THESIS

DO FATS ISOLATED FROM DIFFERENT TROPHIC LEVELS AFFECT THE AEROBIC POTENTIAL OF TERRESTRIAL MAMMALS?

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ABSTRACT

DO FATS ISOLATED FROM DIFFERENT TROPHIC LEVELS AFFECT THE AEROBIC POTENTIAL OF TERRESTRIAL MAMMALS?

Fats serve as a primary fuel source for the metabolism of animals. Certain fatty acids, like polyunsaturated ones, can influence myoglobin concentration and enzymatic activity. This can enhance oxygen utilization in muscle, which is essential for animals relying on aerobic metabolism. Shifts in dietary composition, such as shifts in fatty acids, can have significant implications regarding the physiological adaptations of various species, especially in response to climate change. This study aimed to investigate if fats isolated from different trophic levels, specifically fish and krill, would have physiological differences in mammalian cell culture. C2C12 mouse skeletal muscle cells supplemented with krill and fish oil had significantly reduced media fat concentrations after the seventh day of differentiation, with no crude fat measured in the media for cells supplemented with fish oil. Intracellular crude fat levels mirrored these observations, with the highest concentrations in the fish treatment, followed by krill, and then the lipid cocktail. Fish oil was also measured to have the greatest variation of fatty acids, while krill oil had significantly higher concentrations of omega-3 fatty acids other than docosahexaenoic acid. This led to the hypothesis that cells supplemented with fats isolated from fish would reflect increased markers of aerobic capacity, specifically myoglobin concentration and citrate synthase activity. An increase trend of myoglobin expression in all treatment conditions was observed, aligning with previous work, but only the cells supplemented with the standard lipid cocktail showed a statistically significant increase compared to the control. An

observed trend of decreased citrate synthase enzymatic activity supported that crude fat levels cell culture media had passed the established 5% threshold from previous studies known to induce cell death. This work developed a reliable method for creating sterile lipid-supplemented media, ensuring consistent incorporation of hydrophobic lipids into aqueous cell culture systems, and highlights how understanding the impact of different fatty acids on skeletal muscle metabolism is crucial for comprehending the effects on metabolism in a changing environment.

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TABLE OF CONTENTS

ABSTRACT……………………………………………………………………………………....ii
ACKNOWLEDGEMENTS…………………………………………………………………iv
Chapter 1 – Introduction…………………………………………………………..……….......1
Chapter 2 – Establishing Experimental Conditions Between Fats Isolated from Different Trophic Levels (Fish and Krill) In C2C12 Skeletal Muscle Cell Culture……………...…….……...…....20
Chapter 3 – Is Aerobic Capacity Influenced by Lipids from Different Trophic Levels in C2C12 Cell Culture? ……………………………...…………...…...54
Chapter 4 – Conclusions and Future Directions……………………………………….….…..68

CHAPTER 1: INTRODUCTION

Global patterns linked to climate change are fundamentally reshaping ecosystems worldwide, leading to complex, cascading effects that influence organisms at every level of biological organization, from cellular function to entire populations and communities (Blois et al., 2013; Grimm et al., 2013). Calls for changes in policy are driven by global environmental impacts, which include intensifying droughts, worsening water scarcity, more frequent and destructive wildfires, accelerating glacial and polar ice melt, and a marked decline in global biodiversity (Nadeau et al., 2022; Pörtner et al., 2023). A more subtle yet profoundly important consequence of climate change is its impact on the availability, composition, and quality of nutrients across trophic levels within ecological food webs, and how this will influence organismal physiology and behavior (Cross et al., 2015). Shifts in the distribution, behavior, and nutritional profiles of organisms at the base of the food web that serve as important prey to many animals have been described as additional consequences from climate change (Rosenblatt, 2018; Soares et al., 2019). Of these changes, shifts in dietary quality and composition, such as changes in fatty acids, which are components of fats, have often been overlooked despite their important physiological roles.

The importance of fat as a dietary resource becomes even more apparent when considering that many essential fatty acids cannot be synthesized by most endothermic vertebrates and therefore must be obtained from food (Pough et al., 1999). These essential fatty acids are not only energy substrates but also play a crucial role in maintaining the fluidity of cellular membranes, thereby enabling effective nerve function, modulating inflammatory responses, and optimizing mitochondrial efficiency (Das, 2006). Therefore, it can be predicted

that any climate-driven shift in the availability, type, or balance of these fatty acids in prey species can have direct, measurable impacts on the health and fitness of consumer organisms, from impaired aerobic capacity to reduced reproductive output and weakened resilience to environmental stress (Karasov & del Rio, 2007). Studies have begun to investigate how climate change affects the fatty acid composition at the base of aquatic food webs, where phytoplankton, primary producers vital to many ecosystems, have been noted to alter their lipid profiles under warming conditions, a change that ultimately impacts organisms that consume them, including seabirds, marine mammals, and even humans (Hixson & Arts, 2016). Shifts in prey lipid content, for instance, have already been linked to changes in diving ability and reproductive success in marine mammals (Trumble & Kanatous, 2012), and similar concerns apply to migratory birds, which depend on fat-rich diets to sustain long-distance flights (McWilliams et al., 2004).

Despite these insights, the interaction between climate-induced shifts in dietary quality, such as changes in fat, and their impact on organismal physiology remains understudied, particularly in the context of aerobic performance and ecological fitness. This knowledge gap is problematic, as the ability of animals to meet energy demands in a changing world depends not only on the quantity of food available but also on its quality, particularly the types of fats they can access and metabolize. To better understand these implications, the first chapter of this work will introduce fat as a critical and efficient energy substrate for endothermic vertebrates. We will examine how these shifts may affect organismal physiology and behavior, especially in species that depend heavily on sustained aerobic activity, specifically endothermic vertebrates. By investigating environmental, physiological, and biochemical perspectives, this chapter aims to introduce how climate change may fundamentally alter the physiology and behavior of various species.

Fat Serves as Optimal Fuel for Endothermic Vertebrates

Given the central role of fat metabolism in supporting performance and survival across endothermic vertebrates, the consequences of climate-driven shifts in dietary fat composition have the potential to have major impacts. Climate change alters the abundance and composition of primary producers, which in turn impacts the lipid profiles available to higher trophic levels (Cross et al., 2015). As a result, organisms reliant on fat to support aerobic-based activities may face challenges in meeting their energetic needs, which can potentially affect their foraging success, migratory timing, reproductive investment, and overall fitness (Hixson & Arts, 2016; Huey & Kingsolver, 2019). Since fats are important in supporting vital activities associated with fitness, dietary fatty acids have also been of interest in studies investigating physiological feats in endothermic vertebrates such as migration, torpor, hibernation, and diving conducted by Aves and both terrestrial and marine mammals.

Amongst endothermic vertebrates, studies have focused on birds, small mammals, and humans. Within the Aves, the impact of dietary fats on migration and flight has been of focus, emphasized by data supporting that migrating birds have been recorded relying primarily on fats to fuel to sustain flight during migration (McWilliams et al., 2004). Studies have suggested Aves are reliant on fat for up to 90% of the energy to fuel migratory flight, and that it is the preferred nutrient to stock up on in preparation for migration (Bairlein & Hüppop, 2004; Guglielmo, 2010). With an energy yield of approximately 9 kilocalories per gram, more than twice that of carbohydrates or proteins, fats represent the most energy-dense macronutrient available to these organisms (Pond, 1978; Young, 1976). Fat as the primary fuel source contributes minimally to weight and is energy-dense, making it crucial for optimal performance during the non-stop aerobic activity migratory flight can be for many avian species (Jenni & Jenni-Eiermann, 1998;

McWilliams et al., 2004). While building up fat deposits pre-migration is important, many migratory species are unable to refuel for periods of time due to factors such as crossing the Gulf of Mexico, making it essential to rely on optimal fat storage or optimal refuel foraging (Deppe et al., 2015). As a hydrophobic and nonpolar structure, fat can be stored without water, which influences an organism's weight (Pond, 1978). However, excessive fat intake can lead to large deposits that negatively impact flight dynamics, which are reliant on weight limitations (Hou & Welch, 2016); therefore, selecting the right type and amount of fat for optimal performance is crucial. Cornelius et al., 2021 analyzed fat scores of both facultative and obligate migratory species and noted that both types showed distinct peaks in fat deposits in preparation for their long-distance flight stages that exceeded nesting stage deposits.

In addition to sustained long-distance flight associated with migration, muscular interactions with fats have been observed to sustain non-migratory flight activities. Infante et al., 2001 observed that hummingbird pectoralis muscles, considered large flight muscles with high aerobic capacity, exhibited fatty acid-rich membranes and increased Ca^{2+}-ATPase activity, suggesting specific types of fats play an essential role in maintaining membrane fluidity and function during repeat contraction activities. The various roles of fats in sustaining both migratory and non-migratory flight support the importance of dietary choice and fat in influencing optimal performance in the energetically demanding activity of flight.

Similarly to Aves, mammals have also been studied to understand how the choice of diet affects fat composition and influences factors such as physiology and health. Mammals utilize fats in sustaining not just flight (in the case of Chiropterans) but also prolonged locomotory activities such as walking, running, climbing, and swimming. High proportions of fatty acids in the skeletal muscles of brown hares have been recorded, which supports their ability to run at

fast speeds by facilitated rapid, sustained movement (Valencak et al., 2003). Additionally, Ruf et al., 2006 compiled the muscular fatty acid profiles of 26 mammals across eight different taxonomic orders and observed a positive relationship between muscle phospholipids and running speed. Studies on rats have shown that diets high in specific fatty acids can lead to improvements in endurance performance, such as increased time to exhaustion on a treadmill (Ayre & Hulbert, 1997). In addition to sustaining locomotion, research on dietary fats, specifically polyunsaturated fatty acids (PUFAs), has revealed their important role in energy regulation and physiological efficiency in mammals, particularly in states such as torpor and hibernation, with fats playing a significant role in regulating metabolic efficiency and energy conservation during torpor (Dark, 2005; Geiser & Kenagy, 1987; Ruf & Arnold, 2008). Several studies investigating the influence of dietary PUFAs on torpor have suggested that small mammals provided with a moderate amount of PUFAs through enriched diets enhanced torpor expression and decreased the energetic cost of hibernation (Florant, 1998; Frank, 1992; Geiser & Kenagy, 1987; Ruf & Arnold, 2008). Fats are therefore important in sustaining locomotion of various types in mammals, beyond other physiological activities such as hibernation.

For marine mammals such as pinnipeds, diving is sustained through aerobic, fat-based metabolism supported through their diet (Kanatous et al., 1999). These species have evolved enhanced oxygen storage capacities, bradycardia, and mitochondrial efficiency to optimize the aerobic oxidation of fats during dives (Trumble & Kanatous, 2012; Kanatous et al., 1999). Fat stores are not only crucial for energy, but also for thermoregulation and buoyancy in aquatic environments. Since fat provides more than twice the energy per gram compared to carbohydrates, and its hydrophobic nature allows storage without added weight from water, this provides an advantage for both flying birds and diving mammals (Pond, 1978). However,

disruptions in the type or availability of dietary fats could impair physiological performance, aerobic capacity, and resilience in a changing environment (De Miranda et al., 2012; Rosenblatt & Schmitz, 2016).

For many of these endothermic vertebrates, fats not only serve as a primary energy source but also as a key structural and signaling component in biological systems (Chong et al., 2006; Hulbert et al., 2005; Simopoulos, 2007). Fatty acids are fundamental components of cell membranes, contributing to membrane fluidity and permeability, which in turn affect cellular communication, nutrient transport, and signal transduction (Hulbert et al., 2005; Simopoulos, 2007; Valencak & Azzu, 2014; Weber, 2009). Specific types of fatty acids support membrane flexibility, allowing cells to maintain optimal function across a range of temperatures and environmental conditions (Dey et al., 1993; Schuchardt et al., 2011). Additionally, fatty acids serve as precursors for a variety of molecules, including hormones, signaling lipids, and inflammatory mediators, all of which can impact the physiology of an organism (Calder, 2015).

The fatty-acid composition in the human diet has also been and continues to be well-studied. Bischoff-Ferrari et al. 2025 conducted a clinical trial investigating the impact of omega-3 and exercise on aging, with results showing that omega-3 supplementation positively affected DNA methylation measures of biological aging. Additional potential positive effects of omega-3 supplementation on human health that have been investigated include endothelial function, inflammation, arrhythmia prevention, and improved heart rate (Mohebi-Nejad & Bikdeli, 2014). Therefore, continuing to explore the benefits of fats available to all endothermic vertebrates is a vital avenue not only for conservation efforts in animals but also for advancements in our understanding of the impact of diet choice and quality on human health.

So, What Is the Right Type of Fat?

While fats are widely recognized as essential for supporting an organism's physical performance, particularly in energetically demanding activities such as locomotion, as well as overall health and cellular function, an even more critical factor lies in the quality and specific composition of dietary fats consumed. The fat content in an organism's diet can be highly variable, encompassing a diverse array of lipid molecules that are further metabolized into fatty acids and ultimately converted into usable energy in the form of ATP via the process of beta-oxidation (Karasov & del Rio, 2007). Understanding the biochemical nature of these fats and their metabolic pathways is fundamental to appreciating how dietary quality affects physiology and fitness.

At a molecular level, fatty acids are composed of long chains of carbon and hydrogen with a carboxyl group (-COOH) at one end. The presence and position of double bonds within these chains determine if the fatty acid is considered saturated or unsaturated, and further into monounsaturated and polyunsaturated, influencing their properties and role in the body (De Carvalho & Caramujo, 2018). Saturated fatty acids contain no double bonds, resulting in straight chains that pack tightly together, which influences their physical and biological properties. In contrast, unsaturated fatty acids contain one or more double bonds. Unsaturated fatty acids are further subdivided into monounsaturated fatty acids (MUFAs), with a single double bond, and polyunsaturated fatty acids (PUFAs), which have two or more double bonds (De Carvalho & Caramujo, 2018).

A brief outline of general differences between unsaturated, monounsaturated, and polyunsaturated fatty acids is outlined in Table 1-1. Among dietary unsaturated fats, two groups of particular physiological and ecological importance are omega-3 and omega-6 fatty acids (Das, 2006). General nomenclature for these fats is recorded as a ratio of the number of carbons in the

chain to the number of bonds (Chow, 2007). Omega-3s are polyunsaturated fatty acids (PUFAs) with their first double-bond occurring at the third carbon position from the methyl end, and include fatty acids such as alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) (Covington, 2004). In contrast, omega-6's first double bond occurs at the sixth carbon position and include fatty acids such as linoleic acid, arachidonic acid, and gamma-linolenic acid (Dubois et al., 2007). Both omega-3 and omega-6 fatty acids are essential nutrients because many organisms cannot synthesize them and must obtain them through diet. Maintaining an optimal ratio of PUFAs is crucial as the proportion directly influences physiological function, overall health, and performance. Imbalances can lead to chronic inflammation or impaired cellular function, which can negatively impact growth, reproduction, thermoregulation, and endurance (Simopoulos, 2002). This balance is therefore of particular interest in ecological and physiological research, especially when considering how climate-driven changes in food webs might shift fatty acid availability and composition, ultimately affecting the health and performance of organisms across trophic levels.

There are additional differences in the different fatty acids that impact physiological ability. While most fat digestion occurs in the small intestine, the initial stages of fat digestion occur in the stomach, where triglycerides are broken down into free fatty acids through hydrolysis. Fatty acids are then broken down in the mitochondria by oxidation of acetyl-CoA, which is further oxidized in the citric acid cycle to generate ATP (Kennelly et al., 2022). When comparing the different types of fatty acids, unsaturated fatty acids have been recorded to be esterified at a higher rate than saturated fatty acids of similar length (Gangl et al., 1980). In addition to this, when compared to SFAs of similar length, MUFAs and PUFAs require fewer moles of oxygen to be metabolized (Trumble & Kanatous, 2012). Since effective aerobic

locomotion relies on sufficient oxygen delivery and the proper distribution of energy sources, the quality of one's diet is essential for maintaining optimal performance. With many studies specifically highlighting PUFAs, how do lipids, specifically this type of fatty acid, support physiology, such as maintaining aerobic capacity, and what occurs if it is not available in the diet?

Table 1-1. A list of highlighted common fatty acids categorized into saturated, monounsaturated, and polyunsaturated, and general differences in structure and β-oxidation.

Fatty Acids				**B-Oxidation**		
Type	Common name	Common sources	Structure	Pathway differences	Enzymes	Estimated mol of oxygen for complete oxidation
Saturated						
14:0	Myristic acid	Nutmeg, butter, and coconut oils	No double bonds, all carbons are saturated with hydrogen	Removal of 2-carbon units from fatty acid chains in sequential order	Acyl-CoA dehydrogenase, enoyl-CoA hydratase, hydroxyacyl-CoA dehydrogenase, thiolase	Stearic acid – 26 mol
16:0	Palmitic acid	Common in animal and plant fats				
18:0	Stearic acid	Animal and plant fats (more abundant in animal fat)				
Monounsaturated						
16:1n-7	Palmitoleic acid	In many fats	One double bond in the cis configuration	Requires additional modifications at the double bond site	Acyl-CoA dehydrogenase, enoyl-CoA hydratase, hydroxyacyl-CoA dehydrogenase, thiolase, and Enoyl-CoA isomerase	Oleic acid – 25.5 mol
18:1	Oleic acid	Olive oil, very common in natural fats				
Polyunsaturated						
Omega-3						
18:3n-3	a-Linolenic acid (ALA)	Associated with linoleic acid	Two or more double bonds in the cis configuration	Requires additional modifications for each double bond site	Acyl-CoA dehydrogenase, enoyl-CoA hydratase, hydroxyacyl-CoA dehydrogenase, thiolase, and Enoyl-CoA isomerase and reductases	ALA – 25 mol
20:5n-3	Eicosapentaenoic acid (EPA)	Fatty fish				
22:6n-3	Docosahexaenoic acid (DHA)	Fatty fish				
Omega-6						
18:1n-9	Linoleic acid	Plant oils (corn, peanut, soy, etc.)				
18:3n-6	Gamma-linolenic acid	Plant oils				
20:4n-6	Arachidonic acid	Animal fats				

Lipids As a Driver of Aerobic Capacity

Lipids, specifically fatty acids, have been demonstrated to influence aerobic ability in mammalian models, as mentioned previously. One physiologically important protein to aerobic metabolism is myoglobin, a monomeric heme protein that has the ability to reversibly bind oxygen and, therefore, act as storage for muscular oxygen (Rayner et al., 2009; Wittenberg & Wittenberg, 1989). While the regulation of this protein is not yet fully understood, more and more studies are being conducted regarding myoglobin regulation and stimulation. Understanding the factors that influence myoglobin expression is key to understanding how mammals adapt to varying oxygen demands, such as during exercise or at high altitudes. Skeletal muscle studies have shown a positive correlation between myoglobin expression in skeletal muscle cells and lipid supplementation. For example, it was established that increasing the amount of lipid up to a certain percentage of volume in differentiation media upregulated myoglobin expression in both terrestrial and marine mammal cells, driving its expression (De Miranda et al., 2012; Schlater et al., 2014). This suggests that fatty acids can act as signaling molecules, influencing gene expression and protein synthesis related to oxygen storage. For instance, De Miranda et al. (2012) demonstrated that the addition of palmitic acid to cell cultures of bovine muscle cells resulted in a significant increase in myoglobin protein levels. It has also been revealed that myoglobin can bind to fatty acids, enhancing the solubility of hydrophobic fatty acids within the aqueous cytosol (Sriram et al., 2008). This interaction may facilitate the transport of fatty acids into mitochondria for beta-oxidation, the process by which fatty acids are broken down to generate energy. The expression of myoglobin can and has been measured in various mammalian species, and its contributions to aerobic capacity make it a relevant protein

for investigating the primers of oxidative efficiency in mammalian models in relation to the effects of lipid supplementation.

An additional relevant indicator of aerobic capacity is citrate synthase. Citrate synthase is a key mitochondrial enzyme that utilizes acetyl-CoA and oxaloacetate to form citrate in the mitochondrial membrane, which participates in energy production in the citric acid cycle and is linked to the electron transport chain (Srere, 1975). The citric acid cycle is a central metabolic pathway that oxidizes acetyl-CoA derived from carbohydrates, fats, and proteins, generating high-energy electron carriers (NADH and FADH2) that fuel the electron transport chain (Kennelly et al., 2022). Since it is the rate-limiting enzyme of the citric acid cycle, it can be used to measure aerobic capacity in cells, such as aerobic lipid-based metabolism with higher citrate synthase activity generally indicates a greater capacity for oxidative metabolism (Kanatous et al., 2002). These measurements can provide insight into comparing the impact that different isolated fatty acids, such as PUFAs, have on aerobic ability and what shifts in dietary fats may mean on an organismal scale.

Do Shifting Climates Have a Fat Chance of Shifting Fats?

Shifts in temperatures associated with climate change have severely impacted the ecological distribution and availability of many organisms that serve as vital food sources for many organisms, including humans, with multiple studies indicating shifts in fat composition (Cross et al., 2015; Rosenblatt & Schmitz, 2016). One of the most well-documented regions experiencing these changes is the Antarctic, where rising temperatures and shifting sea-ice patterns are predicted to have wide-ranging ecological consequences. For example, several fish species that serve as key prey for Antarctic predators are changing their lipid profiles. Changes in the Antarctic climate have been predicted to have various environmental effects, including

affecting the lipid composition of fish species that serve as primary prey for many Antarctic organisms (Phleger, Nelson, et al., 1999; Phleger, Nichols, et al., 1999). These studies demonstrated that changes in water temperature and ice dynamics can alter the lipid content and fatty acid composition of these fish, potentially reducing their nutritional quality. Since many Antarctic organisms rely heavily on these lipid-rich fish to fuel energetically demanding activities like reproduction, thermoregulation, and diving, small changes in prey quality could impact predator performance and fitness.

Fish are not the only prey species investigated for fatty acid shifts, as studies have also predicted shifts in fatty acid production in phytoplankton, which are considered a main source of omega-3 fatty acids in aquatic ecosystems. Hixson and Arts (2016) conducted a comprehensive analysis of over 900 fatty acid profiles across six major phytoplankton taxa, drawing from a wide range of studies and ecosystems. Their analysis found a trend that as water temperatures rise, levels of highly unsaturated omega-3 fatty acids, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), tend to decrease, while concentrations of saturated fatty acids increase. Since DHA and EPA are essential for the development, reproduction, and metabolic efficiency of many aquatic and terrestrial animals, and cannot be synthesized by most consumers, these shifts in the biochemical quality of primary producers pose a serious threat to higher trophic levels including predators such as pinnipeds.

Trumble & Kanatous, 2012 highlighted the importance of diving mammal’s ability to obtain proper fatty acids in their diet. This suggested that even minor alterations in the fatty acid composition of prey fish could impair the diving efficiency of these seals. This reduction in foraging performance could then force seals to shift to alternative, potentially less optimal prey sources, which could set off a chain reaction throughout the ecosystem (Figure 1-1). Such

predator-prey mismatches may disrupt trophic relationships, alter competitive dynamics, and reduce the resilience of marine food webs.

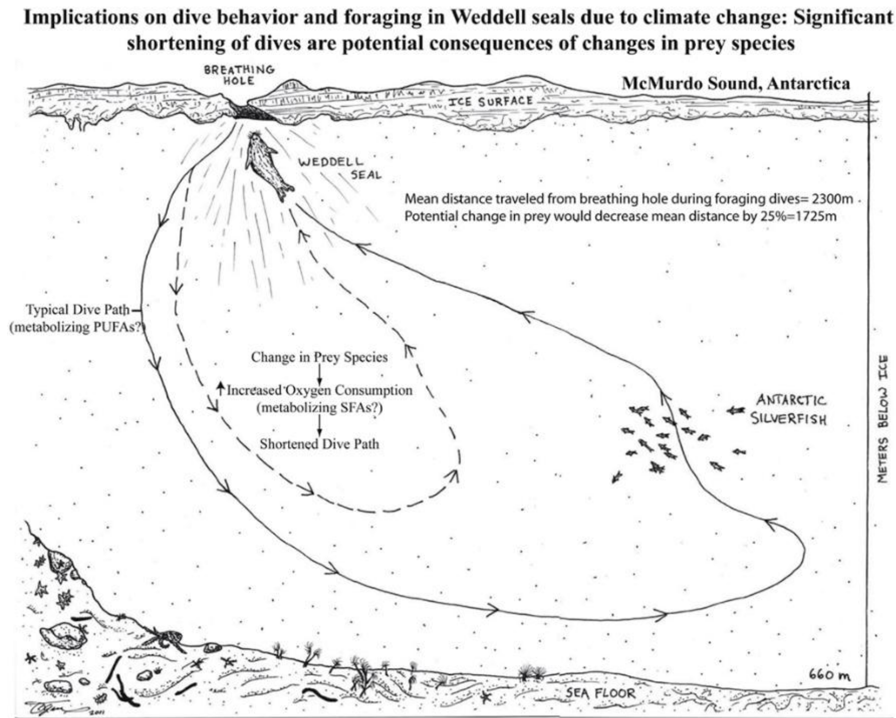

Figure 1-1. Illustration outlining the typical dive path of a Weddel seal when Antarctic silverfish are available versus the proposed shortened dive path as a result of a shift in prey's fatty acid composition availability due to changing climates. (Trumble & Kanatous, 2012)

Therefore, investigating the coordination of physiological processes to assess whole-organism performance in an ecological context is a key factor in understanding the interactions between organisms and their environment and the impact of climate change. Beyond ecological and organismal conservation, fatty acids are important to pharmaceuticals, cosmetics, plastics, food, and feedstock in human economics (Cerone & Smith, 2021). Despite this, when examining the responses of food webs to climate change, shifts in the nutrient content of resources, particularly the quality and types of fats, are often overlooked or underemphasized. Traditional

studies tend to focus primarily on changes in species abundance or distribution, neglecting the biochemical and nutritional dimensions that critically influence organismal health and ecological interactions. These shifts must be investigated as they have potential organismal (Figure 1-2) effects that impact fitness but could eventually develop into ecosystem-wide effects that impact humans.

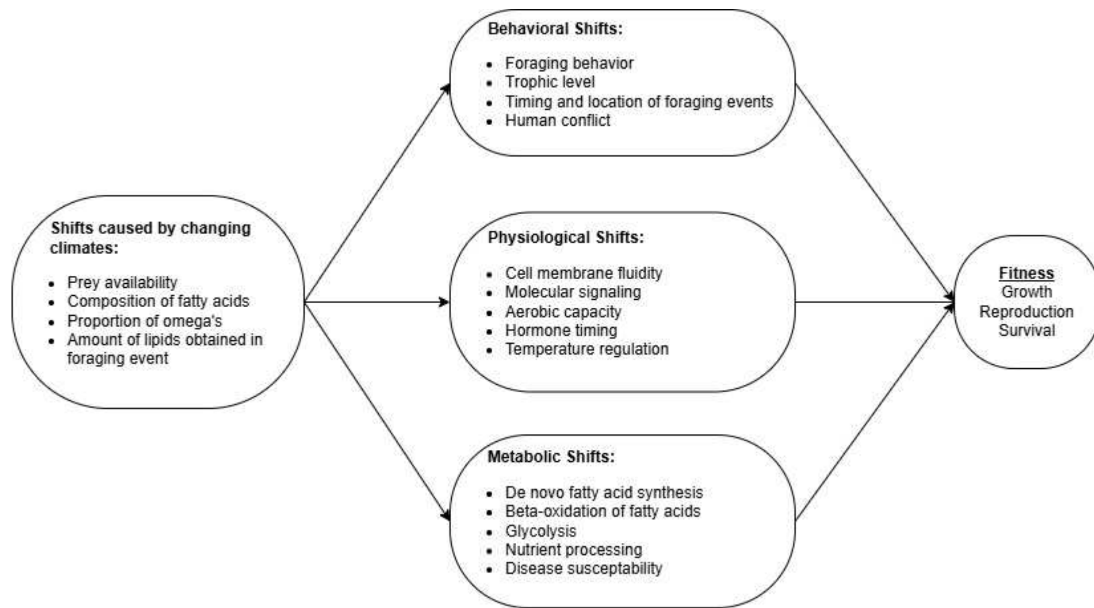

Figure 1-2. Summary of potential shifts in fatty acids on behavior, metabolism, and physiology that ultimately influence fitness traits for organisms. Behavioral shifts such as changes in the location of foraging events, such as Weddell seals shifting to shallower waters due to shifts away from PUFAs, have the potential not only to impact an individual’s fitness but also create ecosystem-wide disturbances.

Project Aims

To understand the effects of these shifts in fats on organismal physiology, a protocol was created to incorporate fats isolated from different trophic levels, specifically fish and krill, into C2C12 skeletal muscle cell culture. The project’s first objective was to integrate lipid (as

commercially available supplements) into C2C12 mouse skeletal muscle cell culture media. The lipids chosen were a generic lipid cocktail, fish oil, and krill oil. Fish and krill as the target sources were chosen as they are prey items commonly consumed by a variety of organisms. Initially, equal volumes of the control, lipid cocktail, fish oil, and krill oil were added to the media, as per previous literature, at a 2.5% volume in the differentiation media. Crude fat concentration analyses revealed that the fat amounts indicated by product manufacturers did not match an actual 2.5% concentration and, additionally, were not significantly different from control fat concentrations. Therefore, lipid treatment values were raised to 5% concentration in 500 mL of differentiation media. The second objective was to see if crude fat concentrations differed between treatments. Once this concentration was established, it was hypothesized that crude fat concentrations of lipid-treated cells would be different compared to the control, but would not differ between lipid, fish, and krill treatments.

Since the overarching aim of this study was to understand if fats isolated from different trophic levels would have physiological differences in mammalian cell culture, after lipid treatment protocols were established, indicators of aerobic capacity were then compared. Lipid uptake in both media and intracellular levels was significant in fish, and a complete uptake of crude fat was observed. This led to the hypothesis that cells supplemented with fats isolated from fish would exhibit increased markers of aerobic capacity, specifically myoglobin concentration and citrate synthase activity. Fish is a significant source of PUFAs, specifically omega-3s, and a primary prey source for many organisms, not just terrestrial mammals. The establishment of this protocol and these findings lay the foundational groundwork for measuring the physiological impact of shifts in fats caused by changing climates on organisms.

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CHAPTER 2: ESTABLISHING EXPERIMENTAL CONDITIONS BETWEEN FATS ISOLATED FROM DIFFERENT TROPHIC LEVELS (FISH AND KRILL) IN C2C12 SKELETAL MUSCLE CELL CULTURE

Introduction

Fats, specifically their derivatives known as fatty acids, are an essential nutrient that endothermic vertebrates use to fuel activities such as locomotion, foraging, and reproduction (Chong et al., 2006; Karasov & del Rio, 2007). Most endothermic vertebrates cannot synthesize many essential fatty acids and therefore must obtain them from their food (Pough et al., 1999). This process of obtaining fatty acids through diet can be affected by climate change, as it is reshaping ecosystems globally, triggering complex biological impacts from cellular to community levels (Blois et al., 2013; Grimm et al., 2013). A less visible but critical consequence is the disruption of nutrient quality across food webs, particularly affecting fatty acids essential to endothermic vertebrates (Cross et al., 2015; Rosenblatt, 2018; Soares et al., 2019). Climate-driven shifts in fatty acid availability due to changes in primary producers, like phytoplankton, can directly impair health, reproduction, and aerobic capacity across trophic levels (Hixson & Arts, 2016; Trumble & Kanatous, 2012; McWilliams et al., 2004).

These shifts can have consequences for organisms relying on fats via diet, not just to obtain their essential nutrients, but also to support aerobic capacity and activities. For example. studies have suggested that many migratory bird species are reliant on fat for up to 90% of the energy to fuel migratory flight, and that it is the preferred nutrient to stock up on in preparation for migration (Bairlein & Hüppop, 2004; Guglielmo, 2010; Pond, 1978). These studies in Aves highlight the need for the availability of prey and the nutrient quality of the prey for the birds to

be supported during migration. Fats have also been noted to play a key role in energy conservation during torpor and hibernation in small mammal species (Geiser & Kenagy, 1987; Ruf & Arnold, 2008). In terrestrial mammals, fats are utilized as fuel to support endurance activities such as running (Valencak et al., 2003; Ruf et al., 2006). Marine mammals, such as pinnipeds, also rely on fats like their terrestrial counterparts, but additionally utilize fats for support in diving, thermoregulation, and buoyancy (Kanatous et al., 1999; De Miranda et al., 2012). These examples highlight the importance of dietary fats in supporting aerobic activities across different endothermic vertebrates; therefore, disruptions in dietary fat quality and availability have the potential to impact performance, such as diving duration in the face of limited oxygen. While dietary fats are noted to be important as fuel, they also serve roles beyond energy provision, which can be impacted by shifts in quality and availability.

Beyond energy, fats serve as structural and signaling molecules that are essential for cellular communication, temperature regulation, and hormonal function (Chong et al., 2006; Hulbert et al., 2005; Simopoulos, 2007). The physiological impact of fats depends on their molecular structure. Saturated fatty acids (SFAs) lack double bonds and are less metabolically efficient than monounsaturated (MUFAs) or polyunsaturated fats (PUFAs), which require less oxygen for breakdown and are more suited for high-performance aerobic activity (De Carvalho & Caramujo, 2018; Trumble & Kanatous, 2012). Fatty acid composition has also been noted to influence health and performance in humans as well, with omega-3s, a type of PUFA, shown to improve aging biomarkers and cardiovascular health (Bischoff-Ferrari et al., 2025; Mohebi-Nejad & Bikdeli, 2014). The importance of fatty acids in sustaining locomotion, supporting thermoregulation, and influencing reproductive success emphasize the importance of furthering our understanding of how climate change is altering fatty acid composition and the impacts the

changes will have on both physiological and ecological levels for endothermic vertebrates, including humans. (Karasov & del Rio, 2007; Covington, 2004; Dubois et al., 2007).

Building on the broader ecological significance of fats, further experimental studies are needed to continue exploring how the availability of specific lipidscan influence physiological traits to understand how shifts may affect organisms. Previous work has demonstrated a positive correlation between myoglobin expression and lipid supplementation in mammalian skeletal muscle cell culture studies.(De Miranda et al., 2012; Schlater et al., 2014). Myoglobin is a physiologically important protein to aerobic metabolism, as it is a monomeric heme protein that has the ability to reversibly bind oxygen and, therefore, act as storage for muscular oxygen (Rayner et al., 2009; Wittenberg & Wittenberg, 1989). Myoglobin consists of a single polypeptide chain and contains one heme group that includes an iron ion (Fe^{2+}) at its center, which is the site where oxygen binds (Kendrew et al., 1958). Myoglobin's structure allows it to reversibly bind oxygen, which enables myoglobin to act as a localized reservoir of oxygen within muscle cells that rely heavily on aerobic metabolism by picking up and releasing oxygen molecules when needed, such as during strenuous exercise (Chen et al., 2008, Jürgens et al., 2000; Wittenberg & Wittenberg, 1989). The role of myoglobin becomes especially important under conditions where oxygen delivery from the blood is temporarily insufficient. During sustained exercise, when muscle cells consume oxygen faster than it can be replenished by circulation, myoglobin releases its stored oxygen to help maintain cellular respiration and energy production (Chung et al., 2005; Jürgens et al., 2000). As myoglobin is an essential protein in oxygen storage, understanding how its expression could be influenced by lipid supplementation could provide further insight into muscle metabolic adaptations.

In skeletal muscle cell culture work, it was established that increasing the amount of lipid up to a certain percentage of volume in differentiation media upregulated myoglobin expression in both terrestrial and marine mammal cells, driving its expression (De Miranda et al., 2012; Schlater et al., 2014). Previous work had also established a baseline addition of lipid into differentiation media to be 2.5% by volume before cell death occurred (De Miranda et al., 2012). However, in the previous mammalian culture work, a generic lipid cocktail solution was used (2µg/ml arachadonic acid; 10µg/ml each of linoleic, linolenic, myristic, oleic, palmitic, and stearic fatty acids, Sigma Aldrich) for experimental treatments, which contained common fatty acids but were not entirely reflective of an organism's diet out in the wild. Therefore, building on these previous findings between lipid supplementation in cell culture and on the broader goal of understanding the effects of different fats on organismal physiology, the present project aims to refine the previous approaches by incorporating lipids from different trophic levels to take steps in better reflecting mammalian diets in cell culture work.

The project's first objective was to integrate lipid (as commercially available supplements) into C2C12 mouse skeletal muscle cell culture media. The lipids chosen were a generic lipid cocktail, fish oil, and krill oil. Fish and krill as the target sources were chosen as they are prey items commonly consumed by a variety of organisms, including mammals, and are representative of two different trophic levels (Everson, 2001; Power, 1990; Traugott et al., 2021). Initially, equal volumes of the control, lipid cocktail, fish, and krill oils were integrated media according to previous literature of 2.5% volume in differentiation media. However, crude fat concentration analyses revealed that the fat amounts indicated by product manufacturers did not match a true 2.5% concentration, suggesting inconsistencies between labeling and actual concentrations. Additionally, these values were not significantly different from control fat

concentrations. Therefore, lipid treatment values were raised to 5% concentration of crude fat in 500ml of differentiation media.

Since the selected lipid treatments represented different trophic levels and the generic lipid supplement used in previous studies, the second objective was to investigate whether crude fat concentrations differed between the three lipid treatments. It was hypothesized that, after seven days, the uptake of lipids from the culture media would be equal across all treatments based on the equal crude fat between lipid treatments. We also expected to see equal uptake amongst treatments in the internal cellular lipid concentration. Fatty acid profiles of each treatment was also analyzed using gas chromatography to investigate if variation in fatty acids was associated with increased markers of aerobic capacity as seen with previous studies investigating lipid supplementation in cell culture (which were measured in Chapter 3).

Methods

Crude Fat Extraction

Crude fat analysis was conducted to calculate the crude fat percentages of lipid cocktail (Sigma-Aldrich), fish oil (Grizzly Pollock Oil Supplement; Pollock Oil, Mixed Tocopherols, Rosemary Extract), and krill oil (Grizzly Krill Oil Supplement; Omega-3 Krill Oil, Astaxanthin) supplements to integrate into the media. Crude fat in lipid-treated media was measured at creation to confirm equal values. Crude fat percentages were also calculated once the supplements were integrated into the media before exposure to cell culture (n=3 per experimental round of each media sample). 13 150mm (P150 plates) cell culture plates were harvested after 7 days of growth per experimental round per treatment, with media collected from all 13 plates and combined. Crude fat percentages were calculated for each combined media sample after

exposure to cell culture (n=3 per experimental round of each media sample). Harvested cells from the 13 plates were also combined, with the resulting supernatant additionally measured to observe intracellular crude fat percentages of the cells between treatments.

Crude fat analysis was performed based on a revised protocol from Folch et al., 1957 to calculate the crude fat percentage samples. 15ml of 2:1 Chloroform: Methanol was added to 1ml of each lipid supplement. The resulting mixture was agitated on a vortex for 30 seconds. Samples were gently inverted, then 4ml of 0.9% NaCl was added to each sample tube. Samples were covered and stored in a 4°C refrigerator overnight. After this phase separation period, the separated lipid layer from the tubes was collected after a 15-minute chilling period in a desiccator oven and transferred into pre-weighed glass scintillation vials. Samples were placed in a nitrogen evaporator (NEvap) until dry, dried in a hood for two hours, and then placed into a dry matter oven at 60°C overnight. After a 30-minute cooling period in a desiccator oven, the vials were weighed again the following day. The weight of the dried fat sample was calculated by subtracting the final weight from the initial weight. The percentage of crude fat in the sample was calculated by dividing the final crude fat weight by the original sample weight. These values were used to measure and establish equal crude fat volumes in lipid-treated media. All samples were run in triplicate for crude fat analysis.

After crude fat measurements were conducted, each lipid supplement additionally had fatty acid components outlined through gas chromatography (GC) after alkaline saponification of triglycerides and derivatization of the fatty acids to methyl esters (FAME) (Cruz-Hernandez & and Destaillats, 2009; Pavan & Duckett, 2007). All samples were run in duplicate.

Cell Culture

Cell growth and differentiation were conducted according to Kanatous lab protocols (Davis & Kanatous, 1999; Kanatous et al., 2002). C_2C_{12} immortalized mouse skeletal muscle myoblasts were grown under standard normoxic (21% O2) conditions and incubated at 5% CO_2 and 37 degrees Celsius, as previously described by Schlater et al., 2014. To achieve proliferation, cells were grown with standard growth media containing high glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich), 20% fetal bovine serum (FBS), 1% penicillin/streptomycin as an antibiotic, and 1% sodium pyruvate. Cells were originally plated onto P60 plates, then were passed at ~50% confluence to P100 plates. At ~50% confluence, they were passed onto 0.1% gel-coated P150 plates.

When cells reached 90-95% confluency, the standard growth media was removed via vacuum, and standard differentiation media was applied to elicit differentiation of the myoblasts into myotubes. Control muscle differentiation media (HIT media) consisted of 2% Horse Serum, 10ug/mL of 4mg/mL insulin, 10ug/mL of 20mg/ml Transferrin, and High Glucose DMEM. The differentiation media for the lipid groups additionally contained an added lipid mixture, separated into three different treatments of different volumes outlined in Table 2-1. The lipid cocktail consisting of 2 µg/ml arachidonic and 10 µg/ml each of linoleic, linolenic, myristic, oleic, palmitic and stearic acid, 0.22 mg/ml cholesterol from New Zealand sheep′s wool, 2.2 mg/ml Tween-80, 70 µg/ml tocopherol acetate and 100 mg/ml Pluronic F-68 solubilized in cell culture water (Sigma-Aldrich). Grizzly Omegas Pet Products were used for the fish (Grizzly Pollock Oil Supplement; Pollock Oil, Mixed Tocopherols, Rosemary Extract) and krill (Grizzly Krill Oil Supplement; Omega-3 Krill Oil, Astaxanthin) oil supplements. Differentiation media was filtered to prepare the lipid treatment groups, and then the respective volumes for each lipid

treatment were aliquoted out and replaced with lipid. All media were warmed in a 37 degrees Celsius water bath and shaken to integrate lipids into the solution before media changes.

Harvesting took place after seven days of differentiation with 0.01M phosphate buffered saline (pH 7.4, Sigma). Plates were scraped with a rubber policeman, and cells were harvested and then frozen at -80 degrees Celsius. Samples were thawed after 24 hours and centrifuged for 10 minutes at 10,000g and 4 degrees Celsius. The supernatant was isolated from the pellet and aliquoted for further analyses of myoglobin concentration, enzymatic activity, and crude fat measurements.

Statistical Analysis

Paired t-tests and a one-way analysis of variance (ANOVA) with Tukey's *post hoc* test were utilized for statistical data analysis using RStudio. All data presented as means±standard error, with significance reported for values at $P \leq 0.05$. Figures were also rendered with RStudio.

Results

Establishing Crude Fat Conditions

Initially, equal volumes of the lipid cocktail, fish, and krill oils were integrated into the media according to previous literature of 2.5% volume in differentiation media (Table 2-1), or 12.5 mL of lipid integrated in 500 mL of media. Crude fat measurement analyses revealed that the fat amounts indicated by product manufacturers did not match a true 2.5% concentration and, additionally, were not significantly different from control fat concentrations (Figure 2-1). Therefore, lipid treatment values were raised to 5% calculated concentration of crude fat in 500ml of differentiation media. Control growth media, media without the addition of any

supplemented lipids, was kept the same as the original values from previous experiments (Figure 2-2).

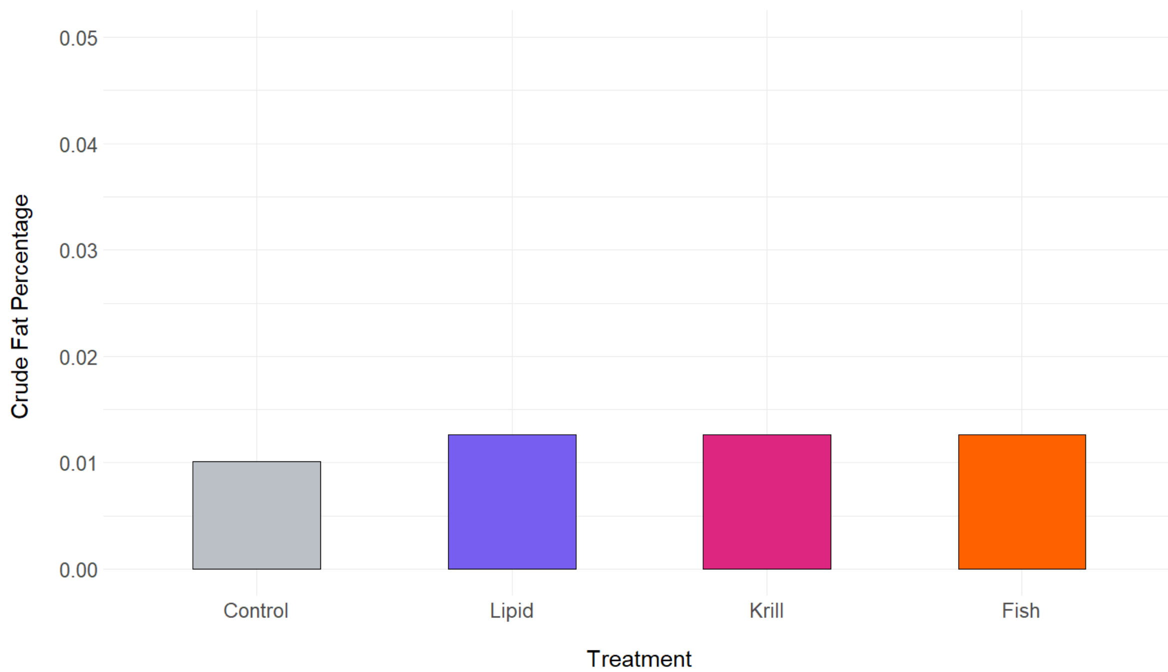

Figure 2-1. Crude fat percentages of lipid supplements did not match the manufacturer's values of 2.5% crude fat. Comparison of crude fat percentages between literature-based 2.5% volume in media based on crude fat analysis revealed less crude fat than stated by the products' information, and no difference between control (media with no lipid supplement) and lipid supplements.

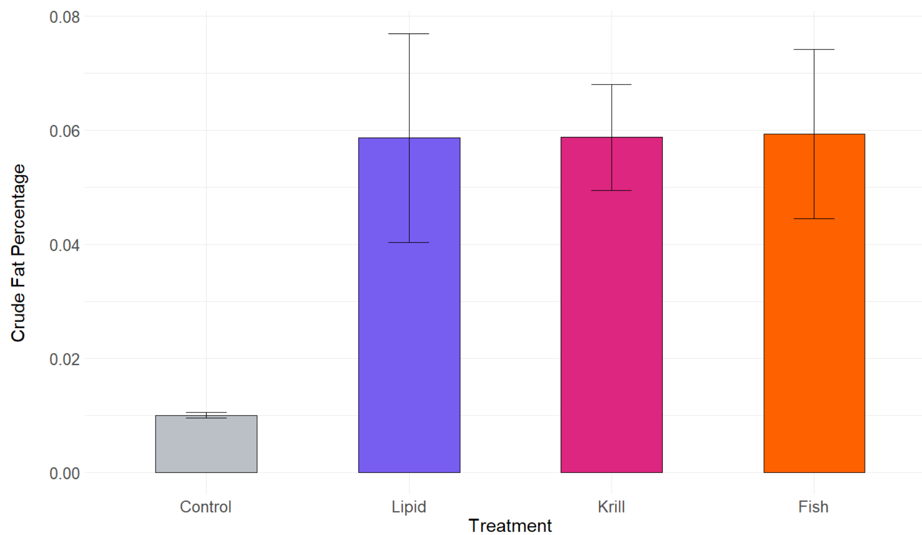

Figure 2-2. Updated calculated concentration of crude fat in culture differentiation media. No significant differences were determined between crude fat percentages of updated volumes of lipid supplements before exposure (PRE) to cell culture treatments, with significance determined at $p \leq 0.05$. n=3 with all samples run in triplicate.

Table 2-1. Established Values Across Lipid Treatments**.** Initial volumes were based on previous literature of a 2.5% treatment (equaling 12.5ml of lipid cocktail in 500ml of cell growth media). Updated values are based on the amount of crude fat determined to be in 1mL of supplement, which was determined through a revised protocol from Folch et al., 1957.

	Lipid Cocktail (Sigma Aldrich)	**Fish Oil** (Grizzly Products)	**Krill Oil** (Grizzly Products)
Previous 2.5% Volume (ml in 500ml of media)	12.5	12.5	12.5
Crude fat in 1ml (mg crude fat)	102	883	895
2.5% Volume by Crude Fat Calculation (ml in 500ml of media)	12.5	1.44	1.42
Final Updated 5% Volume (ml in 500ml of media)	25	2.88	2.84

Crude Fat Uptake in Media

Fat uptake by media analysis showed a trend of decreasing lipid in media across lipid-treated samples compared to the control values after seven days (Figure 2-3). However, paired t-test analysis did not demonstrate a significant decrease in lipid percentage within the media for control values after seven days (PRE: 0.0101, POST: 0.0088; t = -0.92, p = 0.389)

While cells treated with lipid cocktail had a decrease in lipid in the growth media, it was determined not to be statistically significant (PRE: 0.0587, POST: 0.034; t = -1.32, p = 0.239). In comparison, there was a significant decrease in lipid in the media after seven days for cells treated with krill oil (PRE: 0.0588, POST: 0.0050; t = -5.70, p = 0.000992) and fish oil (PRE: 0.0593, POST: 0; t = -4.01, p = 0.0102). Additionally, the media supplemented with fish oil demonstrated a complete depletion of detectable lipid in the media compared to the other treatments and the control (Table 2-2). For these samples, follow-up crude fat analyses on scintillation vials used for measurements supported no crude fat present after analyses.

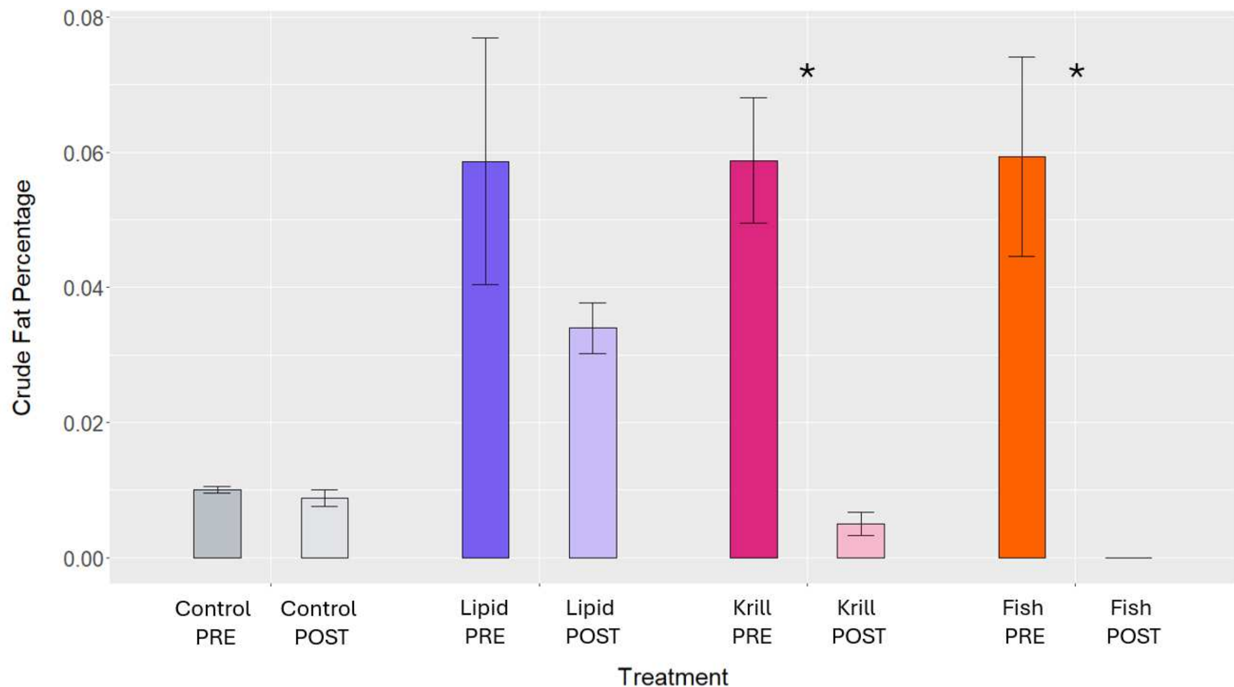

Figure 2-3. Media uptake comparison of fats across treatments after seven days, showing values of media before treatment and after. n=3 with all samples run in triplicate. (* denotes significant difference, significance determined at $p \leq 0.05$).

Table 2-2. Treatment means and paired t-test comparison between PRE values and POST values of the samples (* denotes significant difference, significance determined at $p \leq 0.05$). n=3 with all samples run in triplicate.

Treatment	**PRE** (Mean±SE)	**POST** (Mean±SE)	**p-value** (PRE vs POST)
Control	0.0101±0.0004	0.00883±0.003	0.389
Lipid	0.0587±0.017	0.0340±0.0084	0.239
Krill	0.0588±0.014	0.00501±0.005	0.000992*
Fish	0.0593±0.009	0±0	0.0102*

Cell Culture

Lipid concentrations in C2C12 skeletal muscle cell culture samples were examined after seven days of growth (POST). Crude fat was significantly higher in all lipid-supplemented cell culture samples when compared to the control, with lipid cocktail ($t(4.25) = -3.19$, $p = 0.031$), krill oil ($t(2.71) = -4.33$, $p = 0.028$), and fish oil ($t(6.93) = -4.15$, $p = 0.0044$) demonstrating statistically significant increases in lipid content through paired t-test analysis (Table 2-3).

Table 2-3. Means and paired t-test comparison of lipid treatments (lipid cocktail, krill, and fish) to control values, showing significant differences in intracellular lipid concentration (* denotes significance $P \leq 0.05$). n=3 with all samples run in triplicate.

Treatment	**Crude Fat** (Mean±SE)	**p-value** (vs. Control)
Control	0.00075±0.00022	
Lipid	0.0024±0.0031	0.031*
Krill	0.0028±0.00018	0.028*
Fish	0.0044±0.00076	0.0044*

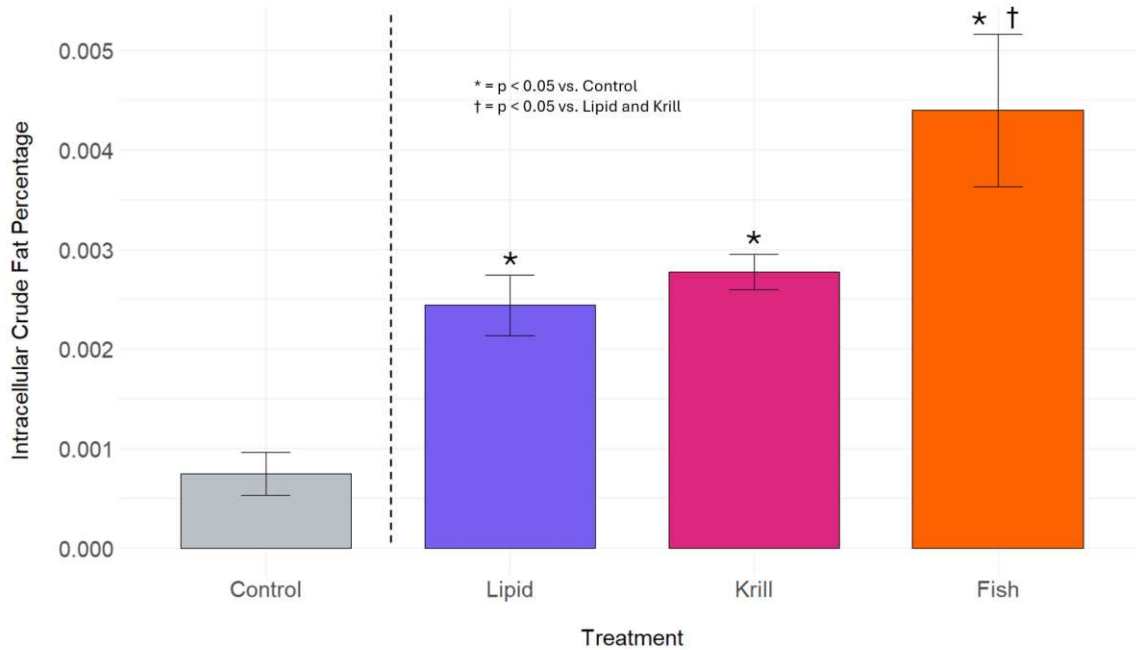

Figure 2-4. Intercellular crude fat percentages across treatments. * denotes significance from the control, with significance determined at p≤ 0.05. † denotes significance of lipid and krill intracellular crude fat from fish intracellular crude fat. n=3 with all samples run in triplicate.

A one-way ANOVA revealed that there was a significant effect on treatment on crude fat percentage ($F(2, 21) = 5.90$, $p = 0.009$). Tukey's post-hoc test demonstrated that the cells

supplemented with fish oil had significantly higher lipid than both cells treated with lipid cocktail (p = 0.0091) and krill oil (p = 0.032). However, there was no significant difference between lipid and krill treatments (p = 0.81). While fat concentrations within the cell increased, the crude fat concentration was observably less than the actual concentration measured in media. Overall, there was a significant trend of increased fat concentration within the cells with the addition of lipid into the differentiation media with fish demonstrating a significant crude fat percentage in comparison to the other treatments and the controls (Figure 2-4).

Table 2-4. One-way ANOVA with Tukey's post-hoc test between treatments demonstrated that cells supplemented with fish oil had significantly higher crude fat than lipid and krill-treated cells, while lipid and krill did not significantly differ. (* denotes significance $P \leq 0.05$). n=3 with all samples run in triplicate.

Comparison	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**	**Mean Diff**	**95% CI Lower**	**95% CI Upper**	**Tukey adj. *p***
ANOVA									
Treatment	2	1.492e-05	7.459e-06	5.904	0.00923				
Residuals	21	2.653e-05	1.263e-06						
TukeyHSD									
Krill – Fish						-0.0016	-0.0031	-0.00013	0.0315*
Lipid – Fish						-0.00196	-0.0035	-0.00046	0.0091*
Lipid – Krill						-0.00033	-0.0017	0.00100	0.8062

Fatty Acid Profiles

The fatty acid composition showed variation in both presence and concentration among the different fatty acids in the added lipid supplements (Figure 2-5). Fish oil as a supplement contained the highest amount of fatty acids, as indicated by gas chromatography, followed by

krill oil and differentiation media without any additional lipid supplement, with the lipid cocktail having the lowest amount of recognized fatty acids (Table 2-5).

When looking at three specific omega-3 fatty acids, α-Linolenic acid (C18:3 N3, ALA), Eicosapentaenoic acid (C20:5 N3, EPA), and Docosahexaenoic acid (C22:6 N3, DHA), one-way ANOVA analysis revealed differences between the concentrations of fatty acids ALA (p = 0.0186) and EPA ($p = 1.63 \times 10^{-5}$) among the three supplements, while DHA showed no variation across the supplements (Figure 2-6). A post-hoc Tukey test demonstrated that krill oil had significantly higher levels of ALA when compared to the concentration in lipid cocktail (p = 0.0243) and fish oil (p = 0.0247). Krill also had a significantly higher concentration of EPA when compared to the lipid cocktail ($p < 0.001$) and fish oil ($p < 0.001$), but fish oil and lipid oil had no significant difference in concentration when compared to each other. No DHA was detected across all three supplements (Figure 2-6, Table 2-5).

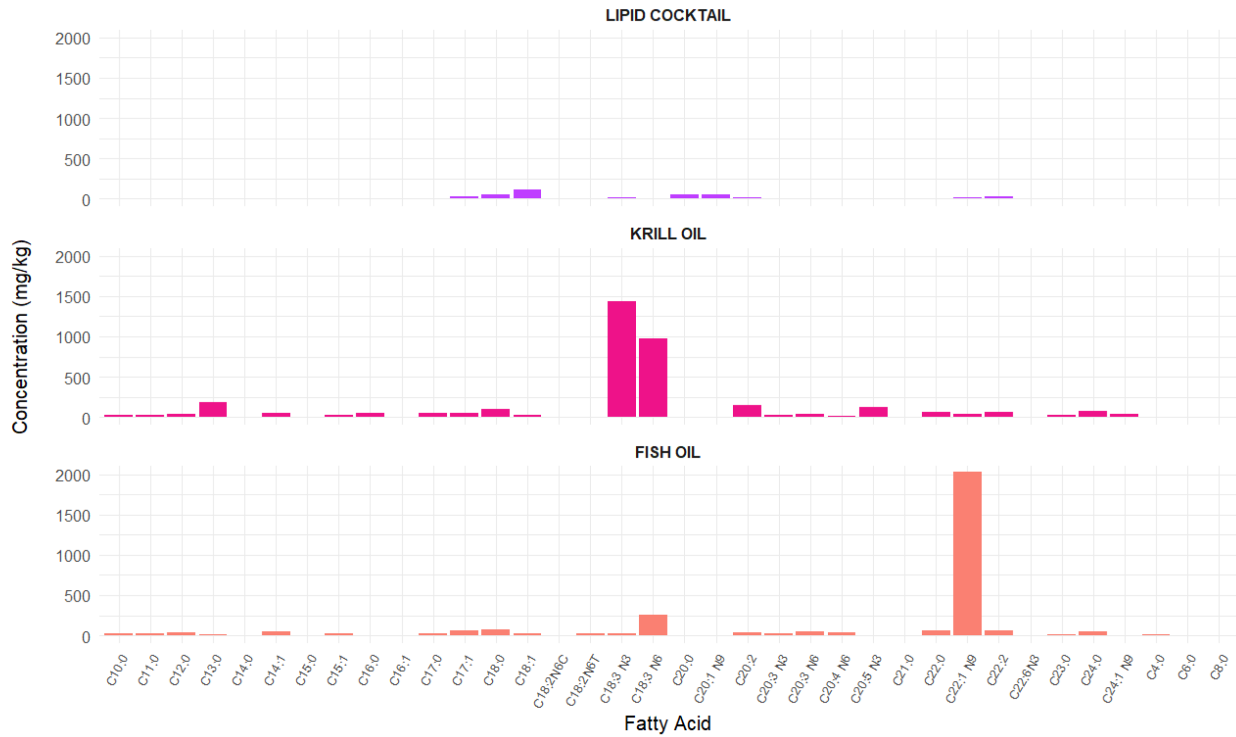

Figure 2-5. Concentrations of measured fatty acids through gas chromatography in the three lipid supplements added to the media. All samples run in duplicate.

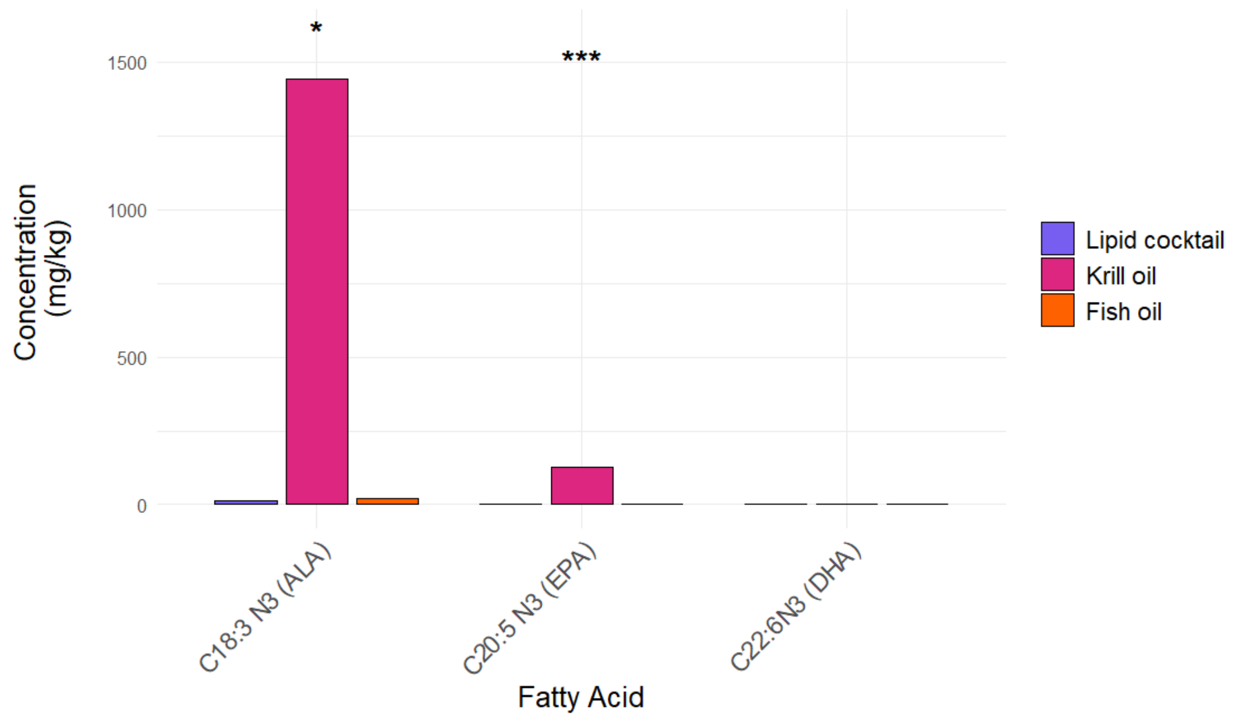

Figure 2-6. Concentrations of selected omega-3 fatty acids in the three treatments: α-Linolenic acid (C18:3 N3), Eicosapentaenoic acid (C20:5 N3), and Docosahexaenoic acid (C22:6 N3). Krill oil had significantly higher levels of ALA and EPA (statistically significant at $p < 0.05$, $p < 0.001$, respectively) compared to the lipid cocktail and fish oil. Asterisks indicate statistical significance based on one-way ANOVA followed by a Tukey's post hoc test (*$p < 0.05$, ***$p < 0.001$). All samples were run in duplicate.

Table 2-4. One-way ANOVA with Tukey's post-hoc test between treatments demonstrated differences in fatty acid concentrations between the three supplements, with krill oil having significant differences in ALA and EPA concentrations (* denotes significance $P \leq 0.05$).

Fatty Acid	Comparison	Mean Difference	Adjusted p-value
C18:3 N3 (ALA)	*ANOVA*		p = 0.0186*
	Krill - Lipid	1426.72	0.0243*
	Fish - Lipid	7.48	0.9995
	Fish - Krill	-1419.24	0.0247*
C20:5 N3 (EPA)	*ANOVA*		p = 1.63e-5*
	Krill - Lipid	127.04	<0.001*
	Fish - Lipid	-3.55e-15	1.000
	Fish - Krill	-127.04	<0.001*
C22:6N3 (DHA)	-	-	-

Table 2-5. Comparison of the presence of the different fatty acids supplemented in the differentiation media without the addition of lipid treatments, as well as the composition of lipid treatments.

Fatty acid	**Differentiation Media** (Control PRE)	**Treatment**		
		Lipid Cocktail	**Krill**	**Fish**
C4:0		X		X
C6:0		X		
C8:0				
C10:0	X		X	X
C11:0			X	X
C12:0	X		X	X
C13:0	X		X	X
C14:0	X			
C14:1	X		X	X
C15:0	X			X
C15:1	X		X	X
C16:0	X		X	

C16:1	X			
C17:0	X		X	X
C17:1	X	X	X	X
C18:0	X	X	X	X
C18:1	X	X	X	X
C18:2N6C				
C18:2N6T	X			X
C18:3 N6	X	X	X	X
C18:3 N3 (ALA)	X	X	X	X
C20:0		X		X
C20:1 N9	X	X	X	X
C20:2	X	X	X	X
C20:3 N6	X		X	X
C21:0				X
C20:3 N3	X		X	X
C20:4 N6	X	X	X	X
C20:5 N3 (EPA)	X		X	
C22:0	X		X	X
C22:1 N9	X	X	X	X
C22:2	X	X	X	X
C23:0			X	X
C24:0	X	X	X	X
C22:6N3 (DHA)				
C24:1 N9		X	X	

Methodology Protocols

FOLCH CRUDE FAT ANALYSIS

Modified from CSU Ruminant Nutrition Laboratory - Engle Lab

Before integrating oils into media, crude fat analysis must be conducted to calculate how much crude fat is in each oil. After this value is calculated and oil is added to the media, this analysis must be conducted **again** to check crude fat between the treatment medias. This method will be conducted again to calculate crude fat in the media after a seven-day growth period and to measure intracellular crude fat. Samples must be run in triplicate.

<u>DAY 1</u>

- Label clean tall glass tubes appropriately, you will need 3 per sample

- Place 1ml of samples into labeled tubes, get as much possible out of pipette tip if working with oils (suspend above tube to let drip)
- Use Eppendorf Repeater Pippetor with 50ml combi-tip to add 20ml 2:1 Chloroform: Methanol
- Add 15ml 2:1 Chloroform: Methanol to each sample
- Vortex tubes for 30sec or until the sample is completely mixed
- Let samples sit for at least 15 minutes
    - Before adding NaCl, some separation may occur, so agitate samples to get to mix
- Add 4ml of 0.9% NaCl solution (0.9g NaCl to 100ml DI water) to each tube with repeat pipettor
- Cover sample tubes and place in 4°C refrigerator and let stand overnight
- Prepare materials for second day:
- Weigh and label the appropriate number of GLASS 20ml scintillation vials with caps
- Place vials without caps in dry matter oven at 60°C

Figure 2-7. Picture of samples after Day 1of Folch crude fat analysis demonstrating phase separation.

DAY 2

- Place glass scintillation vials into the desiccator without caps on still, and let chill for at least 15 minutes
- Once chilled, weigh scintillation vials and record weight
- Remove the bottom layer (lipid) without getting the intermediate layer by using a glass pipette
    - Note: The solvent will have separated into two phases - the total lipid extract is in the LOWER phase
    - Use glass pipette to remove the layer
- Transfer lipid into the pre-weighed and labeled scintillation vial associated with that sample
- Place samples in nitrogen evaporator until dry (visual observation)
    - For viscous oil samples, if sample does not reduce after an hour remove from evaporator and continue analyses
- Let air dry for at least 2 hours under lab hood to remove all traces of chloroform which will combust in the drying oven (UNCAPPED)
- Place in Dry Matter Oven at 60°C for at least 16 hours (UNCAPPED)

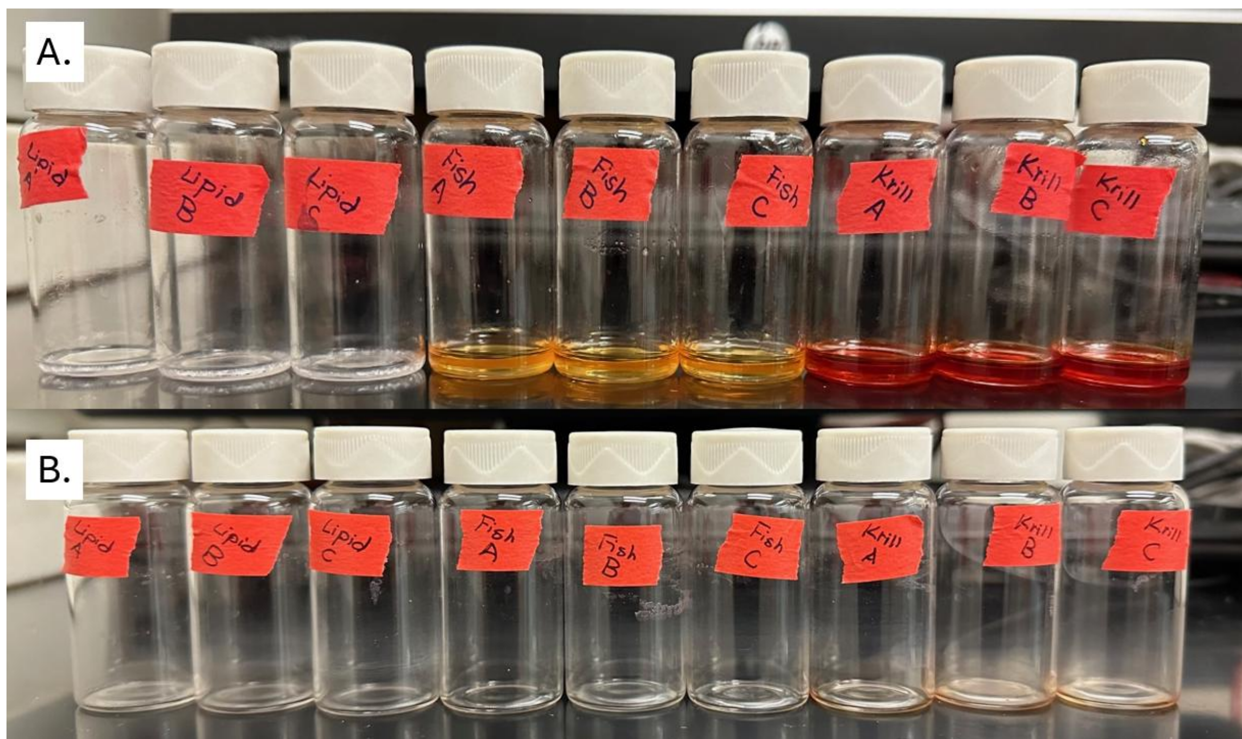

Figure 2-8. Pictures of A) crude fat samples of lipid cocktail, fish oil, and lipid oil supplements and B) POST media samples in scintillation vials after Day 2 of Folch crude fat analysis.

<u>DAY 3</u>

- Remove samples from oven, and place in the desiccator with cap off until completely cool (at least 15 minutes)
- Weigh on the scale, record final weight, and save data
- Store capped if needed for methylation or cholesterol analysis
    - Fill with liquid nitrogen if you will not analyze the samples within a month

BF3 FAME METHYLATION

Modified from CSU Ruminant Nutrition Laboratory – Engle Lab

- Add 1 mL of 0.5 N KOH in MeOH into each 20mL glass scintillation vial containing processed Folch samples
- Tightly cap and put samples in 70°C water bath for 10 min
    - While samples are in the water bath, label new tall glass tubes and scintillation vials
- Add 1 mL of 14% BF_3 in MeOH
- Tightly cap and put samples in a 70°C water bath for 30 min
    - While heating, prepare tubes for Step 10 by appropriately labeling and adding ~800 mg Na_2SO_4 into each tall glass tube
- Take tubes out of water bath and allow to cool to room temperature
- Add 2mL HPLC grade hexane to scintillation vials
- Add 2 mL saturated NaCl to scintillation vials
- Cap and vortex each vial for 30 seconds
- Pipette the upper hexane layer into it's associated glass tube (do not dispose scintillation vial)
- Back extract vials by adding 2mL of hexane, briefly vortex, allow phases to separate, then pipette upper layer into tube
- Add 1mL hexane to tube with Na_2SO_4 in it.
- Vortex briefly
- Transfer hexane to labeled scintillation vials and place samples in nitrogen evaporator for ~20 minutes
- Once done, add 500ul to scintillation vials
- Pipette 400ul of this solution into 2mL autosampler vial containing 1.6mL HPLC grade hexane
- Run gas chromatography according to machine protocols for type of fat analysis

CELL CULTURE

Modified from CSU Kanatous Laboratory Protocols

Unfreezing and Plating Cells

- Defrost frozen C2C12 skeletal muscle cells and warm media in 37℃ water bath
- Once defrosted, pipette cells into sterilized microcentrifuge tubes
- Centrifuge cells at 1500rpm for 5 minutes at 4℃
- Remove supernatant using pipette without disturbing the pellet
- Pipette 1mL of growth media into microcentrifuge tubes and resuspend the pellet by triturating
- Pipette media onto starting size plate
- Add growth media to bring plate to volume (p60 – 2mL, p100 – 6mL, p150 – 16mL)
- Disperse cells by gently moving plate and liquid
- Incubate plates at 37℃ for 24 hours

Splitting cells

- After 24 hours, if confluence is >50% plate then split cells
- Use vacuum pump to remove all of the media from plates
- Wash plates twice with sterile Phosphate Buffered Saline (PBS), adjust volume as needed per plate size
- Pipette Trypsin (-20℃) to middle of plate to until bottom of plate is covered, adjust volume as needed per plate size
- Incubate at 37℃ for 3 minutes
- Once removed from incubator, add growth media to plates (amount varies by number of plates)

- Triturate to expel media at an angle to dislodge and unclump cells
- Divide collected media/cells onto new labeled plates
- Add growth media to bring plate to volume
- Disperse cells by gently moving plate and liquid
- Incubate plates at 37°C for 24 hours

Changing media

- After 24 hours have passed since plating cells, if visible growth is occurring but confluence is <50% plate and there is no visible contamination, change media
- Use vacuum pump to remove all of the media from plates
- Add new growth media to bring plate to volume
- Incubate plates at 37°Cfor 24 hours

HIT Media

- Split cells onto gel plates and incubate for 24 hours to allow >95% confluence
- Prepare differentiation media
    - Aliquot prepared media to serve as control
    - All lipid supplements must be warmed in water bath alongside media before adding – leave in water bath to stay warm until right before adding to cells
    - Pipette 25mL of lipid cocktail in 500ml of differentiation media
    - Pipette 2.88mL of fish oil in 500ml of differentiation media
    - Pipette 2.84mL of krill oil in 500ml of differentiation media
- Use vacuum pump to remove growth media from plates and replace with HIT media
- Harvest cells after 7 days of myotube growth

Harvesting cells

- Using the vacuum, remove all media from plates (do one at a time)
- Wash the plate 3x or until clear with warmed, sterile PBS
- Add 250uL homogenization buffer
- Gently shake the plate on the shaker for 5 minutes
- Use the rubber policeman to scrape the cells to one side of the plate
    - Rinse the policeman in EtOH and then distilled water between each plates
- Pipette the cells & buffer into a sterile microcentrifuge tube
- Freeze in -80°C for at least 24 hours
- Centrifuge thawed cells for 10 minutes at 4°C at maximum velocity for complete protein extraction
- Remove supernatant using a pipette and place into a new sterile microcentrifuge tube
- Store at -80°C after aliquoting and labeling.

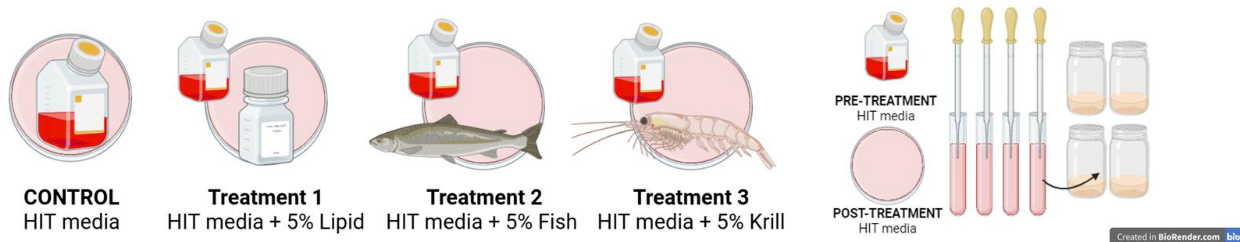

Figure 2-9. A simple schematic of control and lipid treatments, followed by simple visual imagery of crude fat analysis tools. Visual created on BioRender.

Discussion

This work has established the initial protocol for integrating and studying the effects of lipid shifts in C2C12 mouse skeletal muscle cells, demonstrating differences between trophic-

level fats. The main findings of this work were 1) it is important to measure and establish the crude fat levels of supplemented fats in cell culture, 2) there was a significant decrease in lipid levels in the media for the cells treated with fish oil and krill oil compared to control values, 3) intracellular fat concentrations also supported increased trend in fat uptake in lipid-treated cells and specifically with fish, and 4) there was variation in the fatty acid composition of supplements, with krill oil demonstrating significant differences in ALA and EPA concentration, but all lacked DHA. Establishing the same crude fat percentages in incorporated lipid supplements supports observed differences in the experimental outcomes for this work and future work are not due to differences in total fat quantity, therefore adding an additional layer of control to future lipid-based work. Standardization for comparability adds another step in future experiments that supports specific work on the effect of fats incorporated into media, as fatty acids can have dose-dependent biological effects and even result in cell death (De Miranda et al., 2012). Beyond working towards reproducibility and standardization in experiments utilizing lipids, it is essential to try to accurately mimic in vivo lipid environments when modeling physiological conditions.

The differences in fatty acid composition and concentration between the lipid supplements (fish oil, krill oil, and lipid cocktail) and the control group are likely due to factors such as the source of the oils and how they are processed, since these are commercial supplements. The source of the lipids significantly influences the fatty acid profiles of the supplements, which was supported by the fatty acid composition observed for each supplement and control media (Table 2–5). Among the three supplements, fish oil contained the highest total concentration and diversity of fatty acids, which is consistent with previous studies reporting that fish oils are rich in a wide range of PUFAs, including both omega-3 and omega-6 fatty acids

(Parrish, 2009). However, while EPA (C20:5 N3) was detected in the control and krill oil, and was found at significantly higher levels in krill oil compared to the other two supplements ($p < 0.001$), DHA (C22:6N3) was not detected in any of the supplements, including fish oil. Similarly, α-linolenic acid (ALA; C18:3 N3) was present at significantly higher levels in krill oil than in fish oil or the lipid cocktail ($p < 0.05$), further indicating a distinct fatty acid profile for this supplement. These omega-3 findings suggest the possibility of discrepancies between the product label and actual composition. Low PUFA levels have been previously recorded for supplements, with previous work citing high levels of oxidation and poor quality control in manufacturing, in addition to mislabeled content (Albert et al., 2015). The specific fish oil product used claimed a minimum of 540 mg of EPA and 275 mg of DHA per 16 oz bottle, but no DHA was detected, and the manufacturer declined to provide a more detailed compositional breakdown. Notably, the same manufacturer also produced the krill oil supplement.

Krill oil, also a marine-derived product noted to be rich in PUFAs, also yielded the same observation of lacking DHA (Table 2–5). However, it did contain significantly higher concentrations of both ALA and EPA compared to the other treatments (Figure 2-6). This observation may be related to krill oil's unique lipid structure as it predominantly contains phospholipids rather than the triglyceride-based fat content typical of fish oil (Schuchardt et al., 2011). This structural difference may have contributed to a slightly less diverse, but more concentrated, profile of specific omega-3 fatty acids such as EPA and ALA. This suggests that krill oil, despite being marine-derived like fish oil, may have the ability to influence intracellular lipid composition and physiology differently due to both its chemical structure and fatty acid profile. In contrast to fish and krill oil, the lipid cocktail exhibited the lowest levels of omega-3 fatty acids, with very minimal detection of ALA and EPA, and no DHA present, like the other

two supplements (Figure 2-6). It could be considered understandable that the lipid cocktail would have a minimal fatty acid profile, since cell culture media supplements are usually made of ingredients considered basal components in synthetic media (Yao & Asayama, 2017). This lesser amount of fatty acids present in the composition of the cocktail may be due to the design of the product, aiming to deliver only "essential" fatty acids, or due to low concentrations in the product. Overall, the observed variation in fatty acid profiles and omega-3 concentrations in the supplements strongly suggests differences in their sourcing and possible formulation of the supplements. These differences highlight the importance of investigating added supplements in future lipid experiments before actual incorporation into experiments.

After adding the lipid supplements to the cell differentiation media, the residual crude fat content in the media was measured after seven days to assess the decrease of lipid content in the media. Across all lipid treatments, there was an observed trend of decreased crude fat, which indicates that the cells had likely absorbed a portion of the supplemented lipids (Figure 2-3). The control group did not show significant differences in decreased crude fat. This outcome was expected and supports that the cell culture conditions alone did not lead to unusual lipid loss or uptake in the absence of additional lipid supplementation. While the media treated with the generic lipid cocktail demonstrated a small decrease in crude fat content, the decrease was not statistically significant. This suggests minimal lipid absorption by the cells with this supplemented media. As mentioned previously, generic lipid cocktails are often formulated with baseline fatty acids in minimal quantities (Yao & Asayama, 2017). Therefore, they may not have sufficient concentrations or composition of fatty acids and will not necessarily mimic in vivo lipid exposure. These products are often optimized to maintain general cellular function rather

than to mimic the complexity of physiological lipid environments, which may limit their effectiveness in delivering functionally relevant lipids (Albert et al., 2015).

In contrast, media supplemented with krill oil and fish oil showed a statistically significant reduction in crude fat after seven days, indicating a greater degree of lipid uptake by the cells (Figure 2-3). The higher uptake in these conditions could be attributed to the specific fatty acid compositions of krill and fish oils, both of which are usually documented to be rich in long-chain omega-3 fatty acids such as EPA. However, that amount of EPA was only significantly observed in krill oil and not fish oil (Figure 2-6). Krill oil is also noted to contain fatty acids primarily in phospholipid form, while fish oil is noted to have triglyceride-based fatty acids. Both forms have been recorded to be absorbed by mammalian cells, both marine-derived, and have fatty acids that could be considered “bioavailable”, which may facilitate more efficient transport across cell membranes or more rapid incorporation into cellular processes such as membrane synthesis, signaling, or energy metabolism (Schuchardt et al., 2011).

While the reduction in crude fat levels in the media suggests that the cells were potentially absorbing and utilizing the supplemented lipids, intracellular crude fat analysis that measured how much was retained in the cells after seven days did not match the amount that was supposedly absorbed (Figure 2-4). The difference between the depletion in media and intracellular fat concentration could be caused by factors due to cellular metabolism or experimental processes. In terms of cellular metabolism, once lipids are taken up by cells, they may not be stored in their original form. Instead, the supplemented lipids could have been rapidly metabolized for energy or converted into signaling molecules (Schuchardt et al., 2011). These rapid metabolic processes could result in reduced amounts of the measured crude fat in the cells by the time they were harvested, even if there had been a larger uptake. Future experiments

could investigate if hydrolysis is occurring by measuring elevated lipase activity that could be attributed to the breaking down of the lipids. In addition to this, targeted lipidomics, such as liquid chromatography-mass spectrometry, could provide insight into whether the supplemented lipids were stored, built into membranes, converted into signaling molecules, or oxidized.

Differences in fat content could have been influenced by variations in how the cells stored the supplemented lipids. Some fatty acids may be stored in lipid droplets as neutral lipids like triglycerides, while others are rapidly turned over or directed into biosynthetic pathways. Experimental factors could have also contributed to the differences in crude fat amounts, such as the small sample size (n=3 per experimental round of each media sample). It is worthwhile to note that during cell harvesting, there is the potential for some intracellular lipids to be stuck to plasticware or degraded in the process, leading to underestimation by extraction processes (Canez & Li, 2024). Despite this potential source of error, it is important to note that there were no significant differences in intracellular crude fat variability between replicates, which supports the reliability of the overall measurements. However, future work could simultaneously incubate the different lipid-supplemented treatments in non-experimental plates (without cells) and measure lipid disappearance over time to investigate depletion attributable to non-cellular processes.

Ultimately, the difference in crude fat depletion in media versus the intracellular amount highlights the importance of complementing intracellular lipid measurements with additional metabolic or imaging assays to better understand lipid utilization and compartmentalization within cells. Future experiments could investigate tracing lipid fate through the use of stable isotope-labeled fatty acids incorporated into the media, similarly to how they are traced in whole animal studies (Budge et al., 2004; Takahashi et al., 2017). Additionally, carrier proteins and

cofactors such as albumin and acyl-CoA should be investigated in the media to see if uptake or lack of uptake was caused by a lack of necessary carrier proteins (Doege & Stahl, 2006).

Overall, this work emphasizes the need to investigate true fat concentrations within added supplements to be able to interpret future analyses further when applying this precursor methodology to future lipid-based work. Results and observed trends from this work support the idea that not all lipid sources are equally effective in supplementing cell culture systems. These results emphasize the importance of considering lipid composition, structural form, and source when selecting supplements for in vitro studies. Future work could continue exploring how the bioavailability and molecular forms of fatty acids influence cellular uptake dynamics, such as gene expression related to lipid metabolism.

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CHAPTER 3: IS AEROBIC CAPACITY INFLUENCED BY LIPIDS FROM DIFFERENT TROPHIC LEVELS IN C2C12 CELL CULTURE?

Introduction

Lipids, specifically fatty acids, have been demonstrated to influence aerobic ability in mammalian models of cell culture, both terrestrial and marine. In association with this demonstrated relationship, lipids have also been investigated in serving as a driver for myoglobin expression. Myoglobin is a monomeric heme protein that has the ability to reversibly bind oxygen and, therefore, act as storage for muscular oxygen, therefore making it essential in sustaining aerobic metabolism under limited oxygen availability (Rayner et al., 2009; Wittenberg & Wittenberg, 1989). Specifically, the previous work has demonstrated a positive correlation between myoglobin expression in skeletal muscle cells and lipid supplementation. In this skeletal muscle cell culture work, it was established that increasing the amount of lipid up to a certain percentage of volume, 2.5% before cell death, in differentiation media upregulated myoglobin expression in both terrestrial and marine mammal cells, driving its expression under both normoxic and hypoxic cell culture conditions (De Miranda et al., 2012; Schlater et al., 2014).

The expression of myoglobin can and has been measured in various mammalian species, and its contributions to aerobic capacity make it a relevant protein for investigating the primers of oxidative efficiency in mammalian models in relation to the effects of lipid supplementation. An additional relevant indicator of aerobic capacity is the activity of citrate synthase, a key enzyme in the citric acid cycle. Citrate synthase is a key mitochondrial enzyme that utilizes acetyl-CoA and oxaloacetate to form citrate in the mitochondrial membrane, which participates in energy production in the citric acid cycle and is linked to the electron transport chain (Srere, 1975). Broadly speaking, it is an enzyme that catalyzes the first step, also known as the rate-

limiting step, of the citric acid cycle and therefore has been used to reflect metabolic potential under aerobic lipid-based metabolism (Kanatous et al., 2002). These measurements can provide insight into comparing the impact that different isolated fatty acids have on aerobic ability and what shifts in dietary fats may mean on an organismal scale.

The overarching aim of this study was to understand if fats isolated from different trophic levels would differentially affect the muscle physiology of mammalian cell culture experiments, specifically indicators of aerobic capacity. A protocol integrating the fats was established, and initially, equal volumes of the control, lipid cocktail, fish, and krill oils were added to the media according to previous literature, at a 2.5% volume in differentiation media. Crude fat concentration analyses revealed that the fat amounts indicated by product manufacturers did not match a true 2.5% concentration and, additionally, were not significantly different from control fat concentrations. Therefore, lipid treatment values were raised to 5% concentration in 500ml of differentiation media. Since the overarching aim of this study was to understand if fats isolated from different trophic levels would have physiological differences in mammalian cell culture, after lipid treatment protocols were established, the expression of myoglobin and the activity of citrate synthase were measured and compared between treatments. Differences between uptake in cell culture were observed with fish having higher uptake from media and concentration through intracellular crude fat (see chapter 2). Fish contain many fatty acids, specifically omega-3 polyunsaturated fatty acids, and are also a major prey source for many organisms, not just terrestrial mammals (Phleger et al., 1999; Rimm et al., 2018; Shahidi & Ambigaipalan, 2018). Therefore, we hypothesized that cells supplemented with fats isolated from fish would reflect increased markers of aerobic capacity, specifically myoglobin concentration and citrate synthase activity.

Methods

Cell Culture

Cell growth and differentiation were conducted according to Kanatous lab protocols. C_2C_{12} immortalized mouse skeletal muscle myoblasts were grown under standard normoxic (21% O2) conditions and incubated at 5% CO_2 and 37 degrees Celsius, as previously described (Schlater et al., 2014). To achieve proliferation, cells were grown with standard growth media containing high glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich), 20% fetal bovine serum (FBS), 1% penicillin/streptomycin as an antibiotic, and 1% sodium pyruvate. Cells were originally plated onto P60 plates, then were passed at ~50% confluence to P100 plates. At ~50% confluence, they were passed onto 0.1% gel-coated P150 plates.

When cells reached 90-95% confluency, the standard growth media was removed via vacuum, and standard differentiation media was applied to elicit differentiation of the myoblasts into myotubes. Control muscle differentiation media (HIT media) consisted of 2% Horse Serum, 10ug/mL of 4mg/mL insulin, 10ug/mL of 20mg/ml Transferrin, and High Glucose DMEM. The differentiation media for the lipid groups additionally contained an added lipid mixture, separated into three different treatments of different volumes (Chapter 2, Table 2-1). The lipid cocktail consisting of 2 μg/ml arachidonic and 10 μg/ml each of linoleic, linolenic, myristic, oleic, palmitic and stearic acid, 0.22 mg/ml cholesterol from New Zealand sheep′s wool, 2.2 mg/ml Tween-80, 70 μg/ml tocopherol acetate and 100 mg/ml Pluronic F-68 solubilized in cell culture water (Sigma-Aldrich). Grizzly Omegas Pet Products were used for the fish (Grizzly Pollock Oil Supplement; Pollock Oil, Mixed Tocopherols, Rosemary Extract) and krill (Grizzly Krill Oil Supplement; Omega-3 Krill Oil, Astaxanthin) oil supplements. Differentiation media was filtered to prepare the lipid treatment groups, and then the respective volumes for each lipid

treatment were aliquoted out and replaced with lipid. All media were warmed in a 37 degrees Celsius water bath and shaken to integrate lipids into the solution before media changes.

Harvesting took place after seven days of differentiation with 0.01M phosphate buffered saline (pH 7.4, Sigma). Plates were scraped with a rubber policeman, and cells were harvested and then frozen at -80 degrees Celsius. Samples were thawed after 24 hours and centrifuged for 10 minutes at 10,000g and 4 degrees Celsius. The supernatant was isolated from the pellet, and aliquoted for further analyses of myoglobin concentration, myoglobin expression, enzymatic activity, and crude fat measurements.

Myoglobin Concentration

Functional myoglobin is reported as mg myoglobin per mg protein normalized to the original protein concentration. The protein concentration of samples was measured by absorbance with Pierce Coomassie Plus Protein Assay Reagent (Thermo Scientific) in a Beckman DU series 800 spectrophotometer. All samples were run in triplicate.

Myoglobin concentration was measured via an assay adapted from literature (Kanatous et al., 2002; Reynafarje, 1963). Protein homogenates were diluted with phosphate buffer (0.04 $moll^{-1}$, pH 6.6), and centrifuged at 13.5 g for 120 minutes at 4 degrees Celsius. Samples were bubbled with 99.9% carbon monoxide to convert myoglobin into carboxymyoglobin. Absorbance was read at 538 and 568nm. Concentration was calculated as described in Reynafarje 1963 and Kanatous 2002, and expressed in mg mg^{-1} protein. All samples were performed in triplicate and standardized to mouse soleus or gastrocnemius muscle samples.

Enzyme Assay

Citrate synthase was measured using a CS assay kit (Sigma MAK193) to determine the aerobic capacity of cells. A412 readings were measured using a BioTek Synergy HI Multidetection microplate reader. All samples were run in triplicate.

Statistical Analysis

One-way analyses of variance (ANOVA) with Tukey's post hoc test (if significant) were utilized for statistical data analysis using RStudio. A Levene's test and Mann-Whitney U test were used to determine the assumption of homogeneity of variance and for a non-parametric validation of results of myoglobin concentration. All data presented as means ± standard error, with significance reported for values at $P \leq 0.05$. Figures were also rendered with RStudio.

Results

Myoglobin Concentration

Myoglobin concentration values trended higher in lipid-treated cells, with values trending higher specifically in cells treated with fish oil (Figure 3-1). A paired t-test comparing lipid-treated values to control values showed a significant increase in myoglobin concentration in samples treated with the lipid cocktail ($t(10.68) = 3.05$, $p = 0.011$). This increase was also supported by a Mann-Whitney U test ($p = 0.034$). Krill ($t(8.63) = 1.81$, $p = 0.105$) and fish ($t(8.15) = 1.70$, $p = 0.128$) demonstrated elevated myoglobin concentrations but did not have a significant difference compared to control values (Table 3-1).

A Levene's test indicated that there was no significant difference in variance across the lipid treatment groups ($F(3, 32) = 1.73$, $p = 0.181$), which supported the assumption of homogeneity of variance. A one-way ANOVA revealed no significant differences in myoglobin concentration among treatment groups ($F(3, 32) = 1.49$, $p = 0.237$).

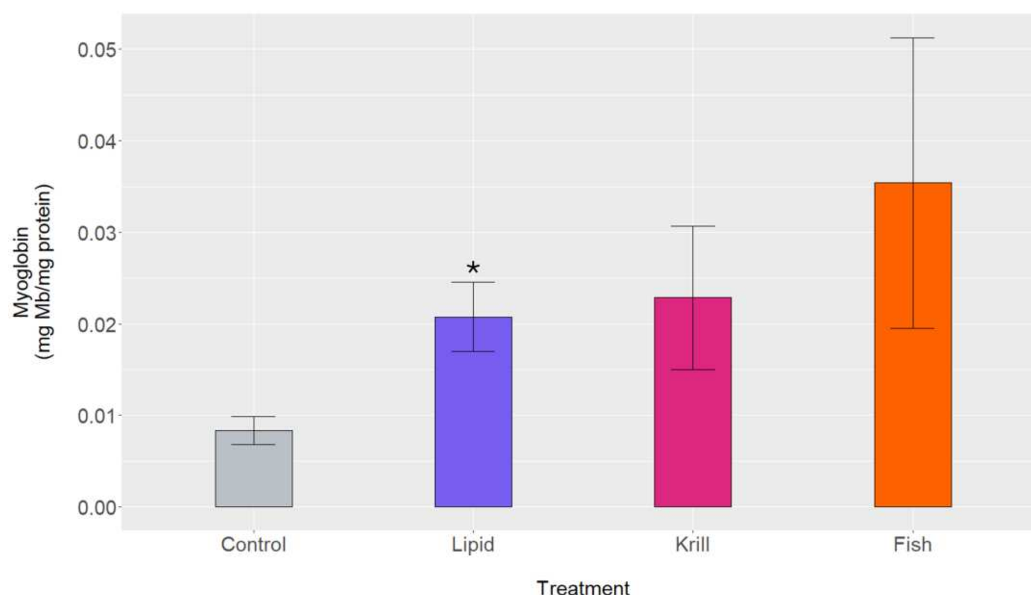

Figure 3-1. Myoglobin concentration comparison between control and treatment samples revealed a trend of increased concentration in lipid-supplemented groups, but a statistically significant difference to the control was only seen in cells supplemented with the generic lipid cocktail. (* denotes significance $p \leq 0.05$). n=3, with all samples run in triplicate.

Table 3-1. Summary of myoglobin concentration of C2C12 skeletal muscle cell control and treatment. Myoglobin concentration reported as (mg mB/mg protein). p-values for pairwise comparison to control values (* denotes significance $P \leq 0.05$), n=3 with all samples run in triplicate.

Treatment	**Myoglobin concentration** (mean±SE)	**p-value** (vs. Control)
Control	0.00835±0.00156	
Lipid	0.02084±0.00276	0.011*
Krill	0.0229±0.00786	0.105
Fish	0.0354±0.0159	0.128

Citrate Synthase

A Levene's test indicated that there was no significant difference in the variance of citrate synthase activity across treatment groups ($F(3, 29) = 0.29$, $p = 0.831$), which supported the assumption of homogeneity of variance. CS activity did not have statistically significant variation between treatments as determined through a one-way ANOVA ($F(3, 29) = 1.95$, $p = 0.144$). A paired t-test demonstrated that lipid ($t(9.57) = -2.00$, $p = 0.075$), krill ($t(15.39) = -1.91$, $p = 0.075$), or fish ($t(15.99) = -1.53$, $p = 0.145$) did not have a significant difference from the control despite trending lower enzymatic activity (Figure 3-2, Table 3-2).

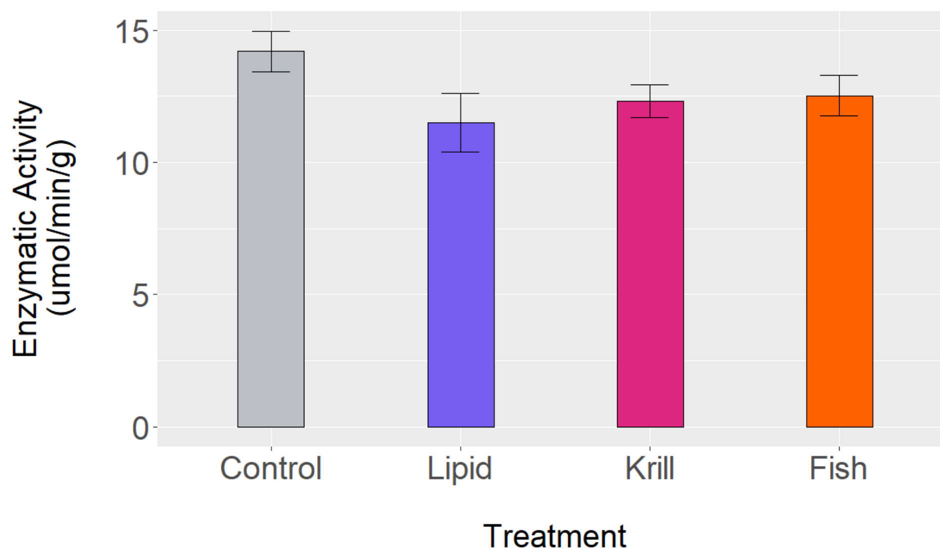

Figure 3-2. Citrate synthase activity for control and treatment samples revealed a trend of decreased activity across lipid supplemented treatments, but no significant difference (determined at *$p \leq 0.05$)* was recorded across treatment groups compared to control values. n=3, with all samples run in triplicate.

Table 3-2. Summary of citrate synthase enzymatic activity of C2C12 skeletal muscle cell control and treatment groups with p-values for pairwise comparison. Enzymatic activity is noted as umol/min/g. n=3, with all samples run in triplicate.

Comparison	**Enzymatic Activity** (mean±SE)	**p-value** (vs. Control)
Control	14.2±0.769	
Lipid	11.5±1.11	0.075
Krill	12.3±0.628	0.075
Fish	12.5±0.780	0.145

Discussion

The results of this study contribute to our growing understanding of how C2C12 skeletal muscle cells' aerobic capacity is influenced by lipid supplementation, specifically by examining the effects of isolated fats from different trophic levels. By supplementing cell culture media with a generic lipid cocktail, fish oil, and krill oil, this work provides detailed insight into how lipid composition can modulate key indicators of aerobic capacity, namely myoglobin and citrate synthase activity. The targeted use of marine-derived oils from fish and krill, which represent two distinct trophic levels, marks a step toward making cell culture studies more ecologically relevant by reflecting the diversity of dietary lipid sources found in natural food webs. This approach advances our ability to assess how fats from different trophic levels can differentially influence muscle physiology in mammalian cell cultures, with a focus on aerobic metabolism. Ultimately, this work not only builds on previous cell culture studies but also lays important groundwork for future research into how diet-driven variation in lipid intake may shape muscle performance, endurance, and physiological adaptation across mammals.

In the previous chapter, the fatty acid profiles of the lipid supplements were analyzed, as well as comparing the concentrations of key omega-3 fatty acids ALA, EPA, and DHA. The fatty acid compositions of the lipid supplements did reveal notable differences, most significantly the absence of detectable DHA across all treatments (see Chapter 2, Table 2-5). This finding is particularly relevant, as DHA has been implicated in enhancing oxidative capacity and mitochondrial biogenesis in skeletal muscle cells (Chow, 2007). Its absence may have limited the metabolic response of the cells, potentially explaining why fish oil supplementation did not lead to a significant increase in markers of aerobic capacity, contrary to our hypothesis. The hypothesis was built on the assumption that fish oil's richness in long-chain PUFAs such as DHA and EPA would enhance cellular oxidative traits such as myoglobin concentration and mitochondrial activity. Our initial hypothesis that fish would have a significant impact on increasing indicators of aerobic capacity was based on the measured intracellular crude fat concentration of those specific cells. Contrary to our hypothesis, fish oil did not have a significant impact on increasing the two measured indicators of aerobic capacity (Table 3-1, 3-2).

Additionally, this study gave mixed results in comparison to other studies using C2C12 skeletal muscle cells with lipid supplementation (De Miranda et al., 2012; Schlater et al., 2014), as there was a significant difference in myoglobin concentration when cells were treated with a generic lipid cocktail but not the other lipid supplements (Figure 3-1). These differences may reflect variations in fatty acid profiles, lipid formulation, or concentration-dependent cellular stress responses. The lipid cocktail used in this study, although generic, may have included specific essential fatty acids in proportions that more effectively supported myoglobin expression or prevented stress responses at the cellular level. The commercial lipid cocktail used may have

included beneficial additives such as vitamins, antioxidants, emulsifiers, or stabilizers which could have reduced oxidative stress or support membrane integrity, indirectly promoting better cell function and differentiation that resulted in supporting myoglobin concentration stability (Albert et al., 2015).

The previous chapter also established that the concentration of crude fat added to the media was higher than the 2.5% volume threshold (see Chapter 2, Figure 2-2) utilized in previous studies (De Miranda et al., 2012; Schlater et al., 2014). Crude fat concentration added to the media was significantly increased beyond this amount, which may in turn have impacted the myoglobin concentration through cell viability and stress. This updated concentration of excessive crude fat very likely introduced confounding variables, such as lipotoxic stress or impaired nutrient diffusion, which can inhibit cellular differentiation and metabolic adaptation (Obaseki et al., 2024). Excessive lipid loading in normoxic conditions has been associated with the induction of cellular stress pathways, reduced mitochondrial efficiency, and even apoptosis in some types of cells (van Herpen & Schrauwen-Hinderling, 2008). This effect could have impacted the cells' ability to respond to supplementation and could partly explain the lack of impact for the measured aerobic markers, despite the observed uptake of certain fatty acids demonstrated in the previous chapter. These results support the need for careful consideration of fatty acid composition, such as the presence of DHA, as well as considering established thresholds to not impact cell viability and influence aerobic capacity markers such as myoglobin concentration.

Citrate synthase activity was also recorded to have lower activity levels across all lipid treatments when compared to the control activity values (Figure 3-2). These activity levels may be lower due to a shift in metabolism from carbohydrate oxidation to fatty acid oxidation due to

the added supplementation of fatty acids. When cells are provided with high levels of fatty acids, they often reduce their reliance on glucose metabolism and the tricarboxylic acid cycle as a primary energy source, which could explain the observed decrease in citrate synthase activity (Kennelly et al., 2022). Cell proliferation and mitochondrial biogenesis could have been negatively affected by lipid supplementation, which was discussed in the previous paragraph to have exceeded the previous 2.5% threshold. Excess lipid exposure has been shown to disrupt mitochondrial function and lower enzymatic activity, potentially through mechanisms such as oxidative stress, altered membrane integrity, or impaired signaling pathways involved in mitochondrial dynamics (Alhindi et al., 2019). It has also been noted that concentrations of certain fatty acids can be lipotoxic, which can lead to a negative impact on mitochondrial function, which would in turn lower citrate synthase activity as a reflection of this impacted function (Li et al., 2008).

Additionally, both myoglobin concentration and citrate synthase activity in these experiments were assessed in differentiated C2C12 skeletal muscle cells, and differentiation was confirmed visually through microscope observation of healthy myotube morphology. This observation ensures that these findings reflect the physiology of mature muscle cells, rather than reduced values caused by undifferentiated myoblasts. Overall, our data suggests that while there were observed trends in the measured markers of aerobic capacity, there was a significant impact from the amount of supplemented lipid to this data.

To better understand the mechanisms that could be driving these results and observed trends in myoglobin and citrate synthase activity levels, future work could incorporate identifying the impact of transcriptional regulators that influence oxidative metabolism, specifically myoglobin expression, in this cell culture work. For example, peroxisome

proliferator-activated receptors are lipid-influenced receptors of interest to investigate, as they impact muscle-specific oxidative protein expression and therefore may influence myoglobin (Neels & Grimaldi, 2014; Sprecher, 2007). Detecting upregulated genes associated with them and measuring the expression of associated proteins through blotting could provide insight into whether these receptors and other pathways were activated in response to the additional lipid supplementation. Myoglobin expression was also not measured in this project, which could provide further insight into whether detecting upregulated genes is the correct direction for future work. Additionally, citrate synthase in previous lipid work has been measured alongside and as a ratio to beta-hydroxyacyl CoA dehydrogenase (De Miranda et al., 2012). This could be a worthwhile next step to expand on this work, as HAD is assayed to determine the capacity of cells to oxidize lipids for energy production. In addition to this, future studies could benefit from increased sample sizes and trials. While the regulation of specifically myoglobin is still not yet understood, these additional investigations could help in increasing our understanding of how lipids influence muscle oxidative capacity and specifically influence indicators of aerobic capacity.

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CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

The overarching aim of this thesis was to investigate whether fats isolated from different trophic levels exhibit physiological effects in mammalian cell culture models. This work was framed around the current global observations of patterns linked to climate change, fundamentally reshaping ecosystems worldwide, causing effects from impacting cellular function to disrupting populations (Blois et al., 2013; Grimm et al., 2013). Shifts in the distribution, behavior, and nutritional profiles of organisms at the base of the food web that serve as important prey to many animals have been described as additional consequences of climate change (Rosenblatt, 2018; Soares et al., 2019). Shifts in dietary quality and composition, such as changes in fatty acids, have often been overlooked despite their important physiological roles, a point underscored by the fact that many essential fatty acids cannot be synthesized by most endothermic vertebrates and therefore must be obtained from an organism's diet (Pough et al., 1999). Therefore, it can be predicted that any climate-driven shift in the availability, type, or balance of these fatty acids in prey species can have direct, measurable impacts on the health and fitness of consumer organisms, from impaired aerobic capacity to reduced reproductive output and weakened resilience to environmental stress (Karasov & del Rio, 2007).

Chapter 2 of this work established the protocol that allows future investigation into the effects of trophic dietary shifts to move forward. The specific lipids chosen for this work were a generic lipid cocktail (Sigma-Aldrich), fish oil (Grizzly Pollock Oil Supplement; Pollock Oil, Mixed Tocopherols, Rosemary Extract), and krill oil (Grizzly Krill Oil Supplement; Omega-3 Krill Oil, Astaxanthin). This particular lipid cocktail was previously utilized in C2C12 skeletal muscle cell culture work involving the effects of lipids, work that was additionally used as the starting reference point of a 2.5% volume integration of lipid into cell culture media (De Miranda

et al., 2012; Schlater et al., 2014). Fish and krill oil were utilized as their sources are prey items commonly consumed by a variety of organisms, including mammals, and are representative of two different trophic levels, reflecting the overarching aim of investigating the effects of shifts in trophic levels (Everson, 2001; Power, 1990; Traugott et al., 2021).

One of the first hurdles of this work was physically integrating the lipid into the skeletal muscle cell culture media. Initial volumes reflecting 2.5% volumes for cell culture media were used across treatments, resulting in cell death in cells treated with fish and krill oil. Utilizing the concentrations provided by the source companies of the lipid supplements, new volumes were drawn up to match the crude fat percentage measured in the 2.5% lipid cocktail cell culture mixture. However, once the lipid supplements were integrated into the media and crude fat of these mixtures was measured, Crude fat measurement analyses revealed that the fat amounts indicated by product manufacturers did not match a true 2.5% concentration and, additionally, were not significantly different from control fat concentrations (Chapter 2, Figure 2-1). Therefore, lipid treatment values were raised to 5% calculated concentration of crude fat in 500ml of differentiation media. Control growth media, media without the addition of any supplemented lipids, was kept the same as the original values from previous experiments (De Miranda et al., 2012; Schlater et al., 2014).

Once the volumes of lipid supplements in the media were determined, C2C12 skeletal muscle cells were grown, differentiated, and exposed to the media for 7 days before being harvested for analysis. Since the concentration of fats was established for each treatment through crude fat analyses of the supplements and media and set to match the crude fat in the lipid cocktail, it was hypothesized that crude fat analyses of the lipid supplements would be higher than the control, but would not differ between lipid, fish, and krill treatments. This hypothesis

was supported, establishing the same crude fat percentages in incorporated lipid supplements, which supports that observed differences in the experimental outcomes for this work and future work are not due to differences in total fat quantity, and adds an additional layer of control to future lipid-based work.

With the established values of crude fat across the lipid treatments, it was hypothesized that there would be equal uptake of fats amongst treatments after seven days, which would be reflected in the internal cellular lipid concentration and crude fat depletion in the media. There was a significant decrease in lipid in the media after seven days for cells treated with krill oil (PRE: 0.0588, POST: 0.0050; $t = -5.70$, $p = 0.000992$) and fish oil (PRE: 0.0593, POST: 0; $t = -4.01$, $p = 0.0102$). Additionally, the media supplemented with fish oil demonstrated a complete depletion of detectable lipid in the media compared to the other treatments and the control (Chapter 2, Table 2-2). While cells treated with lipid cocktail had an observed decrease in lipid in the growth media, it was determined not to be statistically significant (PRE: 0.0587, POST: 0.034; $t = -1.32$, $p = 0.239$).

It was therefore expected that fish would reveal the highest intracellular crude fat, followed by fish, then lipid. Crude fat was significantly higher in all lipid-supplemented cell culture samples when compared to the control, with lipid cocktail ($t(4.25) = –3.19$, $p = 0.031$), krill oil ($t(2.71) = –4.33$, $p = 0.028$), and fish oil ($t(6.93) = –4.15$, $p = 0.004$) demonstrating statistically significant higher intracellular crude fat compared to control values. This enhanced absorption likely reflects differences in fatty acid composition, as commercial lipid sources vary in quality and processing (Jacobsen et al., 2009). While the reduction in crude fat levels in the media suggests that the cells were potentially absorbing and utilizing the supplemented lipids, intracellular crude fat analysis that measured how much was retained in the cells after seven days

did not match the amount that was supposedly absorbed (Figure 2-4). The difference between the depletion in media and intracellular fat concentration could be caused by factors due to cellular metabolism, storage of fat, or experimental processes. In terms of cellular metabolism, once lipids are taken up by cells, they may not be stored in their original form. Instead, the supplemented lipids could have been rapidly metabolized for energy, incorporated into cellular membranes, or converted into signaling molecules (Schuchardt et al., 2011).

Fatty acid composition of each treatment was also analyzed using gas chromatography, and we additionally hypothesized that each treatment would differ in its PUFA composition, with fish oil hypothesized to have the highest concentration of omega-3 PUFAs, specifically docosahexaenoic acid (DHA). Among the three supplements, fish oil contained the highest concentration and diversity of fatty acids, consistent with its reported richness in PUFAs, including omega-3 and omega-6 (Parrish, 2009). However, krill oil had a significantly higher concentration of EPA when compared to the lipid cocktail ($p < 0.001$) and fish oil ($p < 0.001$) and had significantly higher levels of ALA when compared to the concentration in the lipid cocktail ($p = 0.0243$) and fish oil ($p = 0.0247$). Interestingly, there was a lack of detectable DHA in all three supplements, despite it being listed as a guaranteed concentration in the fish and krill oils. The absence of DHA suggests the possibility of discrepancies between the product label and actual composition. Low PUFA levels have been previously recorded for supplements, with previous work citing high levels of oxidation and poor quality control in manufacturing, in addition to mislabeled content (Albert et al., 2015).

The main findings of Chapter 2 were 1) it is important to measure and establish the crude fat levels of supplemented fats in cell culture, 2) there was an increased trend in media uptake in lipid-treated cells compared to the controls, specifically with fish and krill, 3) intracellular fat

concentrations also supported increased trend in fat uptake in lipid-treated cells and specifically with fish, and 4) there was variation in the fatty acid composition of supplements, with krill oil demonstrating significant differences in ALA and EPA concentration, but all lacked DHA (Figure 4-1).

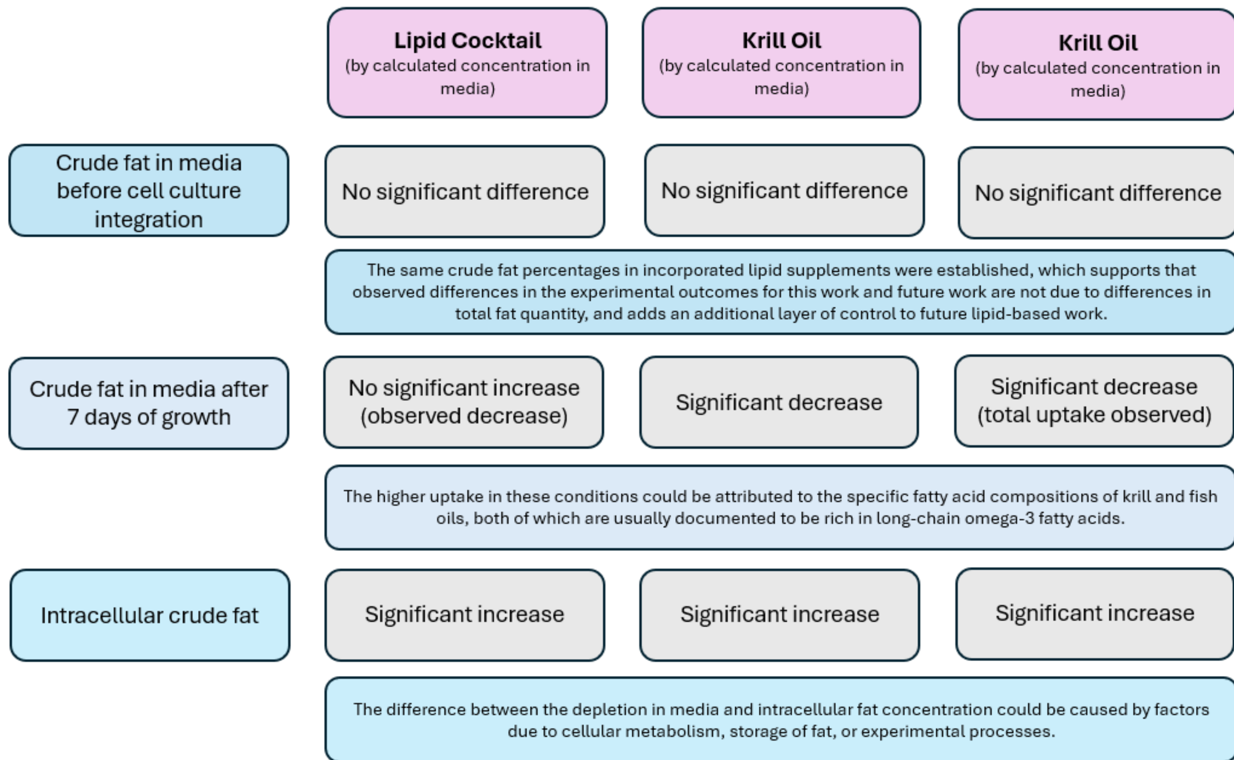

Figure 4-1. Summary of results and proposed explanations for observed trends across the the established crude fat concentrations in media, the crude fat concentrations of media PRE and POST seven days, and intracellular crude fat after seven days.

From these trends and results, Chapter 3 focused on the hypothesis that cells specifically supplemented with fish oil would exhibit significant changes in aerobic capacity, especially with the assumption of fish oil's richness in long-chain PUFAs, which would enhance cellular oxidative traits, including myoglobin concentration and mitochondrial activity. Myoglobin and

citrate synthase were examined to investigate whether the measured lipid uptake trends in Chapter 2, specifically the trends observed in cells treated with fish oil, translated into changes in aerobic capacity indicators. Myoglobin assays showed significant changes in myoglobin for cells treated with the general lipid cocktail compared to controls ($t(10.68) = 3.05$, $p = 0.011$). Krill ($t(8.63) = 1.81$, $p = 0.105$) and fish ($t(8.15) = 1.70$, $p = 0.128$) demonstrated elevated myoglobin concentrations but did not have a significant difference compared to control values (Chapter 3, Table 3-1). Citrate synthase activity in the lipid supplements trended lower than the control, but there was no statistical difference when the control values were compared to lipid ($t(9.57) = -2.00$, $p = 0.075$), krill ($t(15.39) = -1.91$, $p = 0.075$), or fish ($t(15.99) = -1.53$, $p = 0.145$), (Figure 4-2). The hypothesis that there would be significant changes in aerobic capacity indicators in cells supplemented with fish oil was not supported, but cells treated with fish oil did have the highest concentration of myoglobin and mean citrate synthase enzymatic activity, with cells treated with krill oil showing the next highest values.

While there were non-significant trends such as an observed increase in myoglobin and a decrease in citrate synthase activity, these did not align with previous results reported from similar work (De Miranda et al., 2012; Schlater et al., 2014). The observed lack of significant changes in aerobic markers in the additional supplements raises additional questions about the relationship between fatty acid supplementation and proteins like myoglobin. Although a slight increase in myoglobin levels was seen across the fish and krill treated cells compared to controls, this did not reach statistical significance. Similarly, while not significant across all treatments, citrate synthase showed a downward trend.

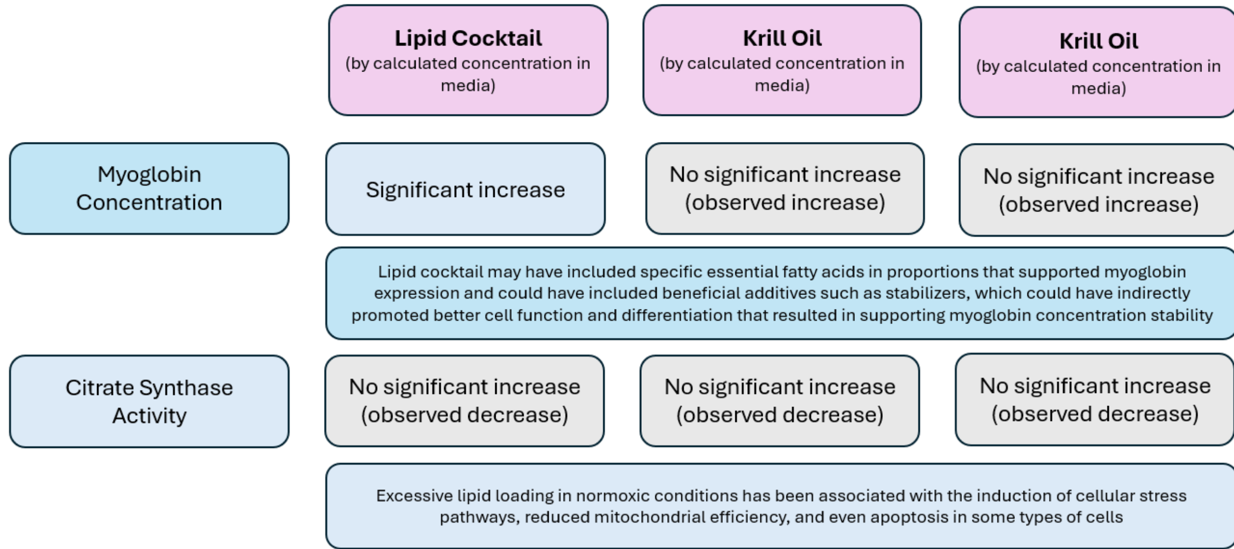

Figure 4-2. Summary of results and proposed explanations for observed trends across the different treatments' myoglobin concentrations and citrate synthase enzymatic activity.

It is worthwhile to restate that Chapter 2 revealed the absence of detectable DHA across all treatments (see Chapter 2, Table 2-5), which was stated on the supplement labels to be present. This finding is particularly relevant, as DHA has been implicated in enhancing oxidative capacity and mitochondrial biogenesis in skeletal muscle cells (Chow, 2007). Its absence may have limited the metabolic response of the cells, potentially explaining why fish oil supplementation did not lead to a significant increase in markers of aerobic capacity, contrary to our hypothesis. While DHA was absent in the lipid cocktail, that was the only lipid supplement to have a statistically higher concentration of myoglobin. The lipid cocktail used in this study was the same used in previous studies investigating lipid supplementation and aligned with previous results of driving increased myoglobin concentration (De Miranda et al., 2012; Schlater et al., 2014). Although generic, when compared to the fish and krill oil, the lipid cocktail may have included specific essential fatty acids in proportions that more effectively supported myoglobin expression and additionally could have included beneficial additives such as

stabilizers, which could have indirectly promoted better cell function and differentiation that resulted in supporting myoglobin concentration stability (Albert et al., 2015). These results suggest that further targeted studies are necessary to clarify how specific fatty acids influence oxidative metabolism in C2C12 skeletal muscle cells.

Chapter 3 revealed observed trends in aerobic capacity may be inhibited by the amount of fat introduced to the media in Chapter 2. The addition of high crude fat levels, exceeding physiological relevance, could have saturated cellular uptake mechanisms or triggered compensatory responses that masked or diminished expected metabolic changes. Excessive lipid loading in normoxic conditions in cell culture has been associated with the induction of cellular stress pathways, reduced mitochondrial efficiency, and even apoptosis in some types of cells (van Herpen & Schrauwen-Hinderling, 2008). Next steps should involve testing supplementation at crude fat concentrations previously established at 2.5%, as used in De Miranda et al., 2012; Schlater et al., 2014, and ensuring that these values correspond to actual crude fat content to reduce variability. Therefore, refining supplementation to mirror physiological or ecologically relevant crude fat concentrations is critical for revealing subtle but meaningful effects on cell metabolism. Future work should aim to refine both the concentration and composition of the lipid supplementation to support alignment with ensuring any effects on oxidative metabolism can be accurately and reliably detected.

Future work discussed in both Chapter 2 and 3 could expand on these findings by examining in greater detail how the bioavailability and molecular forms of fatty acids influence cellular uptake dynamics, such as gene expression related to lipid metabolism. Beyond measuring changes in crude fat levels, future studies can investigate expanding our understanding of the mechanisms driving lipid absorption and processing at the cellular level.

One approach would be to investigate if hydrolysis is occurring by measuring elevated lipase activity that could be attributed to the breaking down of the lipids prior to or during uptake (Thomson et al., 1999). Complementary methods, such as targeted lipidomics like liquid chromatography-mass spectrometry, could provide insight into whether supplemented lipids are stored, incorporated into membranes, converted into signaling molecules, or oxidized (Cajka & Fiehn, 2014). To investigate depletion attributable to non-cellular processes, incubations of lipid-supplemented treatments in non-experimental plates (without cells) could be used to track lipid disappearance over time. These additional investigations could support a more nuanced understanding of how both the chemical form of lipids and the cellular environment shape uptake efficiency and metabolic fate.

Additionally, a critical distinction emerged in Chapter 2 between the fat content remaining in the media and the intracellular lipid concentrations, which could have impacted factors related to myoglobin and citrate synthase in Chapter 3. This distinction raises important questions: Where exactly was the supplemented fat going? Did the cells actively take up all the lipids, or were other mechanisms influencing fat disappearance from the media? If cellular uptake was incomplete or selective, what factors drove the observed changes in crude fat and myoglobin levels? Understanding these dynamics requires more detailed investigations into lipid transport, cellular uptake pathways, and metabolic fate in cultured skeletal muscle cells (Table 4-1).

Building on this foundation, future experiments should also investigate how these uptake patterns translate into functional outcomes, such as changes in oxidative metabolism and gene expression. The observed differences between crude fat depletion in media and intracellular lipid accumulation emphasize the need to pair intracellular measurements with additional metabolic or

imaging assays to better capture lipid utilization and compartmentalization. Future experiments could investigate tracing lipid fate through the use of stable isotope-labeled fatty acids incorporated into the media, similarly to how they are traced in whole animal studies (Budge et al., 2004; Takahashi et al., 2017). Carrier proteins and cofactors such as albumin and acyl-CoA could be investigated in the media to see if uptake or lack of uptake was caused by a lack of necessary carrier proteins (Doege & Stahl, 2006). Detecting upregulated genes associated with lipid-influenced receptors and measuring the expression of associated proteins through blotting could provide insight into whether these receptors and other pathways were activated in response to the additional lipid supplementation (Neels & Grimaldi, 2014; Sprecher, 2007).

Table 4-1. A summary of aims, questions answered, and results. A proposed question to investigate results are expressed in the right-most column.

Overarching aim	Questions	Results	Proposed questions
To investigate whether fats isolated from different trophic levels exhibit physiological effects in mammalian cell culture models	Can fats be integrated into C2C12 cell culture media?	A protocol was successfully established and lays the groundwork for future lipid investigations.	Are modifications for future work investigating the effects of isolated fatty acids needed?
	Do the fats being studied have the same composition at the same concentration?	There were significant variations in EPA and ALA, with krill having significant differences in concentrations. All treatments lacked DHA.	Did the absence of DHA in all lipid treatments limit the metabolic response of the cells?
	Does intracellular crude fat concentration match observations in media uptake?	The concentrations of fat that were reduced in the media were not reflected intracellularly, and were lower, even in the complete uptake of fish oil.	Will elevated lipase activity be measured as a result of hydrolysis, which could be attributed to the breaking down of the lipids? Are the lipids in the media being converted, stored, oxidized?
	Do aerobic markers reflect increased uptake of fats in media?	Trends were observed for both myoglobin and citrate synthase assay, but only lipid cocktail had a significant increase of myoglobin concentration.	Will the results of these aerobic markers be reflected if crude fat amounts are adjusted to previous literature values of 2.5%?

Expanding on these cellular-level findings by discussing them within a broader ecological and physiological context is crucial for gaining a comprehensive understanding of how organisms interact with their environment. This is especially important as these interactions are increasingly influenced by the ongoing effects of climate change (see Chapter 1, Figure 1-2). Climate-driven shifts in global temperatures are causing significant alterations in the distribution, abundance, and nutritional composition of many foundational species within ecosystems (Blois et al., 2013; Grimm et al., 2013; Rosenblatt, 2018; Soares et al., 2019). Such changes have cascading effects throughout food webs, as the availability and quality of primary food sources directly influence the health and survival of higher trophic level consumers (Cross et al., 2015). One of the most critical aspects affected by these environmental changes is the fatty acid composition of prey species, which is closely tied to their habitat conditions and diet (Trumble & Kanatous, 2012). Research has demonstrated that rising temperatures can lead to alterations in the lipid profiles of key organisms, including reductions in essential polyunsaturated fatty acids (PUFAs) such as omega-3 and omega-6 fatty acids (Cross et al., 2015; Rosenblatt & Schmitz, 2016). These shifts in fatty acid profiles can significantly impact the nutritional value of these organisms as prey, thereby influencing the metabolic processes of predators that rely on them (Ericson et al., 2019; Mintenbeck et al., 2012). In this context, fish and krill, which were both primary sources of the oils used in this study, play a pivotal role. These organisms represented two distinct trophic levels and arguably form the dietary backbone for a wide array of aquatic species and have ecological importance that extends to animals as well as humans who depend on seafood for essential nutrients (Everson, 2001; Power, 1990; Traugott et al., 2021).

Beyond ecological and conservation implications, the findings hold significant relevance for human health. Omega-3 and omega-6 fatty acids play essential roles in cardiovascular

function, brain development and cognitive performance, immune regulation, and overall metabolic health. As such, changes in their dietary availability can have wide-ranging effects on disease risk and population health outcomes (Bischoff-Ferrari et al., 2025; Mohebi-Nejad & Bikdeli, 2014). Shifts in the nutritional quality and concern over contaminants of globally traded seafood could complicate efforts to address chronic diseases linked to fatty acid imbalance, such as cardiovascular disease, neurodegenerative disorders, and obesity (Gerber et al., 2012; Marques et al., 2010; Robinson et al., 2022). Understanding how environmental factors influence the composition of these nutrients in food sources is therefore tied to nutritional guidelines and public health policy. Climate change–driven alterations to marine ecosystems may not only affect the abundance of fish species but also their fatty acid profiles, leading to reductions in the quality of seafood as a primary source of long-chain PUFAs such as EPA and DHA (Kang, 2011). These changes could disproportionately affect coastal and subsistence populations, particularly Indigenous communities that rely heavily on marine products as a dietary staple, having their traditional food acquisition disrupted (Andronov et al., 2021). By connecting ecological change to human nutrition, these findings underscore the importance of integrating marine conservation strategies with public health planning to ensure continued access to high-quality dietary lipids in the face of a changing climate.

In addition to these ecological and nutritional implications, the findings also intersect with clinical research, where growing attention is being given to how dietary fats influence aerobic metabolism and their contribution to causing, preventing, or treating metabolic disease. There is growing interest in how dietary fats influence aerobic metabolism in humans, particularly as researchers seek nutritional interventions to enhance fat metabolism and mitigate metabolic diseases such as obesity and type 2 diabetes (Husain et al., 2025; Miller et al., 2005;

Ravindra et al., 2022). Establishing standardized and physiologically relevant protocols for integrating lipid supplements into mammalian cell culture models enables more reliable studies of therapeutic agents targeting fat metabolism before progressing to animal or human trials. This potential for methodological standardization also strengthens conservation-focused research by improving experimental rigor under climate change scenarios and facilitates translational insights into human nutrition and disease. Insights gained from mammalian cell culture models provide a valuable bridge between molecular mechanisms and whole-organism responses. These models enable controlled studies of how specific fatty acids modulate metabolic pathways, which can inform predictions about how wildlife species might respond physiologically to changes in their diets caused by climate-induced shifts in prey composition. Such knowledge is vital for conservation efforts, particularly for species already vulnerable due to habitat loss and environmental stressors.

While this thesis centered on C2C12 skeletal muscle cells, which are a terrestrial mammalian model, our findings establish support for future investigations expanding our understanding of how shifts in dietary fat quality might impact organismal performance across ecosystems by establishing a protocol for lipid work. The methodologies and insights developed here can be adapted for aquatic and marine species, particularly marine mammals that rely heavily on lipid metabolism. Because fish and krill constitute a large part of many marine mammals' diets, comparing the uptake and metabolic effects of specific fatty acid concentrations between terrestrial and marine organisms opens avenues for comparative physiology studies (Everson, 2001; Power, 1990; Traugott et al., 2021). Investigating the lipid uptake mechanisms and transporter expression in marine mammal muscles could reveal how these animals uniquely cope with high-lipid diets in their extreme environments.

In summary, although this work centered on lipid interactions at the cellular level, the development and use of the established standardized protocol in this thesis is impactful beyond the scope of basic laboratory analyses. Reliable and reproducible lipid quantification forms the foundation for linking biochemical processes to organismal performance, enabling more accurate assessments of aerobic metabolism, energy budgets, and physiological limits across species. From an ecological perspective, this approach offers a framework to investigate how climate-driven changes in fat availability and composition influence food web dynamics, predator–prey interactions, and the resilience of ecosystems facing environmental stress. Moreover, by studying lipid metabolism within a standardized and analytical context, this thesis contributes to bridging cellular physiology with applications to ecology, conservation physiology, and even clinical studies of metabolic health. This thesis also contributes to the growing body of knowledge on the role of fats in shaping aerobic capacity and lays a platform for integrative research that spans from cells to the whole organism, to ecosystems.

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