTGTCG	Qiagen Universal Primer	-
omy-miR-143-3p	TGAGATGAAGCACTGTAGCT	Qiagen Universal Primer	-
omy-let-7a-5p	TGAGGTAGTAGGTTGTATAGTT	Qiagen Universal Primer	-
omy-miR-29a-3p	TAGCACCATTTGAAATCGGTTA	Qiagen Universal Primer	-
SeraMir Spike-in	Spike-in Forward primer	Qiagen Universal Primer	-
GAPDH	CAACGGATTTGGCCGTATTG	ATGTACTGCAGGTCGATGAAG	102

2.2.6 Bioinformatic Analysis of Targets for miRNAs

Since each miRNA can have hundreds of different targets, a Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was carried out to determine what pathways might be impacted by any changes observed (Lewis et al., 2005). The MiRanda algorithm was used to find all potential targets for the miRNAs using the standard parameters (Enright et al., 2003). miRNA sequences were acquired from Juanchich et al. (2016) and searched against the *Salmo salar* 3' UTRs extracted from RNA from genomic file on NCBI (GCF_000233375.1_ICSASG_v2/GCF_000233375.1_ICSASG_v2_rna_from_genomic.fna.gz). A cutoff pairing score of > 140 and free-energy score ΔG of < -20 was used to be consistent with other studies in rainbow trout that have used MiRanda for predicted targets (Kostyniuk et al., 2019). Refseq accession numbers were then converted to UniprotKB IDs to be compatible with KEGG. The top 10 pathways impacted were graphed in pie charts to show pathways that are likely to be impacted. All impacted pathways for each miRNA can be found in Appendix B. Uniprot accessions were also run through DAVID Bioinformatics Resource 6.8 to find functional enrichment clusters that are likely to be influenced by the miRNA measured (Huang et al., 2009a, 2009b). Any functional clusters with Enrichment Scores higher than 1.3 were determined to be significantly overrepresented in the list of genes (Huang et al., 2007).

2.2.7 Statistical Analysis

Data was analyzed using GraphPad Prism (GraphPad Software, La Jolla CA) and presented as the average ± standard error of the mean (SEM). Significant differences between relative abundances, cortisol or glucose concentrations were found using one-way analysis of variance tests (ANOVA, $p<0.05$). Normality was determined using a Shapiro-Wilk test and when significant differences were found between groups, Tukey's Post-Hoc Multiple

Comparison Test was used to determine where the differences occurred. When results did not pass the normality test, a Kruskal-Wallis test was carried out and when significant differences were found between groups, Dunn's multiple comparisons test was used to determine where the differences occurred.

2.3 Results

2.3.1 TEM images of trout EVs

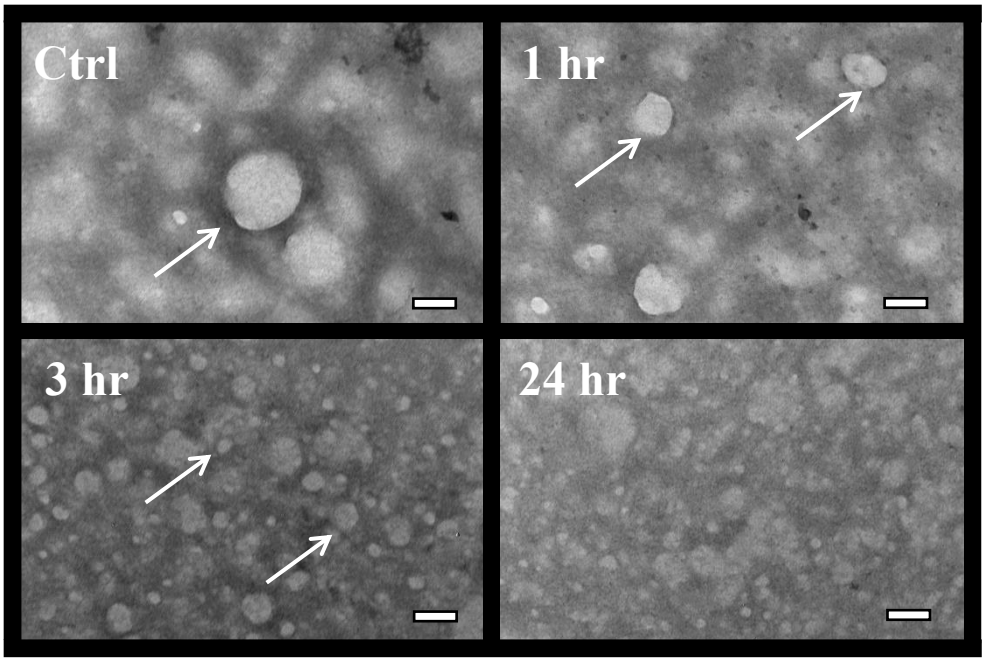


Figure 2.1: Transmission electron microscopy (TEM) of rainbow trout plasma samples during recovery from an acute handling stressor. Ctrl and 1-hr images were generated using undiluted samples while 3- and 24-hr images were generated using samples diluted 1:5. Scale bars in the bottom right of each image are 100 nm in length and arrows are pointing to EVs.

ExoQuick was able to precipitate rainbow trout EVs based on the images generated via TEM (Fig 2.1). Vesicles in the Ctrl and 1-hour timepoint are large (denoted with arrows), reaching around 100 nm in diameter. In the images for the 3- and 24-hour timepoints, vesicles appear to increase in number and smaller vesicles are observed. These samples have been diluted 1:5 as undiluted samples were oversaturated and clear boundaries between vesicles were not observed.

2.3.2 Plasma Cortisol and Glucose concentration

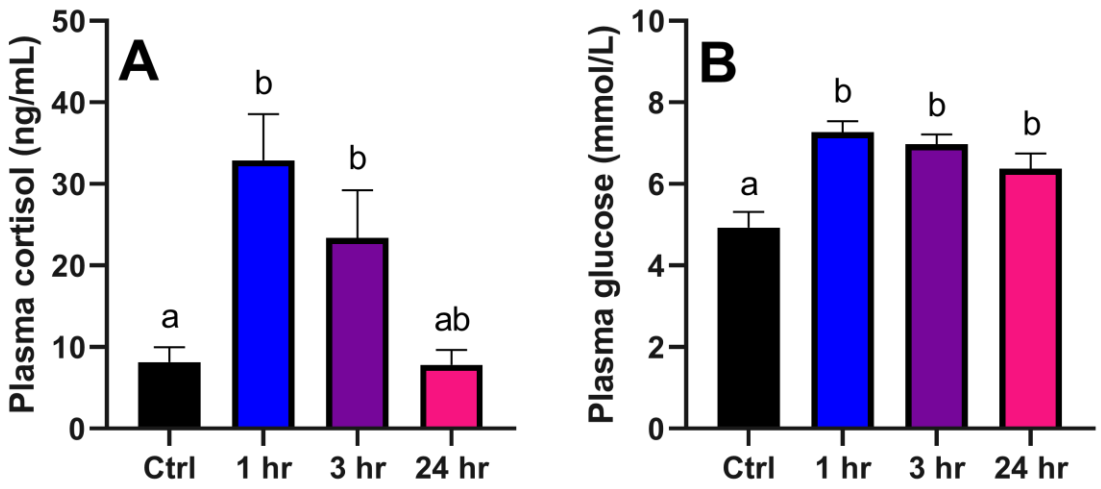


Figure 2.2: Plasma cortisol (A) and glucose (B) levels after exposure to an acute handling stressor. Plasma hormone and metabolite levels were measured 1-, 3-, or 24-hours by either ELISA (Cayman) or metabolite enzymatic assay respectively. Bars that do not share common letters were found to be significantly different (One-way ANOVA, $p < 0.05$, Tukey or Dunnett's Post Hoc, $n = 6$).

Fish exposed to the 3-minute handling stress had significantly elevated cortisol concentrations in circulation that returned to control levels within 24 hours. Cortisol levels increased 4-fold compared to unstressed control after 1-hour (Dunnett, $p = 0.0016$) and were still elevated by almost 3-fold after 3 hours of recovery (Dunnett, $p = 0.0494$).

Fish exposed to the 3-minute handling stress had significantly elevated glucose levels in circulation. These circulating values increased to 7.27 mmol/L (±0.26, Tukey, $p = 0.0003$) 1 hour

after the stress and remained elevated after 3 hours (6.96 mmol/L ±0.25, Tukey, p = 0.0013). Even after 24 hours, these circulating levels remained significantly elevated compared to unstressed fish (6.36 ± 0.38 mmol/L, Tukey, p = 0.024).

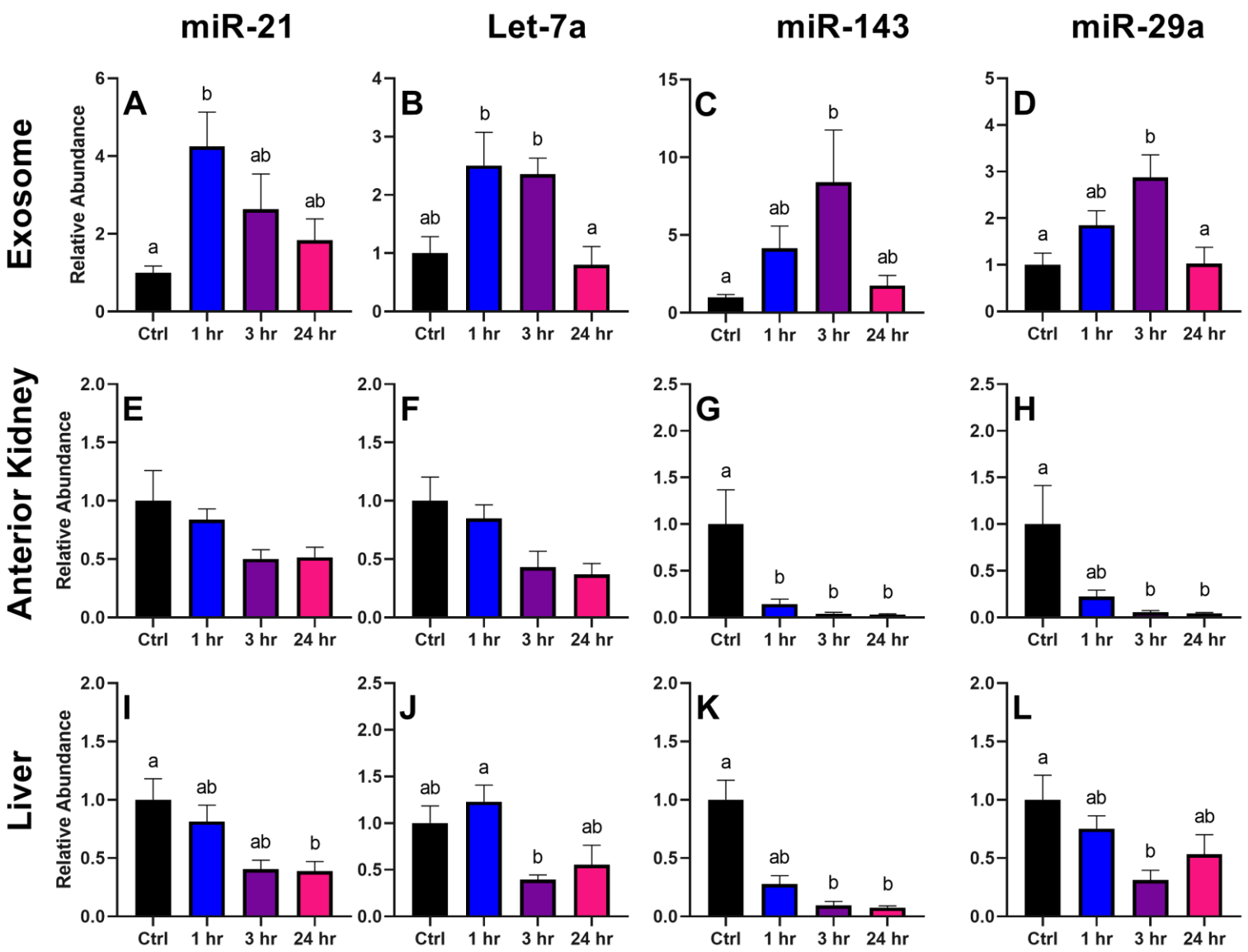

miR-21
Let-7a
miR-143
miR-29a
Exosome
Anterior Kidney
Liver
Relative Abundance
A
B
C
D
E
F
G
H
I
J
K
L
Ctrl
1 hr
3 hr
24 hr

Figure 2.3: miRNA abundances within plasma exosomes (A-D), anterior kidney (E-H), and liver (I-L) after exposure to an acute handling stress. Relative abundances were measured using RT-qPCR and normalized to a spike in for exosomal abundances or GAPDH for tissue samples. Bars that do not share common letters were found to be significantly different while graphs with no lettering denote no significant differences between groups (One-way ANOVA, $p < 0.05$, Tukey or Dunnett's Post Hoc, $n = 6$).

2.3.3 Plasma exosome miRNA abundances

Exosomal abundances of the miRNAs measured tended to increase post-stress at different timepoints (Fig 2.3 A-D). MiR-21 was increased 4-fold higher than control only 1 hour after the handling stress (Dunnett's, $p = 0.0057$) and started returning to normal levels at 3 hours. There was no significant change in the Let-7a levels compared to control. However, there was a significant difference between stressed groups with the 1-hour (Tukey, $p = 0.0235$) and 3-hour (Tukey, $p = 0.0415$) timepoints being significantly higher than the 24-hour post stress timepoint. MiR-143 had a significant increase in circulating exosomes 3-hours post stress (Tukey, $p = 0.0475$), increasing to almost 10 times the control levels. Finally, miR-29a was found to be significantly increased in circulation at the 3-hour timepoint relative to both control (Tukey, $p = 0.0072$) and 24-hours post-stress (Tukey, $p = 0.0081$).

2.3.4 Anterior kidney miRNA abundances

MiRNA abundances measured within the anterior kidney tended to decrease after the handling stressor (Fig 2.3 E-H). MiR-21 and let-7a had similar trends at the timepoints measured but no significant differences were found. However, miR-143 had significantly downregulated abundances at all timepoints post stress, with 1-hour decreasing to 15% of control values (Tukey,

p = 0.0196) and the 3-hour (Tukey, p = 0.0081) and 24-hour timepoint anterior kidneys (Tukey, p = 0.0074) containing less than 5% of control values. A similar trend was seen in miR-29a; however, the 1-hour timepoint was not significantly different from control levels. The 3- (Dunn, p = 0.0087) and 24-hour timepoints (Dunn, p = 0.0020) had around 5% of the control levels of miR-29a.

2.3.5 Liver miRNA abundances

The miRNA levels measured in the liver tended to decrease compared to control and all miRNA levels were significantly impacted by the handling stressor (Fig 2.3 I-L). MiR-21 was found to be significantly decreased after 24-hours (Dunn, p = 0.033) to around 50% of control values. Let-7a relative abundance within the liver was only reduced by 60% at the 3-hr timepoint, yet had recovered by 24-hrs (Dunn, p = 0.0225). The liver abundance of miR-143 followed a similar trend to what was observed in the anterior kidney. The 3-hour post-stress group decreased to around 10% of control abundances (Dunn, p = 0.0031) while the 24-hour timepoint was around 7.5% (Dunn, p = 0.0023). MiR-29a decreased in abundance at the 3-hour timepoint to around 30% of the control values (Dunn, p = 0.0256) while the 24-hour group was statistically similar to the control.

2.3.6 KEGG and DAVID analysis of miRNA targets

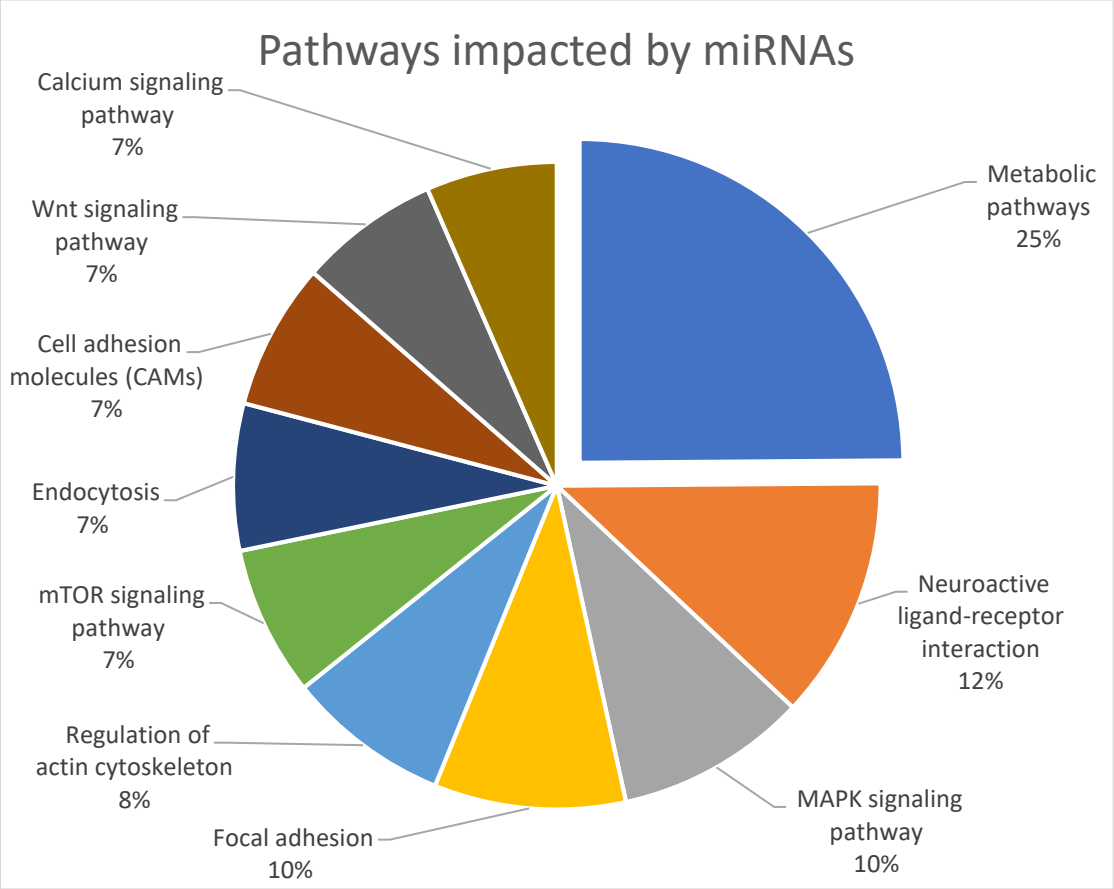


Figure 2.4: Top 10 pathways influenced by targets of miRNAs measured. Targets for miR-21, let-7a, miR-143, and miR-29a were predicted using MiRanda on *Salmo salar* 3' UTRs and run through KEGG pathway analysis. Percentages below each target are related to how many gene targets within this list are represented by the pathway.

KEGG pathway analysis revealed a total of 151 pathways predicted to be impacted by the changes in miRNA measured. The top 10 pathways impacted are shown in Figure 2.4 while the complete lists of predicted targets for each miRNA can be found in Appendix B. 627 genes are

accounted for in the top 10 pathways and 25% of these genes are grouped under metabolic regulation.

MiR-21 is predicted to inhibit 41 target genes associated with metabolic pathways, including methylmalonyl-CoA mutase (*mcm*; A0A1S3MCC1) and pyruvate dehydrogenase E1 component subunit alpha (*pdh*; A0A1S3NHE5) which are involved in carbon metabolism (sasa01200). The next highest grouping was Neuroactive ligand-receptor interaction (sasa04080) which had 19 target genes, including growth hormone secretagogue receptor 1 (*ghs-r*; A0A1S3MSS9) and gonadotropin subunit beta-2 (*gthb2*; C0HA57). Insulin signalling was also predicted to be impacted with 6 target genes, including phosphatidylinositol 3-kinase regulatory subunit gamma (*pi3k*; A0A1S3MNM5) and RAC-gamma serine/threonine-protein kinase (*akt3*; A0A1S3Q7R0). When the list of targets was run through DAVID to determine functional enrichment, no clusters were found to be significant.

Let-7a is predicted to inhibit 69 target genes associated with metabolic pathways, including isocitrate dehydrogenase [NADP] cytoplasmic (*idh*; A0A1S3NZU3) and citrate synthase (CS; A0A1S3P5N2) which are involved in carbon metabolism (sasa01200). Let-7a had 45 target genes in Neuroactive ligand-receptor interaction (sasa04080), including corticotropin-releasing factor receptor 1 (*crfr*; A0A1S3R6V7) and glucocorticoid receptor (*gr*; A0A1S3SWF6). UDP-glucuronosyltransferase 1-2 (*ugt*; A0A1S3N8T1), involved in steroid biosynthesis (sasa00140), was also a target for let-7a. Insulin signalling was also found to be impacted, with 13 target genes including phosphatidylinositol 3-kinase regulatory subunit beta (*pi3k*; A0A1S3LWS3). When the list of gene targets was run through DAVID to determine functional enrichment, proteins involved in cell differentiation and development (Enrichment

Score 2.1), G-protein coupled receptors (Enrichment Score 1.7), and microtubule organization (Enrichment Score 1.5) were found to be significantly overrepresented.

MiR-143 is predicted to inhibit 28 target genes associated with metabolic pathways, including acetyl-coenzyme A synthetase, cytoplasmic (*acs*; A0A1S3LIB5), pyruvate dehydrogenase E1 component subunit alpha, mitochondrial (*pdh*; A0A1S3NHE5), and hexokinase-1 (*hk1*; A0A1S3SXE7) which are involved in glycolysis/gluconeogenesis (sasa00010). MiR-143 also had 11 target genes were predicted to be involved in Neuroactive ligand-receptor interaction (sasa04080), including corticotropin-releasing factor receptor 1 (*crfr*; A0A1S3R6V7). When the list of gene targets was run through DAVID to determine functional enrichment, proteins involved in transmembrane binding (Enrichment Score 4.4) and G-protein coupled receptors (Enrichment Score 2.9) were found to be significantly overrepresented.

MiR-29a is predicted to inhibit 29 target genes associated with metabolic pathways but none directly involved in carbon metabolism. However, 15 target genes were predicted to be involved in Neuroactive ligand-receptor interaction (sasa04080), including growth hormone prepeptide (Q5SDS1). MiRanda also found 9 target genes were predicted to be involved in insulin signalling, including phosphatidylinositol 3-kinase regulatory subunit beta (*pi3k*; A0A1S3R0Y4). When the list of gene targets were run through DAVID to determine functional enrichment, proteins involved in transmembrane binding (Enrichment Score 2.1) and the inflammatory response (Enrichment Score 1.8) were found to be significantly overrepresented.

2.4 Discussion

This study was aimed at determining the miRNA changes that occur both within and between tissues after exposure to a similar acute handling stressor to regular aquaculture practice or commercial fishing. Rainbow trout are the ideal model organism to use for this study due to their extensively studied stress response and metabolism during such responses (Aluru & Vijayan, 2009; Kostyniuk et al., 2019; López-Patiño et al., 2014; Mommsen et al., 1999).

2.4.1 Confirmation of EV Extraction

The protocol for EV extraction used in this study (ExoQuick) had not been attempted in a teleost species. Therefore, EVs were extracted from plasma samples in each treatment group and visualized using TEM (Fig 2.1). The vesicles extracted were within the size range expected for circulating vesicles as exosomes range in size from 30-100 nm and microvesicles range from 50-1000 nm in diameter in mammals (Raposo & Stoorvogel, 2013; van Niel et al., 2018). Rainbow trout exosomes have been established to be 50 nm in diameter on average and the EVs extracted between 50 and 150 nm in diameter, falling within the range expected (Faught et al., 2017). It was also observed that at the 3- and 24-hour timepoints that vesicles decreased in size and increased in number since these samples were diluted 1:5 compared to the control and 1-hour samples. Few studies have looked at changes in EV size, but it is believed that different subpopulations of EVs can be secreted. In human T-lymphocytes, apoptosis and cell activation altered both the size and proteins associated with EVs (Tucher et al., 2018). However, only one sample was extracted from each group so the differences could be individual variation. Future studies should investigate if cortisol stimulation impacts EV size in a similar fashion to T-cell activation.

2.4.2 Confirmation of Stress axis activation

The stress axis is acutely activated in response to changes in the environment or chronically when environmental changes persist. In either case, the increase in the stress hormone cortisol in circulation is used as an indicator of HPI activation (Mommsen et al., 1999). An acute handling stress was used in this study since its been shown to cause physiological stress and is relevant to both aquaculture practices and wild populations (Barton & Iwama, 1991; Twardek et al., 2018). The fish used were in a state of recovery from stress at the 1- and 3-hour timepoints as circulating levels of the hormone were significantly higher than control (Fig 2.2 A). These circulating cortisol levels agree with literature values, where rainbow trout exposed to an acute handling stressor had cortisol levels around 40 ng/mL at 1- and 3-hours post stress (Barton, 2000). Cortisol has many physiological functions during these recovery periods, but increase in circulating glucose is one of the primary ways to provide energy for highly active tissues (Bonga, 1997; Mommsen et al., 1999).

All groups that had been exposed to handling were found to have significantly elevated plasma glucose levels (Fig 2.2 B), supporting the cortisol findings. These values were comparable to literature values for circulating glucose levels after an acute handling stress (Barton et al., 1987). Plasma glucose levels were still significantly elevated 24-hours after the stressor even though cortisol levels had returned to control values. Rainbow trout have a glucose intolerant phenotype, experiencing hyperglycemia when fed a carbohydrate enriched meal (Enes et al., 2009). Recent studies have attributed this to poor regulation of gluconeogenesis in the liver and poor breakdown of glucose by peripheral tissues (Enes et al., 2009; Forbes et al., 2019; Kirchner et al., 2003; Moon, 2001). Other studies have found that even 24 hours after handling rainbow trout can still have elevated plasma glucose levels, indicating that this is likely due to

the poor clearance of the metabolite by this species (Barton et al., 1987). Therefore, it can be confirmed that the handling stressor was sufficient to induce HPI axis activation in the trout used.

2.4.3 MiRNA abundances in EVs

The miRNAs measured from extracted exosomes were found to be altered after the handling stress in a time dependant manner (Fig 2.3 A-D). MiR-21 increased quickly in circulation after 1-hour before returning to normal levels at 3-hours while miR-143 and miR-29a were found to be elevated at the 3-hour timepoint. This shows that there can be a time dependant effect on the secretion of miRNAs into circulation, similar to what has been found in mammalian models (Hergenreider et al., 2012). Let-7a was significantly depleted at the 24-hr timepoint compared to the other stressed group, indicating that there is a time dependant depletion in circulation for other miRNAs during such recovery periods. Rats exposed to acute stress showed depletions of immune-related miRNAs, indicating that miRNAs are regulated in circulation by stress (Beninson et al., 2014).

Figure 2.4 shows the top KEGG pathways impacted by the miRNAs measured. Since the general trend in circulation was to increase miRNA abundance (Fig 2.3 A-D), lists were combined to compare the increases seen with the decreases observed in tissues (Fig 2.3 E-L). Proteins involved in metabolism make up 25% of the top pathway hits and show the importance of regulating energy during stressful events. Neuroactive ligand-receptor interactions are the next most represented and include hormonal signalling, another important pathway during stress responses and the return to homeostasis. The MAPK signalling pathway is an important regulator of cell differentiation and proliferation (Seger & Krebs, 1995). Increases in circulating levels of these miRNAs could be used as a signalling between tissues to inhibit the pathway at later

timepoints during recovery or could be secreted by tissues to upregulate their own ability to respond to signalling.

MiR-21-3p is a highly abundant miRNA in rainbow trout and the miR-21 family accounts for 33% of all the miRNA expressed in this species, being the most expressed miRNA in the brain, anterior pituitary, anterior kidney, and liver (Juanchich et al., 2016). EV associated miR-21 has been associated with tumor progression, angiogenesis, and cardiac contractility (Liu et al., 2016; Mayourian et al., 2018; Zhou et al., 2018). However, these studies have looked at exosomal trafficking in the context of mammalian diseases that might not be transferable to teleost models. The KEGG analysis can give insights into how these alterations are important physiologically in the current study. MiR-21 is predicted to have two targets associated with carbon metabolism, which undergoes drastic changes during stress recovery based on cortisol signalling. Methylmalonyl-CoA mutase (Mcm) is a mitochondrial enzyme involved in the production of succinyl-CoA from amino acids, odd-chain fatty acids, and cholesterol to feed the tricarboxylic acid (TCA) cycle (Takahashi-Iñiguez et al., 2012). Pyruvate dehydrogenase (Pdh) is another enzyme involved in feeding the TCA cycle by aiding in the conversion of pyruvate into acetyl-CoA (Sharma et al., 2005). These genes would be expected to be inhibited by the increase in miR-21 within tissues, so the EV-associated fraction could be acting as a storage to prevent inhibition of vital genes. However, they could also be shuttled to tissues for inhibition of other genes such as those associated with development and reproduction. Growth hormone secretagogue receptor (Ghs-r) have been shown to be inhibited along with its ligand, ghrelin, by stress to regulate appetite during stress in teleosts (Conde-Sieira et al., 2018; Janzen et al., 2012; Tort, 2013). Gonadotropin subunit beta-2 (Gthb2) is involved in signalling gonads to maintain reproductive function, a process which is inhibited by stress and cortisol (Faught & Vijayan,

2018). Studies in exosomal trafficking have determined that miRNAs can be transported between tissues for intercellular communication (Mittelbrunn et al, 2011). MiR-21 could therefore be transported from tissues that are important for metabolic regulation (i.e. liver) and shuttled to tissues involved in suppressing feeding and reproduction (i.e. brain). MiRanda analysis also displayed targets for miR-21 that were involved insulin signalling. Pi3k and Akt3 are important elements in the signalling cascade that lead to upregulation of insulin-responsive functions (Forbes et al., 2019). Another function of shuttling this miRNA could be to remove any inhibitory effects on key insulin responsive proteins so that plasma glucose can be cleared quickly and efficiently. However, without knowing where these miRNAs are coming from or going to, there is no way to know which functions they could be carrying out in circulation.

Let-7a-5p is part of the highly abundant let-7 family of miRNAs, accounting for 4% of all the miRNAs in rainbow trout and the 2nd most expressed miRNA in the anterior pituitary (Juanchich et al., 2016). The let-7 family of miRNAs have been found to be enriched in exosomes from human cancer cells as they can act as tumor suppressors in tissues (Ohshima et al., 2010). The MiRanda predicted targets for this miRNA offer some explanation for the decrease seen at 24-hrs in circulation. Idh is a cytoplasmic enzyme involved in the TCA cycle, converting isocitrate and $NADP^+$ to 2-ketoglutarate and NADPH (Al-Khallaf, 2017). However, this enzyme can also carry out the reverse reaction to help regulate glycolysis in hypoxic melanoma cells (Filipp et al., 2012). The other carbon metabolism target was citrate synthase, an important enzyme involved in the TCA cyle that produces citrate from oxaloacetate and acetyl-CoA (Lemos et al., 2003). Circulating glucose levels were still elevated at the 24 hour timepoint when the decrease in circulating let-7a was observed. This miRNA could be taken up by tissues to prevent overexpression of these metabolic enzymes once circulating glucose levels return to

resting values. Hormone receptors were also among the targets, which could explain the decrease observed at 24-hours. Crf receptors respond to secretion from the hypothalamus to cause ACTH secretion from the anterior pituitary (Mommsen et al., 1999). Peripheral Crf receptors are proposed to be involved in circulating leukocyte activation and regulation of cardiac output (Pepels et al., 2004). The depression at 24-hours could be peripheral tissues uptaking let-7a containing EVs to inhibit these receptors from being overexpressed. Gr is the primary receptor for cortisol that is used by the hormone to activate transcription of stress responsive elements (Aluru & Vijayan, 2009; Charmandari et al., 2005). However, cortisol upregulates Gr mRNA levels while causing decreases in protein levels in the liver 24 hours after exposure (Aluru & Vijayan, 2007; Sathiyaa & Vijayan, 2003). This regulation of protein levels could be aided by the reabsorption of let-7a up to 24-hours after after stress to reduce the liver's response to cortisol. Another predicted target for let-7a is Ugt, a protein involved in the degredation of steroids (Mackenzie et al., 1992). This could be another sink for the miRNA, as cortisol levels had returned to normal so the liver and other organs might be increasing uptake to inhibit overexpression of the enzyme. Let-7a, like miR-21, was also predicted to target Pi3k and could have an effect on insulin signalling when taken up by tissues. The DAVID analysis of all targets showed an overrepresentation of proteins involved in cell differentiation and development, GPCRs, and microtubule organization. Cortisol causes proliferation of chloride cells in anadromous fish and when combined with insulin can stimulate adipocytes to differentiate (Foskett et al., 1983; Mommsen et al., 1999). Microtubules help to transport proteins around cells, so the decrease observed at 24-hours could be tissues uptaking the miRNA to reverse changes that occurred as a result of the stress and return to homeostasis.

MiR-143-3p is the second most expressed miRNA in the brain and anterior pituitary after miR-21 (Juanchich et al., 2016). Exosomally associated miR-143 is involved in signalling between arterial smooth muscle and endothelial cells during pulmonary arterial hypertenstion (Deng et al., 2015). In rainbow trout, miR-143 has been implicated in the insulin response pathway by inhibiting the phosphorylation of Akt (Mennigen et al., 2012). The other miRNAs measured also had connections to the insulin response, so the upregulation in circulation could be a similar removal from tissues to reduce plasma glucose levels. MiRanda did not predict any insulin response targets, but it did find targets involved in glycolysis/gluconeogenesis. Acs produces acetyl-CoA to support lipid synthesis rather than the TCA cycle (Castro et al., 2012; Ikeda et al., 2001). Chronically stressed rainbow trout have increased lipid synthesis indicating that cortisol can play a role in lipid metabolism (Kostyniuk et al., 2018). Therefore, the circulating miR-143 could be stored in the blood to increase lipid metabolism in tissues. MiR-143 and miR-21 were both predicted to inhibit PDH based on MiRanda. Therefore, it could be a tissue specific secretion of the miRNA accounting for these differences if feeding into the TCA cycle is one of the drivers for the circulating differences. The last glycolytic target for miR-143 was hexokinase-1 which is a high affinity enzyme that converts glucose into glucose-6-phosphate (Enes et al., 2009). Hexokinases are present in most tissues and activity of the enzyme increases 2-hours after a handling stress (López-Patiño et al., 2014). The acute increase in this miRNA in circulation could be allowing peripheral tissues to increase their glycolytic potential during the hyperglycemia measured. MiR-143 was also predicted to inhibit Crf receptor similar to let-7a. The two types of the receptor have different functions in digestion; type 1 (predicted to be inhibited by both let-7a and miR-143) has stimulatory properties in gastric contractions in rats while the type 2 receptor modulates type 1 (Nozu et al., 2013). Both chronic and acute stressors

cause appetite suppression which can have an effect on growth (Barton et al., 1987). The increase at 3-hours of this miRNA could be a return to normal gut function by increasing Crf receptor abundance and allowing digestion to continue. The DAVID analysis revealed that many of the miR-143 targets were GPCRs and proteins involved in transmembrane binding. GPCRs are membrane bound, so the increase in circulating miR-143 could indicate that during recovery from a stress response, tissues are trying to upregulate their cell surface receptors to respond to further signalling. However, this warrents further investigation.

The final miRNA measured was miR-29a-3p which was chosen for its role in regulating DNA methylation in rainbow trout (Kuc et al., 2017). Circulating miR-29a has been shown to activate toll-like receptors to upregulate inflammation to improve metastasis in cancer (Fabbri et al., 2012). None of the predicted targets were classified under carbon metabolism, but Table B.4 shows that many of the metabolic pathways are involved in the breakdown of amino acids. Gluconeogenesis creates glucose for energy from non-carbohydrate sources such as amino acids (Walton & Cowey, 1979). Cortisol causes catabolic activity by breaking down proteins to provide substrates for gluconeogenesis, and part of the return to homeostasis is re-establishing anabolic processes (Mommsen et al., 1999). One explanation for the upregulation of circulating miR-29a at 3-hours could be inhibition of amino acid metabolism later in the stress response. MiR-29a was also found to target growth hormone, an important regulator of anabolic processes in teleost fishes (Gahr et al., 2008). In tilapia, cortisol in combination with growth hormone can inhibit some anabolic peptides while upregulating others (Pierce et al., 2011). This miRNA could be secreted later during the stress response to allow for anabolic activity to return. MiR-29a was also predicted to inhibit Pi3k like miR-21 and let-7a, and miR-143 was found to inhibit phosphorylation of Akt. However, since these miRNAs have different trends in circulation, they

could be working in conjunction to increase insulin responses during hyperglycemia. MiRNAs can act in conjunction to inhibit translation more efficiently (Hashimoto et al., 2013). The changes observed could be a result of the miRNAs sharing the load to increase tissue insulin activity so that other targets for each are not overexpressed. DAVID enrichment analysis also revealed that transmembrane binding was overrepresented in the list of genes. This miRNA could be carrying out a similar role to miR-143. By increasing in circulation later in the stress response, it can signal peripheral tissues to upregulate membrane bound proteins for stress recovery. Inflammation related proteins were also found to be overrepresented and inflammatory reactions are inhibited by cortisol (Mommsen et al., 1999). The increase in circulating miR-29a later in the stress response could be to allow tissues to begin returning to homeostasis by recovering immunological function.

2.4.4 MiRNA abundances in tissues

Although the role of these miRNAs in circulation is speculative, changes in tissue can be easier to asses since the organs collected had specific roles during recovery from stress. The tissues collected for this chapter were important regulators of stress in teleosts. The anterior kidney contains interrenal cells that respond to ACTH and secrete cortisol into circulation (Bonga, 1997; Mommsen et al., 1999). However, this organ is heterogenous, containing mostly hematopoietic and immune cells responsible for antigen presentation and cytokine production (Geven & Klaren, 2017; Uribe et al., 2011). This suggests that the miRNA changes are likely involved in immune regulation rather than steroid production. The liver contains mostly hepatocytes which have multiple functions in maintaining homeostasis within teleosts (Andersen et al., 1991; Gelboin, 1980; Hagey et al., 2010; Sundling et al., 2014). This integration of multiple functions can make it difficult to determine what these miRNA changes are regulating.

During stress responses the liver plays a key role in liberating glucose to supply energy for overcoming the perceived stress (Mommsen et al., 1999). Therefore, the two tissues collected have important roles to play during cortisol stimulation and the miRNA changes observed can be explained in these contexts.

Overall, the tissue abundances of the miRNAs decreased compared to control. Figure 2.4 shows that metabolic targets were predicted to be inhibited by the miRNAs measured. It would make sense that these miRNAs would decrease in abundance in the liver to allow for upregulation of metabolic activities. The time dependant decrease in this tissue would point to the specific targets, with miR-21 and miR-143 being downregulated later in the response and let-7a and miR-29a only being downregulated at 3-hours (Fig 2.3 I-L). The recovery at 24-hours of control abundances of let-7a and miR-29a likely show their importance to the tissue's normal function in the absence of cortisol. MiR-21 and miR-143 are likely important for other metabolic roles, such as reducing the circulating glucose levels. In the anterior kidney, miR-21 and let-7a were unimpacted by stress (Fig 2.3 E-H). However, miR-143 and miR-29a were almost completely depleted in the tissue and remained low after 24 hours. Acute stress has been shown to enhance antigen presentation and other immune functions, so the sustained decreases could be facilitating the anterior kidney's primary function (Dhabhar & McEwen, 1996; Dhabhar et al., 2010; Dhabhar & Viswanathan, 2005; Pickford et al., 1971). Genes involved in focal adhesion (10%), actin cytoskeleton (8%), and endocytosis (7%) could all be upregulated in this organ to enhance antigen presentation of macrophages.

Relative abundance of miR-21 in the anterior kidney were unaffected at any timepoint after stress (Fig 2.3 E). MiR-21 has important roles in regulating macrophage polarization, so maintaining constant levels in this immune organ might be necessary to maintain proper function

(Wang et al., 2015). Pi3k/Akt are also important regulators of cytokine and pathogen associated molecular patterns (Vergadi et al., 2017). Maintenance of inhibitor miRNAs (as miR-21 is predicted to do) is vital to prevent unnecessary immune activation. However, in the liver, miR-21 levels decrease at the 24-hour timepoint (Fig 2.3 I). Although this could be to enhance metabolic function, a more likely explanation is to remove inhibition on the insulin response pathway through Pi3k/Akt. On top of being important signalling proteins in the immune response, they are also important regulators of insulin signal transduction, so downregulation of regulatory miRNA could help metabolize glucose and inhibit gluconeogenic activity. This is supported by studies with mice, where miR-21 knockout hepatocytes were more responsive to insulin (Calo et al., 2016).

The anterior kidney had no change in let-7a levels after stress similarly to miR-21 (Fig 2.3 F). This miRNA in humans is involved in macrophage activation and interleukin inhibition, so maintaining its levels in the anterior kidney is important to maintain homeostasis when pathogens are not present (Iliopoulos et al., 2009; Mazumder et al., 2013). Pi3k was a predicted target of this miRNA, and as mentioned above, is an important regulator of immune function. Therefore, limiting changes in let-7a expression will prevent dysregulation of key signalling pathways. In the liver an acute decrease in let-7a was measured at 3-hours post stress. Since this is primarily a metabolic organ, increases in Pi3k at this timepoint would contribute to increase the potential for the liver to respond to insulin. This acute decrease in let-7a could also allow upregulation of Idh and citrate synthase to aid in the clearing of glucose. Decreased citrate synthase activity is associated with glucose intolerance, so by upregulating this enzyme hepatocytes could be increasing glycolytic potential (Alhindi et al., 2019). Ugt could also be upregulated to aid in the degradation of cortisol since the liver is important for steroid

metabolism (Mackenzie et al., 1992). The recovery of control let-7a levels could aid in preventing overexpression of this enzyme and this recovery could be in part due to uptake of circulating let-7a.

MiR-143 was found to significantly decrease at all timepoints post stress and almost be completely depleted at 3- and 24-hours after stress axis activation in both tissues. The most likely reason for this decrease is removal of hexokinase-1 inhibition. Hexokinase-1 is highly expressed in the mammalian and teleost kidney and is important for glycolysis (Enes et al., 2009; Soengas et al., 2006). Since glucose was still elevated at all timepoints post-stress, hexokinase upregulation could aid in clearing the metabolite. Another cell type in the teleost anterior kidney are chromaffin cells which are important for catecholamine secretion (Mommsen et al., 1999). These cells contain Crf receptor which is important for paracrine effects between the cell types (Huising et al., 2007). The receptor could be upregulated by the decrease in miR-143 and allow for increased responsivity during the recovery period. MiR-143 also saw significant decreases in the liver later in the stress response and remained low even after 24-hours. Hepatic miR-143 in rainbow trout is predicted to inhibit Akt phosphorylation by downregulation Orp8 which responds to insulin (Jordan et al., 2011; Mennigen et al., 2012). This could partly explain the drastic decrease seen in the liver of this miRNA as this would help support the insulin signalling cascade. The other metabolic targets for this miRNA could also be benefited by this decrease. Increase in hexokinase expression can increase glycolytic potential of the liver. Acs feeds into the anabolic lipid synthesis from glycolysis, so upregulation of this enzyme can aid in the clearing of plasma glucose (Castro et al., 2012).

MiR-29a had a similar trend to miR-143 in the kidney, where 3- and 24-hours were significantly depleted (Fig 2.3 H). The likely cause of this decrease in the anterior kidney is to

enhance immune function as previously discussed. Acute stressors have been shown to enhance antigen presentation, a key role for macrophages (Dhabhar & McEwen, 1996). The KEGG results for this miRNA (Table B.4) show cytokine-cytokine receptor interactions as highly expressed in the targets. Cytokine levels in the anterior kidney were inhibited 2-hours after cortisol exposure in gilthead seabream, but in rainbow trout cortisol enhanced inflammation-related cytokine action of macrophages after 6-hours (Castillo et al., 2009; Castro et al., 2011). The sustained decrease could aid in enhancing immune function during recovery from stressors. The hepatic levels of miR-29a were decreased at the 3-hour timepoint and returned to normal after 24-hours, like what was measured for let-7a (Fig 2.3 L). This decrease may be attributed to the increase in insulin response through Pi3k upregulation to increase glycolytic activities. MiR-29a inhibition of Pi3k has already been shown in mice, and overexpression of this miRNA inhibits insulin signalling (Pandey et al., 2011). However, since it also influences DNA methylation, maintaining its levels within tissues is important to return to homeostasis. Previous work has shown that this miRNA is a regulator of DNA methylation by targeting DNA methyltransferase 3a (Dnmt3a; Kuc et al., 2017). This could cause an upregulation of DNA methylation, acting to silence gene transcription. Rainbow trout gluconeogenic genes have DNA methylation sites, so by increasing Dnmt3a levels these gluconeogenic transcripts could be downregulated later in the stress response (Marandel et al., 2016). No studies have explored the effect of cortisol on DNA methylation in the teleost liver, but this could be another mechanism of reducing gluconeogenesis during stress recovery.

2.4.5 Conclusions

In conclusion, there are clear time-dependant changes in miRNA levels during recovery from acute stress. In plasma EVs, both increases and decreases in circulating levels of these

miRNAs were measured. Increases were reverted after cortisol levels had returned to normal, linking circulating miRNA levels with the hormone. MiRNA tissue abundances either remained constant or decreased during the recovery period; however, not all miRNA abundances were recovered after cortisol had returned to normal. Drosha, an important enzyme in miRNA biogenesis, has previously been shown to be altered by chronic stress in rainbow trout (Kostyniuk et al., 2018). This decrease in the key miRNA-regulating enzyme could explain the overall trend but whether Drosha is regulated after acute cortisol exposures is unknown and should be investigated in the future. KEGG analysis of the targets for these miRNAs revealed that metabolic pathways are likely impacted by the miRNA abundance changes. This agrees with the literature where metabolic reprogramming is a primary occurrence during the recovery period from stress. However, determining direct relationships for the miRNA changes based on KEGG can be difficult due to the high number of pathways that are predicted to be impacted. Even if no direct relationships can be confirmed, the changes measured are physiologically relevant and provides evidence for the epigenetic regulation that can occur during stress recovery.

Chapter 3

In vitro regulation of microRNA through cortisol stimulation in rainbow trout (_Oncorhynchus mykiss_) hepatocytes

3.1 Introduction

The liver is a multifunctioning organ that is vital to teleost physiology and studying the liver is vital to expanding the knowledge on how fish adapt to their environment. Hepatocytes produce bile and store the solution in the attached gall bladder to aid in the digestion of proteins and lipids (Hagey et al., 2010; Segner, 1998). The liver is also the primary site of clearing for xenobiotic compounds such as pharmaceuticals and other foreign substances (Gelboin, 1980; Kuc et al., 2017; Tapper et al., 2018). In breeding females, hepatocytes produce vitellogenin which is required for healthy egg formation (Sundling et al., 2014). The liver also carries out important roles in regulating metabolism, acting both as a producer and storage of energy, and has essential roles in glycogen storage and breakdown following stressful events (Andersen et al., 1991; Mommsen et al., 1999; Vijayan et al., 1991), which is a focus of this chapter.

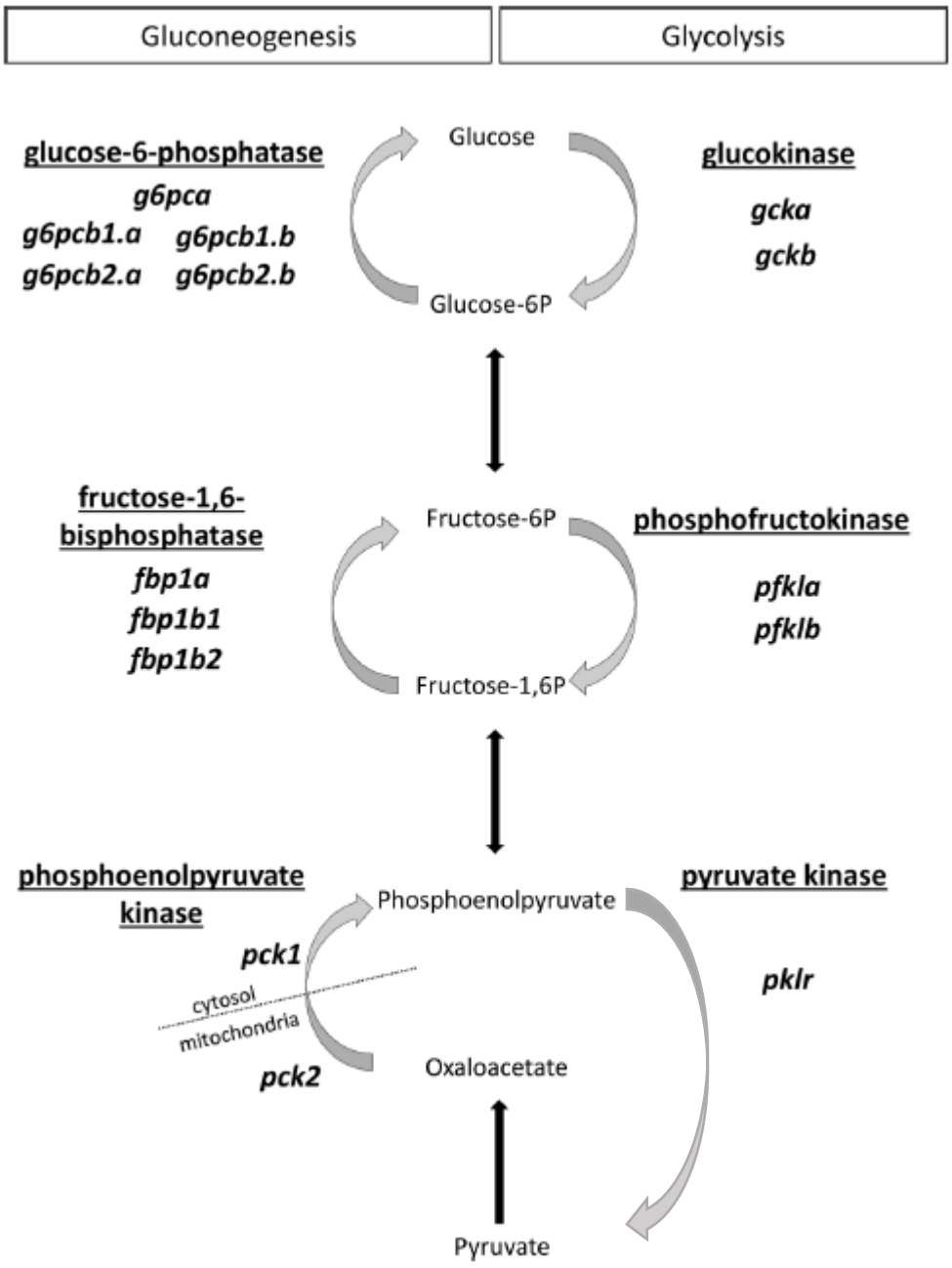


Figure 3.1: Important glucose regulating enzymes in the gluconeogenic (production) and glycolytic (breakdown) pathways. The transcript names below each enzyme are the paralogs in rainbow trout that are differentially expressed. This figure was adapted from Marandel et al., (2016).

Glycolysis is the breakdown of glucose into pyruvate, which can be used by other metabolic pathways such as the Krebs cycle during aerobic respiration (Fig 3.1; Li et al., 2015; Polakof et al., 2012). The first enzyme involved in this pathway is hexokinase (Hk) which converts glucose into glucose-6-phosphate (Enes et al., 2009; Panserat et al., 2001; Polakof et al., 2012). This is the rate limiting step of glycolysis and both activity of the enzyme and expression can be induced by increases in glucose levels (Enes et al., 2009; Panserat et al., 2001; Polakof et al., 2012). Hks have 4 different isozymes that are expressed in different tissues and have different affinities for glucose. Hk 1-3 have high affinities for glucose and are expressed in most tissues within teleosts to aid in the breakdown of circulating glucose for energy (Enes et al., 2009). Hk 4 (also called glucokinase; Gk) is mainly present in the liver and has a lower affinity for glucose but its activity is unimpacted by higher levels of glucose-6-phosphate (Enes et al., 2009; Printz et al., 1993). The glucose-6-phosphate produced by this enzyme can then be further metabolized, eventually being converted into pyruvate by pyruvate kinase (Pk; Enes et al., 2009). Pk activity is regulated by changes in carbohydrate availability in rainbow trout and has lower activity in the liver when compared to other tissues such as muscle and brain (Knox et al., 1980; Panserat et al., 2001). In rainbow trout, Pk has one gene that encodes the liver and erythrocyte version of the enzyme, *pklr* (Enes et al., 2009; Marandel et al., 2016).

Unlike glycolysis which is active in most tissues, the primary site for gluconeogenesis is the liver (Knox et al., 1980). The rate-limiting enzymes for gluconeogenesis act in direct opposition to glycolysis (Fig 3.1; Marandel et al., 2016). This pathway uses non-carbohydrates such as amino acids, lactate, and glycerol to produce glucose that can be used by energy intensive tissues (Walton & Cowey, 1979). Fructose-1,6-bisphosphatase (Fbpase) acts in opposition to phosphofructokinase to convert fructose-1,6-bisphosphate into fructose-6-

phosphate (Enes et al., 2009; Suarez & Mommsen, 1986). Fbpase is primarily active in the liver but has been found in other tissues such as the kidney and muscle (Enes et al., 2009; Knox et al., 1980). Multiple studies have examined the regulation of Fbpase and found in dietary protein and starvation can alter both gene expression and enzymatic activity (Enes et al., 2009; Kirchner et al., 2003; Marandel et al., 2015; Morata et al., 1982; Suarez & Mommsen, 1986). Three genes encode rainbow trout Fbpase (*fbp1a*, *fbp1b1*, and *fbp1b2*) and are differentially regulated by changes in diet and stages of development (Marandel et al., 2016; Song et al., 2018) However, most of the studies on gluconeogenesis investigate phosphoenolpyruvate carboxykinase (Pepck) and its regulation under different treatments. Pepck acts opposite to Pk, converting oxaloacetate to phosphoenolpyruvate which can then feed into the other gluconeogenic enzymes (Knox et al., 1980; Suarez & Mommsen, 1986). Pepck has a mitochondrial and cytosolic form while the other gluconeogenic enzymes are all cytosolic (Enes et al., 2009; Marandel et al., 2019; Marandel et al., 2015; Suarez & Mommsen, 1986). In rainbow trout, the cytosolic (*pepck1*) and mitochondrial (*pepck2a* and *pepck2b*) forms are encoded by different genes and are transcriptionally regulated by treatments such as cortisol and nutritional changes (Enes et al., 2009; Marandel et al., 2019).

Rainbow trout metabolism has been extensively studied from the perspective of nutritional content due to the glucose intolerant phenotype experienced by the species (Moon, 2001). Early studies investigated the relationship between glycolytic and gluconeogenic activities to try and explain the hyperglycemia present after carbohydrate meals (Panserat et al., 2001; Panserat et al., 2001; Polakof et al., 2012; Suarez & Mommsen, 1986). Overall, it was determined that the likely cause of reduced glucose clearance was due to poor peripheral breakdown of the metabolite (Moon, 2001). This was supplemented by more recent findings that show lack of regulation of gluconeogenic enzymes by changes in diet that contribute to the

excess glucose in circulation (Enes et al., 2009; Kirchner et al., 2003; Panserat et al., 2001; Panserat et al., 2001). With the recent sequencing of the rainbow trout genome, multiple gene paralogs of rainbow trout gluconeogenic enzymes show how complicated the regulation of this pathway has become, which is attributed to subsequent genome duplications events in the teleost lineage (Marandel et al., 2016; Marandel et al., 2019; Marandel et al., 2017, 2015; Marandel et al., 2016). Therefore, it is necessary that future studies that look to understand glucose regulation in rainbow trout explore the differential regulation of these gene paralogs.

Cortisol, the primary glucocorticoid, is responsible for regulating glucose levels as a way of supporting recovery from a stressor (Bamberger et al., 1996; Mommsen et al., 1999). This hormone acts through the glucocorticoid receptor to upregulate genes important for the stress response (Aluru & Vijayan, 2009; Charmandari et al., 2005). In addition to impacting growth and reproductive pathways, stress and cortisol have been shown to impact glycolysis and gluconeogenesis. *Gk* gene expression increases 24-hours after cortisol exposure in rainbow trout hepatocytes while also increasing in activity 2-hours after a handling stressor (López-Patiño et al., 2014; Wiseman et al., 2007). *Pk* expression has been shown to be upregulated 1 hour after stress in rainbow trout and Pk activity increased after cortisol exposure in tilapia livers while being unimpacted by handling in rainbow trout (Mommsen et al., 1999; Morales et al., 1990; Vijayan et al., 1997; Wiseman et al., 2007). High levels of circulating cortisol significantly elevated Fbpase activity in carp within 24-hours while rainbow trout exposed to a handling stress had Fbpase increase in activity within 6 hours (Dziewulska-Szwajkowska et al., 2003; Morales et al., 1990). *Pepck* has a well established glucocorticoid responsive element upstream of the gene, making it a good indicator of cortisol activation (Heinrichs et al., 1994; Mommsen et al., 1999). In rainbow trout, *pepck* expression is upregulated 24-hours after an acute handling stress and at

the same timepoint in hepatocytes exposed to the hormone (Aluru & Vijayan, 2007; Sathiyaa & Vijayan, 2003; Wiseman et al., 2007). However, activity of the enzyme was decreased at the same timepoint, potentially linking this enzyme to other forms of regulation post-transcriptionally (Sathiyaa & Vijayan, 2003).

MicroRNA (miRNA), a post-transcriptional regulator of gene expression, have been investigated for their role in metabolic regulation. Multiple miRNAs in rats have been found to regulate *Gk* expression and the potential to target these miRNAs for treatment of diabetes is a possibility (Mirra et al., 2018). Most studies investigate these effects in mammals but evolutionary conservation of these relationships can be quite low, with less than 10% of predicted binding sites being conserved between humans and teleosts (Xu et al., 2013). Therefore, recent studies have begun to investigate miRNA regulation of teleost metabolism to explain physiological differences between species. Rainbow trout have been one of the models for these miRNA studies due to their well studied metabolic physiology. MiR-122 has been found to be altered by developmental stage and feeding within the liver to aid in increasing liver function at key steps (Mennigen et al., 2013; Mennigen et al., 2012). Chronic social stress in rainbow trout has been shown to impact miRNAs associated with lipid and carbohydrate metabolism, linking the effects of cortisol on development and growth with epigenetic changes (Kostyniuk et al., 2018, 2019).

MiRNAs are stable in the extracellular environment by association with vesicles or proteins and have been found in biological fluids such as blood, urine, and saliva (Arroyo et al., 2011; Caby et al., 2005; Kim et al., 2007; Ogawa et al., 2011; Pisitkun et al., 2004; Turchinovich et al., 2011; Vickers et al., 2011). Vesicle-enclosed miRNAs have been shown to be biologically active paracrine factors by influencing distant tissues within organisms (Pegtel et al., 2010;

Raposo & Stoorvogel, 2013; van Niel et al., 2018). However, most of the miRNA in circulation are likely associated with Ago2, a protein complex important for repression of mRNA expression (Arroyo et al., 2011; Turchinovich et al., 2011). Currently, whether these RISC-associated miRNAs are biologically active or just stable in circulation is in question since these Ago2-associated miRNAs remained constant for two months at room temperature (Turchinovich et al., 2011). In humans, circulating levels of miR-122 has been correlated with obesity and insulin resistance (Wang et al., 2015). Zhu *et al.,* (2018) has demonstrated correlations between circulating miRNAs and metabolic changes in trout, indicating that these circulating miRNAs could be biomarkers of fish health. These changes in circulating miRNAs could be biological indicators of metabolism, but whether the liver is contributing to the alterations and actively signalling other tissues is currently unknown.

Studying the changes in miRNA in hepatocytes after cortisol exposure can help expand our knowledge on the metabolic reprogramming that occurs during recovery from stress. It is hypothesized that miRNAs important to gluconeogenesis and glycolysis will be altered within hepatocytes after this exposure and reflected in the circulation as a way of communicating between cells. The miRNAs chosen for this study were predicted to bind to the 3' UTRs for *gk* and *pk* in glycolysis and *fbpase* and *pepck* in gluconeogenesis based on the MiRanda targeting algorithm. This is the first study to connect changes in enzyme activity with the miRNAs that target them in tissue and circulation.

3.2 Materials and methods

3.2.1 Study Animals

Rainbow trout (*Oncorhynchus mykiss*) of mixed sex were purchased from Silver Creek Aquaculture Inc in Erin, ON. Trout were held in 450-gallon flow through tanks at a density of ~35 fish per tank. During the study, a 12-hour light-dark cycle was maintained and temperature, dissolved oxygen, pH, and conductivity monitored. Water quality parameters were constant over the course of the study. Experimental procedures and the use of animals in this study was approved by the University of Waterloo Animal Care Committee and within CCAC guidelines (AUPP 40315).

3.2.2 Hepatocyte collection and culturing

Unless otherwise indicated, all chemicals were purchased through Sigma-Aldrich (Mississauga, ON). Three fish (two male and one female) were collected from the holding tank and immediately euthanized in 0.5 g/L bicarbonate buffered MS-222. The protocol for hepatocyte isolation follows the procedure used by Craig et al. (2013) and recipes for buffers can be found in Appendix A. Fish were opened mid-ventrally to expose the digestive system and the hepatic portal vessel cannulated with a Gilson minipuls 3 peristaltic pump (Mandel). Initially, the pump was operating slowly by perfusing rinsing solution (Basic Hank's, 1 mM EGTA, pH 7.63) through the liver and speed was increased to 2 mL/min. During this time the bulbous arteriosus of the heart was cut to prevent back-pressure and excess solution pooling in the body cavity removed with a plastic pipette. The liver was monitored and massaged until all traces of blood were removed before transferring pump to perfusing collagenase media (50 mL Basic Hank's, 7.5 mg collagenase type IV, pH 7.63) at a rate of 2 mL/min. The collagenase perfusion was monitored for 12 mins with periodic massaging of the liver to aid in even distribution of

perfusate throughout the tissue. After 12 mins the liver became soft and expanded and the pump was turned off. The liver was carefully removed from the peritoneal cavity and the gall bladder carefully cut away to prevent contamination of the tissue. Liver tissue was then diced over rinsing solution and passed through 250 mm and 75 mm mesh screens to separate cells. This suspension of hepatocytes was centrifuged for 2 mins at 800 rpm and 4°C and the supernatant decanted to remove excess rinsing solution. Hepatocytes were rinsed three more times, once with half volume of rinsing solution and half volume of resuspension media (Basic Hank's, 3 mM $CaCl_2$, 1.5% BSA) and twice with only resuspension media before sitting on ice for 1 hour. Cells were then rinsed twice more with Hanks' Culture Media and then cell viabilities determined using a Trypan Blue exclusion assay. Viabilities were all >95%, and therefore hepatocytes were plated in sterilized 48-well tissue culture plates (VWR, Cat# 10861-702) at 10 mg per well (stock solution concentration 25 mg/mL). Cells were monitored each day for loss of attachment and media changed after 24 hours in culture.

3.2.3 Hepatocyte Cortisol Exposure

After 48 hours in culture, cells were exposed to increasing concentrations of cortisol, following a similar protocol to Pierce et al. (2012). Hydrocortisone (Sigma-Aldrich, Cat# H0888) was dissolved in Hanks' culture media supplemented with 0.1% ethanol at concentrations of 3000 ng/mL, 300 ng/mL, 30 ng/mL, or 0 ng/mL (control). Cells were exposed for either 2- or 4-hours and fractions of supernatant and cells were taken to determine miRNA differences between the two. Wells were pooled together to get three samples per concentration at each timepoint. Hepatocyte yields were lower for the second fish, resulting in only 3 wells pooled per sample while the first and third had 4 wells per sample. Supernatant fractions were taken first by aliquoting 300 µL of the cortisol or control media while cell fractions were taken

by washing wells multiple times with the remaining 100 µL of media to remove adhered hepatocytes and combined into pre-weighed tubes. Cells were then centrifuged at 12 000 x *g* for 2 mins to remove excess media before being flash frozen in liquid nitrogen. Supernatant was differentially centrifuged to remove any cellular material from circulating miRNA based on a protocol from personal communication with FroggaBio and Kenigsberg, (2016). Cellular components were removed by centrifuging twice at 4°C, 2500 x *g* for 15 mins. Media supernatant was then centrifuged at 300 x *g* for 10 mins, 2000 x *g* for 10 mins, and lastly 12 000 x *g* for 30 mins to remove other unwanted components. This final fraction was passed through a 0.22 µm filter to clear out larger vesicles and cellular debris that might have remained after centrifugation steps. All samples were subsequently stored at -80°C until analysis.

3.2.4 RNA Extractions

Supernatant RNA was extracted using a commercially available Qiagen miRNeasy Serum/Plasma Advanced Kit (Cat# 217204) and followed without deviation. Cell RNA was extracted using the Qiagen miRNeasy Mini Kit (Cat#: 217004). Pellets weights were weighed, and all fell within the recommended tissue weights for the kit. The extraction protocol was followed based on the manufacturer's guidelines without deviation. RNase-free water (30 µL) was used to elute RNA and all samples stored at -80°C until subsequent analysis.

3.2.5 RT-qPCR

Extracted samples were thawed on ice and RNA concentration determined using a SpectraDrop Micro-Volume Microplate (Molecular Devices). Either 100 ng of supernatant RNA or 500 ng of cellular RNA was used for cDNA synthesis. Qiagen's miScript II RT kit (Cat# 218161) was used since it was able to generate miRNA specific cDNA. The manufacturer's protocol was followed without deviation. HiSpec buffer was used for supernatant samples while

the HiFlex buffer was used for tissue samples since both can be used to make cDNA for mature miRNA but the HiFlex buffer is more ideal for reverse transcribing mRNA. A pooled sample was also reverse transcribed from each tissue in order to generate standard curves.

Standard curves were run for each target in the supernatant and cell fractions. Dilutions within these curves were 4x, 16x, 64x, 256x, and 1024x and standard curves for each primer was found to be linear after the 16x dilution. Therefore, all samples were diluted 20x to reduce qPCR inhibition. Each qPCR reaction contained 2 µL of diluted cDNA (0.5 ng of supernatant cDNA or 2.5 ng of cell cDNA), 5 µL of Biorad SYBR Green Master Mix (Cat# 1725272), 1 µL of 5 µM forward primer for the target miRNA or mRNA (Sigma-Aldritch), 1 µL of 5 µM Universal Primer for miRNAs (Qiagen, Cat# 218073) or 5 µM reverse primer for mRNA, and 1 µL of water. Samples were run on CFX96 Touch Real-Time PCR Detection System (Biorad). Each run consisted of 30 seconds at 95°C initially, followed by 45 repeated cycles of 10 seconds at 95°C and 15 seconds at 60°C. All runs finished off with a melt curve to make sure that only one qPCR product was generated during the run. Primer sequences and amplicon sizes can be found in Table 3.1. Primers for mRNA targets were designed using NCBI's Primer Blast tool. MiRNAs were found to be the top targets based on the total score for these mRNAs based on the MiRanda algorithm using standard parameters (Enright et al., 2003). Relative abundances were calculated by normalizing to the housekeeping gene 18s rRNA using the standard $2^{-\Delta\Delta Ct}$ equation (Livak & Schmittgen, 2001). Samples were further normalized to the 2-hour control of the same fish to determine relative abundance change due to high variation between fish.

Table 3.1: Primer sequences for targets of hepatocyte cortisol exposure study. Forward sequences for miRNAs were acquired from miRbase while reverse primers were proprietary Qiagen sequences (cite for miRbase). Amplicon length was unable to be determined for these sequences due to the proprietary nature of the Qiagen reverse transcription kit. Primers for mRNA sequences were designed using the Primer-BLAST tool (Ye et al., 2012).

Target	Forward Primer Sequence	Reverse Primer sequence	Reference number	Amplicon length
Fructose-1,6-Bisphosphotase	CTGTACGAGTGCAACCCCAT	GCTGTCTCACATGCGTGTCT	XM_021603006.1	186
Phosphoenolpyruvate carboxykinase	TATGAGAACTGCTGGCTGGC	ACCTACTCGTGGAGACGGAA	XM_021568440.1	114
Glucokinase	CTGACGCCTCACTGTGACAT	TAGGTGGGAGGCTGTTACGA	NM_001124249.2	137
Pyruvate Kinase	GGTGACATGGTGATCGTGGT	GAGTGTTTAGGGCACGTGGA	XM_021622264.1	87
ssa-miR-21a-5p	TAGCTTATCAGACTGGTGTTGACT	Qiagen Universal Primer	MIMAT0032533	-
ssa-miR-194a-3p	CCAGTGGAGATGCTGTTACCTGC	Qiagen Universal Primer	MIMAT0032448	-
ssa-miR-33a-5p	GTGCATTGTAGTTGCATTGCA	Qiagen Universal Primer	MIMAT0032618	-
ssa-miR-192a-3p	CCTGTCAGTTATGTAGGCCACT	Qiagen Universal Primer	MIMAT0032441	-
18s rRNA	ATGGCCGTTCTTAGTTGGTG	CTCAATCTCGTGTGGCTGAA	FJ710874.1	145

3.2.6 Enzymatic Activity

Buffers for spectrophotometric enzymatic assays can be found in Appendix A. Hepatocyte pellets were weighed and sonicated in a 20x dilution of extraction buffer using sonic dismembrator model 100 (Fisher). Sonicated hepatocytes were then centrifuged for 10 mins at 12 000 x *g* at 4°C and the supernatant used for enzymatic assays. Activities were measured using 96-well microplates using a SpectraMax 190 Microplate Reader by measuring the change in NAD(P) and NAD(P)H at 340 nm over 30 mins at room temperature. Phosphoenolpyruvate carboxykinase (Pepck, EC 4.1.1.49) was measured in fresh samples since only the cytosolic version of the enzyme was of interest while fructose-1,6-bisphosphatase (Fbpase, EC 3.1.3.11), glucokinase (Gk, EC 2.7.1.2), and pyruvate kinase (Pk, EC 2.7.1.40) were measured after being stored at -80°C. Fbpase activity was measured as per Tranulis et al., (1996). Lactate dehydrogenase (LDH, EC 1.1.1.27) activity was measured in the supernatant to determine hepatocyte viability. Enzyme activities were normalized to the protein concentration of the

sample measured using a Bicinchoninic acid assay (Sigma-Aldritch) with a 6-point bovine serum albumin standard curve. Activities of hepatocyte cell enzymes were further normalized to the 2-hour control of the same fish to determine relative abundance change due to high variation between fish.

3.2.6 Statistical Analysis

Data was analyzed using GraphPad Prism (GraphPad Software, La Jolla CA) and presented as the average ± standard error of the mean (SEM). Significant differences between relative abundances and enzymatic activities were found using two-way analysis of variance tests (ANOVA, $p<0.05$). Normality was assumed and when significant differences were found, Dunnett's Post-Hoc Multiple Comparison Test was used to determine if the cortisol treated hepatocytes differed from their respective time-point control. For LDH activity, Tukey's Post-Hoc Multiple Comparison Test was used to determine differences between fish and timepoints.

3.3 Results

3.3.1 LDH leakage assay

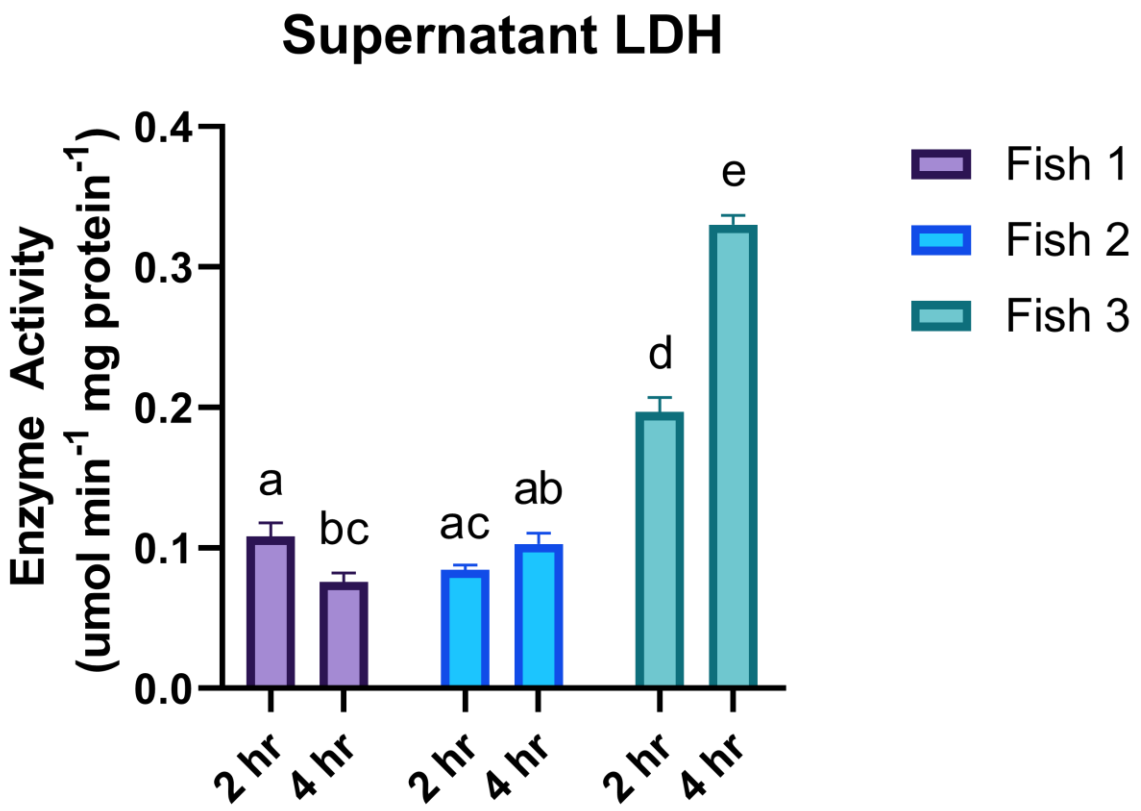


Figure 3.2: LDH enzymatic activity in the supernatant of hepatocytes exposed to cortisol. Activity was measured after 2- or 4-hours of exposure using a spectrophotometric enzymatic assay. Bars that do not share a common letter within the same timepoint were found to be significantly different (Two-way ANOVA, $p < 0.05$ Tukey's Post Hoc, $n = 3$).

Activity of LDH was measured in supernatant samples to assess the level of cell leakage during the exposure. Fish 1 had significantly lower supernatant activity at 4-hours compared to

the 2-hour timepoint (Tukey, p = 0.0478). However, fish 1 and 2 had no significant difference in their LDH activity. Fish 3 not only had significantly elevated LDH activity compared to the other two fish (Tukey, p = <0.0001), but it's 4-hour timepoint had significantly elevated levels of the enzyme compared to it's 2-hour timepoint (Tukey, p = <0.0001).

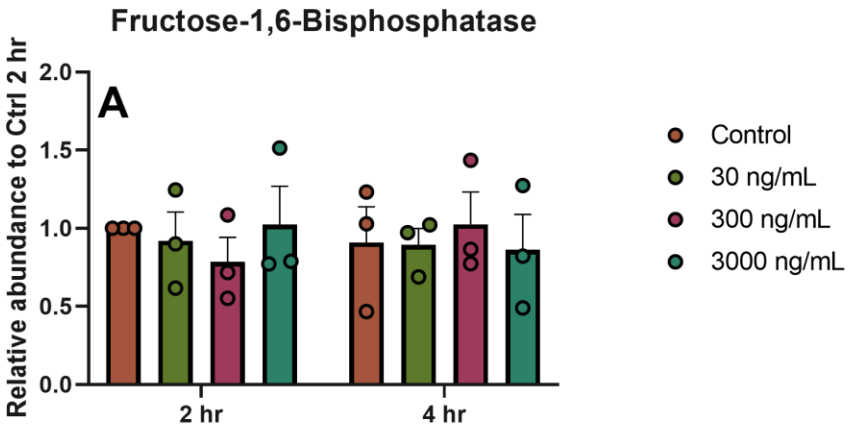
3.3.2 MiRanda miRNA binding prediction

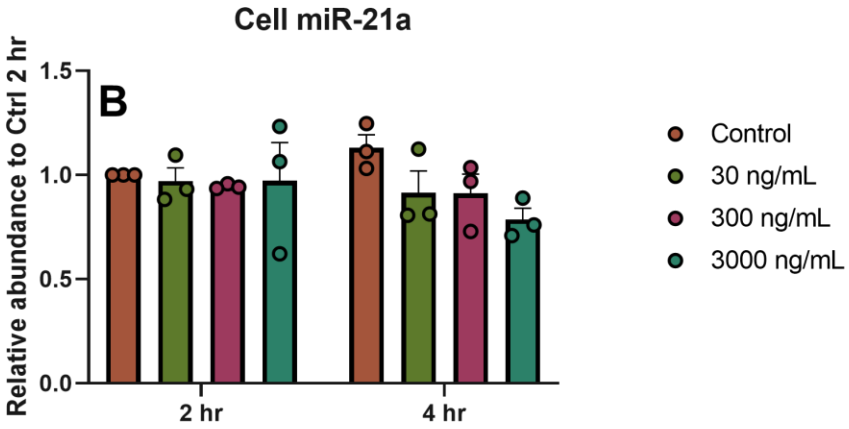
MiRNA predicted to target the enzymes of interest in this chapter were found by using the standard parameters for the MiRanda algorithm (Enright et al., 2003). A cut-off of 190 for the pairing score and free energy score of -40 was used by Mennigen & Zhang (2016) but when applied to the 3' UTRs of interest to this chapter returned no results. Therefore, no cut-off score was used and the top target for each mRNA based on the highest total pairing score was chosen as the most likely miRNA to influence expression. A summary of the MiRanda output can be found in Table 3.2 below.

Table 3.2: Summary of the MiRanda output for miRNA predicted to target key metabolic enzymes. Fructose-1,6-bisphosphatase (*Fbp1b2*) and phosphoenolpyruvate carboxykinase (*pepck1*) are key gluconeogenic enzymes while glucokinase and pyruvate kinase are important glycolytic enzymes. *Salmo salar* miRNAs were used since the sequences were readily available on miRbase. The score generated by MiRanda relates to the likelihood of binding and influencing expression with a higher score being more likely. A lower free energy score is more advantageous for miRNA binding since this relates to the duplex structure formed when a miRNA interacts with its mRNA.

Target	miRNA Name	Score	Free energy (kCal/Mol)
Fructose-1,6-bisphosphatase	ssa-miR-21a-5p	155	-13.53
Phosphoenolpyruvate carboxykinase	ssa-miR-194a-3p	301	-37.89
Glucokinase	ssa-miR-33a-5p	449	-45.13
Pyruvate Kinase	ssa-miR-192a-3p	160	-22.72

The top binding site for a miRNA within the 3’ UTR of fructose-1,6-bisphosphatase (*fbp1b2*; XM_021603006.1) was for ssa-miR-21a -5p (MIMAT0032533) with a pairing score of 155 and free energy score of -13.53 kCal/Mol. When the cytosolic form of the enzyme phosphoenolpyruvate carboxykinase (*pepck1*; XM_021568440.1) was ran through the algorithm, two binding sites for ssa-miR-194a-3p (MIMAT0032448) were found in the 3’ UTR that combined gave a total binding score of 301 and a free energy score of -37.89 kCal/Mol. The glucokinase (*gcka*; NM_001124249.2) 3’ UTR had three predicted ssa-miR-33a-5p (MIMAT0032620) binding sites that gave total binding score of 449 and free energy of -45.13 kCal/Mol. Finally, the pyruvate kinase (*pklr*; XM_021622264) 3’ UTR was found to have one binding site for ssa-miR-192a-3p (MIMAT0032441) that had a binding score of 160 and a free energy score of -22.72 kCal/Mol.


Fructose-1,6-Bisphosphatase
A
Relative abundance to Ctrl 2 hr
2.0
1.5
1.0
0.5
0.0
2 hr
4 hr
Control
30 ng/mL
300 ng/mL
3000 ng/mL


Cell miR-21a
B
Relative abundance to Ctrl 2 hr
1.5
1.0
0.5
0.0
2 hr
4 hr
Control
30 ng/mL
300 ng/mL
3000 ng/mL

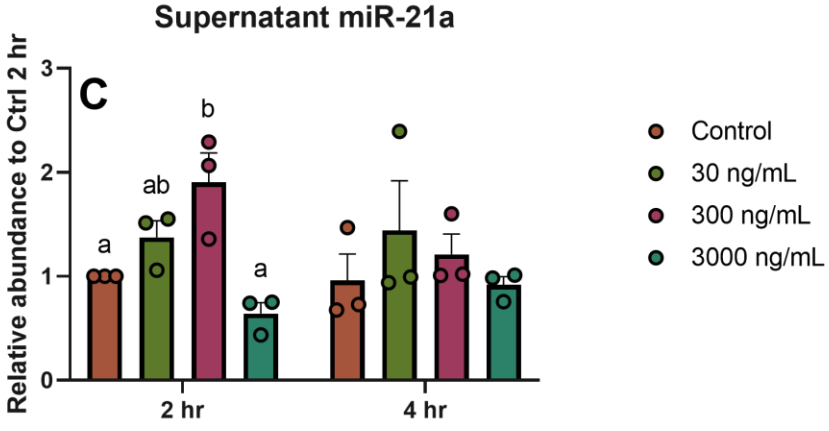
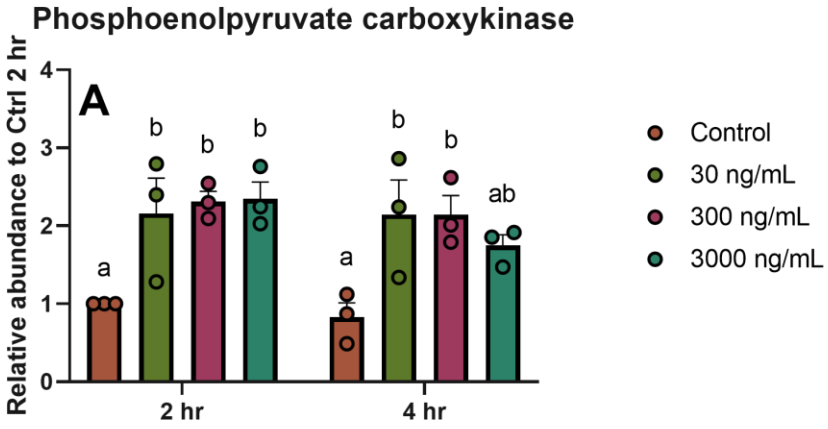
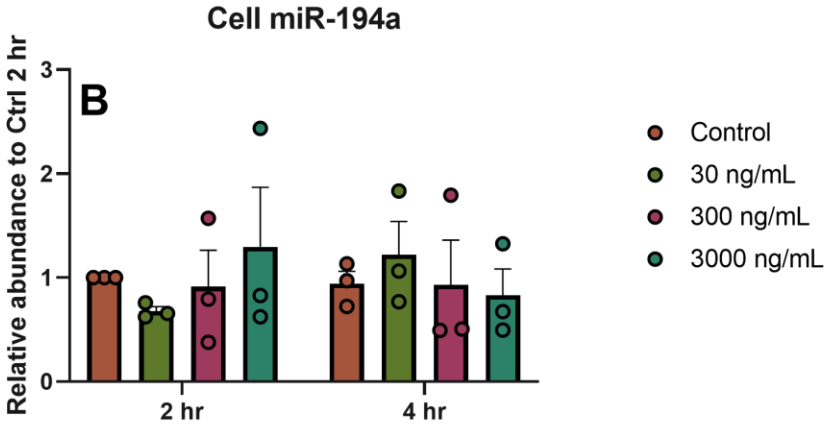

Supernatant miR-21a
C
Relative abundance to Ctrl 2 hr
3
2
1
0
a
ab
b
a
2 hr
4 hr
Control
30 ng/mL
300 ng/mL
3000 ng/mL

Figure 3.3: Relative expression of fructose-1,6-bisphosphate and ssa-miR-21a-5p in hepatocytes exposed to increasing concentrations of cortisol. mRNA of the *fbp1b2* transcript was measured in cell lysates (A) while miR-21a was measured in both the cell lysate (B) and the cell media supernatant (C) after 2- or 4-hours of exposure. Relative abundances were measured using RT-qPCR and normalized to the 18s rRNA expression in both fractions. Relative abundances were subsequently normalized to the 2-hour control of the same fish prior to analysis. Bars that do not share a common letter within the same timepoint were found to be significantly different (Two-way ANOVA, $p < 0.05$ Dunnett's Post Hoc, $n = 3$).

3.3.3 Fructose-1,6-bisphosphatase expression

The transcript levels for *fbp1b2*, one of the paralogs for fructose-1,6-bisphosphatase in rainbow trout, were not significantly influenced by 2- or 4-hours of cortisol exposure (Fig 3.3 A). A similar result was found for ssa-miR-21a which, although being highly likely to influence the expression of *fbp1b2* according to the MiRanda algorithm, was found to be unaffected at the same timepoints (Fig 3.3 B). However, ssa-miR-21a was found to be significantly elevated in the media supernatant of cells exposed to 300 ng/mL cortisol after 2-hours (Fig 3.3 C, Dunnett's Post Hoc, $p = 0.041$). This increased secretion had returned to control values at the 4-hour timepoint.


Phosphoenolpyruvate carboxykinase
A
Relative abundance to Ctrl 2 hr
0
1
2
3
4
a
b
b
b
a
b
b
ab
2 hr
4 hr
Control
30 ng/mL
300 ng/mL
3000 ng/mL


Cell miR-194a
B
Relative abundance to Ctrl 2 hr
0
1
2
3
2 hr
4 hr
Control
30 ng/mL
300 ng/mL
3000 ng/mL

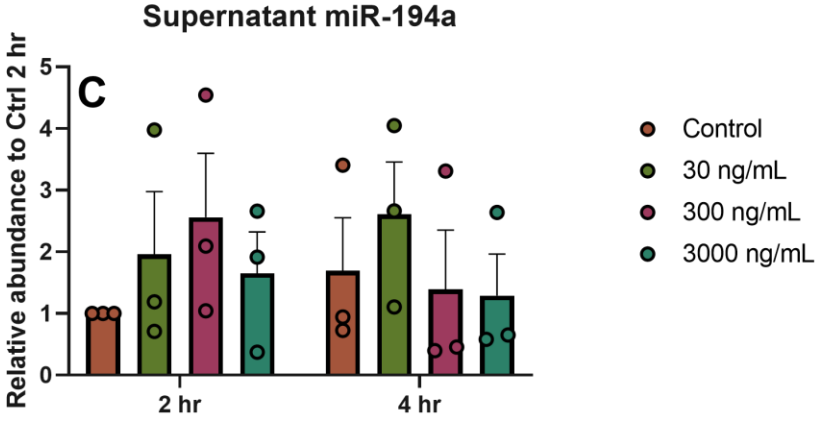
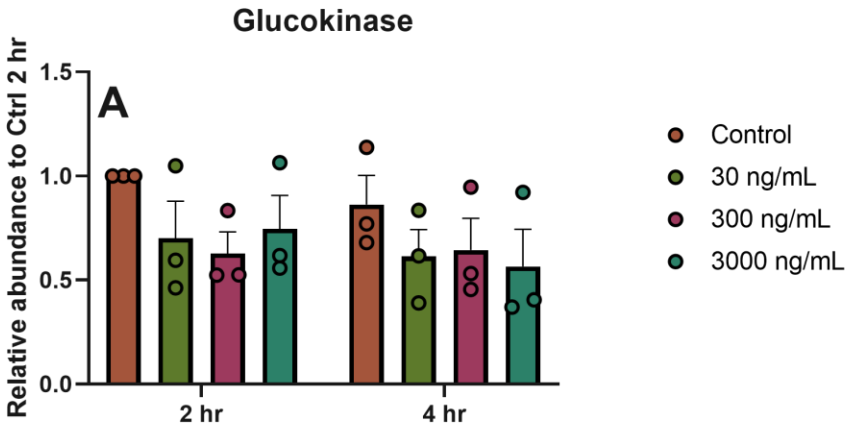
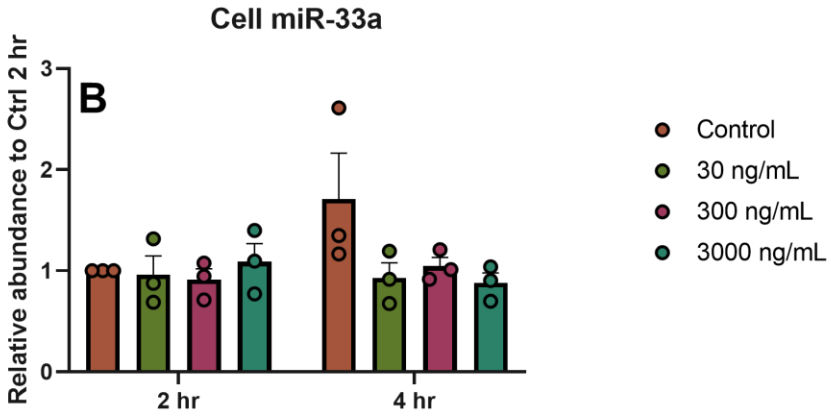

Supernatant miR-194a
C
Relative abundance to Ctrl 2 hr
0
1
2
3
4
5
2 hr
4 hr
Control
30 ng/mL
300 ng/mL
3000 ng/mL

Figure 3.4: Relative expression of phosphoenolpyruvate carboxykinase and ssa-miR-194a-3p in hepatocytes exposed to increasing concentrations of cortisol. mRNA of the *pepck1* transcript was measured in cell lysates (A) while miR-194a was measured in both the cell lysate (B) and the cell media supernatant (C) after 2- or 4-hours of exposure. Relative abundances were measured using RT-qPCR and normalized to the 18s rRNA expression in both fractions. Relative abundances were subsequently normalized to the 2-hour control of the same fish prior to analysis. Bars that do not share a common letter within the same timepoint were found to be significantly different (Two-way ANOVA, $p < 0.05$ Dunnett's Post Hoc, $n = 3$).

3.3.4 Phosphoenolpyruvate carboxykinase expression

Transcript levels for *pepck1*, the cytosolic form of phosphoenolpyruvate carboxykinase, were found to be significantly elevated at both timepoints after cortisol exposure in hepatocytes (Fig 3.4 A). At the 2-hour timepoint, 30 ng/mL (Dunnett's Post Hoc, $p = 0.0203$), 300 ng/mL (Dunnett's Post Hoc, $p = 0.0088$), and 3000 ng/mL (Dunnett's Post Hoc, $p = 0.0075$) significantly increased transcript levels. However, at the 4-hour timepoint, cells exposed to 3000 ng/mL (Dunnett's Post Hoc, $p = 0.070$) had returned to control levels while cells exposed to 30 ng/mL (Dunnett's Post Hoc, $p = 0.0086$) and 300 ng/mL (Dunnett's Post Hoc, $p = 0.088$) cortisol remained significantly elevated. The top miRNA predicted to influence *pepck1* expression, ssa-miR-194a, was not influenced by cortisol exposure in hepatocytes (Fig 3.4 B) or in supernatant (Fig 3.4 C).


Glucokinase
A
Relative abundance to Ctrl 2 hr
1.5
1.0
0.5
0.0
2 hr
4 hr
Control
30 ng/mL
300 ng/mL
3000 ng/mL


Cell miR-33a
B
Relative abundance to Ctrl 2 hr
3
2
1
0
2 hr
4 hr
Control
30 ng/mL
300 ng/mL
3000 ng/mL

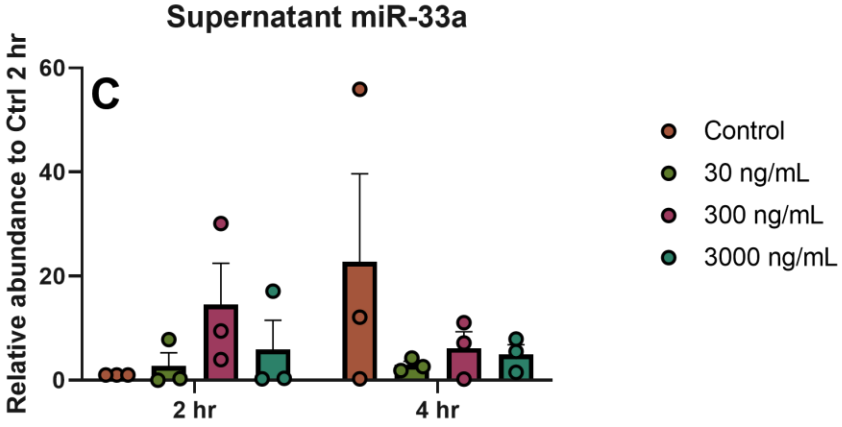
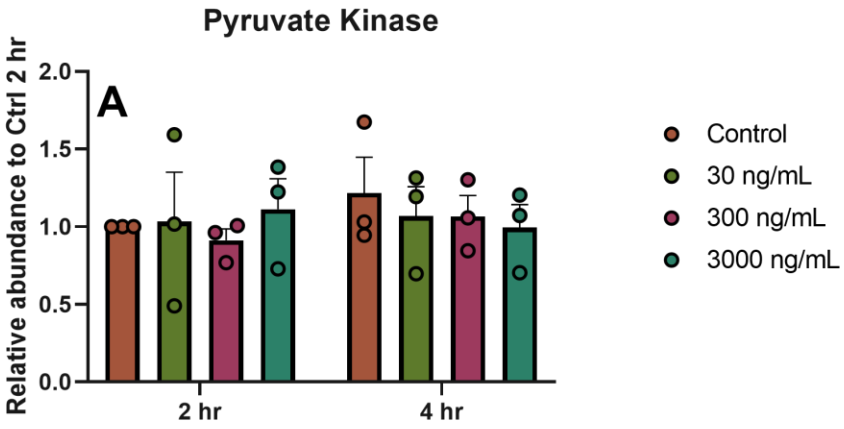
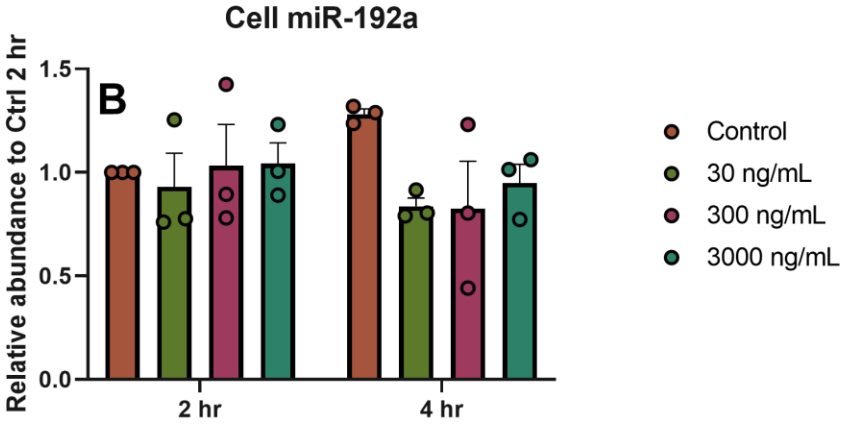

Supernatant miR-33a
C
Relative abundance to Ctrl 2 hr
60
40
20
0
2 hr
4 hr
Control
30 ng/mL
300 ng/mL
3000 ng/mL

Figure 3.5: Relative expression of glucokinase and ssa-miR-33a-5p in hepatocytes exposed to increasing concentrations of cortisol. mRNA of the *gck* transcript was measured in cell lysates (A) while miR-194a was measured in both the cell lysate (B) and the cell media supernatant (C) after 2- or 4-hours of exposure. Relative abundances were measured using RT-qPCR and normalized to the 18s rRNA expression in both fractions. Relative abundances were subsequently normalized to the 2-hour control of the same fish prior to analysis. Bars that do not share a common letter within the same timepoint were found to be significantly different (Two-way ANOVA, $p < 0.05$ Dunnett's Post Hoc, $n = 3$).

3.3.5 Glucokinase expression

Glucokinase transcript levels were found to be unimpacted by cortisol within hepatocytes. The top miRNA predicted to impact expression, ssa-miR-33a, was also unimpacted both in the cell lysates and in cell media supernatant. However, at 2-hours of 300 ng/mL exposure, supernatant levels were found to be increased from 4- to 30-fold times.


Pyruvate Kinase
A
Relative abundance to Ctrl 2 hr
2.0
1.5
1.0
0.5
0.0
2 hr
4 hr
Control
30 ng/mL
300 ng/mL
3000 ng/mL


Cell miR-192a
B
Relative abundance to Ctrl 2 hr
1.5
1.0
0.5
0.0
2 hr
4 hr
Control
30 ng/mL
300 ng/mL
3000 ng/mL

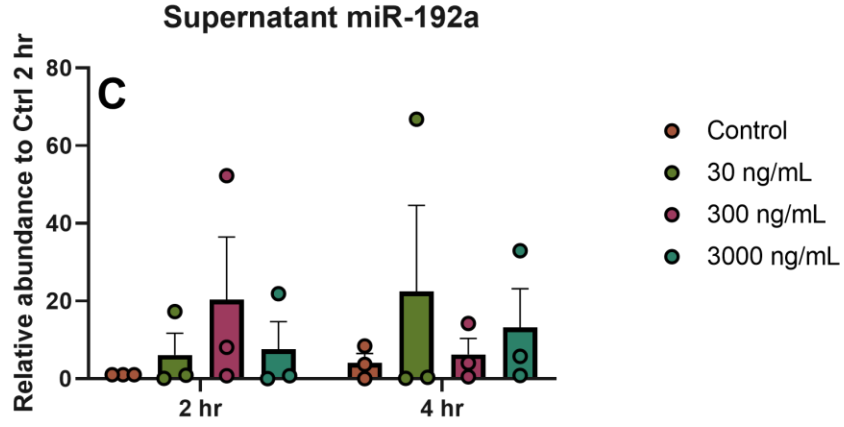

Supernatant miR-192a
C
Relative abundance to Ctrl 2 hr
80
60
40
20
0
2 hr
4 hr
Control
30 ng/mL
300 ng/mL
3000 ng/mL

Figure 3.6: Relative expression of pyruvate kinase and ssa-miR-192a-3p in hepatocytes exposed to increasing concentrations of cortisol. mRNA of the *pklr* transcript was measured in cell lysates (A) while miR-194a was measured in both the cell lysate (B) and the cell media supernatant (C) after 2- or 4-hours of exposure. Relative abundances were measured using RT-qPCR and normalized to the 18s rRNA expression in both fractions. Relative abundances were subsequently normalized to the 2-hour control of the same fish prior to analysis. Bars that do not share a common letter within the same timepoint were found to be significantly different (Two-way ANOVA, $p < 0.05$ Dunnett's Post Hoc, $n = 3$).

3.3.6 Pyruvate kinase expression

Pyruvate kinase transcript levels were found to be unimpacted by cortisol within hepatocytes. The top miRNA predicted to impact expression, ssa-miR-192a, was also unimpacted both in the cell lysates and in cell media supernatant.

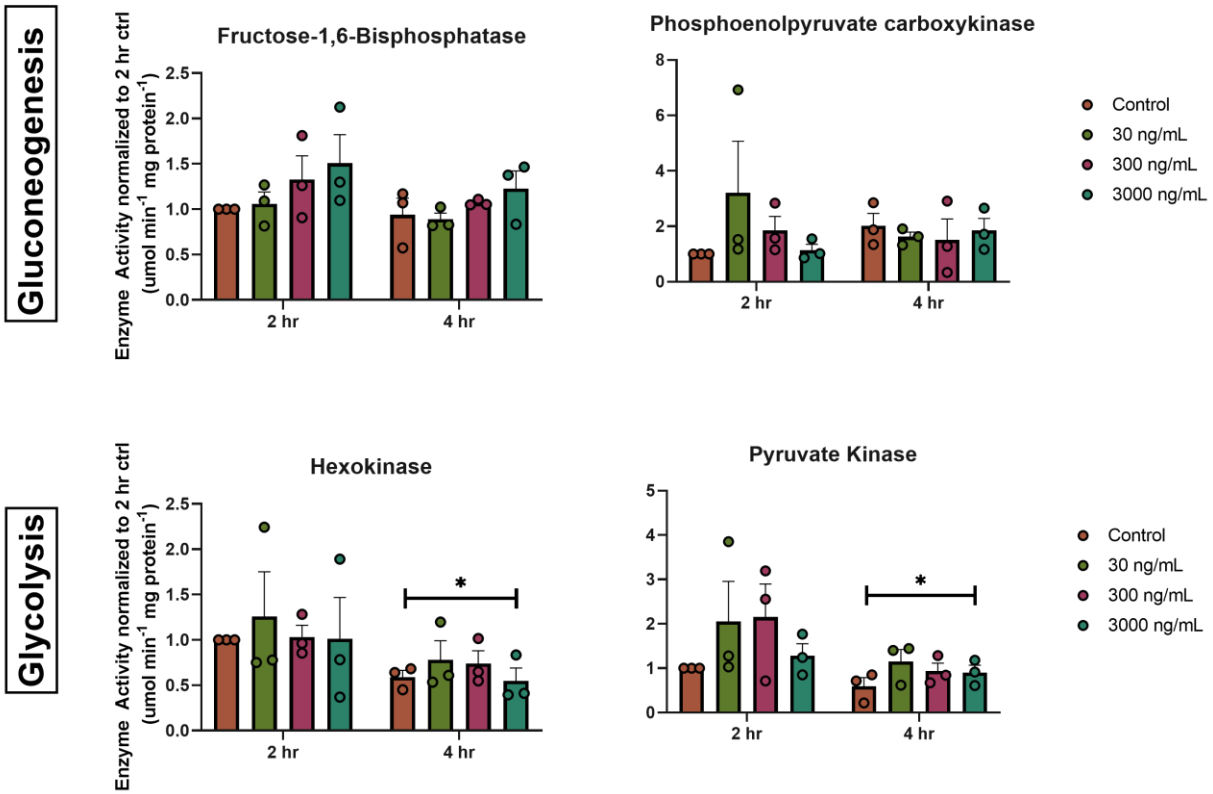


Figure 3.7: Enzyme activity of key metabolic enzymes in hepatocytes exposed to increasing concentrations of cortisol. Activity was measured after 2- or 4-hours of exposure using coupled enzyme reactions and normalized to the 2-hour control timepoint in each fish. Bars overtop of specific timepoints indicate a significant difference between timepoints (Two-way ANOVA, $p < 0.05$, $n = 3$).

3.3.7 Activities of metabolic enzymes of interest

Activities of the enzymes of interest were measured from cell lysates to see how cortisol impacted glucose utilization. Both fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase activities were not impacted by any concentration of cortisol during the timepoints measured. However, hexokinase (Two-way ANOVA, $p = 0.0419$) and pyruvate

kinase (Two-way ANOVA, p =0.0356) had significantly lower activity at the 4-hour timepoint for all groups when compared to the 2-hour timepoint.

3.4 Discussion

This study was aimed at determining the miRNA changes that occur in hepatocytes after exposure to cortisol. These miRNAs were predicted to target key metabolic enzymes involved in glucose regulation and were measured in hepatocytes and their cell media supernatant. Rainbow trout are the ideal model for this study as the glucose metabolism of this species is well studied and recent work has started looking into miRNA roles in this phenotype (Mennigen, 2016). Studies investigating the effect of cortisol on hepatocyte metabolism have used this same species so using rainbow trout hepatocytes can expand the role of miRNAs during stress (Aluru & Vijayan, 2009; Wiseman et al., 2007).

3.4.1 Viability of cultured hepatocyte

Primary culturing of rainbow trout hepatocytes offers an excellent model for studying metabolism in teleosts (Segner, 1998). These cells can still be metabolically active up to 72-hours if cultured in media supplemented with nutrients (Segner et al., 1994). The hepatocytes in this study were maintained at these conditions and exposed within the 72-hours of collection. However, to confirm that cells were viable during the experiment, LDH was measured in the supernatant. LDH is a cytoplasmic enzyme that is released by cells after damage (Kumar 2018). This assay has been used in rainbow trout to confirm consistent cell viability between treatments (Craig et al., 2013; Faught et al., 2017). LDH activity in the supernatant of the cultured hepatocytes shows clear differences within and between fish (Fig 3.2). Fish 1 had a significant

decrease in LDH levels at the 4-hour timepoint while fish 3 had significantly higher LDH activity at the 4-hour timepoint. As well, fish 3 had significantly higher LDH activities compared to the other two specimens. Likely this implies that cells were undergoing necrosis or had plasma membrane leakage. Fish 3 could have been unhealthy, and hepatocytes were unviable at the time of the study, although I did not observe any signs of abnormalities or pathologies during hepatocyte preparation. However, another explanation could be the treatment conditions used in the experiment. In order to expose cells to cortisol, 0.1% ethanol was used to dissolve the hormone as it is hydrophobic (Mommsen et al., 1999). This concentration has been used on tilapia hepatocytes but the authors did not report the viability of cells after exposure (Pierce et al., 2012). Ethanol concentrations up to 1% do not impact viability in mammalian cultures, but other rainbow trout exposures use concentrations of 0.01% (Aluru & Vijayan, 2007; Faught et al., 2017; Sathiyaa & Vijayan, 2003; Timm et al., 2013). If hepatocytes were not healthy at the time of exposure, the higher ethanol concentration could cause excess cell death. Therefore, the results from this chapter might not be physiologically relevant.

3.4.2 Regulation of fructose-1,6-bisphosphatase by cortisol

The first enzyme of interest was Fbpase, which catalyzes the production of fructose-6-phosphate from fructose-1,6-phosphate (Enes et al., 2009; Suarez & Mommsen, 1986). For this study the *fbp1b2* gene was chosen since *fbp1b* is expressed in the liver and *fbp1b2* was found to be influenced by feeding in rainbow trout alevins (Marandel et al., 2015; Marandel et al., 2016). Figure 3.3A shows that transcript abundances for this gene were unaffected by cortisol treatment in the hepatocytes. Most studies have investigated the role of cortisol on activity rather than gene expression after hormonal stimulation, so the enzyme might not be under transcriptional regulation (Dziewulska-Szwajkowska et al., 2003; Morales et al., 1990). However, the activity of

the enzyme was also unaffected by cortisol (Fig 3.7) even though Fbpase activity was shown to be increased 6-hours after handling (Morales et al., 1990). There could be a few reasons for this; either the timepoints were too early to increase activity of the enzyme or cortisol by itself is unable to influence Fbpase activity. When stress is induced *in vivo*, other signalling mechanisms influence altered physiology, including catecholamines and neuroendocrine peptides (Bonga, 1997; Mommsen et al., 1999). Any of these other mechanisms could influence Fbpase activity and the changes reported by Morales et al (1990) are only present during the full stress responses.

MiRanda was used to predict miRNA that could influence expression of Fbpase. The top predicted miRNA was ssa-miR-21a-5p which is a part of the highly expressed miR-21 family of miRNAs in rainbow trout livers (Juanchich et al., 2016). Hepatocyte expression of this miRNA was unaffected by cortisol (Fig 3.3 B) but supernatant expression was significantly elevated at 300 ng/mL cortisol after 2-hours (Fig 3.3 C). This was lost at the 4-hour timepoint where no significant differences in the miRNA were found. MiRNAs are stable in solution for up 2 months at room temperature so they likely did not degrade (Turchinovich et al., 2011). A plausible explanation is that the hepatocytes reabsorbed the miRNA, although no difference in abundance of this miRNA was measured at 4-hours in the cells. The miR-21 family is the most highly abundant miRNA in the liver (Juanchich et al., 2016). Reuptake of this miRNA might not contribute enough to the cellular abundance but then that begs to question: what is the purpose of these secreted miRNAs? They could still be influencing protein levels of Fbpase, although this is not supported by the lack of change in enzymatic activity. Future work would benefit investigating this relationship to see if miR-21a could be impacting Fbpase, through transfection studies.

3.4.3 Regulation of phosphoenolpyruvate carboxykinase by cortisol

Pepck is a well studied enzyme involved in gluconeogenesis that catalyzes the production of phosphoenolpyruvate from oxaloacetate (Knox et al., 1980; Suarez & Mommsen, 1986). Pepck also has three genes that are expressed in different parts of the cell: *pepck1* is cytosolic while *pepck2a* and *pepck2b* are mitochondrial (Enes et al., 2009; Marandel et al., 2019). The cytosolic form has been shown to be transcriptionally altered by cortisol and was therefore chosen as the target for this study (Marandel et al., 2019). Figure 3.4A shows that physiological levels of cortisol (30 and 300 ng/mL) increased *pepck1* expression at 2- and 4-hours while high levels of cortisol (3000 ng/mL) was only elevated at 2-hours. Pepck can only be upregulated transcriptionally since its activity cannot be modified allosterically (Enes et al., 2009). Other studies have found that cortisol upregulates *pepck1* transcript abundance 3-fold after 24-hours but none have looked at acute upregulation at 2-hours (Aluru & Vijayan, 2007). However, Figure 3.7 shows that Pepck activity was unimpacted at either of the timepoints. This could be due to the method of enzyme isolation. The mitochondrial forms of Pepck have been shown to be endogenously expressed and therefore are unimpacted by cortisol signalling (Marandel et al., 2019). To properly measure only the cytosolic form, differential centrifugation should have been performed on homogenates to separate out the different fractions of the enzyme. This could have provided more information on whether any post-transcriptional regulation could be occurring since activity and gene expression of this enzyme are not always correlated (Sathiyaa & Vijayan, 2003).

The top predicted target for *pepck1* was ssa-miR-194a. MiRanda found 2 binding sites in the 3’ UTR of this transcript which resulted in a higher total score. MiRNAs can act synergistically, and multiple miRNAs binding not only allows for greater repression of

translation but allows for more fine tuning of protein expression (Brennecke et al., 2005). This means that subtle changes in miRNA expression could have large changes in *pepck* expression. However, miRNA levels were unimpacted by cortisol stimulation. MiRNAs can act as thresholds to prevent expression until a certain amount of their target mRNA is expressed, so lack of change in transcript abundance does not indicate that *pepck* is not regulated by miRNAs (Leung & Sharp, 2010). Therefore, future studies should use knockdowns, transfections, or luciferase binding assays to determine if *pepck1* is under miRNA regulation.

3.4.4 Regulation of glucokinase by cortisol

Gk is the liver isozyme of Hk that catalyzes the production of glucose-6-phosphate from glucose in glycolysis (Enes et al., 2009; S Panserat et al., 2001; Polakof et al., 2012). *Gk* has two genes in rainbow trout which both increase in expression after carbohydrate loading (Marandel et al., 2017, 2015). However, in the present study, there were no changes in gene expression after cortisol exposure (Fig 3.5 A). Previous work has found that *gk* is upregulated at 24-hours post cortisol exposure, so the transcript might require more than 4-hours for upregulation (Wiseman et al., 2007). Enzyme activity was also unimpacted by cortisol even though stress has been shown to increase activity within 2-hours (Fig 3.7; López-Patiño et al., 2014). The increase seen *in vivo* could be regulated by multiple interacting pathways and changes that occur during recovery from stress as mentioned above. Figure 3.7 also shows that there was a time-dependant effect, where Gk activity was decreased at the 4-hour timepoint compared to the 2-hour timepoint. Likely this decrease was due to the change in media increasing glucose concentrations for hepatocytes to metabolize. This further points to metabolites such as glucose regulating the activity of the enzyme during these times.

MiRanda predicted ssa-miR-33a-5p as the top miRNA to bind since it had 3 different miRNA binding sites in the *gk* 3' UTR. As mentioned above, multiple miRNA binding sites per UTR can act synergistically to fine tune the regulation of the gene (Brennecke et al., 2005). However, miR-33a was not altered at any timepoint post cortisol exposure in the hepatocytes or the supernatant (Fig 3.5 B & C). Supernatant levels of the miRNA were more variable at physiological cortisol concentrations (300 ng/mL) than tissue levels, showing that there might be individual-driven differences in secretion that the low sample size might not be able to demonstrate. The miR-33 family of miRNAs have been shown to impact metabolism both in humans and fish (Kostyniuk et al., 2018; Mennigen, 2016; Mennigen et al., 2012; Mirra et al., 2018). Although no changes were observed, further investigations should determine if *gk* could be another metabolic target for this miRNA.

3.4.5 Regulation of pyruvate kinase by cortisol

Pk is another important glycolytic enzyme that catalyzes the formation of pyruvate from phosphoenolpyruvate, which can then feed into the TCA cycle (Enes et al., 2009). The liver version of this enzyme has been shown to be upregulated 1-hour after a handling stress in rainbow trout (Wiseman et al., 2007). However, *pklr* expression in this study was unimpacted by cortisol (Fig 3.6A). This shows that cortisol is insufficient to induce a transcriptional response and signalling events *in vivo* after stress are necessary for increasing gene expression. Cortisol exposure in Nile tilapia increased Pk enzyme activity; however handling stress of rainbow trout did not have the same impact (Morales et al., 1990; Vijayan et al., 1997). Cortisol was also insufficient in altering Pk activity at the timepoints measured (Fig 3.7). This could be attributed to the difference in timepoints between studies since activity could be controlled in a time dependant manner (Laiz-Carrion et al., 2003; Polakof et al., 2008). Similar to Gk activity, there

was a significant decrease in Pk activity at the 4-hour timepoint that is likely attributed to the change in media. This change could be masking any changes in activity due to cortisol, so future studies should investigate reducing or removing glucose from the media to limit any metabolite-drive effects.

Ssa-miR-192a was predicted to be the top miRNA to bind *pklr*. Although it only had one binding site, the score and free energy fell within the range used by other studies as a cut-off for predicted targets (Kostyniuk et al., 2019). Figure 3.6 B & C show that this miRNA was unimpacted by cortisol at both timepoints in hepatocytes and supernatant. Yet again there is high variability at the physiological cortisol levels in the supernatant so different fish could be responding to these changes by secreting miRNAs. Of course, this could be due to cell leakage (as shown with LDH assay), but further study should see if such changes occur in healthy hepatocytes. MiR-192 has been linked to carbohydrate metabolism by increasing in circulation of pre-diabetic patients and glucose-intolerant mice (Párrizas et al., 2015). Rainbow trout are a model to study glucose intolerance, so this miRNA could have similar responses in circulation.

3.4.6 Conclusion

In conclusion, cortisol has been shown to impact miRNA levels and key genes involved in gluconeogenesis in rainbow trout hepatocytes. This hormone upregulates gluconeogenesis, but has also been shown to impact glycolysis during recovery from stress (Aluru & Vijayan, 2007; Mommsen et al., 1999; Wiseman et al., 2007). In this study enzymatic activities were unimpacted by cortisol, but glycolytic activity could have been masked by changes in metabolites. Unfortunately, hepatocytes might not have responded in a physiological manner due to increased cell leakage. This is especially problematic for a study interested in measuring miRNA secretion, so care in the future should be taken to maintain cell viability. The miRNAs

predicted to target these carbohydrate regulating enzymes are already associated with metabolism, so future studies should investigate if changes in circulation and cell abundances could have impacts on the glucose intolerant phenotype of rainbow trout.

Chapter 4

General Conclusion

The primary objective of this study was to examine how miRNAs are altered following a stressor in rainbow trout. MiRNAs were of interest since they have been shown to respond to environmental change as well as hormonal signalling (Cameron et al., 2015; Craig et al., 2014). Understanding the functional roles miRNAs have in regulating gene expression can expand the current knowledge on how their dysregulation through environmental contamination or pathologies could impact cellular function. Determining the physiological relevance of these small, non-coding RNAs can also provide insight into how stress can be mitigated in teleosts such as the economically relevant rainbow trout.

The two studies carried out in this thesis have yielded interesting results on miRNA regulation following a stressful event in rainbow trout; however, comparing the results can be difficult due to differences in how the studies were conducted. For example, the circulating miRNA were extracted using different methods. Chapter 2 miRNAs were associated with EVs while the miRNAs in chapter 3 were extracted using a general circulating miRNA protocol. Most of the miRNA in circulation have been found to be associated with protein complexes independent of vesicles (Arroyo et al., 2011; Turchinovich et al., 2011). The vesicle-associated fraction have a different miRNA signature than the general circulating miRNAs, but have been shown to biologically active (Pegtel et al., 2010; Turchinovich et al., 2011). Therefore, any changes that could be occurring in vesicles in chapter 3 might be masked by the overall circulating abundance remaining constant, which was not measured in chapter 2. The timepoints are also different between studies, and since the changes in chapter 2 were found to be altered at different times for each miRNA, any alteration of chapter 3 miRNAs might have been missed. The difference in stressors between the two chapters could also be a source of the differences. Responses to handling *in vivo* will impact whole animal physiology using complicated signalling

networks between and within tissues. The *in vitro* study, while valuable in determining specific effects of one aspect of the stress response, is on an isolated cell type in a culture plate which might respond differently when *in vivo*. This all must be considered when comparing the findings of this thesis.

Even so, both chapters stand independently to show that miRNAs can be altered by stress and cortisol. In chapter 2, it was found that vesicle-associated miRNAs were altered in a time-dependant manner depending on the specific miRNA examined. The likely source of these miRNAs are tissues with direct contact to blood, which includes liver, gills, kidney, spleen, endothelial cells, and even the erythrocytes themselves (Turchinovich et al., 2011). KEGG analysis revealed that these altered miRNAs were predicted to inhibit metabolic pathways and metabolic changes are vital during stress recovery. When these same miRNAs were measured in tissues, different trends were observed that could be supporting the tissue in its role during stress recovery. Upon looking at the general trend (Fig 2.3), it was seen that there is an increase in miRNA abundance in EVs in the blood, and a decrease in the abundance of miRNA measured in the tissue, in a time, and miRNA-specific manner. This may imply that following a stressful event, inhibitors are removed from stress responsive tissues to ensure there is an appropriate upregulation in specific stress responsive pathways. In the liver, decreases in miRNA abundances could be aiding in increasing energy availability to other tissues as this is the primary role for the organ during recovery. In the anterior kidney, the miRNAs were either not impacted at all or almost completely depleted which could be due to their role as regulators of immune function. However, the physiological results of these changes need to be explored further to understand how exactly miRNAs support stress recovery in teleosts.

In chapter 3, it was found that miRNAs could be altered by cortisol. Tissue miRNA abundances were unaltered by cortisol exposure, but it was able to upregulate key genes involved in gluconeogenesis. MiRNAs can act as a translational buffer, so although no abundance changes were measured, they could still be preventing overexpression of these metabolic genes (Leung & Sharp, 2010). Cortisol was also found to have a dose-dependant effect on one miRNA in the supernatant, indicating that it can impact miRNA secretion from hepatocytes. However, whether the secretion of this miRNA is related to its predicted binding target in this chapter or some other target requires further study. This miRNA (ssa-miR-21a-5p) has a similar sequence omy-miR-21 which was measured in chapter 2. The miR-21 family is highly abundant in the liver of rainbow trout (Juanchich et al., 2016). Whether abundance of the miRNA families within a tissue could be a factor in their secretion is unknown, but future studies could explore this relationship. Finally, although they were found to be unaltered in this experiment, the miRNA predicted to inhibit metabolic enzymes have been already associated with dysregulation of metabolism (Kostyniuk et al., 2018; Mennigen et al., 2012; Párrizas et al., 2015).

There are many opportunities for future work that can build on the current findings. Chapter 2 shows that there are epigenetic changes occurring during recovery from stress, but it does not fully explain what these miRNAs are doing. Future studies can investigate the abundance changes in other tissues such as the gills or spleen to see how they could be contributing to the circulating complement of miRNAs. Perfusions can also be used as ways of determining the functions of these miRNAs; what functional, phenotypic changes occur if the liver (or other tissues) are exposed to stress produced EVs? Chapter 3 demonstrates that there could be interesting epigenetic changes occurring due to cortisol exposure in hepatocytes, but how this contributes to the liver's role in response stress remains unclear and warrants further

investigation. Repeating the experiment using healthy tissue and testing timepoints that align with current literature can offer more explanation for the role of miRNAs in recovery from stress. Since *in vivo* effects include many interacting signals, factors such as catecholamines or elevated circulating glucose could be accounted for by exposing cells before or after adding cortisol to determine their influence on changes. Finally, the miRNA predicted to target these enzymes are associated with metabolism. Future work should investigate whether their influence on gluconeogenesis/glycolysis plays a role in the glucose intolerance of rainbow trout.

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Appendix A – Recipes for Solutions

Chapter 2 – Handling Stressor Buffers

Table A.1: Glucose spectrophotometry assay buffer. Buffer was combined with rainbow trout plasma to determine glucose levels in circulation. The change in NADP to NADPH was measured for 20 mins at 340 nm.

Chemical	Final Concentration	pH
Hepes Buffer	50 mM	7.4
$MgCl_2$	10 mM	
KCl	100 mM	
NADP	1 mM	
ATP	1 mM	
Glucose-6-Phosphate dehydrogenase	1 unit/mL	
Hexokinase	1 unit/mL	

Chapter 3 – Hepatocyte Exposure Buffers

Hepatocyte Isolation

Table A.2: Hanks' Salts Solution (5x)

Chemical	Final Concentration (mM)
Na_2HPO_4	0.33
KH_2PO_4	0.44
$MgSO_4$ x $7H_2O$	0.8
NaCl	136.9
KCl	5.4
Hepes	5
Hepes x Na	5

Table A.3: Basic Hanks' Solution (pH 7.63)

Chemical	Final Concentration
5x Hanks' salt solution	1x
H_2O	
$NaHCO_3$	5 mM

Table A.4: Rinsing Solution (pH 7.63)

Chemical	Final Concentration
Basic Hanks' Solution	
EGTA	1 mM

Table A.5: Collagenase Media (pH 7.63)

Chemical	Final Concentration
Basic Hanks' Solution	
Collagenase Type IV	31.5 units/mL

Table A.6: BSA Hanks' Resuspension Media (pH 7.63)

Chemical	Final Concentration
Basic Hanks' Solution	
$CaCl_2$	1.5 mM
Bovine Serum Albumin	1.5%

Table A.7: Hanks' Culture Media (pH 7.63). Antibiotics were added after filtering media through a 0.22 µm bottle filter into an autoclaved bottle.

Chemical	Final Concentration
Basic Hanks' Solution	
100x MEM Non-essential amino acids	1x
50x MEM Essential amino acids	1x
$CaCl_2$	1.5 mM
100x Antibiotics solution	1x

Enzyme Assay Reaction Buffers

Table A.8: Enzyme Extraction buffer. Cell pellets were weighed and diluted in 20x buffer before sonication.

Chemical	Final Concentration	pH
Hepes Buffer	20 mM	7
EDTA	1 mM	
Triton X-100	0.10%	
Protease Inhibitor Cocktail (Roche)	1/10 mL	

Table A.9: Lactate Dehydrogenase assay. LDH activity was measured in cell media supernatants to determine leakage. The reaction buffer was added first to sample, and assay started by adding substrate after 1-minute incubation. The change in ratio of NADH to NAD was measured spectrophotometrically at 340 nm for 30 mins.

Chemical	Final Concentration	pH
Imidazole Buffer	20 mM	7.4
NADH	0.15 mM	

Substrate	Final Concentration
Pyruvate	1 mM

Table A.10: Pyruvate Kinase assay. PK activity was measured in cell lysates to determine how cortisol influenced enzymatic activity. The reaction buffer was added first to sample, and assay started by adding substrate after 1-minute incubation. The change in ratio of NADH to NAD was measured spectrophotometrically at 340 nm for 30 mins.

Chemical	Final Concentration	pH
Imidazole Buffer	50 mM	7.4
ADP	5 mM	
KCl	100 mM	
MgCl2	10 mM	
NADH	0.15 mM	
Fructose-1,6-Bisphosphate	10 mM	
Lactate Dehydrogenase	5 units/mL	

Substrate	Final Concentration
Phosphoenol pyruvate	5 mM

Table A.11: Phosphoenolpyruvate carboxykinase assay. Pepck activity was measured in cell lysates to determine how cortisol influenced enzymatic activity. The reaction buffer was added first to sample, and assay started by adding substrate after 1-minute incubation. The change in ratio of NADH to NAD was measured spectrophotometrically at 340 nm for 30 mins.

Chemical	Final Concentration	pH
Tris-HCl Buffer	80 mM	7.4
KCN	1 mM	
MnCl2	1 mM	
MgCl2	1 mM	
Inosine 5'-diphosphate	1.5 mM	
NADH	0.17 mM	
Phosphoenol pyruvate	1.1 mM	
Malate dehydrogenase	19 units/mL	

Substrate	Final Concentration
NaHCO3	0.001 mM

Table A.12: Hexokinase assay. HK activity was measured in cell lysates to determine how cortisol influenced enzymatic activity. The reaction buffer was added first to sample, and assay started by adding substrate after 1-minute incubation. The change in ratio of NADP to NADPH was measured spectrophotometrically at 340 nm for 30 mins.

Chemical	Final Concentration	pH
Imidazole Buffer	20 mM	7.4
MgCl2	5 mM	
1,4-Dithioerythritol	5 mM	
Glucose	100 mM	
NADP+	0.5 mM	
Glucose-6-Phosphate Dehydrogenase	0.5 units/mL	

Substrate	Final Concentration
ATP	1 mM

Table A.13: Fructose-1,6-Bisphosphatase assay. FBPase activity was measured in cell lysates to determine how cortisol influenced enzymatic activity. The reaction buffer was added first to sample, and assay started by adding substrate after 1-minute incubation. The change in ratio of NADP to NADPH was measured spectrophotometrically at 340 nm for 30 mins.

Chemical	Final Concentration	pH
Tris-HCl Buffer	80 mM	8
EDTA	5 mM	
MgSO4	8 mM	
KH2PO4	1 mM	
NAHCO	2 mM	
1,4-Dithioerythritol	2.5 mM	
NADP+	2 mM	
Glucose-6-Phosphate Dehydrogenase	65 mUnits/mL	
6-Phosphogluconate dehydrogenase	65 mUnits/mL	

Substrate	Final Concentration
Fructose-1,6-Bisphosphate	2 mM

Appendix B – KEGG Pathways

Table B.1: Pathways impacted by miR-21. Predicted targets for miR-21 were determined by searching the miRNA sequence through MiRanda with all *Salmo salar* 3' UTRs as the database. Targets were then run through KEGG to determine pathways predicted to be impacted.

KEGG Identifier	Pathway	Number of Targets
sasa01100	Metabolic pathways	41
sasa04144	Endocytosis	23
sasa04080	Neuroactive ligand-receptor interaction	19
sasa05168	Herpes simplex virus 1 infection	14
sasa04070	Phosphatidylinositol signaling system	13
sasa04933	AGE-RAGE signaling pathway in diabetic complications	13
sasa04010	MAPK signaling pathway	13
sasa04150	mTOR signaling pathway	12
sasa04371	Apelin signaling pathway	12
sasa04020	Calcium signaling pathway	12
sasa04510	Focal adhesion	12
sasa04140	Autophagy - animal	11
sasa05132	Salmonella infection	11
sasa04916	Melanogenesis	10
sasa04514	Cell adhesion molecules (CAMs)	10
sasa04310	Wnt signaling pathway	10
sasa04520	Adherens junction	10
sasa04210	Apoptosis	10
sasa04218	Cellular senescence	9
sasa04810	Regulation of actin cytoskeleton	9
sasa04068	FoxO signaling pathway	9
sasa00562	Inositol phosphate metabolism	8
sasa04261	Adrenergic signaling in cardiomyocytes	8
sasa04540	Gap junction	8
sasa04530	Tight junction	8
sasa04270	Vascular smooth muscle contraction	7
sasa00230	Purine metabolism	7
sasa04912	GnRH signaling pathway	7
sasa04012	ErbB signaling pathway	6
sasa04621	NOD-like receptor signaling pathway	6
sasa04217	Necroptosis	6
sasa04141	Protein processing in endoplasmic reticulum	6
sasa04145	Phagosome	6
sasa04910	Insulin signaling pathway	6
sasa03013	RNA transport	5
sasa04625	C-type lectin receptor signaling pathway	5

sasa04920	Adipocytokine signaling pathway	5
sasa04142	Lysosome	5
sasa04370	VEGF signaling pathway	5
sasa04914	Progesterone-mediated oocyte maturation	4
sasa04137	Mitophagy - animal	4
sasa04512	ECM-receptor interaction	4
sasa04115	p53 signaling pathway	4
sasa00534	Glycosaminoglycan biosynthesis - heparan sulfate / heparin	4
sasa03320	PPAR signaling pathway	3
sasa01212	Fatty acid metabolism	3
sasa00280	Valine leucine and isoleucine degradation	3
sasa04744	Phototransduction	3
sasa00520	Amino sugar and nucleotide sugar metabolism	3
sasa04060	Cytokine-cytokine receptor interaction	3
sasa00270	Cysteine and methionine metabolism	3
sasa04620	Toll-like receptor signaling pathway	3
sasa04216	Ferroptosis	2
sasa04622	RIG-I-like receptor signaling pathway	2
sasa04130	SNARE interactions in vesicular transport	2
sasa01200	Carbon metabolism	2
sasa00830	Retinol metabolism	2
sasa01040	Biosynthesis of unsaturated fatty acids	2
sasa00601	Glycosphingolipid biosynthesis - lacto and neolacto series	2
sasa00310	Lysine degradation	2
sasa04120	Ubiquitin mediated proteolysis	2
sasa02010	ABC transporters	2
sasa00561	Glycerolipid metabolism	2
sasa03015	mRNA surveillance pathway	2
sasa04350	TGF-beta signaling pathway	2
sasa00532	Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	2
sasa00510	N-Glycan biosynthesis	2
sasa00564	Glycerophospholipid metabolism	2
sasa00600	Sphingolipid metabolism	2
sasa04136	Autophagy - other	2
sasa00603	Glycosphingolipid biosynthesis - globo and isoglobo series	2
sasa04330	Notch signaling pathway	1
sasa00020	Citrate cycle TCA cycle	1
sasa00072	Synthesis and degradation of ketone bodies	1
sasa00604	Glycosphingolipid biosynthesis - ganglio series	1
sasa00565	Ether lipid metabolism	1
sasa00970	Aminoacyl-tRNA biosynthesis	1
sasa03040	Spliceosome	1

sasa00770	Pantothenate and CoA biosynthesis	1
sasa00640	Propanoate metabolism	1
sasa03010	Ribosome	1
sasa00010	Glycolysis / Gluconeogenesis	1
sasa03022	Basal transcription factors	1
sasa00563	Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	1
sasa00120	Primary bile acid biosynthesis	1
sasa04114	Oocyte meiosis	1
sasa03008	Ribosome biogenesis in eukaryotes	1
sasa00071	Fatty acid degradation	1
sasa04340	Hedgehog signaling pathway	1
sasa00061	Fatty acid biosynthesis	1
sasa01230	Biosynthesis of amino acids	1
sasa00511	Other glycan degradation	1
sasa00062	Fatty acid elongation	1
sasa00620	Pyruvate metabolism	1
sasa00260	Glycine serine and threonine metabolism	1
sasa00051	Fructose and mannose metabolism	1
sasa04260	Cardiac muscle contraction	1
sasa03018	RNA degradation	1
sasa00650	Butanoate metabolism	1
sasa00760	Nicotinate and nicotinamide metabolism	1
sasa04146	Peroxisome	1
sasa00240	Pyrimidine metabolism	1
sasa00983	Drug metabolism - other enzymes	1
sasa04110	Cell cycle	1
sasa03440	Homologous recombination	1
sasa03060	Protein export	1
sasa00630	Glyoxylate and dicarboxylate metabolism	1
sasa00480	Glutathione metabolism	1
sasa00250	Alanine aspartate and glutamate metabolism	1
sasa00515	Mannose type O-glycan biosynthesis	1
sasa00531	Glycosaminoglycan degradation	1

Table B.2: Pathways impacted by Let-7a. Predicted targets for let-7a were determined by searching the miRNA sequence through MiRanda with all *Salmo salar* 3' UTRs as the database. Targets were then run through KEGG to determine pathways predicted to be impacted.

KEGG Identifier	Pathway	Number of Targets
sasa01100	Metabolic pathways	69
sasa04080	Neuroactive ligand-receptor interaction	45
sasa04010	MAPK signaling pathway	31
sasa04510	Focal adhesion	26
sasa04514	Cell adhesion molecules (CAMs)	25
sasa04810	Regulation of actin cytoskeleton	25
sasa04150	mTOR signaling pathway	24
sasa04310	Wnt signaling pathway	23
sasa04020	Calcium signaling pathway	23
sasa04068	FoxO signaling pathway	22
sasa04916	Melanogenesis	21
sasa04261	Adrenergic signaling in cardiomyocytes	19
sasa04530	Tight junction	18
sasa04141	Protein processing in endoplasmic reticulum	17
sasa04933	AGE-RAGE signaling pathway in diabetic complications	16
sasa04914	Progesterone-mediated oocyte maturation	16
sasa04371	Apelin signaling pathway	15
sasa04218	Cellular senescence	15
sasa04144	Endocytosis	15
sasa04140	Autophagy - animal	14
sasa04120	Ubiquitin mediated proteolysis	14
sasa04350	TGF-beta signaling pathway	14
sasa04910	Insulin signaling pathway	13
sasa04210	Apoptosis	13
sasa04625	C-type lectin receptor signaling pathway	13
sasa04012	ErbB signaling pathway	13
sasa04512	ECM-receptor interaction	13
sasa04370	VEGF signaling pathway	12
sasa04114	Oocyte meiosis	12
sasa04540	Gap junction	11
sasa05132	Salmonella infection	11
sasa04912	GnRH signaling pathway	11
sasa04620	Toll-like receptor signaling pathway	10
sasa04060	Cytokine-cytokine receptor interaction	10
sasa00230	Purine metabolism	10
sasa04621	NOD-like receptor signaling pathway	10
sasa04070	Phosphatidylinositol signaling system	10

sasa04520	Adherens junction	9
sasa04260	Cardiac muscle contraction	8
sasa00565	Ether lipid metabolism	8
sasa05168	Herpes simplex virus 1	8
sasa00564	Glycerophospholipid metabolism	7
sasa04115	p53 signaling pathway	7
sasa04270	Vascular smooth muscle contraction	7
sasa00561	Glycerolipid metabolism	6
sasa04110	Cell cycle	6
sasa04145	Phagosome	6
sasa03013	RNA transport	5
sasa00562	Inositol phosphate metabolism	5
sasa04622	RIG-I-like receptor signaling pathway	5
sasa00600	Sphingolipid metabolism	5
sasa04142	Lysosome	5
sasa04340	Hedgehog signaling pathway	5
sasa00510	N-Glycan biosynthesis	4
sasa04137	Mitophagy - animal	4
sasa03015	mRNA surveillance pathway	3
sasa00604	Glycosphingolipid biosynthesis - ganglio series	3
sasa03040	Spliceosome	3
sasa03018	RNA degradation	3
sasa03440	Homologous recombination	3
sasa00310	Lysine degradation	3
sasa01210	2-Oxocarboxylic Acid Metabolism	2
sasa04920	Adipocytokine signaling pathway	2
sasa03410	Base excision repair	2
sasa02010	ABC transporters	2
sasa00515	Mannose type O-glycan biosynthesis	2
sasa00051	Fructose and mannose metabolism	2
sasa00120	Primary bile acid biosynthesis	2
sasa00511	Other glycan degradation	2
sasa00512	Mucin type O-glycan biosynthesis	2
sasa00630	Glyoxylate and dicarboxylate metabolism	2
sasa04330	Notch signaling pathway	2
sasa00534	Glycosaminoglycan biosynthesis - heparan sulfate / heparin	2
sasa04130	SNARE interactions in vesicular transport	2
sasa04146	Peroxisome	2
sasa04744	Phototransduction	2
sasa00240	Pyrimidine metabolism	2
sasa04217	Necroptosis	2
sasa00020	Citrate cycle (TCA cycle)	2
sasa00563	Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	2

sasa00603	Glycosphingolipid biosynthesis - globo and isoglobo series	2
sasa00260	Glycine serine and threonine metabolism	2
sasa00190	Oxidative phosphorylation	2
sasa00830	Retinol metabolism	2
sasa00760	Nicotinate and nicotinamide metabolism	2
sasa00983	Drug metabolism - other enzymes	2
sasa01230	Biosynthesis of amino acids	2
sasa01200	Carbon metabolism	2
sasa00270	Cysteine and methionine metabolism	2
sasa00514	Other types of O-glycan biosynthesis	1
sasa03010	Ribosome	1
sasa00380	Tryptophan metabolism	1
sasa00440	Phosphonate and phosphinate metabolism	1
sasa00480	Glutathione metabolism	1
sasa00052	Galactose metabolism	1
sasa03430	Mismatch repair	1
sasa03320	PPAR signaling pathway	1
sasa00533	Glycosaminoglycan biosynthesis - keratan sulfate	1
sasa03020	RNA polymerase	1
sasa00220	Arginine biosynthesis	1
sasa04216	Ferroptosis	1
sasa03030	DNA replication	1
sasa03460	Fanconi anemia pathway	1
sasa00860	Porphyrin and chlorophyll metabolism	1
sasa00040	Pentose and glucuronate interconversions	1
sasa00140	Steroid hormone biosynthesis	1
sasa00471	D-Glutamine and D-glutamate metabolism	1
sasa00590	Arachidonic acid metabolism	1
sasa00360	Phenylalanine metabolism	1
sasa00910	Nitrogen metabolism	1
sasa00970	Aminoacyl-tRNA biosynthesis	1
sasa00601	Glycosphingolipid biosynthesis - lacto and neolacto series	1
sasa00980	Metabolism of xenobiotics by cytochrome P450	1
sasa00531	Glycosaminoglycan degradation	1
sasa00520	Amino sugar and nucleotide sugar metabolism	1
sasa03420	Nucleotide excision repair	1
sasa04136	Autophagy - other	1
sasa00770	Pantothenate and CoA biosynthesis	1
sasa00650	Butanoate metabolism	1
sasa00350	Tyrosine metabolism	1
sasa00250	Alanine aspartate and glutamate metabolism	1
sasa00053	Ascorbate and aldarate metabolism	1
sasa00620	Pyruvate metabolism	1

sasa03022	Basal transcription factors	1
sasa00532	Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	1
sasa00410	beta-Alanine metabolism	1
sasa00982	Drug metabolism - cytochrome P450	1
sasa03008	Ribosome biogenesis in eukaryotes	1

Table B.3: Pathways impacted by miR-143. Predicted targets for miR-143 were determined by searching the miRNA sequence through MiRanda with all *Salmo salar* 3' UTRs as the database. Targets were then run through KEGG to determine pathways predicted to be impacted.

KEGG Identifier	Pathway	Number of Targets
sasa01100	Metabolic pathways	28
sasa04010	MAPK signaling pathway	12
sasa04080	Neuroactive ligand-receptor interaction	11
sasa04810	Regulation of actin cytoskeleton	10
sasa04510	Focal adhesion	10
sasa04150	mTOR signaling pathway	9
sasa04916	Melanogenesis	8
sasa04514	Cell adhesion molecules (CAMs)	8
sasa04144	Endocytosis	7
sasa04530	Tight junction	7
sasa04310	Wnt signaling pathway	7
sasa04110	Cell cycle	6
sasa04020	Calcium signaling pathway	6
sasa04371	Apelin signaling pathway	6
sasa00230	Purine metabolism	5
sasa04140	Autophagy - animal	5
sasa04910	Insulin signaling pathway	5
sasa00310	Lysine degradation	4
sasa04218	Cellular senescence	4
sasa04114	Oocyte meiosis	4
sasa01200	Carbon metabolism	4
sasa04520	Adherens junction	4
sasa04142	Lysosome	4
sasa04914	Progesterone-mediated oocyte maturation	4
sasa00564	Glycerophospholipid metabolism	3
sasa04012	ErbB signaling pathway	3
sasa04270	Vascular smooth muscle contraction	3
sasa04512	ECM-receptor interaction	3
sasa04540	Gap junction	3

sasa00563	Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	3
sasa00620	Pyruvate metabolism	3
sasa04912	GnRH signaling pathway	3
sasa04145	Phagosome	3
sasa04210	Apoptosis	3
sasa04120	Ubiquitin mediated proteolysis	3
sasa00561	Glycerolipid metabolism	3
sasa00010	Glycolysis / Gluconeogenesis	3
sasa04137	Mitophagy - animal	3
sasa04350	TGF-beta signaling pathway	2
sasa04621	NOD-like receptor signaling pathway	2
sasa04330	Notch signaling pathway	2
sasa05132	Salmonella infection	2
sasa04060	Cytokine-cytokine receptor interaction	2
sasa00600	Sphingolipid metabolism	2
sasa04070	Phosphatidylinositol signaling system	2
sasa04933	AGE-RAGE signaling pathway in diabetic complications	2
sasa03018	RNA degradation	2
sasa04115	p53 signaling pathway	2
sasa03320	PPAR signaling pathway	2
sasa04625	C-type lectin receptor signaling pathway	2
sasa04068	FoxO signaling pathway	2
sasa04261	Adrenergic signaling in cardiomyocytes	2
sasa00630	Glyoxylate and dicarboxylate metabolism	1
sasa00524	Neomycin kanamycin and gentamicin biosynthesis	1
sasa00590	Arachidonic acid metabolism	1
sasa00512	Mucin type O-glycan biosynthesis	1
sasa00270	Cysteine and methionine metabolism	1
sasa00790	Folate biosynthesis	1
sasa00514	Other types of O-glycan biosynthesis	1
sasa00052	Galactose metabolism	1
sasa00020	Citrate cycle (TCA cycle)	1
sasa00640	Propanoate metabolism	1
sasa03022	Basal transcription factors	1
sasa00670	One carbon pool by folate	1
sasa04130	SNARE interactions in vesicular transport	1
sasa04370	VEGF signaling pathway	1
sasa05168	Herpes simplex virus 1	1
sasa00250	Alanine aspartate and glutamate metabolism	1
sasa00471	D-Glutamine and D-glutamate metabolism	1
sasa00520	Amino sugar and nucleotide sugar metabolism	1

sasa00450	Selenocompound metabolism	1
sasa03013	RNA transport	1
sasa00601	Glycosphingolipid biosynthesis - lacto and neolacto series	1
sasa00562	Inositol phosphate metabolism	1
sasa00750	Vitamin B6	1
sasa03040	Spliceosome	1
sasa04920	Adipocytokine signaling pathway	1
sasa00220	Arginine biosynthesis	1
sasa00511	Other glycan degradation	1
sasa04622	RIG-I-like receptor signaling pathway	1
sasa04216	Ferroptosis	1
sasa04141	Protein processing in endoplasmic reticulum	1
sasa00260	Glycine serine and threonine metabolism	1
sasa00500	Starch and sucrose metabolism	1
sasa04136	Autophagy - other	1
sasa00051	Fructose and mannose metabolism	1
sasa00970	Aminoacyl-tRNA biosynthesis	1
sasa00920	Sulfur metabolism	1
sasa04260	Cardiac muscle contraction	1
sasa00480	Glutathione metabolism	1

Table B.4: Pathways impacted by miR-29a. Predicted targets for miR-21 were determined by searching the miRNA sequence through MiRanda with all *Salmo salar* 3' UTRs as the database. Targets were then run through KEGG to determine pathways predicted to be impacted.

KEGG Identifier	Pathway	Number of Targets
sasa01100	Metabolic pathways	29
sasa04080	Neuroactive ligand-receptor interaction	15
sasa04510	Focal adhesion	14
sasa04060	Cytokine-cytokine receptor interaction	11
sasa05168	Herpes simplex virus 1 infection	11
sasa04310	Wnt signaling pathway	10
sasa04010	MAPK signaling pathway	9
sasa04810	Regulation of actin cytoskeleton	9
sasa04514	Cell adhesion molecules (CAMs)	9
sasa04910	Insulin signaling pathway	9
sasa04150	mTOR signaling pathway	8
sasa05132	Salmonella infection	7
sasa04512	ECM-receptor interaction	7
sasa04070	Phosphatidylinositol signaling system	7
sasa04144	Endocytosis	7

sasa04530	Tight junction	6
sasa03015	mRNA surveillance pathway	6
sasa00562	Inositol phosphate metabolism	5
sasa04270	Vascular smooth muscle contraction	5
sasa04068	FoxO signaling pathway	5
sasa04217	Necroptosis	5
sasa04350	TGF-beta signaling pathway	5
sasa04114	Oocyte meiosis	5
sasa04140	Autophagy - animal	5
sasa04218	Cellular senescence	5
sasa03013	RNA transport	5
sasa04120	Ubiquitin mediated proteolysis	5
sasa04145	Phagosome	5
sasa04020	Calcium signaling pathway	4
sasa04620	Toll-like receptor signaling pathway	4
sasa04933	AGE-RAGE signaling pathway in diabetic complications	4
sasa04261	Adrenergic signaling in cardiomyocytes	4
sasa04621	NOD-like receptor signaling pathway	4
sasa04916	Melanogenesis	4
sasa04210	Apoptosis	4
sasa04340	Hedgehog signaling pathway	4
sasa04142	Lysosome	4
sasa00601	Glycosphingolipid biosynthesis - lacto and neolacto series	4
sasa04146	Peroxisome	3
sasa04330	Notch signaling pathway	3
sasa04141	Protein processing in endoplasmic reticulum	3
sasa00512	Mucin type O-glycan biosynthesis	3
sasa04110	Cell cycle	3
sasa04115	p53 signaling pathway	3
sasa04371	Apelin signaling pathway	3
sasa04914	Progesterone-mediated oocyte maturation	3
sasa04012	ErbB signaling pathway	3
sasa04625	C-type lectin receptor signaling pathway	3
sasa03460	Fanconi anemia pathway	2
sasa00603	Glycosphingolipid biosynthesis - globo and isoglobo series	2
sasa00534	Glycosaminoglycan biosynthesis - heparan sulfate / heparin	2
sasa00604	Glycosphingolipid biosynthesis - ganglio series	2
sasa04540	Gap junction	2
sasa04622	RIG-I-like receptor signaling pathway	2
sasa04912	GnRH signaling pathway	2
sasa00240	Pyrimidine metabolism	2
sasa00072	Synthesis and degradation of ketone bodies	2

sasa00650	Butanoate metabolism	2
sasa00564	Glycerophospholipid metabolism	2
sasa02010	ABC transporters	2
sasa00280	Valine leucine and isoleucine degradation	2
sasa00515	Mannose type O-glycan biosynthesis	2
sasa03008	Ribosome biogenesis in eukaryotes	1
sasa04744	Phototransduction	1
sasa00380	Tryptophan metabolism	1
sasa00563	Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	1
sasa00480	Glutathione metabolism	1
sasa04216	Ferroptosis	1
sasa00561	Glycerolipid metabolism	1
sasa00510	N-Glycan biosynthesis	1
sasa00350	Tyrosine metabolism	1
sasa00590	Arachidonic acid metabolism	1
sasa04136	Autophagy - other	1
sasa00330	Arginine and proline metabolism	1
sasa03040	Spliceosome	1
sasa04623	Cytosolic DNA-sensing pathway	1
sasa00740	Riboflavin metabolism	1
sasa04672	Intestinal immune network for IgA production	1
sasa00270	Cysteine and methionine metabolism	1
sasa03018	RNA degradation	1
sasa00360	Phenylalanine metabolism	1
sasa04137	Mitophagy - animal	1
sasa00790	Folate biosynthesis	1
sasa00511	Other glycan degradation	1
sasa00062	Fatty acid elongation	1
sasa00531	Glycosaminoglycan degradation	1
sasa00052	Galactose metabolism	1
sasa03430	Mismatch repair	1
sasa04370	VEGF signaling pathway	1
sasa01212	Fatty acid metabolism	1
sasa00600	Sphingolipid metabolism	1
sasa04920	Adipocytokine signaling pathway	1
sasa00900	Terpenoid backbone biosynthesis	1
sasa03022	Basal transcription factors	1
sasa04260	Cardiac muscle contraction	1