The Effects of Hydraulic Fracturing Flowback and Produced Water on Exercise Capacity and Metabolic Fuel Selection in Rainbow Trout *(Oncorhynchus mykiss)*

by

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ABSTRACT

Hydraulic fracturing is an increasingly popular technique for unconventional hydrocarbon extraction in Canada. As its popularity continues to grow, concerns are being raised regarding its waste product, hydraulic fracturing flowback and produced water (FPW), and its effects on the environment. Over the last decade, several thousand incidents of FPW spills have been recorded in Canada. Many of these releases will eventually make their way into waterways which impact aquatic biota.

This study is the first to investigate the effects of hydraulic fracturing FPW on energetics and swim performance in adult rainbow trout *in vivo.* Using double aortic cannulation in trout exposed to either control (no exposure), diluted FPW, or a salinity-matched solution (SW), swim performance, metabolic fluxes, fuel utilization, and enzyme activity, were assessed.

Swim performance was not significantly impacted by the exposure of fish to either SW (6.64%) or FPW (6.64%). Metabolic fluxes (glycerol and carbohydrate) were also unaffected by SW and FPW. Plasma metabolites presented some noticeable and significant changes as a result of exercise and/or treatment. Both SW-treated and FPW-treated fish had noticeably lower plasma glycerol concentrations, whereas plasma glucose concentration was generally higher but more variable than control. SW-treated fish were the only group showing significant increases in plasma lactate during exhaustive exercise.

Measurement of metabolic enzyme activity produced mixed results. The carbohydrate-related metabolism enzymes, pyruvate kinase, and lactate dehydrogenase were not altered from exposure to either SW or FPW relative to control, whereas lipase enzyme activity was noticeably increased in SW-treated and FPW-treated fish relative to controls.

Oxidative stress biomarkers were also measured, and both SW and FPW exposure induced noticeable and significant alterations in activity. In FPW-treated fish, liver EROD activity significantly increased whereas the same enzyme in gill tissue was noticeably impaired. TBARS showed an inverse response in liver and gill EROD results.

Although there were alterations in some energetic parameters within this study, they were not consistent with the original hypothesis that FPW would impair metabolic fluxes and subsequently alter energetics and swim performance. I interpret these results as either the SW and FPW exposures induced an effect on key parameters measured within the study through direct alterations without altering metabolic fluxes or that FPW and SW induced an indirect metabolic and energetic response via stress axes and compensatory mechanisms.

This thesis is the first to examine the effects of complex hydrocarbon-based pollutants (FPW) exposure on adult rainbow trout metabolic substrate physiology, their energetics, and swim performance responses *in vivo*. Through the culmination of assessing these hazard effects of FPW can we improve risk management and help protect values ecosystems within Canada.

PREFACE

Anika Cyr is the principal researcher for the work within this thesis and therefore assumes all responsibilities for any inaccuracies present. The components of swim performance and fluxes experiment and analyses were modified from previous publications by Turenne and Weber, (2018; doi:10.1242/jeb.171553); Shanghavi and Weber, (1999; doi:10.1242/jeb.202.16.21), and exposure protocols were modified from Weber, (1991) to suit the conditions, resources, and research goals of this work. All experimental work was conducted within the Department of Biological Sciences located at the University of Alberta, North Campus, Edmonton, Alberta, Canada.

Dr. Yueyang (Brian) Zhang provided analyses of enzyme activity, specifically, lipase activity and liver EROD activity. Diane Mielewczyk provided assistance in some surgical cannulation, execution of stepwise swim experiments, tissue collections, and metabolite analyses. Katherine Snihur and Yifeng Zhang provided analyses of the inorganic and organic fractions of the flowback and produced water used in this study respectively. The entirety of this work was conducted under the supervision of Dr. Greg Goss.

All experiments conducted on live organisms (*Oncorhynchus mykiss*) were approved by the University of Alberta Animal Care Committee through AUP00003502 and AUP00001334.

No component of this thesis has been published as of the submission of this work to the University of Alberta.

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Thank you to my committee member Dr. Keith Tierney, your generosity in providing invaluable advice and equipment was key to getting this project off the ground. I would also like to thank Dr. Tamzin Blewett whose support got me (and my work) to the finish line. Thank you both.

Of course, none of this work would have been possible without the use of live animals, for that, I would like to acknowledge and thank the Animal Care staff for caring for our trout who were vital in this work.

Although this work focuses on environmental toxicology, it also focuses on salmonid energetics. My foundational knowledge on this topic stems from my previous research supervisor Dr. Jean-Michel Weber. Your willingness to share your knowledge in the field of energetics gave me the opportunity to discover and pursue this topic with great curiosity. Your impact will not be soon forgotten.

I would also like to thank my lab mates and friends who taught, guided, and supported my work throughout my time at U of A. Thank you Christiana Frost, Diane Mielewczyk**,** Ivy Luu, Abigail Robinson, Chantelle Klaczek, Pat Wang, AJ Abd Elhafiz, Dr. Eric Folkerts, Dr. Marina Mussoi Giacomin, Dr. Alex Zimmer and Dr. Yueyang (Brian) Zhang, and all past and present Goss lab members. As well as my very dear friends back home who have supported me unconditionally, merci Rachel Bergeron and Olivia Pantoja. You were all vital to my success!

Lastly, I would like to acknowledge and thank my supportive partner and parents.

To Daniel Hogan, for the last six years, you have been unconditionally supportive of my aspirations to pursue higher education (BSc + MSc). It can be said that at this point, you most

definitely deserve an honorary Masters degree in this field. Thank you for taking this adventure with me to the ‘west’ coast and learning my research on the fly. Although I tell you often, no words can put into concept the magnitude of your impact on me, this work, and my training as a scientist over all these years. P.S. thank you for letting me turn our apartment into a zoo full of foster animals in the middle of a global pandemic!

Finally, to my lovely and supportive parents, Marthe Cyr and François Cyr. You were both the catalyst of my interest in science and have supported my interest in science with great enthusiasm. I consider myself truly lucky to have had you both as my parents. Cette thèse n’est pas seulement le reflet de mon travail mais aussi de votre dévouement et encouragement inconditionnel. Je vous aimes beaucoup.

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LIST OF ABBREVIATIONS

µL	Microliter
µm	Micrometer
µM	Micromolar
µmol/mL	Micromole per Milliliter
µmol/kg/min	Micromole per kilogram per minute
%	Percentage
AhR	Aryl Hydrocarbon Receptor
Al	Aluminium
As	Arsenic
ATGL	Adipose Triacylglyceride Lipase
ATP	Adenosine Triphosphate
B	Boron
Ba	Barium
β-NADH	Beta-Nicotinamide Adenine Dinucleotide+Hydrogen
BHT	Butylated Hydroxytoluene
BL/sec	Body Length per second
Br(He)	Bromine-Helium
°C	Celsius
C^{14}	Carbon-14

Ca^{2+}	Calcium
Cd	Cadmium
Cl^{-}	Chloride
cm	Centimeter
Co	Cobalt
cpm	Counts per Minute
cpm/mL	Counts per Minute per Milliliter
cpm/kg/min	Counts per kilogram per Minute
Cr	Chromium
Cu	Copper
CYP450	Cytochrome P450
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene Glycol-bis(β-Aminoethyl Ether)-N,N,N′,N′-Tetraacetic Acid
EROD	Ethoxyresorufin-O-deethylase
Fe(He)	Iron-Helium
FPW	Flowback and Produced Water
g	Gravitational Force
g	Gram
g/L	Gram per Liter
H^3	Tritiated Hydrogen

HCl	Hydrochloric Acid
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HF	Hydraulic Fracturing
hr(s)	Hour(s)
HSL	Hormone Sensitive Lipase
K^+	Potassium
KCl	Potassium Chloride
kg	Kilogram
K_3PO_4	Potassium Phosphate
L	Liters
LC_{10}	Lethal Concentration in 10% of an exposed population
LC_{50}	Lethal Concentration in 50% of an exposed population
LDH	Lactate Dehydrogenase
Li	Lithium
Mg^{2+}	Magnesium
Mg-ATP	Magnesium-Adenosine Triphosphate
$MgCl_2$	Magnesium Chloride
MGL	Monoacylglycerol Lipase
mg/L	Milligram per Liter
min	Minute

mL	Milliliter
mL/kg	Milliliter per Kilogram
mL/kg/hr	Milliliter per Kilogram per Hour
mL/min	Milliliter per minute
mM	Millimolar
Mn	Manganese
Mo	Molybdenum
MO_2	Metabolic Rate
MRC	Mitochondria Rich Cells
N_2	Nitrogen
Na^+	Sodium
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaOH	Sodium Hydroxide
NAD^+	Nicotinamide Adenine Dinucleotide
ng/L	Nanogram per Liter
Ni	Nickel
nm	Nanometer
nmol/mL/min	Nanomols per milliliter per minute
P	Phosphorus
PAH	Polycyclic Aromatic Hydrocarbons

Pb	Lead
PBS	Phosphate-Buffered Saline
PCA	Perchloric Acid
PK	Pyruvate Kinase
ppm	Parts Per Million
pV	Plasma Volume
R_a	Rate of Appearance
R_d	Rate of Disappearance
rpm	Rotation per Minute
S	Sulfur
Sec	Second
SEM	Standard Error of Mean
Si	Silicon
SOD	Superoxide Dismutase
Sr	Strontium
SW	Salinity-matched control
TBA	2-Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
TDS	Total Dissolved Solids
Ti	Titanium

TMS	Tricaine mesylate (Tricaine Methanesulfonate)
U_{crit}	Critical Swim Speed
U/mL	Units per milliliter
UCi/kg/min	Micro Currie per Kilogram per Minute
U	Uranium
US	United States
wt/wt	Weight Weight Percentage
Zn	Zinc

CHAPTER 1
GENERAL INTRODUCTION:

HYDRAULIC FRACTURING & FLOWBACK AND PRODUCED WATER:

Hydraulic fracturing (HF) is a common technique used for hydrocarbon extraction in unconventional tight and low permeable rock formations (Becklumb et al., 2015; Stringfellow et al., 2014; National Energy Board, 2011). It was initially developed in the mid-twentieth century to accompany conventional drilling and increase hydrocarbon yields (Becklumb et al., 2015; Stringfellow et al., 2014). HF has since grown in popularity with it predicting to become the primary extraction method in the US (Stringfellow et al., 2014) and Canada by 2035 (Natural Resources Canada, 2020). The transition towards unconventional extraction methods is a culmination of factors, including a decrease in conventional oil production (Rivard et al., 2013), a continuous increase in demand for hydrocarbon (Greene et al., 2006), and the rising price of oil (National Energy Board, 2011). The sum of all these factors has allowed HF to become a highly lucrative technique that continues to be employed with increasing regularity.

HF, specifically horizontal hydraulic fracturing is a multi-step process that uses pressurized fluid injected into bored wells located in low permeable shale/tight oil reserves to liberate and collect hydrocarbons (Soeder, 2020; Wang and Chen, 2019) within a parallel plane of the substrate (Soeder, 2020). The injection fluid consists primarily of water and proppants such as ceramic beads and sand, with a minority stake comprised of a variety of chemical additives (Clancy et al., 2018; Stringfellow et al., 2014). The exact chemical composition of the injection fluid can differ between manufacturers, formations, and well pads, however, they generally contain, biocides, clay stabilizers, friction reducers, surfactants, crosslinkers, pH buffers, and corrosion inhibitors which

in sum assist in maximizing hydrocarbon yield while minimizing damage to extraction equipment (Stringfellow et al., 2014; Vengosh et al., 2014; Wood et al., 2011).

The extraction of hydrocarbons begins with the insertion of an injection fluid into a borehole well (Soeder, 2020). Once filled, the individual wells are pressurized using a wellhead cap which induces aggregates of fissures within the rock substrate (Soeder, 2020). These fissures are maintained open via the aforementioned proppants and allow for the release of hydrocarbon (Stringfellow et al., 2014). Once the pressurized/fractured well is opened, a fluid mixture resurfaces (Stringfellow et al., 2014) and the hydrocarbons of interest as well as hydrocarbon-contaminated wastewater collectively termed flowback and produced water (FPW) are separated (He et al, 2017; Wood et al., 2011). FPW consist of the two major components; the first being the resurfaced injected fluid (flowback water) and the second being produced water, which is incorporated water sourced from endogenous stores within the explored substrate (Soeder, 2020; Stringfellow et al., 2014; Rivard et al., 2013; Wood et al., 2011). Resurfacing FPW tend to act as a transport medium for a variety of compounds from the rock formation, including metals, naturally occurring radioisotopes, a variety of newly formed hydrocarbons (Polycyclic aromatic hydrocarbon, PAH), and very high concentrations of salts (Folkerts et al., 2020; Goss et al., 2015).

Although most of these hydrocarbons stem directly from the rock substrate, others are produced during the fracturing process extended period of high heat and pressure (Folkerts et al., 2020). These further alterations to the initial injection fluid tend to produce very complex mixtures which can be challenging to predict their exact impact on the environment.

The destination of collected FPW can vary. In recent years, the recycling and reuse of FPW has grown to reduce cost and limit water usage in hydrocarbon extraction processes (Goss et al.,

2015; Boschee, 2014). However, when injection fluid can no longer be reused, it will most often be treated in a chemical facility and be disposed of in deep injection wells (Alessi et al., 2017) or in surface tailing ponds (Goss et al., 2015). Although Canadian legislation has been put into place to avoid incidents of untreated FPW release at the well pad, during transit or at disposal, accident still persist throughout Alberta (Goss et al., 2015). For example, 57 and 28 incidents were reported in which produced water in volumes exceeding 1000L was released into the environment between 2021 and 2022 respectively (as of April 2022) (Goss et al., 2015; AER, N.d). Releases of FPW usually stem from the wellpad or during transport or disposal (Vengosh et al., 2014). Regardless of its method of entry into the environment, the presence of FPW in waterways has been shown to cause significant and detrimental perturbations to aquatic life (Weinrauch et al., 2021; Delompre et al., 2019; Blewett et al., 2017; Flynn et al., 2017; Folkerts et al., 2017ab).

FLOWBACK AND PRODUCED WATER; ITS EFFECTS ON AQUATIC BIOTA:

HF has been able to increase the financial value of tight oil reserves and has subsequently resulted in the development of an entirely new sector of the oil and gas industry within Canada and the US (National Energy Board, 2011). However, HF is also tied to serious environmental risks through spills of its waste product: FPW (Blewett et al., 2017; Vengosh et al., 2014; Brittingham et al., 2014; Wood et al., 2011;). Therefore, it is necessary to assess the risks of FPW exposure on susceptible biota to improve risk management of vulnerable ecosystems.

Previous research has observed significant deleterious effects of acute FPW exposure on aquatic biota physiology (He et al., 2018; Blewett et al., 2017; Folkerts et al., 2017ab). These effects were observed in the aquatic vertebrate species zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*). Specifically, impacts on fish cardiorespiratory systems (Folkerts et al.,

2017b), oxidative stress (Blewett et al., 2017), gene expression (He et al., 2018), and energetics (Weinrauch et al., 2021) have been recently evaluated.

In a seminal study, zebrafish acutely exposed to FPW as embryos showed significant cardio-respiratory (Folkerts et al., 2017b) and aerobic performance (Folkerts et al., 2017a) impacts later in life. These impacts include increased developmental malformation (Folkerts et al., 2017b; He et al., 2017), a decline in both heart rate and metabolic rate (MO_2) as well as altered expression of certain cardiac genes (Folkerts et al., 2017b). Interestingly, a significant decline in aerobic swim performance was observed in juvenile zebrafish after having been exposed acutely to FPW as embryos (Folkerts et al., 2017a). In each of these above-mentioned studies, the authors have suggested that the organic compounds present within FPW are the likely culprit for the developmental malformation, cardiorespiratory impacts, reduction in aerobic capacity and fitness in these fish (Folkerts et al., 2017ab).

In addition to cardiac and exercise performance effects, increased oxidative stress and changes in expression of specific oxidative stress and reproduction-related genes have been observed in both zebrafish and rainbow trout (He et al., 2018; Blewett et al., 2017) suggesting possible endocrine and physiological disruption effects by FPW.

Oxidative stress indicators genes such as *cyp1a, cyp1b1, cyp1c1, cyp1c2* were observed to be elevated in zebrafish following exposure to various fractions (solid, organics only) of FPW (He et al., 2018). The alteration of CYP gene activity can be caused by PAHs which are noticeably present within FPW, thereby suggesting that the organic component is causing significant adverse effects on the physiology of the organism (Whyte et al., 2000). Furthermore, downstream oxidative stress enzymes and tissue damage indicators, such as ethoxyresuforin-O-deethylase

(EROD), and thiobarbituric acid reactive substances (TBARS) respectively, were elevated in the liver following acute (24/48hrs) FPW exposures (2.5% and 7.5%) to zebrafish (He et al., 2017). EROD is a crucial enzyme and key biomarker in fish physiology, which represents aryl hydrocarbon receptor (AhR) activation and subsequent activation of the CYP defense cascades, a process of detoxification (Uno et al., 2012; Whyte et al., 2000;). Whereas, TBARS is an indicator of oxidative stress (ROS) damage in the form of lipid peroxidation which affects cellular membrane function (Stepić et al., 2011). Blewett et al., (2017) also observed alterations to oxidative stress enzymes activity, specifically, superoxide dismutase (SOD) in both liver and gill as well as catalase activity in the liver, both following a 48hr exposure in fish. Lastly, estrogenic endocrine genes associated with reproduction, specifically, vitellogenin 1 (*vtg1*) were elevated after FPW exposure in fish (He et al., 2018). This change in gene expression is an indication that a reproductive dysfunction is being induced in fish by the constituents of FPW (He et al., 2018). Together, the changes in gene and enzymatic expression are indicators of physiological perturbations resulting from FPW exposure.

Recently, trout energetics have also been observed to be impacted by FPW exposure (Weinrauch et al., 2021). FPW-treated trout hepatocytes showed a 6.8- and 12.9-fold increase in glucose uptake in acutely (48hr) exposed fish (2.5% and 7.5% FPW) compared to control (Weinrauch et al., 2021). Enzymes such as phosphoenolpyruvate carboxylase (PEPCK), an enzyme associated with gluconeogenesis, was downregulated compared to baseline (control) and parallel salt controls. Although glucose uptake presented no alterations following a 3-week recovery of the same treated fish across all treatment groups when compared to control, this study shows short-term metabolic stress does occur in trout carbohydrate metabolism exposed to FPW (Weinrauch et al., 2021).

FPW exposure has been conclusively demonstrated to cause significant adverse impacts on aquatic vertebrate physiology. While the recent study by Weinrauch et al., (2021) determined that FPW alters metabolic nutrient flux *in vitro* in trout hepatocytes (Weinrauch et al., 2021), nothing is currently known about the impacts of FPW on adult trout metabolism *in vivo*. It is, therefore, crucial to investigate whether the impacts observed in Weinrauch et al., (2021) result in similar changes in metabolic fuel use *in vivo* and whether these impacts result in significant changes in swimming performance during exercise.

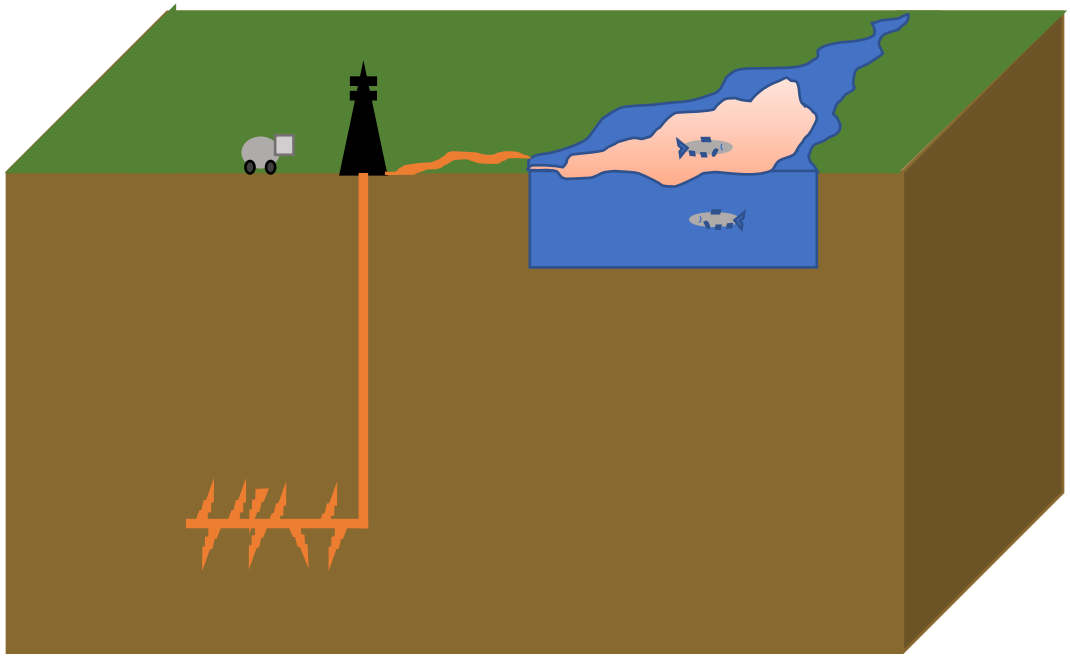


Fig. 1.1. Schematic of flowback and produced water spill into adjacent waterways. A transverse view of a common method of released of FPW into the environment. Salmonids are often located in areas which are near hydraulic fracturing sites and therefore spills of FPW can eventually make its way into adjacent waterways.

ONCORHYNCHUS MYKISS:

Oncorhynchus mykiss is a species within the Salmonidae family (Hardy, 2002) that comprises the residential rainbow trout and the anadromous steelhead trout (Kendall et al., 2015). Both are native to the Pacific coast of North America, with territories extending from Alaska to the southern California coast and Mexico (Hardy, 2002). Rainbow trout also exist extensively as an introduced species and have naturalized populations located worldwide (MacCrimmon, 1971). Rainbow trout can also be found in a unique population located on the inland eastern slope of the Rockies, this population sub-species is known as Alberta's Athabasca rainbow trout (Carl, 1994), a current species at risk (Fisheries and Oceans Canada, 2020; Whyte et al., 2000). Although both residential and anadromous populations participate in distinct lifestyles, they are considered the same species as they can interbreed and their offspring can adopt either behavior (Kendall et al., 2015). Rainbow and Steelhead trout are a vital species and provide an economic, ecological, and scientific value to Canada's waterways.

O. mykiss is a species that contributes significantly to Canada's aquaculture economy (FAO, 2018). Canada has observed a steady increase in revenue from rainbow trout aquaculture since the mid-1970s (FAO, 2018) and, generated over 60 million dollars in annual revenue since 2020 alone (Fisheries and Oceans Canada, 2022). Moreover, rainbow trout, along with other salmonids, contribute significantly to recreational fishing, generating billions in equipment and permit spending by residential and tourist anglers over the last four decades (Brownscombe et al., 2014). Together, the recreational and industrial contribution of *O. mykiss* toward Canada's economy is substantial.

O. mykiss presents significant ecological value within Canada. During migration, *O. mykiss* will navigate towards small tributaries to reach their reproductive site (Kendall et al., 2015; Lucas et al., 2008). During these travels, they deposit nutrients in the form of nitrogen and carbon through the elimination of waste and recycling of carcasses (Naiman et al., 2002). The input of nutrients from migrating trout allows for other organisms located in isolated habitats to acquire valuable resources which would otherwise be unavailable (Naiman et al., 2002). Moreover, as *O. mykiss* travels through various ecosystems they interact within multiple food-webs in which they act as both prey (Stanek et al., 2017; Whyte et al., 2000) and predator (Winfield et al., 2012). The presence of *O. mykiss* provides a source of food for predators, supporting areas that would otherwise be either spatially or temporally nutritionally scarce (Field and Reynolds, 2013), while also controlling insect populations (Winfield et al., 2012) and maintaining ecological equilibrium. All in all, *O. mykiss* is a key sentinel species for the investigation of potential adversely impacted ecosystems.

O. mykiss is also a valuable species for biological research. Rainbow trout have been extensively utilized in ecotoxicological research given that they are considered sensitive to chemical stressors (Teather and Parrot, 2006). The insight into the adverse health effects observed in rainbow trout by anthropogenic pollutants can provide extrapolating data to protect other salmonid species (Teather and Parrot, 2006; Wolf and Rumsey, 1985). The terrestrial and aquatic habitats in Alberta and northern British Columbia have seen a rapid industrial expansion in the past 2 decades because of the development of HF that made tight shale formations for oil and gas extraction available (Rivard et al., 2013). Concurrently, we have seen an increase in industrial releases of waste into the environment affiliated with hydraulic fracturing and hydrocarbon extraction in Canada (COSEWIC, 2014). These releases of waste present a significant risk to future

generations of trout within North America (COSEWIC, 2014) and which rainbow trout can act as a key species model to evaluate the severity of the health effects in aquatic biota.

The sum of these factors provides strong justification for the assessment of the impacts of anthropogenic pollutants (FPW) on salmonid species residing within Canada's waterways. Importantly, the success of this species relies on the success of trout populations completing key tasks such as migration, reproduction, acquiring prey and evading predators. All these tasks are vulnerable to adverse physiological effects induced by hydraulic fracturing and FPW spills. The capacity to successfully complete these tasks is ultimately determined by the ability of trout to access endogenous stores of metabolic fuels in the form of lipids, and carbohydrates and convert them into energy. The potential for FPW to impact fuel utilization in rainbow trout during exercise forms the central thesis of my research.

LIPIDS AND CARBOHYDRATES: THEIR STORAGE AND THEIR MOBILIZATION:

To successfully meet energetic demands during exercise, trout accumulate and subsequently consume endogenous metabolic fuels (Weber, 2011). Lipids and carbohydrates are acquired through diet and are selectively oxidized to synthesize adenosine triphosphate (ATP) (Weber, 2011). Both lipids and carbohydrates are uniquely stored and utilized based on their own characteristics (Weber, 2011).

LIPIDS:

Lipids are a valuable metabolic fuel for *O. mykiss* and other salmonids during exercise (Turenne and Weber 2018; Richard et al., 2002). Following digestion, non-esterified fatty acids and triacylglycerols present within the lumen of the intestines are escorted using chylomicron and lipoproteins in the circulatory system to be stored primarily within adipose tissue, red muscle, and

the liver (Sheridan, 1994; Sheridan, 1988). While small reserves are present in all cells, these storage sites are minimal compared to the aforementioned tissues (Sheridan, 1994). Once lipids arrive at the designated storage location, single fatty acid molecules are stored as long-chained lipids, with the most common form of storage as triacylglycerols (Sheridan, 1988). Fatty acids are bound in triplets to a glycerol-3-phosphate backbone via a series of enzymatic reactions which utilize the following enzymes: glycerol-3-phosphate acyltransferase, 1-acylglycerol-3-phosphate acyltransferase, phosphatidic acid phosphatase and diacylglycerol acyltransferase (Alves-Bezerra and Cohen, 2011; Ahmadian et al., 2007).

Fatty acids characterize themselves with respect to ease of storage within tissues and their energetic density (Weber, 2011). Fatty acids require very little water for long-term storage and yield the highest energetic return of all metabolic fuels (Weber, 2011). Therefore, trout accumulate large volumes of lipids without becoming bulky (Weber, 2011). However, the use of lipids as a metabolic fuel does present some drawbacks. Lipids/fatty acids are slow to mobilize, require carrier proteins to be transported through the circulatory system, and can only be consumed using aerobic respiration (Weber, 2011).

When mobilizing fatty acids from their storage site, individual triacylglycerols are hydrolyzed through a process known as lipolysis (Lass et al., 2011). Lipolysis utilizes a series of enzymes; adipose triacylglyceride lipase (ATGL), hormone-sensitive lipase (HSL,) and monoacylglycerol lipase (MGL) which removes the first, second, and third fatty acid from the glycerol backbone respectively (Lass et al., 2011; Salmerón; 2018). The now liberated non-esterified fatty acids are transported to the mitochondria via carrier proteins to be oxidized (Lass et al., 2011). Once the fatty acids arrive at the mitochondria, they enter a series of metabolic pathways which convert it into metabolic intermediates (Lass et al., 2011). The individual fatty

acids are initially converted into acetyl-COA via β-oxidation, followed by the Krebs cycle, the electron transport chain, and oxidative phosphorylation to eventually synthesize ATP (Weber, 2011).

Given the abundance of lipids within tissues and their energetic density, lipids can be considered the preferred fuel for ATP synthesis in trout during long-distance strenuous aerobic exercise (Turenne and Weber, 2018; Magnoni et al., 2006). The use of fatty acids during strenuous exercise could be viewed as counterproductive, given its slow mobilization speed (Weber, 2011). However, trout and other salmonids mediate this constraint by reducing the lag time for fatty acid delivery (Turenne and Weber, 2018). Salmonids have developed the capacity to mobilize fatty acids at a very high rate (Turenne and Weber, 2018; Magnoni et al., 2008). This high lipolytic rate produces an abundance of fatty acids within the circulation, thus making fatty acids available for immediate consumption during relatively high exercise intensities (Turenne and Weber, 2018).

CARBOHYDRATES:

Carbohydrates are a valuable but limited metabolic fuel for salmonids. In tissues, carbohydrates are primarily stored as glycogen, a large hydrocarbon chained polymer composed of several glucose molecules (Berg et al., 2002). Glycogen reserves are most abundant in hepatocytes and in skeletal muscle (Berg et al., 2002). As a metabolic fuel, glycogen and subsequently glucose presents some strengths and weaknesses (Weber, 2011).

Carbohydrates are considered among the most flexible metabolic fuel, given that they can be utilized during both aerobic and anaerobic conditions as well as can be converted and reduced into lipids for longer-term storage (Dashty, 2013). However, there are some drawbacks, for example, glycogen reserves are limited due to their need to be stored with large volumes of water

(Weber, 2011). Within muscular tissue in fish glycogen reserves only comprises 1% of total muscle weight (Keiffer, 2000). Generally, carbohydrates play a minor role during exercise with the exception of increased use noted during high intensity or burst exercise (Dashty, 2013).

During oxidation, glycogen is broken down into glucose through glycogenolysis (Berg et al., 2002). Glycogen is broken down by glycogen phosphorylase and phosphoglucomutase to produce glucose-6-phosphate, the secondary intermediate within glycolysis (Berg et al., 2002). Glucose-6-phosphate can then enter glycolysis to form pyruvate (Moyes and Schulte, 2016). Alternatively, glucose from the circulation pool can be shuttled and consumed through glycolysis to also form pyruvate (Moyes and Schulte, 2016). Pyruvate then enters the mitochondria and is further converted into high-energy intermediates through the Krebs cycle, the electron transport chain, and oxidative phosphorylation to eventually synthesize ATP (Moyes and Schulte, 2016). Lactate, an indicator of anaerobic metabolism, is produced from glucose in bouts of high-intensity anaerobic exercise (Moyes and Schulte, 2016).

Although carbohydrate reserves are limited, glucose plays a vital role in rainbow trout metabolism. They provide the energy necessary to accomplish the most strenuous exercise (Richard et al., 2002).

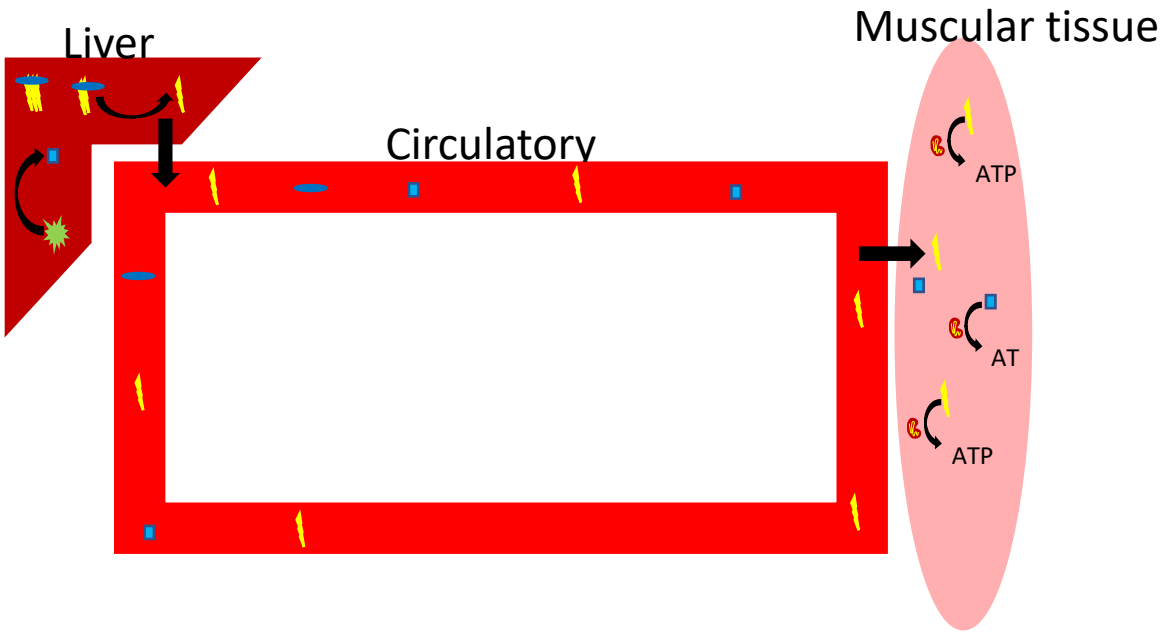

Liver
Circulatory
Muscular tissue
ATP
AT
ATP

Fig. 1.2. Delivery of circulatory metabolic fuels from liver to muscular tissue during exercise. Metabolic fuels are mobilized and selectively oxidized on demand during exercise. While fuels can be stored in all tissues/cells, they are found in large reserves in the liver. Fatty acids (yellow) are mobilized from their storage form, triacylglycerols (yellow and blue) via a series of lipases. These individual fatty acids are subsequently transported through the circulation and enter periphery tissue, such as muscle. Simultaneously as a triacylglycerol becomes fully hydrolyzed, its backbone, glycerol (horizontal blue oval) will be transferred into the circulation to be eventually taken back up and reesterified with other fatty acids into glycerol. Glycogen (green star), the storage form of glucose and will be broken down into glucose via glycogenolysis. The subsequent released carbohydrates also make their way into the circulation where they are also taken up by periphery tissues. These fuels are then converted into ATP via a series of metabolic cascades both adjacent and inside the mitochondria (red and yellow) including, β-oxidation (fatty acids), glycolysis (glucose), electron transport chain and oxidative phosphorylation. Dependance on fuels will vary, however, salmonids depending primarily on fatty acids as a primary fuel of oxidation during most exercise intensities and reserving carbohydrates for more strenuous or anaerobic exercise conditions.

THESIS AIMS:

I propose to evaluate the impacts of FPW exposure on adult rainbow trout metabolism during active swimming. Specifically, I will test if there are changes in lipid and carbohydrate use between FPW-treated rainbow trout and associated control groups during stepwise exercise. Aerobic performance will be evaluated using swim tunnel respirometry and I will assess if exercise performance is impacted as a result of FPW exposure. Moreover, I will examine if there are differences in metabolic fuel selection during exercise between control and FPW-treated fish. I hypothesize that the exposure of rainbow trout to FPW will significantly alter metabolic fuel selection patterns in the form of both metabolite concentration and metabolite flux rate.

Specifically, I hypothesize that there will be a decrease in lipid mobilization/utilization from impaired lipolytic enzymatic activity as a result of FPW exposure which then forces an increase in alternative carbohydrate fuel use in the form of increased flux, enzymatic activity and circulating metabolites to compensate and maintain adequate ATP synthesis in rainbow trout during exercise. I also predict there will be alterations in specific fuel mobilizing enzymes as demonstrated by altered activity. Specifically, lipid mobilizing enzymes are predicted to decrease in activity whereas carbohydrate enzymes are predicted to have a heightened activity following FPW exposure when compared to control-treated fish thereby transitioning fuel mobilization from primarily lipid dependance to carbohydrate dependance. Finally, I predict that because of these changes in fuel selection patterns, trout exposed to FPW will display an reduce swim performance.

CHAPTER 2

INVESTIGATING THE EFFECTS OF HYDRAULIC FRACTURING FLOWBACK AND PRODUCED WATER ON SWIM PERFORMANCE, METABOLISM, AND METABOLIC FUEL SELECTION DURING EXERCISE IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS:*

INTRODUCTION:

Rainbow trout (*Oncorhynchus mykiss*) is a species within the Salmonidae family (Kendall et al., 2015). They are native to the western coast of North America (Hardy, 2002; MacCrimmon, 1971) and present significant economic and ecological value worldwide (FAO, 2018; Field and Reynolds, 2013; Winfield et al., 2012). Rainbow trout are considered an athletic fish species, with certain populations participating in rigorous exercise, such as migrations which can exceed 1000 km in distance, all to complete behavioural or physiological tasks (i.e., catching prey, reproductive migration) (Ohms et al., 2013). Rainbow trout are capable of accomplishing these strenuous tasks thanks to their specialized metabolism.

Salmonids are well adapted to a variety of exercise intensities due to their capacity to store and utilize metabolic fuels efficiently (Turenne and Weber 2018). *O. mykiss* stores the majority of its accessible metabolic fuel reserves as lipids primarily in the liver, adipose, and intramuscular tissue (Turenne and Weber, 2018; Weber, 2011; Moves and West, 1995). Lipids are comprised of large triacylglycerol reserves, a molecule consisting of three fatty acid chains anchored by a glycerol backbone (Salmerón, 2018). This fuel is an ideal resource for a high-performance species, as it is energetically dense and easy to store within tissues given its minimal requirement for water (Weber, 2011; Weber and Haman, 2004). However, the major disadvantage associated with consuming lipids during exercise is their relatively slow mobilization into the circulation and

delivery to target tissues (muscle) (Weber, 2010; Weber and Haman, 2004). To overcome this metabolic shortfall, rainbow trout mobilize abundant levels of fatty acids into the circulation which surpass the fuel demand required to meet most of the animal's energetic needs (Turenne and Weber, 2018).

In addition to lipid use during exercise, rainbow trout present an adequate reserve of carbohydrates in the form of glucose and glycogen (Moyes and West, 1995). These reserves are stored primarily within the liver and intramuscular tissues and are consumed during exercise, albeit to a lower extent than fatty acids (Moyes and West, 1995). Given carbohydrates' relatively quick oxidation speed, and capacity to be consumed in the absence of oxygen, this fuel is preferred in periods of burst exercise, anaerobic conditions or to supplement metabolic demand when fuel such as lipids become insufficient (Moyes and West, 1995).

Salmonid metabolism has evolved to maximize energetic yield within their own metabolic reserves. This, has allowed for this family of fishes to successfully complete challenging high intensity exercise. However, recent evidence suggests that, emerging hydrocarbon-based anthropogenic stressors such as FPW can disrupt this crucial balance in physiology (Weinrauch et al., 2021; Blewett et al., 2017, Folkerts et al., 2017ab).

Waterways that house *O. mykiss* present some direct spatial overlaps with hydrocarbon reserves being currently developed including the Duvernay formation and the Dawson-Montney formation (Fisheries and Oceans Canada, 2020; National Energy Board, 2011). These formations are primarily composed of siltstone, sandstone, and shale and are located between Alberta and northeast British-Columbia (Canadian Energy Regulator, 2013). The exploration of these formations heavily relies on hydraulic fracturing (Teare et al., 2015; Canadian Energy Regulator,

2013) which is a technique that consists of injecting pressurized liquid into rock formations for the extraction of hydrocarbons (Stringfellow et al., 2014). While this technique is effective in extracting otherwise inaccessible oil and gas, it generates large volumes of by-products in the form of FPW (Stringfellow et al., 2014). The risk associated with FPW is the threat of entering the environment through spills (Stringfellow et al., 2014), its propagation and accumulation in adjacent water bodies (Stringfellow et al., 2014; Wood et al., 2011). Numerous incidents attributed to release of hydraulic fracturing waste have been reported within Alberta alone since 2005 (Folkerts et al., 2020) including several reported incidents exceeding 1000L in 2021 and 2022 alone within the region of the Montney formation (AER, 2022).

The spatial overlap of hydraulic fracturing activities and native rainbow trout populations have the capacity to cause harm (Folkerts et al., 2020; Wood et al., 2011). Previous studies have observed adverse impacts of hydraulic fracturing FPW exposure on fish swim performance, metabolic rate (Folkerts et al., 2017a), and oxidative stress (Blewett et al., 2017). Moreover, a recent study by Weinrauch et al., (2021) was the first to demonstrate the impacts of FPW exposure on trout energetics, whereby rainbow trout exposed to FPW presented altered energetic uptake in the liver, a crucial organ for mobilization of circulatory fuels and metabolism. These results are the first to indicate that FPW can alter the availability of metabolic fuels. To our current knowledge, there has been no research assessing whether FPW alters metabolic fuel utilization (fatty acid and glucose availability) within adult trout during exercise *in vivo*. It is therefore imperative to assess the *in vivo* impacts of FPW towards salmonid energetics with the goal of appropriately assessing the hazard from hydrocarbon pollutants in Canada's waterways.

My thesis evaluates the impacts of FPW on adult rainbow trout metabolism during active swimming, specifically examining the changes in fatty acid and glucose dependence during

exercise between treated and control groups. Moreover, aerobic performance was evaluated to assess if exercise performance is impacted because of FPW exposure and metabolic fuel selection. I hypothesize that there will be a decrease in lipid mobilization/utilization from impaired lipolytic enzymatic activity as a result of FPW exposure which then forces an increase in alternative carbohydrate fuel use in the form of increased flux, enzymatic activity and circulating metabolites to compensate and maintain adequate ATP synthesis in rainbow trout during exercise. I predict there will be alterations in specific fuel mobilizing enzymes as demonstrated by altered activity. Specifically, lipid mobilizing enzymes are predicted to decrease in activity whereas carbohydrate enzymes are predicted to have a heightened activity following FPW exposure when compared to control-treated fish. Finally, I predict that because of these changes in fuel selection patterns, trout exposed to FPW will display an impaired swim performance.

METHODS:

RAINBOW TROUT:

Adult rainbow trout were sourced from Allison Creek Hatchery (Crowsnest Pass, Alberta) and were held within the aquatic facility at the University of Alberta (Edmonton, Canada) in appropriately sized flow-through tanks filled with oxygenated dechlorinated City of Edmonton tap water maintained at a temperature of approximately 9-10°C. Fish were cared for daily and fed a salmon pellet diet (EWOS Vita, Surrey, Canada) every other day.

ORGANIC AND INORGANIC ANALYSES OF FLOWBACK AND PRODUCED WATER:

The flowback and produced water sourced within this study was collected within the Montney formation in Dawson, British Columbia. FPW was sourced specifically from Well-P, 02-12-81w6 on June 2nd 2019, 4hrs following the opening of the well. FPW was composed of freshwater (source) and was considered to be an anionic high viscosity composition once recovered. Samples were collected and analyses for both organic and inorganic composition. Organic composition was analyzed via Sun et al., (2019) while inorganic analyses was conducted via inductively coupled mass spectrometry similarly to Boyd et al., (2022).

ESTABLISHING TOXICITY OF FLOWBACK AND PRODUCED WATER: LC_{50} (SERIES 1)

A preliminary LC_{50} toxicity experiment (series 1), was conducted to establish a range of toxicity from the specific batch of hydraulic fracturing FPW used in this study as well as provide a general guideline on appropriate exposure concentrations for later experiments (Weber, 1991). Raw FPW sourced from Well-P (02- 12-81-18w6; June 2, 2019, 4Hrs) located on the Dawson-Montney formation was diluted to 2.5%, 5%, 7.5%, 12%, 15%, and 20% of its original strength (100%) using the city of Edmonton dechlorinated tap water. A 0% control was also present which

consisted entirely of the city of Edmonton's dechlorinated tap water. Each aforementioned concentration was conducted in triplicates in continuously oxygenated 8L tanks with 6 juvenile rainbow trout (approximately 5-7cm) of both sex in each tank. All tanks were fastened with a lid to limit evaporation and avoid fish escaping. Tanks were also partially externally bathed in continuously flowing dechlorinated facility water which maintained the tanks at approximately 10°C. The exposure period was conducted for 96hrs, in which the exposure was static non-renewal, this method was chosen over others given the limited volume of FPW. Throughout the entirety of the experiment, exposure tanks were checked every 24hr, dead trout were removed. Trout which showed significant impairment and were expected to die imminently (i.e., complete loss of equilibrium, very slow operculum movement, little to no reaction to touch) were considered 'dead' and therefore also removed from the exposure tanks. As a result, 100% of the trout in tanks 0%, 2.5% and 5% survived the entirety of the 96hr exposure, fish in tanks consisting of 7.5%, 12% and 15% FPW suffered partial death after 96hrs whereas all fish in 20% exposure tanks were dead by the 96hr mark. From the LC50 toxicity assay, a Gaussian model was utilized via the Toxicity Relationship Analysis Program (TRAP; 1.30a; EPA, Washington, DC, USA) software system and an LC10 was established as 6.64%. The concentration of 6.64% was utilized as the working exposure concentration for the remainder of subsequent experiments.

EXPOSURE PROTOCOL: SERIES 2

Individual adult rainbow trout (0.642 ± 0.025kg) were transferred into continuously oxygenated static non-renewal 15-liter tanks fastened with a lid and black out cover. Fish were exposed to either facility water (city of Edmonton dechlorinated water) as a control, a diluted salinity-matched solution treatment (SW) or diluted raw FPW effluent treatment (series 2). The SW treatment consisted of a stock saline solution composed of nanopure water and laboratory-

grade salts (Sigma-Aldrich: Na+, Cl-, Mg2+, Ca+, K+) at concentrations representative of the salinity of FPW (Blewett et al., 2017). The stock saline solution was then diluted to 6.64% of its original strength using the city of Edmonton dechlorinated water with the goal of matching the FPW saline strength. The saline control allows for differentiation of the physiological response of salt versus organics present within the FPW (Blewett et al., 2017). FPW exposure tanks consisted of diluted raw FPW (6.64%) from the Dawson-Montney Formation (P-well; 02-12-81-18w6; June 2nd 2019, 4hrs) diluted in the city of Edmonton dechlorinated tap water.

Exposures lasted 24hrs and fish were fasted throughout the exposure phase as well as subsequent experimental phases. Following the exposure, each trout was transferred into flowing, clean dechlorinated water for the remainder of the subsequent experiments. Exposure of fish was conducted under the supervision and approval of the University of Alberta Animal Care Service AUP00003502 and AUP00001334.

SURGICAL CANNULATION:

Following exposure treatment, individual adult rainbow trout were anesthetized using TMS (Trimethylsulfonate; 0.1g/L) and buffered with sodium bicarbonate (0.2mg/L). Catheterization was conducted as noted in Turenne and Weber (2018), Haman and Weber (1996), and Haman et al., (1997) with some modifications (see Fig. 2.1). Briefly, anesthetized rainbow trout were catheterized between the 3rd – 4th gill arch and 1st – 2nd gill arch with two catheters (Instech-PE-50). The first cannula measuring approximately 12-14cm was inserted behind the palette between the 3-4th gill arch (Haman and Weber 1996; Turenne and Weber, 2018). The second catheter, measuring approximately 6-8cm and inserted between the 1st-2nd gill arch (Haman and Weber, 1996; Haman et al., 1997; Turenne and Weber, 2018). The first and second catheters play separate

roles for infusion and blood sampling, respectively (Turenne and Weber, 2018). Both catheters were filled with heparinized Cortland saline solution (50U/mL) for the purpose of minimizing the formation of blood clots at the surface and within the catheter (Turenne and Weber, 2018). Following catheterization, rainbow trout were placed into the 90L swim tunnel. They were left to recover for 12-16h at a speed of approximately 0.5 Body length/sec, a speed previously used by Choi and Weber (2016). This allowed for optimal support and oxygenation of the animal while reducing active swimming (Choi and Weber, 2016). The recovery period allows for any heparin to be eliminated from the circulatory system prior to isotopic infusion and swim protocol experiments (Hunn et al., 1968).

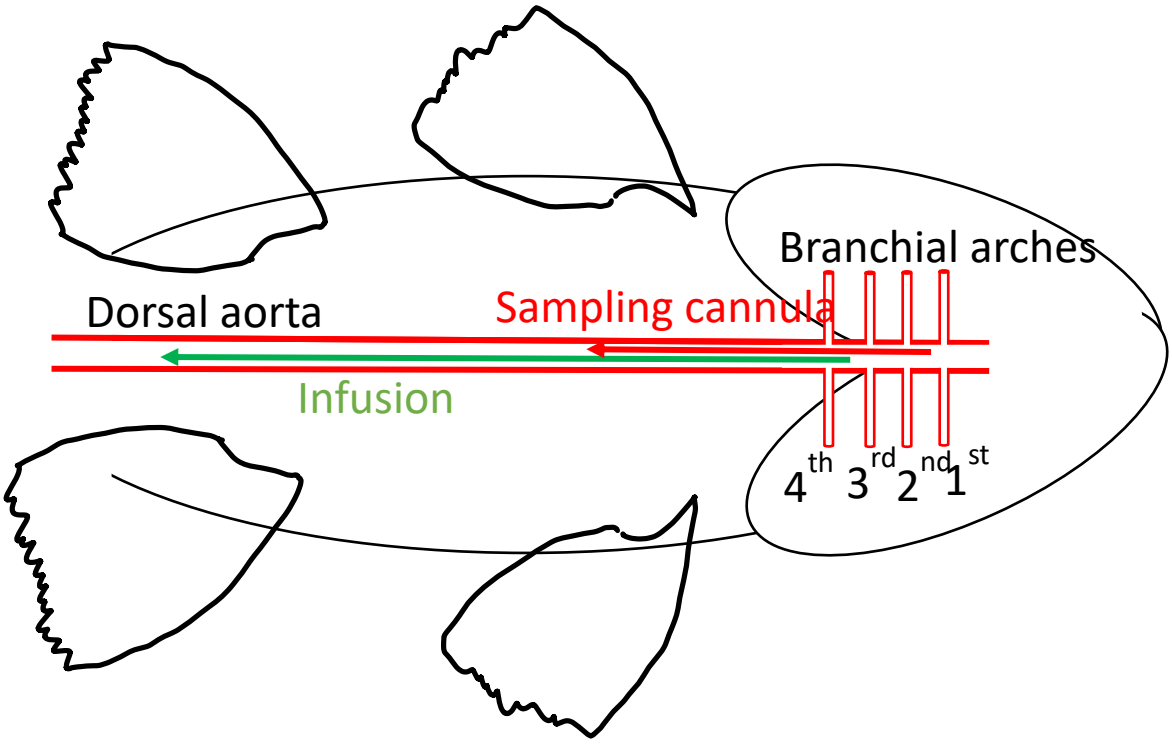


Fig. 2.1. Schematic of double aortic cannulation. Shows placement of catheters inserted into the dorsal aorta. A ventral view of the insertion of cannulae into the dorsal aorta. Arrows present the approximate length and direction of blood flow. The infusion cannula is used to administer radiotracer solutions whereas the sampling cannula was used to collect period blood draws.

RESPIROMETRY:

Oxygen measurements during the stepwise swim protocols in a 90L swim tunnel (see Fig. 2.2) were recorded using a Witrox 1, DAQ- M, and Autoresp software (Loligo format 2.2.0). Oxygen consumption was used as a proxy for metabolic rate (MO_2) as aerobic metabolism requires oxygen to generate ATP and therefore acts as an indicator of energetic requirements (Turenne and Weber, 2018; Shanghavi and Weber, 1999). The temperature of the swim tunnel was measured throughout the swim experiment and was maintained at approximately 10 ± 1°C.

STEPWISE SWIM PROTOCOL:

The performance stepwise swim protocol was conducted through a modified protocol of Turenne and Weber (2018) (see Fig. 2.3). Briefly, baseline swim speed was established as 0.9bodylength/sec (BL/sec) and increased in increments of 0.1BL/sec every 20min over 4h. Critical swim speed (U_{crit}) is described as the animal becoming incapable of keeping up with the current water and is incapable of removing itself from the back grate of the swim tunnel (Turenne and Weber, 2018).

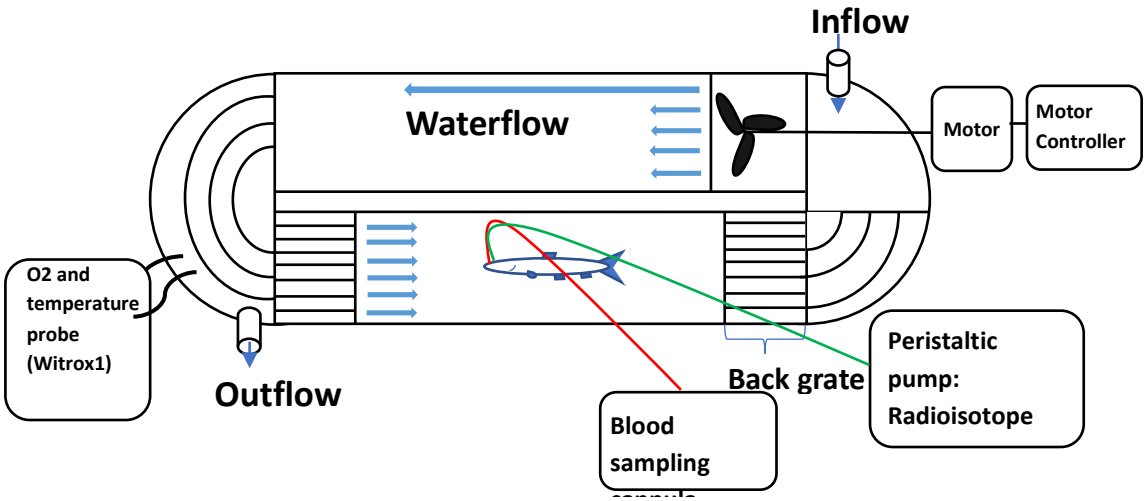


Fig. 2.2. Schematic of swim tunnel. Rainbow trout were inserted into a 90L swim tunnel and subjected to intermittent respirometry. An overhead view of the swim tunnel, its components and the position of the rainbow trout. Components including O_2 measurement equipment (Witrox 1), temperature probes, pumps, motor and motor controller are presented in figure. The entire swim tunnel is covered with a opaque tarp to remove the factor of light on metabolism.

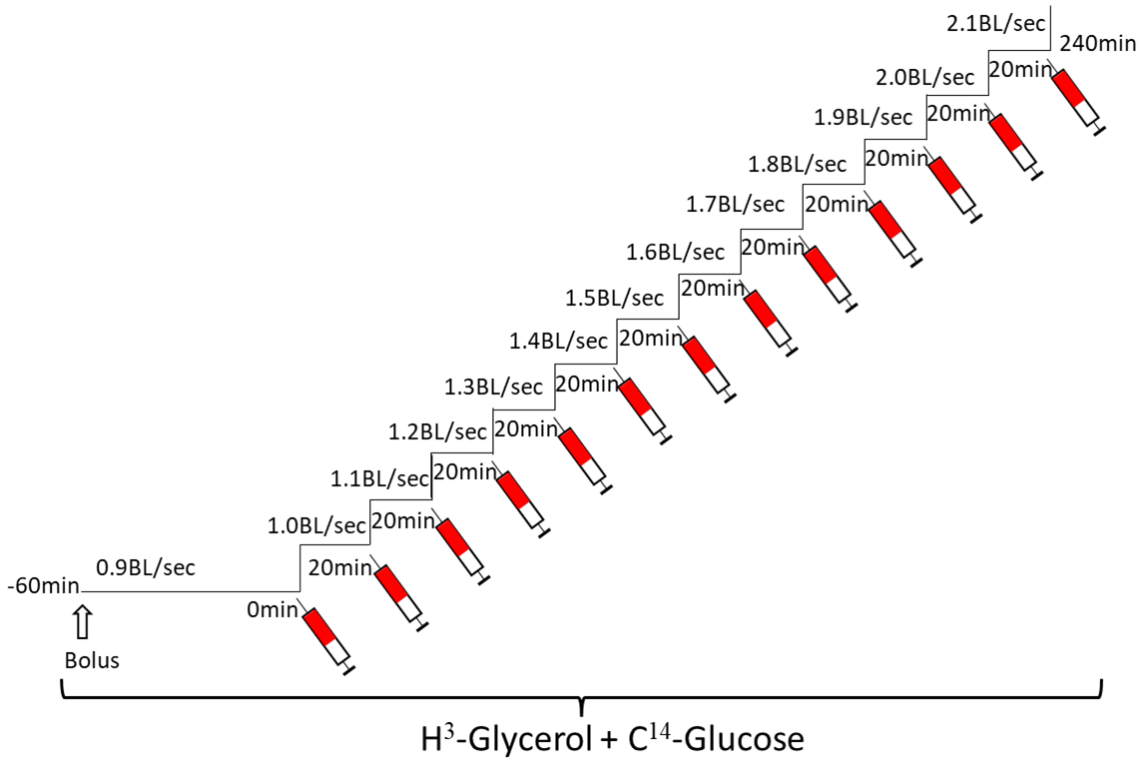


Fig. 2.3. Stepwise swim protocol, infusion, and blood sampling. Figure depicts the administration of radioisotope tracer from bolus to 240minutes (or less dependant on U_{crit}), stepwise swim (BL/sec) and blood sampling protocol (syringes) in one. Briefly, fish were administered a bolus and a continuous infusion 60minutes prior to start as well as throughout the the stepwise swim protocol. Blood samples were taken throughout the experiment to analyze blood metabolites and flux measurements at varying intensity of exercise.

RADIOISOTOPE INFUSION:

An administered bolus of D-C^{14}(U)-glucose followed by continuous infusion of 2-H^3(N)-glycerol and D-C^{14}(U)-glucose protocol was adapted from Turenne and Weber (2018) and Shanghavi and Weber (1999) respectively.

Briefly, both 2-H^3(N)-glycerol (Perkin-Elmer; NET022L001MC) and D-C^{14}(U)-glucose (Perkin Elmer; NEC042X250UC) were prepared by taking an aliquot of both radioisotopes, mixed and dried under N_2 gas to remove suspension fluid (ethanol and water; 9:1). The dried radioisotopes were then resuspended in Cortland saline at a volume of 2mL/kg/hr (Turenne and Weber, 2018; Shanghavi and Weber, 1999). A bolus of D-C^{14}(U)-glucose equivalent of 90min of infusion was prepared using the same drying and resuspension protocol as the 2-H^3(N)-glycerol and D-C^{14}(U)-glucose infusate (Shanghavi and Weber, 2018).

To administer the radioisotope, the bolus dose of D-C^{14}(U)-glucose was first provided through the infusion cannula. Following the administration of the bolus, the glycerol-glucose mixture comprised of 2-H^3(N)-glycerol and D-C^{14}(U)-glucose was administered continuously through the same cannula via a peristaltic pump (see figure 2.1). The administration of the bolus initiated a 1hr priming period prior to the swim protocol. Infusate was administered at a volume rate of 2mL/kg/hr and at a rate of 0.046147 ± 0.00102 μCi/kg/min and 0.018244 ± 0.000391μCi/kg/min for 2-H^3(N)-glycerol and D-C^{14}(U)-glucose respectively (Turenne and Weber, 2018; Shanghavi and Weber, 1999).

BLOOD SAMPLING:

A preliminary blood sample (250uL) was collected prior to the administration of radioactive material, this provided us a baseline for blood metabolites at rest (Turenne and Weber, 2018). Following administration of bolus and during the continuous infusion of the radioactive

glycerol-glucose mixture, individual baseline measurements were collected at -10, -5, 0min (equivalent to 50, 55, and 60min after bolus administration) prior to initiation of the swim protocol (Turenne and Weber, 2018). Following the 0min mark, blood (250μL) was drawn from the sampling cannulae, every 20mins over the course of the swimming protocol for a maximum of 4h (Turenne and Weber, 2018). Blood sampling did not exceed 10% of total animal blood volume as in accordance with the University of Alberta Science Animal Support Services (SASS) guidelines (Dennis et al., n.d.). Each blood sample was deproteinized with 500uL of perchloric acid (PCA) 6% wt/wt (1:2 blood: perchloric acid) (Turenne and Weber, 2018) centrifuged at ~11752g (12000rpm) for 5min (Choi and Weber, 2016), and the supernatant was collected and immediately frozen in liquid nitrogen and stored at -80C for glycerol, glucose and lactate concentration, and metabolite flux measurements (Turenne and Weber, 2018; Shanghavi and Weber, 1999).

TISSUE COLLECTION:

Once the fish were impinged on the back grate of the swim tunnel and considered exhausted, tissue collection was conducted. Fish were euthanized via cephalic blow. This method was used over chemical euthanasia via overdose of tricaine mesylate (TMS) since TMS is known to alter enzyme activity in fatty acid metabolism (Harrington et al., 1991; Hunn et al., 1968). Tissues were collected in the same sequence; gill, liver, and white muscle (located between the dorsal and adipose fin) from every fish. All tissues were placed in precooled aluminum foil and snap-frozen in liquid nitrogen. Samples were then stored at -80C for later enzyme activity analyses.

CARBOHYDRATE AND GLYCEROL FLUXES:

Glycerol fluxes were measured using a modified technique previously noted in Turenne and Weber (2018). Due to H^3 capacity to dissociate from glycerol and bind to glucose and water through biochemical reactions within the organism, both tritiated glucose and water were removed

using an ion-exchange column (Dowex50x8 HCl form, Dowex 1x8 NaOH form and Dowex 1x8 Formic acid form) and evaporation of water using N_2 with heat respectively (see appendix) (Turenne and Weber, 2018). Briefly, to remove tritiated glucose produced within the animal, the supernatant was charged with hexokinase, ATP, glucose-6-phosphate dehydrogenase, and NAD+ for 60minutes (Turenne 2018; Turenne and Weber, 2018). The treated supernatant was then added to the ion exchange column (Chromspec: UCTRFV0004P Reservoir, 4mL; UCTFR10041P, 10um, 1/16", 4mL) prepared with the aforementioned resins, and the column was washed with 5ml of nanopure water, and the eluate was collected in a glass scintillation vial (Turenne and Weber, 2018). Next, samples were allowed to dry in a heated gas box under a continuous stream of N_2 (Turenne and Weber, 2018). Once dry, samples were resuspended in 1mL of nanopure water and 5mL of scintillation fluid (Ultima Gold, Perkin Elmer) (Turenne and Weber, 2018). Samples were allowed to incubate in a dark space for a minimum of 24hrs to reduce chemiluminescence. Samples were then counted using a Hitachi Aloka Accuflex LSC-8000.

Whole carbohydrate activity fluxes (glucose + lactate) were measured by adding 50uL of deproteinated (6% wt/wt perchloric acid) with 5mL of scintillation fluid (Ultima Gold, Perkin Elmer) using a protocol from Blasco et al., (2001) with research appropriate modifications (volume of plasma and scintillation fluid type). Samples were allowed to sit for a minimum of 24hrs prior to reading with the beta counter (Hitachi Aloka Accuflex LSC-8000).

ANALYSES OF METABOLITES:

Glycerol, glucose, and lactate concentrations were assessed using spectrophotometry (Molecular Devices, Versa max, microplate reader) and using previously described protocols (Turenne and Weber, 2018; Shanghavi and Weber, 1999). Briefly, glycerol concentration analyses were comprised of combining 20uL of plasma sample with glycerol phosphate dehydrogenase,

glycerol kinase enzyme solution (Ammonium sulfate; 3.2M; 80U/mL glycerokinase; 1% ethylene glycol), NAD+ (2mM), ATP (2mM), glycerol dehydrogenase (10U/mL) and hydrazine buffer (Hydrazine Monohydrate, 350mM; Hydrazine dihydrochloride; 50mM) and incubated for 80min and spectroscopically measured at 340 nm against a standard curve (Turenne and Weber, 2018). For glucose concentration, plasma samples (5uL) were incubated with hexokinase (7U/mL) and glucose-6-phosphate dehydrogenase (0.3U/mL), NAD+ (2.22mM), and ATP (1.1mM), and then incubated for 40min and spectroscopically measured at 340 nm against a standard curve (Shanghavi and Weber, 1999). Lactate concentrations were determined using lactate dehydrogenase (322U/mL), glycine (0.6M)/hydrazine buffer (0.5M) and NAD+ (2.5mg/mL) incubated with 5uL of plasma sample and were incubated for 50min before spectroscopically measuring concentration at 340 nm against a standard curve (Turenne and Weber, 2018). All assays were run in either duplicate or triplicate and incubated at room temperature.

ENZYME ACTIVITY ANALYSES:

A series of oxidative stress biomarkers were measured to determine activity changes between treatment groups. Oxidative stress markers such as ethoxyresorufin-O-deethylase (EROD) and thiobarbituric acid reactive substances (TBARS) were both analyzed in the gill and liver.

EROD analyses were conducted on both gill and liver tissues. Both tissues were treated the same, briefly, 0.15g of previously frozen tissue was ground in a mortar and pestle chilled with liquid N_2. Once ground with a mortar and pestle samples were suspended in KCl-HEPES homogenization buffer (KCl, 149.96mM; HEPES, 20.02mM; pH 7.4). Samples were homogenized using a sonicator (Virtis Virsonic 100 ultrasonic cell disrupter, 115v, 1amp, 60Hz) while on ice (Blewett et al., 2017). The homogenized tissues were then centrifuged at 10,000***g*** at 4 °C for 20min

(Blewett et al., 2017). 50µL of supernatant was then transferred into a 96well-clear bottom-black plate. 200uL of cold EROD buffer (Tris HCl, 91.8mM; EDTA, 1.0mM; 7-ethoxyresorufin, 2.5µM, pH7.4) (Blewett et al., 2017). An NADPH solution (10uL; 5mM) was added to initiate the reaction. Samples were incubated in darkness on a shaker plate for 20min (Blewett et al., 2017). Samples were read using fluorescence spectrophotometry 535/595nm. Results are presented as fold change relative to control (Blewett et al., 2017).

TBARS analyses in both gill and liver were conducted using the protocol He et al., (2017) and Blewett et al., (2017). Briefly, individual samples of gill or liver were homogenized using a sonicator (Virtis Virsonic 100 ultrasonic cell disrupter, 115v, 1amp, 60Hz) in a phosphate buffer (PBS 100mM, 5mM EDTA, pH 7.5) (Blewett et al., 2017; He et al., 2017). The samples were then centrifuged (1000***g*** for 120sec) and the supernatant was incubated with butylated hydroxytoluene (BHT, 1mM), trichloroacetic acid (50%) and 2-thiobarbituric acid (TBA, 1.3%) (He et al., 2017; Blewett et al., 2017). Samples were then centrifuged again at 13000***g*** for 2min and subsequently incubated for 90min at 60°C (Blewett et al., 2017; He et al., 2017). Samples were then analyzed using a fluorescence spectrophotometer at a 530/533nm (excitation/emission). Samples results are presented as fold change relative to control.

Lactate dehydrogenase activity was analyzed in white muscle. Briefly, white muscle tissues of each fish were homogenized using an extraction buffer (HEPES, 1.0mM; EDTA, 20mM; 0.1% triton X-100). Homogenate was then centrifuged at 16000***g*** at 4°C for 5 minutes. Subsequently the supernatant was collected and combined with imidazole (0.0025mM) dissolved in MilliQ (nanopure) water, pH 7, sodium pyruvate and β-NADH and measured at 340nm every 30sec for 15min. A Bradford assay was also performed to measure proteins.

Pyruvate kinase activity was measured by grinding white muscle tissue using a chilled mortar and pestle (liquid N_2). Processed tissue was suspended in homogenization buffer (K_3PO4, 100mM; EGTA, 1mM; EDTA, 1mM; 0.1% Triton X-100, pH.7.2) at a ratio of 1:10. Samples were homogenized with a handheld motorized homogenizer and centrifuged at 10000***g*** for 20min. 2.5uL of supernatant diluted to 2x its original strength was plated on a clear bottom 96-well plate. 200uL of assay buffer (Phosphoenolpyruvate, 10mM; Mg-ADP, 2.5mM; MgCl2, 10mM; NADH 0.3mM, lactate dehydrogenase 5Units/mL) was added to the plate. Samples were read kinetically on a spectrophotometer at 340nm (Molecular Devices, VersaMax) over 5min in 15sec cycles. A Bradford assay was also conducted to measure proteins.

Lipase activity was measured using a commercially available kit (Lipase Assay Kit; abcam102524). Briefly, red muscle tissue (40mg) previously frozen was homogenized with assay buffer using a sonicator (Virtis Virsonic 100 ultrasonic cell disrupter, 115v, 1amp, 60Hz). The homogenate was then centrifuged at 14,000rpm for 5min at 4°C. Supernatant was then collected and kept on ice. Assay was conducted through combining sample (50uL) with assay buffer (oxiRed Probe, enzyme mixture and lipase substrate). Wells were kinetically measured at 570nm every 2 minutes for 90minutes at 30°C. Data was compared to a standard curve made alongside samples. Lipase activity was presented as fold change.

STATISTICAL ANALYSES:

All data presented was first analyzed for normal distribution via a Shapiro-Wilks or d'Agostino-Pearson test. If the data failed the aforementioned test, it was log transformed and reanalyzed for normality. If data still didn't pass the normality test, statistical analyses were conducted using non-parametric analyses with a Kolmogorov-Smirnov or Kruskal-Wallis test. All physical parameters were measured through a one-way ANOVA using a Tukey *post-hoc* (see

Table 2.3.). The acute toxicity assay (LC_{50}) was first analyzed through the Toxicity Relationship Analysis Program (TRAP; 1.30a; EPA, Washington, DC, USA) software system followed by a two-way ANOVA mixed-effect model), with a Dunnett's Multiple comparison tests using a concentration 0% FPW as baseline (see Figure 2.4.). Swim stamina of adult rainbow trout (see Figure 2.5.) was analyzed using a two-way ANOVA mixed-effect model and inter-treatment (effects of FPW and SW) comparison using a Tukey *post-hoc*. Metabolic rate (see Fig. 2.6.) were analysed using a two-way ANOVA with inter (effects of FPW and SW) and intra-treatment (effects of exercise) analyses using a Tukey and Dunnett's *post hoc* respectively. Intra-treatment comparisons were conducted using each treatment groups baseline value at 0.9BL/sec / 0min. All circulating plasma metabolites (glycerol, glucose and lactate) (see Fig. 2.7-2.9) passed either Shapiro-Wilks or d'Agostino-Pearson normality test. All three metabolites were measured via inter and intra-treatment analyses previously mentioned in metabolic rate. Glycerol flux (R_a and R_d) (see Fig. 2.10.), was first log transformed to meet normality (Shapiro-Wilks) and analyzed using a two-way ANOVA with inter and intra-treatment analyses, similarly to MO2 and plasma metabolites. Net glycerol flux (see Fig. 2.10.) was analyzed via Kolmogorov-Smirnov non-parametric analysis. Carbohydrate flux (glucose + lactate) R_a and R_d (see Fig. 2.11.), did not meet normal distribution and therefore they were analyzed using non-parametric analyses Kolmogorov-Smirnov test. Enzyme activity of EROD, LDH, PK (see Fig. 2.12.; 2.15.; 2.16.) were analyzed using a one-way ANOVA with Tukey *post hoc.* TBARS (gill and liver) (see Fig. 2.13) and lipase (see Fig.2.14) activity were analyzed through non-parametric analyses, Kruskal Wallis with a Tukey *post hoc* comparing SW and FPW-treated groups to the control group as well as comparing each treatment group to each other values. Significances were noted in results.

RESULTS:

LC50 toxicity results showed that juvenile rainbow trout were susceptible to the FPW used in this study. At concentrations below 5%, no rainbow trout died throughout the entirety of the 96hr exposure. At concentrations varying from 7.5 to 15% partial mortality was observed in exposed rainbow trout p = 0.0226-0.8638. Finally, all rainbow trout exposed to a concentration of 20% FPW died within 96hrs of exposure (see Fig. 2.4.).

There were no differences in physical parameters across all treatment groups (p = 0.2921 – 0.8303) (see Table 2.3.).

SW and FPW treatments had no statistically significant effect on swim stamina in adult rainbow trout (see Fig. 2.5.), (p=0.3871). However, SW and FPW-treated fish began to noticeably decline in swim performance earlier (at 1.2BL/sec) relative to control (at 1.4BL/sec). Due to high variability in responses, each treatment group overlapped and this was exacerbated because as speed increased, there was significantly declining participation. Moreover, maximal stamina between all three treatment groups was found to be between 1.9 and 2.1BL/sec.

Metabolic rate (MO_2) (see Fig. 2.6.) is another metric of swim performance that indicates indirectly the required energy necessary to complete a given task (i.e., swimming). Oxygen is required for aerobic conversion of all fatty acids and carbohydrates into ATP. There were no statistically significant differences in oxygen consumption between treatment groups (p = 0.1323-0.999) where SW and FPW treatments did not induce significant alterations in MO_2 during exercise. However, it should be noted that a noticeable trend was observed in which FPW treated fish had consistently higher MO_2 relative to control and SW-treated fish. FPW tended to have a MO2 which was on average 10 to upwards of 150mg O_2/Kg/Hr higher than the control and/or SW treated group throughout the entirety of the experiment.

Exercise intensity significantly increase MO_2 over time within each treatment groups relative to the baseline MO_2 value (0.9BL/sec). The control-treated group had a significantly increased MO_2 relative to baseline at 1.5 to 1.8BL/sec ($p = 0.0139 – 0.0392$). The salinity-matched group had a significant increase in MO_2 relative to baseline from 1.1 to 1.6BL/sec ($p = 0.0052 – 0.0327$) while the FPW-treated group had a significant increase of MO_2 relative to baseline from 1.2 to 1.5BL/sec ($p = 0.0144 – 0.0410$). (see Fig. 2.6.)

Plasma glycerol concentration (see Fig.2.7.), a proxy for fatty acid metabolism, was measured in plasma in all treatment groups during stepwise swimming. Both treatment (FPW and SW) ($p = 0.4274 – 0.9999$) and exercise (SW, $p = 0.2356$-0.9996; FPW, $p = 0.1470$-0.999) had no effect on plasma glycerol concentration within the treatment groups tested. Control-treated fish did present a noticeable but not significant ($p = 0.0889$-0.9997) increase in glycerol concentration throughout the stepwise swim from 1.6 – 1.8BL/sec. Glycerol range at the aforementioned speed in control-treated fish was found to be between 0.5 and 0.66mM, however this trend may be the result of a relatively low sample size at higher swim speeds ($N \leq 3$). SW-treated and FPW-treated fish presented glycerol concentrations which was consistent with other studies (Turenne and Weber, 2018)

Plasma glucose concentrations (see Fig. 2.8.) was measured in all treatment groups during stepwise swimming. There were no significant differences in glucose concentration when comparing treatment groups ($p = 0.1323 – 0.9999$), therefore SW and FPW did not induce any significant alterations to plasma glucose concentration relative to control-treated fish. However, plasma glucose did fluctuate noticeably more in SW-treated and FPW-treated groups whereas control-treated fish had plasma glucose which was more stable. Notably, both SW-treated and FPW-treated fish had higher than normal plasma glucose concentration initially during the

stepwise exercise. This trend dissipated, and FPW-treated fish plasma glucose decreased below normal range as exercise intensity increased, whereas SW-treated had a dip in plasma glucose mid-way through the exercise but quickly increased blood plasma glucose at the highest exercise intensities.

Plasma lactate (see Fig. 2.9.), a metabolite produced as a result of anaerobic metabolism was measured in all treatment groups during stepwise swimming. Both exercise and exposure treatment generated significant increase in plasma lactate at the higher swim speeds. FPW-treated fish had increased plasma lactate at 1.0BL/sec (p = 0.0280) relative to baseline (0.9BL/sec). Additionally, SW-treated fish presented a significant increase in plasma lactate at 1.6BL/sec relative to the same timepoints control-treated fish value (p = 0.0478). There was a noticeable increase in plasma lactate concentration in SW-treated fish from 1.3 to 1.8BL/sec. FPW-treated fish also presented a similar trend in which plasma lactate increased at 1.3BL/sec to 1.5BL/sec. The sample size in total was of N=6 across all treatment groups.

Plasma glycerol flux (see Fig.2.10.); rate of appearance (R_a), rate of disappearance (R_d), and net flux (R_a-R_d) of radiotracer in the circulation were measured during stepwise exercise in all treatment groups. R_a presented no significant differences in fluxes across treatment groups (p = 0.3642), therefore treatment of SW and FPW did not generate any changes in glycerol flux relative to control conditions. R_a did present some significant decreases within treatment groups (as a result of exercise) relative to baseline (0.9BL/sec). Control-treated fish had significantly lower R_a at 1.0 (p=0.0322), 1.2(p=0.0280), and 1.4(p=0.0135) BL/sec, SW-treated fish had significantly lower R_a at 1.0 (p =0.0148), 1.1(p =0.0115),, 1.2 (p =0.0046), 1.5 (p = 0.0041), and 1.6BL/sec(p = 0.0068) relative to 0.9BL/sec. No differences as a result of exercise were observed in fish exposed to FPW (p = 0.1010- 0.8511). R_d of glycerol did not present significant alterations across treatment groups

(SW and FPW-treated) when compared to control-treated fish (p = 0.4224). Intra-treatment (exercise induced) alterations to R_d were observed in which control and SW-treated fish had significantly lower R_d relative to respective baseline values (0.9BL/sec). Control -treated fish presented decreases in the rate of disappearance at 1.0 (p = 0.0129), 1.2 (p = 0.0363), 1.3 (p = 0.0361), and 1.4BL/sec (p = 0.0008). SW-treated fish presented changes relative to their respective baseline (0.9Bl/sec) at 1.0 (p =0.0024), 1.1(p = 0.0231), 1.2(p = 0.0051), 1.5(p = 0.0073), 1.6(p = 0.0043), and 1.7BL/sec (p = 0.0479). Net glycerol flux presented no differences relative to control (SW, p = 0.357-0.999; FPW, p = 0.177-0.999). There were no changes across treatment groups in the net flux of glycerol. Sample size was of N=6 for control and SW-treated fish and N=5 for FPW-treated fish.

Plasma carbohydrate flux (see Fig.2.11), rate of appearance (R_a), rate of disappearance (R_d), and net flux (R_a-R_d) of radiotracers in circulation were measured during stepwise swimming in all treatment groups. There were no significant alterations (p = 0.6803-0.9999) in carbohydrate R_a flux within treatment (as a result of exercise) groups relative to their respective baseline value (0.9BL/sec). R_a of plasma carbohydrate in FPW-treated fish was increased relative to control-treated fish at the same timepoint. FPW at 1.1 (p = 0.0194) and 1.3BL/sec (p = 0.0313) were both significantly higher than control-treated fish. Additionally, a consistent trend of FPW and SW-treated fish having noticeably higher values than control was observed in R_a plasma carbohydrate. R_d was not significantly altered within treatment groups relative to their respective baseline (0.9BL/sec); control-treated p = 0.3472-0.9253; SW-treated fish p = 0.6671- 0.9999 and FPW p = 0.7602 – 0.9999. Nor were there alterations in R_d flux between treatments at the same time points (SW and FPW-treated, p = 0.1082-0.9999). There was however a noticeable trend in which both SW and FPW-treated fish had noticeably higher rate of disposal compared to control-treated fish.

Net flux of carbohydrates presented no changes across treatment groups (p= 0.778-0.9993) or within treatment groups (as a result of exercise) (p = 0.2914- 0.9999). Sample size was of N=6 for each treatment groups and results are presented as means ± SEM.

Oxidative stress enzymes, ethoxyresorufin-O-deethylase (EROD) activity was measured in both gill and liver tissue in fish exposed to control, SW or FPW conditions. Gill EROD activity in SW and FPW were statistically lower than control p<0.0001. Liver tissue in SW treated fish were not statistically different from control, however, FPW treated fish EROD activity were significantly higher than control values p<0.0001. (See Fig. 2.12)

Liver and gill thiobarbituric acid reactive substances (TBARS) (see Fig. 2.13), an indicator of oxidative stress induced cellular (lipid membrane), damage was measured in control, SW, and FPW-treated fish. Gill tissue presented no statistically significant differences TBARS activity across treatment groups (p = 0.3887-0.5123). However, there was a noticeable trend observed in gill tissue in which SW and FPW are approximately 2 and 3 times higher than control values. Liver TBARS showed statistically significant differences in SW-treated fish relative to control values. SW-treated fish had an approximately 2.5x increase in damage relative to control values (p=0.0024). There were no differences in TBARS between control and FPW-treated fish (p = 0.8024).

Lipase activity was not significantly altered from SW and FPW relative to control-treated fish. Noticeable trends were observed in which SW and FPW did present a higher activity than control. SW (p = 0.9999) and FPW (p = 0.999). (See Fig. 2.14). Pyruvate kinase (see Fig. 2.15), a key enzyme in glycolysis, was measured in white muscle in all three treatment groups. There were no differences in specific activity across treatment groups (p = 0.0542 – 0.2968). Lactate

dehydrogenase (see Fig. 2.16), an enzyme responsible for pyruvate to lactate conversion during anaerobic metabolism was also measured in white muscle across all treatment groups. There were no statistical differences in specific activity across treatment groups.

The fig. 2.17 indicates the FPW-treatment groups incapacity to swim. FPW treated fish suffered the most impairment in swimming directly related to exposure type among all treatment groups. While complications such as catheter clotting, and catheter disconnection was prevalent in all treatment groups as a method of failure which excluded fish from this study. FPW treated fish group who presented consistent unwillingness or incapacity to meet the given exercise intensity.

Table 2.1. Ionic composition of raw hydraulic fracturing flowback and produced water (Well-P 02-12-81-18-w6, June 2nd 2019, 4hr) of the Dawson-Montney formation, Alberta, Canada. ND = Non detectable values

Elements	Concentration (ppm)
Total dissolved solids (TDS)	47800
Chloride (Cl-)	18840
Sodium (Na)	13740
Calcium (Ca)	1830
Potassium (K)	590
Magnesium (Mg)	330
Strontium (Sr)	250
Sulfur (S)	170
Bromide (Br(He))	66
Silicon (Si)	33
Iron (He) (Fe(He))	17
Lithium (Li)	9
Boron (B)	9
Manganese (Mn)	3
Barium (Ba)	4
Molybdenum (Mo)	1
Cobalt (Co)	1
Phosphorus (P)	1
Titanium (Ti)	ND
Chromium (Cr)	ND
Aluminum (Al)	ND

Cadmium (Cd)	ND
Nickel (Ni)	ND
Copper (Cu)	ND
Zinc (Zn)	ND
Arsenic (As)	ND
Lead (Pb)	ND
Uranium (U)	ND

Table 2.2. Organic composition of raw hydraulic fracturing flowback and produced water (Well-P 02-12-81-18-w6, June 2nd 2019, 4hr) of the Dawson-Montney formation, Alberta, Canada. ND = Non detectable values

Organic compound	Aqueous (ng/L)	Sediment (ng/L)
1-n-Butylpyrene	ND	ND
1-Ethylnaphthalene	ND	ND
1-Ethylpyrene	ND	ND
1-Methylbenz[a]anthracene	ND	ND
1-Methylfluorene	385.90	158
1-Methylnaphthalene	ND	ND
1-Methylphenanthrene	235.03	186
1-Methylpyrene	7.74	66
2-n-Propylnaphthalene	ND	ND
3,6-Dimenthylphenanthrene	87.14	233
5-Methylchrysene	ND	ND
6-n-Butylchrysene	ND	ND
6-Ethylchrysene	ND	ND
9-n-Butylfluorene	ND	ND
9-n-Butylphenanthrene	ND	ND
9-Ethylfluorene	ND	ND
Acenaphthlene	ND	ND
Anthracene	ND	ND

Ben[a]anthracene	2.44	36
Benzo[a]pyrene	ND	ND
Benzo[g,h,i]perylene	1.85	26
Benzo[b]fluoranthene	2.35	36
Benzo[k+j]fluoranthene	ND	ND
Chrysene	30.48	112
Diben[a,h]anthracene	ND	ND
Fluoranthene	8.99	25
Fluorene	293.16	221
Indeno[1,2,3-cd]pyrene	ND	ND
Naphthalene	ND	ND
Phenanthrene	308.49	115
Pyrene	36.88	139
Retene	8.17	127

Table 2.3. Physical parameters of adult rainbow trout, *Oncorhynchus mykiss* used in swimming performance studies. The sample size was comprised of N= 6 for all treatment groups. Weight p = 0.2217 – 0.4371; Length p = 0.1110 – 0.6236; Depth p = 0.2212 – 0.3162 ; Width $p = 0.5104$; Hematocrit p = 0.1318 – 0.8790. No significant difference between treatment groups across all physical parameters.

Physical Parameters	Control	Saline-Control	Flowback and produced water (FPW)
Weight (kg)	0.664 ± 0.02	0.646 ± 0.022	0.615 ± 0.026
Length (cm)	39.3 ± 0.8	37.3 ± 0.6	38.0 ± 1.4
Depth (cm)	8.4 ± 0.1	8.5 ± 0.2	8.5 ± 0.1
Width (cm)	4.1 ± 0.1	4.1 ± 0.1	3.9 ± 0.1
Hematocrit (%)	26.8 ± 3.3	25.2 ± 2.2	29.4 ± 4.1

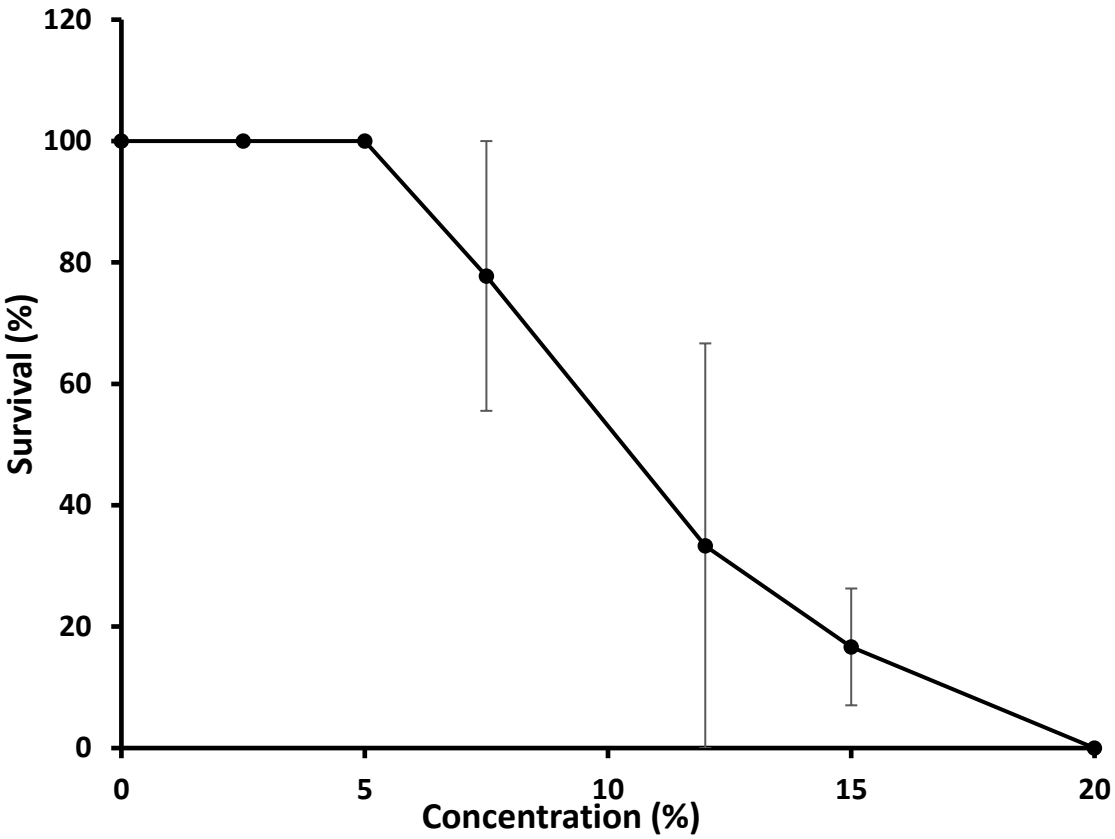


Fig. 2.4. Percent survival of static non-renewal acute (96hr) toxicity analyses test (LC50) of juvenile rainbow trout (*Oncorhynchus mykiss)* exposed to FPW sourced from Dawson-Montney Well-P 02-12-81-18-w6, June 2nd, 2019, 4hr. Tanks at concentrations of 0%, 2.5%, 5%, 7.5%, 12% 15% and 20% FPW were diluted from full strength FPW and diluted using city of Edmonton dechlorinated tap water. Tanks were continuously oxygenated and partially submerged to maintain a consistent temperature of approximately 10°C. Results are percentage means ± SEM. Results are the sum of triplicates per treatment concentration comprised of N=18 in at each concentration (total N=126). LC50 = 10.94% (95% C.I. 9.67 – 12.22%).

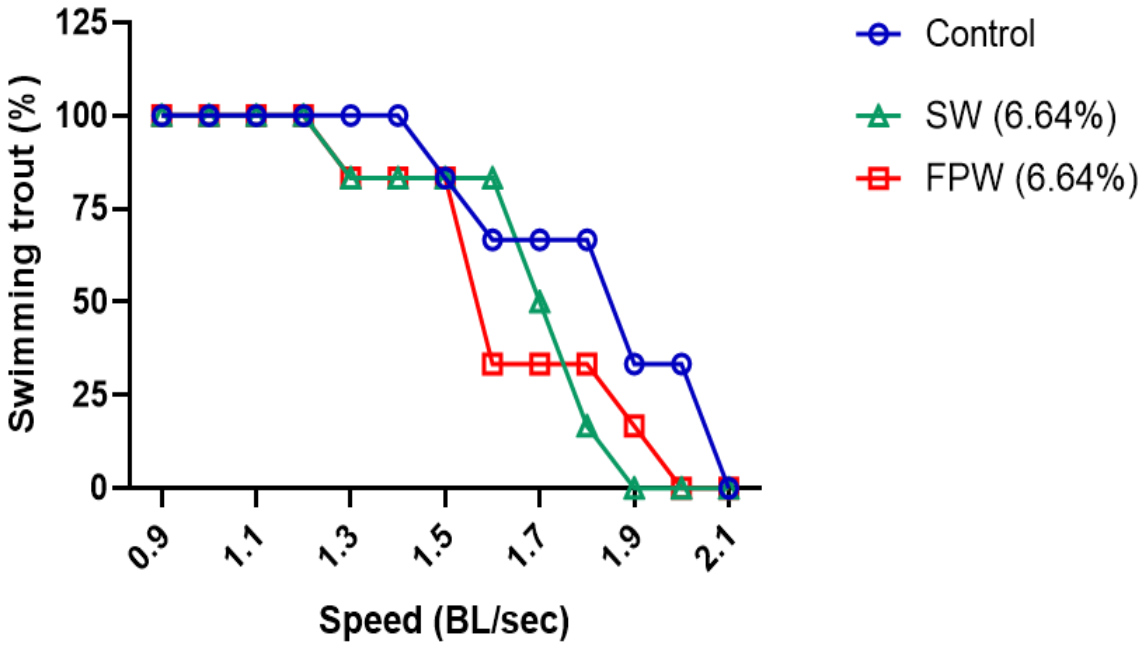


Fig. 2.5. Exercise stamina in adult rainbow trout exposed for 24hr to a salinity-matched solution (SW; 6.64%) or flowback and produced water (FPW; 6.64%) and recovered for 12-16 hours prior to stepwise exercise. Fish were only removed from a swim step when they became impinged onto the back grate and could not keep up with the current. All groups each had an N=6. SW and FPW had no significant effect on swim stamina (p=0.3871). Values are presented as percentage of participants.

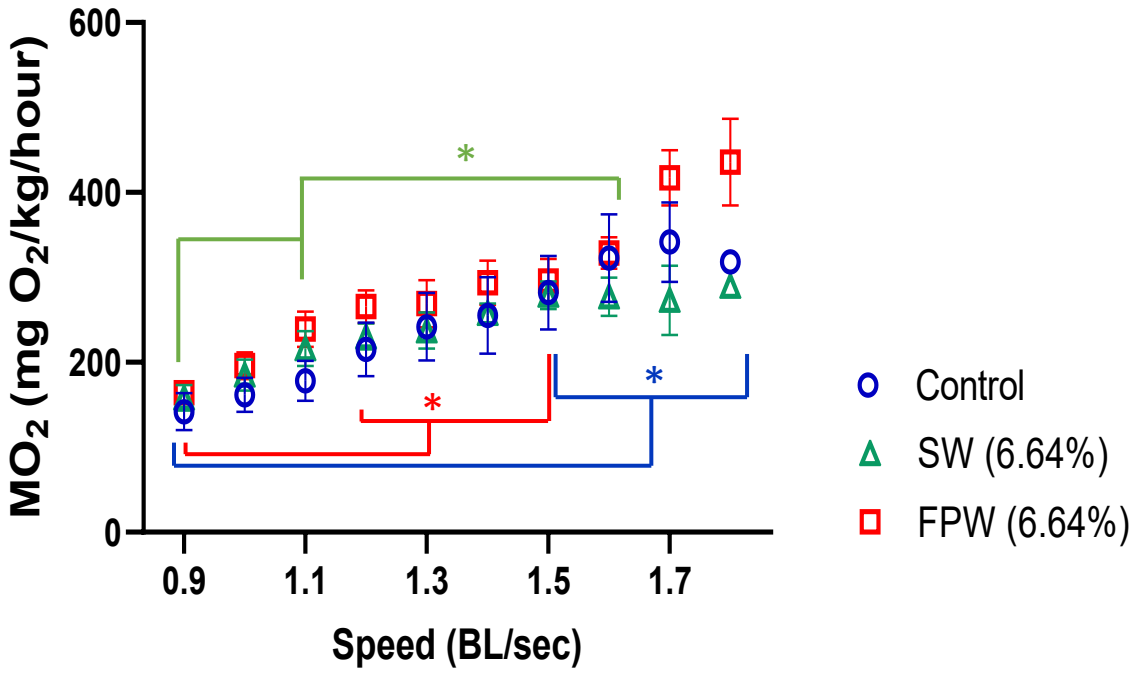


Fig 2.6. Metabolic rate (MO_2) of adult rainbow trout exposed to either a salinity-matched solution l (SW; 6.64%), or flowback and produced water (FPW; 6.64%) for 24hrs and recovered for 12-16hours prior to stepwise swimming. Fish were subjected to 20minute stepwise swim cycle at water current speeds relative to their respective body length per second (BL/sec) All treatment groups presented significant increases in MO_2 as a result of exercise intensity $p<0.05$. Coloured bars with asterisks (blue: control-treated fish ; green: SW-treated fish; Red: FPW-treated fish) indicate a significant increase (in all values within the bar) in MO_2 within a given treatment relative to their respective baseline (0.9BL/sec). No differences between treatment groups $p>0.05$. Error bars on control and SW group 1.8BL/sec too small to visualize. All groups had an N=6. Values are presented as means ± SEM.

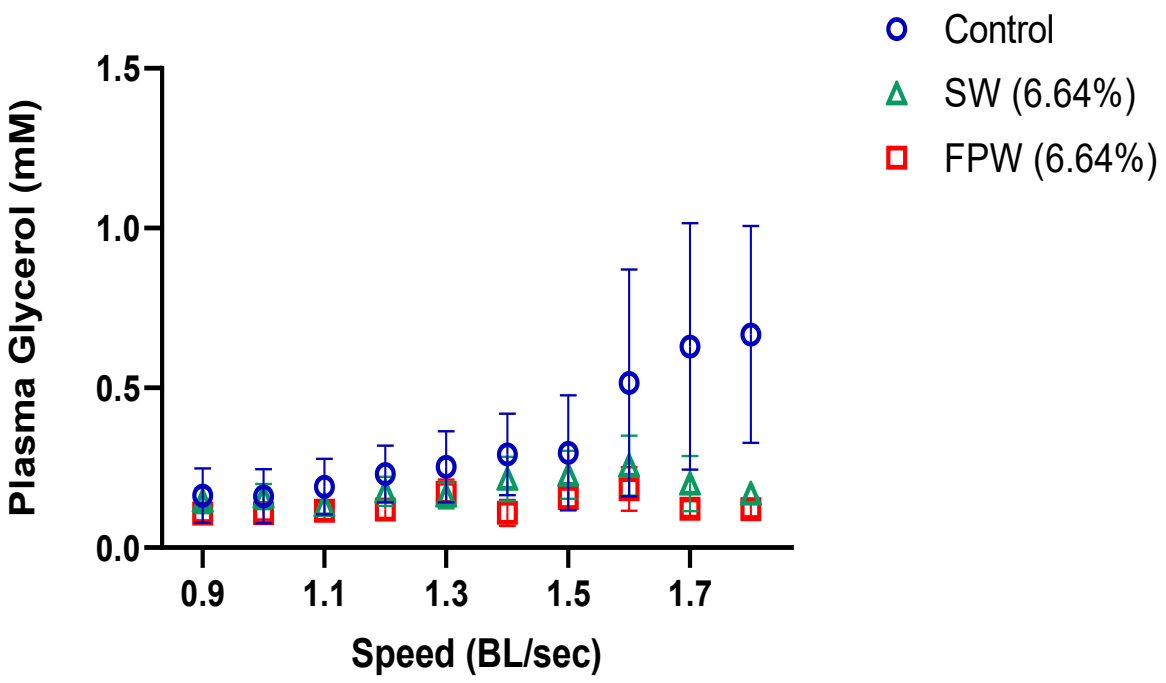


Fig 2.7. Plasma glycerol concentration in adult rainbow trout having been exposed to a salinity-matched solution (SW; 6.64%) or flowback and produced water (FPW; 6.64%) for 24hrs and recovered for 12-16 hours prior to stepwise exercise. Plasma glycerol concentration as determined using periodic blood draws throughout the stepwise exercise experiment. Exercise (p =0.1470-0.9999) and treatment (p = 0.4272-0.9999) (SW and FPW) had no impact on the plasma glycerol concentration among the treatment groups. Significance was defined as changes in plasma glycerol concentration as a result of exercise or treatment which the differences are $p<0.05$. Sample size is N=6 per treatment group. All results are presented in means ± SEM.

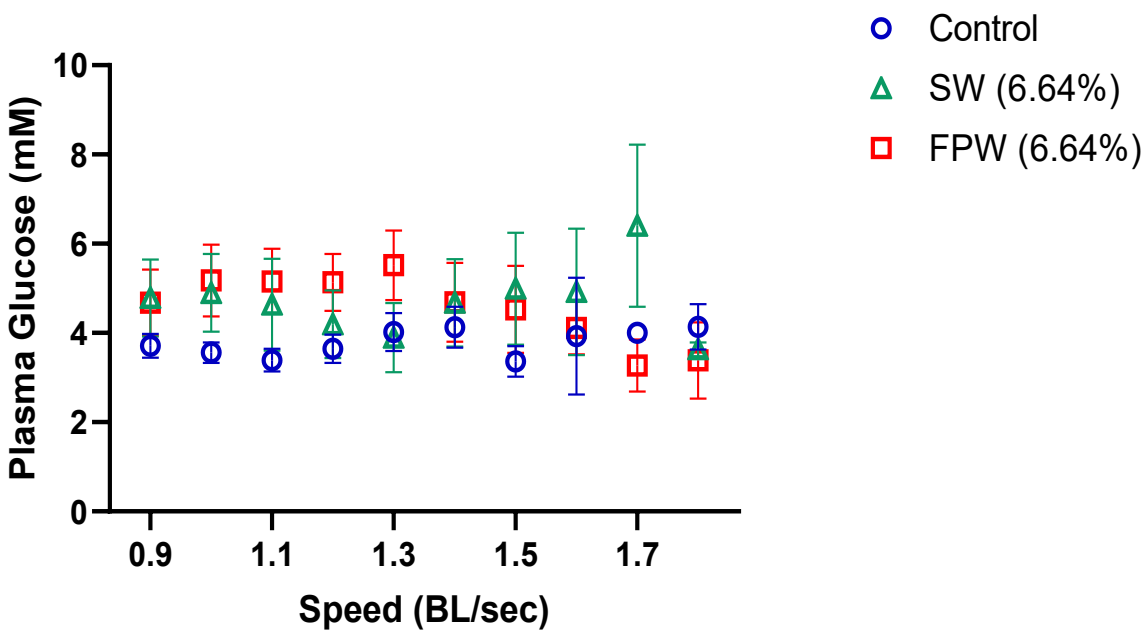


Fig. 2.8. Plasma glucose concentration in adult rainbow trout exposed to a salinity-matched solution (SW; 6.64%) or flowback and produced water (FPW; 6.64%) for 24hrs and recovered for 12-16hrs prior to stepwise exercise. Plasma glucose concentration as determined using periodic blood draws throughout the stepwise exercise experiment. No significant differences were observed as a result of treatment (p =0.1323-0.9999) or exercise (p =0.2959-0.9999) within or across treatment groups. Sample size is N=6 per treatment group. All results are presented in means ± SEM.

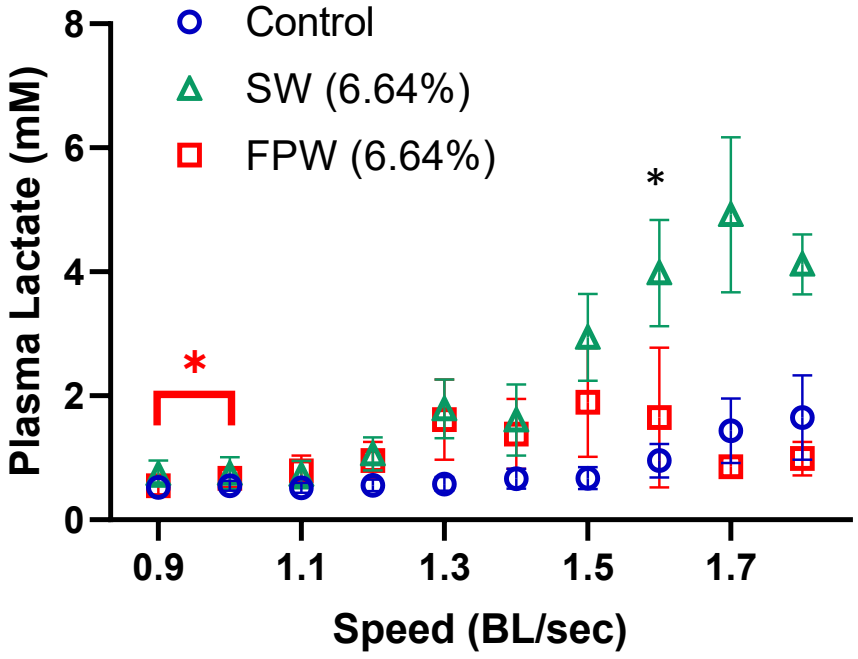
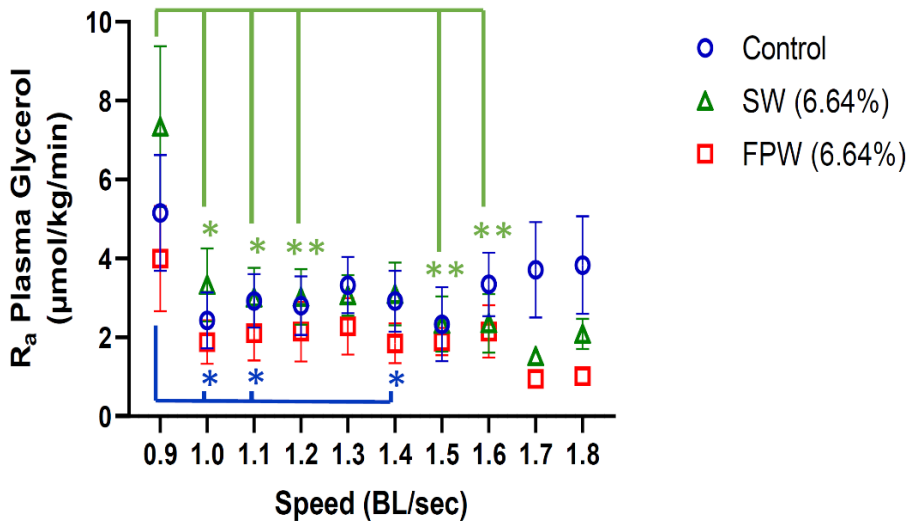
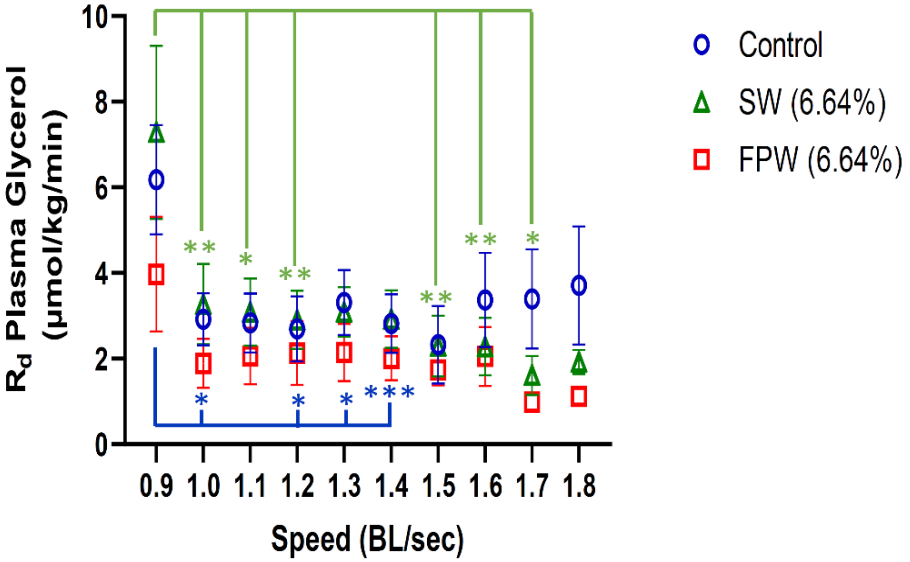


Fig. 2.9. Plasma lactate concentration in swimming adult rainbow trout having been exposed to a salinity-matched solution (SW; 6.64%) or flowback and produced water (FPW; 6.64%) for 24hrs and recovered for 12-16hrs prior to stepwise exercise. Significant differences are defined as changes in plasma lactate concentration as a result of exercise or treatment which are $p<0.05$. Black asterisks indicate differences in plasma lactate between SW-treated fish or FPW-treated fish relative to control-treated fish. Whereas a coloured bar (blue: control-treated fish; green: SW-treated fish; Red: FPW-treated fish) indicates significant differences observed between two speeds within the same treatment group. Plasma lactate was significantly increased in SW-treated fish relative to control-treated fish at 1.6BL/sec. Moreover, FPW-treated fish presented a significant increase in plasma lactate at 1.0BL/sec relative to its own baseline value (0.9BL/sec). Sample size is N=6 per treatment group.


R_a Plasma Glycerol (µmol/kg/min)
Speed (BL/sec)
Control
SW (6.64%)
FPW (6.64%)


R_d Plasma Glycerol (µmol/kg/min)
Speed (BL/sec)
Control
SW (6.64%)
FPW (6.64%)

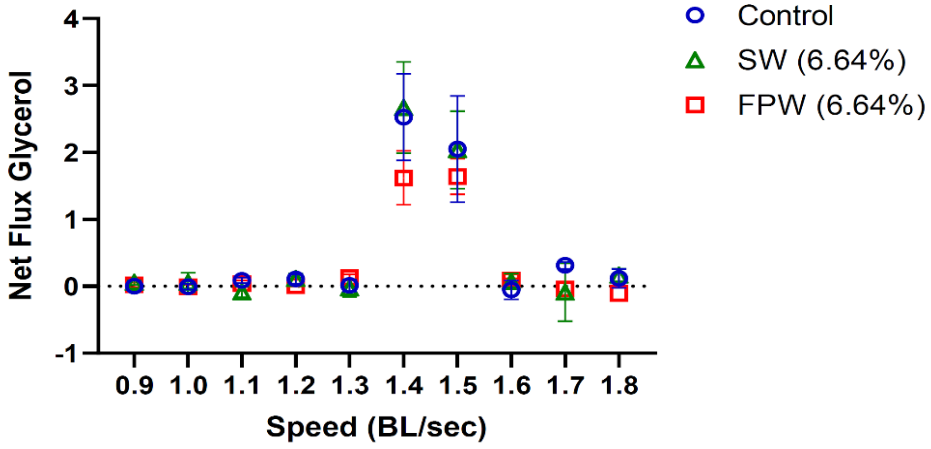
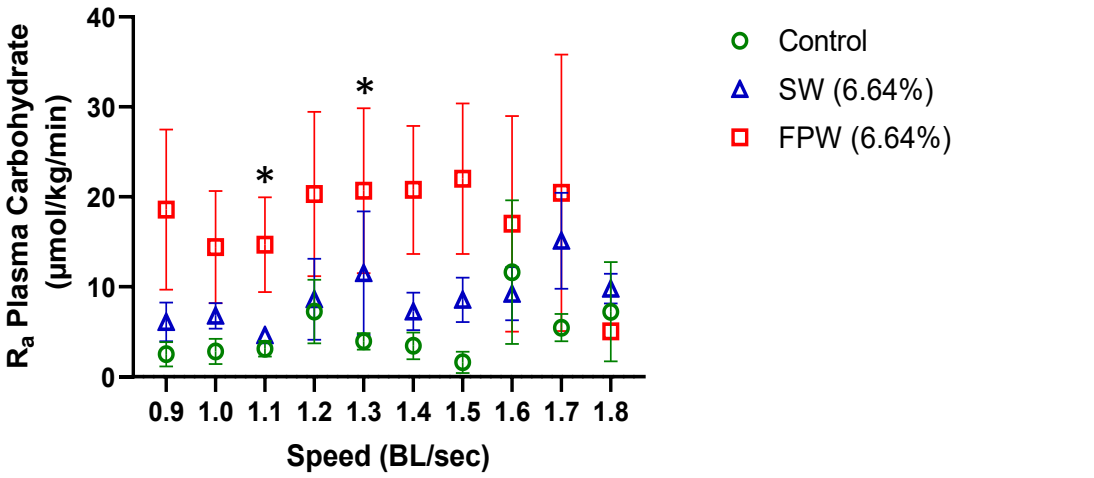
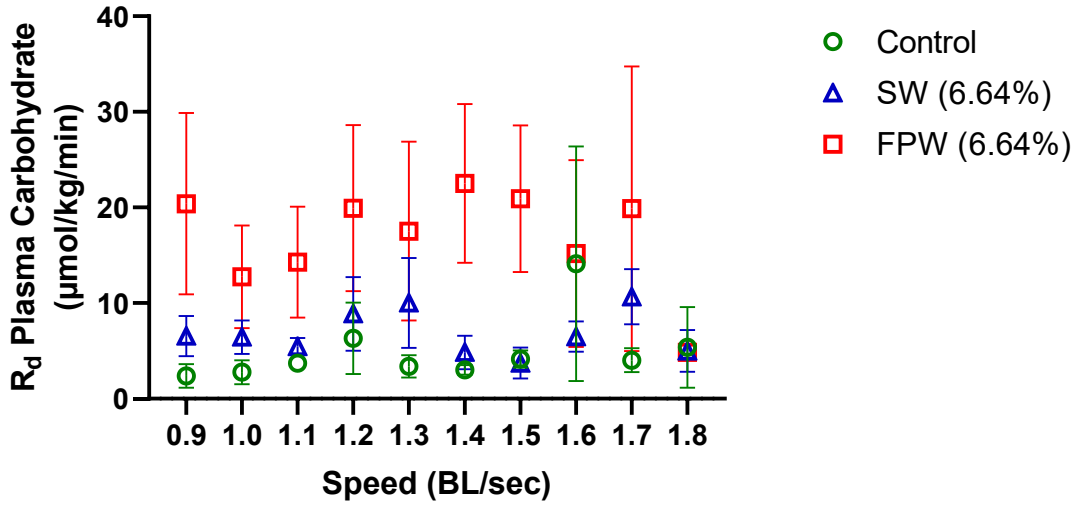

Net Flux Glycerol
Speed (BL/sec)
Control
SW (6.64%)
FPW (6.64%)

Fig. 2.10. Rate of appearance (R_a), rate of disappearance (R_d), and net flux of glycerol in rainbow trout exposed to either a salinity-matched solution (SW; 6.64%) or flowback and produced water (FPW; 6.64%) for 24hrs and recovered for 12-16hrs prior to stepwise swimming. All values were collected and analyzed from periodic blood draws throughout the stepwise exercise experiment. Significance within a figure is indicated by a coloured bar (blue: control-treated fish; green: SW-treated fish; Red: FPW-treated fish). This type of significance indicates changes in the given parameter (exercise) relative to each treatment groups baseline value (0.9BL/sec). R_a and R_d both present significant decreases in SW-treated fish relative to their respective baseline (0.9BL/sec). Additionally, R_a and R_d presented significant differences relative to their respective baseline (0.9BL/sec) Significance is dictated by a $p<0.05$. Data is presented as mean ± SEM. Sample size is of N=6 for control and SW and N=5 for FPW.


R_a Plasma Carbohydrate (µmol/kg/min)
40
30
20
10
0
*
*
0.9 1.0 1.1 1.2 1.3 1.4 1.5 1.6 1.7 1.8
Speed (BL/sec)
Control
SW (6.64%)
FPW (6.64%)


R_d Plasma Carbohydrate (µmol/kg/min)
40
30
20
10
0
0.9 1.0 1.1 1.2 1.3 1.4 1.5 1.6 1.7 1.8
Speed (BL/sec)
Control
SW (6.64%)
FPW (6.64%)

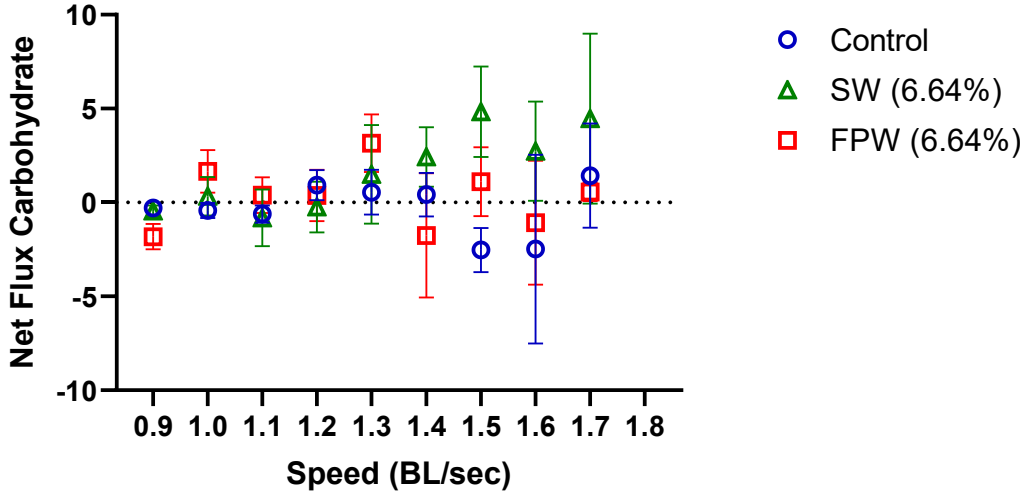

Net Flux Carbohydrate
10
5
0
-5
-10
0.9 1.0 1.1 1.2 1.3 1.4 1.5 1.6 1.7 1.8
Speed (BL/sec)
Control
SW (6.64%)
FPW (6.64%)

Fig. 2.11. Rate of appearance (R_a), rate of disappearance (R_d) of carbohydrate (glucose + lactate) and net flux in adult rainbow trout having been exposed to either a salinity-matched solution (SW; 6.64%) or flowback and produced water (FPW; 6.64%) for 24hrs followed by a 12-16 hours recovery prior to stepwise exercise. Black asterisks indicate differences in plasma lactate between SW-treated fish or FPW-treated fish relative to control-treated fish. R_a of carbohydrate presented significant increase in FPW-treated fish relative to control-treated fish at the same swim speed. Significance is dictated by a $p<0.05$. Data is presented as mean ± SEM. Sample size is of N=6 for each treatment group.

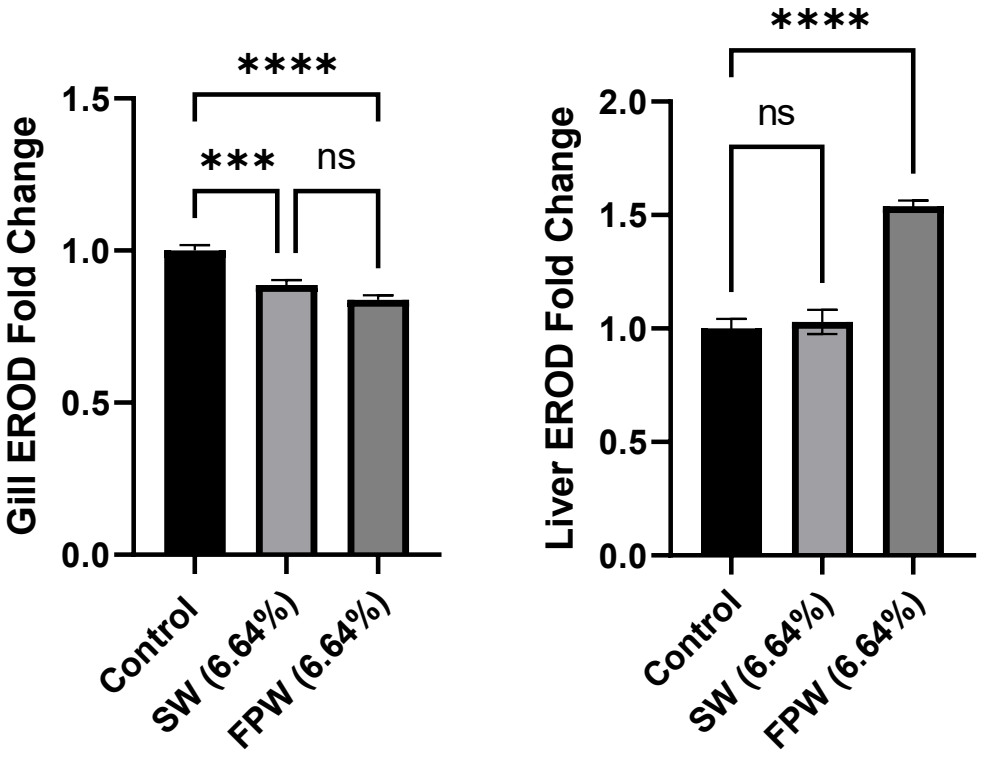


Fig. 2.12. Gill and liver ethoxyresorufin-O-deethylase (EROD) activity in fold change post stepwise swimming in rainbow trout exposed to either a salinity- matched solution (6.64%, SW) or flowback and produced water (6.64%, FPW) for 24hours and after stepwise swimming. Significance is defined as follows: ns= not significant, *** means $p < 0.001$, *** means $p < 0.0001$. Fold change is relative to control values set at 1. Sample size is N = 6 for each treatment group. All data is presented as fold change ± SEM.

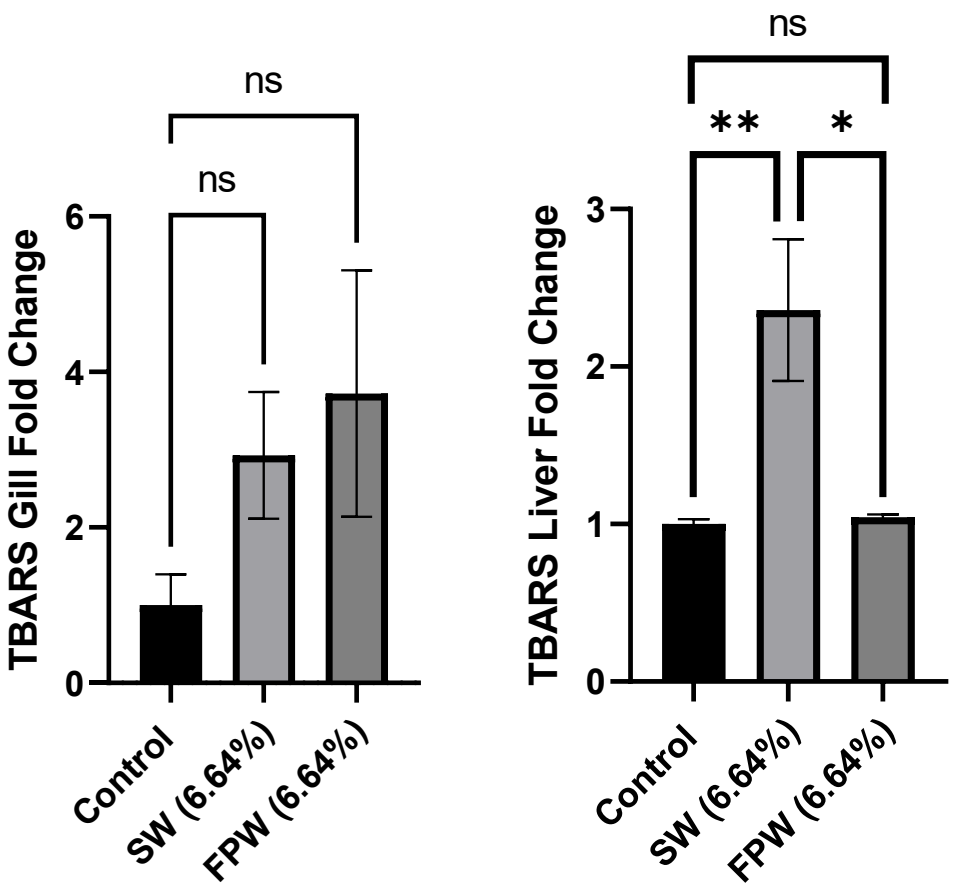


Fig. 2.13. Liver and gill thiobarbituric acid reactive substances (TBARS) activity in fold change post stepwise swimming in rainbow trout exposed to either a salinity-matched solution (SW; 6.64%) or flowback and produced water (FPW; 6.64%) for 24hours and after stepwise swimming. Significance is defined as follows: ns= not significant, * means $p < 0.05$, ** means $p < 0.01$. Fold change is relative to control values set at 1. Sample size in gill is N = 6 for control and FPW and N=5 for SW. Sample size in liver is N=6 for FPW and SW and N=5 for control. All data is presented as fold change ± SEM.

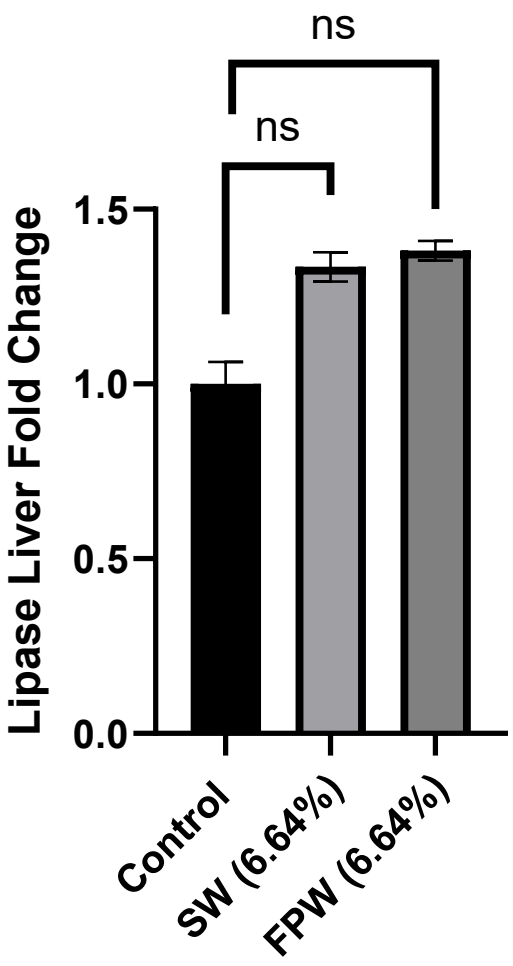


Fig. 2.14. Liver lipase activity in fold change post stepwise swimming in rainbow trout exposed to either a salinity- matched solution (SW; 6.64%) or flowback and produced water (FPW; 6.64%) for 24hours and after stepwise swimming. There were no significant differences in SW (p = 0.9643) and FPW (p = 0.7757) activity relative to control. Fold change is relative to control values set at 1. The indicator ns = not significant in which values comparison to control-treated fish is not $p < 0.05$. Sample size in gill is N = 6 for FPW and SW and N=5 for control. All data was presented as fold change ± SEM.

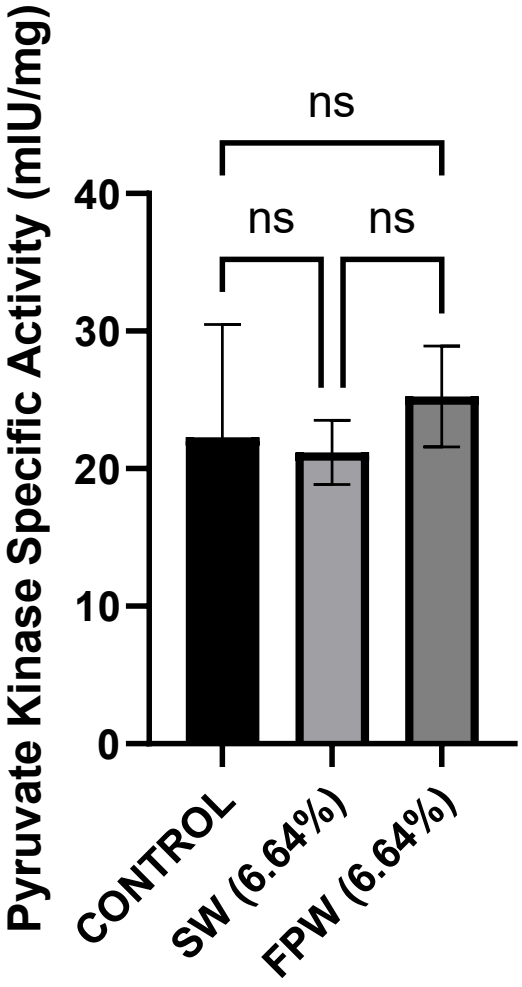


Fig. 2.15. Pyruvate kinase activity in white muscle in rainbow trout post stepwise swimming exposed to either salinity- matched solution (SW; 6.64%) or flowback and produced water (FPW; 6.64%) for 24hours and after stepwise swimming. All data is presented as means ± SEM. Sample size is as follows: control and FPW N=6; SW N=6.

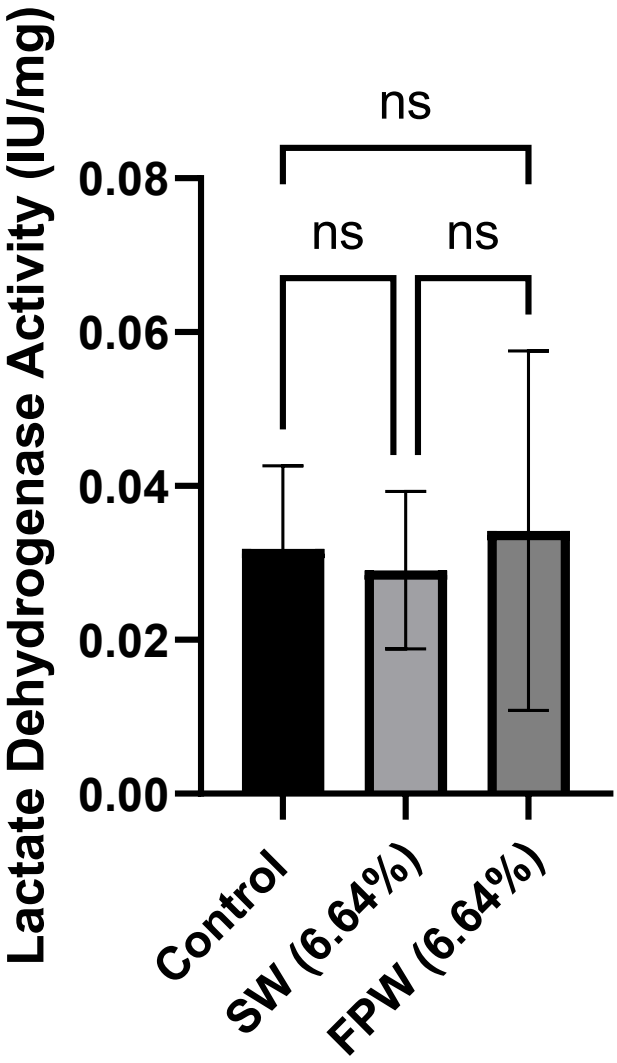


Fig. 2.16. Lactate dehydrogenase (LDH) activity in white muscle of rainbow trout post stepwise swimming who were either exposed to a salinity-matched solution (6.64%) or FPW (6.64%) for 24hours and after stepwise swimming. There were no significant differences between treatment groups. The indicator ns = not significant in which values comparison to control-treated fish is not $p < 0.05$. Data is presented as means ± SEM. Sample size is N=6 for each group (total=18).

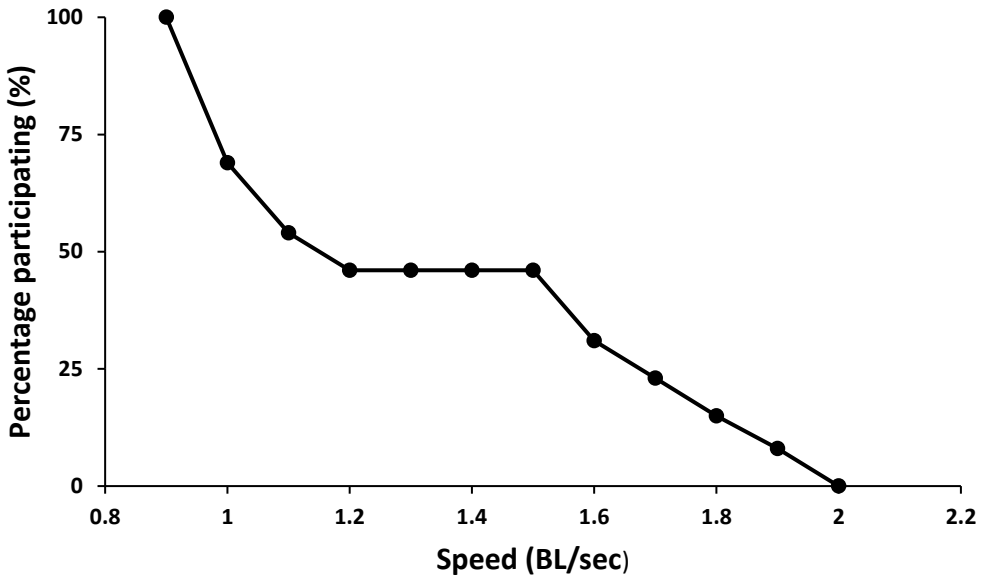


Fig. 2.17. Participating FPW-treated fish exposed for 24hours, both included and excluded from study. FPW-treated fish were the treatment group which presented the greatest struggle for swimming. Minimum threshold to have been considered for the study was for each fish to reach 1.2 BL/sec. Approximately 50% of fish treated with FPW did not meet this criteria as a direct result of unwillingness to swim. This was not observed in other treatment groups.

DISCUSSION:

This study is the first to show the effects of FPW on swim performance and energetics in adult rainbow trout *in vivo*. We assessed whether FPW: 1. Would impair swim performance in rainbow trout through alterations to metabolic rate and stamina. 2. Would alter the circulating concentration of metabolites associated with fatty acid and carbohydrate oxidation. 3. Would decrease lipolysis and fatty acid fluxes thereby increasing reliance for carbohydrates and subsequently increasing glucose and lactate concentrations and fluxes. 4. Would decrease enzyme activity for lipid mobilization and increase activity for enzymes associated with carbohydrate mobilization.

SWIM PERFORMANCE IS NOT SIGNIFICANTLY AFFECTED BY FPW:

In the present study, the exposure of SW or FPW to rainbow trout did not induce significant alteration in exercise stamina and MO_2 relative to control. Trout exposed to FPW did present noticeable increases in MO_2 throughout the experiment, and this trend became more apparent as exercise intensities increased. However, the lack of significant impacts on swim performance was not expected. This may be attributed to the fact that many FPW-exposed fish would/could not swim, even at very low speeds (<1.2BL/sec) and therefore needed to be discarded from the comparative analysis (see Fig. 2.17.). This phenomenon, known as the survivorship bias, persists in other studies which have a sequential series of experimentation in which a require threshold must be met for a sample (in this case live trout) to move on to the next stage (analysis) (Gagliardi et al., 2019). Therefore, the attenuated response observed in this study may be considered the 'best case scenario', given that the most severely impacted individuals didn't meet the minimum threshold requirement. In this case, it can be assumed that the effects of FPW are most likely more severe than what has been observed in this study.

Aside from survivorship bias, other causes which could have attributed to the attenuated but noticeable response towards swim performance across treatment groups are, rainbow trout's capacity to mediate changes in salinity and organics as well as the composition of FPW itself.

In the current study, our observed salinity of undiluted FPW presented a TDS, Na^+ and Cl^- concentration of 47800mg/L, 13500mg/L, and 18800mg/L respectively, approximately 1.37x the salinity of seawater (USGS, 2018). This concentration is much lower than previously reported salinities of FPW in various regions within Canada. Blewett et al., (2017) observed their working FPWs having a salinity concentration of 59,500mg/L and 107,000mg/L for Na^+ and Cl^- respectively. Whereas, Delompre et al., (2019) observed FPW with Na^+ and Cl^- salinity values of approximately 68100ppm and 47500ppm respectively.

Following the dilution of the FPW used in our current study (6.64%) trout were exposed to a concentration of salinity comparable to brackish/moderate saline water (3,000 to 10,000ppm) (USGS, 2018). Therefore, the concentration of salts in which the trout were exposed to were not outside the scope of acclimation capacities since rainbow trout, along with other fish species are a euryhaline (Bonzi et al., 2021). Euryhaline fish species who are acclimated to freshwater conditions present specific physiological behaviours which counteract the continuous loss of ions and passive uptake of water (McCormick, 2001). However, when these fish are transitioned quickly into saline these aforementioned mechanisms much cease and invert to remove excess ions and limit passive water loss (McCormick, 2001). Euryhaline fish are able to do this relatively well through the induction of ion transporters such as Na^+K^+ATPase, ion cotransporters (NKCC) and apical ion channels (Cl-) (Folkerts et al., 2020; McCormick, 2001). For example, Hwang et al., (1989), observed increase in Na^+K^+ATPase activity which helped decrease salinity in plasma in euryhaline species exposed to moderate saline (20% seawater) for 48hr. Delompre et al., (2019)

also observed an induction in Na^+K^+ATPase and H-ATPase activity in solutions which had comparable salinity to the FPW and SW used in our current study. Additionally, impairment of swim performance in salmonids acutely exposed to saline water was only observed when environmental salinities surpassed the concentration of seawater (35,000ppm) (Randall and Brauner, 1991; Brauner et al., 1992; Pedersen and Malte, 2004). Therefore, the presence of salts is unlikely to have caused significant changes in stamina and swim performance, which may be a factor of why exercise capacity was not altered significantly in our study.

Although we observed attenuated changes in exercise capacity, the minor alterations which were present may be attributed to organics. However, the attenuated response observed relative to other studies may be the result of the age of the fish in our study, and the specific composition of FPW.

Folkerts et al., (2017a) and (2020) utilized juvenile fish species to evaluate swim performance in teleost exposed to FPW. Comparatively to our study, Folkerts et al., (2020 and 2017ab) presented much more severe impairment to swim performance, however their study was focused on juvenile stenohaline and euryhaline fish species. Juvenile fish tend to be smaller, have more limited lipid reserves and therefore cannot sequester lipophilic toxicants into lipid storages as effectively (Mohammed, 2013). Moreover, protective physiological mechanisms such as excretion methods may not be fully developed in juvenile fish, as well as smaller fish species have greater surface:volume ratio, making them more susceptible to faster impacts of target toxicants and salts (Mohammed, 2013). In comparison to our study, our fish were euryhaline adult trout which most likely had better mechanisms to cope with an influx of organics thereby making their response less severe and more attenuated when compared to similar research using juveniles.

The composition of FPW also differs between Folkerts et al., (2020) and our study. Folkerts et al., (2020) utilized a FPW fraction comprised of primarily naphthalene and decalin, whereas the FPW used in our current study contains primarily phenanthrene, and pyrene. The differing composition as well as the concentration of organics within the FPW fractions itself could contribute to the lack of response observed in our results as our FPW had lower overall organics saturation than Folkerts et al., (2017ab).

The variability of FPW is a core characteristic of its composition and therefore not all FPW is expected to induce a similar response within an organism. However, the addition of age and species of fish as variables can impact the capacity to compare our study to previous research and may have been a strong factor in the attenuated responses observed in this study.

Finally, the marginal increase in MO_2 observed exclusively in FPW-treated fish could be the result of organic based stressors. The polycyclic aromatic hydrocarbon (PAH), phenanthrene has been observed to increase plasma cortisol concentration in several fish species during both acute and short-term exposures (Haque et al., 2018; Shirmohammadi et al., 2017; Reddam et al., 2017; Oliveira et al., 2007). The release of cortisol from the hypothalamus-pituitary-interrenal axis is often associated with an increase in metabolic rate (Pfalzgraff et al., 2022) and an increase in glucose release into the circulation (Kubilay and Uluköy, 2002). Both factors observed within this study in FPW-treated fish but not in SW-treated or control-treated fish (see Fig, 2.8.).

All in all, exercise capacity was evaluated using swim performance parameters of stamina and MO_2. Our results were non-significant, and noticeable trends were much more attenuated than previous studies investigating similar parameters, however, these results may be attributed to the differing age, species of fish used in this study and composition of working FPW from other

studies. The noticeable trends which were observed may be the result of organic fractions inducing a stress hormone response, however this cannot be confirmed as stress hormones were not evaluated in the scope of this study.

ENERGETICS IN RAINBOW TROUT WERE NOT SEVERELY IMPACTED BY FPW OR SW:

The process of fuel delivery in salmonid is highly regulated (Weber, 2011). Mobilized and circulating fatty acids (and glycerol) and carbohydrates are heavily intertwined and make up the majority of fuel consumption during exercise (Weber, 2011). The delivery of metabolic fuels is crucial for organisms to meet specific physical demands (i.e., exercise) (Weber, 2011). Within the scope of this study, mixed results were observed, SW-treated and FPW-treated fish presented noticeable alterations in plasma metabolite concentrations relative to control-treated fish. However, metabolite fluxes (glycerol and carbohydrate fluxes) remained unchanged relative to the control group. Moreover, metabolism-affiliated carbohydrate enzymes (PK and LDH) presented no significant differences while fatty acid mobilizing lipases did present some noticeable increase in activity. Factors that could be affiliated with the noticeable trends observed in this study are, stress hormones, direct effects from the constituents of FPW (salt or organics) or physiological compensatory mechanisms.

METABOLIC ENZYMES:

As observed in the present study, a generalized increase in lipase activity in the liver was noticed in both SW-treated and FPW-treated fish relative to control-treated fish. Little is known on the effects of FPWs constituents in liver lipase activity in rainbow trout. Given that both SW and FPW-treated fish presented a noticeable increase in lipase activity, it can be deduced that the saline component of the exposures induced changes in lipase activity (see Fig.2.14).

Studies have shown that salinity can alter fatty acid composition in aquatic organisms (Chen et al., 2021; Tocher et al., 1995). While lipolysis was not directly measured, Chen et al., (2021), suggested that the altered fatty acid composition in pacific oyster tissues exposed to varying environmental salinity is the result of changes in triacylglycerol hydrolysis which is mediated by AMPK and lipases. Alternatively, the transfer of a freshwater acclimated fish (trout) into a moderate saline solution may be sufficient to initiate an abrupt catecholamine release which upregulates lipolysis. Walker et al., (1989) observed that *Catostomus commersoni* exposed to a hypersaline environment had a several fold increase in catecholamines (epinephrine and norepinephrine) within the circulation. Although *Catostomus commersoni* are stenohaline fish, a similar but more attenuated response may persist in euryhaline fish acclimated to freshwater conditions, like those in the present study. The abrupt change in environmental conditions followed by a course of strenuous exercise (stepwise) (Reid et al., 1998) can exacerbate the stress response and increase catecholamine sufficiently to induce an increase in lipase activity (Magnoni et al., 2008). It is valuable to note that epinephrine is the only catecholamine which induces a stimulatory effect on lipolysis in fish (Magnoni et al., 2008) and therefore for this response to be possible, circulating epinephrine concentrations must be greater than norepinephrine. Unfortunately, within the scope of this study, catecholamines were not evaluated.

Carbohydrate-associated enzymes, PK and LDH activity in white muscle were not altered by salinity or organics (see Fig.2.15. and 2.16.). These results are consistent with previous studies which investigated LDH and PK activity in hepatocytes and who also observed no alterations in fish exposed to FPW at varying concentrations (Weinrauch et al., 2021). Given that LDH and PK didn't show any signs of disruption from FPW in either tissue (liver or white muscle) at similar concentrations, it may be possible that FPW doesn't have disruptive properties to these specific

enzymes. It doesn't discount that FPW may have effects in other forms of treatment, such as higher acute exposures or lower chronic exposures than those done within Weinrauch et al., (2019) and our present study. It is valuable to note that while PK and LDH didn't have an increase in activity there were increases in lactate and glucose concentration in plasma in both SW and FPW treated fish at some points during our experiments. Therefore, other factors may be at play or activity may be altered sufficiently to induce the observed changes in circulating metabolites but not enough to be detected within the scope of our analyses.

CIRCULATING PLASMA METABOLITES:

Plasma metabolites concentration in both SW-treated and FPW-treated fish were noticeably different than control-treated fish during exercise (see Fig. 2.7., 2.8., 2.9.). These effects may be the result of fuel availability and the interconnection of fatty acid/glycerol and carbohydrate (glucose and lactate) delivery and oxidation in presence of stressors such as exercise and treatment as well as survivorship bias.

Rainbow trout normally depend on fatty acids as their primary fuel during exercise, using almost exclusively fatty acids until approximately 2BL/sec (Turenne and Weber, 2018). This trend was observed in control-treated fish through a gradual but non-significant increase in glycerol concentration and no change in glucose and lactate concentration through the entirety of the experiment (see Fig. 2.7.). Whereas, SW and FPW-treated fish had a stagnant glycerol concentration but had simultaneously increased concentrations in both glucose and lactate (See Fig.2.8. and Fig. 2.9.) which were also more variable. The increase in glucose and lactate circulating in the plasma could be an indication of a stress response (Kubilay and Uluköy, 2002) as well as the incorporation of anaerobic metabolism respectively (Moves and West, 1995). SW

and FPW-treated fish were subjected to an environmental stressor in the form of high salt and high organic exposure (FPW), which can in turn increase stress hormones such as cortisol and subsequently increase glucose concentration (Haque et al., 2018; Shirmohammadi et al., 2017; Reddam et al., 2017; Oliveira et al., 2007).

Additionally, fish exposed to SW presented higher plasma lactate concentrations compared to FPW-treated fish. This may be the result of a survivorship bias (previously noted) in which fish treated with FPW would have had very high lactate concentration in plasma during exercise but were excluded from the study because they were unable to meet swim performance minimal threshold. Whereas there was no apparent survivorship bias in SW-treated fish and control-treated fish and therefore the presence of minor and major impairment which could indicate high plasma lactate concentration are more clearly indicated.

Given the complimentary use of circulating metabolites and the general preference for fatty acids by salmonids (Weber, 2011). The presence of discrepancies in fuel use observed in SW and FPW-treated fish relative to control may be the result of a stress response and alterations in fuel availability via stress hormones, thereby causing an indirect alteration in fuel availability within the circulation.

ENERGETIC FLUXES:

Glycerol fluxes was not altered as a result of exercise or treatment (SW or FPW) relative to control values. This response contrasts our initial hypothesis and prediction, that FPW would impair lipolytic fluxes and force carbohydrate fuels fluxes to mediate the reduced substrate availability. Rather lipid fluxes remained unchanged across treatment groups relative to control.

We can therefore assume that FPW was either insufficient to generate a response, that increased lipase activity may neutralize any impaired flux or that survivorship bias altered observable results.

The observed lack of response in lipolytic rate may be the result of the fish's capacity to mediate environmental stressors such as FPW. Salmonid lipolytic rate have been observed to have varying degrees of susceptibility to environmental stressors. For example, Turenne and Weber, (2018) observed that salmonids lipolytic rate was unaltered as a result of exercise. Whereas, Haman et al., (1997) presented an altered lipolytic rate, specifically a decrease as a result of hypoxia. The divergent response to two types of environmental stressor may be the result of the animal's capability to mediate the stressor. Salmonids are an athletic fish species and therefore exercise, even at high intensity would be insufficient to cause disruptions in lipolytic rate. Whereas, the same salmonids are highly intolerant to hypoxic conditions and therefore suffer severe alterations in energetics as a result (McClelland, 2004; Haman et al., 1997). Acute exposure to FPW, may find itself in the middle of the two extreme responses in which salmonids are capable of mediating acute exposures to salts (present within FPW) but may be susceptible to organic exposure, generating a direct or indirect response. Within the scope of this study the exposure time and concentration may have been insufficient to generate a strong enough stressor (like hypoxia) to generate any detectable changes to lipolytic rate but it doesn't mean that FPW as a whole won't induce alterations in glycerol fluxes. To confirm whether this is true, future studies should investigate lipolytic rate and fluxes in different exposure conditions.

Lipase activity may have also muted any impairment (if any) which may have occurred in glycerol flux. Against the initial predictions of this study, lipase activity increased rather than decreased in activity. It is stipulated that this may be the response of direct induction from salts found in the FPW (Chen et al., 2021) or indirect alterations in enzyme activity as a result of stress

hormone responses (Magnoni et al., 2008). Regardless, of the impacts, lipase activity increased noticeably (see Fig. 2.14.) but lipolytic flux didn't (see Fig. 2.10), which could indicate that there may be some form of neutralization on the part of lipase activity which is masking any impairment of lipolytic flux.

Lastly, survivorship bias may have contributed to the trends observed in our fluxes. Rainbow trout exposed to FPW and SW who were not evaluated in our study due to the unmet requirements for minimum swimming capacity (>1.2BL/sec) may have had observable alterations in fluxes, however, given that these results were not analyzed, trends which may have been consistent with our predictions were overlooked.

Carbohydrate fluxes also remained stable with no significant alterations in activity. There was a noticeable increase in plasma carbohydrates fluxes R_a and R_d which are congruent with our initial hypothesis and prediction that FPW would induce an increase in plasma carbohydrates concentration and increase carbohydrate fluxes. However, the increase FPW carbohydrate R_a and R_d were much more variable than the other treatment groups which may indicate a wide range of responses within the sample of fish evaluated in this study. Moreover, given that these alterations in carbohydrate fluxes were not congruent with the expected predictions regarding lipolytic fluxes (see Fig. 10.), glycerol concentrations (see Fig. 2.7) and carbohydrate affiliated metabolizing enzymes activity (see Fig. 2.15. and 2.16.), the observed increase in carbohydrate R_a and R_d is most likely not related to direct impairment of lipolytic flux as previously hypothesized. Rather, an increase in carbohydrate flux may be the result of an indirect stress response from FPWs organics (given that it was the only noticeably increased group) or increase demand for energy in tissues to combat oxidative stress, alterations in pathways affiliated with carbohydrate metabolism.

The presence of cortisol as a result of the presence of organics (Haque et al., 2018 Shirmohammadi et al., 2017) has been observed to increase circulating plasma glucose (Kubilay and Uluköy, 2016; Foster and Moon, 1986) in fish acutely stressed (for example during our graded swim protocol) (Jentoft et al., 2005) which would contribute to the increase R_a observed in the circulation across the experiment. Additionally, in mammals, stress responses increase a variety of stress hormones including cortisol and epinephrine which in sum increased glucose turnover rate generated by increased hepatic glucose mobilization (Horton and Beisel, 1994) similarly to the trends observed in our study. Therefore, the contribution of a stress response may have influenced the increased turnover of carbohydrates observed within this study.

Additionally, Weinrauch et al., (2021) also observed an increase in R_d in isolated hepatocytes but no alterations in PK and LDH activity. Given the congruence between Weinrauch et al.,'s (2021) results and the results observed in our study, similar physiological responses may be present. Specifically, Weinrauch et al., (2021) speculates that an increase in glucose uptake at the tissues may be the result of mediating processes to uptake carbohydrates from the circulation to replenish depleted carbohydrate stores in tissues as a result of the processing of pollutants (organics) for excretion. This thought is plausible within the scope of this study because both oxidative stress biomarkers presented noticeable and significant increases in activity, further indicating their need for energy to remove organic compounds.

In sum, glycerol (lipolytic) flux and carbohydrate flux did not entirely follow the initial hypothesis and predictions. It is therefore necessary to determine what other factors may be at play in influencing salmonid energetics in this study. Among the factors which may be at play are: insufficient exposure to FPW, mediating lipase activity neutralizing changes in lipolytic fluxes,

stress hormones (cortisol or catecholamines), increase dependance in carbohydrates to mediate pollutants biotransformation and excretion.

ACUTE FPW EXPOSURE INDUCES OXIDATIVE STRESS IN ADULT RAINBOW TROUT:

Hydraulic fracturing FPW has a well-established record of negatively affecting aquatic organisms (He et al., 2016; Blewett et al., 2017, Delompre et al., 2019; Folkerts et al., 2017ab; Folkerts et al., 2019). Its major common effect across species is the induction of oxidative stress biomarkers such as EROD and TBARS (He et al., 2016; Blewett et al., 2017). It has been widely accepted that EROD activity acts to remove xenobiotics through the addition of polar (water soluble) functional groups to pollutants to promote excretion (Whyte et al., 2000; Schlenk et al., 2008). As hydrocarbons enter cells, they bind to aryl hydrocarbon receptors which initiate downstream activation of the cytochrome P450, *CYP1A* genes and eventually upregulate EROD synthesis to initiate phase I biotransformation (Matsuo et al., 2008).

In the current study, liver EROD was significantly increased following the 24h FPW exposure and short recovery period (comprised of recovery of surgical cannulation and stepwise exercise). The results in this study are congruent with several other studies investigating the effects of FPW on aquatic vertebrates (He et al., 2016; Blewett et al., 2017; Delompre et al., 2019). Given that SW and FPW EROD activity results in the liver were dissimilar, it can be inferred that the organic fraction within the FPW is the inducer of EROD activation within this tissue (see Fig.2.12.). Polycyclic aromatic hydrocarbons are key contributors for EROD induction, for example, phenanthrene. Phenanthrene is a PAH significantly present within the FPW used in this study and has records of inducing EROD activation at low concentrations within the liver of teleost (Correia et al., 2007).

Within gill tissue, EROD activity (see Fig.2.12.) has a history of upregulating in the presence of high acute FPW exposures (He et al., 2017; Jönsson et al., 2009). However, this was not observed within the present study, rather, EROD activity was downregulated in both SW and FPW-treated fish, with latter being noticeably more prominent. Therefore, it can be assumed that both salts and organics are contributing to the impairment of EROD activity in gills.

While phenanthrene has a record of inducing EROD activity at low concentration, it also has a record of inhibiting EROD activity at high concentration. For example, Correia et al., 2007 observed high concentrations of phenanthrene induced an inhibition of EROD activity in the liver in fish. While Kang et al., (2022) observed the same trends in carp liver and brain. Given that the primary site of entrance between organics and the fish are the gills (Correia et al., 2007) it can be theorized that their high vascularization and large surface:volume ratio make them susceptible to accumulating organics and possibly inhibition of EROD activity through PAHs such as phenanthrene.

Salinity can also have a significant effect on enzyme activity in tissues. An acute increase in environmental salinity has the capacity to abruptly increase internal salinity in fish (Delompre, 2019). The increase transport of salts via the gills could cause alterations in enzyme activity in the same tissue. For example, superoxide dismutase (SOD), an oxidative stress enzyme responsible for reducing superoxide anions were observed to downregulate in the presence of increasing salinity in killifish (Blewett et al., 2017). Moreover, EROD activity has been observed to decrease in *Oreochromis mossambicus* exposed to increasing environmental salinity (Amutha and Subramanian, 2010). Therefore, the same processes may be occurring in the gill and result in the trends observed within this study.

The sum of both the organics and salts within FPW had the capacity to induce stress responses whether activation or inhibition of antioxidant pathways.

Concurrent with EROD inhibition at the gills, TBARS, a biomarker for lipid peroxidation damage at the gills was noticeably elevated (although not significantly) in the liver. TBARS is a marker of cellular damage caused by perturbing xenobiotics which cannot be neutralized by antioxidant mechanisms. Its primary effects are through cellular membrane lipid peroxidation (Stepić et al., 2012). Our TBARS results in both gill and liver are inverse to the trends observed in EROD. Similar to EROD, it appears that salinity as well as organics have induced some lipid peroxidation in both gill and liver (see Fig.2.12).

PAHs have been recorded to induce TBARS related damage (Ji et al., 2012), however, the highest presence of TBARS was in tissues and treatments which had low EROD induction. This is consistenty with the idea that TBARS is only produced when oxidative damage surpasses antioxidant capacities. Therefore, consistent with our study, when EROD activity was attenuated in gill and liver, in both SW and FPW-treated fish, TBARS activity was observed to be high. (see Fig. 2.12.)

SUMMARY:

In summary, previous literature has observed direct and significant impacts on fish swim performance, and energetics. In our study, results were generally attenuated and mixed. It can be said with certainty that FPW does generate a physiological impact at the concentration tested within this study given that there was an upregulation of oxidative stress hormones in SW-treated and FPW-treated fish. However, as a result of several factors such as: survivorship bias, stress hormones induction, compensatory mechanisms by lipases, or a lack of measurable response could, in part or in sum produced the results observed in this study. Although not all predictions

established within this study were supported, the trends observed in the results will provide valuable insight on the susceptibility of salmonid physiology to FPW and its individual constituents during exercise.

CHAPTER 3:
GENERAL CONCLUSIONS:

This study merges energetics research with environmental toxicology in the investigation of the effects of hydraulic fracturing FPW on aquatic vertebrate energetics and metabolism. Within this research, swim performance, circulating fuels, metabolic fluxes, as well as oxidative stress and metabolic enzymes were evaluated *in vivo* in adult rainbow trout after an acute (24hr) exposure to either control waters, a diluted FPW solution (6.64%), or a salinity-matched solution (SW, 6.64%) followed by stepwise exercise. I originally hypothesize that there will be a decrease in lipid mobilization/utilization from impaired lipolytic enzymatic activity as a result of FPW exposure which then forces an increase in alternative carbohydrate fuel use in the form of increased flux, enzymatic activity and circulating metabolites to compensate and maintain adequate ATP synthesis in rainbow trout during exercise.

The results found within this study did not sufficiently support the aforementioned hypothesis. Physiological responses were heavily attenuated across all measured parameters, with only noticeable trends and some significant differences being visible in the results.

Overall, trout swim performance was minorly affected by FPW. Swim performance remained fairly consistent between treatment groups, and only marginal impairments in stamina and minor increases in MO_2 among FPW-treated fish. These results could be comforting, in the concept that trout may not be heavily affected by FPW in acute situations however, this ideology would overshadow the significant survivorship bias which consistently impacted FPW-treated fish. Although, with that in consideration, rainbow trout did present a suspected stress response in their MO_2 rates during exercise induced by organics (PAHs).

Trout plasma metabolites were measured to determine changes in available fuels. Across plasma glycerol, glucose and lactate concentrations throughout the experimentation, only lactate presented significant changes as a result of exercise and treatment. Glucose remained mostly stable, noticeable but variable increases in plasma glucose were noticed in SW-treated and FPW-treated fish. Whereas plasma glycerol concentration remained stagnant in SW and FPW-treated groups whereas control treated fish had a gradual increase in glycerol within their circulation. This response is most likely due to a stress response from indirect FPW or a possible complementary change in fuel availability in the circulation between fatty acids and carbohydrates. Marginal changes in circulating metabolic fuels alone were not a strong enough argument to conclude that fuel availability was altered. Therefore, the prediction within our thesis was not entirely refuted nor was it completely supported.

Within the same experiment, metabolic fluxes were measured in trout during stepwise exercise to determine changes in fuel availability in conjunction with circulating fuels metabolites (noted above). Across both glycerol and carbohydrate fluxes, there were no significant changes as a result of treatment to SW or FPW. Glycerol fluxes remained stable across treatment groups and it is suspected that the lack of response may be as a result of survivorship bias, an increase in lipase activity which may have neutralized some observable trends or FPW was insufficient in generating a disruptive effect. Carbohydrate flux was however noticeably altered in FPW-treated fish when compared to the control group. This noticeable but variable increase in FPW may be the result of a stress response as a result of the combination of exposure to organics and exercise, as well as the possibility that increasing biotransformation activity in tissues could increase turnover of carbohydrate-based fuels, as suspected by Weinrauch et al., (2021).

All in all, glycerol flux was not consistent with the initial hypothesis and predictions, whereas carbohydrate fluxes were partially consistent but not supported via the predictions noted in this thesis. Therefore, future research should be conducted to get a clearer idea of whether FPW and SW at the concentration tested within this study can induce changes in energetics.

Metabolic enzymes, PK and LDH activity were measured in white muscle while lipases were measured in liver. Both PK and LDH did not have any changes in enzyme activity. The lack of response is consistent with Weinrauch et al., (2021) who saw similar effects in the liver but unexpected given the strenuous exercise and incorporation of anaerobic metabolism observed in SW-treated fish. Given that fuel fluxes didn't change and circulating metabolites didn't fluctuate outside the scope of normal carbohydrate plasma concentrations, PK and LDH may simply have not been impacted enough to see a response. This lack of change is not what we expected given our predictions that enzyme activity in carbohydrate metabolism would be significantly upregulated as a result of the increased dependance in carbohydrate fuels and inhibition of fatty acid availability. There however, may be other metabolic cascades which may be affected by FPW who have not yet been evaluated.

On the other hand, lipase activity was upregulated noticeably which opposed the original hypothesis. However, the increase activity may have been affiliate to stress induced activation of lipolysis via catecholamine (epinephrine). Catecholamine can be released from strenuous exercise or through environmental stressors. Alternatively, an increase in salinity may have increased lipase activity in the tissues evaluated, a trend which has been observed in other studies (Chen et al., 2022). Therefore, the upregulation, while unexpected and against our initial prediction, it not outside the scope of possibilities.

Lastly, oxidative stress enzymes were mostly consistent with previous literature. Both gill and liver activity of EROD and TBARS were analyzed. There were increases in liver EROD activity which were caused by the organic fraction of FPW, whereas gill EROD activity was downregulated, which is suspected to have been caused by environmental salts or organics which have been known to impair enzyme activity. TBARS followed complementary but inversely to EROD results, being upregulated in SW in liver but not FPW in the same tissue, while gill tissue showed an increase in TBARS where EROD tissue showed impairment. This response is fairly consistent with the literature in which excess salts and organics cause perturbations within aquatic organism physiology.

FUTURE DIRECTIONS:

While the merging of both environmental toxicology and energetics research continues to persist, additional studies should be conducted to provide a clearer idea of the effects of hydrocarbon-based pollutants on aquatic organism energetics. Future research should focus on stress hormones, analyzing additional metabolic enzymes, evaluating the contribution of cardiorespiratory impairment in energetics, and removing/limiting the survivorship bias.

Stress hormones have the capacity to influence energetics during rigorous exercise. Stress hormones such as cortisol (Pfalzgraff et al., 2022) and catecholamines (Reid et al., 1998) have been observed to increase during period where fish may be exposed to pollutants, or strenuous exercise/environments. The release of stress hormones within the circulation has the capacity to alter energetics through changes in regulation of certain metabolic cascades such as glycogenolysis, glycogenogenesis and lipolysis (Pfalzgraff et al., 2022; Magnoni et al., 2008). While stress hormones were not evaluated in the scope of this study, determining the magnitude of their influence on energetics is crucial to get a better understanding of FPW in salmonids. Future

research should therefore attempt to qualify and quantify the presence of stress hormones such as catecholamines and cortisol as a result of exercise and xenobiotics exposure on fish. By better assessing the contribution of stress hormones in fish exposed to FPW and rigorous exercise, we will get a better understanding of its influence on energetics.

Within the scope of this study, only a handful of metabolic enzymes activity could be measured (pyruvate kinase, lactate dehydrogenase and lipase family). While these enzymes provide the first insight into glycolysis, lactate synthesis and lipolysis in the presence of FPW, they do not necessarily portray the full image of metabolic enzyme activity in salmonids. Future research should therefore exploit a variety of analyses in several other metabolic enzymes which participate in the mobilization, oxidation and storage of metabolic fuels. By measuring the activity, protein abundance and gene expression of metabolic enzymes associated with metabolic fuel to ATP conversion, we can get a better idea of which cascades are most affected by FPW. For example, measuring the enzymatic activity, protein abundance and gene expression of enzymes associated with triacylglycerol synthesis, Cori cycle (lactate recycling), glyconeogenesis, glycogenolysis and the Krebs cycle would all be crucial parameters to evaluate in future studies. By better understanding the impacts of FPW on enzymatic function of energetics-associated cascades, a clearer picture of salmonid energetics can be produced which demonstrate the true fate of metabolic fuels *in vivo* across a number of tissues when exposed to FPW.

FPW often contains a myriad of organics (PAHs) which have been observed to cause deleterious effects on the cardiorespiratory system (Folkerts et al., 2020). Pericardial edema, altered stroke volume and heart rate have all been seen in fish exposed to hydrocarbon-based pollutants (Folkerts et al., 2020). Given that the cardiorespiratory system is a crucial contributor

to energetics and swim performance through its transport and delivery of oxygen. Its impairment could be a key factor in altering energetics in salmonids during exercise.

Moreover, previous research has observed that sediment, can alter oxygen transport in fish. Sediment is a major component in FPW and fish will likely come into contact with this fraction when exposed to FPW. Sediment can bind to gill tissue, causing an increase in the diffusion distance of ambient oxygen to the fish's circulatory system (Hess et al., 2015) while also contributing to alterations in the anatomical structure of gill lamellae (often reducing it) (Hess et al., 2017; Blewett et al., 2017) which in sum can contribute to a reduced oxygen delivery system in fish.

If oxygen were to become limited salmonids may not be able to sufficient produce ATP via fatty acids catabolism, thereby limiting their capacity to meet energetic demand and reducing swim performance. Alternatively, limited delivery of oxygen via reduced stroke volume or heart rate could force alternative fuels like carbohydrates, which don't depend on oxygen as heavily to become a primary fuel in ATP synthesis. Thereby contributing to the trends, we attempted to evaluate in this study. Therefore, it would be beneficial to determine the changes in stroke volume, heart rate and gill tissue structure and function as well as overall cardiorespiratory and swim performance in adult salmonids exposed to FPW acutely. Having a better understanding of the effects of FPW on adult salmonids cardiorespiratory system could give us a better idea of the magnitude of its impact on energetics in swim performance.

Finally, survivorship bias is a critical factor which must be controlled in similar studies in the future. Future work should evaluate all fish regardless of their recovery, to determine the prominence of oxidative stress and stress hormone impacts following FPW exposure and exercise.

Additionally, the above-mentioned analyses in the cardiorespiratory system and tissue specific enzymatic affiliated markers (activity, gene expression and protein abundance) would all provide more information on which exact physiological parameters are being most impaired and relate those effects to direct or indirect impacts of FPW. Getting more information is key in minimizing survivorship bias. While some fish may not be able to meet certain swim performance requirements, understanding why these fish can't meet certain tresholds is key in understanding the short and long term effects of FPW on salmonid swim performance and whole organism physiology.

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APPENDICES

PREPARATION OF RESIN FOR ION EXCHANGE COLUMN CHROMATOGRAPHY:

The resin was prepared as per noted in Turenne (2018) and Turenne and Weber (2018) with modifications. The use of ion-exchange resin is necessary to separate tritiated glycerol from other compounds like water and glucose which may become tritiated as a result of the metabolization of tracer material. Therefore to acquire true measurement of glycerol flux the removal of tritiated water and tritiated glucose is necessary.

Materials:

3 beakers 100mL

Dowex 50Wx8 (50-100Mesh)

Dowex 1x8 (100-200mesh)

1M Formic acid

2M hydrochloric acid (HCl)

2M Sodium Hydroxide (NaOH)

Waste plastic container(s) (4L)

pH meter (calibrated)

Nanopure water (deionized)

Initial Resin Preparation:

1. Place an equal desire mass (25-50g) of Dowex1x8 into 2 beakers and Dowex50Wx8 into 1 beaker.
2. Add sufficient water to cover resin. Mix resin with glass stir stick (one per beaker) for one min.
3. Allow to stand for 20min. Larger resin particles should fall to the bottom whereas smaller particles should remain in suspension.
4. After 20min, decant water from all beakers in respective waste plastic containers (one per beaker).

5. Replace with fresh water and allow to stand for 10min. Repeat 3 more times or until no colour can be extracted from the resin and smaller particle have been removed.

Hydrogen form resin:

1. After initial resin preparation, add sufficient 2M HCl to the Dowex 50Wx8 (50-100mesh) resin to cover.
2. Gently mix with respective stir stick for 1 minute.
3. Allow to stand for 10min.
4. Decant HCl and pour fresh HCl.
5. Repeat step 2 to 4, 3 more time.
6. Replace acid with water, stir gently for 15-20sec and allow to stand.
7. Decant water and repeat step 6 until pH of decanting water is above 5.
8. Once resin pH is above 5, transfer resin to glass vial with lid, cover resin in vial with water.
9. Fasten vial lid and wrap with parafilm to avoid evaporation of water.
10. Refrigerate until use

Formate form resin:

1. After initial resin preparation, add sufficient 1M formic acid to the Dowex 1x8 (100-200mesh) resin to cover.
2. Gently mix with respective stir stick for 1min.
3. Allow to stand for 10min.
4. Decant formic acid and pour fresh formic acid.
5. Repeat step 2 to 4, 3 more time.
6. Replace acid with water, stir gently for 15-20sec and allow to stand.
7. Decant water and repeat step 6 until pH of decanting water is above 5.
8. Once resin pH is above 5, transfer resin to glass vial with lid, cover resin in vial with water.
9. Fasten vial lid and wrap with parafilm to avoid evaporation of water.
10. Refrigerate until use

Hydrogen form resin:

1. After initial resin preparation, add sufficient 2M NaOH to the Dowex 50Wx8 (50-100mesh) resin to cover.

2. Gently mix with respective stir stick for 1min.
3. Allow to stand for 10min.
4. Decant NaOH and pour fresh NaOH.
5. Repeat step 2 to 4, 3 more time.
6. Replace acid with water, stir gently for 15-20sec and allow to stand.
7. Decant water and repeat step 6 until pH of decanting water is above 5.
8. Once resin pH is below 9, transfer resin to glass vial with lid, cover resin in vial with water.
9. Fasten vial lid and wrap with parafilm to avoid evaporation of water.
10. Refrigerate until use

Preparation of ion-exchange columns:

1. In a upright fasten 4mL plastic elution column (UCT RFV004P), place a frit (UCT 1/16" 10micron/4mL) at the base of the column (near spout).
2. Add approximately 400-500uL of resin formate form into the column.
3. Place a frit over the resin.
4. Add approximately 400-500uL of resin hydroxide form into the column.
5. Place a frit over the resin.
6. Add approximately 400-500uL of resin hydrogen form into the column.
7. Place a frit over the resin.
8. Wash column with minimum 2mL of deionized water, allow eluate to collect in plastic scintillation vials and discard.
9. Assure the resin remains moist at all times.
10. If water does not elute during waste, using plunger from a 3mL syringe needle (18gauge) and a needle. Insert plunger to push water through column and form droplets through spout.
11. Place syringe needle between plunger and column to break vacuum.
12. Remove plunger while assuring droplets of water remains in spout.
13. Repeat if water does clear from column.

CALCULATION OF FLUXES IN GLYCEROL AND CARBOHYDRATE METABOLISM:

Calculation of Rate of appearance of glycerol and carbohydrate (lactate + glucose) was sourced from Shanghavi (2001) and Wolfe (1992) and Turenne (2018).

$$\text{Rate of Appearance } (R_a)\ (\mu mol/kg/min) = \frac{F - pV\,[(C_1+C_2)/2][(SA_2-SA_1)/(t_2-t_1)]}{((SA_1+SA_2)/2)}$$

$$F\ (\text{Infusion Rate})\ (cpm/kg/min) = \frac{\text{Infusate Activity (cpm/mL)} \times \text{Infusion Speed (mL/min)}}{\text{Body Mass (kg)}}$$

$$pV\ (mL/kg) = (1-(\text{decimal hematocrit})) \times (80 mL/kg)$$

$$\text{Rate of Disappearance } (R_d) = R_a - pV[(C_2-C_1/\ (t_2-t_1)]$$

Rate of appearance, rate of disappearance, infusion rate and plasma volume utilize the following parameters:

C_1 and C_2: concentration of plasma metabolite

SA_1 and SA_2: Corrected Specific activity (cpm/mL)

$t_1$and t_2: Time (min)

CHROMATOGRAMS OF ORGANIC ANALYSES:

Organic Analyses were conducted as per Sun et al., (2019). Figures show chromatograms of organic compounds present within the FPW used in this study.