Investigation of PGC-1α Function in Zebrafish

By

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Abstract

Animal life must carefully balance energetic resources with physiological demands to promote growth and ensure survival. Energy serves as a valuable resource to cope with ever-changing environmental demands and requires complex regulatory networks to ensure efficient energy utilization. In mammals, PGC-1α (Peroxisome Proliferator Activated Receptor Co-Activator 1 α) is a master regulator of metabolism coordinating many essential metabolic processes. However, the importance of PGC-1α is currently unknown amongst lower vertebrates, despite controversy suggesting divergent roles for PGC-1α in teleost species. Here, I describe the creation of a mutant zebrafish line dedicated to uncovering the role of PGC-1α in zebrafish. In chapter 2, I disrupted an evolutionary conserved region upstream of the PGC-1α promoter, simultaneously increasing PGC-1α expression in skeletal muscle 4-fold and decreasing PGC-1α expression in cardiac muscle 4-fold. This mutation increased O_2 consumption in white muscle fibres and doubled the resting metabolism in juvenile zebrafish demonstrating that PGC-1α retains its role as a metabolic regulator in fish. In chapter 3, I hypothesized that PGC-1α mutant zebrafish experience impaired growth due to having a decreased metabolic efficiency. Mutant zebrafish larvae displayed decreased heart rate alongside increased yolk fatty acid (FA) content, indicating decreased FA metabolism during early development. Surprisingly, mutant adult zebrafish had increased FA metabolism, resisted growth when presented with a high-fat diet and decreased Dihomo-Gamma-Linoleic-Acid in skeletal muscle, a FA that prevents mitochondrial leakage. In summary, this mutation greatly impedes the metabolic function of zebrafish and provides a promising model for the continued study of PGC-1α in lower vertebrate muscles.

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The statement concerns the following publication:

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List of Abbreviations

Acetyl CoA – Acetyl Coenzyme A

ADP – Adenosine Diphosphate

ATF2 – Activating Transcription Factor 2

ATP – Adenosine Triphosphate

BAT – Brown Adipose Tissue

CCCP - Carbonyl Cyanide M-Chlorophenyl Hydrazone

COX IV – Cytochrome c Oxidase (Complex IV)

CPT – Carnitine palmitoyltransferase

CREB – cAMP Response Element-Binding Protein

CRISPR – Clustered Regularly Interspaced Palindromic Repeats

CS – Citrate Synthase

DSPC – Debiased Sparse Partial Correlation

DGLA – Dihomo-Gamma-Linolenic acid

Dpf – Days Post Fertilization

ERR – Estrogen Related Receptor

ETS – Electron Transport System

FA – Fatty Acid

FABP – Fatty Acid Binding Protein

FAO – Fatty Acid Oxidation

FCR – Flux Control Ratios

FoxO1 – Forkhead Box Class-O

GC-MS – Gas Chromatography Mass Spectrometry

GpDH – Glycerol-3-phosphate Dehydrogenase

gRNA – Guide RNA

HOAD – 3-Hydroxyacyl-CoA Dehydrogenase

HRM – High Resolution Respirometry

LC-MS – Liquid Chromatography Mass Spectrometry

M – Malate

MCAD – Medium-chain acyl-coenzyme A dehydrogenase

MEF2 – Myocyte Enhancer Factor 2

MMR – Maximum Metabolic Rate

NR – Nuclear Receptor

NRF – Nuclear Respiratory Factor 1

Oct – Octanoylcarnitine

P – Pyruvate

Pfi – Permeabilized Muscle Fibres

PGC-1α – Peroxisome Proliferator Activated Receptor Gamma Coactivator 1 Alpha

PGC-1β – Peroxisome Proliferator Activated Receptor Gamma Coactivator 1 Beta

PMG – Pyruvate-Malate-Glutamate

PPAR – Peroxisome Proliferator Activated Receptor

qPCR – Quantitative Polymerase Chain Reaction

RMR – Resting Metabolic Rate

Rot – Rotenone

S – Succinate

SDA – Specific Dynamic Action

TF – Transcription Factor

Chapter 1: Introduction

1.1 Bioenergetics and Energy Importance

All organisms require energy in order to survive and thrive in their environment. Every animal, regardless of size, must therefore be able to derive energy from dietary nutrients and utilize these resources strategically to maximize energy gain while minimizing energy loss. Although a simple concept at the surface level, the process of energy management relies on many factors such as external stimuli (diet, environmental conditions and predation) and internal processes (energy reserves, genetic factors and cellular pathways). Metabolic diseases arise from impaired nutrient usage in living cells, resulting in morbidities such as chronic obesity, heart disease, Parkinson's disease amongst others (Neves et al., 2014; Ostojic, 2017; Spiegelman & Flier, 2001). Although many of these diseases have allowed for remarkable advancements in both medical knowledge and the importance of bioenergetics in everyday life, they continue to become more prevalent within our population with each passing day (Saklayen, 2018).

In 1998, the discovery of the metabolic coactivator termed 'Peroxisome Proliferator Activated Receptor Gamma Co-activator 1 α (PGC-1α) led to rapid increases in our understanding of metabolic regulation (Puigserver et al., 1998). The PGC-1α protein is expressed in a variety of metabolically active tissues and coordinates 1000's of genes involved in tissue remodeling and bioenergetic processes. Researchers have studied PGC-1α using *in vitro* and *in vivo* approaches featuring human cell lines and mammalian models, however, many subtle functional aspects of PGC-1α remain unclear (Austin & St-Pierre, 2012; Besse-Patin et al., 2019; Geng et al., 2010; Russell et al., 2004). This ambiguity largely stems from the complexity of working with an advanced evolutionary model, as mammalian PGC-1α is implicated in a variety of key metabolic pathways that exponentially complicates the study of the role of the protein. An alternative

approach focusing on an evolutionarily distant model might therefore provide additional information on this coactivator by excluding many of the later acquired regulator roles of the protein. Hence, the study of PGC-1α in zebrafish may help to identify key core regulatory roles that are unable to be detailed in mammals, or to provide further clarification on currently controversial aspects of PGC-1α regulation.

1.2 Nutrients

Energetic resources must first be obtained, consumed and metabolized in order to provide energy for animals. All nutrients are derived from living organisms, such as plants or animals. They can be categorized into two major groups according to their general abundance and origin. Micronutrients (vitamins, minerals, and trace minerals) are present in limited quantities and serve various roles throughout the body, often associated with the immune system and enzyme function. In contrast, macronutrients (carbohydrates, lipids, and proteins) are plentiful and serve as primary building blocks for cellular structures in addition to being used as energy resources (Chen et al., 2018). All macromolecules can persist as either monomers or polymers as defined by the cell, allowing for them to readily assessable for catabolism or to be stored for extended periods of time (Deberardinis & Thompson, 2012). While similar in these aspects, each macromolecule monomer is structurally unique, creating a need for multiple energy pathways that occur at the cellular level.

Carbohydrates serve as the main macronutrient for humans and are often described as large repetitive molecules, consisting of simple sugars or starches produced by plant photosynthesis (Stetten & Topper, 1955). After consumption, carbohydrates are broken down into simple sugars (e.g., glucose) that can be imported into cells for immediate energy or converted into glycogen by the liver for storage. Glycogen molecules are stored in the liver and skeletal muscle and can

provide glucose during long fasting periods or in response to physical exercise, effectively acting as a carbohydrate buffer for the body. In vertebrates, glycogen stores are adequately maintained by the pancreas through insulin secretion in response to rising blood sugar levels, ensuring that glycogen stores are fully replenished following carbohydrate rich meals. However, with excessive carbohydrate consumption insulin can also promote the conversion of glucose into fatty acids in adipose tissue to further reduce blood sugar levels (Aronoff et al., 2004; Kersten, 2001; Roach et al., 2012). These negative feedback mechanisms work together in order to satisfy immediate energetic demands while also creating additional energy reserves for future use during unfavourable conditions.

Dietary lipids, specifically triglycerides, are exceptional molecules for energy storage as they contain a high caloric density in comparison to carbohydrate molecules. Triglyceride molecules can be obtained either through dietary means or created *de novo* following lipogenesis. They are built with glycerol backbone bound to single or multiple hydrocarbon chains in a variety of lengths with distinct hydrophobic and hydrophilic regions. In addition, lipids can also contain double bonds between carbon molecules, allowing for them to be further categorized as either saturated or unsaturated fatty acids. These combined characteristics allow for the creation of >1000 structurally distinct lipids, with unique properties to fulfill specialized roles in the cell such as maintaining membrane stability and acting as signaling molecules (Tracey et al., 2018). Aside from their multitude of cellular uses, lipids are energetically dense and can also serve as an important fuel source for tissues, by being stored and released from adipose cells into the bloodstream as free fatty acids (FFAs) which can then be transported into cells and metabolized.

In addition to carbohydrates and lipids, proteins may also provide energy, however, proteins are much more complex in both structure and function when compared to the previous two

macromolecules. Unlike carbohydrates or lipids that consist of varying lengths of carbon molecules, proteins are made from amino acids. This group is comprised of 20 molecules that differ in size, charge and hydrophobicity to create a vast number of functional proteins (Ellington & Cherry, 1996). All amino acids share amino and carboxyl regions that can be linked together following condensation reactions, resulting in large chains of amino acids that can be folded to create functional proteins. Folded proteins maintain their conformation due to strong intermolecular bonds and their unique characteristics allow for proteins to have unique functions that are crucial for cellular function, but many of these processes rely on cellular energy in order to be effective. In periods of extreme starvation (or excess protein intake), amino acids can also be broken down further by energy-deprived cells following glucogenic or ketogenic pathways (Gurina & Mohiuddin, 2020).

1.3 Nutrient Breakdown

Energy rich macromolecules must be first be broken down by a series of enzymatic reactions in order to release usable energy for the cell. Although macromolecules differ in their chemical structure, they all contain strongly linked covalent bonds that release large amounts of energy when broken (Cooper & Klymkowsky, 2013). This energy is captured by carrier molecules (either NADH or FADH) and later used to create ATP (Adenosine Triphosphate) molecules, a stabilized cellular energy molecule used throughout the body. Nutrients can be metabolized by aerobic (requiring oxygen) or anaerobic (without oxygen) degradation pathways depending on substrate structure, energetic demands and oxygen availability. Aerobic pathways are more efficient in extracting macronutrient energy in comparison to anaerobic pathways, however this efficiency requires a lengthy series of chemical reactions in order to maximize energetic output with O_2 usage. In contrast, anaerobic pathways are much faster and release energy without

needing to rely on O_2 levels but produces metabolic wastes. These aspects have restricted lipids and ketones to aerobic pathways as they contain many bonds and require extensive degradation to liberate energy. In contrast, glucose is structurally simpler and can be easily broken down into pyruvate following glycolysis; which can then be degraded by anerobic fermentation for immediate energy release or processed aerobically for higher energetic returns in the mitochondria (Cantrell & Mohiuddin, 2020; Sahlin et al., 1998).

Glycolysis occurs in the cytosol where individual glucose molecules are broken down into two pyruvate molecules. This process requires 2 ATP molecules to initiate the cleavage of each glucose, however the 2 resulting pyruvate molecules can release additional energy by either anaerobic fermentation or aerobic oxidative phosphorylation. Fermentation reduces pyruvate into lactic acid in order to provide 4 ATP molecules in the absence of oxygen, effectively doubling the cell's initial energy investment (Lunt & Vander Heiden, 2011). However, this amount is greatly reduced when compared to the total energetic output of oxidative phosphorylation, a process that generates 32 ATP molecules for each glucose molecule. This increased energy gain is due to the extended degradation of pyruvate occurring inside the mitochondria.

1.4 Mitochondrial Structure

Mitochondria are commonly referred to as the 'powerhouse' of the cell, as their primary role is to convert energy from electron carriers into ATP using a system of transcellular proteins and O_2 molecules. Mitochondria are densely populated around the endoplasmic reticulum, as close proximity allows for lipid exchange, fission/fusion, and mitochondrial-dependant signalling pathways to be initiated following input form the host cell (Marchi et al., 2014). Additionally, mitochondria are highly abundant in skeletal muscle tissues and aggregate between myofibrils where ATP is heavily needed. Mitochondria have two distinct compartments separated by two

membranes; the outer mitochondrial membrane, the intermembrane space, the inner mitochondrial membrane and the internal matrix (McCarron et al., 2013). The outer membrane contains many porins, open channels that allow for the passage of ions and small molecules into the mitochondrial intermembrane space from the cytoplasm. Once inside, molecules must bind to specific transporter proteins in order to cross the inner mitochondrial membrane. These transporter proteins help to restrict ion movement across the inner mitochondrial space in order to maintain an electrochemical gradient required for powering oxidative phosphorylation. Finally, the core of the mitochondria contains the mitochondrial matrix, a space containing a variety of enzymes that power the Krebs cycle, a series of reactions that harvest electrons from macromolecules prior to oxidative phosphorylation.

1.5 The Krebs Cycle

The Krebs cycle is a fundamental part of oxidative metabolism, as it provides a universal degradation pathway for all 3 macromolecules (carbohydrates, lipids and proteins). Prior to entering the Krebs cycle, macronutrients must first be converted into acetyl CoA (Acetyl Coenzyme A), which can be generated following glycolysis (from carbohydrates), β-oxidation (from lipids) or from deamination (from proteins). Upon entering the Krebs cycle, Acetyl CoA is readily converted with endogenous oxaloacetate to form citrate; the first of 9 stepwise modifications that ultimately result in 1) the release of 2 CO_2 molecules and 2) 4 electrons deposited in the form of NADH and $FADH_2$ and a Guanosine Triphosphate energy molecule. Following the release of CO_2, oxaloacetate is returned to renew the reaction process for additional Krebs cycles, allowing for more Acetyl CoA molecules to be degraded for energy harvesting (Gnaiger, 2014). The electrons contained by NADH and $FADH_2$ molecules can then

be transported to the Electron Transport System (ETS) where they can power oxidative phosphorylation and create energy in the form of ATP.

1.6 Electron Transport System

The Electron Transport System consists of 6 separate proteins (Complex I-IV, ubiquinone and cytochrome c) that work together in order to power oxidative metabolism through electron transport. Electrons gained from the Krebs cycle travel through the ETS proteins forcing unidirectional movement of hydrogen ions from the mitochondrial matrix to the inner mitochondrial space, establishing an electrochemical gradient across the inner membrane. As this gradient increases with subsequent electron transfer, hydrogen ions flux back to the mitochondria matrix by passing through ATP synthase (Complex V), the final protein in the ETS generating cellular energy in the form of Adenosine Triphosphate (ATP) (Maloney et al., 1974). These ATP molecules can then be used for a wide variety of cellular processes such as intercellular signalling, DNA/RNA synthesis, ion gradient maintenance and muscle contraction (Dunn & Grider, 2020). As ATP is a vital resource for the cell, mitochondrial function is crucial to provide a constant and reliable stream of energy for the organism.

1.7 Origin of Mitochondria

The origin of mitochondria is believed to stem approximately 1 billion years ago following an endosymbiotic event where two unicellular organisms fused, creating the first common ancestor of all eukaryotic life (Chernikova et al., 2011; Martin & Müller, 1998). These two organisms consisted of a larger prokaryotic cell dependent on the phagocytosis of smaller microorganisms and a smaller hydrogen-dependant bacterium capable of producing its own energy at the cost of expelling toxic reactive oxygen species. Following endosymbiosis, the two cells developed a mutual relationship where the host prokaryotic cell would provide protection to the smaller

bacterium by neutralizing oxidative stress while also becoming energetically self-sufficient (Roger et al., 2017). With time, the two organisms continued to survive as a single entity as an independent organism capable of producing its own cellular energy.

Throughout the billions of years following this initial endosymbiosis, the cell and mitochondria have fostered an intimate relationship due to the major restructuring of genetic regulatory pathways. Most of the mitochondrial genes were relocated to the nucleus of the cell, leaving the modern day animal nucleus responsible for producing over 1500 mitochondrial proteins in comparison to the 22 unique tRNA molecules, 2 rRNA subunits and 13 ETS proteins still encoded by the mitochondrial genome (Jadiya & Tomar, 2020). This genetic shift has gradually allowed for the cell to gain control over mitochondrial mechanisms allowing for increased energy production and efficiency that is required for giving rise to multicellular organisms and specialized tissue types. As a result, eukaryotic life has become incredibly diverse with many adaptations that are heavily reliant on the power produced by the mitochondria. However, with great power also comes great responsibly as cells must regulate these energetic pathways in an efficient but reliable manor to ensure viability for both independent cells and the organism itself.

1.8 Nuclear Receptors & Transcription Factors

The nucleus is responsible for storing the cell's genomic DNA and regulating the expression of genes required for normal cell function. In multicellular organisms, the genomic DNA is quite large and must be physically compressed using histone proteins, leaving only a small portion of the genome freely accessible to cellular machinery at any given moment (Evans & Williams, 2002). These regions can then be activated by Transcription Factors (TFs), a class of proteins responsible for facilitating transcription; the process of converting DNA into mRNA transcripts typically used for protein synthesis. This process is highly complex, as TFs often work together

alongside co-activator,co-repressor and chromatin remodelling proteins to consolidate the regulation of every genetic element in the host cell (Kuntz et al., 2012; Spitz & Furlong, 2012). Additionally, many of these regulatory pathways are primarily activated by external ligands through a subset of TFs known as the Nuclear Receptors (NRs). These NRs often serve as the initial activation point for activating gene expression as their ligands are representative of environmental and internal cellular conditions (Avior et al., 2013).

To date, 48 NRs have been identified in humans and further categorized into 3 subgroups based on their physiological functions and corresponding ligands; Endocrine Receptors, Orphan Receptors and Adopted Orphan Receptors (Sonoda et al., 2008; Weikum et al., 2018). Endocrine Receptors are regulated by circulating hormones and vitamins, many of which are well documented in the literature due to their prominent effects in both normal hormone-induced development and in various pathologies. In contrast, the appropriately named 'Orphan Receptors' have no known ligands, resulting in limited information regarding their physiological function and role. With recent advancements in cellular biology, many of these ligands have been discovered and paired with their respective NRs, allowing for some of the Orphan Receptors to be reclassified as 'Adopted Nuclear Receptors' (De Vera, 2018). Although some of the Adopted Nuclear receptors are activated by non-biological molecules (xenobiotics), a vast majority are activated by dietary lipids such as phospholipids, cholesterol and fatty acids (Sonoda et al., 2008). Given that these dietary lipids reflect the metabolic state of the organism, it should be of no surprise that many of these receptors play important roles in the regulation of cellular bioenergetics.

The cell relies on mitochondrial-specific TFs in order to regulate both energetic and non-energetic mitochondrial processes between the nuclear and mitochondrial genomes (Kelly &

Scarpulla, 2004; Leigh-Brown et al., 2010). As these genomes are physically separated in the cell, mitochondrial genes can only be activated in the presence of specific mitochondrial TFs, which are transcribed by the nucleus. Each mitochondrial TF is responsible for the activation of a subset of mitochondrial genes in response to a particular activating ligand (Table 1). Although some TFs feature redundant roles, different ligands allow for the cell to selectively respond to specific stimuli. These ligands allow for TFs to adjust mitochondrial performance across multiple cell types in accordance with energetic demands, serving a vital role for multicellular organisms. As such, these TFs remain highly conserved amongst animal species and must be tightly regulated to ensure metabolic stability.

Table 1-1: Metabolically relevant transcription factors and their associated ligands

Transcription Factors	Activating Ligand	Regulatory Roles
Nuclear Respiratory Factor 1 & 2 (NRF-1 & NRF-2)	Environmental Stimuli (Exercise) and various hormones	Transcription of ETS proteins, Heme synthesis, mtDNA transcription & replication machinery (Activation of TFAM), Mitochondrial biogenesis, Mitochondrial protein transporters and ion channels (Kelly & Scarpulla, 2004; Mattingly et al., 2008)
Estrogen Related Receptor (ERRα)	Unknown	Transcription of FAO enzymes (MCAD), FA transport, Glucose/Glycogen transport, Mitochondrial biogenesis, Mitochondrial respiratory chain enzymes, Muscle contraction elements (Huss et al., 2004; Mootha et al., 2004)
Thyroid Hormone T_3 Receptor (THR)	Thyroid Hormone (T_3)	Inner mitochondrial membrane proteins, Mitochondrial biogenesis (Activation of NRF-1), Mitochondrial fat and carbohydrate transporters, Mitochondrial uncoupling (Thermogenesis), mtDNA transcription (Activation of TFAM), Mitochondrial ion channels, Mitochondrial fat and carbohydrate transporters (Cioffi et al., 2013; Harper & Seifert, 2008)

Transcription Factor Mitochondrial A (TFAM)	None (TF)	Mediates mtDNA transcription & replication (Kang et al., 2007; Leigh-Brown et al., 2010)
Peroxisome Proliferator Activated Receptor α/β/γ (PPARα/β/γ)	Various Lipids	Transcription of FA transporters and FAO enzymes (Berger & Moller, 2002; Grygiel-Górniak, 2014; Tyagi et al., 2011)

1.9 Transcriptional Co-Regulators & PGC-1α

Transcriptional co-activators and co-repressors provide the cell with an alternative approach to modifying and coordinating TF and NR activity. These proteins are arguably the most intricate players for transcriptional regulation as they are tightly regulated by the cell in relatively low abundance to oversee the activity of TFs or NRs (Bulynko & O'Malley, 2011; Rosenfeld et al., 2006). Ultimately, these proteins serve as the final decision makers towards enabling specific transcriptional patterns with small differences in their expression leading to vast phenotypic differences. From a metabolic perspective, an organism must be capable of balancing both energy acquisition and expenditure in order to grow and survive. These extremely important roles require intricate control over the various metabolic pathways in the cell, as activation of conflicting pathways could present lethal consequences to individual cells and compromise the overall health of the organism (Giudici et al., 2015).

One of the most notorious regulators of overall metabolism is Peroxisome Proliferator Activated Receptor Gamma Co-activator 1 α (PGC-1α), a protein responsible for controlling the expression

of a vast number of metabolic genes (Puigserver & Spiegelman, 2003). With its discovery in 1998, researchers determined that murine PGC-1α directly promoted thermogenesis by upregulating UCP-1 (Uncoupling Protein) in Brown Adipose Tissue (BAT), effectively modifying mitochondria to dissipate heat instead of cellular energy (Puigserver et al., 1998). This discovery led to increased PGC-1α research in the following years, which uncovered many metabolic roles associated with PGC-1α's activation of TFs and NRs. This includes roles in fatty acid oxidation, muscle fibre-type switching, mitochondrial biogenesis and heart development (Table 2). As a result, PGC-1α is often regarded as a 'master regulator' of overall metabolism as it coordinates many complex metabolic pathways to provide energetic stability throughout the entire body. Additionally, two paralogues were later discovered and termed PGC-1β and PRC (PGC-related co-activator), however both proteins have been highly overshadowed by PGC-1α resulting in relatively limited knowledge about their regulatory roles (Austin & St-Pierre, 2012; J. Lin et al., 2005). This is mainly due to irregularities in PGC-1α expression acting as a hallmark of many metabolic diseases, indicating that PGC-1α function is imperative when it comes to understanding the underlying causes of cardiovascular diseases, neurodegenerative diseases, cancer, obesity and diabetes (Austin & St-Pierre, 2012; Handschin & Spiegelman, 2008; Wenz, 2011).

The immense complexity of the PGC-1α regulatory network presents unique challenges for researchers wishing to study this important co-activator. Early PGC-1α studies utilized adenoviral vectors to overexpress PGC-1α in murine tissues and cell culture lines to provide experimental approaches to PGC-1α discovery. However, as a metabolic co-activator PGC-1α activity is heavily influenced by both environmental and cellular factors which must both be controlled in order to examine the true roles of the protein. Furthermore, later findings

demonstrated that PGC-1α could be influenced by cross-tissue regulatory networks, effectively limiting the knowledge gained by traditional isolation techniques (Balampanis et al., 2019). As a result, modern PGC-1α research is primarily conducted in multicellular organisms with altered PGC-1α expression profiles using genetic modification techniques, such as CRISPR, morpholino injections, or Cre-Lox recombination (J. Lin et al., 2004). These techniques aim to toggle or disable PGC-1α expression to explore distinct phenotypes and genetic pathways associated with PGC-1α activity. Surprisingly, -/- PGC-1α whole body knockouts in mice remain viable despite its relatively important roles in individualized tissues, suggesting that PGC-1α might activate only in response to alterations in metabolic states (Arany et al., 2005; J. Lin et al., 2004; Wende et al., 2007).

Further studies on PGC-1α have examined the protein with excessive (or absence) of bioenergetic stimuli to provoke further regulatory behaviour. Such conditions included long fasting periods, overfeeding with high caloric or fat diets and extensive exercise; all implemented in the genetic models discussed previously. As expected, these studies were successful at evoking additional PGC-1α phenotypes in wildtype and +/+ PGC-1α mice proving that PGC-1α responds to changes in diet and exercise (Geng et al., 2010; Handschin & Spiegelman, 2008; Wende et al., 2007; Wilson, 2017). These results are not surprising as both diet and exercise directly alter nutrient consumption and energy output, however they suggest that PGC-1α plays an important role in mediating energetic ‘supply and demand’. These studies have been very helpful in the determination of overall PGC-1α function; however the underlying regulatory mechanisms (TF activation) remain variable in the literature. Overall, these results indicate that further research on the PGC-1α protein is necessary.

Table 1-2. Cell culture experiments featuring PGC-1α interactions with transcription factors and their resulting phenotypes.

Tissue Type	Transcription Factor	Phenotype
Brown Fat	PPARγ, THR (Thyroid Hormone Receptor), ERα (Estrogen Receptor), RARα (Retinoic Acid Receptor α)	Increased Thermogenesis (Puigserver et al., 1998)
Cardiac Muscle	ERRα, ERRγ	Increased Mitochondrial Biogenesis, Decreased Mitochondrial Uncoupling (Lehman et al., 2000)
	PPARα	Increased FAO (Huss et al., 2002)
Skeletal Muscle	NRF-1, NRF-2	Increased Mitochondrial Biogenesis, Thermogenesis (Wu et al., 1999)
	PPARβ	Increased FAO (Y. X. Wang et al., 2003)
Liver	HNF-4a (Hepatocyte Nuclear Factor 4 α)	Increased Gluconeogenesis (Herzig et al., 2001; Yoon et al., 2001),
	ERα	N.D. (Tcherepanova et al., 2000)
	PXR (Pregnane X Receptor)	Glucose/Cholesterol Metabolism (Bhalla et al., 2004)
	PPARγ, HNF-4a, FNX (Farnesoid X Receptor)	Increased FAO (Zhang et al., 2004)
Kidney	PPARα	Increased FAO (Vega et al., 2000)
	PPARβ	Increased FAO (Y. X. Wang et al., 2003)
	ERRα	Increased FAO (Schreiber et al., 2003)
White Fat	PPARβ	Increased FAO (Y. X. Wang et al., 2003)

Table 1-3: Phenotypes Produced by Various PGC1a Mouse Models

Tissue Type	Genetic Modification (Overexpression)	PGC-1α Expression (Overexpression)	Phenotype (Overexpression)	Genetic Modification (Knockout)	PGC1a Expression (Knockout)	Phenotype (Knockout)
Whole Body	Insertion of human PGC-1α transcript (Liang et al., 2009)	1.5 - 2.5x Increase (across all tissues) (Liang et al., 2009)	Increased insulin sensitivity (muscle), absence of cardiac edema (heart), Increased gluconeogenic/mitochondrial enzymes (liver), Decreased FA content (liver), Fiber type switching (muscle), Increased mitochondrial enzymes (muscle) (Liang et al., 2009)	Neomycin-disruption of PGC-1α (-/- PGC-1α) (Leone et al., 2005)	Absent in Heart, BAT, Muscle (Leone et al., 2005)	Decreased thermogenesis with matched UCP-1 expression (BAT), No difference in blood glucose/sensitivity in females but greater sensitivity in males (blood), loss in brain structural integrity (brain), Decreased heartrate and left ventricle fractional shortening following exercise (heart), Decreased cardiac output at rest (heart), Fatty liver after fasting (liver), Reduced FAO through S2+S3 (liver), Reduced tissue mass (heart, muscle), Fewer / Smaller Mitochondria and fewer mitochondrial enzymes (muscle), Reduced S3 O_2 consumption (muscle), Reduced physical endurance following exercise (whole body), Lower body mass following birth but higher during adulthood (whole body), Higher % body fat during adulthood more-so in females (whole body) (Leone et al., 2005)
				Cre-Lox mediated knockout + Recombination (-/- PGC-1α) (Arany et al., 2005; J. Lin et al., 2004; Wende et al., 2007)	Absent in Liver, Muscle, BAT (Arany et al., 2005; J. Lin et al., 2004; Wende et al., 2007)	Resistant to obesity (adipose), Decreased thermogenesis (BAT), Striatum lesions (brain), Decreased neurite branches (brain), Decreased gluconeogenic enzymes (liver), Decreased FAO/Mitochondrial enzymes (muscle), Increased O_2 consumption (whole body) (J. Lin et al., 2004) Increased glucose and glycogen levels post-exercise (muscle), Decreased GLUT4 expression (muscle), Decreased running distance (muscle) (Wende et al., 2007), No difference in cardiac structure or mitochondrial content (heart), Decreased CPT1, CPT2 proteins and 'lower' PPARα, ERRα, NRF-1 expression (heart), Decreased cardiac performance (heart), Decreased fractional shortening (heart), Decreased ATP and PCr (heart), Increased cardiac stress and dysfunction at late age (heart), slight increase in oxidative fibres (muscle), decreased mitochondrial enzymes in isolated cells (muscle) (Arany et al., 2005)

Tissue Type	Genetic Modification (Overexpression)	PGC-1α Expression (Overexpression)	Phenotype (Overexpression)	Genetic Modification (Knockout)	PGC1a Expression (Knockout)	Phenotype (Knockout)
Heart	Overexpression via α-MHC promoter (Lehman et al., 2000)	N.D	Increased mitochondrial biogenesis, cardiac edema (Lehman et al., 2000)			
	Induced overexpression via TRE promoter (Russell et al., 2004)	N.D	Increased mitochondrial density and volume, Decreased myofibrillar density, Increased NRF-1 expression, No difference in FAO CPT-1 or MCAD expression, Cardiac biventricular dilation, Increased mortality via cardiac disfunction, Reduced fractional shortening (Russell et al., 2004)			
Muscle	Transgenetic Overexpression and Knockout via TRE-promoter (Wende et al., 2007)	N.D	Increased mitochondrial enzymes, Increased type I fibre related genes, More prominent redness in mixed-muscle, Increased cyt. c, myoglobin expression in white muscle (J. Lin et al., 2002)	Transgenetic Overexpression and Knockout via TRE-promoter (Wende et al., 2007)	N.D	Reduced muscle glycogen levels (Wende et al., 2007)
	Transgenetic Overexpression via MCK-promoter (J. Lin et al., 2002)	Increased expression in type II muscle (plantaris) matching WT type I muscle (soleus) (J. Lin et al., 2002)	Increased mitochondrial enzymes, Increased type I fibre related genes, More prominent redness in mixed-muscle, Increased cyt. c, myoglobin expression in white muscle (J. Lin et al., 2002)	Cre-Lox Mediated Knockout (-/-PGC-1α) (Geng et al., 2010)	0.2x Reduction (Geng et al., 2010)	Exercise induced mitochondrial biogenesis, Increased physical endurance following exercise, No effect on exercise induced fibre type switching (Geng et al., 2010)

1.10 Evolutionary Differences in PGC1α

The majority of current PGC-1α research has been conducted in mammals, however the function of the protein remains elusive in other vertebrate models (LeMoine et al., 2010; Siepel et al., 2005). Vertebrates have diverged greatly over the past 370 million years following multiple whole genome duplication events (Kumar & Hedges, 1998). These events created functionally identical copies of pre-existing genes, increasing genetic redundancy and eventually resulting in the divergence of duplicated genes resulting in various outcomes including retained and newly derived functions (Force et al., 1999; Meyer & Schartl, 1999). Excluding mammals and birds, most vertebrates (fish, amphibians and reptiles) are ectothermic and lack BAT or endogenous capacity to produce heat, relying on heat from the environment to maintain body temperature (Else & Hulbert, 1981; Zotin, 2018). Primitive forms of the PGC1 family genes can be found in invertebrate species (drosophila, mosquito and sea squirt lineages), however the divergence of PGC-1α/β was believed to occur after the first whole genome duplication event during early chordate evolution. An extensive study examining the phylogeny of PGC-1α transcripts revealed that the amino and carboxyl termini are highly conserved throughout vertebrates, however the internal segment featuring the NRF-1 and MEF2c binding domains experienced increased variation in ray-finned fish species (LeMoine et al., 2010). Additionally, multiple fish species contain serine and glutamine insertions inside the NRF-1 and MEF2c domains that could alter PGC-1α interactions with these proteins, as suggested by studies that have examined NRF-1 and PGC-1α activity in goldfish (Katharina Bremer et al., 2012; LeMoine et al., 2008, 2010). Ultimately, these variant sections of the PGC-1α protein suggest that PGC-1α regulation in lower vertebrates may not be identical to those reported in mammalian models (LeMoine et al., 2008, 2010). With the majority of PGC-1α research focused on mice and human cells, our knowledge

of PGC-1α in other vertebrates is limited. Hence, there is a need for a genetically altered organism from the vertebrate linage to provide a suitable model for the investigation of PGC-1α in non-mammalian species.

1.11 Zebrafish – A Teleost Model for PGC-1α Research

One promising organism for the study of PGC-1α in fish species is the zebrafish, a model organism highly valued due to its extensively annotated genome, relatively low caretaking costs and high reproductive output (Bailone et al., 2020; Suurväli et al., 2020). Collectively, these features position the zebrafish as an attractive model for high-throughput research and the creation of mutant lines serving as the perfect teleost analogue of the traditional mouse model. Additionally, zebrafish organs are highly reminiscent of human organs making them exceptional models organisms for biomedical and metabolic disease research (Befyaeva et al., 2010; Goldsmith & Jobin, 2012; Seth et al., 2013; Teame et al., 2019). Currently, few publications have examined the role of PGC-1α in zebrafish, however no consensus has been reached with regards to the actual role of this co-activator in teleost species. Some of the first investigations of PGC-1α disruption in zebrafish were accomplished by inserting human PGC-1α transcript downstream of the actc1b promoter, effectively creating a mutant zebrafish with localized PGC-1α expression in muscle tissue. Consequently, this mutant experienced 8-fold increases in muscle PGC-1α expression and phenotypes that resembled previously described PGC-1α-overexpressed murine models; featuring ‘red meat’ muscle tissue, increased mitochondrial biogenesis, increased physical endurance, fibre-type switching and mitochondrial restructuring (Parisi et al., 2018). Although these results show that mammalian PGC-1α can elicit a phenotypic response in a teleost organism, it does not necessarily suggest that similar phenotypes occur in response to teleost PGC-1α overexpression. This is largely due to differences between teleost-mammalian

PGC-1α transcript (mentioned previously) and the relative expression of actc1b in comparison to endogenous PGC-1α. Additionally, both zebrafish and human PGC-1α transcripts were expressed simultaneously through different promoters, suggesting that both co-activators may be responsible for creating a hybrid phenotype.

Another approach to identifying the role of PGC-1α in zebrafish featured the administration of anti-sense morpholino to effectively silence PGC-1α expression in larval zebrafish. Using specially redesigned siRNA, it is possible to temporally prevent the transcription of gene expression into function proteins. Recent work in our laboratory successfully created 2 independent knockdowns to identify functional differences in larval zebrafish with decreased PGC-1α and PGC-1β expression. Fundamentally, this work suggested that zebrafish PGC-1α may be interacting with ERRα, but not NRF-1, strongly supporting the idea of evolutionary divergence in PGC-1α function between teleosts and mammals (Northam & LeMoine, 2019). Unfortunately, the research potential for zebrafish larvae is limited in comparison to the later life stages of juvenile and adult zebrafish due to the decoupling of exponential growth in early development and the presence of distinct tissues. Furthermore, zebrafish larvae are small and fragile, limiting the extent of performance and advanced physiological procedures that are required for determining both muscle and mitochondrial performance. Despite these differences, this investigation of PGC-1α activity in larval zebrafish provides sufficient evidence that the PGC-1α co-activator is divergent from that of mammalian species and suggests that further research is necessary to determine if these roles are the same using fully developed adult zebrafish.

1.12 Thesis Objectives

The main objective of my thesis is to examine the role of the metabolic co-activator PGC-1α in zebrafish to investigate underlying mechanisms of metabolic regulation in lower vertebrates. Currently, much of our understanding of cellular metabolism is based on mammalian models; with lower vertebrates obtaining very little attention. To accomplish this, I sought to create a zebrafish mutant line with modified PGC-1α expression to determine the molecular pathways and tissue-specific phenotypes promoted by this co-activator protein. In my 1st study, I successfully created this mutant zebrafish line by disrupting a newly discovered evolutionary conserved regulatory promoter region. This mutation vastly altered heart and muscle PGC-1α expression simultaneously, suggesting that this sequence holds a specialized function in muscle tissues. In addition, this mutation increased zebrafish O_2 consumption in both isolated white muscle tissue fibres and whole juvenile zebrafish at rest, marking increased basal metabolism as a primary phenotype of this mutant line.

In the 2nd study, I utilized the newly established PGC-1α mutant zebrafish line to determine if an increased basal metabolism would negatively impact the growth of mutant zebrafish during both larval and adult life stages. Newly hatched mutant larval zebrafish had a decreased heart rate with increased yolk sac content, suggesting decreased FA metabolism with decreased tissue growth at this early life stage. During the adult stage, mutant zebrafish displayed an increased ability to metabolize FAs but still experienced diminished growth even when supplemented with a high-fat diet, demonstrating that PGC-1α mutant zebrafish face impaired growth throughout their lifespan. In summary, the work presented here indicates that the regulatory networks coordinated by zebrafish PGC-1α are not entirely matched to those of mammalian PGC-1α,

however the regulatory region investigated may have conservative features worth further investigation in higher vertebrates.

Chapter 2: An evolutionary conserved regulatory sequence dictates PGC-1α expression in Zebrafish striated muscles

Kurchaba N, Charette M and LeMoine CMR. In preparation for journal submission.

2.1 Chapter Summary

The peroxisome proliferator activated receptor gamma co-activator 1 alpha (PGC-1α) is central to the regulation of cellular and mitochondrial energy homeostasis in mammals, but its role in other vertebrates remains unclear. Indeed, previous work suggests extensive structural and functional divergence of PGC-1α in teleosts but this remains to be directly tested. Here, we describe the characterization of a heterozygous PGC-1α mutant zebrafish line created by CRISPR-Cas9 disruption of an evolutionary conserved regulation region of the PGC-1α proximal promoter. Using qPCR, we confirmed the disruption of PGC-1α gene expression in striated muscles, leading to a simultaneous 4-fold increase in mixed skeletal muscle PGC-1α mRNA levels and a contrasting 4-fold downregulation in cardiac muscle. In mixed skeletal muscle, most downstream effector genes were largely unaffected yet two mitochondrial lipid transporters, carnitine palmitoyltransferase 1 and 2, were strongly induced. Conversely, PGC-1α depression in cardiac muscle reduced the expression of several transcriptional regulators (estrogen related receptor alpha, nuclear respiratory factor 1 and PGC-1β) without altering metabolic gene expression. Using high resolution respirometry, we determined that white muscle exhibits increased oxidative capacity with little difference in mitochondrial function. Finally, using whole animal intermittent respirometry, we show that mutant fish exhibit a 2-fold higher basal metabolism than their wildtype counterparts. Altogether, this new model confirms a central but complex role for PGC-1α in mediating energy utilization in zebrafish and we propose its use as a valuable tool to explore the intricate regulatory pathways of energy homeostasis in a popular biomedical model.

2.2 Introduction

The energetic demands of an animal are continuously adjusted to respond to environmental and physiological disturbances, requiring a dynamic equilibrium of energy acquisition and energy use. Under aerobic conditions, mitochondrial metabolism is central to an animal's energy homeostasis. In the presence of oxygen, the organelle oxidizes fuel substrates to establish an electrochemical gradient and produce cellular energy. Thus, throughout an animal's lifetime mitochondrial abundance and biochemical characteristics must be modulated to accommodate changes in bioenergetic demands (Chan, 2012; Woods, 2017). However, mitochondrial proliferation and maintenance is an intricate process that requires the synchronous regulation of hundreds of genes. This is further complicated by the shared responsibility of the nuclear and mitochondrial DNA for encoding all genes necessary for normal mitochondrial function (Francis et al., 2003; Jornayvaz & Shulman, 2010; Kang et al., 2007). At the transcriptional level, a suite of transcription factors and their coregulators collectively carry out this complex regulation and ensure metabolic homeostasis (Scarpulla, 2008, 2010).

Particularly prominent among metabolic regulators, multiple members of the nuclear receptor family exert essential roles in mitochondrial control (Francis et al., 2003; Mazaira et al., 2018). Indeed, various members of the superfamily have been intensively studied in mammals due to their role in normal physiological function and various pathological states (e.g., cancer, heart failure, diabetes) (Robinson-Rechavi et al., 2003). In particular, the peroxisome proliferated activated receptor family (PPARα, PPARβ, and PPAR) plays an essential role in lipid metabolism, while the estrogen-related receptor a (ERRα) regulates both nuclear and mitochondrial genes essential to the organelle's function (Ranhotra, 2015; Tyagi et al., 2011; Youssef & Badr, 2013). Beyond the nuclear receptor family, other important mitochondrial

regulators, such as the transcription factors Nuclear Respiratory Factor 1 (NRF-1) and NRF-2, facilitate mitochondrial biogenesis in mammalian cells (Wu et al., 1999). Although each of these transcription factors individually carry regulatory functions, their respective transcriptional activities are also tightly regulated by interactions with co-activator and co-repressor proteins.

Central to this additional level of regulation, over the past decades the PPARγ Coactivator 1α (PGC-1α) has emerged as a master regulator of mammalian metabolic activity. As a co-activator, PGC-1α can bind to and regulate a multitude of TFs associated with metabolic genes (Ventura-Clapier et al., 2008). In addition to the PPARs, Estrogen Related Receptor α (ERRα) and Nuclear Respiratory Factor 1 (NRF-1) serve as upstream activators for PGC-1α in mammals, controlling mitochondrial biogenesis, gluconeogenesis, β-oxidation, and muscle fiber remodeling (Finck & Kelly, 2006; Jornayvaz & Shulman, 2010; J. D. Lin, 2009). In mammalian systems, the coactivator drives many of the necessary mitochondrial adjustments in response to changes in energy demand, including cold-induced thermogenesis, fuel mobilization in fasting, or muscle adaptation to exercise (see Fernandez-Marcos and Auwerx, 2011 for review). The various stimuli responsible for PGC-1α induction apparently converge towards a handful of transcription factors. In particular, myocyte enhancer factor 2 (MEF2), forkhead box class-O (FoxO1) activating transcription factor 2 (ATF2) and cAMP response element-binding protein (CREB) all upregulate PGC-1α transcription by binding to upstream regulatory sites in its proximal promoter (Akimoto et al., 2005; Handschin et al., 2003; Herzig et al., 2001; J. Lin et al., 2002; Sajan et al., 2018).

Interestingly, while this ‘master controller’ is certainly central to metabolic plasticity in mammals, not all of these characteristics appear to be conserved by PGC-1α in other vertebrates. Indeed, the PGC-1α amino-acid sequence shows increased divergence amongst teleosts,

particularly with respect to the NRF-1 binding motif (LeMoine et al., 2010). Furthermore, dietary and temperature manipulations in goldfish showed alteration of mitochondrial function decoupled from PGC-1α but not the NRF-1 axis (LeMoine et al., 2008). Conversely, both PPARα and ERRα PGC-1α-binding motifs appear to be highly conserved throughout evolution, suggesting that these two TFs could still maintain interactions with PGC-1α and collectively act as important metabolic regulators in non-mammalian vertebrates (LeMoine et al., 2010).

Recently, the use of reverse and forward genetics fostered the investigation of PGC-1α's regulatory activity in non-mammals (Dumesic et al., 2019). Recently, morpholino silencing of PGC-1α in larval zebrafish suggested that metabolic cascades were negatively affected by PGC-1α knockdown in early development (Northam & LeMoine, 2019). Thus, it appears that some PGC-1α-regulated roles remain partially conserved among vertebrates, but further experimental work is required to validate this argument. Unfortunately, the transient nature of morpholino knockdowns limited observations to early developmental time points and were insufficient to assess many of the potential roles of the co-activator in later life stages. Thus, given the complexity of PGC-1α regulation, we here developed a different approach to study endogenous function of PGC-1α in a teleost species throughout its lifetime.

The use of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology has provided an invaluable tool for investigating gene function in living model organisms. Such techniques allow for site-directed deletions occurring within the promoter region of target genes often leading to impaired function of the protein and cellular cascade imbalances resulting in a reprogramming of metabolic processes (Spiegel et al., 2019). Although the deletion of vital genes often leads to premature death, alternative approaches can be used to target regulatory regions (such as TF binding sites) to provide insight on endogenous regulatory mechanisms

(Babaei et al., 2019). The zebrafish has been used in a variety of CRISPR studies as a model organism due to its well-documented genome, high fecundity and external fertilization allowing for simplified CRISPR delivery (Cornet et al., 2018; Hruscha & Schmid, 2015). Furthermore, its use as a model organism is widespread in toxicology, comparative genetics, and biomedical research making it an ideal target to generate gain or loss of function models for broad use (Befyaeva et al., 2010; Goldsmith & Jobin, 2012; Gonzales & Joanna Yeh, 2014; Teame et al., 2019).

Thus, to investigate the role of PGC-1α in a lower vertebrate, we created a CRISPR zebrafish model targeting an evolutionary conserved region in a recently discovered regulatory region of the PGC-1α promoter (Dumesic et al., 2019). Our deletion disrupted putative TF binding sites, resulting in a 4-fold increase in PGC-1α mRNA expression in mixed skeletal muscle and 4-fold decrease in the heart. Our results indicate that PGC-1α expression potentially increases skeletal muscles' fatty-acid (FA) transport through the carnitine palmitoyltransferase (CPT) axis and promotes increased mitochondrial capacity in white muscle. These in turn affect whole animal metabolism by effectively doubling their routine metabolic rate (RMR). With these properties, we propose that our mutant zebrafish line presents a valuable biomedical model for the investigation of various metabolic disorders and to further our understanding of PGC-1α as a metabolic coactivator in an established model organism representative of teleost species.

2.3 Methods

2.3.1 Caretaking

Commercially obtained adult zebrafish were maintained and bred for several generations with ongoing introductions in our in-house colony. The fish were housed in an aquatic housing system (Aquaneering, San Diego, CA) maintained at 27°C with a 14:10hr light-dark cycle with two

feedings of Zeigler Adult Zebrafish Complete Diet and one feeding of *Artemia naupii* daily for the preparation of the F_0 generation of mutant zebrafish. Embryos were obtained using breeding traps in communal holding tanks and collected shortly after the beginning of the light cycle. Embryos were washed with 0.05% bleach solution and rinsed with dechlorinated water twice prior to micro-injection. Injected embryos were kept at 28°C in E3 solution (5mM NaCl, 0.17mM KCl, 0.33mM CaCl2, 0.33 mM MgSO4, 0.0006 mM methylene blue) for 5 days with daily medium changes before being moved into 300ml beakers filled with aged dechlorinated system water. Approximately 1/3 of the water was replaced daily following two feeding events; one with phase I/II larvae feed (<50/<200 microns) and another with *Artermia naupii*. On day 21, the F_0 zebrafish were moved into the aquatic housing system. All zebrafish were screened for mutations following the CRISPR HRM protocol described below. The F_0 fish with considerable DNA deletions were then bred to produce the F_1 generation. Care for the F_1 generation of zebrafish was as described above. All procedures follow the guidelines of the Canadian Council of Animal Care and were approved by the Brandon University Animal Care Committee.

2.3.2 CRISPR Design and Delivery

The CRISPR gRNA was designed using the Benchling software to target potential regulatory sequences upstream of the PGC-1α promoter (*Benchling*, 2019). Two gRNAs with on-target and off-target scores above 60 were selected, synthesized and tested before opting for the guide with highest on-target mutation rate for the creation of the mutant line. The gRNA synthesis was performed using a GeneArt™ Precision gRNA Synthesis Kit following standard procedures with the use of custom template primers (Table S1). Prior to injection, the gRNA was mixed with GeneArt™ Platinum™ Cas 9 Nuclease in a 3:1 ratio with phenol red dye (20mg/µl) to visualize the site of injection. The first generation (F_0) embryos received 100pl of injection mix via

microinjection using 1.0mm borosilicate glass micropipette needles into the yolk during the single cell stage with the use of picopump (PV830 Pneumatic Picometer, World Precision Instruments, Saratosa, FL) and micromanipulator (MM3301R, World Precision Instruments, Saratosa, FL) (Northam & LeMoine, 2019). We removed all embryos beyond the 2-cell stage post-injection to limit mosaicism.

2.3.3 CRISPR Screening

Fin tissue samples (adults) or whole embryos (larvae) were collected in order to identify potential mutant zebrafish. Prior to tissue collection, adult zebrafish were briefly exposed to 100mg/L of tricaine solution until movement had ceased and gill movement had significantly slowed. Zebrafish were promptly removed from the tricaine solution and placed in a water filled petri plate for fin clipping before being returned to the aquatic system. All zebrafish were isolated and monitored for up to 1 week post-amputation to ensure adequate recovery before transfer to communal tanks in the same aquatic system. Fin clips were submerged in 10µl of NaOH and heated to 100°C for 10 minutes for crude DNA extraction as described previously (Samarut et al., 2016). Zebrafish embryos were humanely euthanized with 100mg/L of tricaine solution prior to DNA extraction following the same protocol as fin clippings.

After DNA extraction, High Resolution Melt (HRM) analysis was used to examine the DNA melt curves of individual zebrafish (Qiagen, Hilden, Germany) (Samarut et al., 2016). A single primer set was generated using Primer3 to target a 200bp region surrounding the CRISPR site (Table S1, Untergasser et al., 2012). A standard concentration of DNA (50ug/µl) was used as suggested by the manufacturer's protocol. Using Qiagen RoterGene Q® software (V2.3.5, Qiagen, Hilden, Germany) we detected mutants using normalized melt curves by identifying deviations from wildtype zebrafish DNA profile (Figure S1). Multiple peaks and shifted melt

curves following normalization procedures allowed for the detection of mutant zebrafish and were used as diagnostics to establish the F_1 generation.

2.3.4 Sequencing

Genomic DNA from 9 HRM-confirmed mutant zebrafish were selected for Sanger Sequencing to validate the position and identity of the CRISPR modified genomic DNA. PCR amplified DNA samples were cloned into plasmid vectors using NEB® PCR Cloning Kit (New England Biology, Whitby, Canada) following heat-shock absorption by competent *E. coli* plated on nutrient agar. Five individual colonies from each plate (45 colonies total) were subcultured in liquid media overnight prior to centrifugation to obtain a plasmid-rich liquid fraction and later purified using an EZ-10 Spin Column Plasmid DNA Minipreps Kit (Biobasic, Markham, Canada). Plasmid extracts were validated by gel electrophoresis and sent to Genome Quebec (Montréal, Canada) for Sanger sequencing. Sequence data was retrieved in FASTA format and aligned using Geneious Prime (Geneious, Auckland, New Zealand) to create consensus sequences. The consensus sequence was uploaded to rVista 2.0 to identify regulatory motifs using both mouse and human reference genomes (Loots & Ovcharenko, 2004). The region of interest was then used to retrieve similar sequences from representative vertebrate lineages, and aligned in Multalin (Corpet, 1988).

2.3.5 RNA Extraction, Reverse Transcription and qPCR

Twenty-four adult F_1 PGC-1α mutant and wildtype zebrafish (12 and 12 respectively) were humanely sacrificed by concussion and severance of spinal cord, and dissected to obtain tissues (brain, gills, heart, liver, muscle, and gut) for RNA extraction. Samples consisted of 6 male and 6 female control fish (300 ± 36 mg), and 9 male and 3 female mutant fish (308 ± 23 mg). All extractions were performed using an Animal Tissue Extraction Kit (Norgen, Thorold, Canada) in

accordance with the manufacturer's protocol and each sample was inspected using a NP80 Nanophotometer (Implen®, Munich, Germany) to validate RNA quantity and purity. A total of 500ng of RNA was used to generate cDNA using the GoScript™ Reverse Transcription System (Promega, Madison, USA). The resulting cDNA was diluted prior to using Quantinova SYBR® Green PCR (Qiagen, Hilden, Germany) to assess the transcript levels of 14 genes (see Table S1). Target genes included transcriptional regulators (PPARα, ERRα, NRF-1), ETS components (COX IV), Krebs cycle enzyme (CS), lipid β-oxidation enzymes (HOAD, MCAD) and lipid transporters (FABP3, CPT-1, CPT-2). Each primer set was checked for efficiency using standard curves ensuring efficiency values of 1.00 +/-0.20 (Northam & LeMoine, 2019). Gene expression values were calculated using the ΔΔCT method using the geometric mean of EF1a and Rpl1 as housekeeping genes (Ibarra et al., 2017). Statistical differences were detected using the Student's t-test after confirming both normality and equal variance of the datasets. In instances where the data was not normally distributed, the data was normalized by log transformation and retested using the Student's t-test. Genes that were normalized but failed to show equal variance (Heart PPARα, CS) were exclusively tested using the Welch t-test.

2.3.6 High-Resolution Respirometry

Twelve adult F_1 zebrafish were sacrificed to individually assess mitochondrial function of red and white muscle. Sampling consisted of 6 mutants (3 males, 3 females; 480 ± 30mg) and 6 wildtypes (4 males, 2 females; 446 ± 44 mg). High resolution respirometry was performed using the Oroboros O2K following the SUIT-002 (D006) protocol (Doerrier et al., 2018). Muscle fibers were quickly dissected and separated before being placed into ice-cold BIOPS buffer (10 mM Ca-EGTA buffer, 0.1 µM calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM MgCl2, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1) and cut into small

2mm x 2mm squares. Tissue fibers were permeabilized in saponin solution (5mg/ml) for 20 minutes on ice with moderate shaking before being rinsed in BIOPS buffer for an additional 20 minutes. Fiber bundles were blotted dry on filter paper prior to weighing and then directly inserted into the O2K chamber containing MiRO5 respiration medium (0.5mM EGTA, 60mM K+ Lactobionicate, 3mM $MgCl_2$, 20mM Taurine, 10mM KH_2PO_4, 20mM HEPES, 110mM D-Sucrose, adjusted to pH 7.1 with KOH; with addition of 1g/l BSA). Pressurized O_2 provided sufficient oxygenation of the muscle fibers throughout the experiment. All values were background corrected using the DatLab Anaylsis Template. Statistical differences were calculated using Student's t-test (Sigmaplot 14.0) between all specific flux values and flux control ratios (FCRs).

2.3.7 RMR and MMR in Juvenile Zebrafish

The resting metabolic rate (RMR) and maximum metabolic rates (MMR) of juvenile zebrafish were measured with intermittent flow-through respirometry. Sixteen zebrafish, consisting of 8 mutants (40.2 ± 4.6 mg), 8 wildtypes (48.0 ± 4.2 mg), were individually chased for 5 minutes before being transferred to borosilicate tubes equipped with an oxygen sensor and two lines for water exchange. Measurements were conducted using AutoResp Software (Loligo Systems, Denmark) with a controlled flush loop (600s flush, 90s wait, 900s measurement). The measurement periods were further trimmed to 360s to focus on the linear segment of oxygen consumption. Data analysis was conducted with the use of RespR (v1.1.0.0, Harianto & Carey, 2019) by using the linear auto_rate function to determine the MMR using the first loop and the RMR using the lowest 10^{th} percentile of the subsequent loops. Statistical tests were performed using the Student's t-test following normalization by log transformation.

2.4 Results

2.4.1 CRISPR target

Throughout this study we used an HRM approach to identify CRISPR mutated Zebrafish. To confirm the adequacy of this screening technique, we randomly selected several F_1 fish showing a mutant phenotype on our HRM screen and sequenced the region of interest. The sequencing results confirmed the targeting of our CRISPR mutation to the PGC-1α proximal promoter. Moreover, we identified an extremely well conserved region of the PGC-1α promoter, as the site of several-overlapping mutations in CRISPR-F_1 zebrafish (Figure 1). The most prevalent mutation consisted of a 7-bp deletion occurring near the 3' end of the gRNA site. In addition, a smaller 4-bp and larger 13-bp deletion were also identified in several fish, in close proximity to the 7-bp deletion (Figure 3-1A). Using rVista 2.0, we identified *in silico* several putative regulatory regions within the overlapping regions of the DNA mutations including putative binding sites for MINI19/20, RUNX2, NFE2L1, and a highly conserved ATF/CREB response element. Comparing this region in various representative species indicates that the ATF/CREB response element is extremely well conserved in a majority of representative vertebrate species (Figure 3-1B).

2.4.2 Gene Expression

We sampled major tissues in adult fish and could only detect a significant impact of the mutation on PGC-1α mRNA levels in striated muscle tissues. In particular, CRISPR-mutants exhibited an overall 4-fold increase in mixed skeletal muscle and a contrasting 4-fold decrease in cardiac muscle (Figure 2-2A). To investigate the downstream effects of PGC-1α dysregulation in these two tissues, we examined multiple genes associated with metabolism and regulatory pathways. In skeletal muscle, none of the transcription factors assessed were significantly affected, though

PPARα levels were marginally increased. In contrast, we identified a net 4-fold increase in gene expression of CPT-1, paralleling the increase in PGC-1α levels, and a 29-fold change in CPT-2 mRNA levels (Figure 2-2B). All other effectors assessed (CS, COX IV, HOAD, MCAD, FABP3) seemed unaffected by PGC-1α overexpression in mixed skeletal muscle. In contrast, the reduction of PGC-1α transcript levels in cardiac muscle was accompanied by 50-70% decrease in gene expression of three central metabolic regulators: ERRα, NRF-1, and PGC-1β (Figure 2-2C). While not significant, a trend for downregulation of CS and COX IV was also apparently in cardiac muscles, while fatty acid metabolism enzymes were virtually identical in mutant and wildtype fish.

2.4.3 High-Resolution Respirometry

In order to measure mitochondrial activity in response to individual substrates, we assessed potential differences in red and white muscle mitochondrial physiology by measuring oxygen flux between permeabilized muscle fiber types isolated from wildtype and mutant fish. Overall, mitochondrial capacity per tissue weight amongst control fish was higher in red muscle, with an average doubled capacity for fatty acids respiratory flux, Complex I-III activities and for total electron transport chain capacity. As individual differences in mitochondrial efficiencies could be obscured by total fluxes and sample preparations, we also compared flux corrected ratios (FCRs) between muscle types. The FCRs of red and white muscles showed similar oxidative profiles suggesting that mitochondrial populations in the two tissues have similar functional characteristics in wildtype fish. In red muscle, although there seemed to be a trend for higher fluxes in mutant fish, none of the substrate/inhibitors additions significantly affected respiratory fluxes whether corrected for tissue weight or maximal flux (Figure 3A). In contrast, in the white muscle of mutant fish, fatty acid oxidation was marginally increased ($p= 0.09$), and there was a

30-40% significant increase in flux though Complex I-III per tissue weight (Figure 2-3B). In addition, mass corrected total electron transport chain capacity was 30% higher in mutant fish (Figure 2-3B), along with a parallel 25% increase in flux through Complex IV (data not shown). Once again, when corrected for maximal flux rates, the FCRs were identical in permeabilized white muscle of both mutant and wild type fish.

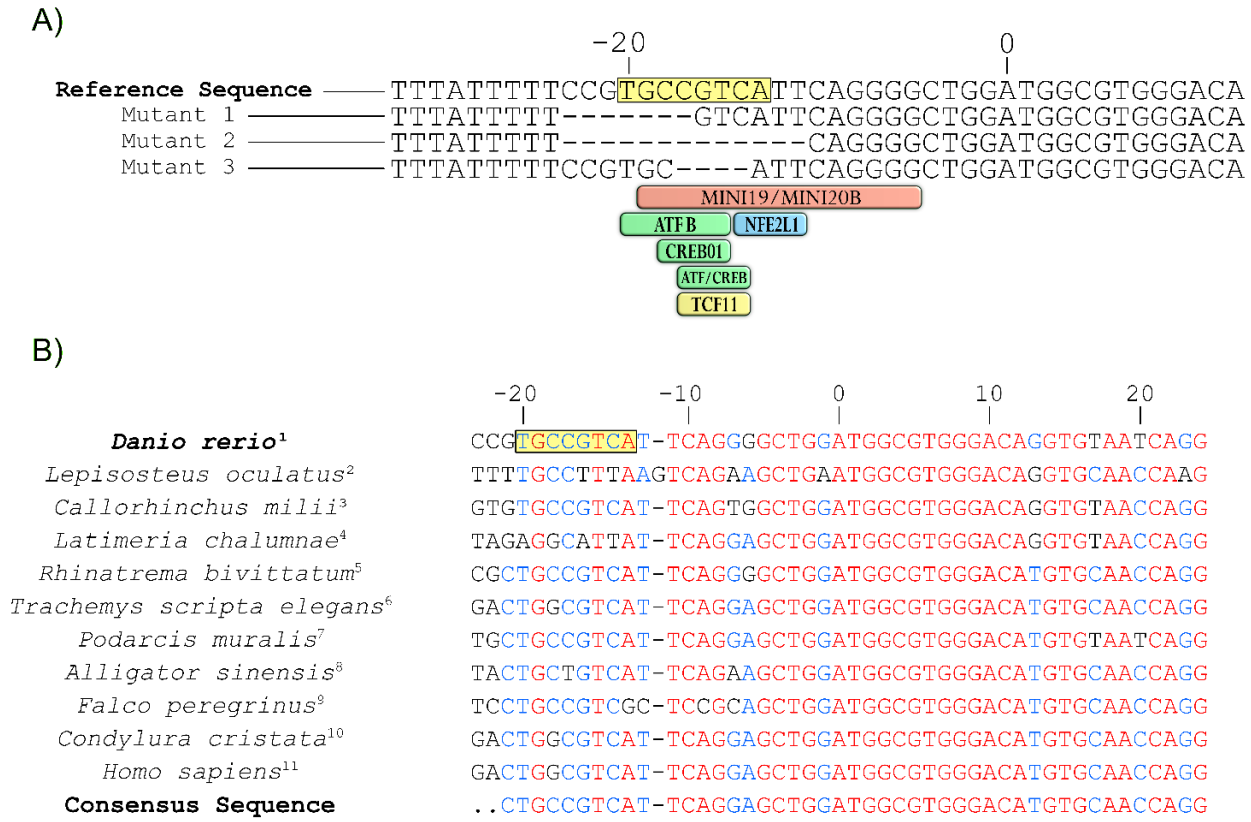

Figure 2-1: The CRISPR-Cas9 mutation occurred in an evolutionarily conserved region hosting multiple regulatory elements. A) Sanger sequencing alignment of individual mutants with the PGC-1α reference sequence, with the 0-position indicating the beginning of the start codon. Three different mutations were identified amongst mutant zebrafish with each mutation disrupting the conserved ATF/CREB response element (TGCCGTCA). B) Comparative alignment of the deleted region shows a high degree of sequence conservation between vertebrate species. Accession numbers for the included genetic transcripts: 1 - XM_017357140.2, 2 - XM_006629820.2, 3 - XM_007889043.1, 4 - XM_005997866.2, 5 - XM_029590174.1, 6 - XM_034770966.1, 7 - XM_028743340, 10 - XM_004681371.2, 8 - XM_025205580.1, 9 - XM_027795074, 10 - XM_004681371.2, 11 - NR_148984.2.

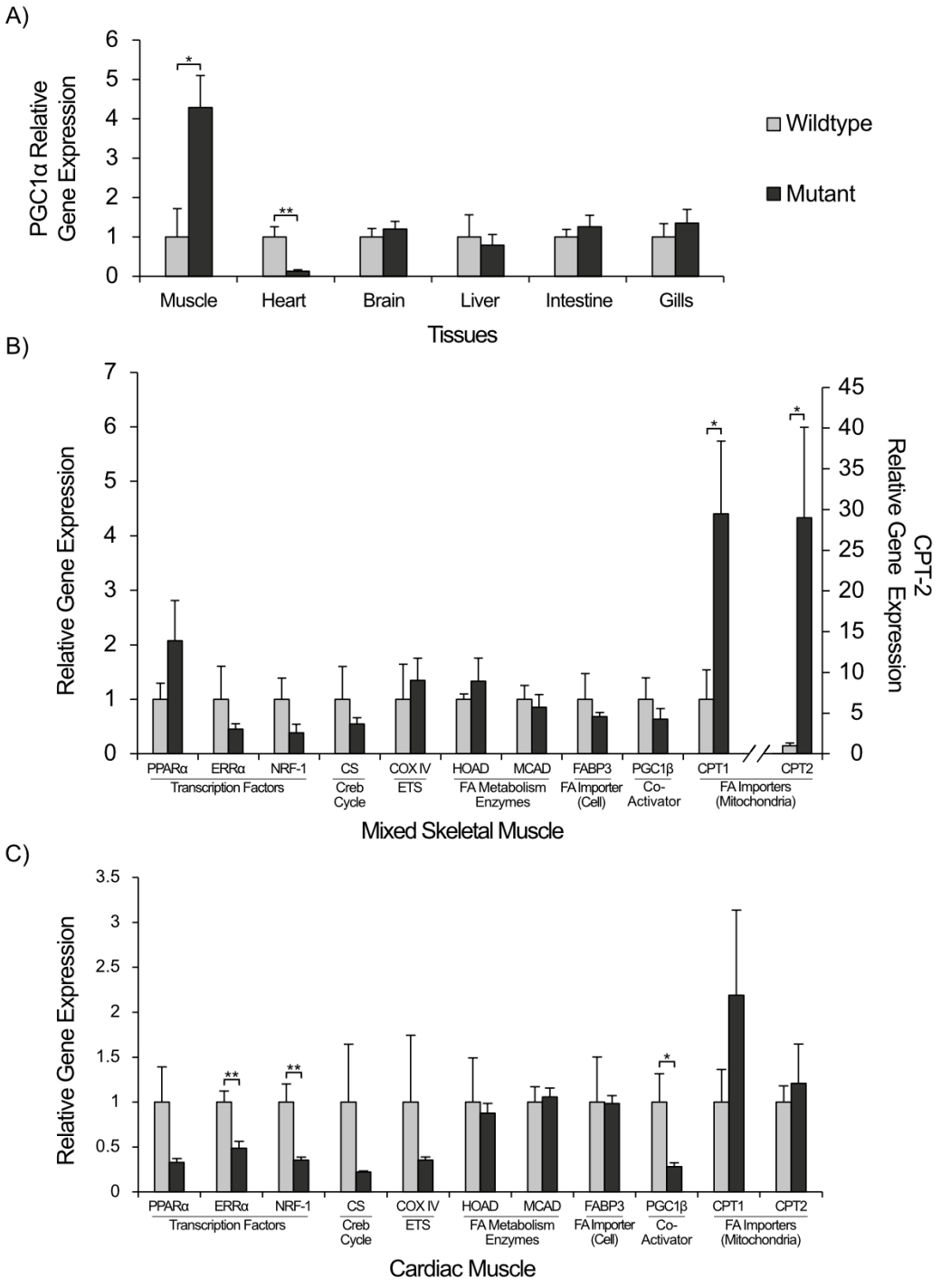

Figure 2-2: The CRISPR-Cas9 mutation altered PGC-1α expression in striated muscle tissues. A) PGC-1α expression across multiple tissues in both mutant and wildtype individuals. Sample sizes varied between tissue types (Muscle = 6, Heart = 5x2, Liver = 4, Intestine = 4, Gills = 3). PGC-1α expression was upregulated in muscle tissue 4-fold ($p = 0.009$) and downregulated in heart tissue 4-fold ($p = 0.012$). B) Increased PGC-1α expression in muscle tissue greatly increased CPT-1 (0.023) and CPT-2 ($p = 0.009$) gene expression 4-fold and 29-fold respectively. C) Decreased PGC-1α expression in cardiac tissue reduced ERRα ($p = 0.011$), NRF-1 ($p = 0.016$) and PGC-1β ($p = 0.045$) expression. Fold-increases were calculated by using the geometric mean of EF1α and Rpl-13a reference genes.

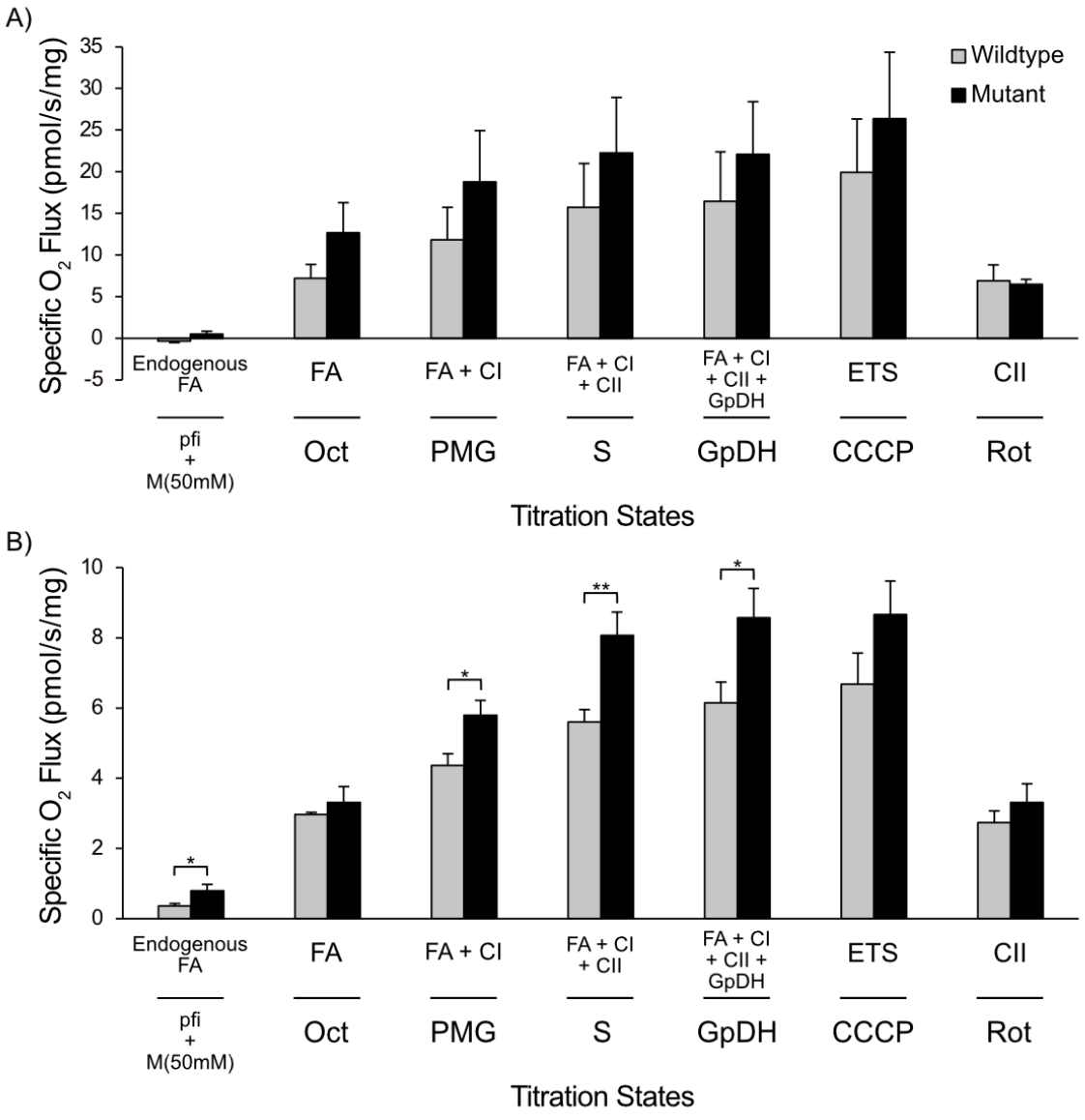

Figure 2-3: Mutant zebrafish maintained similar oxygen fluxes in permeabilized red muscle fibres, with increased oxygen fluxes in permeabilized white muscle fibres. A) Mutant zebrafish displayed similar flux values in red muscle fibres during each step of the SUIT-002-005 protocol. B) Mutant zebrafish maintained higher specific flux values immediately once placed into the O2K ($p = 0.038$), and when FA and CI pathways were activated following PMG addition ($p = 0.014$). Further activation of the mitochondria following S addition and the saturation of the FA, CI and CII pathways produced greater specific flux differences between mutant and wildtype zebrafish ($p = 0.005$), with GpDH addition also maintaining differences in specific flux ($p = 0.025$). M – Malate, Oct – Octynolcarnitine, PMG – Pyruvate-Malate-Glutamate, GpDH - Glycerol-3-phosphate dehydrogenase, CCCP - Carbonyl cyanide m-chlorophenyl hydrazone, Rot – Rotenone.

2.4.4 Whole Animal Respirometry

Given the impact of the PGC-1α mutation on skeletal muscle oxidative phenotype, we explored the impact our genetic manipulation could have on whole organismal metabolism. We first assessed a proxy of mass-specific maximal metabolic rate (MMR) by performing a 5-minute chase of the fish prior to oxygen consumption rate measurement. Overall, there was no observable difference in oxygen consumption between mutants and wildtype fish under these conditions. In contrast, when we measured mass-specific resting metabolic rate (RMR) overnight, mutant fish exhibited an oxygen consumption rate two-fold higher than their wildtype counterparts (Figure 2-3). Consequently, mutant fish exhibited an approximate 7% reduction in estimated aerobic scope (MMR-RMR), but this metric was not deemed statistically significant.

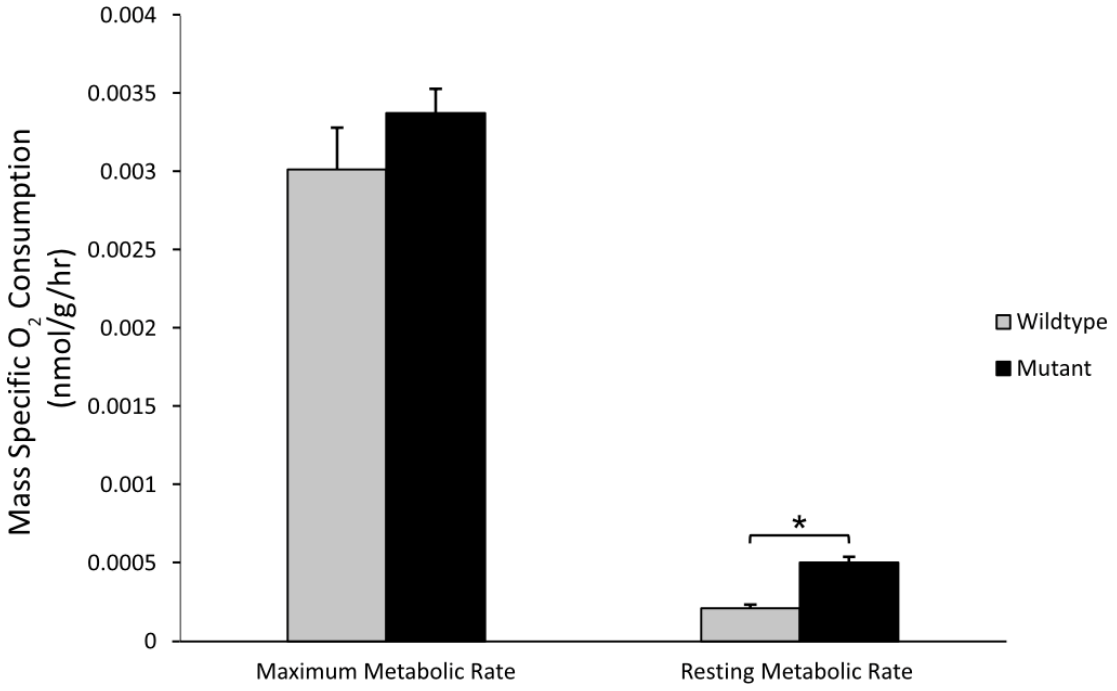

Figure 2-4: Mutant juvenile zebrafish maintain a higher resting metabolism in comparison to wildtype zebrafish. The maximum metabolic rate held near identical O_2 consumption rates between mutant and wildtype zebrafish, however mutant zebrafish maintained a 2-fold increase in O_2 consumption during the comparison of routine metabolic rates ($p = 0.018$).

2.5 Discussion

In mammals, the PGC-1 family of coactivators is of prime importance to the regulation of a plethora of metabolic pathways. In particular, the founding member of the family, PGC-1α, is ubiquitously expressed in mammals and plays a predominant role in muscle homeostasis in both health and disease. Until recently, the role of this co-activator in other biomedical model organisms such as the zebrafish remained elusive, hindering the use of this animal model in comparative metabolic and biomedical research. In the current study, we show that mutations in a highly conserved region of the PGC-1α promoter disrupts muscle-specific expression of this energetic co-activator. More specifically, this mutation drives skeletal and cardiac muscle PGC-1α gene transcription in opposite directions resulting in broad metabolic implications at the genetic, mitochondrial and whole organismal level.

2.5.1 A Conserved Regulatory Sequence Controls PGC-1α Expression in Striated Muscle

In order to modulate PGC-1α gene expression we opted to target the zebrafish PGC-1α proximal promoter, a region characterized by several regulatory sites in mammals (see Fernandez-Marcos and Auwerx, 2011). This region contains notable response elements for the human PGC-1α start codon such as MEF2 (-1,400 bp), FOXO1 (-500bp) and ATF/CREB (-130 bp) (Akimoto et al., 2005; Handschin et al., 2003; Sajan et al., 2018; Zhao et al., 1999). Through our CRISPR design, we specifically targeted a site immediately (-20bp) upstream of the PGC-1α start codon. Upon further *in silico* exploration of this area, we identified several putative transcription factor binding sites, including motifs for ATF/CREB/TCF and MINI19B/20B. From a regulatory perspective, the Muscle Initiator Sequences (MINI19B/20B) is interesting though relatively undescribed, but may serve as a repressor in muscles since its motif is exclusive to genes expressed in muscle tissue (Frith et al., 2004) and should be certainly explored further. The other predominant regulatory site, the putative ATF/CREB/TCF motif has an extensive repertoire of

roles devoted to metabolic regulation. Indeed, these motifs are highly abundant in eukaryotic promoters with ATF/CREB postulated to regulate expression of approximately 25% of all genes (Moore et al., 2016), playing a critical role in the regulation of cellular growth and metabolism (Shanware et al., 2010; Wen et al., 2010). More specifically, the CREBP-mediated activation of the mammalian cAMP regulatory cascade manages injury, regeneration, and intense exercise related pathways in muscle tissue (Berdeaux & Hutchins, 2019; Egan et al., 2010; Popov et al., 2019; Stewart et al., 2011; Widegren et al., 1998). Additionally, this pathway serves as a central regulatory pathway of PGC-1α expression in brain and liver (Handschin et al., 2003; Herzig et al., 2001; Lee et al., 2018; Liu et al., 2014). The CREBP pathway is also an important mediator of muscle homeostasis in zebrafish, as CREBP-targeted knockouts negatively impact muscle structure and function (Fauquier et al., 2018). In addition, the TCF regulatory element disrupted by our mutation is also closely linked to the CREB pathway with evidence suggesting that TCF may act as a co-repressor when present with CREB (Barker et al., 1999). Interestingly, the PGC-1α CRISPR-induced mutations resulted in differential expression of the co-activator in opposite direction in the heart and mixed skeletal muscle of zebrafish, with no impact on other tissues (see below). Certainly, while some transcription factors have ubiquitous effects across tissue types, others may be more restricted to certain tissues allowing inter-tissue variability in regulatory pathways (Qian et al., 2005; Sonawane et al., 2017; Vaquerizas et al., 2009). Furthermore, recent work suggests that a region in the 5'-URF is responsible for muscle PGC-1α repression in fish (Dumesic et al., 2019). Here we contend that this repression sequence can be further focused to a narrower region directly targeted by our CRISPR mutation. We propose that this promoter region may disrupt the transcriptional pathways of striated muscles as evidenced by the opposing effects of the mutation in cardiac and skeletal muscles.

2.5.2 Gene Expression

In order to gain insight on the effects of PGC-1α dysregulation in striated muscles we explored the expression levels of three important regulatory factors (PPAR, NRF-1 and ERRα) that regulate key metabolic genes in conjunction with PGC-1α (Huss et al., 2002; Vega et al., 2000; Wu et al., 1999). Based on their respective roles and previous work in zebrafish (Northam & LeMoine, 2019), we hypothesized that levels for these regulators would parallel changes in PGC-1α expression. Surprisingly, transcript levels of NRF-1/ERRα, but not PPARα, were downregulated in the heart due to PGC-1α suppression. In contrast, although PPARα levels seemed elevated, no significant changes amongst transcription factor expression were detected in skeletal muscle tissues overexpressing PGC-1α (Figure 2B). While the overall roles of these transcription factors are thought to be conserved throughout vertebrates, their respective interactions with PGC-1α have been questioned. Indeed, while the PPAR/ERRα binding site of PGC-1α is highly conserved in vertebrates, the putative NRF-1 binding motif is highly divergent in teleosts (LeMoine et al., 2010; Parisi et al., 2018). Considering that at least some of the interactions of PGC-1α with PPAR, NRF-1 or ERRα involves positive autoregulatory loops, the patterns we see here suggest a dissociation between PGC-1α and its binding partners. Interestingly, PGC-1β is strongly downregulated in the heart suggesting a regulatory relationship between the two coactivators. Indeed, previous observations in whole zebrafish larvae have demonstrated that PGC-1α knockdown has marginal effects on PGC-1β levels in whole zebrafish larvae (Northam & LeMoine, 2019). Considering that PGC-1β has been suggested as a modulator of some NRF-1 cascades in several fish models (Katharina Bremer et al., 2012; LeMoine et al., 2008; Northam & LeMoine, 2019), our results suggest that it may be the subsequential knockdown of PGC-1β that causes downregulation of some of the transcription

factors observed in cardiac muscle. Furthermore, given the stark contrast between the transcriptional patterns of the two muscle types, it is also very likely that these regulatory relationships are tissue specific. It should be noted that there have been instances of a dissociation of the mammalian PGC-1α/NRF-1 axis in previous knockout experiments in rodents. For example, muscle specific PGC-1α knockout mice failed to affect NRF-1 but resulted in a 50% reduction of ERRα expression and mitochondrial abundance (Handschin et al., 2007). Thus, while genetic PGC-1α models offer insight in the function of the coactivator, it is clear that some variability exists presumably due to the variety of approaches (e.g., whole body vs. single tissue knock-in/out), and animal model employed. However, we still propose our mutant line as a promising starting point to evaluate these regulatory cascades in zebrafish.

To further probe PGC-1α function, we assessed the expression of putative targets of the NRF-1/PPARs/ERRα pathways. In skeletal muscle, the only genes affected were the mitochondrial lipid transporters CPT-1 and 2. These two transporters are thought to be regulated through a PGC-1α/PPARα axis in mammals (Huss et al., 2004), though interestingly MCAD and HOAD are also regulated via that pathway but seem unaffected by PGC-1α overexpression. However, it is important to note that in zebrafish, as in most teleosts, several genes including the PPARs have experienced additional duplication events, leading to the presence of several isoforms (Den Broeder et al., 2015). Thus, it is likely that some redundancy exists in these regulatory pathways and it is possible that FAO associated enzymes in skeletal muscle are regulated via parallel pathways, such as another PPAR member or via other nuclear hormone receptors (e.g., ERRα, (Villena & Kralli, 2008). Furthermore, it is also possible that the PGC-1α promoter controlling CPT-1/2 expression is of prime importance under basal condition in muscle, given the lack of response elicited by the remaining target genes.

In cardiac muscle, none of the effector transcripts were significantly affected. Although treatment variability was high amongst certain genes, increasing our sample size failed to reduce it, thus we are confident in the overall pattern presented here. Given the overall lack of response in cardiac metabolic gene expression, it seems that both PGC-1α and β have a lesser influence on the metabolic phenotype of this tissue under basal conditions. It should be noted that due to reduced tissue and animal availability, we only were able to assess mixed skeletal muscles in this study, likely obscuring some of the shifts seen in gene expression. However, current efforts to generate stable homozygous mutant lines should further clarify some of these regulatory relationships and the differences between the two types of skeletal muscles.

2.5.3 High-Resolution Respirometry

To fully investigate the effects of PGC-1α upregulation on mitochondrial function, we examined aspects of red and white muscle oxidative capacity using high-resolution respirometry in adult fish. This enabled us to assess mitochondrial activity by controlled sequential activation and deactivation of the various mitochondrial components (Doerrier et al., 2018). In particular, we aimed to determine putative changes in FAO capacity in mutant zebrafish as suggested by the significant upregulation of FAO transporters gene expression (see above). For our analysis, we compared raw oxygen consumption as an estimate of overall mitochondrial density and calculated FCRs to provide insight on mitochondrial performance within each tissue and genotype (Porter & Wall, 2012). As expected, the highly oxidative red muscle exhibited much higher respiratory rates than white muscle. In zebrafish, red muscle tissue lies as a superficial lateral thin band of muscle and is responsible for slow-twitch movements for prolonged activity including general locomotion and feeding, while the majority of the axial musculature is made up of white muscle used for spontaneous movements such as burst swimming (Dou et al., 2008;

Luna et al., 2015; van Raamsdonk et al., 1982). While the difference was not as dramatic as described before for other teleosts, the overall pattern is comparable to previous investigation in zebrafish (Moyes et al., 1989; Teulier et al., 2018).

In white muscle, we observed a pronounced increase in raw oxygen flux across multiple substrates with minimal differences between intrinsic FCRs. Consequently, it appears that PGC-1α mutants demonstrate a higher white muscle oxidative capacity with overall little intrinsic differences in mitochondrial function. While the molecular regulatory cascade responsible for these changes is largely unclear at this point, upregulation of PGC-1α in skeletal muscle clearly provoked mitochondrial proliferation in white muscle, a phenotype reminiscent of mammalian models of PGC-1α muscle overexpression (Geng et al., 2010; Huss et al., 2004). Red muscle did not exhibit much response but given the typically high mitochondrial density in this tissue, up to 40% per volume, there may be little room for further increases in organelle density (Battersby & Moyes, 1998; Suarez, 1996).

In mutant fish, neither of the skeletal muscles showed differences in maximal FAO capacity despite the large upregulation of CPT-1 and 2. However, octanoylcarnitine, the FA substrate used in our assay, bypasses CPT-1 and thus is more indicative of the capacity to oxidize fatty acids rather than the process of transporting them into the organelle (Ojuka et al., 2016). Nonetheless, we observed a doubling in raw oxygen flux in response to low concentrations of malate and ADP prior to the addition of any substrates. Since malate is essential for β-oxidation to proceed when no other electron donators are present (Gnaiger, 2014), we propose that endogenous FA may have been more readily mobilized in mutant zebrafish white muscle potentially resulting in an increased metabolic output with little changes in individual mitochondrial performance.

2.5.4 Whole Animal Respirometry

Considering that white muscle constitutes approximately 90% of total muscle mass (Kiessling et al., 2006; Rovira et al., 2017), which itself likely accounts for large proportion of total body mass, we hypothesized that a 20% increase in white muscle mitochondrial activity would have a nominal impact on whole organism metabolism. Thus, we assessed if mutant zebrafish display a heightened metabolic rate as a result of our mutation. We measured metabolic activity at minimum and maximum points of activity to assess the metabolic scope of juvenile fish (Claireaux & Lefrançois, 2007). To evaluate routine O_2 consumption, our aim was to observe individual zebrafish overnight to obtain the lowest basal rate possible (Chabot, Steffensen, et al., 2016; Drown et al., 2020). During this period mutant PGC-1α zebrafish maintained an average two-fold increase in metabolic output, indicating that baseline metabolism was forcibly increased during routine activity. Conversely, we chased the fish to exhaustion to evaluate their maximum metabolic rate (MMR), while we recognize that this technique presents caveats (Andersson et al., 2020), it remains a useful proxy to evaluate MMR in our model (Little et al., 2020). Further, while RMR is typically relatively consistent within species, MMRs can vary between individuals due to its forced activation of muscle tissue (Norin & Clark, 2016; Weibel & Hoppeler, 2005). As such, we expected that mutant PGC-1α zebrafish would exhibit an increased metabolic rate in comparison to their wildtype counterparts to mimic the RMR comparison. Despite differences in mean MMRs across both treatments, we were unable to identify an increase in maximal performance due to the induced PGC-1α mutation. In turn, this would suggest that mutant PGC-1α fish exhibit a relatively smaller aerobic scope, further making them an interesting model to assess the impact of aerobic scope on whole animal performance. However, while we present some interesting observations, it is clear that many other performance variables should be

assessed in further detail, such as swimming capacity and maximal active metabolic rate during sustained swimming.

2.6 Conclusion

Our findings suggest that a mutation of an evolutionary conserved regulatory sequence was sufficient to upregulate PGC-1α expression in skeletal muscle resulting in an increased resting metabolism. At the tissue level, this translated into an elevated mitochondrial capacity and fatty-acid mobilization in adult zebrafish muscle. As differences in muscle tissues can have vast impacts on the overall organismal health, we propose that our mutant zebrafish line has the potential to serve as a unique biomedical model for the study of metabolic disorders, particularly those involving lipid metabolism. Furthermore, we will certainly be able to capitalize on this model from a comparative perspective to elucidate the significance and evolution of these complex metabolic regulatory pathways in vertebrates.

Chapter 3: Altered PGC-1α expression in Zebrafish limits growth irrespective of nutrient consumption

Kurchaba N, Duncan E, LeMoine CMR. In preparation for journal submission.

3.1 Chapter Summary

Animals must carefully budget energy derived from nutrients in order to survive and grow. This process requires the coordination of metabolic pathways with respect to environmental and internal physiological conditions, however the underlying mechanisms controlling energy allocation for growth remain complex and relatively unexplored. The metabolic co-activator PGC-1α plays a central role in regulating growth in response to caloric reduction and abundance in mammalian species, however few reports have examined its role in other vertebrates. In this study, we examined the role of PGC-1α in zebrafish growth using a previously established PGC-1α mutant zebrafish line with increased expression in muscle tissue and decreased expression in cardiac tissue. We observed mutant zebrafish larvae during early development (4-6 days post fertilization) and observed decreased heart rate post-hatching and a reduction in yolk lipid utilization amongst mutants, indicating that PGC-1α may serve as an important mediator for growth and fatty acid (FA) metabolism during early development. Conversely, adult mutant zebrafish maintained a higher metabolic rate following a high-fat meal during the specific dynamic action period. This increased metabolic rate likely delayed growth in adult zebrafish exposed to either a standard or high-fat diet over a 3-week period, with male zebrafish experiencing less growth than females. Upon viewing the FA profiles of adult male zebrafish, we identified a mutant-specific deficiency of Dihomo-gamma-Linolenic Acid, a FA responsible for reducing mitochondrial proton leakage. These results suggest that PGC-1α plays an important role in FA metabolism in zebrafish with important consequences for both larval and adult growth.

3.2 Introduction

The overall health of an animal is heavily dependant on the nutritional diet consumed during its lifespan. In nature, animals rely on the environmental abundance of nutrients to fuel ongoing bioenergetic processes in addition to maintaining pre-existing tissue mass. Fluctuations in environmental conditions often affect nutrient availability, requiring animals to modulate their metabolism constantly to survive. For example, during extreme fasting periods or starvation, animals may restrict their movement or metabolic processes (Heilbronn & Ravussin, 2003; Novak et al., 2005). Conversely, when nutrients are consumed in excess, animals can carefully reinvest their energetic surplus into future fuel reserves (fat) or initiate growth by building new tissue mass (Sibly et al., 2015; West et al., 2001). However, prolonged overfeeding combined with a sedimentary lifestyle can be detrimental to an animal's well-being, as fat accumulation can lead to metabolic dysfunction (for example: obesity, diabetes and stroke) (Kern & Mitmesser, 2018; Saklayen, 2018). As such, healthy growth requires a careful balance between immediate energetic demands and investment into additional tissue mass.

In mammals, PGC-1α (Peroxisome Proliferator Activated Receptor Gamma Coactivator 1 α) serves as a mediator between energy reserves and energetic demands. With the use of genetic mouse models, the PGC-1α protein has been described as a key protein involved in many metabolic processes, such as thermogenesis, mitochondrial biogenesis, fatty acid oxidation (FAO), glucose metabolism and muscle fibre-type switching (Huss et al., 2002; Lin et al., 2002; Puigserver et al., 1998; Wang et al., 2003; Wende et al., 2007). Although many of these processes are vital to an organism's survival regardless of environmental status, current evidence suggests that PGC-1α regulates all these processes in response to energetic cues. Indeed, caloric restriction with forced PGC-1α expression shifts mitochondrial fuel preference to endogenous

lipid reserves with enhanced mitochondrial respiration. Additionally, forced PGC-1α expression in cell cultures impairs growth despite increases in energetic output, suggesting that much of this energy is dissipated elsewhere (Miller et al., 2019; Villena, 2015).

The role of PGC-1α in mammalian models (mouse and human cells) has been documented extensively in biomedical literature, however very few studies have examined the role of PGC-1α in other vertebrates. Many of these other vertebrates (amphibians, reptiles and fish) are ectothermic and lack the capacity for thermogenesis, resulting in reduced metabolic activity during sub-optimal temperatures to maintain thermal homeostasis (Else & Hulbert, 1981; Seebacher et al., 2009). Examination of PGC-1α transcripts across vertebrate phylogeny demonstrates that the PGC-1α protein structure is preserved within vertebrates, however several teleost species contain various insertions in some of the transcription factor binding motifs (LeMoine et al., 2010). Interestingly, research examining the role of PGC-1α in various fish species suggests that teleost PGC-1α carries both similar and divergent roles from those previously described in mammals (Bremer et al., 2016; Bremer et al., 2012; Northam & LeMoine, 2019; Parisi et al., 2018).

Recently, we established a stable PGC-1α-mutant zebrafish line in hopes to further understand the role of PGC-1α in a teleost species. Our mutant zebrafish line contains a deletion in a recently discovered regulatory region found upstream of the PGC-1α promoter, resulting in a simultaneous increase in white muscle PGC-1α expression and down-regulation of heart PGC-1α expression. Reminiscent of previous mammalian studies, we found that zebrafish with forced muscular PGC-1α expression increased the expression of fatty acid (FA) transporter proteins CPT-1/2 alongside increased whole-body metabolic rate and higher maximal O2 consumption in permeabilized white muscle fibers. However, we observed no differences in the expression of

transcription factors (ERRα, NRF-1, PPAR-α), mitochondrial enzymes (CS, COX V) or FAO enzymes (HOAD, MCAD) that are known targets of PGC-1α regulation in mammals. Clearly, the PGC-1α protein remains elusive and requires further investigation to fully understand the consequences of metabolic dysregulation.

In this study, we examined the connection between increased metabolic rate and increased FAO with respect to growth using our PGC-1α zebrafish model. Our investigation began by examining yolk-limited FA metabolism and heart rate during early development of zebrafish larvae and identified decreased lipid metabolism in addition to decreased overall heartrate. During the adult stage, zebrafish fed a high-fat meal demonstrated a 2-fold increase in overall O_2 consumption during the post-prandial period, with an extended SDA (Specific Dynamic Action) window suggesting heightened fatty acid (FA) metabolism. In addition, wildtype and mutant zebrafish were fed standard or *Artemia*-supplemented (high-fat) diets for 3 weeks to monitor the effect of diet and genotype on the growth of individual adult zebrafish, and the metabolite profile of their muscle tissues. Interestingly, mutant zebrafish exhibited impaired growth, even with high caloric supplementation. Finally, targeted metabolomic analyses revealed that male mutant zebrafish fed a high fat diet had reduced levels of Dihomo-Gamma-Linolenic Acid (DGLA), a FA that reduces proton leak in the mitochondria. With our findings, we propose that our mutant zebrafish line resists growth through increased metabolism induced by PGC-1α-regulated mitochondrial dysfunction.

3.3 Methods

3.3.1 Caretaking

All zebrafish were derived from an initial stock of zebrafish that were commercially obtained and housed in our aquatic Aquaneering system (Aquaneering, San Diego, USA) maintained at

27°C with a 14:10hr light-dark cycle. Stock zebrafish were bred and genetically altered by CRISPR to produce F_0 subsequently F_1 PGC-1α-dysregulated mutant zebrafish as described previously (see Chapter 2). Following breeding all embryos were rinsed in 0.05% bleach solution before being rinsed twice with dechlorinated water. Larval zebrafish were transferred to E3 medium (5mM NaCl, 0.17mM KCl, 0.33mM CaCl2, 0.33 mM MgSO4, 0.0006 mM methylene blue) for 5 days with daily solution changes before being transferred to 300ml beakers containing aged dechlorinated water. Zebrafish were fed twice daily with phase I/II larval feed (<50/<200 microns) until 21 days post-fertilization (dpf), with daily replacement of 1/3 total water volume. Zebrafish were transferred into a recirculating system until adulthood. Water quality in the recirculating system was monitored daily to ensure optimal holding conditions during the experiment. Zebrafish were fed Zeigler Complete Diet pellets (Zeigler, Gardner, USA) twice daily and freshly hatched *Artemia naupii* (OceanStar, Snowville, USA) each morning. Both wildtype and mutant F_1 zebrafish were raised identically as littermates, and only separated at adulthood upon genotype determination. All procedures follow standards of the Canadian Council for Animal Care and were approved by the Brandon University Animal Care Committee.

All F_1 mutant zebrafish were genotyped to validate their genetic identity. We followed a previously described High Resolution Melt (HRM) protocol that allows for the quick detection of mutants by observing the melting temperature of 200bp segments surrounding the CRISPR target site (see Chapter 2). Individual zebrafish were removed from the tank and exposed to tricaine solution (100mg/L) until movement ceased, then transferred to a small petri plate for the collection of a small sample of the caudal fin. Subsequently, zebrafish were returned to the recirculating system and kept isolated for 1 week to ensure optimal recovery. Each caudal fin

clipping was subjected to crude DNA extraction for genotyping using High Resolution Melt (HRM) analysis using a Type-It HRM PCR kit (Qiagen, Aarhus, Denmark) (Samarut et al., 2016). Upon analysis of the HRM diagnostic, fish were separated into two groups based on their genotype, termed ‘Wildtype’ and ‘Mutant’ prior to beginning any of the after mentioned experiments.

3.3.2 Monitoring Larval Heartrate

The heartrate of larval zebrafish was measured over a 3-day period (4– 6dpf) to mirror the developmental timepoints of the lipid content assay. In order to minimize temperature fluctuations during the experiment, we set up a temperature-controlled arena consisting of water pump, custom glass arena, and a water bath maintained at 28C°. This arena was mounted below a microscope fitted with an AmScope MU300 camera (AmScope, Irvine, USA) to capture video recordings of individual zebrafish larvae using AmScope software (v3.70). Inside the arena, we used aquarium grade silicone and plastic dividers to create two sections inside the arena; one containing 50mg/L of tricaine-E3 solution, a concentration deemed to have minimal consequences on attenuating heart rate in larval zebrafish (Northam & LeMoine, 2019; Tzaneva & Perry, 2016), and another with E3 solution. Prior to assessing the heart rate of individual zebrafish, we placed each zebrafish in the tricaine-E3 solution for three minutes to attenuate movement. After the three-minute period, zebrafish larvae were transferred to the E3 medium and orientated for the recording of zebrafish heartrate. All videos were recorded at 24fps using the lowest video quality to maintain visual smoothness for accurate heart rate measurements. Between each measurement period, zebrafish were thoroughly washed in E3 solution to prevent excess exposure to tricaine. At the end of the experiment, all zebrafish were collected for crude DNA extraction and HRM genotyping.

3.3.3 Lipid Content in Zebrafish Larvae

To evaluate yolk lipid content, zebrafish larvae (3dpf) were exposed to Nile Red solution (5ng/ml) for 8hrs as described previously (Jones et al., 2008; Miyares et al., 2014). For three subsequent days, we monitored yolk fluorescence and area using an MVX10 microscope (Olympus, Waltham, USA) using the included Green Fluorescent Protein (GFP) filter. Larval zebrafish were anaesthetized with cold 50mg/ml tricaine solution prior to photographing and were returned to fresh E3 medium afterwards. Lateral images of zebrafish larvae were analyzed using ImageJ software to calculate the area and relative fluorescence of the yolk (Schneider et al., 2012). All zebrafish larvae used for yolk measurements were isolated throughout the entire experiment to allow for individual-level analysis. The experiment was terminated on 6dpf and zebrafish larvae were sacrificed for whole-body DNA extraction and HRM genotyping.

3.3.4 Post-Prandial Metabolic Scope

Post-prandial metabolic state of adult wildtype and mutant zebrafish was assessed over a 10-hour period using intermittent respirometry. Both wildtype and mutant zebrafish were starved 24h prior to the beginning of the experiment where they received 300mg of hatched *Artemia naupii* as a morning meal in their holding tank with the recirculation system turned off for a 15-minute period. Fish were then swiftly transferred to a Loligo® Systems swim tunnel respirometer (Loligo Systems, Viborg, Denmark), fitted with a 170mL chamber and 2 oxygen dipping probes to measure oxygen consumption in the chamber and a background measurement from the surrounding water. The swim tunnel and oxygen probe were configured using AutoResp 2.0 software (Loligo Systems) and programmed according to a 1-hour loop (2910s flush, 90s wait, 600s measure) to collect hourly measurements. During the measurement period, the internal swim tunnel motor was used at minimal speed to maintain water recirculation but with no

adverse effect on the fish during the experiment. The water in the system was kept at 26°C with a submersible heater, and water quality was maintained with a standard aquarium water filter. The swim tunnel and the surrounding water was manually cleaned prior to start of each experiment and 1/3 of the swim tunnel water was replaced with fresh dechlorinated water. After 23 hours in the respirometer, the fish was removed from the chamber and sacrificed to obtain weight and length measurements. The metabolic activity of the zebrafish was analysed using RespR (v1.1.0.0), to allow subtraction of background measurements and rate conversions as previously described (Harianto & Carey, 2019; Chapter 2).

3.3.5 Prolonged High-Fat Dietary Exposure

The diet of both wildtype and mutant zebrafish was manipulated over a 3-week period to assess the effects of a high-fat diet on PGC-1α-dysregulated mutants. Zebrafish were anesthetized using tricaine solution (100mg/L) to record initial weights and lengths prior to transfer to individual tanks in the recirculating system. Four treatments were derived by exposing each genotype to 2 separate dietary regimes; referred to as 'Standard' and High-Fat' diets. Both diets received an identical morning meal consisting of 5 Ziegler Complete Diet pellets and 5mg of hatched brine shrimp, with high-fat diet zebrafish receiving 2 extra 30mg portions of brine shrimp 4 and 8hrs after the initial morning meal. Zeigler pellets were always presented prior to brine shrimp to ensure that daily nutrient requirements were met. Recirculation was turned off for 15 minutes to allow zebrafish to eat *ad libitum* while preventing the removal of the brine shrimp, after which water circulation was restarted. On a weekly basis and prior to their daily morning meal, zebrafish were anesthetized in tricaine (100mg/L) for weight and length measurements to track individual growth throughout the experiment. On the final measurement day, zebrafish were humanely euthanized by a blow to the head and severance of the spinal cord. Muscle samples

were quickly dissected, frozen in liquid nitrogen, and stored in an ultra cold freezer prior to further manipulation. In total, the experiment was repeated 5 times yielding 54 individual zebrafish (15 WT-Standard, 12 WT-High-Fat, 11 M-Standard, 16 M-High-Fat) that were analyzed both together and separated by sex (Male – 6 WT-Standard Female, 8 WT-High-Fat, 8 M-Standard, 8 M-High-Fat and Female – 9 WT-Standard, 4 WT-High-Fat, 3 M-Standard, 8 M-High-Fat) . The Fulton's Conditioning Factor (hereafter referred to as the K-value) was calculated for each time point by relating overall mass and length measurements using Equation 1 (Mozsár et al., 2015).

$$\frac{Mass\ (g)\ \times 100}{Length\ (cm)^3} = K\ Value$$

Equation 1: Calculation for determining the Fulton's Conditioning Factor (K-Value) of individual zebrafish.

3.3.6 Metabolomics

Tissue samples obtained from the standard/high fat diet zebrafish were pulverized in liquid nitrogen and weighed to obtain the wet mass of the isolated tissues. The tissues were then dehydrated for 2 hours at -50°C and 0.05mBar using a FreeZone® 4.5 Liter Freeze Dry System (Labconco, Kansas City, USA). Tissue samples were then removed and weighed an additional time for evaluation of dry-mass tissue values. Both wet and dry mass tissue values were used to calculate the water content of the muscle tissue for each sample. Freeze-dried tissue samples were then packaged and shipped to University of North Texas for biomass sequential extraction (Gas Chromatography, UV Spectrophotometry and Liquid Chromatography). These methods were used to identify total protein/fat content as well as the individual levels of saturated FAs (SFAs), mono-unsaturated FAs (MUFAs), poly-unsaturated n-3 FAs (n-3 PUFAs) and polyunsaturated (n-6) FAs (PUFAs). The resulting dataset was also analysed using

MetaboAnalyst Software to perform statistical tests on FA levels and Debiased Sparse Partial Correlation (DSPC) network analysis to identity upregulated pathways impacted by FA changes. An additional DSPC guided analysis modeled a disease network using human data to identify increased metabolic disease susceptibility induced by dietary changes (Pang et al., 2020; Xia et al., 2011).

3.3.7 Statistical Methods

The data obtained from the yolk assay, zebrafish high-fat diet experiment and post-prandial O_2 consumption were analysed using SigmaPlot 14.0 (Systat Software, San Jose, USA). Yolk size and fluorescence data was compared using the Student's t-test for each comparison ($p = 0.05$). Zebrafish lengths, masses and K-values were analysed using Two-Way ANOVA tests with parametric Holm-Sidak post-hoc tests for datasets passing normality and equal variance. Datasets that did not meet these conditions were exponentially transformed and datasets that still failed to meet these conditions were subjected to non-parametric tests instead. Post-Prandial data was examined by using Two-Way ANOVA Repeated Measures ensuring normality and variance once again before adhering to parametric post-hoc tests, with overall O_2 consumption evaluated with matched hourly comparisons ($p < 0.05$). The overall maximum peak was identified by comparing the 4-hour (highest) and 10-hour (lowest) readings irrespective of genotype, with 4-hour and 7-hour measurements serving as notable peaks of metabolic activity due to having increased statistical significance ($p < 0.01$) between genotypes at the same time period. Metabolic data analysis was handled by the MetaboAnalyst software using the Two-Factor analysis method after normalization by log transformation and range-scaling of the dataset. All FA comparisons were conducted using Two-Way ANOVA Type 1 comparison models.

Individual FAs were normalized by first applying a log transformation to all values followed by range scaling, while subclass FAs were normalized according to the median of the dataset.

3.4 Results

3.4.1 Larval Zebrafish Heartrate is Decreased During Early Development

Mutant zebrafish larvae showed decreased heart rates in comparison to wildtype larvae initially, however no difference in heartrate was found by the end of the experiment (Figure 3-1A). The largest difference between the two genotypes was apparent at 4DPF where mutant heart rate was 5.3% lower than that of the wildtype group. By 5DPF, heart rate in mutant had recovered and was indistinguishable from the wildtype group. At 6DPF, the heart rates of both genotypes increased slightly from 5DPF values but showed no differences according to genotypes. Overall, wildtype zebrafish had a lower variance of heart rate in comparison to the mutant zebrafish during each of the experimental time points.

3.4.2 Larval Yolk Size is Larger in Mutant Zebrafish and Displays Higher Lipid Content

Mutant zebrafish experienced a noticeable decrease in lipid abundance over a 3-day period (Figure 3-1B). Nile Red fluorescence decreased with each subsequent day allowing for individual lipid consumption tracking by direct comparison to the 4DPF timepoint. Although lipid fluorescence decreased daily in all treatments, mutant zebrafish maintained higher lipid content in comparison to their wildtype counterparts. When comparing 5DPF and 6DPF Nile Red fluorescence values to the initial 4DPF timepoint (5DPF/4DPF, 6DPF/4DPF), mutant zebrafish experienced less decay in fluorescence than wildtypes. While yolk sac size was similar at 4DPF, mutant larvae had 13.9% larger yolks at 5DPF and 9.4% larger yolks at 6DPF (Figure 3-1C).

3.4.3 Mutant Zebrafish Maintained Higher O_2 Consumption and Displayed an Increased SDA Window After Consuming a High-Fat Meal

Mutant zebrafish had an overall increased metabolic scope in comparison to wildtype fish after feeding on a morning meal of high-fat *Artemia nauplii*. Mutant zebrafish had a higher average O_2 consumption rate (48.19 ±3.11µmol/g/hr) than wildtype zebrafish (25.72 ±1.29 µmol/g/hr) over the entire 10-hour period. All hourly measurements were determined to be significantly different between the two treatments except for the 1-hour 10-hour timepoints ($p < 0.05$), with 2 notable peaks occurring at the 4 and 7-hour time points in the mutant treatment ($p < 0.01$) (Figure 3-2). We could not detect significant differences between hourly measurements within each treatment, however oxygen consumption rates at hour 4 and hour 10 irrespective of genotype were significantly different ($p = 0.05$).

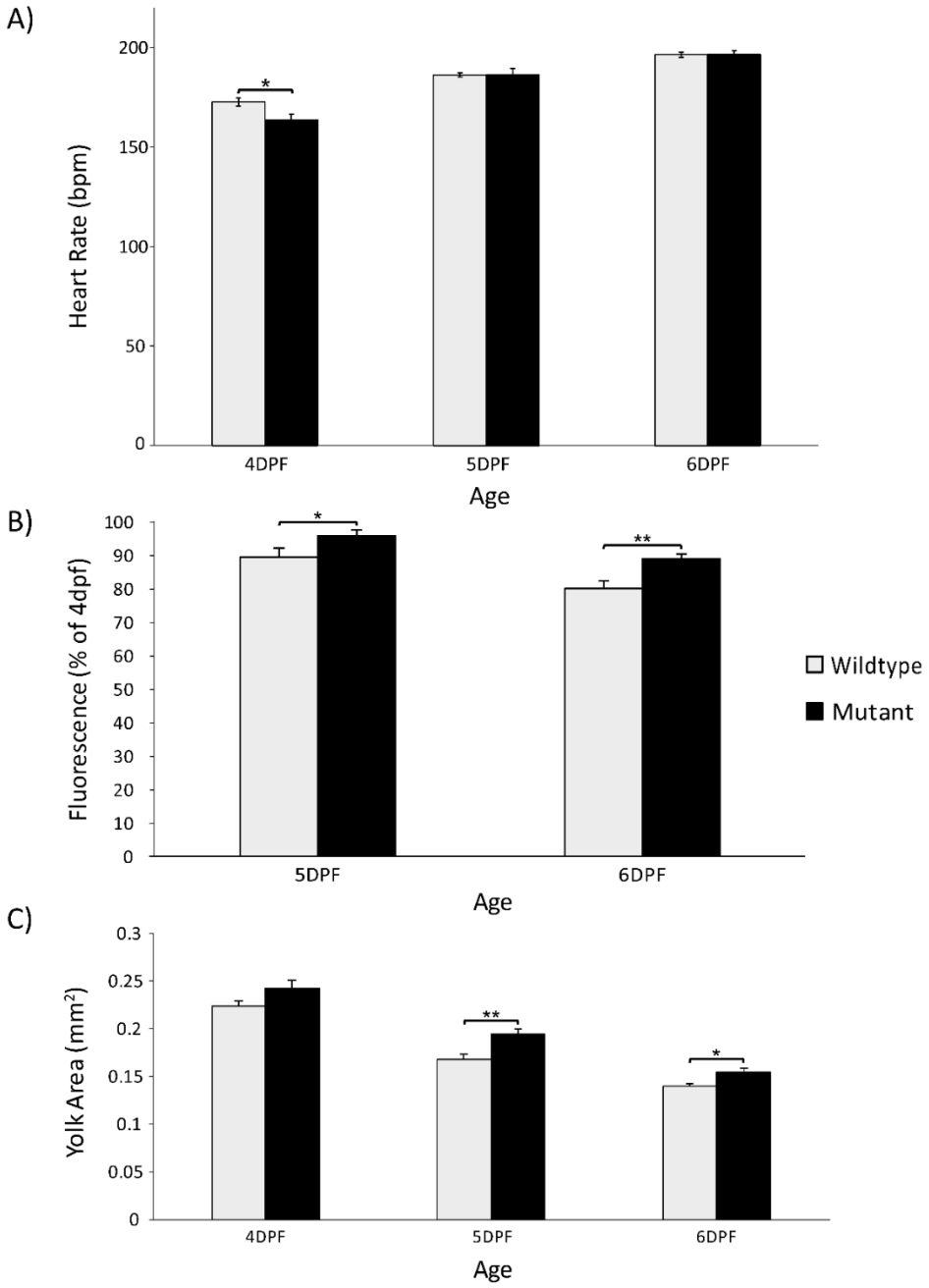

Figure 3-1: Mutant zebrafish larvae experience decreased heartrate, decreased yolk size and increased yolk lipid content during early development. A) At 4DPF, mutant zebrafish larvae experienced a 5.28% lower heart rate when compared to wildtype zebrafish ($p = 0.019$). B) Yolk fluorescence at 5/6DPF was compared to the initial fluorescence values at 4DPF. Mutant zebrafish yolk had 6.3% higher fluorescence readings at 5DPF ($p = 0.05$), with 9.04% higher fluorescence readings at 6DPF ($p = 0.01$). C) Mutant zebrafish maintained larger yolk sizes at 5DPF (0.001) and 6DPF ($p = 0.05$).

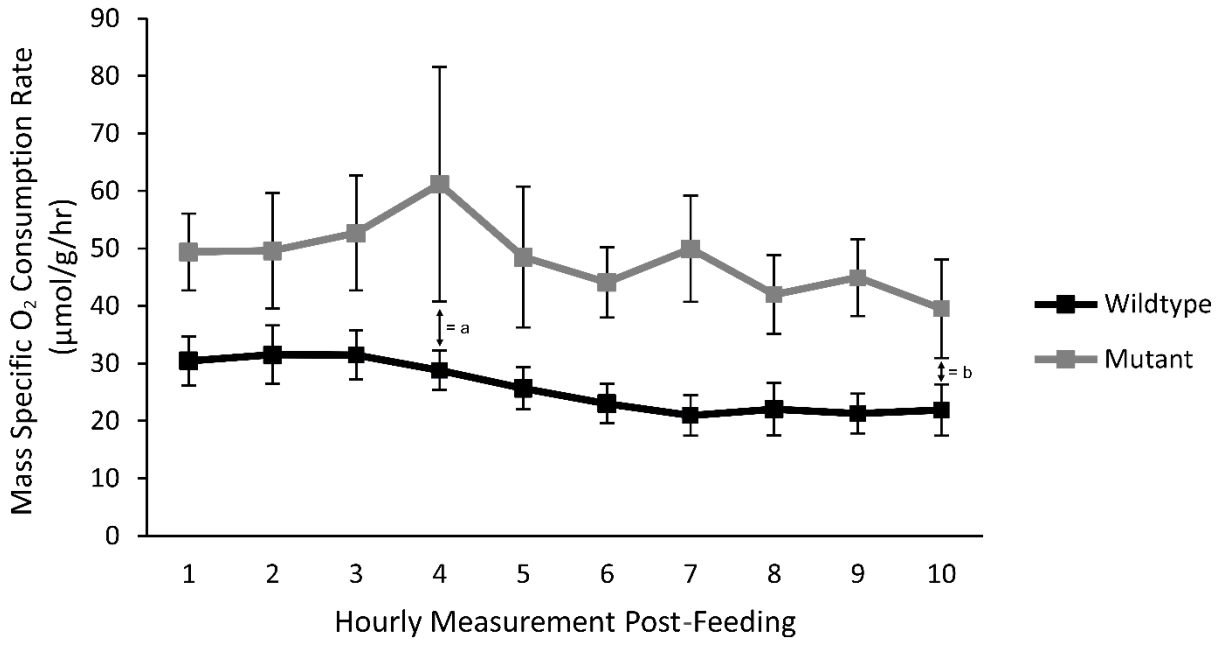

Figure 3-2: Mutant zebrafish have a higher post-prandial O_2 consumption rate following a high-fat meal. All timepoints between Wildtype and Mutant zebrafish were deemed significant following Two-Factor ANOVA Repeated Measurement analysis, except for the 1-hour and 10-hour timepoints ($p = 0.05$), with higher significance identified at the 4-hr and 7-hr timepoints ($p = 0.001$). Timepoint comparisons, irrespective of genotype, between the 4-hr (a) and 10-hr (b) timepoints were also identified as significantly different ($p = 0.05$).

3.4.4 Adult Mutant Zebrafish Show Decreased Mass and Length Increases in Response to a High-Fat Diet

Mutant zebrafish displayed impaired growth despite maintaining mass and length increases throughout 3-week experimental period and experienced minimal gains in the presence of a high-fat diet. Irrespective of diet and sex, we found that mutant zebrafish were shorter than their wildtype counterparts at week 2 and week 3 ($p < 0.001$ and 0.002 respectively, Figure S2). In general, WT-High-Fat zebrafish experienced the largest longitudinal growth (+11.4%) followed by WT-Standard diet zebrafish (+10.2%), M-High-Fat zebrafish (+8.5%) and finally M-Standard zebrafish (+6.6%). Interestingly, female M-High-Fat zebrafish were longer than M-Standard diet zebrafish (+4.9%), but this trend was reversed amongst male zebrafish as M-High-Fat zebrafish were shorter than M-Standard zebrafish (-3.7%). Despite this male-specific trend, we were only

able to detect diet-induced changes between our treatments in our female cohort at week 2 ($p = 0.027$, Figure 3-3).

With respect to zebrafish mass, we found that mutant zebrafish were significantly smaller than wildtype zebrafish across all weigh-in periods. Irrespective of sex, we found that WT-High-Fat zebrafish maintained the highest mass increases at week 3 (+48.3%) followed by M-High-Fat (+41.3%), WT-Standard (+34.4%), and M-Standard zebrafish (+14.6%, Figure S3). Interestingly, we noticed that female WT-Standard and M-High-Fat zebrafish masses converged at week 3 where they became indistinguishable from one another. A similar phenomenon occurred amongst male zebrafish between M-High-Fat and M-Standard zebrafish, as both zebrafish gained similar amounts of weight by the end of the experimental period (Figure 3-4). Similar to length comparisons, we found that female zebrafish mass was greatly impacted by the inclusion of a high-fat diet especially due to mass increases amongst WT-High-Fat diet zebrafish.

Finally, we compared the K-value of individual zebrafish to quantify the effect of genotype and diet on the health of individual zebrafish. By week 3, sex-independent K-value increases largest were highest amongst WT-High Fat zebrafish (14.9%) followed by M-High-Fat zebrafish (+10.9%), WT-Standard zebrafish (+4.5%) and finally M-Standard zebrafish (-2.9%). Overall, we found that genotype had a negligible effect on the K-value of our zebrafish in comparison to diet (Figure S4). The effect of diet was most evident amongst female zebrafish, specifically in WT-Standard zebrafish that continued to increase throughout the experiment in comparison to the other three treatments. With male zebrafish, we observed very few differences across all treatments and failed to identify dietary-induced differences until the final measurement point where M-Standard zebrafish exhibited an overall decrease in K-value (Figure 3-5).

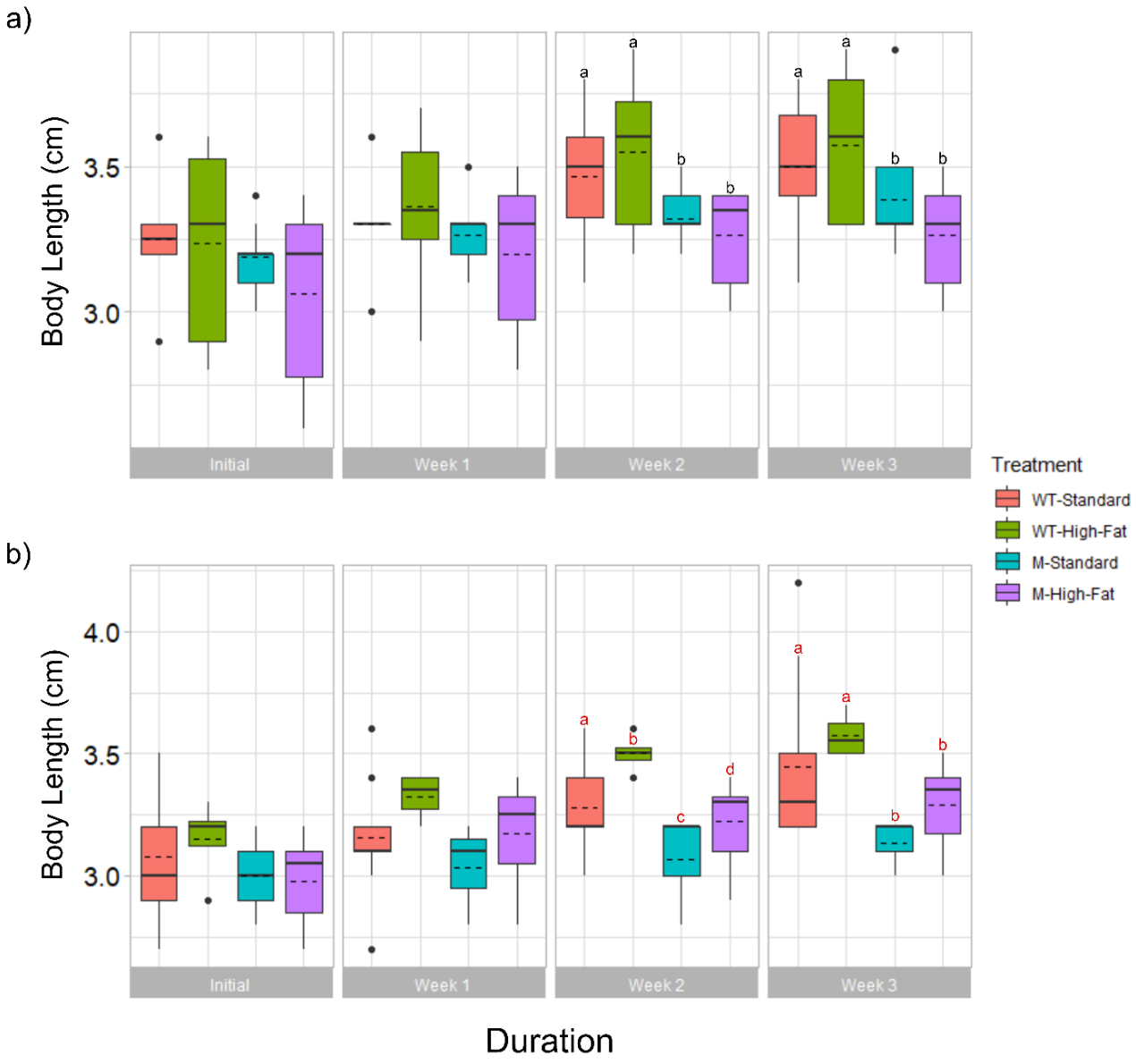

Figure 3-3: Sex and genotype effect on length growth in zebrafish fed standard and high-fat diets for 3-weeks. a) Male zebrafish experienced genetic-based differences in length at Week 2 ($p = 0.006$) and Week 3 ($p = 0.016$). B) Female zebrafish experienced diet and genetic linked mass differences at Week 2 (Diet $p = 0.027$, Genetic $p = 0.06$), with genotype differences at Week 3 ($p = 0.018$). Significance markings in red indicate the use of ranked testing for when normality and equal variance were not met.

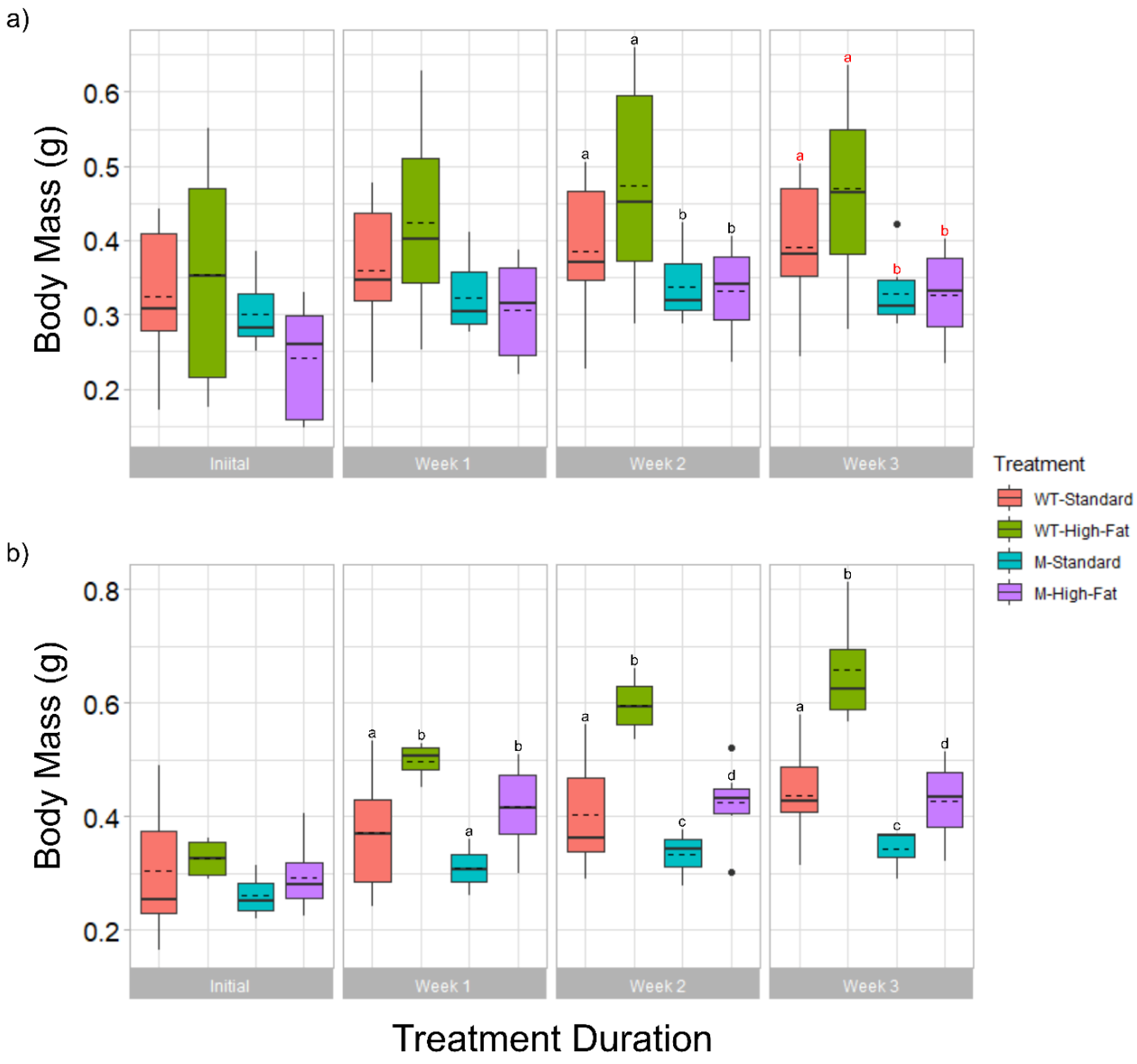

Figure 3-4: Individually tracked mass increases in both male and female zebrafish in response to standard and high-fat diets over a 3-week period. a) Male zebrafish experienced genetically induced mass differences at Week 2 ($p = 0.011$) and Week 3 ($p = 0.004$). B) Female zebrafish experienced dietary linked mass differences at Week 1 ($p = 0.06$) with diet and genetically induced differences at Week 2 (Diet $p = <0.001$, Genetic $p = 0.002$) and Week 3 (Diet $p < 0.001$, Genetic $p < 0.001$). Significance markings in red indicate the use of ranked testing for when normality and equal variance were not met.

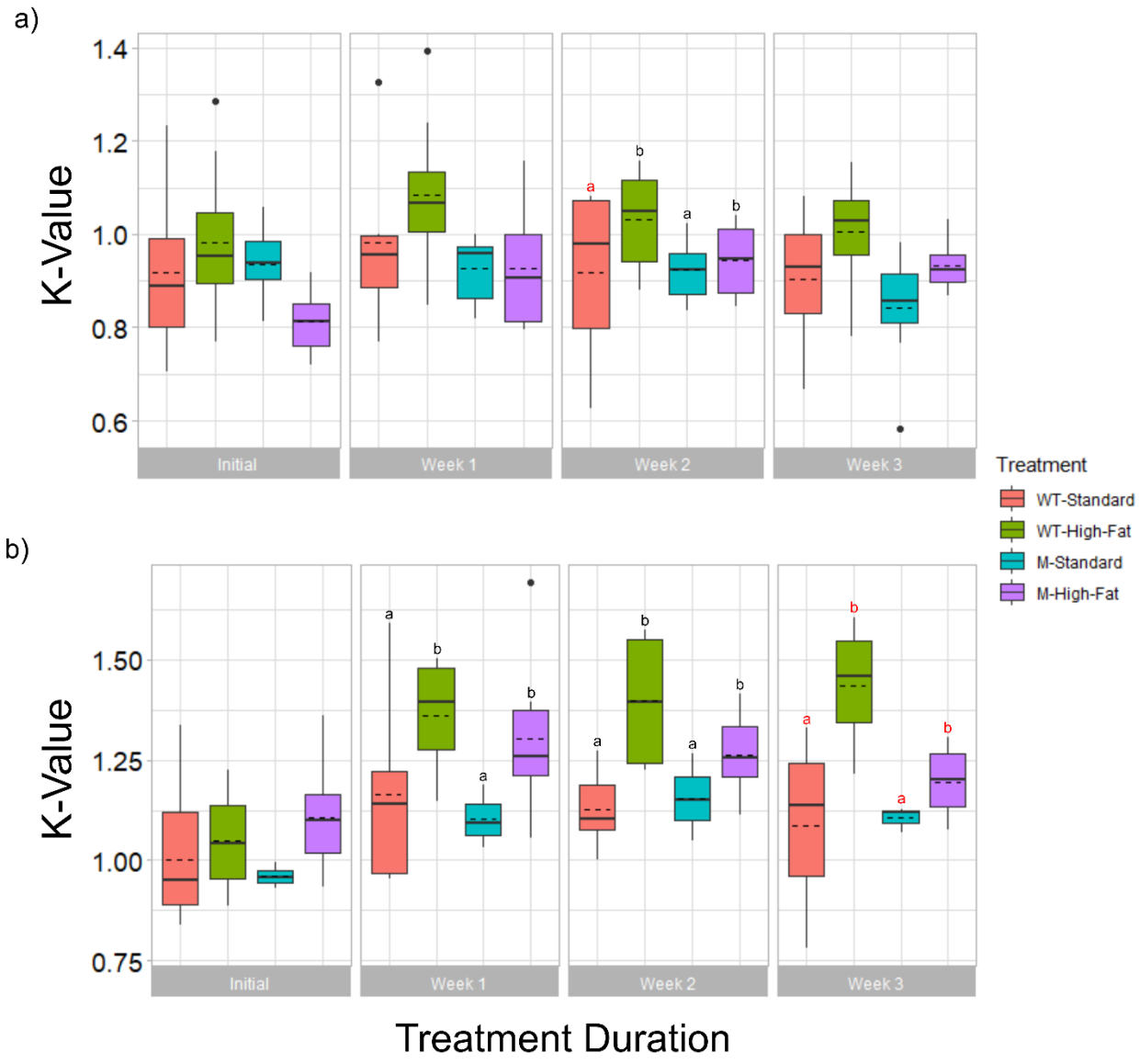

Figure 3-5: Condition factor increases in both male and female zebrafish in response to standard and high-fat diet over a 3-week period. a) Male zebrafish experienced dietary-induced K-Values at Week 2 ($p = 0.032$). b) Female zebrafish experienced dietary linked differences in K-Values at Week 1 ($p = 0.03$), Week 2 ($p = 0.002$) and Week 3 ($p = 0.004$). Significance markings in red indicate the use of ranked testing for when normality and equal variance were not met.

3.4.5 Metabolomics

Regardless of diet and genotype, all male zebrafish in our study had similar levels of total fat/protein content, however there were notable differences when examining the FA subclasses between high-low fat diets. Saturated FAs and poly-unsaturated (n-3) FAs were increased in response to a high fat diet, with decreased poly-unsaturated (n-6) FAs and no detectable difference found in mono-unsaturated FAs (Table 3-1). Of the 25 individual FAs measured in this study, 8 FAs (1 SFAs, 2 MUFAs, 4 PUFAs-3, 1 PUFA-6) were found at increased levels in high-fat diet zebrafish while 12 FAs (5 SFAs, 3 MUFAs, 1 PUFAs-3, 3 PUFAs-6) were decreased when compared to low fat diet zebrafish (Figure 3-6 & Table 3-2). Although we were unable to find differences in FA composition solely based on genotypes, we found that DGLA (Dihomo-gamma-linolenic acid; C20:3n6) was decreased in mutant zebrafish exposed to the high-fat diet.

Following DSPC metabolite network analysis we uncovered 19 enriched metabolic pathways, the most prominent being increased biosynthesis of Unsaturated Fatty Acids (UFAs) ($P = 4.0x10^{-8}$) and increased glycolysis/gluconeogenesis ($p = 1.81x10^{-5}$). In addition, FA restructuring pathways (FA biosynthesis $p = 0.0226$, FA elongation $p = 0.0288$ and FA degradation $p = 0.0392$) and sugar metabolism pathways (Glyoxylate and dicarboxylate metabolism $p = 0.00379$, Amino sugar and nucleotide sugar metabolism $p = 0.0228$, Galactose metabolism $p = 0.0404$) were also highly prominent in our dataset (Table 3-3). The extended DPSC disease network analysis identified 4 metabolic diseases (schizophrenia, isovaleric acidemia, hypertension and stroke) that were influenced by 6 of the previously mentioned enriched metabolic pathways (Figure S5). The most significant risk factor associated with all 4 metabolic diseases was the increased biosynthesis of UFAs ($p = 7x10^{-8}$).

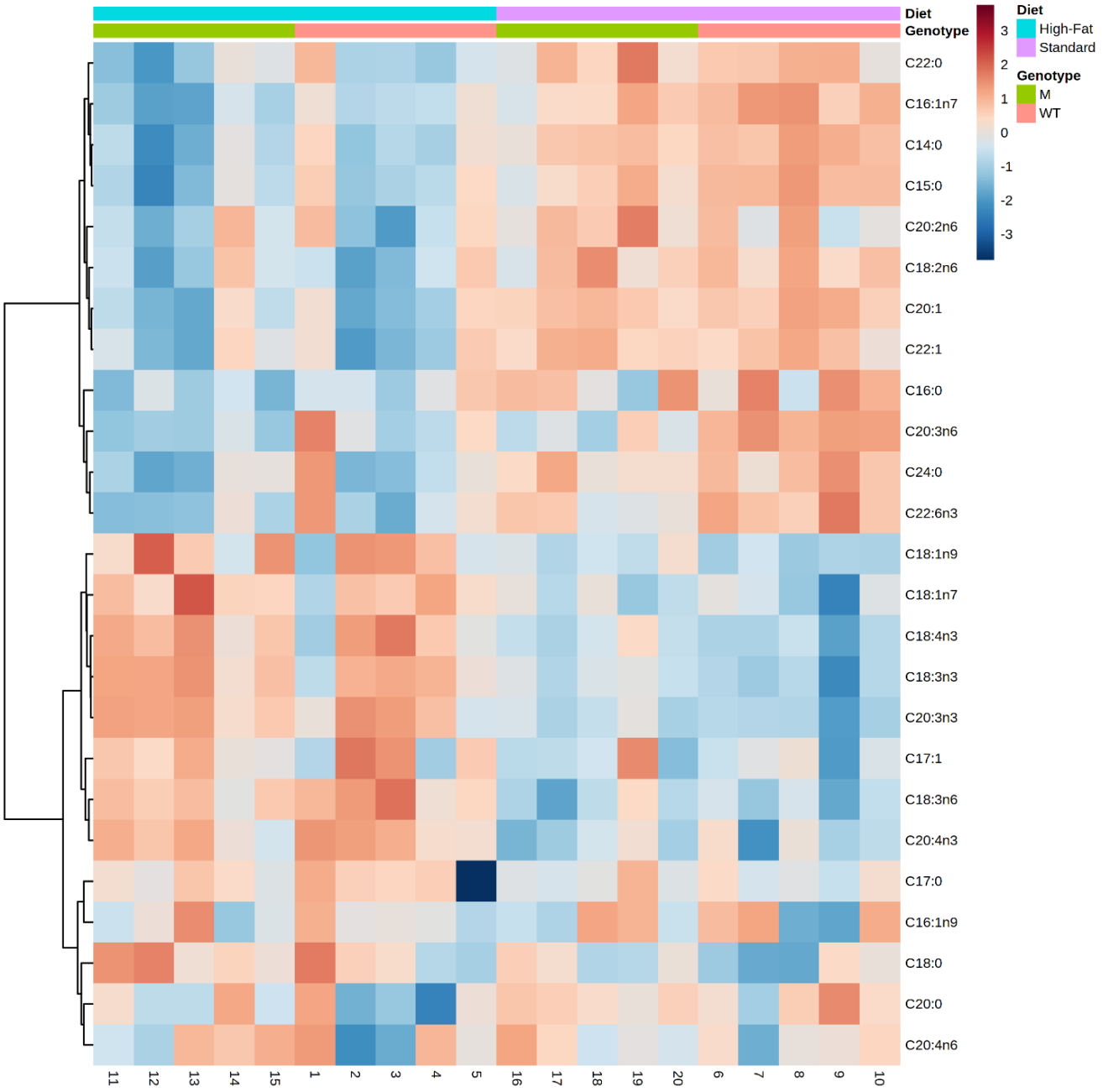

Figure 3-6: Metabolomic heatmap of individual FAs changes in male zebrafish fed Standard and High-Fat diets for 3 weeks.

Table 3-1: Total FA subgroups altered by either diet or genotype. Saturated FAs and PUFAs (n-6) were decreased, whilst PUFA (n-6) were increased in response to a High-Fat diet, with no overall differences being attributed to genotype. The overall abundance of FA subgroupings was determined via GC-MS/LC-MS and were thereafter normalized (log transformation, range scaled) and compared via Two-Factor ANOVA by MetaboAnalyst. The interaction between Diet and Genotype was determined to be non-significant for all comparisons (p = 0.94659).

Total Fatty Acid Abundance	WT-Standard (% Normalized)	M-Standard (% Normalized)	WT-High-Fat ((% Normalized)	M-High-Fat (% Normalized)	Diet p-value (adj)	Genotype p-value (adj)
Saturated FAs	1.304913 (± 0.03356)	1.289011 (± 0.03107)	1.171211 (± 0.06817)	1.141916 (± 0.05340)	1.7211E-5	0.6488
PUFA (n-3)	0.695984 (± 0.03205)	0.710989 (± 0.03107)	0.828789 (± 0.06817)	0.858084 (± 0.05340)	1.7211E-5	0.6488
PUFA (n-6)	0.373655 (± 0.01629)	0.37077 (± 0.02011)	0.336431 (± 0.02811)	0.338986 (± 0.02726)	0.006186	0.9876

Table 3-2: Individual FAs altered by either diet or genotype. A total of 19 FAs were affected by diet whilst 1 FA was affected by both diet and genotype. The overall abundance of individual FAs (%) were determined via GC-MS/LC-MS and were thereafter normalized (log transformation, range scaled) and compared via Two-Factor ANOVA by MetaboAnalyst. The interaction between Diet and Genotype was determined to be non-significant for all comparisons (p = 0.95551).

Individual Fatty Acid Abundance	WT-Standard (% Normalized)	M-Standard (% Normalized)	WT-High-Fat (% Normalized)	M-High-Fat (% Normalized)	Diet p-value (adj)	Genotype p-value (adj)
Saturated FAs						
C14:0 Myristic Acid	0.039138 (± 0.002671)	0.034976 (± 0.003209)	0.02658 (± 0.005792)	0.022481 (± 0.00513)	2.01E-4	0.3248
C15:0 Pentadecanoic Acid	0.006037 (± 0.000309)	0.005272 (± 0.000622)	0.004463 (± 0.000884)	0.003783 (± 0.000728)	6.97E-4	0.2021
C16:0 Palmitic Acid	0.165346 (± 0.009089)	0.161858 (± 0.009671)	0.155739 (± 0.005991)	0.149638 (± 0.005196)	0.009	0.4420
C18:0 Stearic Acid	0.058616 (± 0.006349)	0.062954 (± 0.004369)	0.065347 (± 0.008028)	0.069629 (± 0.00563)	0.0361	0.4166

C22:0 Behenic Acid	0.045868 (± 0.002999)	0.04569 (± 0.005268)	0.038641 (± 0.00518)	0.036049 (± 0.004562)	0.0018	0.7064
C24:0 Lignoceric Acid	0.017551 (± 0.00127)	0.016546 (± 0.001114)	0.014796 (± 0.002672)	0.013762 (± 0.001687)	0.0045	0.5189
Mono-Unsaturated FAs						
C16:1n7 (Palmitoleic Acid)	0.063155 (± 0.003554)	0.057636 (± 0.004783)	0.050774 (± 0.003759)	0.04465 (± 0.004479)	8.818E-5	0.0796
C18:1n7 (Vaccenic Acid)	0.043975 (± 0.004663)	0.045438 (± 0.002721)	0.050681 (± 0.00438)	0.053247 (± 0.004848)	0.0034	0.5718
C18:1n9 (Oleic Acid)	0.180327 (± 0.004672)	0.188534 (± 0.007175)	0.203316 (± 0.020821)	0.210511 (± 0.018363)	0.005	0.5189
C20:1 (11-Eicosenoic Acid)	0.041872 (± 0.003885)	0.039697 (± 0.002594)	0.028055 (± 0.007644)	0.026711 (± 0.006175)	4.362E-4	0.7851
C22:1 (Cetoleic Acid)	0.027525 (± 0.003978)	0.027894 (± 0.003472)	0.0178 (± 0.006982)	0.018178 (± 0.005622)	0.0026	0.8865
Poly-Unsaturated FA (n-3)						
C18:3n3 (Alpha-linoleic Acid)	0.054358 (± 0.011235)	0.070981 (± 0.007753)	0.102801 (± 0.027551)	0.120224 (± 0.020437)	2.012E-4	0.2021
C18:4n3 (Stearidonic Acid)	0.012974 (± 0.002066)	0.015834 (± 0.002875)	0.022995 (± 0.008438)	0.024667 (± 0.004297)	0.0014	0.442

C20:3n3 (Dihomo-alpha-linoleic Acid)	0.003763 (± 0.000487)	0.004349 (± 0.000513)	0.006318 (± 0.00142)	0.006763 (± 0.000852)	8.818E-5	0.4166
C20:4n3 (Eicosatetraenoic acid)	0.012518 (± 0.00115)	0.012384 (± 0.00083)	0.014526 (± 0.000818)	0.014097 (± 0.00092)	0.0014	0.7851
C22:6n3 (Docosahexenoic Acid)	0.09928 (± 0.011157)	0.082913 (± 0.009218)	0.076548 (± 0.020342)	0.064339 (± 0.009586)	0.004	0.2021
Poly-Unsaturated FA (n-6)						
C18:2n6 (Linoleic Acid)	0.077839 (± 0.002533)	0.076922 (± 0.004369)	0.069494 (± 0.005528)	0.069908 (± 0.00561)	0.004	0.946
C18:3n6 (Gamma-linolenic Acid)	0.001706 (± 0.000348)	0.001786 (± 0.000512)	0.003237 (± 0.000798)	0.002794 (± 0.000365)	2.632E-4	0.7896
C20:2n6 (Dihomo-linoleic Acid)	0.003226 (± 0.00018)	0.003317 (± 0.000167)	0.003052 (± 0.000262)	0.003042 (± 0.000218)	0.0361	0.8022
C20:3n6 (Dihomo-gamma-linoleic acid)	0.003566 (± 0.000105)	0.002888 (± 0.000254)	0.003051 (± 0.000471)	0.002631 (± 0.000155)	0.0091	0.0133

Table 3-3: Summary table of metabolite-metabolite network analysis. A total of 19 metabolic pathways were upregulated in zebrafish fed with the high-fat diet. Metabolite-metabolite associations were obtained using MetaboAnalyst using the MetPriCNet module with data from the STITCH database.

Pathway	Total	Expected	Hits	P. Value
Biosynthesis of unsaturated fatty acids	36	1.77	12	4.05E-08
Glycolysis or Gluconeogenesis	26	1.28	8	1.81E-05
Pyruvate metabolism	22	1.08	5	0.00344
Glyoxylate and dicarboxylate metabolism	32	1.57	6	0.00379
Riboflavin metabolism	4	0.197	2	0.0134
Citrate cycle (TCA cycle)	20	0.984	4	0.0144
Linoleic acid metabolism	5	0.246	2	0.0217
Fatty acid biosynthesis	46	2.26	6	0.0226
Arginine biosynthesis	14	0.689	3	0.0282
Fatty acid elongation	36	1.77	5	0.0288
Amino sugar and nucleotide sugar metabolism	36	1.77	5	0.0288
Glycerophospholipid metabolism	36	1.77	5	0.0288
Arachidonic acid metabolism	36	1.77	5	0.0288
Fatty acid degradation	39	1.92	5	0.0392
Galactose metabolism	27	1.33	4	0.0402
Glycerolipid metabolism	16	0.787	3	0.0404

Retinol metabolism	16	0.787	3	0.0404
Alanine, aspartate and glutamate metabolism	28	1.38	4	0.0451
Glutathione metabolism	28	1.38	4	0.0451

3.5 Discussion

Our experiment focused on examining the impact of altered PGC-1α expression on the growth and metabolic function of larval and adult zebrafish. Our initial characterization of mutant PGC-1α zebrafish revealed increased basal metabolic rates during the juvenile life stage, alongside increased mitochondrial activity and lipid transport in adult white muscle tissue (see Chapter 2). Since an increase in basal metabolism reduces the amount of energy used for other physiological processes, we hypothesized that our PGC-1α mutant would exhibit stunted growth in comparison to wildtype zebrafish. Furthermore, as PGC-1α is commonly associated with mediating energetic demand, we sought to determine whether PGC-1α zebrafish displayed increased rates of FA metabolism and how growth may be impaired following a standard zebrafish diet compared to a high-fat diet.

3.5.1 Mutant Zebrafish have Larger Yolks with Higher Fluorescence Values (Increased Lipid Content), But Also Display Decreased Heart Rate

We first assessed growth and FA utilization in zebrafish embryo by tracking individual lipid consumption in the larval yolk sac. The zebrafish has served as a well-known model for developmental growth due to its transparent chorion and rapid growth following fertilization. These processes rely on the nutrient-rich yolk sac provided by the mother, a finite energy resource rich in lipids and protein content (Sant & Timme-Laragy, 2018). For these reasons,

larval zebrafish have become a useful model in the study of lipid metabolism and dyslipidemias (Miyares et al., 2014). One advantage of the zebrafish model is the ability to view lipophilic dyes through the transparent chorion to monitor lipid consumption. Furthermore, these dyes can also be visualized in laterally orientated zebrafish larvae allowing for yolk size and lipid fluorescence to be accurately measured by area measurements (Minchin & Rawls, 2017; Tingaud-Sequeira et al., 2011). We used 4-6dpf fish to focus on rapid growth and nutrient uptake, as the 3dpf timepoint provides consistent lipid content amongst siblings and prolonging our measurement period past 6dpf would result in yolk sac depletion (Jones et al., 2008). We observed decreased lipid consumption and larger yolk sacs in mutant zebrafish across all timepoints, suggesting that overall nutrient usage (and lipid-specific usage) is impaired by our PGC-1α mutation. These results contrast with our previous findings in PGC-1α adult mutants that had increased mitochondrial content and CPT-1/2 transporters in skeletal muscle, suggesting that the function of PGC-1α may change with development (see Chapter 2). The zebrafish yolk is rich in lipid content making it an adequate fuel source for β-oxidation (Fraher et al., 2016; Quinlivan & Farber, 2017), yet we observed decreased fat consumption across the 3-day development period with no increases in developmental fatality or deformations

In addition to measuring larval nutrient consumption, we also monitored the heart rate of larval zebrafish to assess cardiac performance. The transparency of zebrafish larvae greatly benefits the study of cardiac development by allowing for non-invasive monitoring of heart rate, cardiac output and structural deformities making the zebrafish an excellent model organism for studying cardiac function (Asnani & Peterson, 2014; Genge et al., 2016). Cardiac development is paramount to embryo development due to its ability to translocate nutrients and oxygen throughout the zebrafish embryo, becoming apparent at 24hpf, structurally complete at 72hpf,

and fully functional by 92hpf (Dyballa et al., 2019; Kimmel et al., 1995). Indeed, the heart plays an important role in mediating growth during early development in both zebrafish and amongst other vertebrates by promoting angiogenesis and the creation of new tissue mass associated with organogenesis (Burggren, 2013; Goenezen et al., 2012; Pelster & Burggren, 1996). As larval zebrafish receive adequate blood oxygenation by diffusion, we believe that our experimental timeframe presents a unique environment where heart-specific PGC-1α activity can be linked to individual growth impairment in individual zebrafish larvae.

Our results demonstrate that mutant zebrafish experience lower heart rates at 4DPF but quickly recover at 5DPF, resulting in near-identical heart rates consistent with previously reported values (Gore & Burggren, 2012; Kopp et al., 2005). The ability for larval zebrafish to recover and match the heartrate of wildtype zebrafish is intriguing, as PGC-1α-deficient mice face lifelong cardiac impairment with decreased heart size, heart rate and cardiac output, with conflicting results suggesting altered mitochondrial density (Arany et al., 2005; Leone et al., 2005). Previously, we examined the mRNA transcripts of our mutant zebrafish line and found that adult zebrafish PGC-1α mutants have profound alterations in gene expression of several regulators (ERRα, NRF-1, PPARα, and PGC-1β), reminiscent of PGC-1α-knockout mice, however no mitochondrial transcripts were altered suggesting that mitochondrial density in the zebrafish heart is unchanged (see Chapter 2). Overall, it appears that PGC-1α still plays an important role in the larval zebrafish heart, however we suspect that this mechanism is different from that observed in mammalian species.

3.5.2 Mutant Zebrafish Have a Higher Metabolic Rate After Consumption of a High-Fat Meal

Considering the effects of the PGC-1α mutation on yolk metabolism, we wanted to assess whether adult zebrafish would have similarly altered metabolism following a high-fat meal. We

starved adult zebrafish 24-hrs prior to feeding them a high-fat meal (600mg of *Artemia*) to observe individual metabolic rates following a high-fat meal. Food consumption leads to increased energy expenditure and heat production due to digestion processes such as enzymatic activity, macromolecule breakdown and nutrient adsorption (Chabot et al., 2016). These processes, referred to as Specific Dynamic Action (SDA) start immediately after feeding and last up to 72hrs, with the energetic peak appearing more prominently when food intake is high and the animal is leaner (Chabot et al., 2016; Feher, 2017). Despite this extensive time period, the majority of energetically intensive digestion occurs during the first 8-hours following meal consumption. To capitalize on this timeframe, we allowed individual zebrafish to feed for 15-minutes *ad libitum* before immediate transfer to the respirometry system. Our results indicated that PGC-1α mutant zebrafish experience a near two-fold increase in metabolic activity across the entire post-prandial period, with an additional SDA-peak near the end of the experimental window. Although the timing of peak SDA activity can vary between species and meal quality, we found that our 10-hour window was sufficient for examining peak O_2 consumption in zebrafish (Chabot et al., 2016; Fu et al., 2005). Furthermore, our wildtype zebrafish had similar SDA O_2 consumption rates observed before, and was 20% higher than previously documented basal metabolic rates, despite immediate transfer of the zebrafish into the respirometer after the initial feeding period (Ferreira et al., 2019; Lucas & Priede, 1992; Silva, 2013).

Our initial characterization of PGC-1α mutant zebrafish described a 2-fold increase in the resting metabolic rate of juvenile zebrafish, however our examination of the SDA peaks show that fat metabolism is also increased by altered PGC-1α expression. Irrespective of genotype, both the 4-hour (peak) and 10-hour (constant) time points were significantly different, suggesting the 4h mark represents SDA peak activity for both treatments. We also found that mutant zebrafish had

a prolonged SDA period with an additional peak present at the 7-hour time point suggesting that the duration of SDA activity is nearly doubled in mutant zebrafish. Overall, these factors suggest that mutant zebrafish have increased metabolic activity during the post-prandial window and further supports the notion that PGC-1α plays a key role in zebrafish fat-metabolism.

3.5.3 Mutant Adult Zebrafish Resist Growth with Decreased Length and Mass, Even When Fed High-Fat Diet

An effective method of encouraging growth in adult zebrafish is to increase the daily amount and frequency of *Artemia* feedings. Artemia have long been used as a laboratory feed for zebrafish due to their high fat and protein content, with overconsumption resulting in rapid onset of obesity (Landgraf et al., 2017; Léger et al., 1987; Oka et al., 2010). The increased consumption of *Artemia* increases daily caloric intake that may contribute to growth or become stored as internal fat reserves. To account for these aspects, we recorded the length and mass of individual zebrafish over a 3-week period. Length and mass measurements are commonly used to represent overall health in laboratory experiments and serve as base metrics for growth in nutrition-based studies, allowing for us to find comparable mass and lengths for both our standard and high-fat diet wildtype zebrafish (Landgraf et al., 2017; Parichy et al., 2009). Overall, we found that mutant zebrafish were lighter and shorter than mutant zebrafish, suggesting that mutant zebrafish are smaller in both metrics even when provided with additional food. (Lugert et al., 2016). Upon seeing these differences, we hypothesized that these mass increase rates may have adverse affects on zebrafish health. Certainly, progressive or ‘natural’ growth is a characteristic of good health but delayed or rapid growth in response to nutrient availability can lead to reduced physical activity or increased susceptibility to metabolic syndrome (Kern & Mitmesser, 2018; Novak et al., 2005).

Moving forward, we decided to examine the K-values of individual zebrafish to see if overall health was impaired by either PGC-1α dysfunction or the presence of a high-fat diet. Typically, the K-value is used as an indicator of overall fish energy reserves, where $K \geq 1$ indicates that an organism has excess energy. However, the K-value can also provide an effective estimation of fat content in freshwater fish indiscriminately of both protein and water content (Mozsár et al., 2015). Therefore, decreased K values may indicate starvation or malnutrition and increased K values may represent onset obesity. Unsurprisingly, both genotypes experienced increased K-values when subjected to the high-fat diet likely due to the increased FA intake, approaching previously reported values (Landgraf et al., 2017). No significance was attributed to genotype for either female or male zebrafish, however wildtype fish appeared to be more susceptible to K-factor increases when subjected to the high-fat diet. In particular, female WT-High-Fat fish experienced a near 2-fold increase in K-value when compared to M-High-Fat zebrafish. Sex-linked differences in response to high-fat diets have been previously identified in zebrafish and other higher vertebrates (Hwang et al., 2010; Navarro-Barrón et al., 2019) likely because of reproductive activity such as oocyte production, resulting in increased mass gains due to excess nutrient availability (Augustine et al., 2011; Quinlivan & Farber, 2017). This suggests that our dietary differences in weight gain may be due to increased clutch sizes in wildtype zebrafish due to excess lipid intake and that mutant female zebrafish experience higher levels of fat metabolism and therefore have decreased lipids available for oocyte production. Therefore, our findings suggest that PGC-1α dysregulation has strong metabolic implications for female zebrafish, however these sex-specific traits are difficult to explain based on body mass gains alone and therefore would warrant further investigation. With these differences in mind, we focused our metabolic analysis exclusively towards male zebrafish skeletal muscle tissue.

3.5.4 Overall Fat and Protein Content is Similar Between Treatments, but Individual FA Content is Impacted by Both Diet and Genotype.

The metabolomic profile of high-fat zebrafish resembles that of the *Artemia* feed with decreased levels of non-essential FAs (SFAs and MUFAs) and increased essential FAs (PUFAs). Although the nutritional profile of *Artemia* varies due to origin and enrichment processes, most *Artemia* sources tend to have low levels of SFAs (~20%), moderate levels of MUFAs (~32%) and high levels of PUFAs (~45%) (Chakraborty et al., 2007; Léger et al., 1987). Both SFA and MUFA are classified as non-essential fatty acids due to their ability to be biosynthesized *in vivo*, however their representation, particularly in the case of SFAs may signify lipid metabolism disorders. Indeed, increased SFA levels in muscle tissue can lead to localized inflammation and increased insulin resistance following the disruption of PGC-1α activity, both resulting in obesity and further metabolic disruption in mammals (Kennedy et al., 2009). We found that our high-fat diet zebrafish had decreased levels of SFAs with negligible changes in MUFAs, suggesting that wildtype male zebrafish may have experienced healthy levels of growth in the presence of high-fat overfeeding.

Zebrafish also exhibited a shift amongst essential fatty acids when exposed to a high-fat diet, with decreased levels of n-6 PUFAs and increased levels of n-3 PUFAs. Freshwater fish are naturally rich in n-6 PUFAs but *ad libitum* feeding of *Artemia* presumably increased n-3 PUFAs amongst high-fat diet zebrafish, which may promote anti-inflammatory processes and provide protection against metabolic diseases (Cheng et al., 2015; Sahena et al., 2009). Various studies have shown that n-3:n-6 imbalances are found in a number of metabolic diseases such as chronic obesity, CVD and depression (Dinicolantonio & O'Keefe, 2018; Fowler et al., 2020). Furthermore, the beneficial effects of n-3 PUFAs also extend to decreased weight loss in

mammals, an effect also seen in female zebrafish using fish oil supplementation (Huang et al., 2016; Meguro & Hasumura, 2018). Although most of the n-3 PUFAs were increased in response to a high-fat diet, we found that C22:6n3 (Docosahexaenoic acid; DHA) levels were decreased as well. Docosahexaenoic acid is the longest n-3 PUFA and is commonly regarded as an 'evolutionary pacemaker' due to its beneficial properties supporting oxidative metabolism. Animals with higher metabolic rates have increased levels of DHA in their plasma membranes, which may raise β-oxidation rates by increasing ion permeability in the mitochondria (Manzi et al., 2015; Price et al., 2018). Although generally n-3 levels were higher in the high-fat diet, decreases in DHA could indicate a decreased metabolic capacity allowing for rapid weight gain that may result in obesity and FA imbalances with continued feeding.

While we were unable to find any individual FAs that were solely impacted by PGC-1α-dysregulation, DGLA levels were significantly decreased in mutant zebrafish and high-fat diet zebrafish. Dihomo-gamma-linolenic acid is a n-6 PUFA originating from either dietary intake or the elongation of Gamma-linolenic acid (C18:3n6) (Sergeant et al., 2016). Afterwards, DGLA is actively converted into various eicosanoids that help regulate immunological and metabolic processes locally; increasing adipogenesis and FA translocase/CD36 activity by PPARγ/α activation in mice (Dennis & Norris, 2015). Additionally, DGLA is also reducing proton leakage in the inner mitochondrial membrane, increasing mitochondrial efficiency by reducing O_2 flux associated with proton re-entry (Brand & Nicholls, 2011; Gallagher et al., 2019). In our previous work we found increased O_2 flux in PGC-1α mutant white muscle tissue with fully saturated mitochondria but observed no further change in O_2 flux following mitochondrial uncoupling. Therefore, it is possible that mutant zebrafish experience increased proton leakage, effectively increasing their basal metabolic rate and limiting their energetic surplus required for growth.

To gain further insight on the metabolic pathways affected by the high-fat diet, we mapped our metabolic dataset using DSCP analysis. Multiple FA specific pathways (FA biosynthesis, lengthening and breakdown) were upregulated and likely responsible for the shift from n-6 PUFAs to n-3 PUFAs alongside decreased SFA levels. Coincidentally, decreased SFAs may also explain why increased glucose production (glycogen breakdown and gluconeogenesis) was also reported by the network analysis, as high SFA levels lead to insulin resistance in muscle tissue (Sears & Perry, 2015). The additional metabolic pathways upregulated by the high-fat diet show increases in nutrient catabolism through the metabolism of riboflavin, pyruvate and various amino acids, lipids and simple sugars. Ultimately, these pathways indicate that our high-fat diet promotes growth by providing increased usage energy to both mutant and wildtype zebrafish.

As increased nutrient intake can lead often to unfavourable metabolic diseases later on in life, we decided to model our high-fat diet dataset to create a predictive disease-network to identify increased risk to metabolic diseases. Although our network analysis relies on a human-orientated database and lacks tissue specificity, similarities between zebrafish and human signalling pathways are explored extensively in biomedical studies (Goldsmith & Jobin, 2012; Seth et al., 2013). In addition, skeletal muscle serves as one of the final storage depots for excess FAs, suggesting that these levels could be considered as ‘conservative estimates’ for other tissues such as adipocytes (Hausman et al., 2014). Overall, the high-fat diet FA profile was associated with four different disorders with a high predictability regarding schizophrenia and fewer relations regarding isovaleric acidemia, hypertension and stroke. We failed to observe any behavioural differences amongst individual zebrafish, however human schizophrenia patients share similar clinical markers with obesity; such as high SFA levels, increased insulin resistance and low DHA levels in brain tissue (Dipasquale et al., 2013; Peet, 2004; Sakai et al., 2018). Isovaleric

acidemia is a genetic disease characterised by the inability to properly metabolize leucine and is unlikely to be present amongst our mutant zebrafish. However, increases in leucine levels can promote weight loss, lean muscle growth and oxidative glucose metabolism in skeletal muscle (Binder et al., 2014; Layman & Walker, 2006). The remaining two diseases, hypertension and stroke, suggest that a high-fat diet would have adverse physiological effects on the zebrafish heart, however it is currently unknown how these pathologies would affect mutant zebrafish with silenced PGC-1α expression in the heart.

3.6 Conclusion

PGC-1α plays an important role in the regulation of mammalian metabolism and growth, however this relationship has not been fully addressed amongst lower vertebrates. In periods of nutrient limitation, mammalian PGC-1α maintains basal metabolism by upregulating FA metabolism in the mitochondria, effectively reducing growth while maintaining adequate energy supply. Using our newly established zebrafish PGC-1α model, we have confirmed that many of these processes are present in a teleost fish species. Overexpression of PGC-1α in adult zebrafish muscle tissue increased FA metabolism during the post-prandial period and decreased overall growth in response to a standard and high-fat diet. Decreased DGLA levels amongst mutant zebrafish suggests that mitochondrial leakage may release excess energy liberated from increased FA metabolism as the underlying mechanism of PGC-1α growth impairment. Conversely, the larval phenotype depicting delayed lipid metabolism suggests that growth may still be impaired, likely through a different mechanism as shown in the later adult stages. This suggests that PGC-1α may have additional relationships with growth and metabolism that clearly warrants further attention.

Chapter 4: Conclusion

4.1 Apparent Evolutionary Similarities and Differences Between Teleost and Mammalian PGC-1α

The PGC1 family of co-activators has demonstrated a crucial importance in the regulation of metabolic activity in mammalian species, however few studies have examined these regulatory roles in other vertebrates including fishes. The few studies on fish PGC1α collectively suggest that the coactivator contains distinct structural differences with potentially divergent roles to that of mammalian PGC-1α, presenting the need for a genetically modified fish model to further investigate the function of this regulator. Using CRISPR technology, I created a mutant zebrafish line displaying a unique PGC-1α expression profile with PGC-1α overexpression in skeletal muscle tissue and down-regulated PGC-1α expression in heart tissue. Additionally, using a variety of genetic and physiological techniques, I characterized the metabolic phenotypes associated with the disruption of PGC-1α in this new biomedical model.

In the 1st study, I disrupted a newly discovered evolutionary conserved region that controls PGC-1α expression in the heart and skeletal muscle tissue of the zebrafish. Hence, this mutation allowed for the examination of tissue-specific patterns of PGC-1α-mediated pathways in the zebrafish for comparison to previous mammalian PGC-1α studies. In response to decreased PGC-1α expression in heart tissue, PGC-1α-mediated transcription factors (ERRα and NRF-1) and the metabolic co-activator PGC-1β were downregulated, yet none of their downstream metabolic genes were presumably affected. Mammalian PGC-1α knockouts describe similar decreases in PGC-1α-mediated TFs (ERRα, NRF-1) that result in multiple metabolic side effects such as increased CPT-1/2 expression, reduced cardiac function and increased cardiac stress in later life stages (Arany et al., 2005). These phenotypes can be detrimental in mammals, however

the zebrafish heart has regenerative properties that could alleviate these potentially unwanted phenotypes (Asnani & Peterson, 2014; Gemberling et al., 2013). Although the identification of a cardiac-specific phenotype for our mutant zebrafish would have been ideal, the zebrafish heart is incredibly small and the few milligrams of tissue that we managed to isolate were not suitable for use in high-resolution respirometry. Due to these physical limitations and no alteration of effector genes in the heart tissue, the remainder of chapter 2 focused on the observation of PGC-1α overexpression in skeletal muscle.

The gene expression profile of mutant skeletal muscle tissue was surprising as PGC-1α-mediated TFs (ERRα, NRF-1, PPARα) expression were unaffected despite a 4-fold increase in PGC-1α expression. In PGC-1α-overexpressed mammalian models, these TFs are upregulated and increase the expression of the metabolism effector proteins (COX IV, CS, MCAD, FABP3 and CPT-1/2) (Supruniuk et al., 2017; Wende et al., 2007; Wu et al., 1999). In the mutant zebrafish, we found that CPT-1 and CPT-2 expression increased independently of PGC-1α-mediated TFs, suggesting that these mitochondrial proteins are regulated by an alternative pathway exclusive to the zebrafish. Increased CPT-1 and CPT-2 expression suggested that PGC-1α mutant zebrafish have an increased affinity for FA substrates, hence leading to the assessment of mitochondrial performance in isolated muscle fibres. Indeed, mutant zebrafish white muscle fibres displayed increased O_2 consumption following maximum load of individual mitochondria, indicating increased mitochondrial abundance or decreased mitochondrial efficiency. Increased O_2 consumption was also observed amongst juvenile mutant zebrafish at rest, providing further evidence that mutant zebrafish face metabolic consequences due to PGC-1α disruption. Altogether, these muscle-based phenotypes suggest that PGC-1α retains its role as a master

regulator in zebrafish, despite alternative regulatory patterns as demonstrated in both the muscle and heart tissues.

In my 2nd study, I utilized the newly established model to examine how altered PGC-1α expression may impact growth in the zebrafish. Decreased weight and size is a prominent phenotype amongst mammalian PGC-1α models and this phenotype may also persist amongst PGC-1α mutant zebrafish (Leone et al., 2005; J. Lin et al., 2004). During the larval stage, mutant zebrafish had a decreased heart rate with increased yolk-lipid content. These observations suggested that FA metabolism and growth were impaired in PGC-1α mutant zebrafish larvae, as yolk-derived lipids are vital for driving organogenesis during this critical developmental time point (Goenezen et al., 2012; Gore & Burggren, 2012). However, this decrease in FA metabolism was unexpected since CPT-1 and CPT-2 transcripts were upregulated in adult mutant muscle tissue, which provoked further investigation of FA metabolism in adult mutant zebrafish. Indeed, a 2-fold increase in O_2 consumption was identified during the SDA period of adult mutant zebrafish following a high-fat meal, indicating that PGC-1α mutants have temporal shifts in their ability to metabolize FA substrates. This increased FA metabolism negatively impacted the growth of mutant zebrafish in our prolonged dietary experiment, where mutant zebrafish experienced minimal increases in length and mass in the presence of a standard diet. When presented with a high-fat diet, mutant zebrafish were still incapable of matching the rapid growth observed in wildtype fish. In fact, mutant zebrafish fed with a high-fat diet experienced similar growth increases as wildtype fish following our standard diet. When examining the metabolomic profile of mutant zebrafish muscle, we found decreased DGLA levels in mixed muscle tissue, a FA responsible for reducing proton leakage in the mitochondria. With a decrease in muscular DGLA, mutant zebrafish may experience increased mitochondrial leakage resulting in

heightened O_2 consumption to maintain the hydrogen gradient required for ATP synthesis. Altogether, the information presented over these two chapters might suggest that the CPT-1 and CPT-2 proteins are upregulated to saturate the FA pathway of the mitochondria to maintain the proton gradient as a method for compensating the increased leakage found in the mitochondrial membrane. Alternatively, one could suggest that CPT-1 and CPT-2 proteins are upregulated in order to recover DGLA levels in the mitochondria of skeletal muscle, but ultimately face degradation before being incorporated into the mitochondrial membrane.

4.2 Future Directives for the Study of PGC-1α in Zebrafish

With additional research, the characterisation of the PGC-1α zebrafish model could certainly be further defined. Using mixed muscle fibres in our initial assessment of gene regulation restricted us from identifying fibre specific patterns, which clearly display different phenotypes in response to altered PGC-1α expression as demonstrated by high-resolution respirometry. Red and white fibres display opposing phenotypes in both fish and mammals, as red muscle is more oxidative and slow twitch, while white muscle is typically more glycolytic and fast twitch. Additionally, white muscle fibres greatly outnumber those of red muscle fibres, however red muscle fibres also contain a much higher density of mitochondria (Buss & Drapeau, 2000; Glancy & Balaban, 2011). Hence, the separation of both red and while muscle fibre types to investigate differences in gene expression could have revealed differences that may have been obscured in this study.

The generation of a true homozygous mutant PGC-1α line would allow for greater clarity in assessing the true roles of the zebrafish PGC-1α protein in both cardiac and skeletal muscle. In the work presented here, we utilized heterozygous mutants primarily due to time constraints to perform the initial characterization of the zebrafish line. As a result, the mutant zebrafish line examined here is comprised of a mixture of 3 unique deletions spanning 4-bp, 7-bp, and 13-bp

segments respectively across 6 regulatory elements associated with metabolic regulation. Conveniently, 5 of these regulatory elements appear to be disrupted, either entirely or partially by each of the 3 mutations apart from NFE2L1, a binding element for NRF-1 that remains uninterrupted by the 7-bp deletion. Despite this ambiguity amongst our mixed genotypes presentr in our mutant zebrafish line, we were still successful in identifying many phenotypes with the current model, such as decreased heart rate and lipid consumption in larvae, decreased overall growth and increased O_2 consumption in juveniles and isolated white fibres and while these phenotypes help validate the establishment of this mutant model it is also possible that the integral roles of PGC-1α remain hidden due to genetic compensation by the wildtype PGC-1α promoter (El-Brolosy et al., 2019; El-Brolosy & Stainier, 2017). Using homozygous mutant zebrafish would also allow for simplified experimental design as it removes the need for individual genotype screening and provides more zebrafish for experimentation. Homozygous mutants would also provide increased reliability for high-throughput genetic assays, delivering rich datasets that can be computationally analyzed and catalogued to further build upon the phenotypes associated with PGC-1α pathologies (Kaliyappan et al., 2012; Z. Wang et al., 2009).

Although our approach was successful in modifying PGC-1α-expression in both the heart and muscle tissue, other metabolically active tissues such as the brain and liver are equally relevant in the overall study of PGC-1α. Certainly, the investigation of PGC-1α in brain tissue would be beneficial to neurological decay and disease research as previous studies have indicated neuroprotective roles of PGC-1α in mammals (Tsunemi & La Spada, 2012; Zheng et al., 2010; Zheng et al., 2017). The liver is also heavily impacted by PGC-1α expression, directly controlling gluconeogenesis through the expression of hepatic insulin receptors (Besse-Patin et al., 2019). Additionally, the zebrafish gills may also be an area of interest considering that this

respiratory organ is absent in other vertebrates but maintains a high mitochondrial density (Dawson et al., 2020; Lin & Sung, 2003). Whole body PGC-1α knockouts may reveal additional phenotypes associated with inter-tissue PGC-1α signaling pathways, a recent discovery noted in mammalian models (Balampanis et al., 2019). Certainly, additional zebrafish PGC-1α models should be investigated for the continued research of PGC-1α in teleosts.

Finally, one application of the work presented here is the implementation of this CRISPR-induced mutation into other vertebrate models. The genetic region modified in the mutant zebrafish is highly conserved throughout vertebrate lineage and suggests that this CRISPR-induced mutation could be easily incorporated into other vertebrates (reptiles, amphibians and birds) and mammalian species for the investigation of PGC-1α activity in skeletal muscle and cardiac tissues. Although the primary goal of this thesis was to identify divergent roles of the teleost PGC-1α protein, implementing this mutation in other model organisms would greatly accelerate our understanding of this co-activator in both an evolutionary and biochemical perspective. While multiple mammalian PGC-1α models have been established thus far, our model provides an interesting hybrid of increased muscle/heart expression that is currently unexplored. As such, it would be interesting to examine if this mutation presents viable offspring in a mammalian model with alternative or similar phenotypes as those detailed here in the zebrafish.

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Appendix

Table S1: Primer *list for CRISPR gRNA synthesis, HRM analysis and gene expression.* Grey lettering indicates template regions required for the synthesis of the gRNA (black lettering).

Primer	Forward Primer (5'-3')	Reverse Primer (5'-3')
EF1α [15]	GTGCTGTGCTGATTGTTGCT	TGTATGCGCTGACTTCCTTG
RPL 13a [15]	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG
PGC1a [15]	ACCAACCATCTTGCCACTTC	ATTACTCAGCCTGGGCCTTT
HOAD	CCACAGGACATTCAGTGGTG	GTCAGTGCCATGAACGACAG
MCAD	CAGAAAGAGTTCCAGGAGGTG	TGTCCGTTCATTAGACCCAG
ERRα	AGATGTGGCATCTGGCTACC	CCAAGCGAACTCCTTCTTTG
NRF-1	AGGCCCTGAGGACTATCGTT	GCTCCAGTGCCAACCTGTAT
CS	ATCCGTTTCCGTGGTTACAG	AGACAGCCAACTGACCTGCT
COX IV	CAAGTTTGTGCAGCAGCTG	CAAAGAAGAAGATTCCTGCAAC
CPT1	TATGACCGTTCAGACGCAGA	TACAGGCAGATGTGGCAGAG
CPT2	AATGGATTGGGTGCAACGTG	TGAGTTCTAACCTTCAGGCTC
FABP3	TCAGCTCAAACATGGCAGAC	CAAAGCCAACACCAATTCCT
PPARα	ACCAACCATCTTGCCACTTC	ATTACTCAGCCTGGGCCTTT
PGC1B	CAGCGAAGAGGAGATTACGG	GAGTCTGCTCAAAGGGCTTG
PGC-1α-gRNA	TAATACGACTCACTATAG AGCCCCTGAATGACGGCA	TTCTAGCTCTAAAAC TGCCGTCATTCAGGGGCTGG
PGC1a-HRM	GCGAGGGAACGAGTGGATTTTC	ATTACACCTGTCCCACGCCATC

Table S2: Flux control ratios calculated by the DatLab analysis excel template to examine individual differences in mitochondrial performance. No significant differences were found when examining all substrate additions in both tissues.

Substrate	White Muscle FCR (Wildtype)	White Muscle FCR (Mutant)	p-value (Student's t-test)	Red Muscle FCR (Wildtype)	Red Muscle FCR (Mutant)	p-value (Student's t-test)
Malate (50mM)	0.0655 (+/- 0.0208)	0.1054 (+/- 0.0349)	0.274	-0.0172 (+/- 0.0115)	0.236 (+/- 0.0203)	0.161
Octanoylcarnitine	0.4695 (+/- 0.0947)	0.4132 (+/- 0.0804)	0.588	0.3696 (+/- 0.0559)	0.4321 (+/- 0.0474)	0.326
Glutamate	0.6766 (+/- 0.0659)	0.6958 (+/- 0.0662)	0.302	0.5744 (+/- 0.0418)	0.6698 (+/- 0.0329)	0.0885
Succinate	0.8884 (+/- 0.0925)	0.9584 (+/- 0.0744)	0.103	0.7499 (+/- 0.0247)	0.8644 (+/- 0.0402)	0.0744
Glycerophosphate	0.9562 (+/- 0.0637)	1.0038 (+/- 0.0555)	0.0998	0.7780 (+/- 0.0404)	0.8753 (+/- 0.0393)	0.284
CCCP	1	1	-	1	1	-
Rotenone	0.4041 (+/- 0.0573)	0.3976 (+/- 0.0781)	0.743	0.3413 (+/- 0.0282)	0.3367 (+/- 0.0844)	0.883

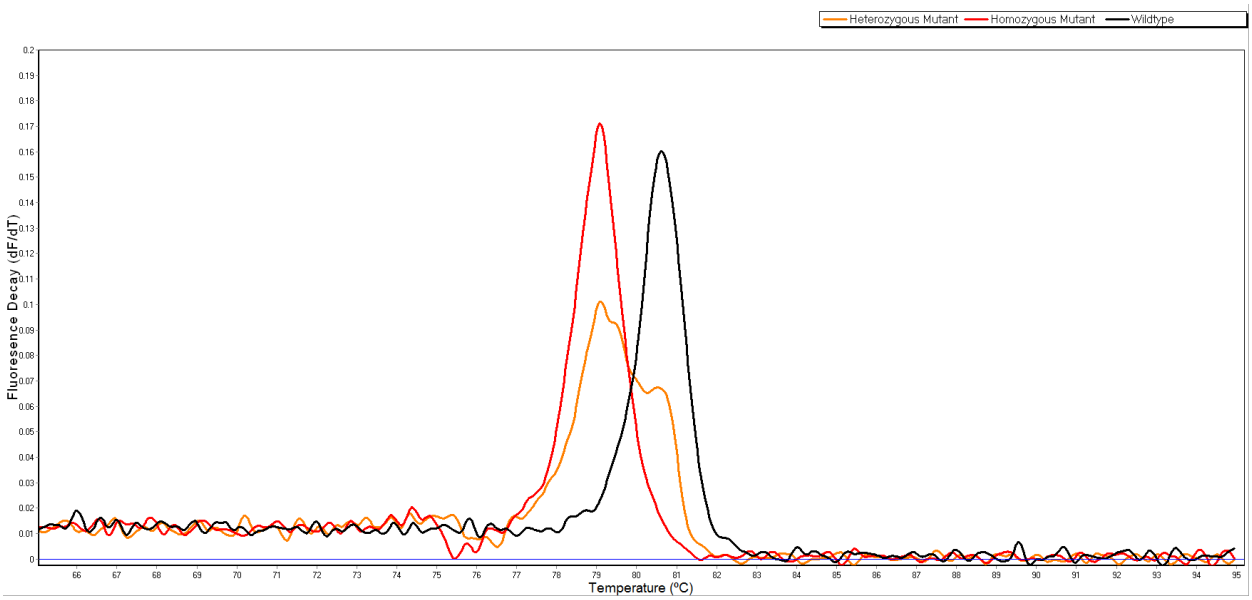

Figure S1: Mutant zebrafish displayed altered melt curves serving as the initial characterization of the mutant line. Mutant zebrafish displayed either shift peaks (termed 'Homozygous', Red) or multiple peaks (termed 'Heterozygous', Orange) when compared to wildtype peaks (Black) in response to successful CRISPR-induced deletions. Homozygous zebrafish were used exclusively for the development of the F1 generation of mutant zebrafish to provide heterozygous mutant zebrafish for experimentation.

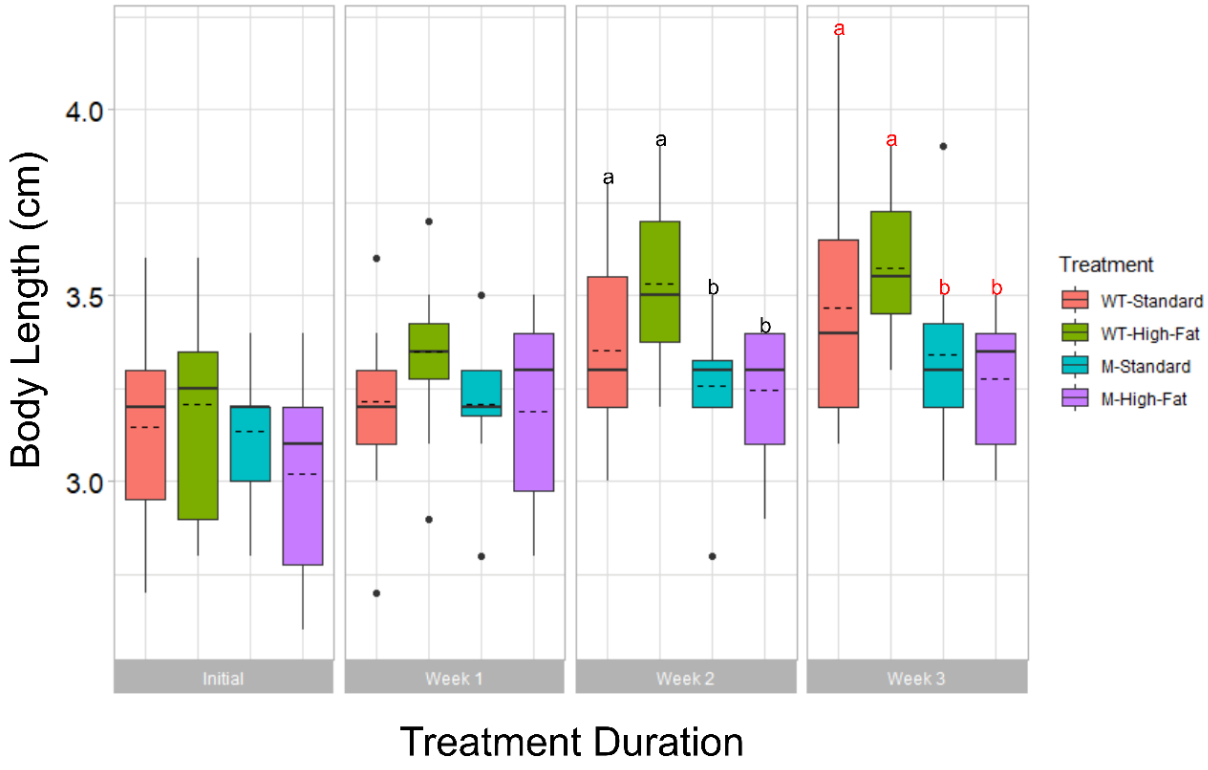

Figure S2: Individually tracked length increases in mixed sex zebrafish in response to standard and high-fat diets over a 3-week period. Zebrafish experienced dietary linked differences in length at Week 2 ($p = <0.001$) and genetic differences at Week 3 ($p = 0.002$). Significance markings in red indicate the use of ranked testing for when normality and equal variance were not met.

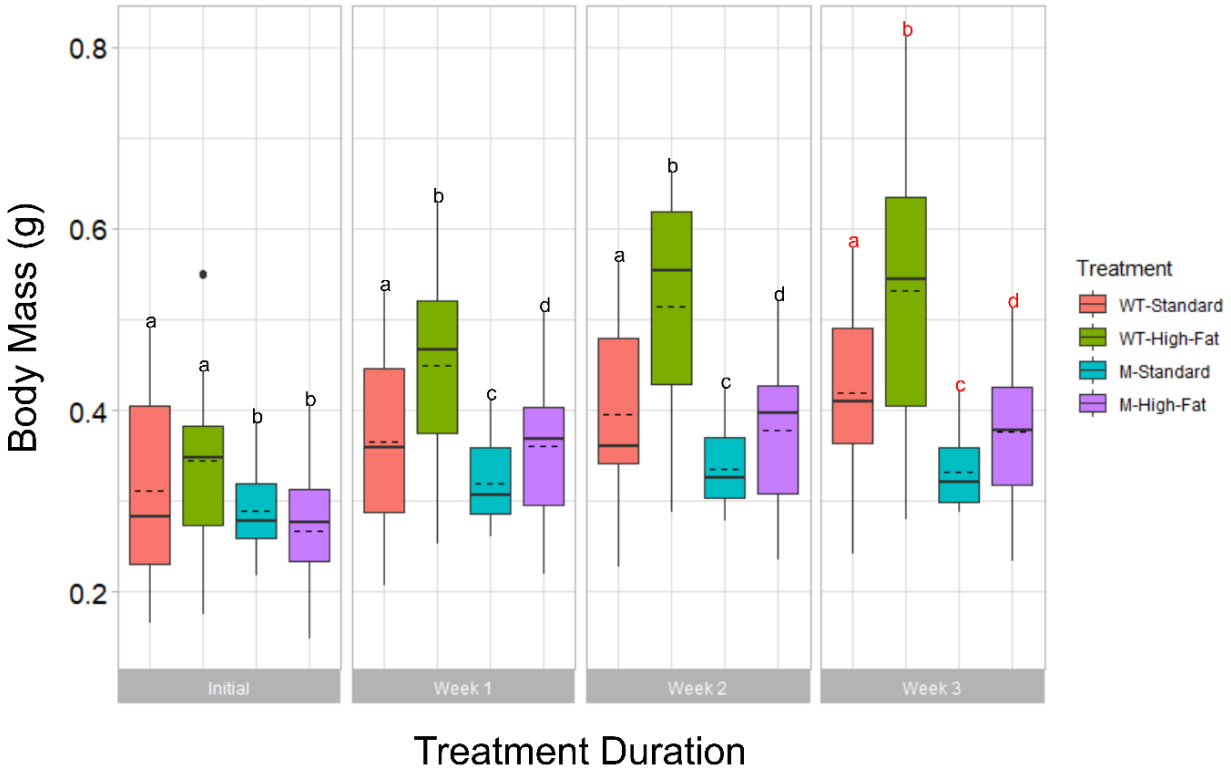

Figure S3: Individually tracked mass increases in both male and female zebrafish in response to standard and high-fat diets over a 3-week period. Zebrafish experienced genotype linked differences in mass initially ($p = 0.041$) with dietary and genetic linked differences at Week 1 (Diet $p = 0.012$, Genotype $p = 0.019$), Week 2 (Diet 0.002, Genotype $p < 0.001$) and Week 3 (Diet $p < 0.005$, Genotype = <0.001). Significance markings in red indicate the use of ranked testing for when normality and equal variance were not met.

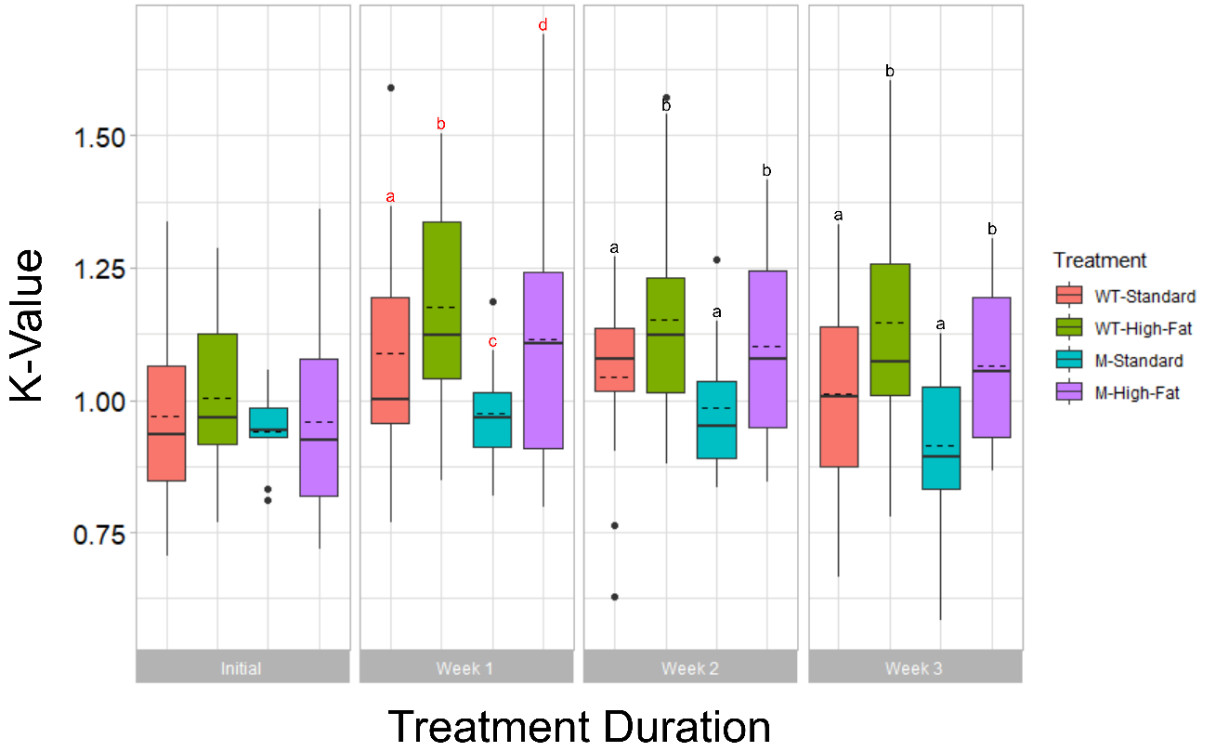

Figure S4: Individually tracked K-Value increases in both male and female zebrafish in response to standard and high-fat diets over a 3-week period. Zebrafish experienced dietary and genetic linked differences in K Value at Week 1 (Diet $p < 0.001$, Genotype $p < 0.001$) with genotype differences at Week 2 (0.027) and Week 3 ($p = 0.009$). Significance markings in red indicate the use of ranked testing for when normality and equal variance were not met.

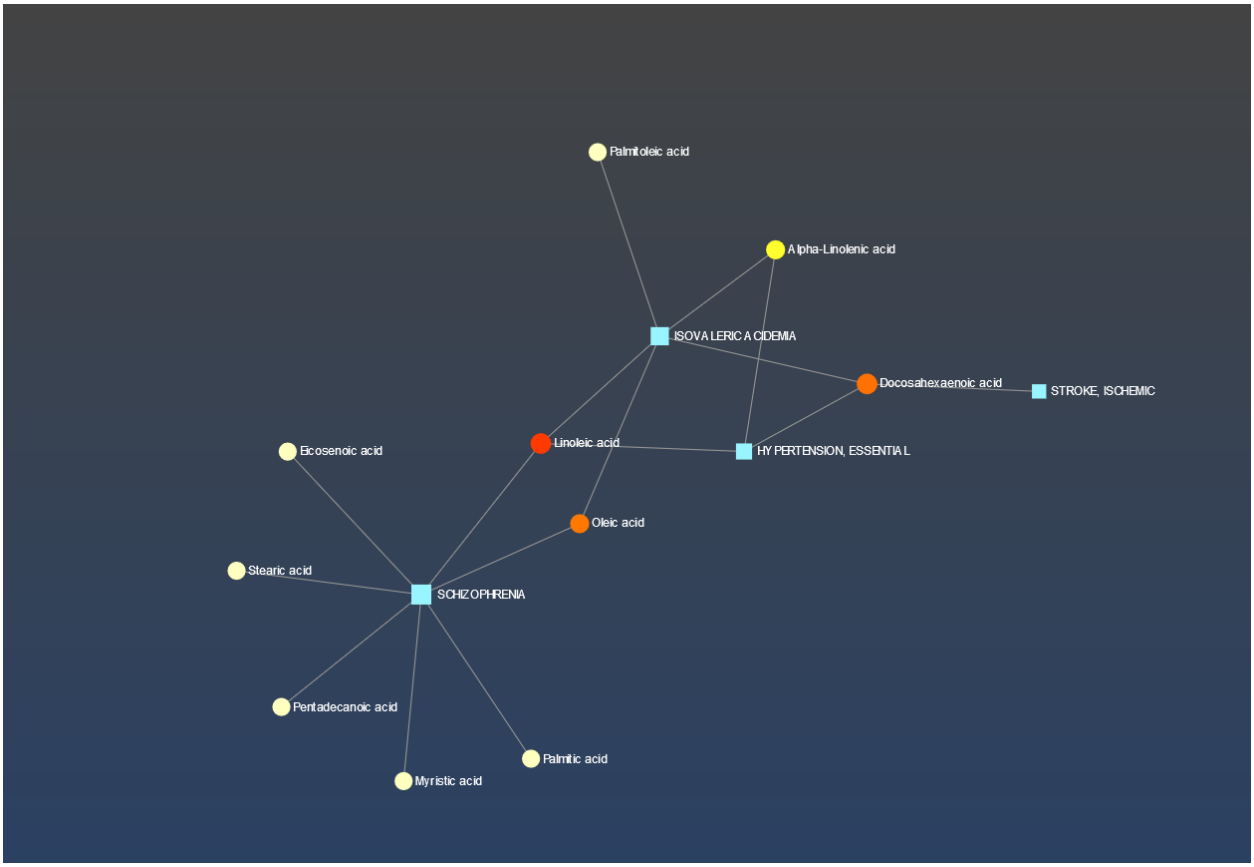

Figure S5: Summary diagram of metabolite-disease network analysis. A total of 5 metabolic diseases were linked to 10 of the upregulated/downregulated FAs found in the high-fat diet zebrafish.