Dietary Manipulation of Fatty Acid Biosynthesis and Metabolism to Improve Fish Performance and Fillet Content With an Emphasis on Omega-3 LC-PUFA and Arachidonic Acid When Feeding High Plant oil Feeds

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Abstract

Fish oils have been used as the primary source of dietary lipid in aquafeeds, especially for salmonids and marine species, which require the supply of long-chain polyunsaturated fatty acids (LC-PUFA) such as eicosapentaenoic acid (20:5n-3, EPA), docosahexaenoic acid (22:6n-3, DHA) and arachidonic acid (20:5n-6, ARA), considered essential fatty acids for many species. However, fish oils have been in increasingly higher demand in the aquaculture industry, while fixed or diminishing supplies have resulted in higher prices, creating the need for alternative oils. In recent decades, extensive research has been conducted to find alternative, sustainable lipid sources for aquafeeds. Plant-derived oils such as soybean, canola, and linseed oil have been widely used and studied in the aquaculture field because they are reasonably cost-effective and readily available. However, the increasing use of such oils has resulted in the decline of beneficial omega-3 fatty acids in the fish fillet, thereby reducing the nutritional value to the final consumer. This thesis aims to investigate fatty acid metabolism by supplementing cholesterol, which plays a vital role in stimulating fatty acid β-oxidation and the conversion of α-linolenic acid (ALA) to DHA, to plant oils (soybean and linseed oils) widely used in aquafeed. Furthermore, this thesis includes an investigation of a novel lipid source to replace fish oil in the trout diet and to better understand fatty acid metabolism in an effort to increase beneficial omega-3 fatty acids in the fish fillet through metabolic processes. Following these studies, experiments were conducted to examine the effects of ARA, known as an essential fatty acid in some marine carnivorous species, on fatty acid metabolism, growth performance, health, and inflammatory responses of different size classes of trout. The first study demonstrated that fish fed 100% of linseed oil with cholesterol supplementation had significantly higher weight gain and feed intake compared to other plant-based diets. In addition, cholesterol supplementation numerically increased EPA and DHA levels in fish fillet when linseed oil was provided above 50%. The second study was conducted over a complete production cycle to evaluate a new transgenic canola oil (Latitude™ oil; Cargill) high in EPA, DPA and, DHA compared with conventional canola oil as a substitute for fish oil in rainbow trout feeds. The results of that study demonstrated that Latitude™ oil improves fish growth and yields elevated n-3 long-chain polyunsaturated fatty acid content in the fillet, making it a candidate lipid source

for use in rainbow trout feeds. However, both intestinal inflammation and size and number of absorptive vacuoles showed significant differences among diet with the distal intestine of fish fed 100% Latitude^{TM} oil having a reduction in size and number of absorptive vacuoles and signs of inflammation. The last two studies, evaluating the effect of ARA in two different stages, swim-up and sub-adult, demonstrated that dietary ARA neither negatively nor positively affected growth performance during both stages. However, dietary ARA improved the immune and inflammatory response to hypoxia and high temperature by enhancing the activation of antioxidant pathways and inhibiting pro-inflammatory responses.

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Dedication

I would like to dedicate this dissertation to my excellent wife, Yujin Kim, who has stuck by me throughout the entire doctorate program. To my wonderful son, Gio, you are my inspiration to achieve greatness. I would also like to dedicate to my parents for the never-ending support, encouragement, and love and for making me who I am. I hope that I have made you proud. Without them, I would not be where I am today

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Chapter 1:
Review of Literature

Fish Oils use in Aquafeed

Introduction

Aquaculture is recognized as the fastest and largest growing food sector. Each year total capture fisheries production remains steady or decreases while total aquaculture production increases. The Food and Agriculture Organization of the United Nations (FAO) reported that aquaculture production reached 87.5 million tonnes in 2020, 6% higher than in 2018. At the same time, capture fisheries production declined to 90.3 million tonnes, a 4% decrease compared to the average over the previous three years (Figure 1.1). Aquaculture began to be recognized as an important food production sector three to four decades ago that could bridge the gap between supply and demand for food fish. As the aquaculture industry grows, the importance of replacing fishmeal and fish oil has been highlighted for decades. It was estimated that the aquaculture sector utilized 3,724,000 and 835,000 metric tons of fishmeal and fish oil, approximating 68.2% and 88.5% of global production, respectively, in 2006 (Tacon and Metian, 2008). The overuse of fishmeal and fish oil is often considered a waste of valuable primary resources (Naylor et al., 2000; Aldhous, 2004; Turchini et al., 2010). In the early stages of aquaculture development, only fishmeal was considered a potential limiting factor in the development of the sector, and fish oil became important only in the last decade, with the development of high-lipid extruded salmonid feeds and the growing inclusion of EPA and DHA. (de Decker et al., 1998; Turchini et al., 2010). A meta-analysis reported in 2020 merged over ten years of experiments and data from aquaculture operations and documented that lowering fish oil in aquafeeds would substantially drop pelagic fish demand more than lowering fishmeal levels since it takes more biomass to produce one tonne of fish oil than one tonne of fishmeal (Cottrell et al., 2020). Aquaculture uses around 75% of the global fish oil supply, and the price of fish oil is expected to continue to rise (Bachis, 2017; Shepherd, 2011; Naylor et al., 2021). In addition to the economic issue of the increasing fish oil price, the aquaculture industry has been heavily criticized by scientists and environmental groups for its excessive use of fishmeal and fish oil (Naylor et

al., 2000; Worm et al., 2006; Turchini et al., 2009). Therefore, it is urgent to find sustainable alternatives to fish oil in aquaculture feeds.

Fish oil production

Fish oil is produced through a process called the reduction process, which includes the cycling of cooking, pressing, extraction, and drying to separate the fishmeal and fish oil (IFFO, 2021). In general, via the reduction process, 100 kg of fresh-fish raw material yields an average of 5-10 kg of fish oil (Turchini et al., 2010; Naylor et al., 2021). Over the last few years, the reduction industry has used approximately 25% of the world's capture fisheries. However, this volume of raw material has been causing ongoing controversy, with the advocacy being that this raw material should be utilized for direct human consumption, particularly in the developing world (Tacon and Metian, 2009).

A number of different species are used as whole fish, mainly small pelagic fish, such as Peruvian anchovy (*Engraulis ringens*), menhaden (*Brevoortia* spp.), blue whiting (*Micromesistius poutassou*), capelin (*Mallotus* spp.), sardine (*Sardinops sagax*), mackerel (*Trachurus/Scomber* spp.) and herring (*Clupea harengus*), Peruvian anchovy is the most utilized species for fishmeal and fish oil production among them. All of these species are considered fatty fish, having a fat content of 8% or more (Turchini et al., 2010).

The percentage of world fisheries destined for fish oil production has decreased over the last 20 years. Fish oil production amounts vary between 0.8 and 1.3 million tonnes yearly (IFFO, 2021). The variations from year to year are significantly affected by the supply of forage fish; the Peruvian anchovy fisheries are the world's largest in volume (varying between 3 and 7 million tonnes/year). The fluctuation of fish oil production is due to changes in the catches of those species linked to El Nino climate events, which bring warm water into the upwelling areas affecting stock quantity. The amount used for the reduction process to fishmeal and fish oil production peaked in 1994 at over 30 million tonnes and then decreased to less than 14 million tonnes in 2014. In 2018, it went up to about 18 million tonnes due to increased catches of Peruvian anchovy before dropping in the subsequent two years to reach over 16 million tonnes in 2020 (FAO, 2022). Continued reduction in supply has been associated with an increased demand caused by a fast-growing aquaculture industry, as well as pig and poultry farming and the pet food and nutraceutical industries (FAO, 2022). The

increasing demand for fishmeal and fish oil led to an increase in their price because of the fact that supply is lower than demand.

Uses of fish oil in aquaculture

Fish oil is considered the most nutritious oil source, being a primary source of beneficial omega-3 fatty acids such as EPA and DHA, for farmed fish. Most of these omega-3 fatty acids are not made by the fish but are concentrated in fish through the food chain from the marine phytoplanktons that synthesize them. Fish evolved in environments that have limited availability of simple carbohydrates. In consequence, fish metabolism is adapted to depend on lipids as the main energy source compared to terrestrial animals (Hemre et al., 2002; Sargent et al., 2002; Polakof et al., 2012; Turchini et al., 2022). In the wild, carnivorous fish eat other fish to obtain omega-3 fatty acids in their diet, which the fish do not produce de novo. Fish oil from wild forage fish allows aquaculture feed to mimic the natural diets of carnivorous species and produce healthier and more valuable farmed seafood.

Fish oil is mainly used for aquaculture feeds, especially for carnivorous fish such as salmonids (salmon and trout) and marine species. According to the Marine Ingredients Organization (IFFO) estimates, in 2020, about 86% of fishmeal was used in aquaculture, 9% was destined for pig farming, 4% for other uses (mainly pet food), and 1% for poultry. In the same year, about 73% of fish oil was destined for aquaculture, 16% for human consumption, and 11% for other uses (including pet food and biofuel) (Figure 1.2).

Lipids in Fish Nutrition

Introduction

For fish, lipids are an important source of energy and essential fatty acids for regular growth, health, reproduction and bodily functions. Lipids also serve as structural components of biomembranes, carriers of fat-soluble vitamins, precursors to eicosanoids, hormones and vitamin D, and as enzyme co-factors (Higgs and Dong 2000; Turchini et al., 2009). The importance of lipids to fish production has become more critical recently concerning the production and implementation of high-lipid, energy-dense diets. Improvements in growth, feed utilization efficiency, and nutrient retention in fish fed such energy-dense diets benefit the fish farmer and the environment (Sargent et al., 2002). Insufficient dietary lipid levels in

feeds may increase the use of protein for energy and, as a consequence, increase ammonia excretion and thus water pollution (Kaushik and Cowey, 1991). On the other hand, excessive dietary lipid was reported to reduce feed intake and growth performance of several fish species (Peres and Oliva-Teles, 1999; Regost et al., 2003; Kim et al., 2006).

Lipid classes & Structure

Lipid can be divided into two main classes, nonpolar lipids and polar lipids. Nonpolar lipids include monoacylglycerols, diacylglycerols, triacylglycerols, free fatty acids, steryl esters, and wax esters, while the major polar lipids include phospholipids and sphingolipids (Turchini et al., 2022).

Monoacylglycerols, Diacylglycerols, and Triacylglycerols

Glycerol, also known as glycerides, are a simple polyol compounds containing three carbon atoms with hydroxyl (-OH) groups bound to it. Monoacylglycerols are a type of glycerol that are made up of glycerol and one fatty acid chain. Monoacylglycerols are found naturally in almost all foods in small amounts. They can be either saturated or unsaturated depending on what type of fatty acid is esterified to a molecule of glycerol. Diacylglycerol, or diglyceride, is glycerol consisting of two fatty acid chains bound to a glycerol molecule through ester linkages. Monoacylglycerols and diacylglycerols can be extracted from plant or animal fats and oils as they are intermediates in the biosynthesis of triacylglycerols and other lipids and intermediates in catabolic processes, including the digestion of triacylglycerols (Turchini et al., 2022). Triacylglycerols comprise three fatty acids esterified with a glycerol backbone and are the most abundant lipid compound specifically used for energy storage. Many different fatty acids can be esterified to any site (sn-1, sn-2, and sn-3) of glycerol, enabling a great variety of triacylglycerol combinations. However, saturated fatty acids and monounsaturated fatty acids are commonly located in the sn-1 and sn-2 sites, whereas polyunsaturated and long-chain polyunsaturated are preferentially found in the sn-2 site of glycerol (Bell and Koppe, 2011). Accordingly, most of the EPA (50%) and DHA (80%) in Atlantic salmon (*Salmo salar*) were reported to be located in the sn-2 site of triacylglycerol (Ruiz- Lopez et al., 2015).

Wax esters and Sterols

A wax ester is an ester of fatty acid and fatty alcohol. Wax esters are particularly widespread in marine environments where they are present in animals, plants, and microbial tissues, performing a wide range of functions from energy storage to serving as waterproofing agents (Turchini et al., 2022). Wax esters are significantly abundant in marine zooplankton, such as calanoid copepods. Copepods are able to store substantial amounts of lipid at certain times of the year (Falk-Petersen et al., 1982, 2000; Turchini et al., 2009). Wax esters can contribute significant amounts of energy-rich fatty alcohols, including the long-chain monounsaturated 20:1 and 22:1 alcohols, which are generally oxidized upon absorption in fish intestine to long-chain monounsaturated fatty acid, preferred substrates for energy production (Henderson, 1996).

Sterols are tetracyclic hydrocarbon compounds, and cholesterol is the most abundant sterol in animals. Cholesterol has a unique structure consisting of four linked hydrocarbon rings forming the bulky steroid structure. A hydrocarbon tail is linked to one end of the steroid and a hydroxyl group is linked to the other. Cholesterol balances the fluidity of cell membranes, which is essential for cell membranogenesis, growth and differentiation, is a crucial structural element of muscle, brain, and the nervous system, and is the precursor for many physiologically active compounds, including sex and molting hormones, adrenal corticoids, bile acids and vitamin D (Vlahcevic et al., 1994, Kritchevsky, 2008; Parish et al., 2008; Zhang et al., 2009; Norambuena et al., 2013). Cholesterol is found only in small amounts in plants. On the other hand, phytosterols are present in plants such as campesterol, stigmasterol, sitosterol, and brassicasterol. It is known that phytosterols inhibit intestinal cholesterol absorption (Ostlund, 2004). Like cholesterol, phytosterols can occur as free sterols or as steryl esters, and there are significant variations between different vegetable oils in total sterol content (Verleyen et al., 2002)

Phospholipids and Sphingolipids

Phospholipids are amphiphilic molecules comprising a glycerol backbone with a hydrophilic polar head group and two hydrophobic fatty acyl tails (Sebaaly et al., 2019). The most well-known constituents of phospholipids are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol, which are esterified

to choline, ethanolamine, serine, and inositol, respectively (Turchini et al., 2010). Among them, the most common phospholipid is phosphatidylcholine, a principal constituent of cellular membranes. Phosphatidylcholine, also referred to as lecithin, has numerous roles, including providing a source of catabolic energy during embryogenesis in eggs and larvae (Finn et al., 1996), a source of essential fatty acids, phosphorous, and choline (Fraser et al., 1988) and an essential component in plasma lipoprotein synthesis involved in intestinal lipid absorption (Fontagne et al., 1998).

The major sphingolipid is sphingomyelin, a phospholipid but not based on a phosphatidic acid backbone. Instead, sphingolipids consist of a sphingoid base backbone and an amino alcohol. The phospholipids and sphingolipids function a major structural role in vertebrate cell membranes where they have several functions, including transport of ions, endo- and exocytosis, and providing an optimized environment for membrane-bound receptors and enzymes (Turchini et al., 2010). It was reported that lower sphingolipid content was seen in fish meat compared to red meat and poultry (Hellgren, 2001). The most abundant fatty acids in fish sphingomyelin were 24:1n-9 and 22:1n-9, whereas in red meat and poultry, 16:0 and 18:0 were the primary fatty acids (Hellgren, 2001).

Fatty acids

Fatty acids are comprised of hydrocarbon chains terminating with carboxyl (-COOH) groups. The most commonly occurring fatty acids found in fish possess even numbers of carbon atoms (C12-C22). The fatty acids are named on the basis of the length of the carbon chain, the number of double bonds, and the position of the double bounds.
The simplest fatty acids are the saturated form, referring to the fact that the carbon chains have no double bonds, and are designated 14:0, 16:0, etc., with the first number being the number of carbon atoms and the second being the number of double bonds. Saturated fatty acids are the predominant fatty acids in the highest amount of 92% of total fatty acids in coconut oil, mainly lauric acid (C12:0) and myristic acid (C14:0) (Kostik et al., 2013). Saturated fatty acids levels in rapeseed oil and rice bran oil are reported to be 6.3% and 22.5% of total fatty acids, respectively (Orsavova et al., 2015). Palmitic acid (C16:0) is the predominant saturated fatty acid in rice bran oil (20%) (Orsavova et al., 2015).

Monounsaturated fatty acids are chemically categorized as fatty acids possessing a double bond. These fatty acids are present in fish and have chain lengths from C14 to C24. Monounsaturated fatty acids generally form the main part of the fatty acid composition in vegetable oils used to replace fish oil, such as 72.8% in rapeseed oil and 44% in rice bran oil, oleic acid (C18:1-9) being the most abundant monounsaturated fatty acids in these oils (Orsavova et al., 2015).

Polyunsaturated fatty acids are hydrocarbon chains containing two or more double bonds. The definition of polyunsaturated fatty acids as either n-3 polyunsaturated fatty acid or n-6 polyunsaturated fatty acid refers to the position of the first double bond close to the methyl group of the fatty acid. Polyunsaturated fatty acids, including n-3 and n-6 fatty acids, are plentiful in fish oils and fewer quantities are present in some meat. The most important and abundant polyunsaturated fatty acids in fish are generally the n-3 fatty acids, whereas in freshwater fish, n-6 polyunsaturated fatty acids are often found at similar or higher concentrations (Karapanagiotidis et al., 2007). The primary n-3 fatty acids in marine fish are long-chain polyunsaturated fatty acids such as EPA and DHA. Long-chain polyunsaturated fatty acids refer to polyunsaturated fatty acids with 20 or more carbon atoms and three or more double bonds. The main n-6 polyunsaturated fatty acids in fish are linoleic acid (18:2n-6) and its metabolic product arachidonic acid (20:4n-6).

Essential fatty acids in fish

The term 'essential' refers to those polyunsaturated fatty acids that must be provided in the diet because they cannot be synthesized de novo. According to the precise meaning of this term, only linoleic acid (18:2n-6) and alpha-linolenic acid (18:3n-3) can theoretically be considered essential fatty acids since fish do not possess the enzyme, $\Delta 12$ and $\Delta 15$, that are involved in converting oleic acid to linoleic acid and to alpha-linolenic acid (present only in plants). Almost all vertebrates potentially have the ability to convert linoleic acid and alpha-linolenic acid into arachidonic acid and EPA and DHA in vivo, respectively, by elongation and desaturation (Nakamura and Nara, 2004). However, these abilities seem to be lost in many fish as they adapted to environments where long-chain polyunsaturated fatty acids are abundant. As a result of this, these species exhibit a dietary requirement for LC-PUFA (Sargent et al., 2002).

The essential fatty acid requirements of fish can vary within a species according to their developmental stage and the environments where they evolved. It was documented that cold water freshwater fish such as salmonids have an essential requirement for n-3 fatty acids, mainly alpha-linolenic acid, whereas warm water freshwater fish such as tilapia require linoleic acid, and fish like catfish (*Ictalurus punctatus*) and common carp (*Cyprinus carpio*) require both n-3 and n-6 fatty acids (De Silva and Anderson, 1995; Tocher 2010). The requirements for n-6 fatty acid can be met by supplying linoleic acid, which is adequately present in almost all terrestrial and marine sources.

In contrast to freshwater species, studies on marine fish indicate that the essential fatty acid requirements cannot be met by either linoleic acid or alpha-linolenic acid; instead, EPA and DHA are required. Levels of n-3 LC-PUFA of less than or up to 1% of diet dry weight can meet the requirements for juvenile turbot (*Psetta maxima*), red sea bream (*Pagrus major*), European sea bass (*Dicentrarchus labrax*), red drum (*Sciaenops ocellatus*), and Korean rockfish (*Sebastes schlegeli*). Other species including silver bream (*Thabdosargus sarba*) and yellowtail flounder (*Pleuronectes ferrugineus*) appear to require levels above 1% (Tocher, 2010; NRC, 2011). In recent years, the role of arachidonic acid is becoming increasingly crucial for the aquafeed industry. The importance of ARA was overlooked for years because it was supplied in abundance in feeds containing fishmeal and fish oil, but it has become an important fatty acid since the industry started replacing fishmeal and fish oil in aquafeeds (Bell and Sargent, 2003). Some studies have shown that ARA plays crucial roles in marine fish growth and survival (Xu et al., 2010; Yuan et al., 2015; Rombenso et al., 2016; Torrecillas et al., 2018), reproduction (Bromage et al., 2001; Kowalska and Kowalski, 2014) and stress and disease resistance (Koven et al., 2001, 2003; Martins et al., 2013), hence playing a vital role across the entirety of the fish life cycle (Hong et al., 2022).

Alternative Oils in Aquafeed

Introduction

Aquaculture production has been increasing steadily in the past few decades while during the same period fishery resources have declined due to overfishing. In 2020, global capture fisheries production was 90.3 million tonnes, including 78.8 million tonnes from marine waters and 11.5 million tonnes from inland waters; a decline of 4% compared to the

average of the previous three years. On the other hand, global aquaculture production in the same year reached a record 122.6 million tonnes. Around 54.5 million tonnes were farmed in inland waters and 68.1 million tonnes were obtained from marine and coastal aquaculture (FAO, 2022). With the growth of aquaculture industry, global consumption of aquatic foods increased at an average annual rate of 3% from 1961 to 2019 (FAO, 2022). The continued growth of aquaculture production along with global consumption has supported a high demand for fish oil, leading to an increase in its price. Because of the fluctuations in production and price variations, studies are focusing on finding and evaluating alternative oil sources of polyunsaturated fatty acids. In addition to the price issue, environmental issues have been emerging. High-value species such as salmon and trout require a relatively higher lipid level in their diets, which traditionally depend on fish oil extracted from wild forage fish resources. Most forage fisheries are fully or over exploited, and landings are now strictly managed to support stock recovery and sustainable harvest (Alder et al., 2008, Naylor et al., 2009). Most of the forage fish catch is processed into fishmeal and fish oil instead of directly consumed by humans. In natural systems, forage fish consume microscopic plants and animals called plankton and convert them into food for larger fish such as salmon and cod, seabirds, and marine mammals (Cury et al., 2000). Their critical role in the marine food web makes forage fish important to wildlife. Cury et al. (2011) recommended that at least a third of forage fish biomass needs to be left in the water in order to maintain seabird populations.

Despite many efforts to replace fishmeal and fish oil in aquafeed, many consumers, producers, and buyers are questioning whether these alternatives are suitable substitutes. In order to be a realistic substitute for fish oil, nutritional quality and sustainability of ingredients need to be considered. Furthermore, these ingredients must be competitively priced and be selected considering the impact on fish health and the environment (Naylor et al., 2009).

Vegetable oils

Vegetable oils have been used extensively as energy sources in aquaculture feeds. They include palm oil, coconut oil, soybean oil, linseed oil corn oil, camelina oil, and cotton seed oil. Vegetable oils have different fatty acid profiles than fish oil. They lack n-3 long-chain polyunsaturated fatty acids but contain higher levels of other fatty acids such as n-3

polyunsaturated, n-6 polyunsaturated, monounsaturated, and saturated fatty acids. Olive oil, rapeseed, and canola oils are rich in monounsaturated fatty acids, whereas sunflower oil, corn oil, and soybean oil possess higher levels of n-6 polyunsaturated fatty acids.

Global production of fish oil has remained stagnant over the past several decades. In contrast, production of vegetable oils, such as palm oil, soybean oil, and canola oil, has increased considerably, and these oils have been used progressively in aquafeeds as alternatives to fish oil. Global production of crude palm oil has almost doubled between 2007 and 2017, and global production of soybean oil and canola oil has increased by nearly 50% (USDA, 2018). Other oils produced commercially in lower amounts, such as sunflower, cottonseed, coconut, and linseed oils, have been used in aquafeeds.

Most vegetable oils are relatively poor sources of long-chain polyunsaturated fatty acids in comparison to fish oil. Instead, they are rich sources of monounsaturated fatty acids, mainly oleic acid, with the exception of linseed oil and coconut oil, which are rich in alpha-linolenic acid and lauric acid (C12:0), respectively.

Linseed oil is high in alpha-linolenic acid, a precursor of the n-3 long-chain polyunsaturated fatty acid series. Linseed oil may have potential as an alternative to fish oil, but the production scale is not comparable with other vegetable oils (USDA, 2018).

Echium oil has been investigated as an alternative to dietary fish oil in marine and freshwater fish. Stearidonic acid (18:4n-3), the product of the delta-6 desaturation of alpha-linolenic acid, was shown to be readily elongated to eicosatetraenoic acid (20:4n-3) and eventually converted to EPA in fish (Ghioni et al., 2002), suggesting that stearidonic acid-rich oils could be used in aquafeed formulations. As it is rich in stearidonic acid, echium oil has the potential to increase eicosatetraenoic acid, a precursor of EPA, in fish tissue (Alhazzaa et al., 2019). However, this oil's current low production volume leads to a higher price than other practical alternatives to fish oil in aquaculture (Alhazzaa et al., 2019).

Depending on the fish species, fish have varying abilities to convert 18:3n-3 to longer and more unsaturated fatty acids in vivo (Sargent et al., 2002). However, particularly for marine carnivorous species, the quantity of n-3 long-chain polyunsaturated fatty acids produced in vivo is not enough to support the optimal growth and health of the fish (Mourente et al., 2005). As a result, vegetable oil and fish oil blends are typically used in

aquafeeds, with the blending ratio considering the price and developmental stage of the fish (Naylor et al., 2009)

Terrestrial animal fat

Terrestrial animal fat has been utilized as an alternative lipid source in aquafeeds for decades due to stable production and its significantly lower cost than vegetable oils. Animal fats generally contain high levels of saturated fatty acids and monounsaturated fatty acids, ranging from 31.2% in poultry fat to 52.1% in tallow and 40.5% in tallow to 44.9% in lard, respectively. Animal fats are attractive lipid sources as a fish oil replacer since they are inexpensive and good sources of dietary energy for fish. However, they contain high levels of saturated fatty acids and therefore have low digestibility at cold temperatures suggesting that these fats need to be blended with polyunsaturated fats to improve digestion (Naylor et al., 2009). Increased replacement of fish oil with rendered terrestrial animal fats rich in saturated fatty acids results in increased levels of saturated fatty acid in the edible portion of fish fillet. In conclusion, the use of terrestrial animal fat in aquafeeds can reduce the use of fish oil but cannot be a complete solution to fish or consumer needs.

Nowadays, insects are regarded as the most promising and sustainable ingredient to supply protein and lipid in aquafeeds mainly because of their nutritional value, fast life cycles, and ability to grow on various substrates, yielding well-balanced amino acid profiles and highly unsaturated fatty acids, as well as vitamins and functional compounds (Tacon and Metian, 2008; Gasco et al., 2016; Gasco et al., 2020; Shafique et al., 2021). In Europe, the interest in insects as a feed ingredient primarily emerged in 2017 when the European Union authorized the use of insects in aquafeeds (Daniso et al., 2020). The use of insects as an ingredient for aquafeeds and a potential substitute for fishmeal is currently widely accepted (Huis and Tomberlin, 2016; Tran et al., 2015). In terms of substitutes for fish oil, a low quantity of polyunsaturated fatty acids in insects reduces their suitability as marine fish feed. However, rearing the insects on substrate rich in n-3 polyunsaturated fatty acids can increase the n-3 polyunsaturated fatty acids content in insects (Zarantoniello et al., 2020).

Single-cell oils

Single-cell ingredients are a class of materials encompassing bacterial, fungal (yeast), and microalgal-derived products (Glencross et al., 2020). Much research has been done on single-cell ingredients, and now they are being used as amino acid sources, n-3 long-chain polyunsaturated sources, and sources of bioactive molecules such as astaxanthin and peptidoglycans (Guedes and Malcata, 2012; Cohen and Ratledge, 2005). The lipid content in fungal and bacterial ingredients tend to be relatively lower than in microalgae; therefore, there has been less interest in the fatty acid profile of these resources (Glencross et al., 2020). On the other hand, single-cell oils extracted from microalgae grown under heterotrophic conditions contain significant amounts of n-3 long-chain polyunsaturated fatty acids (van Ginneken et al., 2011; Borowitzka, 2013). The application of thraustochytrid biomass has been studied extensively over the last 20 years, and recent studies showed that thraustochytrid completely replaced fish oil resulting in comparable growth while increasing the levels of EPA and DHA in fish fillet (Carter et al., 2003; Miller et al., 2007; Sprague et al., 2015). However, the current inconsistent supply and overly high production costs of these resources constrain the use of these alternatives in aquafeeds. A potential cost-effective strategy to use single-cell oils in aquafeed is to incorporate these alternatives to finishing diets for the 6-12 weeks prior to harvesting. This approach would enhance the nutritional value of the final product with higher levels of EPA and DHA for human consumption.

Genetically modified oilseed plants

As plant-based oils cannot fully replace fish oil, commercial feeds generally include a minimum level of fish oil required to meet n-3 LC-PUFA requirements for normal fish growth, with the remaining energy demands met by terrestrial ingredients. This meets the fish's health and growth requirements but results in lower levels of n-3 LC-PUFA in the edible portion of fish, which deteriorate nutritional value for human consumption in terms of EPA and DHA contents (Sprague et al., 2016).

There has been considerable interest in developing genetically modified plants that produce n-3 long-chain polyunsaturated fatty acids within their seeds. As terrestrial plants are not capable of producing n-3 long-chain polyunsaturated fatty acids, genetic modification is the only possible option to alter the fatty acid composition of oilseeds to produce novel n-3

long-chain polyunsaturated fatty acids. Transgenic plants are typically created by editing their genome by adding or deleting existing innate genes within the plant's genome or incorporating external nonhost DNA through splicing (Osmon and Colombo, 2019). Until now, two oilseed crops have been engineered to produce EPA and DHA: camelina (*C. sativa*) and canola (*B. napus L.*). Two different industry co-operations (BASF and Cargill; Nuseed, CSIRO and GRDC) have developed genetically modified canola oil, and both plants produce oils high n-3 long-chain polyunsaturated fatty acids content (~12%), suggesting that these genetically modified oils can be effective alternatives to fish oil (Napier et al., 2019).

Although the fatty acid composition of plant ingredients has been enhanced by gene modification, the use of genetically modified plant ingredients in aquafeed is strictly regulated by certain parts of the world, especially in Europe. The major concern about genetically modified ingredients has been the expression of the modified gene in targeted species, but there is no solid evidence of horizontal gene transfer from genetically modified ingredients to the fish or gut-associated microflora (Sissener et al., 2011). Generally, protein and genetic materials are removed by the process of oil extraction from the seeds of genetically modified ingredients, ensuring that inserted genes will not be included in aquafeeds (Alhazzaa et al., 2019).

Fish oil Replacement by Plant-Based Oils

Effects on fish growth

Over the past decades, there has been an increase in the use of terrestrial plant-based oils, such as canola, soy, linseed, and palm oils, to replace fish oil in aquafeeds. The increasing cost of fish oil has mainly driven extensive research to evaluate replacement oil sources. In the 1980s and 1990s, the cost of fish oil was lower than that of vegetable oils, but since 2001, fish oil has been more expensive (Naylor et al., 2009). Since 2001, considerable efforts have been made to evaluate vegetable oils inclusion in aquafeeds for a variety of farmed species.

In general, dietary fish oil can be replaced with vegetable oils without compromising fish growth and feed efficiency in almost all finfish species, if fish essential fatty acid requirements are met (Turchini et al., 2009). In salmonids species such as Atlantic salmon (Salmo salar), rainbow trout (*Oncorhynchus mykiss*), and charrs (*Salvelinus* sp.), essential

fatty acid requirements are met with n-3 polyunsaturated fatty acids, mainly from linseed oil, and therefore vegetable oils are being used as primary lipid sources in salmonids diets without detrimental effects on fish growth (Turchini et al., 2009; Tocher et al., 2010; Shahrooz et al., 2018; Dupont-Cyr et al., 2022). However, most experimental diets for fish oil replacement study contain fish meal as a protein source and fishmeal contains some residual fish oil that supplies a small but significant amount of n-3 long-chain polyunsaturated fatty acids. Regardless of which lipid material is used, if a large amount of fish meal is included in the diet, it may be unnecessary to supply essential fatty acids through fish oil in the feed (Turchini et al., 2009). Fish meal commonly retains 8-10% residual fat containing around 20 to 35% n-3 long-chain polyunsaturated fatty acids, depending on the species (Martin et al., 2000, Turchini et al., 2009). Therefore, if 30% fishmeal is included in the feed, 0.5 to 1% of dry diet n-3 long-chain polyunsaturated fatty acids will be supplied, which may meet or exceed the fatty acid requirement of currently farmed fish regardless of the dietary lipid source in the diet (Turchini et al., 2009).

Interestingly, although salmonids are classified as anadromous fish, they possess a 'freshwater fish pattern' in the metabolism of alpha-linolenic acid to EPA and DHA. In addition, salmonids can store a relatively large amount of lipid in muscle tissue compared to other fish species that is used to supply metabolic energy. These features of salmonids make it possible to effectively replace fish oil in these species. It has been shown that various vegetable sources can replace fish oil by up to 100% without compromising fish growth, but it results in a lowering of the n-3 long-chain polyunsaturated fatty acid content in the fillet. The effects of fish oil replacement in other farmed species differ from those reported for salmonids species.

Marine species generally require n-3 polyunsaturated fatty acids; in some species, n-6 polyunsaturated fatty acid requirements, the only commercially utilized source of these fatty acids is fish oil (Turchini et al., 2009). Recently, algae oil has been used in a finishing diet for salmon, but the amount added to the diet is not significant. Many studies on marine species have investigated the effects of replacing dietary fish oil with different vegetable oils. In gilthead sea bream (*Sparus aurata*), 80% substitution of fish oil with linseed oil or soybean oil significantly reduced fish performance (Monoto et al., 2004). Also, it was reported that in European sea bass (*Dicentrarchus labrax*), one of the most important marine

carnivorous species farmed in Europe, 50-60% of dietary fish oil could be replaced by vegetable oils without any detrimental effect on growth parameters (Izquierdo et al., 2003; Montero et al., 2005). Similar results were observed in a study with juvenile barramundi (*Lates calcarifer*) of 19 g average initial weight. Soybean, canola, or linseed oils were used to replace fish oil, which resulted in a significant reduction in the growth when those vegetable oils substituted fish oil (Raso and Anderson, 2003). Japanese sea bass of 5.8 g average initial weight were fed on diets containing soybean and corn oils at 50% replacements level of fish oil (Xue et al., 2006). After 10 weeks, growth performance parameters and survival rates were similar compared with fish fed exclusively on fish oil as the dietary lipid.

However, several studies have shown that vegetable oils can totally substitute fish oil in some marine fish species without adverse effects on fish growth. Partial or complete fish oil replacement using a vegetable oil blend of palm and linseed oil was conducted for 22 weeks in juvenile (39 g average initial weight) greater amberjack (*Seriola dumerili*) and revealed that replacement of fish oil did not compromise fish growth performance (Monge-Ortiz et al., 2018). A similar result was observed in a yellowtail (*Seriola quinqueradiata*) study of 252 g average initial weight fed for 40 days on diets containing fish oil or olive oil (Seno-O et al., 2008). It is worth mentioning that most studies on fish have been conducted in a relatively short feeding period (6-12 weeks), and it is challenging to detect nutritional and composition effects over such a short period.

Effects on fatty acid metabolism

Fish oil replacement by vegetable oils in aquafeed significantly alters the fatty acid composition of fish since fish fatty acid profiles generally reflect dietary fatty acid profile. The most significant issue with regard to replacing fish oil with vegetable oils is that it results in lowering the n-3 long-chain polyunsaturated fatty acids content in the fish fillet, which is essential for human health. Almost all fish have the ability to bioconvert alpha-linolenic acid into EPA and DHA through desaturase and elongase enzymatic processing (Sargent et al., 2002; Nakamura and Nara, 2004). However, fish cannot convert oleic acid to linoleic acid and alpha-linolenic acid as they do not possess the vital $\Delta 12$ and $\Delta 15$ desaturases (Figure 1.3). Therefore, most fish require linoleic acid or more extended and unsaturated fatty acids, which must be provided in the diet.

Despite the fact that fish are theoretically able to biosynthesize n-3 long-chain polyunsaturated fatty acids through the desaturation and elongation from alpha-linolenic, their lipid metabolism has long been adapted to n-3 long-chain polyunsaturated fatty acid rich environment. Therefore, fish possess a low capacity to utilize this fatty acid biosynthetic pathway. Even freshwater fish, which can efficiently convert alpha-linolenic acid to EPA and DHA, showed higher expression levels of desaturase and elongase when fish oil was substituted by vegetable oil compared to fish fed fish oil-based diet and had significantly lower amounts of n-3 long-chain polyunsaturated fatty acids, suggesting that no matter how high the fatty acid bioconversion rate in the fish is, the fatty acids deposition in the fish body is insufficient compared to the fish fed an n-3 long-chain polyunsaturated fatty acid-rich diet.

Nile tilapia fry (*Onreochromis niloticus*) of the initial weight of 0.5-2 g were fed on diets containing 3% of either linseed oil, a mixture of linseed oil with palm oil, or fish oil as controls. After a 20-week feeding trial, results have demonstrated that replacing fish oil with vegetable oils lowered EPA and DHA tissue concentrations and consequently produced a product of lower nutritional value for consumers (Karapanagiotidis et al., 2007). Similar results were observed in the studies with anadromous and marine fish. Dupont-Cyr et al. (2022) reported that the complete substitution of fish oil with linseed oil in char feed had no impact on growth rate but yielded a decrease in the content of EPA and DHA in fish fillets compared to the fish fed a fish oil diet. Mourente et al. (2005) pointed out that the bioconversion rate of radiolabeled α-linolenic acid (14C-ALA) to eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) was quantitatively very limited in hepatocytes of European sea bass fed a diet containing a partial substitution of dietary fish oil with blends of rapeseed, linseed, and palm oil. Therefore, it is obvious that European sea bass possesses a 'marine fish pattern' in the metabolism of alpha-linolenic acid to EPA, and therefore, they require relatively high dietary levels of n-3 long-chain polyunsaturated fatty acids in the diet to meet their fatty acid requirements (Mourente and Dick, 2002; Mourente et al., 2005). In gilthead sea bream (*Sparus aurata*), the total substitution of fish oil with vegetable oil modified the fatty acid profile of their tissues (Benedito-Palos et al., 2010), given their inefficient n-3 long-chain polyunsaturated fatty acid biosynthesis pathway (Mourente and Tocher, 1993; Izquierdo et al., 2008).

There are several practical and feasible methods to solve the aforementioned issue. One of the methods is the use of new n-3 long-chain polyunsaturated fatty acid-rich alternative lipid sources. For instance, microalgae are regarded as a promising alternative to fish oil as they are rich sources of n-3 long-chain polyunsaturated fatty acids and pigments. Although they are considered a suitable alternative from a nutritional point of view, insufficient supply and the high prices are obstacles to using these new sources. The incorporation of transgenic oilseed crops in aquafeed and the development of transgenic fish have shown outstanding results, but negative public perception inhibits the advancement of this technology in aquaculture.

Another approach to guarantee high levels of n-3 long-chain polyunsaturated fatty acids in fish is by implementing a finishing diet. This strategy has been adopted where fish initially fed vegetable-based diets for the grow-out phase are fed diets containing only fish oil as the dietary lipid source at the final phase of their rearing period (Jobling, 2003; Turchini et al., 2007, Stone et al., 2011). The fish oil-finishing method has been extensively evaluated in many farmed fish such as Atlantic salmon (*Salmo salar*) (Bell et al., 2004; Torstensen et al., 2004; Codabaccus et al., 2012), rainbow trout (*Oncorhynchus mykiss*) (Stone et al., 2011; Cleveland et al., 2018; Yıldız et al., 2018), European sea bass (*Dicentrarchus labrax*) (Mourente et al., 2005; Mourente and Bell, 2006), Medierranean yellowtail (*Seriola dumerili*) (Bordignon et al., 2020). In most of the aforementioned studies, a finishing diet could restore the DHA level up to between 70 and 90% compared to fish that continuously consumed feed containing fish oil. However, regardless of the duration of the experiments, it has been reported that the high level of some fatty acids derived from vegetable oils, especially linoleic acid, are stored in the fish body until the end of the production period. Although the finishing diet method can partly improve the final fatty acid profile of fish fed alternative oil sources earlier, this is only a temporary solution, not a permanent solution, because a considerable amount of fish oil is still needed.

Dietary supplementation can be another potential option for increasing n-3 long-chain polyunsaturated fatty acids content in fish by improving their fatty acid biosynthesis. It has been examined with bioactive fatty acids (conjugated linoleic acid, 3-thia acids, petroselinic acid), cholesterol, and other botanicals, such as the lignin sesamin (Kennedy et al., 2006; Moya-Falcón et al., 2004; Randall et al., 2013; Ruyter et al., 1997; Trattner et al.,

2008 a, b; Norambuena et al., 2013; Tocher, 2015), but these studies have shown that the increase in bioconversion rate has been too low for this approach to be a viable strategy.

Selective breeding is also one of the strategies to enhance n-3 long-chain polyunsaturated fatty acid levels in fish. Leaver et al. (2011) reported that fillet n-3 long-chain polyunsaturated fatty acid level is a highly heritable trait in Atlantic salmon and that specific hepatic mRNA expression patterns are related to high fillet n-3 long-chain polyunsaturated fatty acid, which shows possible mechanisms for family-dependent deposition in the fish fillet. Horn et al. (2018) have also reported genetic variation in the level of individual fatty acids in salmon fillets; thus, modifying its fatty acid composition by selective breeding is viable. Hence, there is potential in using selective breeding as a tool to increase levels of n-3 long-chain polyunsaturated fatty acid in salmonids muscle by improving the salmon's natural ability to convert α-linolenic acid from alternative oil sources into n-3 long-chain polyunsaturated fatty acid and to deposit these fatty acids in the muscle more efficiently.

Effects on fish health and inflammatory response

The effects of fish oil replacement on fish health have been extensively studied. In recent years, there has been increasing concern among aquaculture producers about the adverse outcomes of stress and feeding unbalanced diets, which result in increased production costs due to effects on disease susceptibility.

Replacement of fish oil high in n-3 long-chain polyunsaturated fatty acids by vegetable oils typically high in n-6 polyunsaturated fatty acids alters fatty acid profiles of fish tissues and cells, influencing the general fish health in various manners, including innate and adaptive immunity as well as eicosanoid production (Montero et al., 2015). It is also known that substituting fish oil with vegetable oil affects the n-3/n-6 ratio, which is crucial to health maintenance of fish (Simopoulos, 2008). Furthermore, Montero and Izquierdo (2010) demonstrated that higher substitution levels by a single vegetable oil affected fish immune response over lower substitution levels or blends of vegetable oil. Despite the fact that dietary fatty acids modify the immune system and health status of fish, the changes in fatty acid composition do not always result in reduced disease resistance since pathogen infection is dependent on intricate interactions between the environment, pathogen, and fish (Montero

et al., 2010). The dietary content of the lipid as well as the fatty acid composition of the diet, fish species, the feeding duration, environmental conditions, and the presence of pathogens in the environment can affect fish immunity and resistance to a pathogen (Montero and Izquierdo, 2010).

In general, replacing fish oil with vegetable oil commonly high in linoleic acid increases arachidonic acid (20:4n-6) content in fish via an n-6 fatty acid pathway. As a result, arachidonic acid and its metabolites (eicosanoids) have attracted much attention with regard to inflammatory processes and disease resistance (Bell and Sargent, 2003; Martins et al., 2012; Norambuena et al., 2015; Asil et al., 2017). Eicosanoids are an important class of biologically active molecules derived from the metabolism of polyunsaturated fatty acids, mainly arachidonic acid and EPA. When the membrane phospholipids are cleaved by phospholipases, primarily phospholipase A2, lipooxygenase (LOX), and cyclooxygenase (COX) enzymes function on free fatty acids to produce pro- or anti-inflammatory mediators. Due to the functions of the LOX and COX enzymes in converting n-3 and n-6 polyunsaturated fatty acids, the two classes of fatty acids compete for these enzymes. In addition, it is known that the fatty acid content of fish generally reflects the fatty acid composition of the diet, suggesting that fish oil replacement influences the levels of particular metabolites. COX enzymes have more affinity for arachidonic acid, a precursor for the pro-inflammatory processes, producing series-2 prostanoids, whereas EPA provides precursors for the production of the anti-inflammatory mediator family of series-3 prostanoids. (Lenihan-Geels et al., 2013). Even though inflammation is a crucial process in relation to innate immunity, excessive production of pro-inflammatory metabolites during chronic inflammation can lead to adverse effects and increase vulnerability to disease. These processes occur through an increase in reactive oxygen species and cellular stress (B Vendramini-Costa and E Carvalho, 2012). Few studies have explicitly focused on the effects of arachidonic acid on disease resistance in fish. Improvement of fish survival during pathogen challenge by moderate levels of dietary arachidonic acid has been observed in Atlantic salmon (Dantagnan et al., 2017), rabbitfish (Nayak et al., 2017), and guppy (Khozin-Goldberg et al., 2006).

Another health concern can arise when excess dietary lipid or energy exceeds the capacity of hepatocytes to oxidize fatty acids. This results in production and accumulation of

triglycerides causing the histological condition, steatosis (Montero and Izquierdo, 2010). The effects of various vegetable oils on liver histology have been widely studied in Atlantic salmon (Ruyter et al., 2006; Kjaer et al., 2008), rainbow trout (Caballero et al., 2002; Figueiredo-Silva et al., 2005), gilthead sea bream (Wassef et al., 2007; Benedito-Palos et al., 2008), and European sea bass (Mourente et al., 2005, 2007; Torrecillas et al., 2017). The liver morphological alterations derived from different dietary vegetable oils seem to depend on the fish species and type of vegetable oil; marine species are more susceptible to the signs of steatosis when n-6 polyunsaturated fatty acids-rich vegetable oils are incorporated into the diet (Montero and Izquierdo, 2010).

Defining the effect of dietary vegetable oils on fish health and immunity is complicated because of the complex relationships between nutrition, health, and environment and the insufficient data available for different species. Thus, further studies are necessary to clarify the most suitable oil blends for each fish species to promote optimum health and immunity. According to the literature published to date, blends of vegetable oil balancing the n-3/n-6 ratios have been shown more suitable in terms of fish health and disease resistance than using a single vegetable oil source (Montero and Izquierdo, 2010).

Effects on human health

Fish consumption has attracted substantial interest in the past few decades regarding human health benefits. Fish with high levels of EPA and DHA and a high n-3/n-6 ratio is essential to a healthy human diet. As mentioned earlier, fish consumption is growing globally, and most governmental food agencies recommend its consumption. An EPA+DHA intake of at least 250 to 500 mg day^{-1} is recommended and the American Heart Association recommends 1000 mg of EPA+DHA day^{-1} for reducing coronary heart disease (USDA, 2019). As fish and seafood are considered the primary provider of EPA and DHA in the human diet, their health-beneficial characteristics must be maintained (Pickova and Mørkøre, 2007). However, as the world's population and consumption of aquatic products increase and wild fishery resources decrease, an increasing proportion of fish consumed by humans is farmed fish. In fact, aquaculture has been the fastest-growing animal food production sector for the past few decades, supplying more than 50% of the world's fish and seafood consumed by humans today. (FAO, 2022).

Atlantic salmon (*Salmo salar L.*) is an increasingly popular fish species in the world seafood market due to its high market value. Atlantic salmon is marketed for its health-promoting characteristics and high nutritional content, mainly its high n-3 long-chain polyunsaturated fatty acids. However, it is clear that this property will diminish as alternative sources such as vegetable oils replace fish oils in salmonid feeds. Similar to human fatty acid metabolism, salmon are inefficient at bioconverting shorter n-3 chain fatty acids, such as alpha-linolenic acid, to EPA and DHA, similar to findings in cold-water marine fish. Therefore, n-3 long-chain polyunsaturated fatty acids must be included in the diet to maintain healthful levels in fillets (Tocher, 2015; Sprague et al., 2016). Several reports have shown that the higher use of plant-based ingredients in salmon diets is associated with higher n-6 levels in farmed fish fillets (Bell et al., 2004; Torstensen et al., 2005; Nichols et al., 2014). Despite the fact that n-6 fatty acids such as linoleic and arachidonic acid play important roles for humans, there is already a very high intake in the human diet of oils having lower n-3/n-6 ratios, affecting the metabolism of beneficial n-3 long-chain polyunsaturated fatty acids (Simopoulos, 2008; Calder, 2014; Marventano et al., 2015; Simopoulos, 2016).

Bell et al. (2004) reported that the development of sustainable aquaculture feeds with increased use of terrestrial materials, primarily oilseed-derived, did not significantly affect the health or growth performance of salmon. However, modifications in dietary oil sources pose challenges for the aquaculture industry since the fatty acid composition of fish muscle reflects the fatty acid composition of the diet (Turchini et al., 2022). The use of plant-based alternatives in aquafeeds has resulted in increased levels of terrestrial-origin fatty acids such as oleic, linoleic, and to some extent, alpha-linolenic acid. At present, commercial aquafeeds increasingly incorporate blends of plant-based and fish or other oils to fulfill the energy and fatty acid requirements of fish, reducing pressure on marine resources. However, this results in a compromise of the health benefit to the human consumers. For instance, the inclusion levels of fish oil in Norwegian salmon diets fell from 24% in 1990 to 11% in 2013 (Ytrestøyl et al., 2015). According to Sprague et al. (2016), while EPA and DHA content fell by approximately half, oleic, linoleic, and alpha-linolenic acid tended to rise from 2010, when the use of vegetable oil in the salmon diet began to grow, and the values of 15%, 5%, and 2% recorded in 2010, doubled in 2015 to ~30%, 10%, and 5%, respectively.

Conclusion and Objectives

Aquaculture expansion is limited primarily by its reliance on marine resources such as fish oil derived predominantly from forage fisheries (Turchini et al., 2009). Therefore, in order to overcome the limitation and continue expanding the aquaculture industry, there is an urgent need for further efforts to find suitable and sustainable alternatives to produce high-quality fish products. As such, the research presented in this dissertation sought to evaluate dietary manipulation of fatty acid metabolism and biosynthesis with an emphasis on fillet omega-3 LC-PUFA content and fish performance.

The objectives and hypotheses were as follows:

1. The use of a low-phytosterol oil, such as soy-oil, in combination with a high ALA rich oil, such as linseed oil, and supplemental cholesterol could effectively replace fish oil in salmonid feeds.
2. Latitude oil containing high EPA and DPA would effectively replace fish oil in trout feeds without detrimental impact on fish performance and fatty acid composition in fillets over a complete production cycle, from fingerling to market weight.
3. Dietary ARA supplementation in swim-up and sub-adult rainbow trout would improve growth performance, feed utilization, and resistance to stressors, such as hypoxia, high temperature and density.

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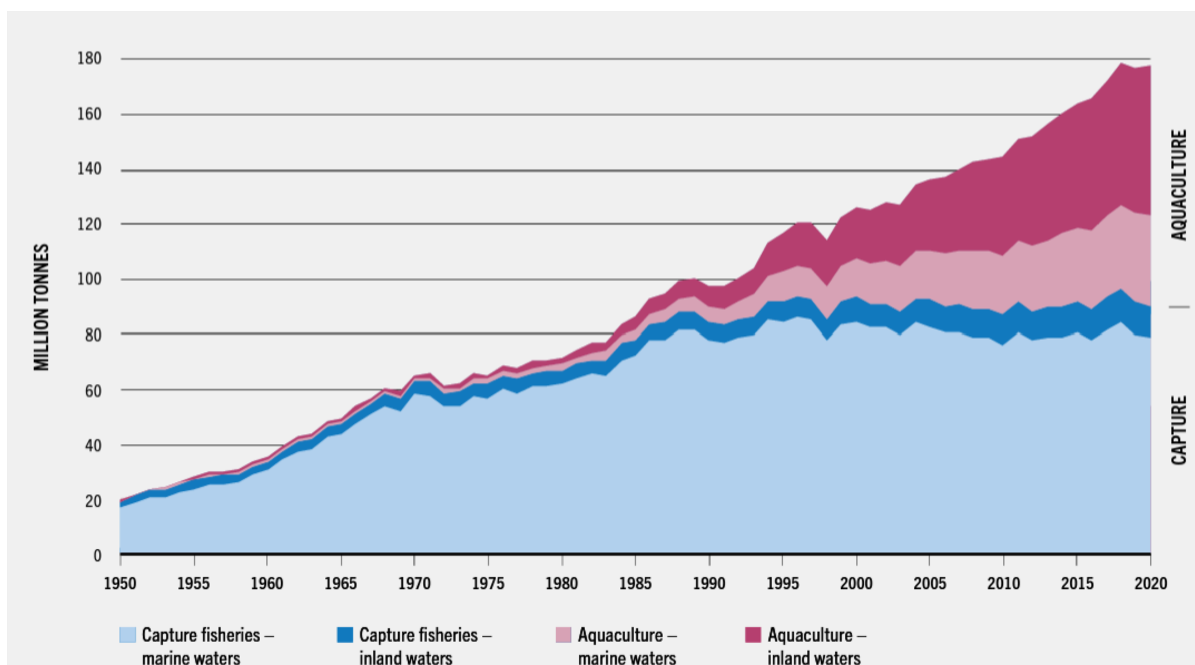

Figure 1.1. World capture fisheries and aquaculture production. Notes: Excluding aquatic mammals crocodiles, alligators, caimans and algae. Data expressed in live weight equivalent. Source: FAO

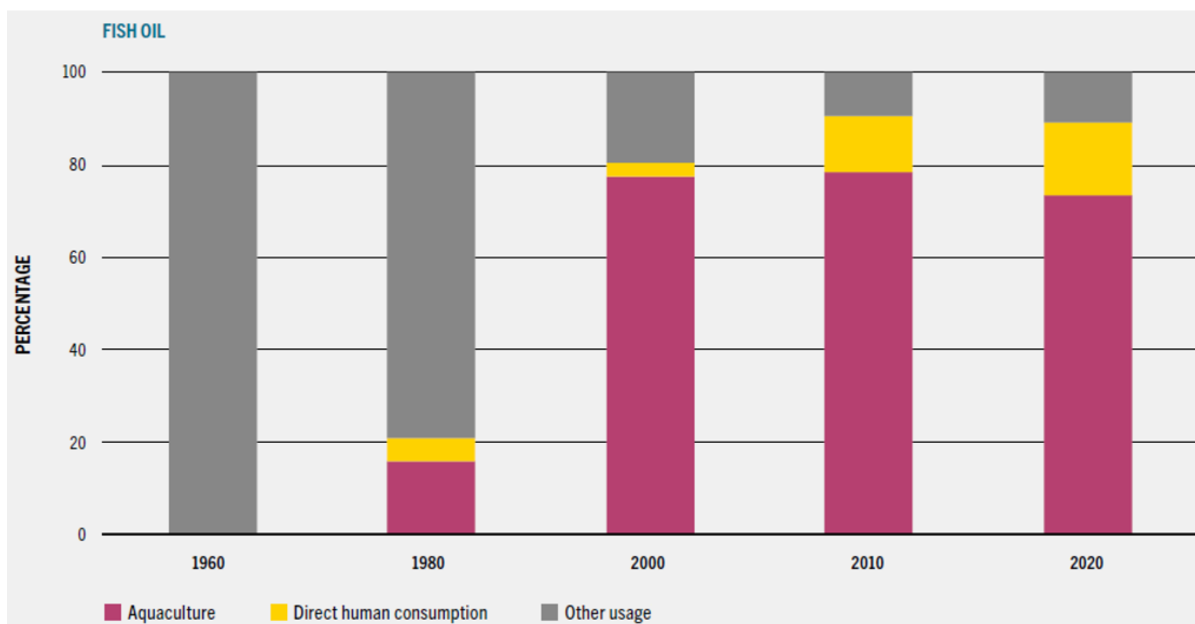

Figure 1.2. Utilization of fish oil. Source: IFFO

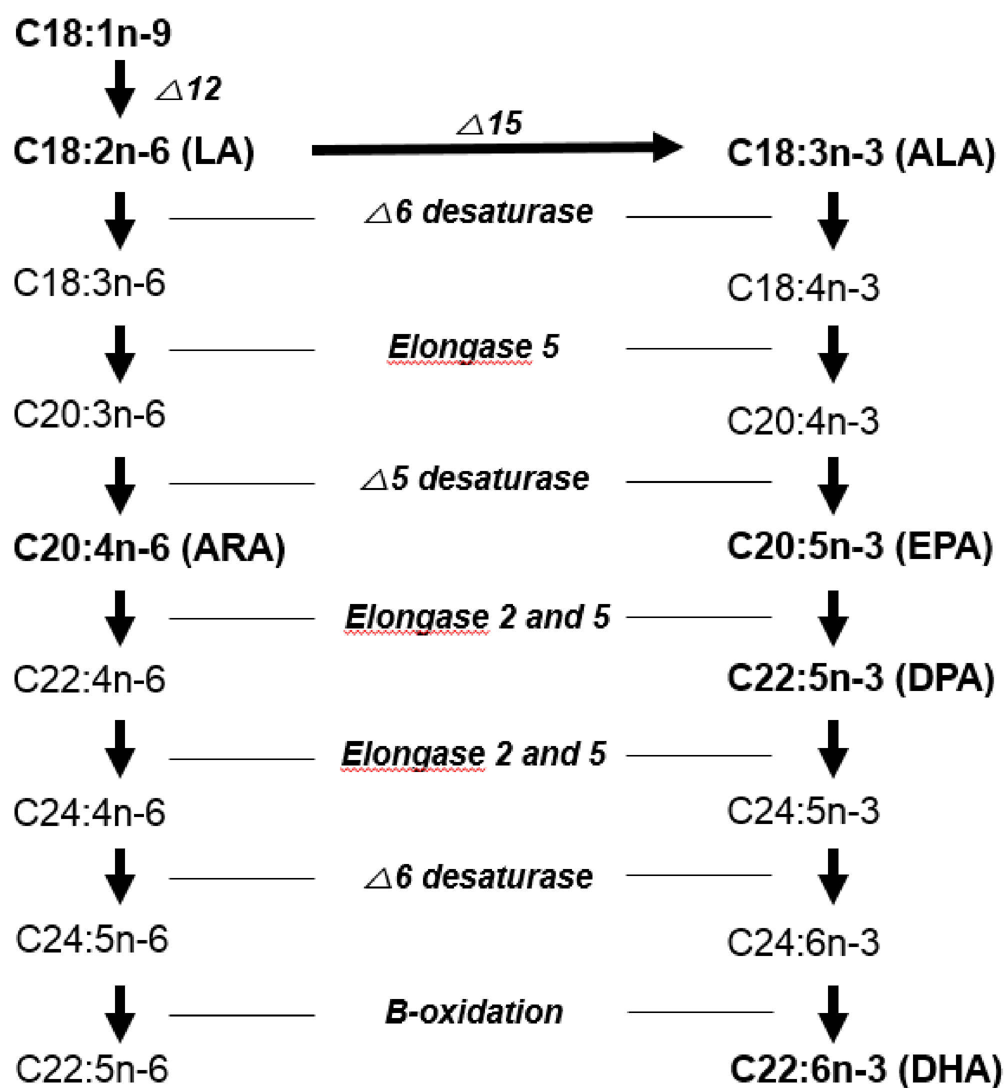

Figure 1.3. Polyunsaturated fatty acid biosynthesis pathway. Δ12 and Δ15 desaturases are found only in plants and some invertebrates, thus C18:2n-6 and C18:3n-3 cannot be synthesized *in vivo*.

Chapter 2:

Balancing Dietary Lipid and Cholesterol to Increase Fillet Omega-3 Deposition in Rainbow Trout Fed a Soy-Based Diet

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Abstract

We hypothesized that a low phytosterol oil, such as soybean oil, in combination with an ALA rich oil, such as linseed oil, and supplemental cholesterol could effectively replace fish oil in salmonid feeds. A such, the primary objective was to blend soybean oil with linseed oil and supplemental cholesterol to maintain fish growth and increase de novo production of the omega-3 fatty acids (n-3 FA) EPA and DHA in trout muscle, since EPA and DHA have been identified as beneficial nutrients in human nutrition, and farmed seafood must meet consumer needs and expectations. To accomplish this, we used soy oil for its low phytosterol properties in combination with linseed oil for its n-3 FA content, with and without cholesterol to improve utilization. Upon conclusion of the study, our results did not support our hypothesis. Trout fed all-plant based diets containing any amount of soy oil had reduced growth performance. Only fish fed the all-plant diet with 100% linseed oil plus supplemental cholesterol grew as well as the fishmeal/fish oil positive control group. With regard to fillet EPA and DHA content, no improvements were seen with soybean oil. Dietary cholesterol supplementation improved plasma cholesterol levels ($P<0.05$), and numerically, but not statistically ($P>0.05$), increased EPA and DHA levels in fillets when linseed oil was provided above 50% of total oil content. Gene expression results supported the results of fatty acid analysis, indicating no significant enhancement by soybean oil or cholesterol. Although, our hypothesis failed and soybean oil was found to be less accepted by the fish, resulting in lower growth and feed intake, we were successful in developing a high soy-protein diet (35%) with all-plant ingredients using linseed oil and supplemental cholesterol that resulted in equal fish performance to fish fed a fishmeal/fish oil control diet. More research is needed to identify a cost-effective EPA/DHA source to meet consumer needs and expectations for n-3 FA in trout fillets.

Introduction

Aquaculture is often touted as a solution to ensuring sufficient fish products for a rapidly increasing global population (FAO, 2014). A great deal of research in the aquaculture field now emphasizes the need to make fish farming more sustainable (FAO, 2014). A large effort in this regard is to develop feeds that reduce reliance on capture fisheries so supply fish meal and fish oil. Success in this regard has brought with it a complication for human health, which is lower omega-3 (n-3) fatty acids (FA) in fish feeds leading to lower n-3 FA in the

consumable flesh of the fish produced (Sprague et al., 2016). Omega-3 fatty acids, particularly EPA (20:5n-3) and DHA (22:6n-3) have been identified as beneficial nutrients in human nutrition for heart and brain health (Calder, 2014). For this reason, farmed seafood must meet consumer expectations as healthful products. One-for-one substitutions of fish oil with plant oils currently is not enough to ensure healthful levels of EPA and DHA in farmed fish products. Alternative feeding strategies, coupled with improved plant and fish genetics, must be developed to increase n-3 FA in farmed fish products.

Over the past decade, considerable research has been conducted on fatty acid metabolism in rainbow trout, a model species for other carnivorous fish species and an important food fish. This is mostly driven by increased demand, stagnant production, rising costs of fish oil and the inevitable modification of the final n-3 long-chain polyunsaturated fatty acid (LCPUFA) content of fish fillets when fish oil is replaced with more economical and sustainable plant oil alternatives. The thrust of much of the research has been on the capability of fish to biosynthesize DHA (22:6n-3) through the desaturation and elongation of α-linolenic acid (ALA, 18:3n-3). The problem appears to be adaptation by the fish to abundant DHA in their natural diet leading to reduced or even “dormant” n-3 biosynthetic capacity (Gregory at el., 2016). As such, a better understanding of FA metabolism has evolved from recent research evaluating FA fate in fish fed alternative dietary oil sources (Turchini and Francis, 2009; Gregory at el., 2016; Teoh and Ng, 2016).

It is now clear that a lack of dietary n-3 LCPUFA leads to an increase in both elongase and desaturase activity and transcription rates (Gregory at el., 2016). However, research has demonstrated that this compensation in fish is insufficient to increase n-3 LCPUFA tissue concentrations to levels adequate to result in human health benefits. In a study by Turchini and Francis (2009) where rainbow trout were fed two different dietary treatments of fish oil or linseed oil, fish fed the linseed oil diet showed elevated delta-6 (Δ-6) and delta-5 (Δ-5) desaturases and commensurate increases in EPA and DHA. However, tissue levels of EPA and DHA were still 5- and 3-fold lower, respectively, than in fish fed the fish oil diet. In light of recent findings by Gregory et al. (2016), it is important to note that both dietary treatments contained fishmeal in the Turchini and Francis (2009) study. Gregory et al. (2016) concluded that when optimizing aquaculture feeds containing vegetable oils and/or fish oil or fishmeal, one must consider both the amount of dietary ALA and DHA.

Their results suggest that dietary DHA has a large negative effect on downregulating both elongases and desaturases, and when no DHA was present in a diet high in ALA, expression levels of Δ-6 and Δ-5 desaturases and elongase-5 and -2 were highest. Cholesterol appears to also play a significant role in regulating FA metabolism by stimulating fatty acid β-oxidation and the conversion of ALA to DHA (Norambuena et al., 2013). Even so, cholesterol use in optimizing fish feed formulations for maximizing n-3 LCPUFA biosynthesis has been limited and not previously reported for fish fed an all-plant based diet replacing fish oil with soy oil. This is important since cholesterol's activity may be affected by phytosterols in plant oils, as phytosterols are known to inhibit intestinal cholesterol absorption (Ostlund, 2004). The deemphasized role of physosterols to date may have confounded much of the research done on FA metabolism and n-3 LCPUFA biosynthesis in trout because the focus has been on maximizing dietary ALA for conversion to DHA by using linseed oil in the diets. While linseed oil is the richest source of ALA among common plant oils, soy oil has about two-thirds the phytosterol content (300 mg/100 g; Verleyen et al., 2002) of linseed oil (467 mg/ 100 g; Yang et al., 2019).

Further development and utilization of soy-based feeds will likely rely on the formulation of diets composed of all plant-derived feed ingredients, including plant oils. However, substituting fish oil with plant oils lowers the levels of EPA and DHA compared to levels in fish fed diets containing fish oil. Omega-3 fatty acids are essential nutrients for fish and inclusion of 5-6% fish oil is sufficient to meet the dietary requirements of salmonids. However, this level of dietary fish oil is not sufficient to ensure levels of EPA and DHA in fillets meet consumer expectations and dietary intake recommendations. Among alternative lipid sources for fish feeds, plant oils high in ALA are of interest because ALA can serve as a precursor for the biosynthesis of EPA and DHA. We hypothesize that the use of a low-phytosterol oil, such as soy-oil, in combination with a high ALA oil, such as linseed oil, and supplemental cholesterol can be used to improve EPA + DHA biosynthesis and fillet content for human consumption. Salmonids possess the capacity to synthesize EPA and DHA from ALA, but the rate of bioconversion is extremely low. The objective of the proposed research was to provide a low amount of phytosterol oil in combination with a high amount of ALA oil and supplemental cholesterol in diets fed to rainbow trout as components of a fishmeal/fish oil free diet (low in LC-PUFA's) and demonstrate increased conversion of

ALA to DHA in the edible fillet. If successful, the results would have significant implications for salmonid diet formulation and warrant future investigations with non-salmonid species while improving the human health benefits associated with consuming DHA and EPA in fillets.

Materials and Methods

Dietary Treatments and Formulation

Diets were formulated to contain 48% protein, 21% lipid and 22.5 MJ/kg energy, and meet or exceed the published minimum nutrient requirements for rainbow trout (NRC, 2011). Experimental diets were cold pelleted at the University of Idaho's Hagerman Fish Culture Experiment Station (HFCES) using a laboratory-scale California pellet mill fitted with a 2.4-mm die. Feeds were dried in a forced-air dryer at 35°C to < 10% moisture, then stored at ambient temperature (20-22°C) until being fed. Samples of the diets were collected for chemical analyses. Dietary cholesterol supplementation was at a level of 1.43 mg/g of diet to mimic the amount of cholesterol in a fish oil-based diet.

Experimental diets: 12 experimental diets were formulated as follows (Table 2.1):

1. Diet 1 (Control): Fishmeal based control diet
2. Diet 2 (F0100): All-plant protein, soy-based diet with 100% fish oil
3. Diet 3 (SO100): All-plant protein, soy-based diet with 100% soy oil
4. Diet 4 (SO100+C): Diet 3 supplemented with 1.43 mg/g diet cholesterol
5. Diet 5 (SO75/25LO): All-plant protein, soy-based diet with 75% soy oil/25% linseed oil
6. Diet 6 (SO75/25LO+C): Diet 5 supplemented with 1.43 mg/g cholesterol
7. Diet 7 (SO50/50LO): All-plant protein, soy-based diet with 50% soy oil/50% linseed oil
8. Diet 8 (SO50/50LO+C): Diet 7 supplemented with 1.43 mg/g cholesterol
9. Diet 9 (SO25/75LO): All-plant protein, soy-based diet with 25% soy oil/75% linseed oil
10. Diet 10 (SO25/75LO+C): Diet 9 supplemented with 1.43 mg/g cholesterol
11. Diet 11 (LO100): All-plant protein, soy-based diet with 100% linseed oil
12. Diet 12 (LO100+C): Diet 11 supplemented with 1.43 mg/g cholesterol

Fish and Feeding Trial

The fish feeding trial was conducted at the University of Idaho's Hagerman Fish Culture Experiment Station in Hagerman, Idaho. All fish handling and sampling, plus the experimental protocols used in this project were approved in advance by the University of Idaho's Institutional Animal Care and Use Committee (IACUC-2018-65).

Rainbow trout (initial body weight: 18.8 ± 0.3g) of the USDA-UI Hagerman strain crossed with Donaldson strain, selected for improved growth on an all-plant protein feed (Diet 2), were stocked into each of 36, 145-L tanks at 25 fish per tank. Each tank was supplied with 8-10 L/min of constant temperature (15 °C) spring water fed by gravity to the fish rearing laboratory. Each diet was assigned randomly to three tanks in a completely randomized design. Fish were hand-fed to apparent satiation two times per day, six days per week for 12 weeks. Photoperiod was maintained at 14 h light: 10 h dark with fluorescent lights controlled by electric timers. At the end of 12 weeks, 16-hour postprandial, three fish per tank were anesthetized with tricaine methanesulfonate (MS-222, 100 mg/L, buffered to pH 7.0). Plasma was collected from the caudal vessels of fish with 1-ml heparinized syringes fitted with 25G 3/4-inch needle for cholesterol analysis. Upon euthanizing those fish with MS-222, liver and white muscle were excised for gene expression analysis. Another three fish per tank were euthanized and filleted for muscle fatty acid analysis. Tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until analysis.

Fatty Acid Analysis

Fatty acid analysis of feeds and muscle was conducted using gas chromatography. Lipids were extracted for fatty acid analysis following a modified Folch method (Folch et al.,1957; Clark et al., 1982). Extracted lipids were derivatized to prepare fatty acid methyl esters (FAME) using tert-butyl methyl ether (TBME) and trimethylsulfonium hydroxide in an Agilent 7696A Sample Prep WorkBench (Agilent Technologies (Shanghai) Co. Ltd, Shanghai, China). FAME was then analyzed using an Agilent 7890B GC System. Fatty acids in samples were identified by comparing the retention times with those of commercial fatty acid analytical standards. Results were expressed on a relative percentage basis, then normalized and reported as % of FAME (fatty acid methyl ester).

Real-time qPCR

Total RNA was isolated from each tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) extraction method following the manufacturer's suggested protocol. Purity and quantity of extracted RNA were assessed by Nanodrop ND-1000 spectrophotometer (260/230 and 260/280 ratios >1.8). Briefly, extracted RNA was treated with DNAse, then 1 µg of total RNA was reverse-transcribed using the iScript™ cDNA Synthesis kit (BioRad, Hercules, CA). Real-time quantitative PCR was carried out on a CFX96 Real-Time System (BioRad) in a 10 µL total volume reaction using iTaq SYBR Green Supermix (BioRad) and 500 nmol primers according to the protocol provided by the manufacturer. PCR cycling conditions for all genes were as follows: 95 °C for 5 s followed by 55 °C for 30s over 40 cycles with an initial denaturation step of 95 °C for 3 min. For each fish, PCR reactions were run in duplicate on RNA samples. Relative expression values for genes constituting the FA transport, oxidation, desaturation, elongation, and incorporation, including delta-5 fatty acyl desaturase (*d5fad*), delta-6 fatty acyl desaturase (*d6fad*), fatty acid elongase 2 (*elovl2*), fatty acid elongase 5 (*elovl5*), fatty acid bind protein 2 (*fabp2*), and enoyl-coa hydratase and 3-hydroxyacyl coa dehydrogenase (*ehhadh*) were determined using primers designed from rainbow trout sequences in the NCBI Genbank® database. In addition, a cellular mRNA control was selected from a set of two reference genes (*arp* and *elf1α*). Primer PCR efficiency was calculated by including six serial dilutions of a standard (pooled from each experimental sample for a given tissue) and utilized for PCR correction for all primer pairs (Pfaffl, 2001). Normalized data were analyzed using the relative quantification method described by Pfaffl (2001).

Calculations

Using the live-weight and feed consumption data, the following indices were calculated.

Weight gain (WG, g/fish)

= (g mean final weight–g mean initial weight)

Specific growth rate (SGR,%/d)

= [(ln mean final weight–ln mean initial weight)/number of days] x 100

Survival (%)

= (number of fish at the end of the trial/number of fish at the beginning) 100

Average feed intake (FI, g/fish)

= g total dry feed intake/number of surviving fish

Feed conversion ratio (FCR)

= g total feed consumed/ (g final biomass – g initial biomass + g dead fish weight)

Statistical analysis

Tank means were used for statistical analysis. Fish growth and feed utilization indices, physiological parameters, and gene expression data were tested for normality and homogeneity of variance prior to one or two-way factorial Analysis of Variance (ANOVA). If significant differences were found by ANOVA, data were subjected to Tukey's HSD test to separate the means with a significance level of $P<0.05$. IBM SPSS (Version 21 for Window; IBM SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

Results

Diets

The proximate composition and energy content of the twelve experimental diets used in the growth trial are presented in Table 2.1. Experimental diets were isonitrogenous, isolipidic and isocaloric, and differed in total cholesterol (55 – 2040 mg/kg), total phytosterol content (1090 – 2350 mg/kg; Table 2.3) and fatty acid content (Table 2.2). FM/FO and PM/FO diets contained EPA (6.59 - 6.79%) and DHA (4.98 - 5.32%) and the rest of the diets did not contain EPA and DHA. Linoleic acid (LA) content decreased as soy oil level dropped to 75, 50, 25 and 0 % (49.5 - 16.4%). On the contrary, alpha-linolenic acid (ALA) content increased as linseed oil increased from 25 to 100% (6.87 - 45.1%) (Table 2.2).

Growth Trial

Rainbow trout juveniles were fed diets containing different ratios of soybean oil and linseed oil and with or without added cholesterol for twelve weeks. The growth performance and feed utilization of the fish are presented in Table 2.4. Survival (93.3% to 100%) and feed conversion ratios (1.02 to 1.09) were similar among dietary treatments groups ($P>0.05$).

Weight gain of fish fed diet FM/FO, PM/FO or L100+C was the highest ($P<0.05$) compared with the fish fed other diets. Different ratios of SO/LO did not affect trout weight gain; however, the addition of cholesterol to the L100+C diet significantly increased fish weight gain. The interaction between the two main factors (Cholesterol and SO/LO ratio) significantly affected feed intake (Table 2.6, $P<0.05$). Fish fed L100+C showed increased feed intake but had no significant effects on FCR or survival ($P>0.05$).

Whole-body Proximate Composition

Whole-body proximate composition of rainbow trout juveniles fed the experimental diets are presented in Table 2.5. Dry matter of fish whole-body varied from 31.8% (SO50/LO50+C) to 33.3% (L100+C). Percent crude protein of fish whole-body ranged from 15.3% (FM/FO) to 16.0% (SO25/LO75, L100) on wet basis. Percent crude fat of fish whole-body ranged from 13.7% (SO50/LO50+C) to 15.2% (LO100+C) on wet basis. Ash content of fish whole-body ranged from 1.90% (SO100+C) to 2.20% (PM/FO) on wet basis. Gross energy of fish whole-body ranged from 27.2 MJ/kg (SO100) to 28.9 MJ/kg (L100+C) on wet basis. There were no differences in whole-body proximate composition among the treatment groups. All of these values are within expected ranges for rainbow trout of this size.

Chemical Analysis

The result of the chemical assessment of plasma is presented in Table 2.7. Total cholesterol in plasma of fish fed diets ranged from 5.47 mmol/L (SO75/LO25) to 8.73 mmol/L (SO50/LO50+C, L100+C) on wet basis. Plasma cholesterol of fish fed L100+C diet was significantly higher than fish fed SO100 and SO75/LO25 diets. Plasma cholesterol of fish fed diet supplemented with cholesterol showed a higher level than those that were not supplemented with cholesterol.

Fatty Acid Analysis

Fatty acid composition of rainbow trout juveniles fed the experimental diets are presented in Table 2.8 and 2.9. LA (C18:2n-6) of fish fillet decreased as soy oil level dropped to 75, 50, 25 and 0% (33.0 - 4.66%), while ALA (C18:3n-3) of fish fillet increased as linseed oil increased from 25 to 100% (0.64 – 24.9%). EPA (C20:5n-3) of fish fillet

ranged from 0.80% (SO100, SO100+C) to 5.34% (FM/FO). DHA (22:6n-3) of fish fillet varied from 3.68% (SO100+C) to 13.3% (PM/FO). The interaction of the two main factors (Cholesterol x SO/LO ratio) significantly affected EPA (P=0.051) and DHA content in fish fillet.

Gene Expression

Relative gene expression in the liver and muscle of rainbow trout juvenile fed experimental diets is presented in Fig.2.4 and 2.5. *Elovl2* expression in liver and muscle varied from 1.07 (FM/FO) to 1.60 (SO100 and SO50/LO50) and 0.70 (FM/FO) to 1.27 (SO100), respectively. *Elovl5* expression in liver and muscle ranged from 0.84 (PM/FO) to 1.20 (LO100+C) and 0.55 (FM/FO) to 1.63 (SO100 and SO50/LO50+C), respectively. Expression of *elovl2* and *elovl5* was lowest with the control diets (FM/FO and PM/FO) but did not differ among diets containing vegetable oils. Similar trends were observed for *d5fad* and *d6fad* in both tissues. *D5fad* expression in liver and muscle varied from 1.02 (FM/FO) to 1.44 (SO100+C) and 0.68 (PM/FO) to 1.27 (SO100+C), respectively. D6fad expression in liver and muscle ranged from 1.74 (PM/FO) to 3.24 (SO100+C) and 3.26 (FM/FO) to 4.68 (SO75/LO25+C), respectively. On the contrary, expression of fabp2 was highest in the control treatments (FM/FO and PM/FO). Expression of *ehhadh* was not affected by diets ($P > 0.05$).

Discussion

The results of this study validate the use of supplemental cholesterol to support growth performance in rainbow trout fed all-plant protein diets. At the same time, phytosterol concentration did not affect the fish growth performance or circulating cholesterol. The central hypothesis of this study was that a plant-based diet would be responsible for increased energy expenditure for de novo cholesterol biosynthesis, and thus would have an impact on fish performance and apparent in vivo fatty acid metabolism. However, in the present study, only fish fed LO100+C showed higher growth performance than fish fed other plant-based diets. One would expect that rainbow trout require a sufficient level of ALA (C18:3n-3) to boost their growth performance. In the 12-week feeding trial with rainbow trout juveniles, replacing fish oil with different ratios of SO/LO showed reduced growth rates except for fish

fed L100+C diet. In Atlantic salmon, no effects of dietary cholesterol supplementation were observed on growth performances when fed high inclusion levels of fish meal and fish oil diets (Bjerkeng et al., 1999); similar observations were reported for channel catfish fed casein-based diets (Twibell and Wilson, 2004). However, improved growth performance was observed in response to supplementing dietary cholesterol when channel catfish were fed soybean-based diets (Twibell and Wilson, 2004). In hybrid striped bass (Morone chrysops x M. saxatilis) fed diets containing a large amount of fish meal and fish oil, no effect of cholesterol supplementation on growth performance was recorded (Sealey et al., 2001). These works all used experimental diets containing abundant fish meal and fish oil; thus, even the control treatments provided large amounts of dietary cholesterol. Furthermore, it was shown that the weight gain and feed intake in fish fed a fishmeal-based diet without plant protein ingredients were not influenced by cholesterol supplementation, as reported in Atlantic salmon (Bjerkeng et al., 1999) and Japanese flounder (Deng et al., 2010). However, in the present study, fish fed LO100+C diet had significantly higher weight gain and feed intake compared with those fish fed the other plant-based diets without cholesterol supplementation. Therefore, cholesterol supplementation may be beneficial when fish are fed plant-based diets with 100% linseed oil.

Dietary cholesterol supplementation had no effects on performance of fish fed diets containing soybean oil, although we hypothesized low phytosterol content would facilitate cholesterol uptake. This may be due to the lack of ALA in the diets that are required for bioconversion into EPA and DHA and this study showed that there were interactions between cholesterol and the ratio of SO and LO. Fish, like all vertebrates, cannot synthesize PUFA *de novo* as they lack the essential *Δ12* and *Δ15 FAD* to convert Oleic acid (C18:1n-9) to LA (C18:2n-6) and ALA (C18:3n-3). However, the growth performance of fish fed S100 and L100 diet were not significantly different. Similarly, in juvenile sharpsnout seabream (*Diplodus puntazzo*) there were no significant differences in fish growth between fish fed soybean oil and linseed oil treatments (Piedecausa et al., 2007). On the other hand, differences in feed intake and weight gain were significant between soybean oil diets and control groups. The decrease in fish growth of fish fed soybean oil diets can be explained by reduced feed intake, likely a result of the absence of n-3 LCPUFA. Roy et al. (2020) showed that rainbow trout could discriminate between feeds with different levels of n-3 LCPUFA.

In this study, a hypocholesterolemic effect was observed in the plasma of fish fed the plant-based diet without cholesterol supplementation compared with the fish fed control treatments (FM/FO and PM/FO) and diets supplemented with cholesterol. It is reported that plant protein sources, such as soybean meal, soy protein concentrate, corn protein concentrate and wheat gluten meal, generally known for low-cost plant protein ingredients, have a hypocholesterolemic effect in rainbow trout, turbot, gilthead sea bream, Atlantic cod and parrot fish fed these ingredients compared to the fish fed fish meal-based diets (Yamamoto et al., 2007, Regost et al., 1999, Venou et al., 2006, Hansen et al., 2007, Lim and Lee, 2009)

Another aspect of the present study was assessing if dietary cholesterol affected fatty acid metabolism. In several species, the key enzymes involved in the fatty acid synthesis pathway are known to be affected by several physiological and nutritional factors, including dietary fatty acid composition and cholesterol. In rats, dietary supplementation of cholesterol reduced the expression of *D5fad* and *D6fad* (Muriana et al., 1992). On the contrary, in the Norambuena (2013) study on rainbow trout, a positive effect was shown in expression of *d6fad* and *elovl5* and resulted in the modification of the whole-body fatty acid composition in fish fed a high cholesterol diet. In the present study, supplementation of cholesterol did not affect the expression of any genes in either tissue evaluated, liver and muscle, but there was an increasing trend in the gene expression of elongase and desaturase with cholesterol supplementation when linseed oil was provided above 50%. Had the study contained a higher level of cholesterol, this difference may have increased to a statistically significant level.

In conclusion, the results of the present study showed that: 1) A plant-based diet without added cholesterol resulted in a reduction in growth and induced a hypocholesterolemic effect in the plasma of juvenile rainbow trout; 2) fish fed 100% of linseed oil with cholesterol supplementation had significantly higher weight gain and feed intake compared with other plant-based diets; 3) cholesterol supplementation numerically increased EPA and DHA levels in fish fillet when linseed oil was provided above 50%; and 4) growth performance in trout fed an all-plant diet high in soy protein (35%) can be achieved by supplementing with cholesterol and using a plant oil high in α-linolenic acid (ALA, 18:3n-3).

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Table 2.1. Ingredient and nutrient composition of the experimental diets fed to rainbow trout juveniles (%, as-fed basis)

Ingredients (%)	Diets											
	FM/FO	PM/FO	SO100	SO100 +C	SO75 LO25	SO75 LO25 +C	SO50 LO50	SO50 LO50 +C	SO25 LO75	SO25 LO75 +C	L100	L100 +C
Fishmeal, sardine	30.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Poultry by-product meal	8.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Blood meal, spray dried	2.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Corn protein concentrate	14.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00
Soybean meal	0.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00
Soy protein concentrate	5.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00
Wheat gluten meal	5.97	11.60	11.60	11.60	11.60	11.60	11.60	11.60	11.60	11.60	11.60	11.60
Wheat flour	17.90	6.60	6.60	6.46	6.60	6.46	6.60	6.46	6.60	6.46	6.60	6.46
L-Lysine HCL	0.00	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99
DL-methionine	0.00	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
L-Threonine	0.00	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Vitamin premix 702[1]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Choline chloride	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60
Stay-C (vitamin C, 35%)	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Trace mineral premix[2]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Dicalcium phosphate	0.00	4.43	4.43	4.43	4.43	4.43	4.43	4.43	4.43	4.43	4.43	4.43
Fish oil	15.20	19.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Soybean oil	0.00	0.00	19.00	19.00	14.30	14.30	9.51	9.51	4.76	4.76	0.00	0.00
Linseed oil	0.00	0.00	0.00	0.00	4.76	4.76	9.51	9.51	14.30	14.30	19.00	19.00
Cholesterol	0.00	0.00	0.00	0.14	0.00	0.14	0.00	0.14	0.00	0.14	0.00	0.14
Composition (%, as fed basis)												
Dry matter (%)	92.2	92.5	92.7	92.9	92.1	92.5	92.7	91.8	92.9	91.7	94.3	92.1
Crude protein (%)	47.5	48.1	48.2	48.8	47.9	48.0	47.9	47.1	47.8	47.4	48.1	47.4
Crude fat (%)	21.5	21.3	21.2	21.2	21.4	21.5	21.3	21.3	21.3	21.3	21.3	21.6
Ash (%)	6.79	6.15	5.99	5.86	5.82	6.10	5.93	5.97	5.87	5.75	6.03	5.82
Gross energy (MJ/kg)	22.5	22.5	22.4	22.5	22.4	22.5	22.8	22.3	22.5	22.5	22.7	22.2

Table 2.1 Cont.

Cholesterol (mg/kg)	2040	1430	55	1290	89	1230	93	1220	106	1270	92	1150

[1]Vitamin premix supply the following to the diet (mg/kg diet): D calcium pantothenate, 46.47; pyridoxine (pyridoxine HCl), 13.68; riboflavin, 9.58; niacinamide, 21.78; folic acid, 2.49; thiamine (thiamine mononitrate), 9.1; inositol, 599; biotin, 0.33; vitamin B_{12}, 0.03; menadione sodium bisulfite complex, 1.1; vitamin E (DL α-tocopherol acetate), 131.9 IU; vitamin D_3 (stabilized), 6594 IU; vitamin A (vitamin A palmitate, stabilized), 9641 IU; ethoxyquin, 198.

[2] Trace mineral premix supply the following to the diet (mg/kg diet): Zn (as $ZnSO_4$ $7H_2O$), 50; Mn (as $MnSO_4$), 7.5; Cu (as $CuSO_4$ $5H_2O$), 2.5; I (as KIO_3), 1; selenium, 0.05.

[*]FM, Fish meal; FO, Fish oil; PM, Plant meal; SO, Soybean oil; LO, Linseed oil; C, Cholesterol.

Table 2.2. Fatty acid composition (% of total fatty acids) of the experimental diets

Fatty acids (%)	Diets											
	FM/FO	PM/FO	SO100	SO100 +C	SO75 LO25	SO75 LO25 +C	SO50 LO50	SO50 LO50 +C	SO25 LO75	SO25 LO75 +C	L100	L100 +C
C14:0	4.30	4.23	0.22	0.08	0.09	0.08	0.07	0.07	0.06	0.06	0.04	0.06
C16:0	14.2	12.7	10.2	9.94	9.00	9.01	7.79	7.86	6.70	6.57	5.43	5.59
C18:0	3.08	2.62	3.53	3.52	3.50	3.52	3.45	3.49	3.47	3.38	3.17	3.48
C16:1n-7	4.68	4.30	0.28	n.d.	0.09	n.d.	0.09	0.10	n.d.	n.d.	0.08	0.08
C18:1n-7	3.16	3.27	1.20	1.11	1.01	1.01	0.89	0.89	0.79	0.76	0.63	0.68
C18:1n-9	16.1	14.3	18.2	18.1	18.2	18.3	18.2	18.4	18.5	18.2	17.8	18.8
C18:2n-6	6.86	6.51	48.8	49.5	41.4	41.9	33.0	33.3	24.7	24.7	19.6	16.4
C18:3n-6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C18:3n-3	1.02	1.12	6.87	7.01	15.2	15.8	25.2	25.2	35.2	35.6	43.3	45.1
C20:5n-3	6.59	6.79	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C22:6n-3	4.98	5.32	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total SFA[1]	22.7	20.6	15.9	15.5	14.5	14.6	13.4	13.3	12.2	11.8	10.3	11.1
Total MUFA[2]	24.0	22.0	19.7	19.2	20.5	20.4	19.2	19.4	19.3	18.9	18.5	19.5
Total n-3 PUFA[3]	12.6	13.2	6.9	7.0	15.2	15.8	25.2	25.2	35.2	35.6	43.3	45.1
Total n-6 PUFA[4]	6.86	6.51	48.8	49.5	41.4	41.9	33.0	33.3	24.7	24.7	19.6	16.4
Total n-3/n-6 PUFA[5]	1.84	2.03	0.14	0.14	0.37	0.38	0.76	0.76	1.43	1.44	2.22	2.75

[1] Sum of saturated fatty acids, includes C10:0, C11:0, C12:0 and C13:0.

[2] Sum of monounsaturated fatty acids, includes C14:1 and C20:1n-9.

[3] Sum of omega-3 polyunsaturated fatty acids

[4] Sum of omega-6 polyunsaturated fatty acids

[5] Ratio of total omega-3 polyunsaturated fatty acids to total omega-6 polyunsaturated fatty acids.

Table 2.3. Sterol concentration of the experimental diets

Phytosterols (mg/kg)	Diets											
	FM/FO	PM/FO	SO100	SO100 +C	SO75 LO25	SO75 LO25 +C	SO50 LO50	SO50 LO50 +C	SO25 LO75	SO25 LO75 +C	L100	L100 +C
Brassicasterol	20	20	0	0	0	10	0	10	10	10	10	10
Campesterol	190	200	320	370	350	370	370	400	380	410	420	420
Campestanol	70	80	80	80	80	80	80	80	80	80	90	90
Stigmasterol	40	60	150	160	150	140	140	130	130	120	120	110
Unidentified sterols	30	30	40	50	40	50	50	50	50	50	50	50
Sitosterol	500	660	960	1000	1030	980	1070	1030	1080	1070	1130	1080
Sitostanol+ delta-5-avenasterol	210	270	290	290	320	190	350	320	370	350	430	380
Delta-5,24-stigmastadienol	10	10	20	20	20	20	30	20	30	30	20	20
Delta-7-stigmastenol	10	20	40	50	40	40	40	40	40	40	40	30
delta-7-Avenasterol	10	30	40	50	40	40	40	40	40	40	40	40
Total plant sterols + plant stanols	1090	1380	1940	2070	2070	1920	2170	2120	2210	2200	2350	2230
Cholesterol	2040	1430	55	1290	89	1230	93	1220	106	1270	92	1150

Table 2.4. Growth performance and feed utilization of rainbow trout juveniles fed all 12 experimental diets for 12 weeks[1]

	Diets											
	FM/FO	PM/FO	SO100	SO100 +C	SO75 LO25	SO75 LO25 +C	SO50 LO50	SO50 LO50 +C	SO25 LO75	SO25 LO75 +C	L100	L100 +C
Initial weight (g)	18.6±0.10	18.8±0.30	18.9±0.29	18.8±0.30	18.8±0.23	18.8±0.23	18.8±0.20	18.9±0.25	18.9±0.20	18.8±0.18	18.8±0.22	18.7±0.13
Final weight (g)	220±0.30^{ab}	222±1.30^{a}	211±2.44^{c}	212±0.69^{c}	209±1.26^{c}	211±1.73^{c}	211±2.70^{c}	210±2.84^{c}	210±1.40^{c}	210±2.59^{c}	213±2.00^{bc}	220±2.45^{ab}
Weight gain (g/fish)	201±0.26^{a}	203±1.26^{a}	192±2.15^{b}	194±0.81^{b}	190±1.21^{b}	193±1.50^{b}	192±2.57^{b}	191±2.60^{b}	191±1.30^{b}	191±2.48^{b}	195±1.86^{b}	201±2.42^{a}
SGR (%/day)	2.97±0.01^{a}	2.97±0.02^{a}	2.91±0.01^{c}	2.92±0.02^{c}	2.90±0.01^{c}	2.91±0.01^{c}	2.91±0.01^{c}	2.90±0.00^{c}	2.90±0.01^{c}	2.91±0.01^{c}	2.93±0.01^{bc}	2.97±0.01^{ab}
Feed intake (g/fish)	190±2.83^{a}	181±1.65^{ab}	174±3.55^{b}	174±3.48^{b}	171±1.92^{b}	172±4.66^{b}	177±0.68^{b}	175±8.22^{b}	172±3.10^{b}	173±2.73^{b}	177±5.05^{b}	192±4.13^{a}
FCR	0.94±0.01	0.89±0.01	0.90±0.01	0.90±0.01	0.90±0.00	0.89±0.02	0.92±0.01	0.91±0.04	0.90±0.02	0.91±0.02	0.91±0.01	0.96±0.02
Survival (%)	98.7±1.89	96.0±0.00	97.3±1.89	94.7±4.99	100±0.00	98.7±1.89	97.3±3.77	97.3±1.89	100±0.00	93.3±4.99	97.3±1.89	97.3±1.89

[1]Mean±SE (n=3) in the same row that share the same superscript are not statistically different (*P*> 0.05; Completely Randomized Design, One-way ANOVA; Tukey's HSD test).

Table 2.5. Whole-body proximate composition (%, wet basis) of rainbow trout juveniles fed experimental diets for 12 weeks[1]

	Diets											
	FM/FO	PM/FO	SO100	SO100 +C	SO75 LO25	SO75 LO25 +C	SO50 LO50	SO50 LO50 +C	SO25 LO75	SO25 LO75 +C	L100	L100 +C
Proximate composition												
Dry matter (%)	32.1±0.96	33.0±0.75	32.1±0.51	32.8±0.29	32.1±0.22	32.3±0.14	32.1±0.73	31.8±0.99	32.2±0.85	32.8±0.84	32.7±0.45	33.3±0.31
Crude protein (%)	15.3±0.30	15.9±0.28	15.8±0.15	15.9±0.64	15.8±0.34	15.9±0.17	15.6±0.21	15.5±0.45	16.0±0.25	15.6±0.51	16.0±0.59	15.7±0.33
Crude fat (%)	14.1±0.89	14.4±0.70	13.9±0.64	14.3±0.51	13.8±0.21	14.0±0.39	13.9±0.47	13.7±1.37	13.9±0.81	14.6±0.85	14.6±0.78	15.2±0.10
Ash (%)	1.97±0.19	2.20±0.08	2.06±0.04	1.90±0.07	1.91±0.09	1.95±0.05	1.94±0.08	2.00±0.11	1.93±0.08	1.94±0.09	1.91±0.06	1.97±0.08
Gross energy (MJ/kg)	28.7±0.22	28.4±0.19	27.2±2.08	28.4±0.40	28.5±0.20	28.5±0.20	28.4±0.21	28.5±0.61	28.2±0.20	27.9±1.16	28.7±0.26	28.9±0.14

[1]Mean±SE (n=3); three fish from each tank were used for whole-body analysis. Proximate composition was not significantly different (*P*> 0.05; Completely Randomized Design, One-way ANOVA; Tukey's HSD test).

Table 2.6. Two-way ANOVA of growth performance and feed utilization of rainbow trout juveniles fed 10 experimental diets (w/o FM/FO, PM/FO) for 12 weeks[1].

Diets	Initial weight (g/fish)	FBW (g/fish)	WG (g/fish)	SGR (%/day)	Survival (%)	FI (g,DM/fish)	FCR
Means of main effects							
Cholesterol (mg/kg)							
0	18.8	211[b]	192[b]	2.91[b]	98.4	201	0.91
1430	18.8	213[a]	194[a]	2.92[a]	96.3	202	0.91
Soy oil / Linseed oil (%)							
100 / 0	18.8	211[b]	193[b]	2.91[b]	96.0	199[b]	0.90
75 /15	18.8	210[b]	191[b]	2.91[b]	99.3	196[b]	0.90
50 /50	18.8	210[b]	192[b]	2.91[b]	97.3	200[b]	0.92
25 / 75	18.6	210[b]	191[b]	2.91[b]	96.7	197[b]	0.90
0 / 100	18.8	217[a]	198[a]	2.95[a]	97.3	213[a]	0.93
Multi factors ANOVA (P Value)							
Cholesterol	0.794	**0.046**	**0.033**	**0.024**	0.111	0.573	0.367
Soy oil / Linseed oil	0.976	**<.001**	**<.001**	**<.001**	0.563	**<.001**	0.064
Cholesterol x Soy oil / Linseed oil	0.976	0.178	0.121	0.090	0.464	**0.042**	0.227

[1] Main effect means followed by a different letter are significantly different at $P < 0.05$, emphasized by bold *P values* in the ANOVA table.

Table 2.7. Plasma cholesterol content of rainbow trout juveniles fed experimental diets for 12 weeks[1,2]

	Diets											
	FM/FO	PM/FO	SO100	SO100 +C	SO75 LO25	SO75 LO25 +C	SO50 LO50	SO50 LO50 +C	SO25 LO75	SO25 LO75 +C	L100	L100 +C
Total Choelsterol												
Plasma (mmol / L)	8.28 ± 0.27^{ab}	7.72 ± 0.29^{abc}	5.79 ± 0.58^{bc}	7.67 ± 0.47^{abc}	5.47 ± 0.11^{c}	7.45 ± 0.37^{abc}	6.06 ± 0.11^{abc}	8.73 ± 1.18^{a}	6.24 ± 0.24^{abc}	7.59 ± 0.61^{abc}	6.11 ± 0.52^{abc}	8.73 ± 2.04^{a}

[1]Mean±SE (n=9 fish per treatment) in the same row that share the same superscript are not statistically different (*P*> 0.05; Completely Randomized Design, One-factor ANOVA; Tukey's HSD test).

[2]Three fish from each tank were used for chemical analysis.

Table 2.8. Fillet fatty acid composition (% of total fatty acids) of rainbow trout juveniles fed experimental diets for 12 weeks[1]

Fatty acids (%)	Diets											
	FM/FO	PM/FO	SO100	SO100 +C	SO75 LO25	SO75 LO25 +C	SO50 LO50	SO50 LO50 +C	SO25 LO75	SO25 LO75 +C	L100	L100 +C
C14:0	3.27 ± 0.12	2.94 ± 0.31	0.61 ± 0.13	0.48 ± 0.03	0.55 ± 0.07	0.43 ± 0.04	0.48 ± 0.10	0.42 ± 0.05	0.43 ± 0.05	0.34 ± 0.06	0.55 ± 0.21	0.54 ± 0.06
C16:0	15.8 ± 0.31	15.5 ± 0.67	14.2 ± 0.74	13.6 ± 0.26	13.1 ± 0.34	12.3 ± 0.02	11.9 ± 1.04	12.1 ± 0.52	10.7 ± 0.20	10.7 ± 0.24	10.6 ± 0.65	11.0 ± 0.28
C18:0	3.93 ± 0.03	3.23 ± 0.11	4.78 ± 0.13	4.91 ± 0.01	4.29 ± 0.17	4.81 ± 0.24	4.44 ± 0.15	4.46 ± 0.18	4.46 ± 0.09	4.03 ± 0.06	3.27 ± 0.12	4.06 ± 0.10
C16:1n-7	4.37 ± 0.07	3.61 ± 0.22	0.94 ± 0.22	0.74 ± 0.08	0.94 ± 0.01	0.97 ± 0.17	0.67 ± 0.11	0.91 ± 0.17	0.79 ± 0.07	0.70 ± 0.28	0.92 ± 0.12	0.79 ± 0.12
C18:1n-7	2.84 ± 0.06	2.75 ± 0.20	1.20 ± 0.00	0.99 ± 0.06	1.02 ± 0.02	1.00 ± 0.03	0.85 ± 0.04	0.86 ± 0.05	0.83 ± 0.02	0.76 ± 0.03	0.76 ± 0.03	0.72 ± 0.02
C18:1n-9	14.6 ± 0.42	13.6 ± 0.67	15.2 ± 1.30	16.2 ± 0.79	16.5 ± 0.17	17.3 ± 0.48	14.6 ± 1.44	15.5 ± 1.18	16.1 ± 0.39	14.4 ± 2.29	16.2 ± 0.22	14.5 ± 1.00
C18:2n-6	4.66 ± 0.10[g]	4.65 ± 0.09[g]	29.3 ± 1.24[ab]	33.0 ± 2.18[a]	27.4 ± 0.06[b]	29.6 ± 0.59[ab]	21.2 ± 2.74[c]	21.6 ± 1.59[c]	17.5 ± 0.46[cd]	16.4 ± 1.81[de]	12.0 ± 0.35[ef]	10.8 ± 0.83[f]
C18:3n-6	n.d.	n.d.	1.02 ± 0.09	1.01 ± 0.13	0.78 ± 0.03	0.70 ± 0.08	0.50 ± 0.02	0.49 ± 0.00	0.39 ± 0.01	0.33 ± 0.02	0.25 ± 0.03	0.24 ± 0.03
C18:3n-3	0.64 ± 0.04[f]	0.74 ± 0.02[f]	3.25 ± 0.17[e]	3.66 ± 0.16[e]	8.33 ± 0.18[d]	8.78 ± 0.50[d]	13.1 ± 1.62[c]	13.1 ± 1.13[c]	19.4 ± 0.78[b]	18.3 ± 1.52[b]	24.9 ± 0.52[a]	22.5 ± 1.06[a]
C20:5n-3	5.34 ± 0.21[a]	5.11 ± 0.24[a]	0.80 ± 0.10[e]	0.80 ± 0.13[e]	1.21 ± 0.06[de]	1.17 ± 0.07[de]	1.70 ± 0.18[cd]	1.74 ± 0.16[cd]	1.90 ± 0.19[c]	2.14 ± 0.19[c]	2.23 ± 0.12[bc]	2.79 ± 0.19[b]
C22:6n-3	12.8 ± 1.13[a]	13.3 ± 1.54[a]	4.35 ± 0.34[bcd]	3.68 ± 0.36[d]	5.18 ± 0.31[bcd]	4.05 ± 0.10[cd]	5.50 ± 0.59[bcd]	5.41 ± 0.62[bcd]	5.69 ± 0.39[bcd]	6.27 ± 0.75[bc]	5.40 ± 0.69[bcd]	6.72 ± 0.51[b]
Total SFA[2]	26.0 ± 0.24[a]	25.2 ± 1.04[ab]	23.3 ± 0.59[abc]	22.3 ± 0.32[bcd]	21.1 ± 0.43[cde]	20.4 ±0.28[cdef]	20.5 ± 1.50[cdef]	20.2 ± 1.05[def]	18.7 ± 0.23[ef]	19.0 ± 1.55[ef]	18.1 ± 0.31[f]	19.8 ± 0.43[ef]
Total MUFA[3]	21.9 ± 0.55[a]	20.1 ± 1.07[ab]	18.2 ± 0.66[b]	18.0 ± 0.69[ab]	18.2 ± 0.53[ab]	19.3 ± 0.68[ab]	16.2 ± 1.46[b]	17.3 ± 1.24[b]	17.7 ± 0.47[ab]	15.9 ± 2.58[b]	17.9 ± 0.34[ab]	16.1 ± 1.10[b]
Total n-3 PUFA[4]	18.7 ± 1.32[c]	19.2 ± 1.77[c]	8.40 ± 0.46[e]	8.13 ± 0.34[e]	14.7 ± 0.30[d]	14.0 ± 0.55[d]	20.3 ± 1.10[c]	20.3 ± 0.97[c]	27.0 ± 0.80[b]	26.7 ± 0.88[b]	32.6 ± 0.64[a]	32.0 ± 0.37[b]
Total n-6 PUFA[5]	5.09 ± 0.20[g]	5.91 ± 0.10[g]	36.1 ± 1.02[ab]	39.6 ± 2.36[a]	32.3 ± 0.32[b]	34.1 ± 0.67[b]	24.8 ± 2.42[cd]	25.3 ± 1.57[c]	20.2 ± 0.46[de]	17.8 ± 2.59[de]	12.7 ± 0.58[f]	11.6 ± 1.29[f]
Total n-3/n-6 PUFA[6]	3.68 ± 0.18[a]	3.24 ± 0.24[ab]	0.23 ± 0.01[f]	0.21 ± 0.02[f]	0.46 ± 0.01[ef]	0.41 ± 0.01[ef]	0.82 ± 0.04[e]	0.80 ± 0.05[e]	1.34 ± 0.03[d]	1.53 ± 0.20[d]	2.58 ± 0.08[c]	2.79 ± 0.30[bc]

[1] Mean±SE (n=9 fish per treatment) in the same row that share the same superscript are not statistically different (*P*> 0.05; Completely Randomized Design, One-factor ANOVA; Tukey's HSD test).

[2] Sum of saturated fatty acids, includes C10:0, C11:0, C12:0 and C13:0.

[3] Sum of monounsaturated fatty acids, includes C14:1 and C20:1n-9.

[4] Sum of omega-3 polyunsaturated fatty acids

[5] Sum of omega-6 polyunsaturated fatty acids

[6] Ratio of total omega-3 polyunsaturated fatty acids to total omega-6 polyunsaturated fatty acids.

Table 2.9. Two-way ANOVA of fillet fatty acid composition of rainbow trout juveniles fed 10 experimental diets (w/o FM/FO, PM/FO) for 12 weeks[1]

Diets	C18:2n-6	C18:3n-3	C20:5n-3	C22:6n-3	Total n-3 PUFA	Total n-6 PUFA
Means of main effects						
Cholesterol (mg/kg)						
0	21.5	13.8	1.57^{b}	5.22	20.6	25.2
1430	22.3	13.3	1.73^{a}	5.22	20.2	25.7
Soy oil / Linseed oil (%)						
100 / 0	31.1^{a}	3.45^{e}	0.80^{d}	4.01^{c}	8.28^{e}	38.0^{e}
75 /15	28.5^{a}	8.55^{d}	1.19^{c}	4.61^{bc}	14.4^{d}	33.2^{d}
50 /50	21.4^{b}	13.1^{c}	1.72^{b}	5.46^{ab}	20.3^{c}	25.0^{c}
25 / 75	17.0^{c}	18.9^{b}	2.02^{b}	5.98^{a}	26.9^{b}	19.0^{b}
0 / 100	11.4^{d}	23.7^{a}	2.51^{a}	6.06^{a}	32.3^{a}	12.1^{a}
Multi factors ANOVA (P Value)						
Cholesterol	0.245	0.205	**0.025**	1.000	0.229	0.430
Soy oil / Linseed oil	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**
Cholesterol x Soy oil / Linseed oil	0.109	0.174	**0.051**	**0.019**	0.972	0.070

[1] Main effect means followed by a different letter are significantly different at $P \leq 0.05$, emphasized by bold *P values* in the ANOVA table.

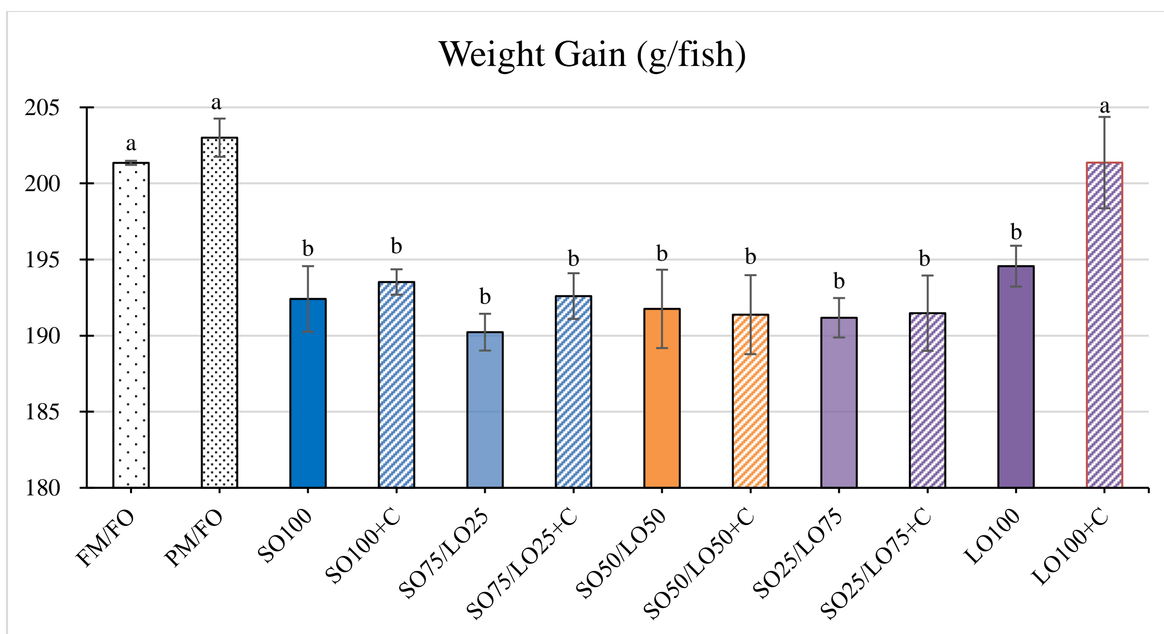


Figure 2.1. Weight gain (g/fish) of rainbow trout juveniles fed experimental diets for 12 weeks. Bars represent mean ± SE of triplicate samples.

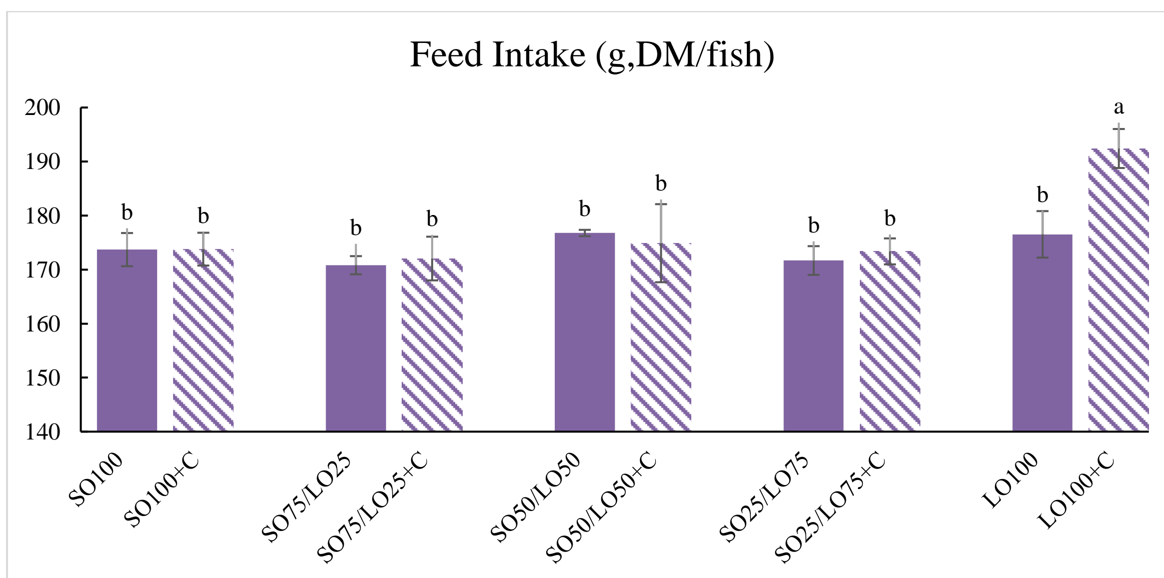


Figure 2.2. Feed intake (g, DM/ fish) of rainbow trout juveniles fed experimental diets for 12 weeks. Bars represent mean ± SE of triplicate samples. Two-way ANOVA indicated a significant Cholesterol x Soy oil / Linseed oil ratio interation ($P < 0.05$).

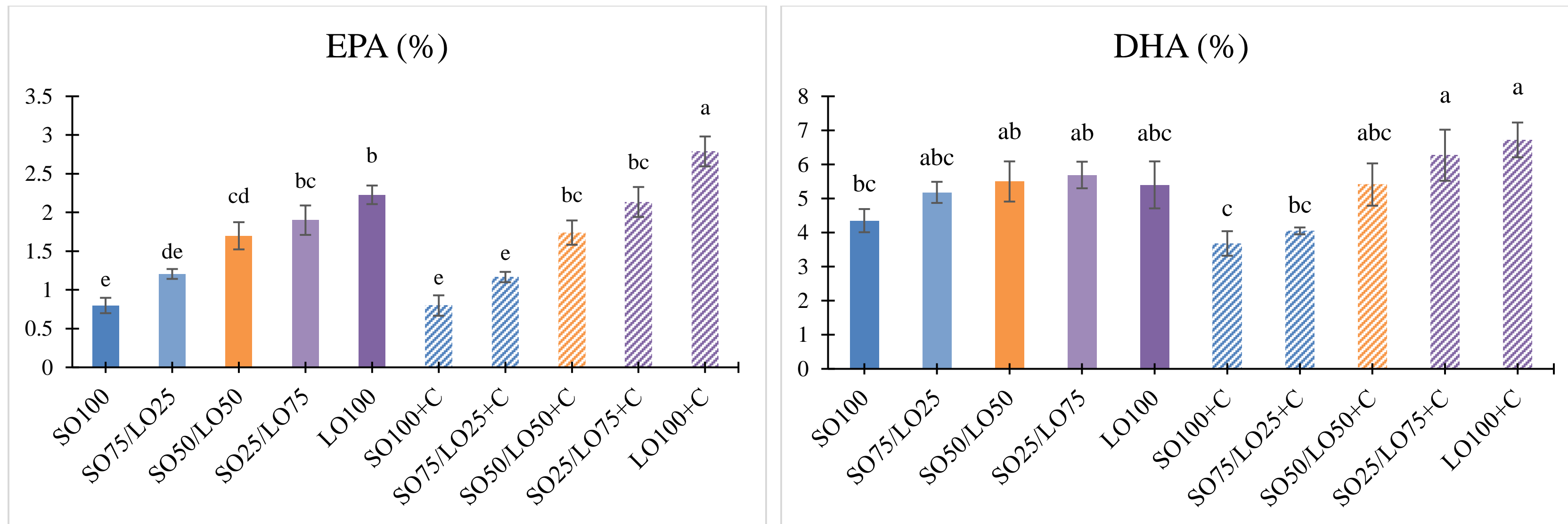
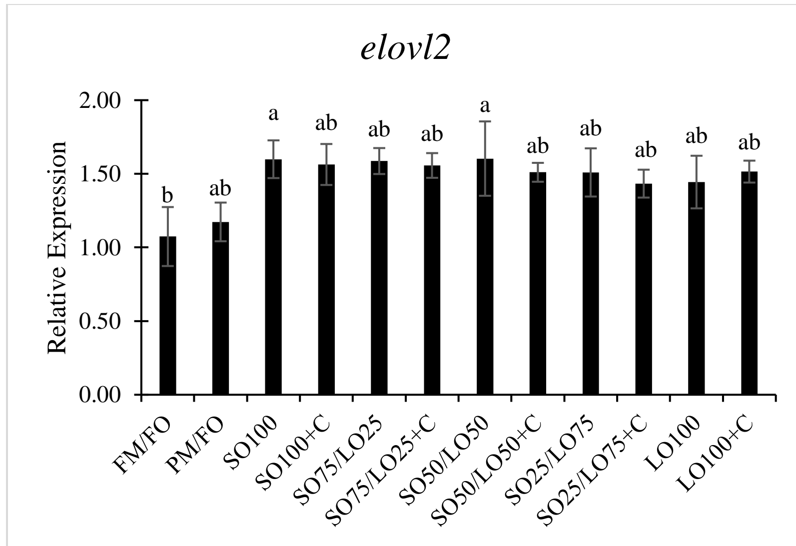


Figure 2.3. EPA and DHA contents in fillet of rainbow trout juveniles fed experimental diets for 12 weeks. Bars represent mean ± SE of triplicate samples. The interaction of the two main factors (Cholesterol x Soy/Linseed oil ratio) significantly affected EPA (P = 0.051) and DHA content in fish fillet ($P < .05$).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1.00
0.50
0.00
b
ab
a
ab
ab
ab
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ab
ab
FM/FO
PM/FO
SO100
SO100+C
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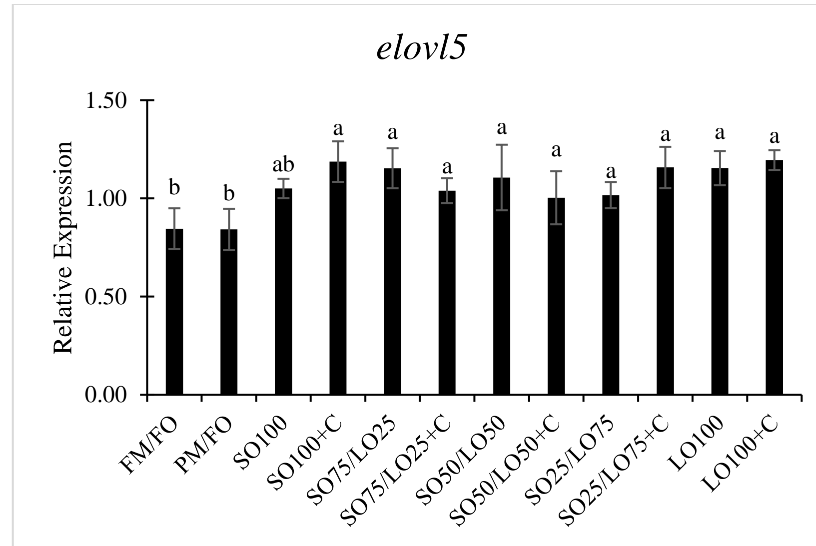
SO25/LO75
SO25/LO75+C
LO100
LO100+C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a
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a
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a
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a
FM/FO
PM/FO
SO100
SO100+C
SO75/LO25
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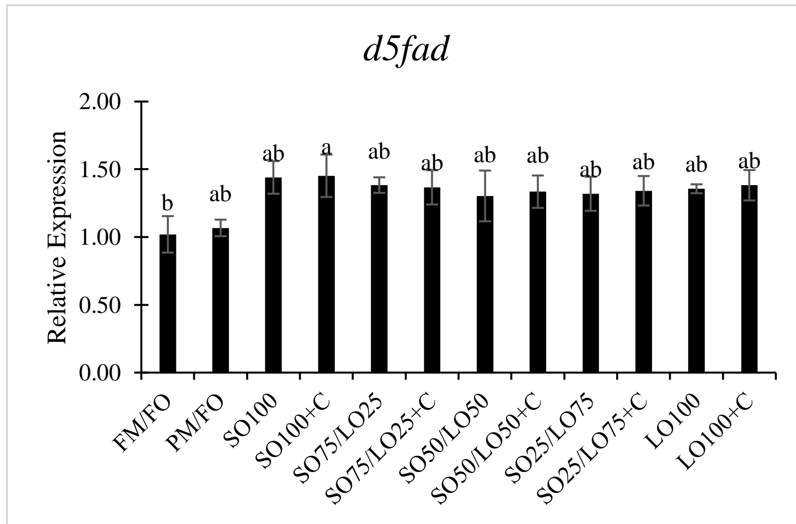
SO25/LO75
SO25/LO75+C
LO100
LO100+C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Relative Expression
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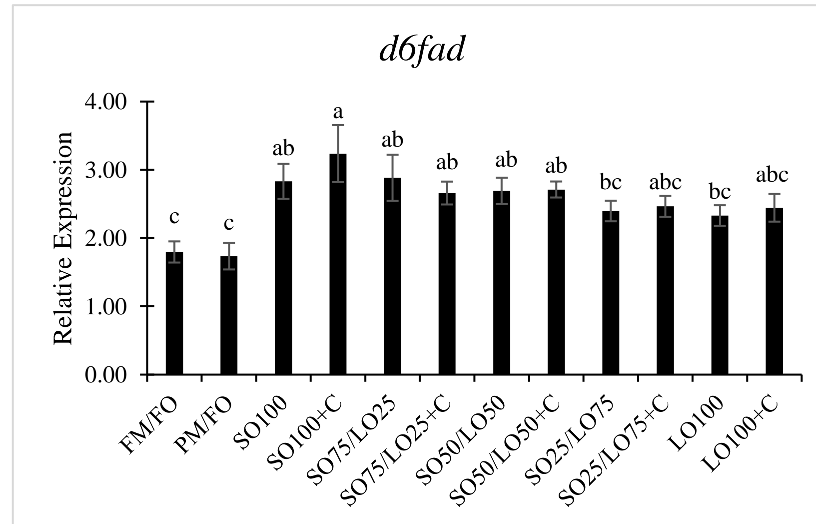
ab
ab
ab
ab
FM/FO
PM/FO
SO100
SO100+C
SO75/LO25
SO75/LO25+C
SO50/LO50
SO50/LO50+C
SO25/LO75
SO25/LO75+C
LO100
LO100+C


d6fad
Relative Expression
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3.00
2.00
1.00
0.00
c
c
ab
a
ab
ab
ab
ab
bc
abc
bc
abc
FM/FO
PM/FO
SO100
SO100+C
SO75/LO25
SO75/LO25+C
SO50/LO50
SO50/LO50+C
SO25/LO75
SO25/LO75+C
LO100
LO100+C

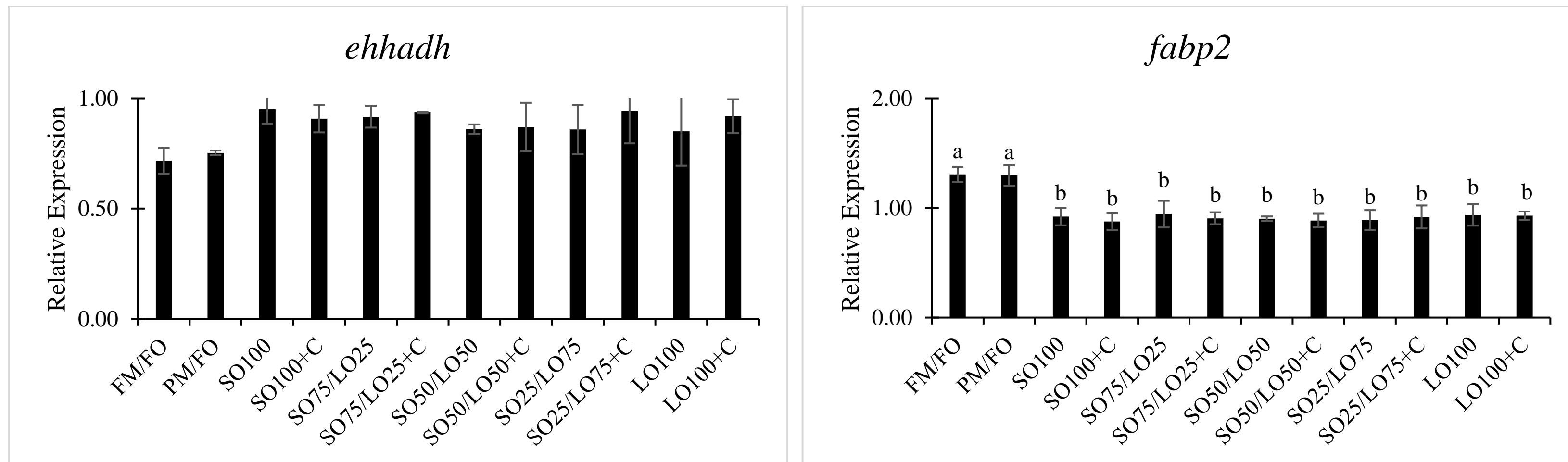
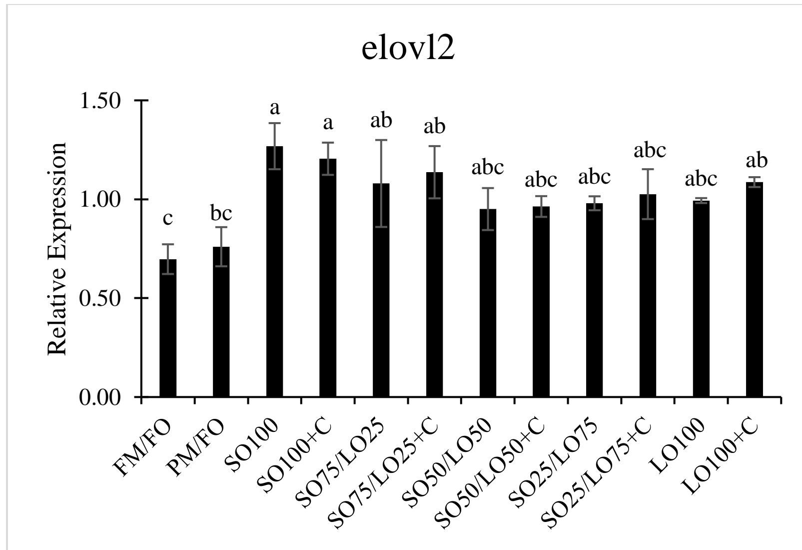


Figure 2.4. Relative mRNA expression of genes (normalized against *elf1α*) involved in elongase (*elovl2* and *elovl5*), desaturase (*d5fad* and *d6fad*), β-oxidation (*ehhadh*) and fatty acid transport (*fabp2*) of liver of rainbow trout juveniles fed experimental diets for 12 weeks. Mean ± SE (n=9 fish per treatment) in the same row that share the same superscript are not statistically different ($P > 0.05$; Completely Randomized Design, One-factor ANOVA; Tukey's HSD test). elovl2: Elongation of very long chain fatty acids-like 2, elovl5: Elongation of very long chain fatty acids-like 5, d5fad: Delta-5 fatty acid desaturase, d6fad: Delta-6 fatty acid desaturase, ehhadh: Enoyl-coa hydratase and 3-hydroxyacyl coa dehydrogenase), fabp2: Fatty acid binding protein-2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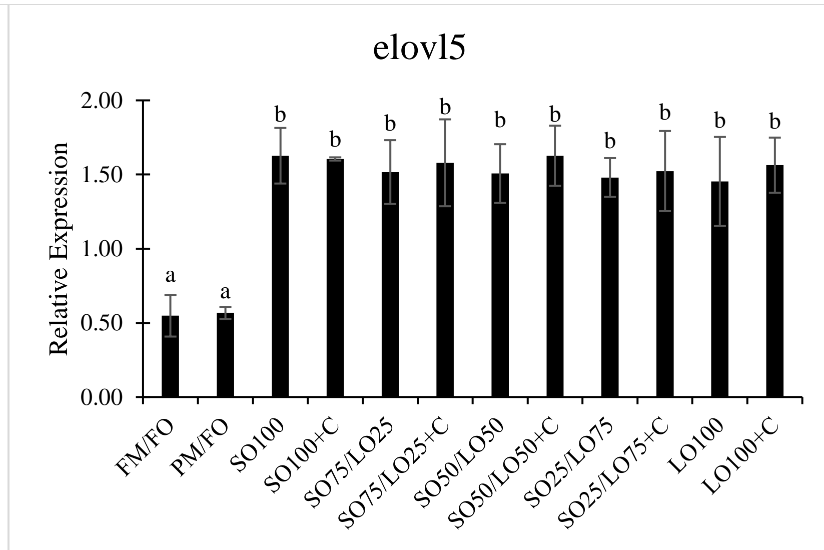
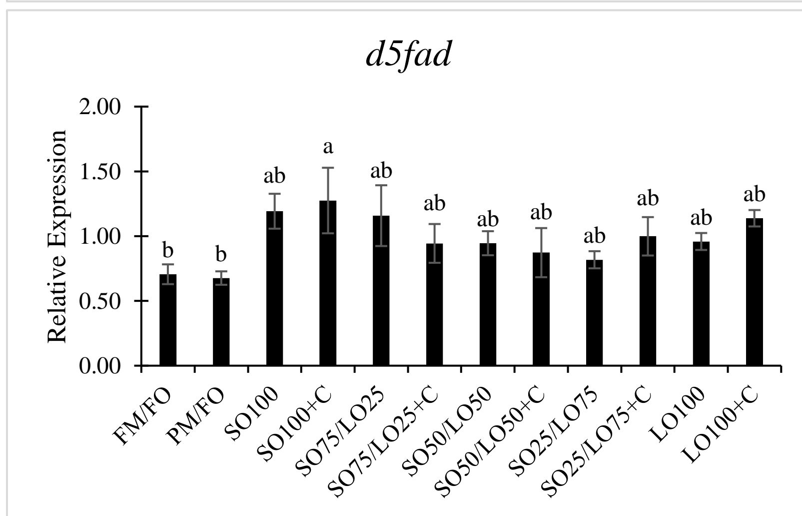
1.00
0.50
0.00
c
bc
a
a
ab
ab
abc
abc
abc
abc
abc
ab
FM/FO
PM/FO
SO100
SO100+C
SO75/LO25
SO75/LO25+C
SO50/LO50
SO50/LO50+C
SO25/LO75
SO25/LO75+C
LO100
LO100+C


elovl5
Relative Expression
2.00
1.50
1.00
0.50
0.00
a
a
b
b
b
b
b
b
b
b
b
b
FM/FO
PM/FO
SO100
SO100+C
SO75/LO25
SO75/LO25+C
SO50/LO50
SO50/LO50+C
SO25/LO75
SO25/LO75+C
LO100
LO100+C


d5fad
Relative Expression
2.00
1.50
1.00
0.50
0.00
b
b
ab
a
ab
ab
ab
ab
ab
ab
ab
ab
FM/FO
PM/FO
SO100
SO100+C
SO75/LO25
SO75/LO25+C
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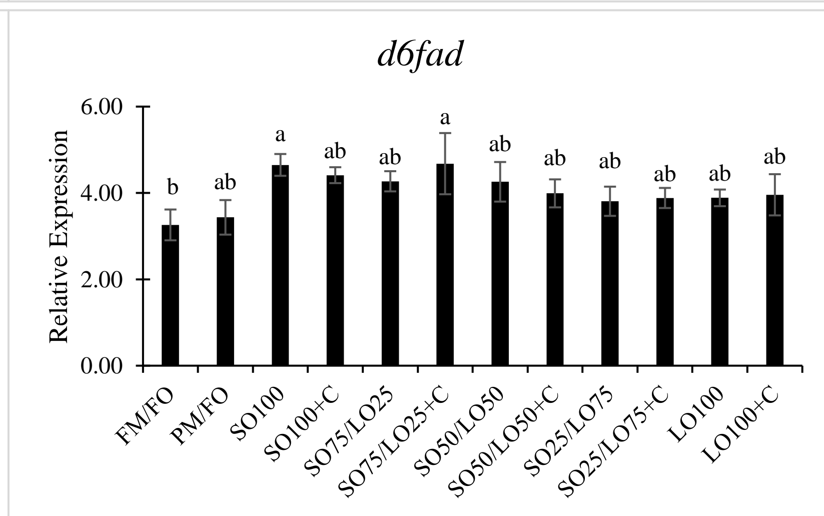
SO25/LO75
SO25/LO75+C
LO100
LO100+C


d6fad
Relative Expression
6.00
4.00
2.00
0.00
b
ab
a
ab
ab
a
ab
ab
ab
ab
ab
ab
FM/FO
PM/FO
SO100
SO100+C
SO75/LO25
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LO100
LO100+C

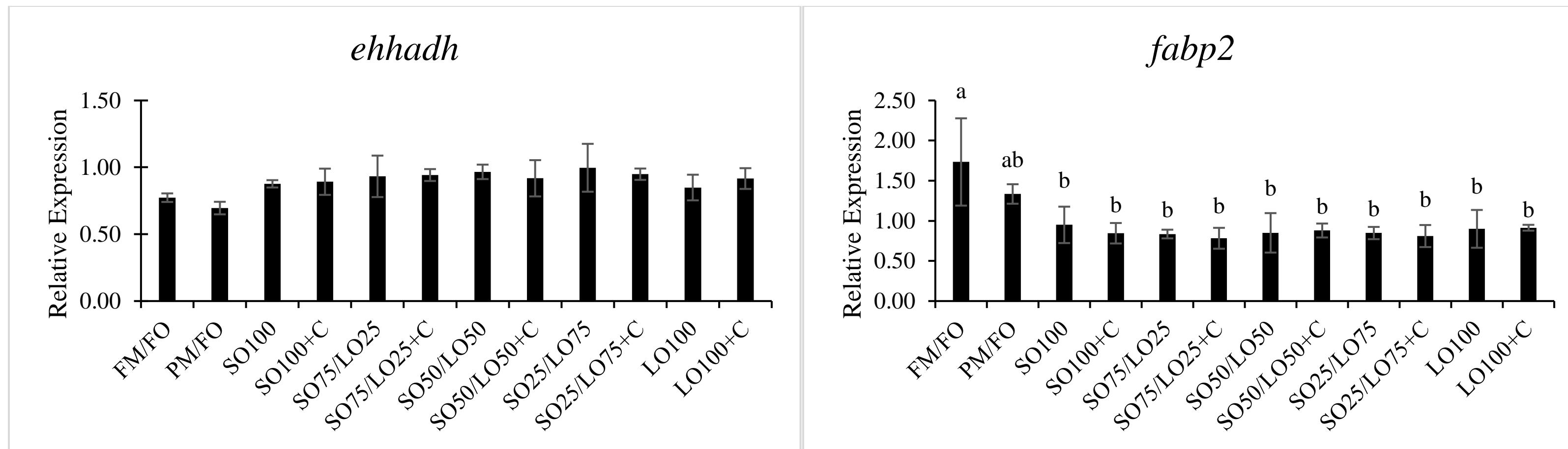

Figure 2.5. Relative mRNA expression of genes (normalized against *elf1α*) involved in elongase (*elovl2* and *elovl5*), desaturase (*d5fad* and *d6fad*), β-oxidation (*ehhadh*) and fatty acid transport (*fabp2*) of muscle of rainbow trout juveniles fed experimental diets for 12 weeks. Mean ± SE (n=9 fish per treatment) in the same row that share the same superscript are not statistically different ($P > 0.05$; Completely Randomized Design, One-factor ANOVA; Tukey's HSD test). elovl2: Elongation of very long chain fatty acids-like 2, elovl5: Elongation of very long chain fatty acids-like 5, d5fad: Delta-5 fatty acid desaturase, d6fad: Delta-6 fatty acid desaturase, ehhadh: Enoyl-coa hydratase and 3-hydroxyacyl coa dehydrogenase), fabp2: Fatty acid binding protein-2

Chapter 3:

Latitude™ Oil as a Sustainable Alternative to Dietary Fish Oil in Rainbow trout (*Oncorhynchus mykiss*): Effects on Filet Fatty Acid Profiles, Intestinal Histology, and Plasma Biochemistry

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Abstract

The aim of this study was to evaluate the effects of LatitudeTM oil (transgenic canola) fed to rainbow trout, Oncorhynchus mykiss, for 52 weeks on growth performance, non-specific immune responses, histology, and filet omega-3 fatty acid content. LatitudeTM oil (LO) has high lipid digestibility (93%), and contains omega-3 fatty acids eicosapentaenoic acid (EPA, C20:5n-3), docosapentaenoic acid (DPA, C22:5n-3), and docosahexaenoic acid (DHA, C22:6n-3). Three isonitrogenous (49%), isolipidic (20%) and isocaloric (24.2 MJ kg^{-1}) diets differing by lipid source (0, 8, or 16% LO, replacing fish oil and poultry fat) were fed over an entire production cycle beginning with 19 g juvenile fish. At the end of the 52-week feeding trial, final body weight, weight gain and specific growth rate of fish fed 8%LO (LO-8) and 16%LO (LO-16) diets were significantly higher than those fed the 0% LO (LO-0) diet ($P < 0.05$). Phagocytic respiratory burst in fish fed the LO-16 diet was significantly higher than those fish fed the other 2 diets ($P < 0.05$). There were no differences in superoxide dismutase, catalase and lysozyme. Histological examination of the distal intestine indicated reduced inflammation in fish fed the LO-8 diet but not the LO-0 and LO-16 diets. Filet DHA content of fish fed the LO-8 and LO-16 diets were similar to those of fish fed the LO-0 diet. As these diets had lower DHA content, this suggests dietary EPA and DPA from LO was converted to DHA and deposited in the filet. This is supported by increased expression of genes involved in fatty acid elongation, desaturation and beta oxidation in both liver and muscle of fish fed LO ($P < 0.05$). Total EPA+DHA content of the edible filet ranged between 1,079–1,241mg 100 g^{-1} across treatments, each providing the recommended daily intake for human consumption (500–1,000mg day^{-1}). Overall, this study demonstrated that LO fed over an entire production period is a highly digestible lipid source suitable and sustainable for meeting the fatty acid requirements of rainbow trout, as well as consumer expectations for filet omega-3 fatty acid content.

Introduction

Over the past decade, dramatic increases in fishmeal (FM) and fish oil (FO) prices have driven feed manufacturers across the aquaculture industry to lower the use of FM and FO in aquafeed for virtually all farmed fish species. For salmonid diets, this has meant a reduction of marine ingredients in the diet by as much as 60% (Ytrestøyl et al., 2015). The transition away from marine ingredients toward plant-based ingredients has afforded the industry the ability to increase production while reducing feed costs and the impact of aquaculture on wild fisheries. However, it is not without costs, in that it has resulted in a substantial reduction in the levels of omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA), specifically eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) in fish tissues. Therefore, it is significantly vital to produce more sustainable oil sources that can be used to meet the increasing demand for oil ingredients high in n-3 LC-PUFA.

Marine microalgae that are able to synthesize EPA and DHA directly are the world's primary producers of these fatty acids, which are then accumulated through the aquatic food webs. For this reason, fish is considered the primary dietary source of n-3 LC-PUFA, EPA and DHA for humans (Betancor et al., 2017; Osmond and Colombo, 2019). Many health agencies worldwide recommend 500–1000mg day^{-1} of total EPA + DHA for reducing cardiovascular disease (Aranceta and Pérez-Rodrigo, 2012).

There is considerable interest in novel sources of n-3 LCPUFA to supplement the limited supplies of fish oil. These sources include othermarine organisms such as krill or copepods, fermentation of algae, and the genetic engineering of microbes such as yeasts (Napier et al., 2019; Betancor et al., 2021).However, these technologies pose economic challenges formass production (Betancor et al., 2017; Tocher et al., 2019; Fabris et al., 2020). Recently, genetically modified canola oil has emerged as an alternative and sustainable oil source for aquafeed. Transgenic canola oil contains arachidonic acid (ARA, C20:4n-6) (~2.3%), EPA (~9.1%), docosapentaenoic acid (DPA, C22:5n-3) (~2.3%) and DHA (~0.9%), which is uncommon in other terrestrial oils. This oil has recently been used as a component of a terrestrial oil blend (40% transgenic canola oil) in rainbow trout (*Oncorhynchus mykiss*) and has shown no adverse effects on growth performance (Hossain et al., 2021).

DPA is an indispensable n-3 LC-PUFA found in large amounts in FO, and even larger amounts in salmon filets (0.4 g 100 g^{-1} filet) (Calder, 2018; Lozano-Muñoz et al., 2020). DPA has attracted recent attention due to its importance in human health. DPA has been found to be a crucial component of phospholipids found in animal cell membranes and shown to lower plasma cholesterol levels (Kaur et al., 2016; Drouin et al., 2019). In addition, it has been reported that DPA reduces the expression of inflammatory genes (Backes et al., 2016). DPA is an intermediate between EPA and DHA in the n-3 LC-PUFA pathway, which may act as a reservoir for EPA and DHA (Dyall, 2015) and is of current interest for its putative capacity to either be converted to DHA or retro-converted to EPA (Drouin et al., 2019). In vertebrates, the synthesis of DHA from ALA requires three desaturation and three elongation steps (Sprecher, 2000). First, synthesis of EPA from a-linolenic acid (ALA, C18:3n-3) is achieved by *Δ6fad* to produce C18:4n-3 that is elongated to C20:4n-3 followed by *Δ5fad*. Then, *elovl2* and *elovl5* are required for elongation of EPA to DPA. Next, DPA is elongated to C24:5n-3 by *elovl2* and *elovl5*, then desaturated to 24:6n-3 by *Δ6fad*, and finally converted to DHA by a peroxisomal chain shortening step (Li et al., 2010; Gregory and James, 2014).

Rainbow trout (*Oncorhynchus mykiss*) is a commercially important salmonid species and an experimental model species since this species requires LC-PUFAs due to limited ability to convert ALA to EPA or DHA. The total n-3 LC-PUFA dietary requirements of rainbow trout and Atlantic salmon, including ALA, EPA, and DHA has been reported to range from 0.4 – 2.0% of the diet (NRC, 2011). These estimates were determined based on the amount required to prevent classical deficiency and nutritional pathology, and many of these studies were performed on small fish fed diets low in lipid for relatively short periods of time (Tocher, 2015). However, it has been proposed that modern high-energy (lipid) diets and higher fish growth rates necessitate a reassessment of these requirements (NRC, 2011), suggesting that higher requirements for essential fatty acids (EFA) may now exist to support faster growth and optimumhealth over the entire production cycle of the fish (Tocher, 2015; Bou et al., 2017). Furthermore, some suggest the requirement should not be set only for optimal fish growth and health, but also to meet the daily recommended n-3 LC-PUFA intake for human consumers (Simopoulos, 2000; Tocher, 2009).

The aim of this study was to evaluate a new transgenic canola oil containing EPA, DPA and DHA as a substitute for FO in rainbow trout feeds reflecting current commercial feed formulation in terms of growth performance, health and n-3 LC-PUFA composition over a complete production cycle, from fingerling to market weight. We hypothesized that Latitude oil (LO) containing high EPA and DPA would effectively replace fish oil in trout feeds without detrimental impact on fish performance. Furthermore, we hypothesized that filet EPA+DHA level of fish fed LO-8 or LO-16 would be similar to filet EPA + DHA content of fish fed LO-0 diet meeting or exceeding the recommended daily intake of EPA + DHA for consumers. To our knowledge, there are no reported studies on transgenic canola oil throughout the entire production cycle in rainbow trout. The ultimate objective was to formulate feeds that not only support early growth, but improve long-term health and survival of the fish, and result in a product that meets the nutritional needs of consumers while being sourced from more environmentally and economically sustainable sources.

Materials and Methods

Experimental Design and Diets

Three experimental diets were prepared and extruded (Bozeman Fish Technology Center, Bozeman, MT) in various sizes from 2.5 to 4.5mm, and were formulated to be isonitrogenous (49% crude protein), isolipidic (20% crude lipid) and isocaloric (24.2 MJ kg^{-1}): a control diet (FO 6.43%, Poultry fat 9.57%) and two experimental diets that replace FO by 50 or 100% with LO. All three diets were formulated to reflect commercial feed formulations for rainbow trout and thus included 20% FM. Poultry fat and LO were gifted by Tyson and Cargill, respectively. The three experimental diets were formulated to maintain 3% EPA + DHA content (% of the diet). To accomplish this, the following proportion of the oils were used, LO-0 (6.43% FO + 9.57% Poultry fat), LO-8 (3.21% FO + 4.79% Poultry fat + 8% LO) and LO-16 (16% LO). The proximate and fatty acid composition of experimental diets are shown in Tables 3.1, and 3.2. Although formulated to maintain 3% EPA+DHA content, diets ranged from 2.78–3.32% of diet.

In vivo digestibility was determined for LO fed to rainbow trout. A reference diet containing practical ingredients and 0.1% yttrium oxide was prepared. A batch of test diet containing 20% test ingredient and 80% reference diet mash (combined on a dry matter

basis) was prepared and analyzed. All ingredients for the digestibility trial were mixed and cold pelleted at the University of Idaho's Hagerman Fish Culture Experiment Stations (HFCES) using a laboratory-scale California pellet mill fitted with a 4-mm die. After 36h drying in a hot-air dryer at 37◦C, the feeds were stored at ambient temperature (20–22◦C) until fed.

Experimental Fish and Feed Trial

Rainbow trout fingerlings were hatched from eggs obtained from a global aquaculture supplier. Rainbow trout juveniles (initial body weight: 18.5 ± 0.3 g) were randomly stocked into each of nine, 145-L tanks at 40 fish per tank. Constant temperature spring water (15°C) was supplied at 8–10L min−1 to each experimental tank. Each diet was assigned randomly to three tanks in a completely randomized design. Fish were hand-fed to apparent satiation three times per day, 6 days per week for 24 weeks. Photoperiod was maintained at 14 h light: 10 h dark with fluorescent lights controlled by electric timers. At week 18, one of the tanks from Diet 2 was removed from the study due to a valve failure resulting in a period of no incoming water flow overnight followed by poor fish performance and symptoms consistent with bacterial gill disease, reducing this treatment to two replicate tanks. After 24 weeks, all fish were moved to an outdoor facility and stocked into each of eight, 1,300-L tanks for another 28 weeks (52 weeks total) under natural light from the 10th January 2020 to the 29th July 2020.

Sample Collection and Proximate Analysis

At the end of 52 weeks, 24-h postprandial, all the fish were counted and weighed to calculate weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR) and survival. After the final weighing, three fish per tank were anesthetized with tricaine methanesulfonate (MS-222, 100mg L^{-1}, buffered to pH 7.0). Then, individual body weight and length of fish was measured, and condition factor (CF) was calculated. Whole blood was collected from the caudal vasculature of fish identified above with 1-ml heparinized syringes fitted with a 24G 1.5- inch needle and centrifuged at 1000 x G for 10min to collect plasma for antioxidant enzyme activity and non-specific immune parameters. Upon euthanizing those fish with additional MS- 222, liver and viscera from the same fish used for plasma

collection were dissected for gene expression, fatty acid analysis, and proximate analysis and weighed individually to calculate hepatosomatic index (HSI) and viscerosomatic index (VSI). From the same fish, liver and distal intestine were excised for histology. Another three fish per tank were euthanized for whole-body proximate analysis. Tissue samples were snap-frozen in liquid nitrogen and stored at −80℃ until analysis.

Experimental feeds, liver, muscle, and whole-body fish samples were analyzed for proximate composition and energy content. Fish samples were pooled by tank and homogenized using an industrial food processor. Samples were dried in a convection oven at 105℃ for 12 h to determine moisture level according to AOAC (Association of Official Analytical Chemists) (2000). Dried samples were finely ground by mortar and pestle and analyzed for CP (total nitrogen × 6.25) using combustion method with a nitrogen determinator (Elementar nitrogen analyzer, Ronkonkoma, NY). Crude lipid was analyzed by subjecting samples to acid hydrolysis using an ANKOM HCL (ANKOM Technology, Macedon, NY) and extracting them with petroleum ether using an ANKOM XT15 extractor. Ash was analyzed by incineration at 550℃ in a muffle furnace for 5h. The energy content of samples was determined using an isoperibol bomb calorimeter (Parr 6300, Parr Instrument Company Inc., Moline, IL).

The fatty acid composition of the liver and filet samples were determined in line with the modified AOAC method 991.39. Briefly, samples were dried for 5–6 h under an N2 stream at 50℃ (OA-SYS heating system, Organomation Associates, Inc., Berlin, MA, USA). Thereafter, 2mL of 0.5N NaOH was added for sample saponification at 70◦C for 60min. Following sample cooling, the free fatty acids were methylated by the addition of 2mL 14% BF3 (Boron trifluoride) in methanol and incubated at 70℃ for 60min. After the samples were allowed to cool, 2mL of hexane was added, inverted repeatedly for 60 s, and 1mL of saturated NaCl was added. The samples were again inverted repeatedly for 60 s and then centrifuged at 2000 × G for 5min. An aliquot (100 µL) of the clarified hexane extract was diluted in hexane (1:10) and put into autosampler vials for gas chromatography/mass spectroscopy (GC/MS) analysis. The injection mode with a helium flow rate and the column temperature was as described by Overturf et al. (2013). All the analyses were done in

duplicate. Filet results are provided as mg 100 g^{-1}, assuming a 100 g portion size for human consumption.

Antioxidant and Non-specific Immune Assays

Oxidative radical production by phagocytes during respiratory burst was measured through nitro blue tetrazolium (NBT) assay described by Anderson and Siwicki (1995). Briefly, whole-blood and NBT (0.2%) (Sigma-Aldrich, St. Louis, MO, USA) were mixed in equal proportion (1:1) and incubated for 30min at room temperature. Then 50 μL was taken out and dispensed into glass tubes. One milliliter of dimethylformamide (Sigma-Aldrich) was added and centrifuged at 2000 × G for 5min. Finally, the optical density of supernatant was measured at 540 nm using a microplate reader (InfiniteR m200 PRO, Tecan Trading AG, Switzerland). Dimethylformamide was used as the blank.

Commercially available kits (Cayman Chemical, Ann Arbor, Michigan) were used to measure superoxide dismutase (SOD) (Itemno: 706002) and catalase (CAT) (Item no: 707002) activities at 25◦C. SOD activity was determined at 450 nm based on xanthine and xanthine oxidase to produce superoxide radicals. SOD activity was measured based on the inhibition rate of this reaction. One unit of SOD activity is equal to 50% inhibition of decrease in 2-(4-iodophenyl)-3-(4nitrophenole)- 5-phenyltetrazolium chloride according to the experimental conditions. CAT activity was measured by determining the decrease in absorbance of H_2O_2 at 540 nm. The reaction mixture containing 50mM K-phosphate buffer (pH 6.5) and 50mM H_2O_2 was diluted in 80mM K-phosphate buffer (pH 6.5). Calculation of activity was done by determining the extinction coefficient for H_2O_2 (a = 40 M^{-1} cm^{-1}).

Lysozyme activity in plasma was analyzed with a lysozyme assay kit (Sigma-Aldrich). Micrococcus lysodeikticus (0.75mg mL^{-1}) was suspended in phosphate buffer (0.1 M, pH 6.24), 800 uL of suspension was placed in each well of 48-well plates, and 30 μL plasma was added subsequently. The reduction in absorbance of the samples was recorded at 450 nm after incubation at room temperature for 0 and 30min in a microplate reader (Infinite® m200 PRO, Tecan Trading AG, Switzerland). A reduction in absorbance of 0.001 min^{-1} was regarded as one unit of lysozyme activity.

Histological Analysis

Tissue samples, about 1 cm in length, from anterior section of the distal intestine and liver were fixed in 10% neutral buffered formalin. All samples were processed by increasing dehydration step, cleared in xylene and embedded in paraffin wax, according to the standard procedures. Longitudinal sections were cut at 5μm thickness and stained with haematoxylin and eosin (H&E). Examination for any pathological alterations was performed using a light microscope at different magnifications. For the evaluation of the effects of the experimental diets on the microscopic anatomy of the liver and intestine, a ranking system was employed, based on the criteria presented in Table 3.9.

RNA Extraction and Quantitative PCR

Total RNA was isolated from each tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) extraction method following the manufacturer's suggested protocol. Purity and quantity of extracted RNA were assessed by Nanodrop ND-1000 spectrophotometer (260/230 and 260/280 ratios >1.8).

Extracted RNA was treated with DNAse, then 1 µg of total RNA was reverse transcribed using the iScript™ cDNA Synthesis kit (BioRad, Hercules, CA). Real-time quantitative PCR was carried out on a CFX96 Real-Time System (BioRad) in 10 µL total volume reactions using iTaq SYBR Green Supermix (BioRad) and 300 (*Δ6fad* and *elovl2*) or 500 nmol (*Δ5fad, elovl5, acox, gapdh* and *arp*) primers according to the protocol provided by the manufacturer. PCR cycling conditions for all genes were as follows: 95℃ for 5 s followed by 55℃ for 30s over 40 cycles with an initial denaturation step of 95℃ for 3min. For each fish, PCR reactions were run in duplicate. Relative expression values for genes constituting the fatty acid oxidation, desaturation and elongation, including delta-5 fatty acyl desaturase (*Δ5fad*), delta-6 fatty acyl desaturase (*Δ6fad*), fatty acid elongase 2 (*elovl2*), fatty acid elongase 5 (*elovl5*) and acyl-Coa oxidase (*acox*) were determined using primers designed from rainbow trout sequences in the NCBI database. Primer sequences for genes are given in Table 3.3. Two genes (*gapdh* and *arp*) were tested as internal reference genes, though only *arp* proved stable across experimental factors and was therefore chosen as the reference gene for normalization of target gene expression in both tissues. Primer PCR efficiency was calculated for each primer set using a six-step serial dilution of a pooled

sample (pooled from each experimental sample for a given tissue). Data were analyzed using the relative quantification method, including efficiency correction following the method of Pfaffl (2001).

Calculation and Statistical Method

Using the live-weight and feed consumption data, the following indices were calculated.

Weight gain (WG, g / fish)

= (g mean final weight–g mean initial weight)

Specific growth rate (SGR, % / d)

= [(Ln mean final weight–Ln mean initial weight)/number of days] x 100

Survival (%)

= (number of fish at the end of the trial/number of fish at the beginning) x 100

Average feed intake (FI, g / fish)

= g total dry feed intake/number of surviving fish

Feed conversion ratio (FCR)

= g total feed consumed / (g final biomass – g initial biomass + g dead fish weight)

Condition factor (CF)

= (g body weight) / (cm body length)^3 x 100

Hepatosomatic index (HSI)

= (g liver weight) / (g whole body weight) x 100

Viscerosomatic index (VSI)

= (g visceral weight) / (g whole body weight) x 100

ADC diet = $1 - [(F / D) \times (Di / Fi)]$ - where D = % lipid of diet, F = % lipid of feces, Di = % digestion indicator of diet, Fi = % digestion indicator of feces

ADC ingredient = $ADCT + [((1 - s) DR) / s DI] \times (ADCT - ADCR)$

where ADCT = ADC of test diet, ADCR = ADC of reference diet, DR = % lipid of reference diet,

DI = % lipid of test ingredient, s = proportion of test ingredient in test diet (0.2)

Tank mean values (n=3; except diet 2, n=2) were used for statistical analysis. Fish growth and feed utilization indices, physiological parameters, and gene expression data were tested for normality and homogeneity of variance prior to one-way Analysis of Variance (ANOVA). If significant differences were found, data were subjected to Tukey's HSD test to separate the means at a significance level of $P < 0.05$. SPSS (Version 21 for Window; IBM SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Principal components analysis (PCA) was performed by dimension reduction and visual mapping of samples based on non-specific immune response parameters and filet fatty acid composition using R v4.0 (The R Foundation, Vienna, Austria). A Kruskal-Wallis test followed by Wilcoxon post-hoc analysis was used for testing of histological results because the values did not meet parametric assumptions of normality.

Results

Growth Performance and Feed Utilization

The growth performance and feed utilization of the fish are presented in Table 3.4. The final weight, weight gain, and SGR of fish fed diet LO-8 or LO-16 were the greatest ($P < 0.05$) compared with the fish fed LO-0. The survival rate (74.8 % - 83.8 %) and feed conversion ratio (1.27 - 1.32) were similar among dietary treatments groups ($P > 0.05$). Results also showed that CF, HSI and VSI were not significantly affected by dietary treatments ($P > 0.05$). ADC for crude lipid of LO was 93% ± 0.2. Changes in the mean body weight of three groups during 52 weeks of feeding is presented in Fig. 3.1. Growth rate began to separate at 24 weeks and became significantly different after 48 weeks.

Whole-Body, Liver and Filet Proximate Composition

Whole-body, liver and filet proximate composition of rainbow trout juveniles fed the experimental diets are presented in Table 3.5. There were no consistent dietary effects for percent crude protein, crude fat, or gross energy across tissues. Additionally, no significant differences in whole-body, liver, and filet proximate composition and gross energy were detected among treatment groups.

Antioxidant and Non-specific Immune Responses

Results of the non-specific plasma immune assays are presented in Table 3.6. Phagocytic oxidative radical production (NBT activity) in whole-blood of fish fed diet LO-16 was significantly higher than fish fed the other two diets ($P < 0.05$). Plasma SOD, CAT and lysozyme activities were higher for rainbow trout fed diet LO-16, but they were not statistically different ($P > 0.05$).

Histological Examination

The results of the histological evaluation from the final sampling are presented in Table 3.7. With regard to distal intestine tissue, it is interesting to note that both inflammation and size and number of absorptive vacuoles showed significant differences among diets. Distal intestine of fish fed diet LO-16 (Diet 3) showed a reduction in size and number of absorptive vacuoles and signs of inflammation, whereas there was no significant difference in liver histological evaluation. PCA of histology and plasma assay results together showed clear separation of dietary treatment groups, with eigenvectors indicating plasma assays were tightly correlated with one another, yet liver and intestinal histology showed opposing patterns of variance across diets (Fig. 3.2 A).

Fatty Acid Composition of Filet

Filet fatty acid composition of rainbow trout juveniles fed the experimental diets are presented in Table 3.8. Linoleic acid (C18:2n-6) of fish filet of fish fed diet LO-16 (795 mg 100 g^{-1}) was significantly higher than those of fish fed diet LO-8 (648 mg 100 g^{-1}) ($P < 0.05$). ALA, ARA, EPA and DPA content was significantly higher in LO-16 diet group compared to other groups ($P < 0.05$). The filet DHA content of fish fed diet LO-8 was numerically higher than those of fish fed other two diets ($P > 0.05$). EPA + DHA contents tended to increase as Latitude™ oil inclusion level increased to 8% and 16%, but were not statistically different ($P > 0.05$). The overall filet fatty acid profile differed as a whole across dietary treatment according to PCA (Fig. 3.2 B), with the DHA eigenvector explaining separation of the LO-8 group and other n-3/n-6 LC-PUFA concentrations explaining the most variance in the LO-16 group.

Gene Expression

The relative mRNA (RT-qPCR) expression of fatty acid metabolism related genes, fatty acid elongases 2 and 5 (*elovl2 and elovl5*), desaturases (*Δ5fad* and *Δ6fad*) and acyl-CoA oxidase (*acox*) in liver and muscle of rainbow trout fed experimental diets is presented in Fig. 3.3 and 3.4, respectively. The hepatic gene expressions of *elovl2 and elovl5* were unaffected by the diet ($P > 0.05$) (Fig. 3.3), however those genes were significantly upregulated ($P < 0.05$) in the LO-16 group compared to LO-0 group in muscle (Fig. 3.4). The fish fed LO-8 or LO-16 diet showed a significantly higher expression of *Δ6fad* as well as *acox* in both liver and muscle compared to the fish fed LO-0 diet, while the relative mRNA expression of *Δ5fad* was not significantly different among the dietary treatment group ($P > 0.05$).

Discussion

Growth Performance and Feed Utilization

This study is the first to address the impact of the substitution of FO by transgenic canola oil, up to 100% substitution, on rainbow trout performance, health, and n-3 LC-PUFA tissue composition over a complete production cycle, from fingerling to the marketing size (52 weeks). In the present study, EPA+DHA contents (% of the diet) were formulated to be 3%. Results were promising, demonstrating that both inclusion levels of LO (8% and 16%) proved to be effective. Remarkably, the fish fed diet LO-8 and LO-16 showed significantly increased growth performance and fillet EPA + DHA concentrations, similar to those achieved in fish fed diet LO-0. While unexpected, the growth results suggest improved lipid utilization in fish fed the diets containing LO compared to fish in the LO-0 diet group. It is worth noting that growth rates began to separate among treatments at 24 weeks and became significantly different after 48 weeks (Fig. 3.1), supporting the need for long-term studies. It is known that the feeding duration in dietary studies can lead to significant growth differences, and it is not easy to compare directly with results from the experiments of shorter duration (Weatherup and McCraken, 1999; De Francesco et al., 2004).

One possible reason for improved growth (final weight, WG, SGR) of fish fed the LO-8 and LO-16 diets could be related to the higher ARA content in LO. Recently, the importance of n-6 LC-PUFA, particularly ARA, has been highlighted (Bell and Sargent, 2003; Norambuena et al., 2015; Xu et al., 2017; Torrecillas et al., 2018; Araújo et al., 2019).

Some studies have shown that ARA plays crucial roles in marine fish growth and survival (Xu et al., 2010; Yuan et al., 2015; Rombenso et al., 2016; Torrecillas et al., 2018), reproduction (Bromage et al., 2001; Kowalska and Kowalski; 2014) and stress and disease resistance (Koven et al., 2001; Koven et al., 2003; Martins et al., 2013), hence playing a vital role across the entirety of the fish life cycle. On the contrary, other studies have demonstrated that the inclusion of dietary ARA did not improve fish growth performance (Koven et al., 2001; Asil et al., 2017; Chee et al., 2020). These different results may be due to distinct experimental conditions, fish size, and duration of the feeding trial. It is known that rainbow trout have a limited capacity to bioconvert ALA to DHA as well as linoleic acid to ARA, similar to many marine carnivorous fishes. Moreover, the preference of desaturase and elongase for n-3 over n-6 substrates leads to ARA synthesis being limited (Bell and Sargent, 2003), suggesting that ARA could be required in adult rainbow trout. Most studies have focused on the functions of ARA in juveniles, but little research has examined the influences of ARA on growth in sub-adult and adult fish.

Antioxidant and Non-specific Immune Responses

Although not observed in the current study, reports with larval fish suggest that ARA may be responsible for enhanced fish survival (Bessonart et al., 1999; Atalah et al., 2011; Yuan et al., 2015). Despite the fact that ARA may be a critical omega-6 fatty acid for some fish species to achieve proper growth, development, and survival, other studies remind us that generation of reactive oxygen species (ROS) at high levels produced by activating NADPH-oxidase can cause toxicity through oxidative stress, as a potential result of an imbalance between antioxidant defenses and ROS generation when excessive levels of ARA are provided in diet (Cury-Boaventura and Curi, 2005; Schrader and Fahimi, 2006; Sakin et al., 2011; Chee et al., 2020). This, together with observations of elevated levels of whole-blood phagocytic oxidative radical production (NBT activity), a measurement of oxidative radical production, in the present study suggest a need for more research on ARA as a functional fatty acid in fish feeds across life stages.

Lysozyme, a non-specific innate immune molecule present in the plasma and body fluids of fish, plays a considerable role in host protection against microbial invasion (Li et al., 2021). While not significant (P=0.075), higher plasma lysozyme activity in fish fed LO-16

diet are consistent with the conclusions of Chee et al. (2020), in which lysozyme activity increased with increasing dietary ARA levels. The increased lysozyme activity with increasing ARA levels could be a result of increased production of leukotriene B_4, a stimulator of release of lysosomal enzymes and superoxide in neutrophils, derived from ARA (Samuelsson, 1983; Chee et al., 2020). Well-known indexes of antioxidant defense status, SOD and CAT activities, were not significantly different in the present study; however, higher activities have been reported in studies with juvenile Japanese seabass (Xu et al., 2010) and Malabar red snapper (Chee et al., 2020). Since SOD and CAT are antioxidant enzymes known to scavenge ROS, increased SOD and CAT activities in those studies were likely in response to increased ROS production associated with increased respiratory burst activity.

Histological Examination

Histological evaluation of the distal intestine of rainbow trout in the present study indicated impaired structural morphology when fed the LO-16 diet compared to fish fed the LO-8 diet but not the LO-0 diet. Specifically, fish fed the LO-16 diet exhibited increased inflammation and reduced size and number of absorptive vacuoles compared to fish fed LO-8 diet. Again, this might be explained by the higher level of ARA. Very little has been reported regarding the effects of ARA on fish intestinal histology in fish. Qi et al. (2016) reported that moderate levels of dietary ARA (0.51% of dry matter) had positive effects on the number of goblet cells and the length of intestinal villus, whereas higher ARA levels (0.88 and 0.96%) had detrimental effects in juvenile golden pompano (*Trachinotus ovatus*). Yu et al. (2019) also reported that dietary ARA supplementation disrupted the intestinal physical barrier of tiger puffer, showing that the gene expression of tight junction proteins, claudin-4 and 7 and zonula occludens-1, was down-regulated in groups supplemented with ARA.

Fatty Acid Composition of Filet

An important aspect of the present study was to assess if LO influenced fatty acid metabolism, as it contains relatively high EPA and DPA levels. In the present study, muscle fatty acid profiles generally reflected those of the diets, as reported previously in other

studies in fish (Montero et al., 2005; Turchini et al., 2009). Interestingly, muscle of fish fed the LO-8 diet showed lower levels of C16:0, C18:1n-9, C18:2n-6 (linoleic acid, LA) fatty acids and DPA and higher levels of DHA compared to the diet, suggesting that the decrease and low retention of these fatty acids could have occurred due to utilization as an energy source by the β-oxidation pathway and DPA being converted to DHA. It has been reported that LA, C16:0, C16:1, and C18:1n-9 fatty acids are preferred substrates for β-oxidation and energy generation in the mitochondria (Henderson, 1996; Bell et al., 2004). Drouin et al. (2019) reported that DPA supplementation affected the overall fatty acid profile, significantly increased EPA and DHA in the liver, and resulted in a slight increase of DHA in the heart and red blood cells in rats. Despite the highest level of DPA in diet LO-16, the concentrations of DHA in the fillet of fish fed diet LO-16 was numerically lower than those of fish fed diet LO-8, with perhaps the high inclusion of ARA negatively affecting the conversion of EPA or DPA to DHA, as ARA, EPA and DPA compete for the same enzymes (*elovl2* and *elovl5*) in their synthesis pathways. This is supported by relatively higher fatty acid content of EPA and DHA in the fillet of fish fed diet LO-16. However, it is worth noting that the fillet DHA content of fish fed diet LO-16 was not significantly different compared to the diet LO-0. In the current study, the fillet EPA + DHA contents of fish fed all three experimental diets satisfied the suggested consumption recommendation by the American Heart Association for people without coronary heart disease (500 mg day^{-1}) and with coronary heart disease (1000 mg day^{-1}).

Gene Expression

The same patterns of gene expression were observed in the liver and fillet. It is generally accepted that the expression of *Δ6fad* is highly responsive to dietary levels of n-3 LC-PUFA, being up-regulated when fish are fed low dietary levels of n-3 LC-PUFA, which leads to increased production of EPA and DHA (Thanuthong et al., 2011; Morais et al., 2012). In contrast, in the present study, fish fed diets with either 8% LO or 16% LO showed up-regulation of *Δ6fad* in liver and fillet, which may be associated with higher levels of DPA included in both diets compared to diet LO-0. The rate-limiting step for the LC-PUFA biosynthetic pathway in fish is controlled by *Δ6fad,* as it is the first enzyme involved in the bioconversion of C18 PUFA to longer and more unsaturated fatty acids, including the

conversion of DPA to DHA (Sprecher, 2000). This result suggests that the expression of *Δ6fad* is more affected by the dietary levels of DPA and DHA. Fish fed the LO-16 diet, which had higher EPA and DPA contents than the other two diets, showed up-regulation of *elovl2* and *elovl5* in the fillet, potentially explaining the higher level of DHA in fillet compared to diet. Additionally, Gregory and James (2014) found that, unlike chicken, where both *elovl2* and *elovl5* can elongate DPA (Gregory et al., 2013), elongation of DPA to tetracosapentaenoic acid (24:5n-3), the penultimate precursor of DHA, is limited to *elovl2* in salmonids. For peroxisomal β-oxidation, *acox* is regarded as the rate-limiting enzyme (Morais et al., 2007). In the present study, the expression levels of *acox* were up-regulated in both liver and fillet with increasing levels of dietary DPA, indicating that there was active catabolism of tetracosahexaenoic acid (24:6n-3), the ultimate precursor of DHA. The up-regulated expression of *acox* by LO agrees with the DHA content in the fillet, which may indicate that a higher level of DHA was required by rainbow trout to sustain physiological function. However, to our knowledge, information on the effects of DPA on fish growth and health performance in fish is lacking due to the high cost and limited availability of high-purity DPA for *in vivo* studies (Drouin et al., 2019).

Conclusions

In conclusion, results of the present study demonstrate that Latitude™ oil is highly digestible, improves fish growth, and yields elevated fillet n-3 LC-PUFA content, making it a sustainable, candidate lipid source for use in rainbow trout feeds. However, there was an indication of inflammation in distal intestine showing that 8% LO inclusion appears to be superior to 16% inclusion. Our results also suggest that the unique fatty acid profile of this oil, being high in the fatty acids ARA and DPA, may have functional properties, including but not limited to oxidative stress and LC-PUFA fatty acid synthesis, that require further investigation. Additionally, further studies are needed to refine optimal LO inclusion levels in rainbow trout and salmonid feeds and to identify the mechanisms leading to improved growth when fed to rainbow trout.

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Table 3.1. Formulation and proximate composition of the experimental diets (as fed).

Ingredients (%)	Diets		
	LO-0	**LO-8**	**LO-16**
Fish meal, sardine[a]	20	20	20
PBM, feed grade[a]	12.5	12.5	12.5
Soybean meal[a]	11.5	11.5	11.5
Soy protein concentrate[b]	5.5	5.5	5.5
Wheat gluten meal[a]	1.5	1.5	1.5
Corn protein concentrate[c]	13.5	13.5	13.5
Wheat flour[a]	16.2	16.2	16.2
Dicalcium phosphate[a]	1.4	1.4	1.4
Trace mineral mix[d]	0.1	0.1	0.1
Vitamin Premix[e]	1.0	1.0	1.0
Choline chloride (60%)[a]	0.6	0.6	0.6
Stay C (35%) vitamin[f]	0.2	0.2	0.2
Fish oil[a]	6.43	3.21	0.0
Poultry fat[g]	9.57	4.79	0.0
Latitude™ oil[h]	0.00	8.00	16.0
Composition (% as-fed basis)			
Dry Matter	98.3	98.5	97.9
Protein	50.0	49.4	49.6
Fat	20.3	21.0	20.1
Ash	3.66	3.54	3.14
Gross energy (MJ/kg)	24.3	24.4	23.9

[a] Rangen Inc., Buhl, ID, USA.
[b] Profine VF, The Solae Company, St. Louis, MO, USA
[c] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA
[d] US Fish and Wildlife Service Trace Mineral Premix #3 supplied the following (mg kg^{-1} diet): Zn (as $ZnSO_4 \cdot 7H_2O$), 75; Mn (as $MnSO_4$), 20; Cu (as $CuSO_4 \cdot 5H_2O$), 1.54; I (as KIO_3), 10.
[e] Vitamin premix supplied the following per kg diet: vitamin A, 2.4 mg; vitamin D, 0.15 mg; vitamin E, 267 mg; vitamin K as menadione sodium bisulfite, 20 µg; thiamin as thiamin mononitrate, 32 mg; riboflavin, 64 mg; pyridoxine as pyridoxine-HCl, 64 mg; pantothenic acid as Ca-d-pantothenate, 192 mg; niacin as nicotinic acid, 240 mg; biotin, 0.56 mg; folic acid, 12 mg; vitamin B_{12}, 50 µg; and inositol as meso-inositol, 400 mg.
[f] Skretting USA, Tooele, UT, USA.
[g] Tyson Foods Inc., Springdale, AR, USA
[h] Cargill Inc., Minneapolis, MN, USA

Table 3.2. Analyzed fatty acid profile of the experimental diets[a]

Fatty acids	Diet					
	LO-0		**LO-8**		**LO-16**	
	% FAME	**% Diet**	**% FAME**	**% Diet**	**% FAME**	**% Diet**
C14:0	1.93	0.39	1.19	0.25	0.38	0.08
C16:0	18.6	3.78	13.3	2.80	9.15	1.84
C18:0	5.52	1.12	4.81	1.01	3.84	0.77
C24:0	1.93	0.39	1.19	0.25	0.38	0.08
ΣSFA	28.0	5.68	20.5	4.31	13.7	2.76
C16:1n-7	7.10	1.44	4.30	0.90	1.39	0.28
C18:1n-9	29.0	5.89	27.5	5.76	26.6	5.34
C20:1n-9	2.35	0.48	1.71	0.36	0.95	0.19
C22:1n-9	1.17	0.24	0.66	0.14	0.14	0.03
ΣMUFA	39.6	8.04	34.1	7.17	29.1	5.84
C18:2n-6	15.0	3.04	20.3	4.26	25.9	5.21
C18:3n-6	0.16	0.03	0.99	0.21	1.90	0.38
C20:2n-6	0.15	0.03	0.12	0.02	0.09	0.02
C20:3n-6	0.13	0.03	1.27	0.27	2.48	0.50
C20:4n-6 (ARA)	1.20	0.24	1.93	0.40	2.63	0.53
n-6 PUFA	16.6	3.38	24.6	5.17	33.0	6.64
C18:3n-3	1.22	0.25	2.33	0.49	3.41	0.68
C20:5n-3 (EPA)	7.39	1.50	10.3	2.17	13.1	2.64
C22:5n-3 (DPA)	0.93	0.19	1.67	0.35	2.28	0.46
C22:6n-3 (DHA)	6.29	1.28	5.00	1.05	3.38	0.68
EPA + DHA	13.7	2.78	15.3	3.22	16.5	3.32
n-3 PUFA	15.8	3.21	19.3	4.06	22.2	4.46
n-3 / n-6	0.95		0.79		0.67	

[a]Abbreviations: SFA, saturated fatty acids; MUFA, mono-unsaturated fatty acids; n-6 PUFA, n-6 poly-unsaturated fatty acids; n-3 PUFA, n-3 poly-unsaturated fatty acids; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

Table 3.3. Primers sequences used in real-time qPCR for the determination of gene expression.

Genes	Forward	Reverse	Bases	Gene Accession NO
elovl2	GATGCCTGCTCTTCCAGTTC	CATTGGTGGAGACAGTGTGG	20	KM244737
elovl5	CTATGGGCTCTC TGCTGTCC	TATCGTCTGGGA CATGGTCA	20	AY605100
Δ5fad	GCAGAGAGAACCGAGGATGG	GCAGTGCTTCTG GACCTCTT	20	JD087459
Δ6fad	ACCTAGTGGCTCCTCTGGTC	CAGATCCCCTGACTTCTTCA	20	AF301910
acox	TTCCACGACCAGACCCATGA	AACGGCGTCCACCAAAGCTA	20	BX085367
gapdh	ACTCTGTTGTGTCTTCTG	TTGTCGTTGAAGGAGATG	18	NM_001124246.1
arp	GAAGGCTGTGGTGCTCAT	CAGGGCAGGGTTGTTCTC	18	XM_021610240.2

Table 3.4. Growth performance and feed utilization of rainbow trout fed for 52 weeks*.

	Diets		
	LO-0	**LO-8**	**LO-16**
Initial weight (g)	18.5 ± 0.09	18.5 ± 0.08	18.5 ± 0.08
Final weight (g)	869 ± 18.6[b]	967 ± 36.4[a]	955 ± 10.8[a]
Weight gain (g fish^{-1})	850 ± 18.5[b]	949 ± 36.4[a]	937 ± 10.8[a]
SGR[a]	1.07 ± 0.10[b]	1.10 ± 0.01[a]	1.10 ± 0.00[a]
Feed intake (g fish^{-1})	1076 ± 12.1	1219 ± 65.5	1244 ± 73.5
FCR[b]	1.27 ± 0.04	1.28 ± 0.02	1.32 ± 0.07
Survival (%)	83.8 ± 2.21	83.8 ± 2.70	74.8 ± 4.59
Condition factor (%)	1.19 ± 0.08	1.22 ± 0.06	1.22 ± 0.03
HSI[c]	0.84 ± 0.07	0.88 ± 0.00	0.74 ± 0.05
VSI[d]	9.07 ± 0.78	8.90 ± 0.27	9.09 ± 0.90

*Mean ± SE (n = 3 tanks per treatment) except for diet 2 (n = 2) in the same row that share the same superscript are not statistically different ($P > 0.05$)

[a]SGR: specific growth rate (% day^{-1})

[b]FCR: feed conversion ratio

[c]HSI: hepatosomatic index (%)

[d]VSI: viscerosomatic index (%)

Table 3.5. Whole-body, liver and filet proximate composition and gross energy (wet basis) of rainbow trout fed experimental diets for 52 weeks*.

	Diets		
	LO-0	**LO-8**	**LO-16**
Whole-body			
Dry matter (%)	29.3 ± 3.15	33.3 ± 0.68	28.8 ± 1.06
Crude protein (%)	17.5 ± 1.09	18.2 ± 0.58	18.0 ± 0.64
Crude fat (%)	9.40 ± 2.45	12.8 ± 1.28	8.80 ± 1.45
Ash (%)	2.1 ± 0.31	1.7 ± 0.34	2.3 ± 0.26
Gross energy (MJ kg^{-1})	26.8 ± 0.84	28.2 ± 0.84	26.7 ± 0.78
Liver			
Dry matter (%)	14.7 ± 1.05	15.2 ± 0.93	13.2 ± 0.75
Crude protein (%)	9.63 ± 0.27	9.67 ± 0.63	8.81 ± 0.52
Crude fat (%)	0.83 ± 0.21	0.64 ± 0.12	0.47 ± 0.02
Gross energy (MJ kg^{-1})	22.0 ± 0.17	22.1 ± 0.03	22.0 ± 0.07
Fillet			
Dry matter (%)	21.6 ± 0.67	20.3 ± 0.04	21.3 ± 1.16
Crude protein (%)	16.0 ± 0.81	14.8 ± 0.18	15.7 ± 0.47
Crude fat (%)	4.95 ± 0.12	4.48 ± 0.28	5.00 ± 0.41
Gross energy (MJ kg^{-1})	26.2 ± 0.13	25.8 ± 0.26	26.0 ± 0.19

*Mean ± SE (n = 3 tanks per treatment) except for diet 2 (n = 2) in the same row that share the same superscript are not statistically different (P > 0.05)

Table 3.6. Non-specific immune responses of rainbow trout fed experimental diets for 52 weeks*.

	Diets		
	LO-0	**LO-8**	**LO-16**
NBT[a]	0.28 ± 0.12[b]	0.19 ± 0.01[b]	0.90 ± 0.14[a]
SOD[b]	6.75 ± 0.28	6.40 ± 0.59	7.41 ± 0.63
Catalase[c]	42.0 ± 2.31	46.6 ± 0.55	48.1 ± 2.52
Lysozyme[d]	329 ± 7.22	385 ± 24.7	414 ± 40.2

*Mean ± SE (n = 3 tanks per treatment) except for diet 2 (n = 2) in the same row that share the same superscript are not statistically different ($P > 0.05$)
[a]NBT: Nitro-blue tetrazolium assay (OD 540nm)
[b]SOD: Super oxide dismutase (%inhibition)
[c]Catalase: Catalase activity (nmol min^{-1} ml^{-1})
[d]Lysozyme: Lysozyme activity (Unit mL^{-1} enzyme)

Table 3.7. Histological scoring of rainbow trout distal intestine and liver tissue following different dietary treatment fed for 52 weeks*.

	Diets		
	LO-0	LO-8	LO-16
Distal intestine			
Inflammation	2.89 ± 0.87[a]	2.00 ± 0.00[b]	3.33 ± 0.47[a]
Absorptive vacuoles	2.22 ± 0.92[ab]	1.67 ± 0.75[b]	3.22 ± 0.42[a]
Liver			
Glycogen vacuolation	2.11 ± 0.74	2.83 ± 0.37	2.11 ± 0.57
Perivascular inflammation	1.67 ± 0.67	1.17 ± 0.37	1.33 ± 0.67
Focal inflammation	1.33 ± 0.67	1.17 ± 0.37	1.00 ± 0.00
Nuclear vacuoles	1.33 ± 0.67	1.17 ± 0.37	1.11 ± 0.31

*Mean ± SE (n = 9 fish per treatment) except for diet 2 (n = 6) in the same row that share the same superscript are not statistically different (P> 0.05; Data was analyzed by Kruskal-Wallis test and Wilcoxon's post-hoc test).

Table 3.8. Filet fatty acid composition of rainbow trout fed experimental diets for 52 weeks*.

	Diets					
	LO-0		LO-8		LO-16	
Fatty acids	**mg 100 g^{-1}**	**% FAME**	**mg 100 g^{-1}**	**% FAME**	**mg 100 g^{-1}**	**% FAME**
C14:0	60.3 ± 2.17	1.22 ± 0.04	46.1 ± 13.0	1.03 ± 0.29	43.8 ± 4.79	0.95 ± 0.14
C16:0	845 ± 50.2^{a}	17.1 ± 1.01^{a}	665 ± 22.6^{b}	14.8 ± 0.50^{ab}	668 ± 60.3^{ab}	13.4 ± 1.21^{b}
C18:0	235 ± 18.7	4.75 ± 0.38	185 ± 7.21	4.13 ± 0.16	211 ± 11.2	4.22 ± 0.22
C24:0	59.7 ± 2.97	1.21 ± 0.06	46.1 ± 13.0	1.03 ± 0.29	47.6 ± 7.27	0.95 ± 0.15
ΣSFA	1200 ± 36.3^{a}	24.2 ± 0.73^{a}	942 ± 10.6^{b}	21.0 ± 0.24^{ab}	970 ± 62.2^{b}	19.5 ± 1.28^{b}
C16:1-7	246 ± 23.6^{a}	4.96 ± 0.48^{a}	208 ± 9.04^{ab}	4.65 ± 0.20^{ab}	179 ± 18.5^{b}	3.58 ± 0.37^{b}
C18:1n-9	1297 ± 40.5^{a}	26.2 ± 0.82	1079 ± 31.5^{b}	24.1 ± 0.70	1272 ± 38.2^{a}	25.4 ± 0.76
C20:1n-9	92.5 ± 12.6^{a}	1.87 ± 0.25	49.1 ± 12.7^{b}	1.10 ± 0.28	68.1 ± 3.92^{ab}	1.36 ± 0.08
C22:1n-9	24.3 ± 5.20^{a}	0.49 ± 0.11^{a}	13.5 ± 2.07^{ab}	0.30 ± 0.05^{ab}	8.55 ± 0.29^{b}	0.17 ± 0.01^{b}
ΣMUFA	1660 ± 81.6^{a}	33.5 ± 1.65	1350 ± 55.3^{b}	30.1 ± 1.23	1528± 54.7^{ab}	30.6 ± 1.09
C18:2n-6	710 ± 26.0^{ab}	14.3 ± 0.53	648 ± 17.6^{b}	14.5 ± 0.39	795 ± 27.1^{a}	15.9 ± 0.54
C18:3n-6	6.57 ± 1.46^{b}	0.13 ± 0.03^{b}	12.3 ± 0.27^{b}	0.27 ± 0.01^{b}	26.6 ± 3.78^{a}	0.53 ± 0.08^{a}
C20:3n-6	21.5 ± 2.80^{b}	0.44 ± 0.06^{b}	41.0 ± 2.11^{b}	0.91 ± 0.05^{ab}	81.4 ± 17.6^{a}	1.63 ± 0.35^{a}
C20:4n-6 (ARA)	51.9 ± 7.11^{b}	1.05 ± 0.14^{a}	66.8 ± 2.87^{b}	1.49 ± 0.06^{ab}	93.3 ± 8.32^{a}	1.87 ± 0.17^{b}
n-6 PUFA	823 ± 40.3^{b}	16.6 ± 0.81^{b}	789 ± 18.1^{b}	17.6 ± 0.40^{ab}	1027 ± 61.1^{a}	20.5 ± 1.22^{a}
C18:3n-3	41.0 ± 5.09^{b}	0.83 ± 0.10^{b}	50.2 ± 1.36^{b}	1.12 ± 0.03^{ab}	78.5 ± 8.74^{a}	1.57 ± 0.17^{a}
C20:5n-3 (EPA)	239 ± 4.95^{b}	4.83 ± 0.10^{b}	241 ± 7.24^{b}	5.37 ± 0.16^{b}	416 ± 14.6^{a}	8.32 ± 0.29^{a}
C22:5n-3 (DPA)	71.3 ± 3.00^{b}	1.44 ± 0.06^{b}	70.3 ± 3.30^{b}	1.57 ± 0.07^{b}	133 ± 9.78^{a}	2.66 ± 0.20^{a}
C22:6n-3 (DHA)	840 ± 68.8	17.0 ± 1.39^{b}	934 ± 16.3	20.9 ±0.36^{a}	825 ± 49.2	16.5 ± 0.98^{b}
EPA + DHA	1079 ± 63.9	21.8 ± 1.29^{b}	1175 ± 23.6	26.2 ± 0.53^{a}	1241 ± 62.5	24.8 ± 1.25^{ab}
n-3 PUFA	1191 ± 56.3^{b}	24.1 ± 1.14^{b}	1296± 21.6^{ab}	28.9 ± 0.48^{a}	1453 ± 69.5^{a}	29.1 ±1.39^{a}
n-3 / n-6	1.45 ± 0.14		1.64 ± 0.01		1.42 ± 0.09	

*Values are mean ± SE (n = 3 tanks per treatment) except for diet 2 (n = 2) in the same row that share the same superscript or absence of superscripts is not statistically different (*P* > 0.05).

Table 3.9. Criteria used for the histological evaluation of the distal intestine and liver of rainbow trout at the end of feeding trial

Score	Distal intestine	
	Inflammation	***Absorptive vacuoles***
1	Normal	Normal
2	Mildly increased	Mildly reduced in size and/or number
3	Moderately increased	Moderately reduced in size and/or number
4	Markedly increased	Markedly reduced in size and/or number
5	Severely increased	Severely reduced in size and/or number
Score	**Liver**	
	Glycogen vacuolation	***Inflammation (perivascular & focal) and nuclear vacuoles***
1	Minimal vacuolation	Normal
2	Moderately decreased	Mildly increased
3	Normal	Moderately increased
4	Moderately increased	Markedly increased
5	Severely increased	Severely increased

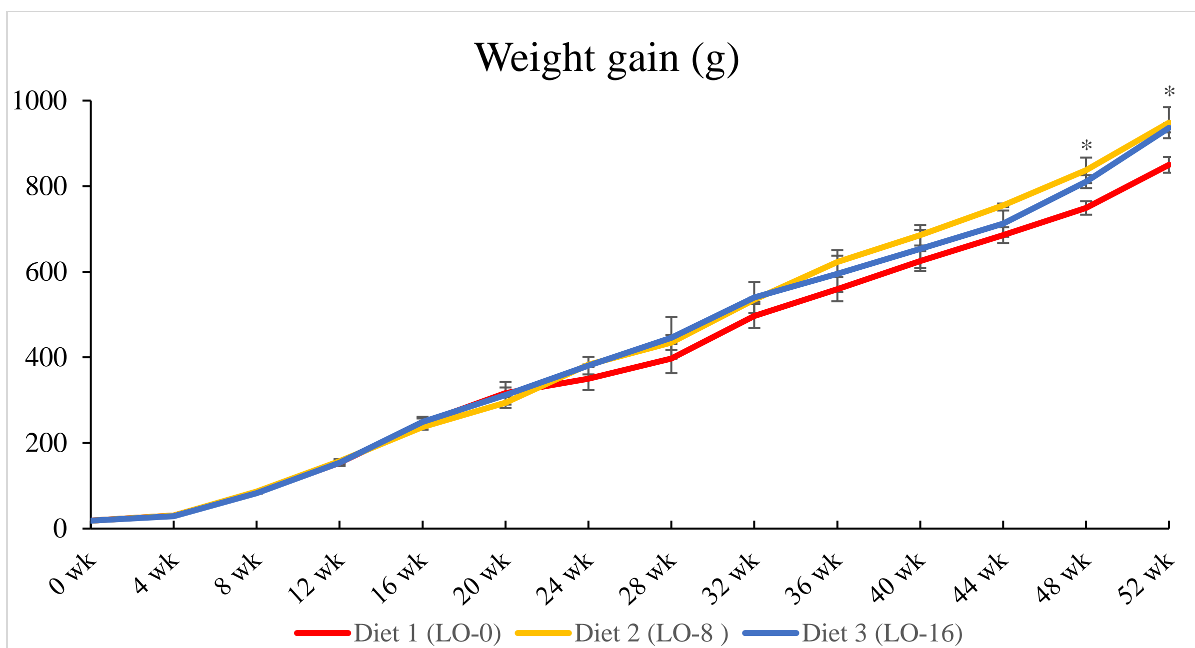

Figure 3.1. Mean body weight over time of rainbow trout fed experimental diets differing in oil source for 52 weeks.

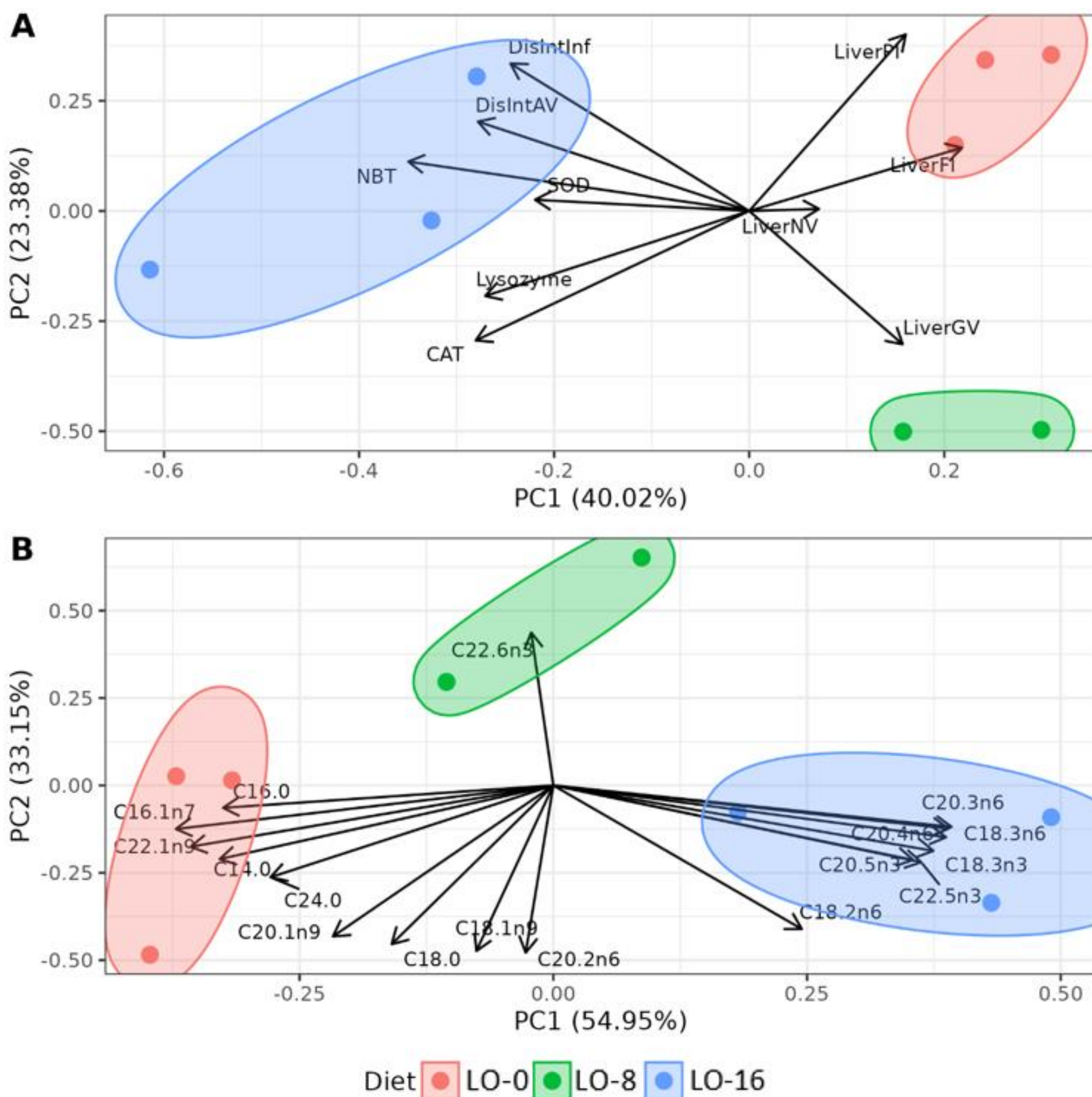

Figure 3.2. Principal component analysis (PCA) of the non-specific immune response parameters (A) and the fillet fatty acid composition (B) of rainbow trout fed experimental diets for 52 weeks. Abbreviations: NBT, nitro-blue tetrazolium; SOD, superoxide dismutase; CAT, catalase; DisInt AV, distal intestine absorptive vacuoles; DisInt Inf, distal intestine inflammation; Liver PI, liver perivascular inflammation; Liver FI, liver focal inflammation; Liver NV, liver nuclear vacuoles; Liver GV, liver glycogen vacuolation.

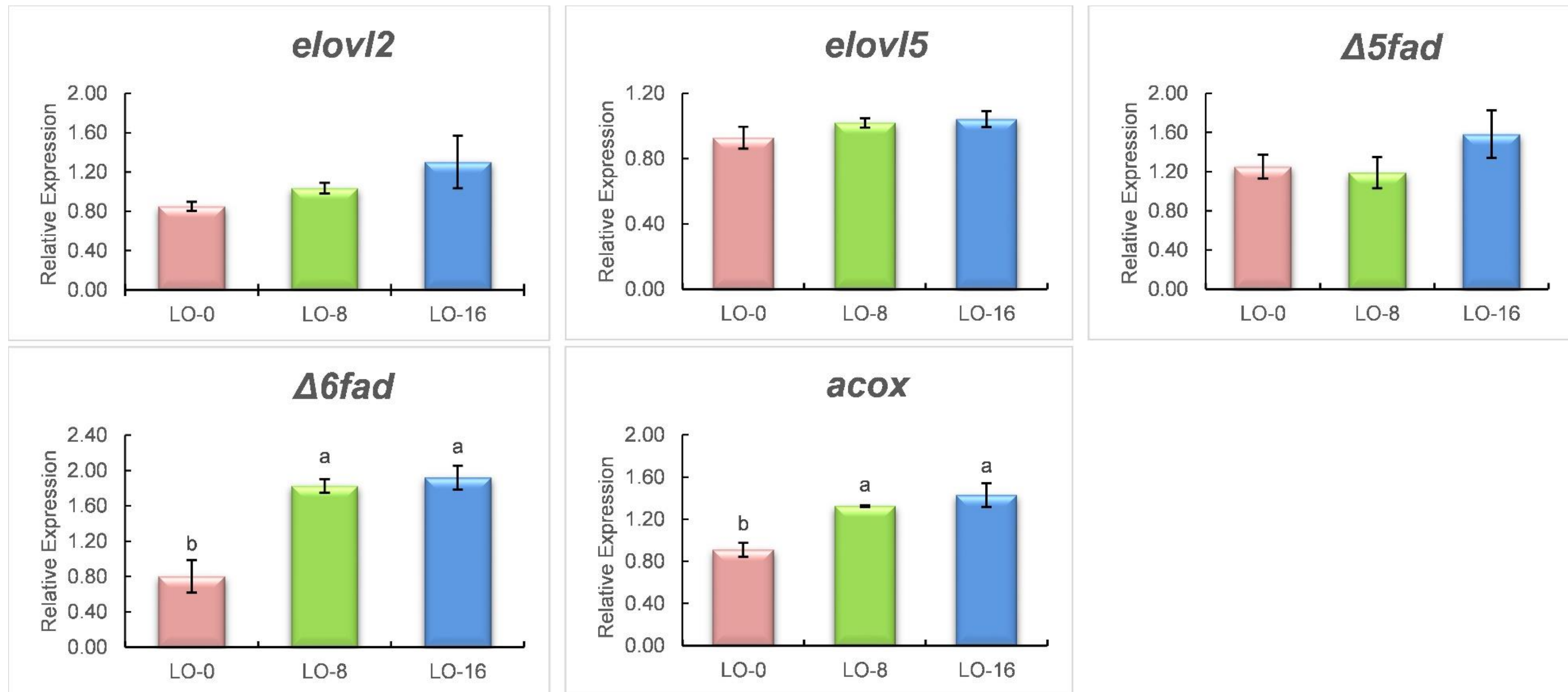

Figure 3.3. Relative mRNA expression (normalized against *arp*) of genes involved in elongation (*elovl2* and *elovl5*), desaturation (*Δ5fad* and *Δ 6fad*) and β-oxidation (*acox*) in liver of rainbow trout fed experimental diets for 52 weeks. Mean ± SE (n = 9 fish per treatment except diet 2, n = 6) in the same row that share the same superscript are not statistically different ($P > 0.05$). Three fish from each tank were used for gene expression. Abbreviations: *elovl2*: Elongation of very long chain fatty acids-like 2; *elovl5*: Elongation of very long chain fatty acids-like 5; *Δ5fad*: Delta-5 fatty acid desaturase; *Δ6fad*: Delta-6 fatty acid desaturase

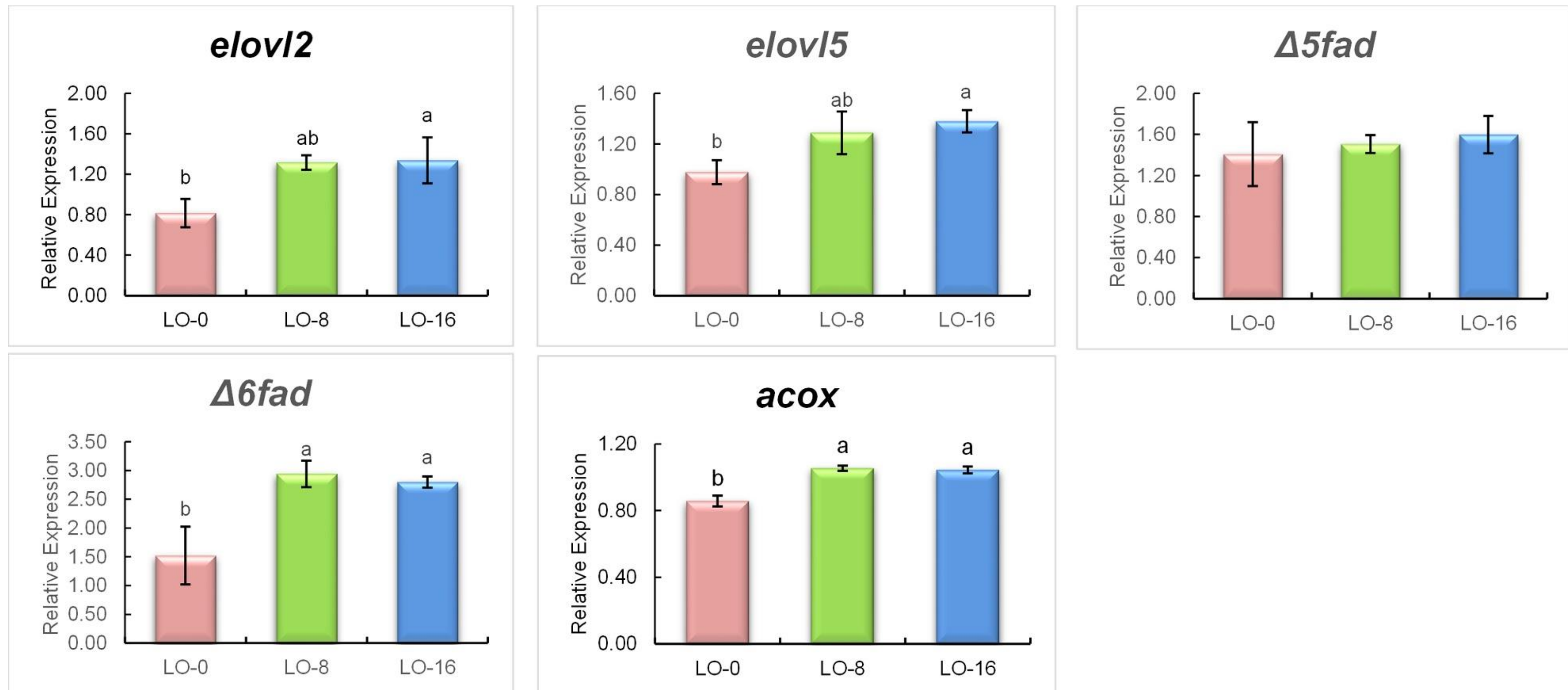

Figure 3.4. Relative mRNA expression (normalized against Arp) of genes involved in elongation (*elovl2* and *elovl5*), desaturation (*Δ5fad* and *Δ6fad*) and β-oxidation (*acox*) in muscle of rainbow trout fed experimental diets for 52 weeks. Mean ± SE (n = 9 fish per treatment except diet 2, n = 6) in the same row that share the same superscript are not statistically different ($P > 0.05$). Three fish from each tank were used for gene expression. Abbreviations: *elovl2*: Elongation of very long chain fatty acids-like 2; *elovl5*: Elongation of very long chain fatty acids-like 5; *Δ5fad*: Delta-5 fatty acid desaturase; *Δ6fad*: Delta-6 fatty acid desaturase

Chapter 4:

Effects of Dietary Arachidonic acid on the Early-Stage Growth Performance, Oxidative Stress and Inflammatory Responses of Rainbow Trout to Hypoxia at two Different Temperatures

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Abstract

The objective of the study was to evaluate the effect of dietary arachidonic acid (ARA) on growth performance, blood biochemical parameters, and antioxidant and inflammatory responses to hypoxia and elevated water temperature in rainbow trout, *Oncorhynchus mykiss*. Four isonitrogenous (51% crude protein), isolipidic (15% crude lipid), and isocaloric (23 MJ/kg) diets were produced, ARA 0 (linseed oil, i.e., the control diet), and three other diets with graded levels of ARA (ARA-L: 3.95%, ARA-M: 7.67, and ARA-H: 14.8% of total fatty acids), and fed throughout this three phase study. In Phase 1, rainbow trout fry (0.17 ± 0.01) were fed to apparent satiation six times a day for 8 weeks to assess early growth performance and feed utilization. Phase 2 continued to evaluate growth performance and feed utilization for another 16 weeks. In Phase 3, fish were raised to approximately 200 g with continued feeding of their respective dietary treatments and exposed to hypoxia at normal (15°C) and elevated (21°C) temperatures. At both 8 weeks (Phase 1) and 24 weeks (Phase 2) the feeding trial demonstrated that dietary ARA did not affect rainbow trout fry and juvenile growth performance and feed utilization ($P > 0.05$). However, Phase 3 demonstrated that ARA supplementation increased the activities of superoxide dismutase (SOD), lysozyme, prostaglandin E_2, and decreased cortisol levels in plasma, all of which were increased in response to hypoxia ($P < 0.05$). SOD and catalase showed an increase with elevated temperature. Two-way and three-way interactions between ARA and hypoxia, and ARA, hypoxia, and temperature were observed for cortisol. The inclusion of ARA in diets affected none of the blood biochemical parameters. Partial pressure carbon dioxide (pCO_2), sodium, glucose, hematocrit, and hemoglobin levels were affected by hypoxia. Whole blood of fish reared at 21℃ revealed increased pCO_2, sodium, and glucose levels. The expressions of cyclooxygenase-2 (*cox-2*), lipoxygenase (*lox*), interleukin-8 (*il-8*), and tumor necrosis factor-alpha (*tnf-α*) were significantly upregulated, whereas interleukin-10 (*il-10*) was significantly downregulated in response to hypoxia. The expression level of transforming growth factor beta (*tgf-β*) showed upregulation as the ARA inclusion level increased ($P < 0.05$), suggesting that ARA had an anti-inflammatory effect. Taken together, dietary ARA appears to improve immune function and reduce the inflammatory response in following hypoxia and elevated water temperature.

Introduction

Long-chain polyunsaturated fatty acids (LC-PUFA) are essential dietary components for fish growth, development, and immune function (Torrecillas et al., 2018; Tian et al., 2014; Kiron et al., 2011). Concerning their major physiological roles, the last few decades have focused on n-3 LC-PUFA, mainly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), due to their level of inclusion in cell membranes. However, it is becoming increasingly urgent for the aquafeed industry to replace fish oil in finfish feeds. Aquaculture's rapid growth and a diminishing supply of fish oil has led to a significant amount of research over the last decade conducted to replace fish oil with alternative lipid sources, primarily vegetable oils, which are more ARA deficient than fish oil.

ARA has a variety of physiological functions, one of the most important of which is that it serves as a primary precursor to several eicosanoids, such as prostaglandins, thromboxane, and leukotriene, produced via pathways of cyclooxygenase and lipoxygenase enzymes (Lund et al., 2008). Eicosanoids play crucial roles throughout the life cycle as they are involved in several physiological processes including growth and development, immune function, stress and inflammatory responses, and reproduction (Sargent et al., 1995; Bell and Sargent 2003; Tocher, 2003; Xu et al., 2022). However, in regard to fish growth, published studies report contradictory results as to whether dietary ARA has a significant effect.

Studies on Atlantic salmon (Dantagnan et al., 2016), barramundi (Salini et al., 2016), grass carp (Tian et al., 2014; Tian et al., 2017), and Malabar red snapper (Chee et al., 2020) showed no significant effects of ARA on growth performance. On the other hand, studies on juvenile cobia (Araújo et al., 2019), juvenile European sea bass (Torrecillas et al., 2017), golden pompano (Qi et al., 2016), and rabbitfish (Nayak et al., 2017) showed that dietary ARA significantly impacted growth performance. Taken together from the studies mentioned above, it is worth noting that primarily high-trophic level species require dietary ARA, whereas low-trophic level species, such as grass carp, do not require dietary ARA in their diets.

ARA is also known to be involved in controlling inflammatory cytokine release due to its essential role as a precursor of eicosanoids (Calder, 2006; Furne et al., 2013; Torrecillas et al., 2017). However, little information has been reported for fish regarding the effects of dietary ARA on inflammation. In a study with rainbow trout, Hong et al. (2022) reported

increased intestinal inflammation and phagocytic oxidative radical production in the distal intestine of rainbow trout fed diets high in ARA (Hong et al., 2022). Despite the fact that n-6 polyunsaturated fatty acid derivatives are generally known to release pro-inflammatory cytokines (Xu et al., 2022), recently reported fish studies have shown that they can also be associated with releasing anti-inflammatory cytokines (Tian et al., 2019; Nayak et al., 2020, Rivero-Ramirez et al., 2020).

With the advancement of aquaculture and the growing demand for seafood in recent years, the scale of intensive aquaculture systems has rapidly expanded. However, high-density fish farming can be stressful to fish, and presents a risk for water quality deterioration. In fact, hypoxia is one of the most common environmental factors that can lead to mortality and retarded growth. During hypoxia, free radicals are produced and accumulate in cells causing oxidative damage (Lushchak, 2011; Zhao et al., 2020). Oxidative stress due to persistent overproduction of ROS accelerates cellular damage and maintains activation of inflammation that triggers the release of pro-inflammatory cytokines such as interleukin-8 (*il-8*) and tumor necrosis factor alpha (*tnf-α*) (Bonga and Wendelaar, 1997; Hyo-Yeon et al., 2011; Jia et al., 2014).

Elevated water temperature can compound the effects of low dissolved oxygen in the water, and can itself negatively affect the fish's physiology. Among the many effects of elevated water temperature, structural changes in the cell membranes and reduced nutrient digestibility have been associated with inhibiting the growth performance of fish (Tocher 2003; Norambuena et al., 2016). Fluidity of the cell membrane is heavily dependent on the fatty acid and phospholipid composition and their associated melting temperatures (Tocher et al., 2004; Araújo et al., 2021). As such, higher levels of dietary ARA are expected to impact cell fluidity and function. It has been reported that rainbow trout can tolerate temperatures between 4°C and 20°C, although the optimum temperature range for growth is very narrow, 15–16℃ (Jalabert and Fostier, 2010). At levels above 20°C, ARA is likely to impact cell membrane composition, which may in turn impact growth and likely the inflammatory response (Norambuena et al., 2016).

Despite the importance of ARA involvement in fish physiological functions, relevant information on immune and inflammatory responses is mostly lacking. Thus, the present study was conducted to investigate the effects of graded levels of dietary ARA on growth

performance of juvenile rainbow trout from first feeding and the oxidative stress and inflammatory responses of juvenile rainbow trout to hypoxia at optimal and elevated temperatures (15 and 21℃).

Materials and Methods

Experimental Diets

Four experimental diets were prepared and extruded (Bozeman Fish Technology Center, Bozeman, MT) in various sizes from #1 to 2.5 mm, and were formulated to be isonitrogenous, isolipidic, and isocaloric. The four diets were as follows: ARA 0 (linseed oil, *i.e*., the control diet), and three other diets with graded levels of ARA oil (ARA-L: 3.95 %, ARA-M: 7.67 and ARA-H: 14.8% of total fatty acids). The desired ARA content was accomplished with a high ARA oil obtained from *Mortierella alpina* (Cabio Biotech, Wuhan, China), which contains a minimum of 40% ARA of total fatty acids. Linseed oil was used in the experimental diets to meet the fatty acid requirements for rainbow trout (NRC, 2011). The proximate and fatty acid composition of experimental diets are shown in Tables 4.1 and 4 2, respectively.

Fish, Feeding Trial and Experimental design

The feeding trial was conducted in three phases at the University of Idaho' Hagerman Fish Culture Experiment Station in Hagerman, Idaho. All fish handling and sampling, plus the experimental protocols used in this study, were approved in advance by the University of Idaho's Institutional Animal Care and Use Committee (IACUC-2021-18).

For Phase 1, rainbow trout eggs were obtained from a commercial supplier. Eggs were reared in a flow-through system supplied with spring water at 15℃. Rainbow trout swim-up fry (initial body weight: 0.17 ± 0.01 g) were randomly stocked into each of the twelve 35-L tanks at 120 fish per tank, and experimental diets were given to fish as the first meal. Constant temperature spring water (15℃) was supplied at 10-12 L min^{-1} to each experimental tank. Each diet was assigned randomly to three tanks in a completely randomized design. Fish were hand-fed to apparent satiation six times per day, seven days per week, for eight weeks (56 days). Photoperiod was maintained at 14 h light: 10 h dark with fluorescent lights controlled by electric timers. At the end of Phase 1 (8 weeks) all the

fish were counted and weighed to calculate weight gain, specific growth rate, feed conversion ratio and percent survival.

For Phase 2, 50 fish were randomly selected from each tank from Phase 1 and distributed into twelve 145-L tanks according to dietary treatment (3 tanks/treatment). Phase 2 extended the growth experiment for another 16 weeks in the redistributed tanks. Fish were hand-fed to apparent satiation three times per day and photoperiod was again maintained at 14 h light: 10 h dark. After a total of 24 weeks, all the fish were again counted and weighed to calculate weight gain, specific growth rate, feed conversion ratio and percent survival. This concluded the assessment of dietary ARA effects on growth performance, as no differences in growth performance were observed.

The fish were then pooled by diet into four tanks and maintained on their respective experimental diets until moved to the recirculating aquaculture system (RAS) for the hypoxia X temperature experiment. During this period, the fish were reared under the same conditions as described for the growth experiment.

For Phase 3, fish (227 ± 8.76 g body weight) were distributed into four tanks on two recirculating aquaculture systems (RAS) to evaluate the effect of dietary ARA on trout response to hypoxia and elevated water temperature. Prior to the acute hypoxia challenge, 40 fish per dietary treatment were split into two groups, 15℃ and 21℃ (1 tank/dietary treatment; 20 fish/tank). After fish were distributed in the RAS system, the water temperature of the 21℃ group was gradually increased at a rate of 0.5℃/day, from 15 ± 0.01℃ to 21 ± 0.05℃, whereas the 15℃ group was maintained at 15 ± 0.01℃. Once system water temperature reached 21℃, the fish were acclimated for an additional 2 weeks. Fish in all tanks were then fasted for 24 hours, followed by a 10-minute acute hypoxic event (20% O_2 saturation) obtained by using a controlled injection of nitrogen gas into system water. A DO meter was used to continuously monitor DO, and nitrogen influx was adjusted accordingly. DO was reduced from 80 % O_2 saturation over 2 min. Then, the fish were at 20% saturation for another 8 min (10 min total stress challenge). After 10 minutes of exposure to hypoxia, DO levels in the tanks were gradually returned to normoxia by influx of O_2 into the tanks (80% O_2 saturation). Five fish per tank were sampled before and after hypoxia exposure and 1 h after recovery, respectively. Initially, these fish were anesthetized with tricaine methanesulfonate (MS-222, 100 mg L^{-1}, buffered to pH 7.0) and blood was

collected from the caudal vasculature of fish with 1-ml heparinized syringes fitted with a 24G 1.5-inch needle. Blood gas and chemistry was measured immediately using i-STAT CG8+ cartridges (Abbot Laboratories, Nepean, Canada) from a drop of blood, and the remaining whole blood was centrifuged at 1000 x G for 10 min to collect plasma for analysis of cortisol, antioxidant enzyme activity, and non-specific immune parameters. The fish were then euthanized with a lethal dose of MS-222, and brain and head kidney were dissected for gene expression. Tissue samples were snap-frozen in liquid nitrogen and stored at -80℃ until analysis.

Proximate and Fatty acid Analysis

Experimental feeds were analyzed for proximate composition and energy content. Feed samples were dried in a convection oven at 105℃ for 12 h to determine moisture level according to AOAC (Association of Official Analytical Chemists) (2000). Dried samples were finely ground by mortar and pestle and analyzed for CP (total nitrogen × 6.25) using combustion method with a nitrogen determinator (Elemental nitrogen analyzer, Ronkonkoma, NY). Crude lipid was analyzed by subjecting samples to acid hydrolysis using an ANKOM HCL (ANKOM Technology, Macedon, NY) and extracting them with petroleum ether using an ANKOM XT15 extractor. Ash was analyzed by incineration at 550℃ in a muffle furnace for 5h. The energy content of samples was determined using an isoperibol bomb calorimeter (Parr 6300, Parr Instrument Company Inc., Moline, IL).

The fatty acid composition of the diets was determined in line with modified AOAC method 991.39. Briefly, samples were dried for 5–6 h under an N_2 stream at 50℃ (OA-SYS heating system, Organomation Associates, Inc., Berlin, MA, USA). Thereafter, 2mL of 0.5N NaOH was added for sample saponification at 70◦C for 60min. Following sample cooling, the free fatty acids were methylated by the addition of 2mL 14% BF3 (Boron trifluoride) in methanol and incubated at 70℃ for 60min. After the samples were allowed to cool, 2mL of hexane was added, inverted repeatedly for 60 s, and 1mL of saturated NaCl was added. The samples were again inverted repeatedly for 60 s and then centrifuged at 2000 × G for 5min. An aliquot (100 μL) of the clarified hexane extract was diluted in hexane (1:10) and put into autosampler vials for gas chromatography analysis. The injection mode with a helium flow

rate and the column temperature was as described by Overturf et al. (2013). All the analyses were done in duplicate.

Antioxidant, Non-specific Immune Assays and Prostaglandin E_2 Analysis

Commercially available kits (Cayman Chemical, Ann Arbor, Michigan) were used to measure superoxide dismutase (SOD) (Itemno: 706002) and catalase (CAT) (Item no: 707002) activities at 25℃. SOD activity was determined at 450 nm based on xanthine and xanthine oxidase to produce superoxide radicals. SOD activity was measured based on the inhibition rate of this reaction. One unit of SOD activity is equal to 50% inhibition of decrease in 2-(4-iodophenyl)-3-(4nitrophenole)- 5-phenyltetrazolium chloride according to the experimental conditions. CAT activity was measured by determining the decrease in absorbance of H_2O_2 at 540 nm. The reaction mixture containing 50mM K-phosphate buffer (pH 6.5) and 50mM H_2O_2 was diluted in 80mM K-phosphate buffer (pH 6.5). Calculation of activity was done by determining the extinction coefficient for H_2O_2 (a = 40 M^{-1} cm^{-1}).

Lysozyme activity (LYS) in plasma was analyzed with a lysozyme assay kit (Sigma-Aldrich). Micrococcus lysodeikticus (0.75 mg mL^{-1}) was suspended in phosphate buffer (0.1 M, pH 6.24), 800 uL of suspension was placed in each well of 48-well plates, and 30 μL plasma was added subsequently. The reduction in absorbance of the samples was recorded at 450 nm after incubation at room temperature for 0 and 30min in a microplate reader (Infinite® m200 PRO, Tecan Trading AG, Switzerland). A reduction in absorbance of 0.001 min^{-1} was regarded as one unit of lysozyme activity.

The levels of prostaglandin E_2 (PGE_2) and cortisol (COR) in plasma were measured using a specific kit (Cayman monoclonal Competitive Enzyme Immunoassay (ELISA) kit, Cat. No 514010, Cayman Chemicals, MI, USA) and cortisol ELISA assay kit (DRG International Inc., Springfield, NJ) according to the manufacturer's instructions, respectively.

Blood Biochemical Parameters

Blood samples were evaluated with the i-STAT Portable Clinical Analyzer (Abbot Laboratories) and CG8+ cartridges (Abbott Laboratories). After collecting whole blood, the needle was removed and one drop of whole blood was dispensed to the CG8+ cartridge, followed by sealing the cartridge port, and inserting the cartridge into the i-STAT Portable

Clinical Analyzer. This CG8+ cartridge provides values of sodium (Na, mmol/L), potassium (K, mmol/L), ionized calcium (iCa, mmol/L), glucose (Glu, mg/dL), hematocrit (Hct, % Packed Cell Volume [PCV]), pH, partial pressure carbon dioxide (PCO_2, mm Hg), partial pressure oxygen (PO_2, mm Hg), oxygen saturation (sO_2,%), and hemoglobin (Hb, g/dL).

RNA Extraction and Quantitative PCR

Total RNA was isolated from brain and head kidney using TRIzol reagent (Invitrogen, Carlsbad, CA) extraction method following the manufacturer's suggested protocol. Purity and quantity of extracted RNA were assessed by Nanodrop ND-1000 spectrophotometer (260/230 and 260/280 ratios >1.8).

Extracted RNA was treated with DNAse, then 1 µg of total RNA was reverse transcribed using the iScript™ cDNA Synthesis kit (BioRad, Hercules, CA). Real-time quantitative PCR was carried out on a CFX96 Real-Time System (BioRad) in 10 µL total volume reactions using iTaq SYBR Green Supermix (BioRad) and 500 nmol primers according to the protocol provided by the manufacturer. PCR cycling conditions for all genes were as follows: 95℃ for 5 s followed by 55℃ for 30s over 40 cycles with an initial denaturation step of 95℃ for 3min. For each fish, PCR reactions were run in duplicate. Relative expression values for genes constituting the pro- and anti-inflammatory response and conversion of ARA to eicosanoids, including tumor necrosis factor alpha (*tnf-α*), interleukin-8 (*il-8*), transforming growth factor beta (*tgf-β*), interleukin-10 (*il-10*), cyclooxygenase (*cox*), and lipoxygenase (*lox*) were determined using primers designed from rainbow trout sequences in the NCBI database. Primer sequences for genes are given in Table 4.3. The two reference genes, elongation factor 1α (*ef1α*) and actin related protein (*arp*) were used to calculate the geometric mean of the two reference genes evaluated. Primer PCR efficiency was calculated for each primer set using a six-step serial dilution of a pooled sample (pooled from each experimental sample for a given tissue). Data were analyzed using the relative quantification method, including efficiency correction following the method of Pfaffl (2001).

Calculation and Statistical Method

Using the live-weight and feed consumption data, the following indices were calculated.

Weight gain (WG, g / fish)
= (g mean final weight–g mean initial weight)
Specific growth rate (SGR, % / d)
= [(Ln mean final weight–Ln mean initial weight)/number of days] x 100
Survival (%)
= (number of fish at the end of the trial/number of fish at the beginning) x 100
Average feed intake (FI, g / fish)
= g total dry feed intake/number of surviving fish
Feed conversion ratio (FCR)
= g total feed consumed / (g final biomass – g initial biomass + g dead fish weight)

Statistical analysis of growth performance and feed utilization results from Phase 1 and Phase 2 used tank mean values (n=3) with a one-way analysis of variance (ANOVA) to identify differences between the dietary treatments. If significant differences were found, data were subjected to Tukey's HSD test to separate the means at a significance level of $P < 0.05$. Individual fish data were used for evaluating statistical differences in Phase 3, regarding blood gas and biochemical parameters, gene expression, and plasma levels of non-specific immune parameters, cortisol and prostaglandin E_2 (n=5). For these analyses, differences were tested by either one-way or multi-factorial ANOVA followed by the Tukey's HSD test to separate the means at a significance level of $P < 0.05$. All the data were tested for normality and homogeneity of variance prior to one-way or multi-factorial ANOVA. SPSS (Version 21 for Window; IBM SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

Results

Growth Performance and Feed Utilization

The growth performance and feed utilization of fish during Phase 1 (swim-up) and Phase 2 (juvenile) stages are presented in Table 4.3 and 4.4, respectively. There were no significant differences among dietary ARA treatments ($P > 0.05$) for final weight (Phase 1: 6.46 - 7.14; Phase 2: 47.9 - 52.4 g), SGR, feed intake, FCR, and survival (Phase 1: 94.7 - 95.6; Phase 2: 82.7 - 91.3 %).

Antioxidant, Non-specific Immune Assays and Prostaglandin E_2 Analysis

The results of the non-specific immune assays are presented in Table 4.6. The increase of ARA level from 0.10 to 14.8 (% of total fatty acid) significantly increased the activities of SOD, LYS, and PGE_2 concentration ($P < 0.05$). However, increasing levels of ARA resulted in significant reduction in COR level. Hypoxia resulted in increased levels of SOD, LYS, PGE_2, and COR. Increasing temperature from 15℃ to 21℃ significantly increased SOD and CAT activities, with the highest level observed at 21℃; however, temperature did not affect the activity of LYS and PGE_2 concentration. A two-way interactive effect of ARA level and hypoxia was obtained on COR concentration ($P < 0.05$). Furthermore, a three-way interaction ($P = 0.003$) of ARA level, hypoxia, and temperature was also detected for COR. There were no three-way interactive effects of ARA, hypoxia, and temperature on SOD, CAT, SYS, and PGE_2.

Blood Biochemical Parameters

The effect of four levels of dietary ARA (0.10, 3.95, 7.67, and 14.8 % total fatty acid), three hypoxic conditions (normoxia, hypoxia, and 1h recovery), and two different water temperatures (15℃ and 21℃) on whole-blood biochemical parameters (pH, pCO_2, PO_2, sO_2, Na, K, iCa, Glu, Hct, and Hb) are presented in Table 4.7. Dietary ARA levels did not influence any of the blood biochemical parameters in the current study. Regardless of dietary ARA levels, hypoxia and temperature impacted the levels of Na and Glu. Blood Na levels were significantly reduced in response to hypoxia whereas an increased level was observed at elevated temperature. Blood Glu levels were significantly increased as a result of hypoxia and elevated temperature ($P < 0.05$). With regards to Hct and Hb levels, only

hypoxia had significant independent effect. Blood pH, PO_2, sO_2, K, and iCa levels were not affected by any of the factors ($P > 0.05$)

Gene Expression

Relative gene expression in the brain and head kidney of rainbow trout fed four experimental diets is presented in Table 4.8. Three main factors (ARA level, hypoxia, and temperature) independently affected the expression of *cox-2* in the brain, with its highest level observed in ARA-H group during hypoxia phase at 21℃, but no interactive effects were detected. With regards to *lox* expression in the brain, only ARA level had significant independent effect ($P < 0.05$), but a two-way interactive effect between ARA level and temperature was observed. Two main factors, ARA level and hypoxia, independently impacted the expression levels of *lox*, *il-8*, and *tnf-α*, demonstrating that both dietary ARA and hypoxic condition increased the expression of those genes. Although only the expression of *il-10* was unaffected by ARA level, hypoxia decreased the expression of *il-10*, and elevated water temperature increased the expression. Higher expression level of *tgf-β* was recorded in fish fed the ARA-M or ARA-H diet. A three-way interaction was not observed in relative gene expression involved in inflammatory response ($P > 0.05$).

Discussion

Most studies in fish examining the effects of ARA on growth and health have been performed in marine fish species due to their limited ability to bioconvert linoleic acid (LA) to ARA. However, as reported in a recent review paper (Xu et al., 2022), high-trophic level fish species have considerably lower ARA levels than low-trophic species in muscle and liver tissues, such as muscle and liver, which suggests that the capacity of bioconversion may be due to their trophic level. In addition, numerous aquaculture species, mainly marine fish, rely on live feed for morphogenesis at early developmental stages (Rønnestad et al., 2013; Boglino et al., 2014). Most common live feeds used for larval development (i.e., rotifer and *Artemia*) are known to be high in ARA, suggesting that ARA could be a crucial fatty acid at the early developmental stages of fish. Currently, the literature integrating ARA and health in fish is lacking, with studies primarily conducted using live feeds on growth, survival, and stress response. Therefore, we hypothesized that dietary ARA in swim-up (first-

feeding) rainbow trout would affect fish growth performance and feed utilization. It was also hypothesized that a continuous supply of ARA would provide protective effects against stressors that fish could easily encounter, such as hypoxia and high temperature.

In the current study, the growth performance and survival were unaffected by dietary ARA levels at the end of both the Phase 1 (swim-up to 8 weeks) and Phase 2 feeding trial (8 – 24 weeks), evidencing that dietary ARA neither negatively nor positively affected early development and growth of rainbow trout. These results are in line with findings from various fish species such as Atlantic cod *Gadus morhua* (Lie et al., 2016), yellowtail flounder *Limanda ferruginea* (Copeman et al., 2002), and gilthead seabream *Sparus aurata* (Martin et al., 2012). On the other hand, studies with European seabass *Dicentrarchus labrax* (Atalah et al., 2011), Japanese flounder *Paralichthys olivaceus* (Esteves et al., 1997), and summer flounder *Paralichthys dentatus* (Willey et al., 2003) showed that dietary ARA promoted growth performance of fish larvae. Further, some of these studies also showed that an oversupply of dietary ARA inhibited fish growth. These studies demonstrate that the impact of ARA on growth performance displays conflicting results and appears to segregate on whether evaluation occurs in freshwater or marine fish species. However, controversial results were observed among studies, even for the same species. For instance, although many studies in Senegalese soles did not show a significant effect of dietary ARA on growth, Boglino et al. (2012) found that dietary ARA contributed to growth improvement during the Artemia feeding period. Most of the studies mentioned above used live feeds, mainly Artemia, with very few employing microdiets. The most significant benefit of using microdiets is that narrows possible effects of ARA by eliminating other confounding factors, such as the presence of EPA or DHA in live feeds, which are known to modulate the effects of ARA (Norambuena et al., 2016).

The non-specific immune system serves as the first line of defense, protecting against oxidative stress, eventually leading to cellular damage (Martínez-Álvarez et al., 2005). In the current study, SOD, LYS, PGE2, and cortisol levels in the plasma of rainbow trout were affected by either ARA level, hypoxia, or temperature. The mechanism by which ARA influences non-specific immunity is known via activation of the pathway of eicosanoid production (Rowley et al., 1995). Among several types of eicosanoids, leukotriene B4, an ARA-derived mediator, plays the central role in the production and release of lysosomal

enzymes and superoxide in leukocytes (Samuelsson et al., 1983); therefore, dietary ARA supplementation enhances the activities of LYS, SOD, and CAT. With regards to PGE2, elevated levels were recorded with increasing levels of dietary ARA. PGE2 is a potent inflammatory eicosanoid derived from ARA generated by the action of the cox pathway (Bell and Sargent, 2003). An increase in this eicosanoid implies greater availability of ARA in the cell membrane of trout fed the three diets supplemented with ARA. In the current study, hypoxia significantly increased the results of all plasma assays except for CAT, and elevated temperature to 21℃ resulted in a significant increase in SOD and CAT activities in the plasma of trout. It is well known that severe hypoxic conditions generally cause oxidative stress, inflammation, and immunosuppression (Zhao et al., 2020), thereby adversely affecting fish growth and health, resulting in economic loss. Moreover, the production of eicosanoids is initiated when fish are exposed to stressful conditions or diseases (Arts and Kohler, 2009). Increased water temperature and hypoxia are general challenges in the aquaculture industry that instantly affect fish growth, physiology, and immune response, and can result in fish death (Dan et al., 2014; Zhao et al., 2020).

During times of stress caused by environmental factors such as low dissolved oxygen or rising water temperature, the level of reactive oxygen species (ROS) rises rapidly, causing oxidative stress (Vinagre et al., 2012). This report is in line with our findings that elevated water temperature and hypoxic conditions increased the activity of antioxidant-related enzymes. Interestingly, the cortisol concentration showed a tendency to decrease significantly as the inclusion of dietary ARA increased. Several studies have documented that n-6 LC-PUFAs are involved in cortisol synthesis via the hypothalamic-pituitary-intrarenal cell (HPI) axis and stimulate cortisol release (Bell and Sargent, 2003; Koven et al., 2001). However, a recent study with European sea bass demonstrated that increased dietary levels of ARA induced a significant downregulation of genes in relation to cortisol synthesis, such as *StAR* and cytochrome 11β-hydroxylase (Montero et al., 2015), suggesting that mRNA levels of those genes contributed to lowering basal cortisol level as dietary ARA level increased in the diet, which is in agreement with our results. Another possible factor for decreased basal cortisol levels observed could be the high-dose ARA employed in this study and the duration of feeding. It must be noted that the fish used for hypoxia and high-temperature challenge were reared from first feeding to approximately 200 g on their

respective dietary treatments. In the current study, the high-dose ARA concentration was supplemented at a level comparable to other ARA supplementation experiments. Combining the results of several studies showing that ARA promotes PGE_2 production and decreases cortisol synthesis, it can be speculated that long-term feeding of high-dose ARA diets induced high cortisol synthesis throughout the entire feeding period, thereby repressing the HPI axis and consequently inducing allostatic overload, which takes place due to the cumulative effects of the chronic stress response, leading to health problems (Hundal et al., 2021).

Supplementation of ARA influences the antioxidant cascade and non-specific immunity (Nayak et al., 2017; Torrecillas et al., 2017), but little is known regarding its respiratory effects. Our results demonstrate that ARA has no effects on any of the biochemical parameters in whole blood of rainbow trout fed experimental diets; however, hypoxia and water temperature did affect some of the whole-blood parameters, including pH, pCO_2, Na, Glu, Hct, and Hb. Under hypoxic stress, fish typically reduce oxygen consumption by minimizing movement and increasing Hb levels, which enhances oxygen-carrying capacity (Abdel-Tawwab et al., 2019). In the present study, the effect of hypoxia to increase Hct and Hb concentrations in rainbow trout was significant, in line with studies with goldfish *Carassius auratus* (Roesner et al., 2008) and genetically improved tilapia *Oreochromis niloticus* (Sheng et al., 2019), showing that fish responded by raising the Hb concentration to increase the oxygen-binding capacity of trout blood. Blood Glu levels can also be an indicator of stress in fish (Pacheco and Santos, 2001). In the present study, increased glucose levels were recorded in the fish exposed to hypoxia and elevated water temperature, possibly due to enhanced glycogenolysis and gluconeogenenic reactions to fulfill the increased energy demand under stressful conditions (Shahjahan et al., 2018). The observed changes in Na concentrations were as expected. Blood inorganic ions such as sodium have often been used as stress biomarkers, primarily responsible for total osmolality in teleost (Sampaio and Freire, 2016). When fish are subjected to an acute stressor, an increase in epinephrine induces vasoconstriction and increased cardiac output, leading to an increase in gill diffusion capacity due to improved lamellae perfusion (Mazeaud and Mazeaud, 1981; Randall and Perry, 1992). The increased diffusion capacity increases ion transport in the gills and, thus,

alters blood osmolality, mainly circulating sodium concentration (McDonald and Milligan, 1997).

Studies on the inflammatory response as modulated by dietary ARA, more specifically its metabolites, are of great interest in mammals, whereas studies with fish are incredibly lacking. It is well established that the expression of *cox-2* and *lox* genes are directly linked to eicosanoid synthesis, derived from ARA (Funk, 2001). Interestingly, in the present study, the baseline levels (normoxia) of *cox-2* and *lox* gene expression were unaffected by the ARA content of the diets. According to the finding of Holen et al. (2015), ARA added alone did not cause upregulated expression levels of cox-2 and lox, whereas the combination of EPA and ARA upregulated the expression of these genes (nonsignificantly) in Atlantic salmon head kidney cells. These results are contrary to the known fact that the cox enzymes have a high affinity for ARA as a substrate.

Compared to the terrestrial environment, the aquatic environment is a complex ecosystem with numerous environmental elements interacting with each other, and in particular, hypoxia can exacerbate oxidative stress along with high temperatures, leading to immunosuppression (Sun et al., 2020). This experiment shows that all the genes involved in the inflammatory response were upregulated in response to hypoxia, except for *il-8* and *tnf-α*, which shows that the inflammatory response and oxidative stress are closely related. These effects associated with hypoxia were strongly evidenced by Zhao et al. (2020). The authors conducted a study with largemouth bass *Micropterus salmoides* and observed upregulated antioxidant-related genes such as *Cu/ZnSOD*, *CAT*, and glutathione peroxidase, inflammatory genes including *il-1β* and *il-8*, as well as downregulation of an anti-inflammatory gene (*il-10*) when exposed to hypoxia, together confirming that hypoxia exposure initiated the inflammation process. In the present study, all the genes involved in eicosanoid production (*cox-2* and *lox*) and pro-inflammation (*il-8* and *tnf-α*) were upregulated in response to hypoxia, indicating that hypoxia also induced a pro-inflammatory response in rainbow trout. Interestingly, although there was no difference in baseline expression levels, the head kidneys of fish fed ARA-supplemented diets showed a tendency of higher *cox-2*, *lox*, *il-8,* and *tnf-α* expression levels during hypoxia and *il-8*, and *tnf-α* were more prominent during the recovery phase. It is unclear what role ARA might play in relation to hypoxia, but based on our results, it can be speculated that the fish fed the ARA-supplemented diets reserve

higher ARA content in their cell membranes and releases more ARA under stress conditions, although this needs to be confirmed in further studies.

Both *il-10* and *tgf-β* are well-known anti-inflammatory markers that inhibit excessive activation of the pro-inflammatory responses (Koj, 1998). The significant up-regulation of *il-10* in our study after 1-h recovery from hypoxia suggests this protective mechanism also exists in fish. It is interesting to note that the expression of *tgf-β* was affected only by ARA levels, showing a tendency to increase as the dietary ARA level increased. In line with the results of the current study, ARA supplementation has been reported to alleviate intestinal inflammation caused by plant-based protein ingredients through increased expression of *tgf-β,* as well as reduced pro-inflammatory cytokines such as *il-8* and *tnf-α* (Wei et al., 2021). These findings contradict the results of studies showing that ARA is pro-inflammatory. However, it remains unclear as to why ARA functioned as pro-inflammatory in some studies. It appears that the balance and content of ARA and EPA may be contributing factors in regulating pro-inflammatory and anti-inflammatory processes.

In conclusion, the present study demonstrated the importance of dietary ARA for antioxidant and inflammatory responses in rainbow trout, particularly in response to the oxidative stressor, hypoxia, and increased temperature. These findings provide new insights into the role of ARA in aquafeeds, and support further exploration of ARA to support oxidative stress in fish, especially in the context of current aquaculture efforts to replace fish oil.

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Table 4.1. Formulation and proximate composition of the experimental diets (as fed).

Ingredients (%)	Diets			
	ARA 0	**ARA-L**	**ARA-M**	**ARA-H**
Fish meal, white fish[a]	10	10	10	10
PBM, feed grade[b]	10	10	10	10
Blood meal, spray dried[b]	5.0	5.0	5.0	5.0
Soy meal, sol ext.[b]	15	15	15	15
Soy protein concentrate[c]	5.0	5.0	5.0	5.0
Wheat gluten meal[b]	5.0	5.0	5.0	5.0
Corn protein con., 75% CP[d]	15	15	15	15
Wheat flour[b]	14	14	14	14
L-Lysine HCL[e]	1.2	1.2	1.2	1.2
DL-Methionine[e]	1.0	1.0	1.0	1.0
Monocalcium phosphate[b]	0.9	0.9	0.9	0.9
Trace mineral mix, Trouw[f]	0.1	0.1	0.1	0.1
Vitamin Premix, ARS 702[g]	1.0	1.0	1.0	1.0
Choline chloride (60%)[h]	0.6	0.6	0.6	0.6
Stay C (35%) vitamin[h]	0.2	0.2	0.2	0.2
Linseed oil[i]	**16**	**14.78**	**13.53**	**11.03**
ARA oil[j]	**0.00**	**1.22**	**2.47**	**4.97**
Nutrients (% as-fed basis)				
Dry Matter	95.42	95.15	95.53	95.49
Protein	51.4	51.8	51.6	51.5
Fat	14.9	14.9	14.8	15.5
Ash	4.95	4.96	5.00	5.02
Gross energy (MJ/kg)	22.8	23.2	22.8	23.4

[a] SeaPro 75, BioOregon Protein, Inc. Warrenton, OR, USA.
[b] Rangen Inc., Buhl, ID, USA.
[c] Profine VF, The Solae Company, St. Louis, MO, USA.

[d] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
[e] Sigma Aldrich, St. Louis MO, USA.
[f] US Fish and Wildlife Service Trace Mineral Premix #3 supplied the following (mg kg^{-1} diet): Zn (as $ZnSO_4 \cdot 7H_2O$), 75; Mn (as $MnSO_4$), 20; Cu (as $CuSO_4 \cdot 5H_2O$), 1.54; I (as KIO_3), 10.
[g] Vitamin premix supplied the following per kg diet: vitamin A, 2.4 mg; vitamin D, 0.15 mg; vitamin E, 267 mg; vitamin K as menadione sodium bisulfite, 20 µg; thiamin as thiamin mononitrate, 32 mg; riboflavin, 64 mg; pyridoxine as pyridoxine-HCl, 64 mg; pantothenic acid as Ca-d-pantothenate, 192 mg; niacin as nicotinic acid, 240 mg; biotin, 0.56 mg; folic acid, 12 mg; vitamin B_{12}, 50 µg; and inositol as meso-inositol, 400 mg.
[h] Skretting USA, Tooele, UT, USA.
[i] Barlean's Ferndale, WA, USA
[j] Cabio Biotech, Wuhan, China

Table 4.2. Analyzed fatty acid profile of the experimental diets.

	Diets			
	ARA 0	**ARA-L**	**ARA-M**	**ARA-H**
C14:0	0.19	0.12	0.18	0.15
C16:0	5.24	5.60	5.73	6.07
C18:0	2.84	2.92	2.99	3.35
C18:1n-9	20.7	18.6	17.6	16.5
C18:2n-6	18.4	18.4	18.1	17
C18:3n-3	39.2	36.3	32.4	25.9
C20:4n-6	0.10	3.95	7.67	14.8
C20:5n-3	0.99	1.09	1.09	0.96
C22:6n-3	1.04	0.99	1.04	0.95

Table 4.3. Growth performance and feed utilization of rainbow trout swim-up fed for 8 weeks*.

	Diets			
	ARA 0	**ARA-L**	**ARA-M**	**ARA-H**
Initial weight (g)	0.17 ± 0.01	0.17 ± 0.01	0.17 ± 0.01	0.17 ± 0.01
Final weight (g)	7.14 ± 0.68	6.81 ± 0.17	6.87 ± 0.36	6.46 ± 0.20
Weight gain (g/fish)	6.97 ± 0.68	6.64 ± 0.17	6.70 ± 0.36	6.29 ± 0.20
SGR (%/day)[a]	6.07 ± 0.15	6.00 ± 0.04	6.01 ± 0.09	5.91 ± 0.05
Feed intake (g/fish)	5.22 ± 0.05	4.97 ± 0.08	5.17 ± 0.22	4.80 ± 0.13
FCR[b]	0.75 ± 0.06	0.75 ± 0.01	0.77 ± 0.02	0.76 ± 0.01
Survival (%)	94.7 ± 1.04	95.6 ± 1.04	95.0 ± 1.36	95.6 ± 1.04

*Values are mean ± SE (n=3). In the same row, absence of superscripts is not statistically different (*P* > 0.05; Completely Randomized Design, One-way ANOVA; Tukey's HSD test).
[a]SGR: specific growth rate (% day^{-1})
[b]FCR: feed conversion ratio

Table 4.4. Growth performance and feed utilization of rainbow trout juvenile fed for 16 weeks after redistribution*.

	Diets			
	ARA 0	**ARA-L**	**ARA-M**	**ARA-H**
Initial weight (g)	7.16 ± 0.28	7.17 ± 0.12	7.08 ± 0.29	6.89 ± 0.34
Final weight (g)	48.7 ± 6.66	50.1 ± 2.67	52.4 ± 3.36	47.9 ± 1.61
Weight gain (g/fish)	41.6 ± 6.58	42.9 ± 2.71	45.3 ± 3.62	41.0 ± 1.94
SGR (%/day)[2]	3.54 ± 0.24	3.60 ± 0.11	3.70 ± 0.94	3.59 ± 0.15
Feed intake (g/fish)	31.9 ± 3.10	30.4 ± 2.03	30.8 ± 0.69	28.6 ± 2.50
FCR[3]	0.79 ± 0.14	0.71 ± 0.09	0.68 ± 0.06	0.70 ± 0.06
Survival (%)	82.7 ± 5.73	88.7 ± 6.60	91.3 ± 0.94	90.0 ± 6.53

*Values are mean ± SE (n=3). In the same row, absence of superscripts is not statistically different (*P* > 0.05; Completely Randomized Design, One-way ANOVA; Tukey's HSD test).
[a]SGR: specific growth rate (% day^{-1})
[b]FCR: feed conversion ratio

Table 4.5. Primers sequences used in real-time qPCR for the determination of gene expression.

Genes	Component	Sequences	Gene Accession NO
il-8	Forward Reverse	CTCGCAACTGGACTGACAAA TGGCTGACATTCTGATGCTC	AJ279069
tnf-α	Forward Reverse	CACACTGGGCTCTTCTTCGT CAAACTGACCTTACCCCGCT	NM_001124357.1
il-10	Forward Reverse	CGACTTTAAATCTCCCATCGAC GCATTGGACGATCTCTTTCTTC	AB118099
tgf-β	Forward Reverse	TCCGCTTCAAAATATCAGGG TGATGGCATTTTCATGGCTA	X99303
cox-2	Forward Reverse	GGGCTTTGACATCCTCAACA CATCGGACAAGAACCCTTGA	NM_001124348.1
lox-5	Forward Reverse	CTGTCCTCCCTCTGTTCTATCT AGCATTCTGTAGGACGCTAATG	NM_001165225.1
arp	Forward Reverse	GAAGGCTGTGGTGCTCAT CAGGGCAGGGTTCTC	XM_021610240.2
elf1α	Forward Reverse	ATGCCCCCAAGTTCCTGAAG AACAGCAACAGTCTGCCTCA	NM_001124339.1

Table 4.6. Plasma non-specific immune responses, prostaglandin E_2, and cortisol concentrations of rainbow trout fed the different ARA levels*.

	SOD[a]	CAT[b]	LYS[c]	PGE_2[d]	COR[e]
Means of main effects					
ARA level (% of total FA)					
0.10	5.39[b]	53.6	110[c]	116[d]	15.9[a]
3.95	5.46[ab]	52.5	126[b]	158[c]	13.6[b]
7.67	5.68[a]	44.5	135[b]	173[b]	11.3[c]
14.8	5.68[a]	57.4	147[a]	190[a]	10.0[d]
Hypoxia					
Normoxia	5.24[b]	49.2	114[b]	147[c]	9.65[c]
Hypoxia	5.64[a]	55.8	138[a]	159[b]	17.6[a]
1h recovery	5.77[a]	51.0	137[a]	175[a]	10.8[b]
Temperature					
15℃	5.45[b]	42.6[b]	131	161	12.7
21℃	5.65[a]	61.4[a]	128	158	12.7
Multi factors ANOVA (P Value)					
ARA level	**0.002**	0.184	**< 0.001**	**< 0.001**	**< 0.001**
Hypoxia	**< 0.001**	0.419	**< 0.001**	**< 0.001**	**< 0.001**
Temperature	**0.003**	**< 0.001**	0.123	0.210	0.817
ARA level × Hypoxia	0.957	0.147	0.672	0.904	**< 0.001**
ARA level × Temperature	0.783	0.518	0.702	0.170	0.178
Hypoxia × Temperature	0.645	0.903	0.757	0.100	0.148
ARA level × Hypoxia × Temperature	0.837	0.445	0.314	0.890	**0.003**

*Main effect means followed by a different letter are significantly different at $P < .05$, emphasized by bold P values in the ANOVA table. [a]SOD, superoxide dismutase (%inhibition). [b]CAT, catalase activity (nmol min^{-1} ml^{-1}). [c]LYS, lysozyme activity (unit ml^{-1} enzyme). [d]PGE_2, prostaglandin E_2 (pg/ml). [e]COR, cortisol (ng ml^{-1}).

Table 4.7. Hematological and biochemical characteristics of rainbow trout fed the different ARA levels[*].

	pH	pCO_2 (mmHg)[a]	PO_2 (mmHg)[b]	sO_2 (%)[c]	Na (mmol/L)[d]	K (mmol/L)[e]	iCa (mg/dL)[f]	Glu (mg/dL)[g]	Hct (%PCV)[h]	Hb (g/dL)[i]
Means of main effects										
ARA levels										
0.10	6.89	29.8	8.43	4.71	144	3.95	1.54	77.5	25.4	8.77
3.95	6.93	29.7	9.20	5.45	146	3.57	1.53	80.2	24.0	8.54
7.67	6.93	29.0	8.74	6.10	145	3.75	1.47	68.8	23.8	8.33
14.8	6.93	29.8	9.22	5.55	145	3.94	1.57	77.5	24.7	8.21
Hypoxia										
Normoxia	6.94	27.8[b]	9.07	5.10	145[ab]	3.74	1.56	65.7[b]	21.3[b]	7.40[b]
Hypoxia	6.90	29.5[ab]	8.38	5.65	144[b]	4.01	1.48	86.1[a]	26.5[a]	9.32[a]
1h recovery	6.92	31.4[a]	9.25	5.61	146[a]	3.67	1.54	76.2[ab]	25.5[a]	8.66[a]
Temperature										
15℃	6.91	25.4[b]	8.36	5.03	144[b]	3.91	1.55	70.9[b]	24.7	8.40
21℃	6.94	33.7[a]	9.43	5.87	146[a]	3.70	1.50	81.0[a]	25.2	8.52
Multi-factor ANOVA										
ARA level (A)	0.169	0.857	0.853	0.355	0.057	0.242	0.666	0.241	0.362	0.325
Hypoxia (H)	0.133	**< 0.001**	0.591	0.627	**0.033**	0.159	0.502	**0.001**	**< 0.001**	**< 0.001**
Temperature (T)	0.123	**< 0.001**	0.153	0.117	**< 0.001**	0.160	0.337	**0.017**	0.501	0.681
A × H	0.121	0.298	0.155	0.235	0.754	0.303	0.570	0.363	0.418	0.231
A × T	0.630	0.152	0.670	0.640	0.114	0.600	0.072	0.820	0.101	0.556
H × T	0.084	0.508	0.520	0.998	0.002	0.111	0.151	0.910	0.785	0.882
A × H × T	0.789	0.994	0.393	0.145	0.213	0.178	0.620	0.464	0.637	0.923

[*]Main effect means followed by a different letter are significantly different at $P < .05$, emphasized by bold P values in the ANOVA table. [a]pCO_2, partial pressure carbon dioxide. [b]PO_2, partial pressure oxygen. [c]sO2, oxygen saturation. [d]Na, sodium. [e]K, potassium. [f]iCa, ionized calcium. [g]Glu, glucose. [h]Hct, hematocrit. [i]Hb, hemoglobin.

Table 4.8. Relative mRNA expression of genes involved in eicosanoids production and inflammatory responses*.

	Brain		Head kidney					
	cox-2[a]	*lox*[b]	*cox-2*	*lox*	*il-8*[c]	*tnf-α*[d]	*il-10*[e]	*tgf-β*[f]
Means of main effects								
ARA level (% of total FA)								
0.10	1.15[b]	0.99[c]	1.47[b]	1.24[b]	1.12[c]	1.19[b]	1.03	1.02[b]
3.95	1.15[b]	1.16[bc]	1.82[a]	1.48[ab]	1.16[bc]	1.30[ab]	1.16	1.20[ab]
7.67	1.32[ab]	1.29[ab]	1.93[a]	1.65[a]	1.39[ab]	1.37[ab]	1.19	1.42[a]
14.8	1.38[a]	1.40[a]	1.77[a]	1.67[a]	1.56[a]	1.48[a]	1.16	1.42[a]
Hypoxia								
Normoxia	1.10[b]	1.11	1.38[b]	1.23[b]	1.21[b]	1.14[b]	1.26[a]	1.19
Hypoxia	1.33[a]	1.27	1.93[a]	1.66[a]	1.41[a]	1.47[a]	0.97[b]	1.22
1h recovery	1.31[a]	1.25	1.86[a]	1.64[a]	1.31[ab]	1.39[a]	1.18[a]	1.39
Temperature								
15℃	1.18[b]	1.17	1.56[b]	1.48	1.28	1.27	1.08[b]	1.15
21℃	1.31[a]	1.25	1.89[a]	1.54	1.33	1.40	1.20[a]	1.28
Pooled SE	*0.03*	*0.03*	*0.06*	*0.06*	*0.04*	*0.04*	*0.03*	*0.04*
Multi factors ANOVA (P Value)								
ARA level	**0.004**	**0.005**	**< 0.001**	**0.004**	**< 0.001**	**0.046**	0.207	**< 0.001**
Hypoxia	**0.001**	**0.024**	**< 0.001**	**< 0.001**	**0.044**	**0.002**	**< 0.001**	0.071
Temperature	**0.017**	0.065	**< 0.001**	0.420	0.418	0.079	**0.038**	0.053
ARA level × Hypoxia	0.987	0.703	0.833	0.956	0.809	0.802	0.594	0.986
ARA level × Temperature	0.754	0.095	0.330	0.106	0.965	0.547	0.297	0.321
Hypoxia × Temperature	0.310	0.812	0.253	0.675	0.259	0.992	0.490	0.610
ARA level × Hypoxia × Temperature	0.711	0.564	0.700	0.918	0.996	0.981	0.990	0.985

*Main effect means followed by a different letter are significantly different at $P < 0.05$, emphasized by bold *P* values in the ANOVA table. [a]*cox-2*, cyclooxygenase. [b]*lox*, lipoxygenase. [c]*il-8*, interleukin-8. [d]*tnf-α*, tumor necrosis factor alpha. [e]*il-10*, interleukin-10. [f]*tgf-β*, transforming growth factor beta.

Chapter 5:

Effects of Dietary Arachidonic Acid Supplementation on Growth Performance, Non-specific immunity, Inflammatory Response to Acute Stress During Final Grow-out in Rainbow Trout

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Abstract

A study was conducted to evaluate the effects of graded levels of arachidonic acid (ARA) on growth performance, fatty acid composition of whole-body and ovary, non-specific immunity, and intestinal inflammatory gene expression in response to stress in rainbow trout, *Oncorhynchus mykiss* during final grow-out. Four isonitrogenous (51% crude protein), isolipidic (15% crude lipid), and isocaloric (23 MJ/kg) diets were produced; ARA 0 (linseed oil, i.e., the control diet), and three other diets with graded levels of ARA oil (ARA-L: 3.95%, ARA-M: 7.67, and ARA-H: 14.8% of total fatty acids). Rainbow trout (307 ± 3.64 g) were fed to apparent satiation three times a day for 12 weeks to assess growth performance and feed utilization, then fish were exposed to an acute stress (lowering the water level to 5 cm, just above the eyes, for 30 mins). The 12-week growth trial showed that dietary ARA did not affect rainbow trout growth performance and feed utilization during final grow-out. Whole-body fatty acid profiles reflected those of the diets except for eicosapentaenoic acid (EPA) content being significantly higher in the ARA 0 group, demonstrating that rainbow trout are capable of bioconversion of α-linolenic acid (ALA) to EPA. EPA and docosahexaenoic acid (DHA) contents in the ovary were less affected by dietary ARA supplementation, likely reflecting the selective deposition of those fatty acids in embryonic development. Plasma superoxide dismutase, catalase, and lysozyme activity significantly increased in both pre-and post-stress groups as a result of incremental increases in dietary ARA, perhaps due to Leukotriene B4, a metabolite of ARA, responsible for the production of lysosomal enzymes and superoxide in leucocytes. The expression of *tnf-α* was significantly downregulated in ARA-M and H relative to other treatment groups, suggesting that dietary ARA had an anti-inflammatory effect. These results suggest that supplementation of ARA can boost immune function and inflammatory responses in sub-adult rainbow trout.

Introduction

Arachidonic acid (ARA) is an n-6 long-chain polyunsaturated fatty acid (LC-PUFA), predominantly present in the phospholipids of cell membranes, and its importance has recently been emphasized, mainly due to its role in eicosanoid production (Bell and Sargent, 2003; Norambuena et al., 2015; Torrecillas et al., 2017; Wang et al., 2019; Xu et al., 2022; Hong et al., 2022). ARA is a required fatty acid in some fish species, playing a crucial role in the regulation of growth and skeletal development (de Vrieze et al., 2014; Lie et al., 2016; Ma et al., 2018; Torrecillas et al., 2018; Araújo et al., 2021) reproduction (Sargent et al., 1999; Norambuena et al., 2012; Zupa et al., 2017; Fei et al., 2021), and resistance to multiple stressors (Koven et al., 2001; Van Anholt et al., 2004a,b). However, several contrasting results have been observed in studies demonstrating the effect of ARA supplementation on several fish species. ARA has been shown to facilitate fish growth in marine and freshwater species such as cobia, *Rachycentron canadum* (Araújo et al., 2019), Japanese eel, *Anguilla japonica* (Shahkar et al., 2016), and yellow catfish, *Pelteobagrus fulvidraco* (Ma et al., 2018); In contrast, significant differences in growth were not observed in grass carp, *Ctenopharyngodon idellus* (Tian et al., 2017), gilthead bream, *Sparus aurata L.* (Fountoulaki et al., 2003), and guppy, *Poecilia reticulate* (Khozin-Goldberg et al., 2006). In this regard, Xu et al. (2021) suggested that ARA, similar to eicosapentaenoic acid (EPA) and docosapentaenoic acid (DHA), may also not be sufficiently biosynthesized from precursors (e.g., linoleic acid, 18:2n-6) by high-trophic level fish species.

Rainbow trout, *Oncorhynchus mykiss*, is a carnivorous fish widely farmed in the worldwide and is a popular experimental model species. Numerous experiments on lipid metabolism, predominantly n-3 LC-PUFA, have been performed with rainbow trout (Gregory and James, 2014; Gregory et al., 2016; Santigosa et al., 2020; Roy et al., 2020). However, to the best of our knowledge, there are no reported studies on the effects of ARA supplementation on rainbow trout growth and stress response.

EPA and ARA have similarities in their structure of 20 carbons and are precursors of eicosanoids that compete for enzymes to synthesize different types of eicosanoids. However, despite the high EPA content in fish tissues, enzymes involved in eicosanoid production, such as cyclooxygenases (COX-1 and 2) and lipoxygenase (LOX), have more affinity for ARA. Under normal conditions, ARA is present in the phospholipids of the cell membrane.

When cells perceive stress, particularly an inflammatory stimulus, phospholipids are released from the cell membrane, and with the help of phospholipase A2, ARA is released (Van Dorp, 1975). The free ARA can be transformed into several different metabolites through the action of different enzymes, COX and LOX, thus facilitating inflammatory cascades (Jonge et al., 1996; Murakami et al., 2000; Arts and Kohler, 2009). Compared to studies in mammals, there are few reports on the effect of ARA on the inflammatory response in fish. In a study with rainbow trout fed genetically modified oil containing relatively high levels of EPA, docosapentaenoic acid and ARA, distal intestinal inflammation was observed, and authors suggested that ARA could be responsible for the inflammation (Hong et al., 2022). However, in European sea bass, an ARA-deficient diet induced upregulation of pro-inflammatory genes expression in the distal intestine (Rivero-Ramirez et al., 2020); similarly, in a study with zebrafish, it was reported that n-6 PUFA-rich microalgae as a dietary supplement up-regulated the expression of interleukin 10, an anti-inflammatory cytokine (Nayak et al., 2020). Farmed fish species are often exposed to many different stressors, such as changes in temperatures, oxygen levels, transportation, and handling, which can cause a stress response, and ARA has been associated with alterations in cortisol levels and responses to stress in different fish species (Koven et al., 2003; Lund and Steenfeldt, 2011; Alves et al., 2013). Thus, the present study investigated the effects of graded levels of dietary ARA on the growth performance, fatty acid composition of whole-body and ovary, non-specific immunity, and intestinal inflammatory gene expression in response to stress in sub-adult) rainbow trout *Oncorhynchus mykiss* (~300 g) during final grow-out.

Materials and Methods

Experimental Diets

Four experimental diets were prepared and extruded (Bozeman Fish Technology Center, Bozeman, MT) in various sizes from 2.5mm to 4.5mm, and were formulated to be isonitrogenous, isolipidic, and isocaloric. The four diets were formulated as follows: ARA 0 (linseed oil, *i.e*., the control diet), and three other diets with graded levels of ARA oil (ARA-L: 3.95 %, ARA-M: 7.67 and ARA-H: 14.8% of total fatty acids). The desired ARA content was accomplished with ARA oil obtained from *Mortierella alpina* (Cabio Biotech, Wuhan, China), which contains a minimum of 40% ARA of total fatty acids. The proximate and fatty

acid composition of experimental diets are shown in Tables 5.1 and 5.2, respectively. Linseed oil was used in the experimental diets to meet the fatty acid requirements of rainbow trout (NRC, 2011).

Fish, Feeding Trial, and Experimental design

The feeding trial was conducted at the University of Idaho' Hagerman Fish Culture Experiment Station in Hagerman, Idaho. All fish handling and sampling, plus the experimental protocols used in this study were approved in advance by the University of Idaho's Institutional Animal Care and Use Committee (IACUC-2021-18).

Rainbow trout (initial body weight: 307 ± 3.64g), grown from eggs obtained from a commercial supplier, were randomly stocked into each of the twelve 1300-L tanks at 23 fish per tank. Prior to the feeding trial, all the fish were pit tagged to identify individuals. Constant temperature spring water (15℃) was supplied at 8-10 L min^{-1} to each experimental tank. Each diet was assigned randomly to three tanks in a completely randomized design. Fish were hand-fed to apparent satiation three times per day, six days per week for 12 weeks under natural light from May 10th 2021 to August 3rd 2021. After the 12-week feeding trial, three fish per tank, fasted for 24 hours, were transferred to a tank where the water level had previously been lowered to 5 cm (just above the eyes) and held there for 30 minutes.

Sample collection and Proximate and Fatty acid Analysis

At the end of 12 weeks of feeding, all the fish were individually counted and weighed to calculate weight gain, specific growth rate, feed conversion ratio, and percent survival. After the final weighing, three fish per tank were anesthetized with tricaine methanesulfonate (MS-222, 100 mg L^{-1}, buffered to pH 7.0), and individual body weight and length of fish were measured to calculate the condition factor (CF). These fish were then euthanized with a lethal dose of MS-222, and the liver, ovary, and distal intestine were dissected for hepatosomatic index calculation (HSI), gonadosomatic index (GSI), fatty acid analysis and gene expression, respectively. Before and after the stress challenge, three fish per each replicate tank were also euthanized to collect whole blood from the caudal vasculature of fish with 1-ml heparinized syringes fitted with a 24G 1.5-inch needle and centrifuged at 1000 x G for 10 min to collect plasma for non-specific immune parameters,

lysozyme, prostaglandin E_2, and cortisol analysis. Another three fish per tank were euthanized for whole-body proximate and fatty acid analysis. Tissue samples were snap-frozen in liquid nitrogen and stored at -80℃ until analysis.

Experimental feeds were analyzed for proximate composition and energy content. Feed samples were dried in a convection oven at 105℃ for 12 h to determine moisture level according to AOAC (Association of Official Analytical Chemists) (2000). Dried samples were finely ground by mortar and pestle and analyzed for CP (total nitrogen × 6.25) using the combustion method with a nitrogen determinator (Elemental nitrogen analyzer, Ronkonkoma, NY). Crude lipid was analyzed by subjecting samples to acid hydrolysis using an ANKOM HCL (ANKOM Technology, Macedon, NY) and followed by extraction petroleum ether using an ANKOM XT15 extractor. Ash was analyzed by incineration at 550℃ in a muffle furnace for five hours. The energy content of samples was determined using an isoperibol bomb calorimeter (Parr 6300, Parr Instrument Company Inc., Moline, IL).

The fatty acid composition of the diets, whole-body, and ovary was determined in line with the modified AOAC method 991.39. Briefly, samples were dried for 5–6 h under an N_2 stream at 50℃ (OA-SYS heating system, Organomation Associates, Inc., Berlin, MA, USA). Thereafter, 2mL of 0.5N NaOH was added for sample saponification at 70◦C for 60min. Following sample cooling, the free fatty acids were methylated by adding 2mL 14% BF3 (Boron trifluoride) in methanol and incubated at 70℃ for 60min. After the samples were allowed to cool, 2mL of hexane was added, inverted repeatedly for 60 s, and 1mL of saturated NaCl was added. The samples were again inverted repeatedly for 60 s and then centrifuged at 2000 × G for 5min. An aliquot (100 µL) of the clarified hexane extract was diluted in hexane (1:10) and put into autosampler vials for gas chromatography analysis. The injection mode with a helium flow rate and the column temperature was as described by Overturf et al. (2013). All the analyses were done in duplicate.

Non-specific immune responses, Prostaglandin E_2, and Cortisol analysis

Commercially available kits (Cayman Chemical, Ann Arbor, Michigan) were used to measure superoxide dismutase (SOD) (Item No: 706002) and catalase (CAT) (Item no: 707002) activities at 25℃. SOD activity was determined at 450 nm based on xanthine and

xanthine oxidase to produce superoxide radicals. SOD activity was measured based on the inhibition rate of this reaction. One unit of SOD activity equals 50% inhibition of decrease in 2-(4-iodophenyl)-3-(4nitrophenole)- 5-phenyltetrazolium chloride according to the experimental conditions. CAT activity was measured by determining the decrease in absorbance of H_2O_2 at 540 nm. The reaction mixture containing 50mM K-phosphate buffer (pH 6.5) and 50mM H_2O_2 was diluted in 80mM K-phosphate buffer (pH 6.5). Calculation of activity was done by determining the extinction coefficient for H_2O_2 (a = 40 M^{-1} cm^{-1}).

Lysozyme activity in plasma was analyzed with a lysozyme assay kit (Sigma-Aldrich). *Micrococcus lysodeikticus* (0.75mg mL^{-1}) was suspended in phosphate buffer (0.1 M, pH 6.24), 800 uL of suspension was placed in each well of 48-well plates, and 30 μL plasma was added subsequently. The reduction in absorbance of the samples was recorded at 450 nm after incubation at room temperature for 0 and 30 min in a microplate reader (Infinite® m200 PRO, Tecan Trading AG, Switzerland). A reduction in absorbance of 0.001 min^{-1} was regarded as one unit of lysozyme activity.

The levels of prostaglandin E_2 (PGE2) and cortisol in plasma were measured using a specific kit (Cayman monoclonal Competitive Enzyme Immunoassay (ELISA) kit, Cat. No 514010, Cayman Chemicals, MI, USA) and cortisol ELISA assay kit (DRG International Inc., Springfield, NJ) according to the manufacturer's instructions, respectively.

RNA Extraction and Quantitative PCR

Total RNA was isolated from the washed anterior section of the distal intestine using TRIzol reagent (Invitrogen, Carlsbad, CA) extraction method following the manufacturer's suggested protocol. The purity and quantity of extracted RNA were assessed by Nanodrop ND-1000 spectrophotometer (260/230 and 260/280 ratios >1.8).

Extracted RNA was treated with DNAse, then 1 μg of total RNA was reverse transcribed using the iScript™ cDNA Synthesis kit (BioRad, Hercules, CA). Real-time quantitative PCR was carried out on a CFX96 Real-Time System (BioRad) in 10 μL total volume reactions using iTaq SYBR Green Supermix (BioRad) and 500 nmol primers according to the protocol provided by the manufacturer. PCR cycling conditions for all genes were as follows: 95°C for 5 s followed by 55°C for the 30s over 40 cycles with an initial denaturation step of 95°C for 3min. For each fish, PCR reactions were run in duplicate.

Relative expression values for genes constituting the pro- and anti-inflammatory response and conversion of ARA to eicosanoids, including tumor necrosis factor alpha (*tnf-α*), interleukin-8 (*il-8*), transforming growth factor beta (*tgf-β*), interleukin-10 (*il-10*), cyclooxygenase-2 (*cox-2*), and Lipoxygenase (*lox*) were determined using primers designed from rainbow trout sequences in the NCBI database. Primer sequences for genes are given in Table 5.4. The two reference genes, elongation factor 1α (*ef1α*) and actin related protein (*arp*) were used to calculate the geometric mean of expression of the two reference genes evaluated. Primer PCR efficiency was calculated for each primer set using a six-step serial dilution of a pooled sample (pooled from each experimental sample for a given tissue). Data were analyzed using the relative quantification method, including efficiency correction following the method of Pfaffl (2001).

Calculation and Statistical Method

The following indices were calculated using the live weight and feed consumption data.

Weight gain (WG, g / fish)

= (g mean final weight–g mean initial weight)

Specific growth rate (SGR, % / d)

= [(Ln mean final weight–Ln mean initial weight)/number of days] x 100

Survival (%)

= (number of fish at the end of the trial/number of fish at the beginning) x 100

Average feed intake (FI, g / fish)

= g total dry feed intake/number of surviving fish

Feed conversion ratio (FCR)

= g total feed consumed / (g final biomass – g initial biomass + g dead fish weight)

Condition factor (CF)

= (g body weight) / (cm body length)^3 × 100

Hepatosomatic index (HSI)

= (g liver weight) / (g whole body weight) × 100

Gonadosomatic index (GSI)

= (g gonad weight) / (g whole body weight) × 100

For statistical analysis of data on growth performance, feed utilization, and pre-stress indices tank mean values (n=3) were used for one-way analyses of variance (ANOVA) to identify differences between the dietary treatments. For data collected during the stress challenge, multi-variate ANOVA, with stress and ARA levels as fixed factors, was performed. All data were tested for normality and homogeneity of variance prior to ANOVA. All statistical analysis was performed using SPSS (Version 21 for Window; IBM SPSS Inc., Chicago, IL, USA). If significant differences were found, data were subjected to Tukey's HSD test to separate the means at a significance level of $P < 0.05$.

Results

Growth Performance and Feed Utilization

At the end of the feeding experiment, there were no significant differences in final weight, WG, SGR, feed intake, FCR, survival, CF, HSI, and GSI among fish fed experimental diets (Table 5.3). Fish weight gain and FCR were similar to values observed in commercial production in Idaho, demonstrating that feeds and rearing practices were appropriate for sub-adult rainbow trout. Survival ranged between 79.7 and 87.0% and was not significantly different among diet groups.

Whole-body proximate composition

The whole-body proximate composition of sub-adult rainbow trout fed the experimental diets is presented in Table 5.5. Whole-body dry matter, crude protein and lipid, ash, and gross energy levels were not significantly influenced by the ARA levels ($P > .05$).

Fatty acid composition of whole-body and ovary

The whole-body and ovary fatty acid composition of sub-adult rainbow trout fed the experimental diets are presented in Table 5.6 and 5.7, respectively. The fatty acid composition of whole-body and ovary were significantly affected by the ARA levels ($P < .05$). α-linolenic acid (ALA, C18:3n-3) of whole-body of fish fed diet ARA 0 (29.1 %) was significantly higher than those of fish fed diet ARA-H (24.7 %) ($P < .05$). ARA concentration significantly increased as ARA inclusion level increased (0.43 – 0.85 %) (P

< .05). Whole-body EPA (C20:5n-3) content was significantly higher in the control (ARA 0) and ARA-L groups compared with ARA-H group (*P* < .05). However, DHA (C22:6n-3) was not significantly influenced by the ARA levels (*P* > .05).

The fatty acid composition of the ovary was influenced by the different levels of ARA. Myristic acid (C14:0), EPA, and DHA contents were not significantly affected by dietary treatments. Similar to the results of whole-body fatty acid analysis, the content of ARA significantly increased as the inclusion of ARA increased (7.65 - 12.8 %); whereas, conversely, the content of ALA decreased.

Non-specific immune responses, Prostaglandin E_2, and Cortisol analysis

The results of the non-specific immune assessment, prostaglandin E_2, and cortisol concentrations of plasma are presented in Table 5.8. Stress and dietary ARA level significantly increased plasma SOD, CAT, LYS activity (*P* < 0.05). However, stress had no effect on PGE_2 concentration in plasma (*P* > 0.05); whereas, different ARA levels affected cortisol concentration in plasma (*P* > 0.05). No significant interactions between ARA and stress were detected.

Gene Expression

The relative mRNA (RT-qPCR) expression of genes involved in the conversion of ARA to eicosanoids (*cox-2* and *lox*), and in pro-inflammatory (*il-8* and *tnf-α*) and anti-inflammatory responses (*il-10* and *tgf-β*) in the distal intestine of sub-adult rainbow trout fed the experimental diets is presented in Fig 5.1. The gene expressions of *cox-2*, *lox*, *il-8*, *il-10*, and *tgf-β* were unaffected by the diet (*P* > 0.05). While the expression of *tnf-α* was significantly downregulated (*P* < 0.05) in the ARA-M and H groups compared to other groups.

Discussion

As the aquaculture industry replaces fish oil in aquafeed with mostly vegetable oils, there is a significant reduction of the content of essential LC-PUFAs, EPA and DHA, and enrichment of the content of LA. For this reason, the importance of ARA did not receive much attention until the early 2000s, but it has attracted significant attention in the last ten

years. However, research on the effects of ARA, which has been demonstrated to influence fish growth, survival, immunity, inflammation, and oxidative stress response, has been conducted primarily in marine fish species (Montero et al., 2015; Huang et al., 2016; Cho et al., 2017; Rivero-Ramirez et al., 2020). However, Xu et al. (2022) suggested that high-trophic level freshwater species might require dietary ARA supplementation, whereas the low-trophic level species, such as grass carp, might not require ARA in their diets, perhaps due to more efficient fatty acid conversion biocapacity. Moreover, ARA synthesis is limited due to the affinity of desaturases and elongases for n-3 over the n-6 pathways (Bell and Sargen, 2003). To the best of our knowledge, this study is the first to evaluate the effects of dietary ARA supplementation on growth performance and the stress and immune responses in sub-adult rainbow trout. We hypothesized that dietary ARA would affect fish growth performance, stress, and inflammatory response in sub-adult rainbow trout (300 g). Hence, after 12 weeks of the feeding trial, fish were exposed to acute stress to reveal potential effects of dietary ARA supplementation following a the stress challenge.

Increasing dietary supplementation of ARA did not affect rainbow trout growth performance and feed utilization indices. In agreement with this study, several studies with barramundi *Lates calcarifer* (Salini et al., 2016), Atlantic salmon *Salmo salar* (Dantagnan et al., 2017), gilthead sea bream *Sparus aurata* (Van Anholt et al., 2004b), and Malabar red snapper *Lutjanus malabaricus* (Chee et al., 2020) reported that ARA does not promote growth performance. However, studies with cobia *Rachycentron canadum* (Araújo et al., 2019), golden pompano *Trachinotus ovatus* (Huang et al., 2016), Japanese eel *Anguilla japonica* (Bae et al., 2010), rabbitfish *Siganus rivulatus* (Nayak et al., 2017), and striped bass *Morone saxatilis* (Araújo et al., 2021) demonstrated that dietary ARA improved growth performance. Although no growth differences were observed in the current study, research on mammals has demonstrated that growth performance may be affected by altering the ratio of PGE_2, involved in protein degradation, to prostaglandin $F_{2\alpha}$, involved in protein synthesis (Bell and Sargent, 2003). Furthermore, Norambuena et al. (2016) reported that the inclusion of ARA and EPA significantly improved Atlantic salmon growth performance when compared to fish diets either richer in ARA or EPA, suggesting that ARA/EPA ratio may be linked to improved growth in this species.

In the present study, whole-body and ovary fatty acid profiles generally reflected those of the diets. However, whole-body EPA content was significantly higher in the ARA 0 group compared to the ARA-H group, demonstrating that rainbow trout is capable of biosynthesizing EPA from the substrate, ALA. The capacity to bioconvert ALA to EPA has been reported to be heavily dependent on substrate availability, indicating that higher ALA content in the diet increases EPA biosynthesis in fish (Takeuchi and Watanabe, 1976). In contrast to the results of whole-body EPA content, EPA and DHA contents in the ovary were less affected by dietary ARA supplementation, likely reflecting selective deposition of those fatty acids during vitellogenesis, suggesting their essential roles in embryonic development (Furuita et al., 2007; Bentancor et al., 2015; Torrecillas et al., 2017). According to ovary ARA fatty acid profile results, the ARA-H group did not show a significantly higher ARA content than the ARA-M group. This result may be because, as reported by Norambuena et al. (2016), ARA deposition can only be increased to a maximally optimal level.

Non-specific immune parameters play a crucial role as the first line of defense, especially in fish (Nayak et al., 2017). SOD and CAT are representative antioxidant enzymes; SOD converts superoxide anion to water and hydrogen peroxide, followed by CAT breaking down hydrogen peroxide into water and oxygen molecules (Luo et al., 2012). In this study, SOD, CAT, and LYS activity in plasma significantly increased in both pre-and post-stress groups by incremental levels of dietary ARA. These results were consistent with the findings in Japanese seabass *Lateolabrax japonicus* (Xu et al., 2012), Malabar red snapper *Lutjanus malabaricus* (Chee et al., 2020), and Javelin goby *Synechogobius hasta* (Luo et al., 2012) fed diets with increasing dietary ARA levels. The mechanism by which ARA affects fish immune function is known to be through alterations in eicosanoid production associated with neutrophil activity (Samuelsson, 1983; Crooks and Stockley, 1998). Therefore, increased activities of these two crucial antioxidant enzymes likely counteracted the high level of reactive oxygen species (ROS) that would have resulted from the increased respiratory burst activity.

Taken together, dietary ARA appears to provide several benefits regarding immune response and health in rainbow trout. In fish, lysozyme plays a significant role in host protection against microbial invasion, causing the lysis of pathogen bacteria (Kiron, 2012). In the present study, higher plasma lysozyme activity levels in ARA-supplemented groups

(ARA-L, M, H) are consistent with the findings in Japanese seabass (Xu et al., 2010), rabbitfish (Nayak et al., 2017) and Malabar red snapper (Chee et al., 2020), in which lysozyme activity increased with increasing dietary ARA levels. Leukotriene B4, a metabolite of ARA, is known to be responsible for the production and release of lysosomal enzymes and superoxide in leukocytes (Samuelsson et al., 1983); thus, increased dietary ARA levels facilitate lysosomal enzyme production. In this sense, it is believed that these antioxidants and lysosomal enzymes are released in response to stress to scavenge ROS and suppress further ROS generation. Similar to the results of non-specific immune parameters, a trend towards increasing plasma total PGE_2 level was observed with the provision of dietary ARA in this study. This finding differs from results in cobia (Araújo et al., 2019), where the prostaglandin level was not proportional to the ARA inclusion levels. These different trends may be due to different fish species, sizes, and inclusion level of ARA. In the case of the findings of Araújo et al. (2019), it is not easy to directly compare with this study since EPA and DHA were supplied in the feed to meet the requirements of this marine fish.

The present study showed no statistically significant differences in cortisol levels in response to ARA levels because of the relatively large variability recorded. However, there was a significant difference between the pre-and post-stress groups. Cortisol is commonly used to measure the stress status in fish. The primary physiological function of cortisol in response to stress is to promote coping and adaptation by regulating energy distribution and balancing osmotic pressure (Bonga, 1997).

The expression of several genes related to eicosanoid production and pro- and anti-inflammatory response were measured in the current study. High dietary ARA supplementation significantly downregulated the expression of *tnf-α* ($P < .05$), whereas different levels of ARA supplementation did not significantly influence other target genes. The functions of *cox* and *lox* are mainly to catalyze ARA to derivative eicosanoids, initiating the inflammatory response (Calder, 2006; Holen et al., 2015). This concept appears to be consistent with our findings that there were no changes in *lox* and *cox-2* expression in ARA-supplemented groups, suggesting that the *cox* and *lox* pathways are probably not activated under normal conditions but may be activated only when stress or inflammatory response is detected. Interestingly, the expression of *tnf-α* was significantly downregulated in ARA-M and H groups, suggesting that dietary ARA had an anti-inflammatory effect. This finding is

contrary to the notion that n-6 fatty acids, especially ARA, are generally related to the production of pro-inflammatory cytokines (Xu et al., 2022). In general, ARA-derived eicosanoids in mammals are considered pro-inflammatory, but Calder (2009) reported that PGE_2, a metabolite of ARA, may have both pro-inflammatory and anti-inflammatory effects. Moreover, PGE_2 is known to inhibit lymphocyte proliferation and interferes with the production of *tnf-α, il-1, il-6*, and *il-2* (Calder and Grimble, 2002; Tian et al., 2014). However, further research is required to identify the exact mechanisms of by which ARA and its metabolites influence rainbow trout health.

In conclusion, in this study, dietary ARA did not affect growth performance, survival, and feed utilization indices of sub-adult rainbow trout. In contrast, dietary ARA showed significant increases in non-specific parameters pre- and post-stress, indicating a role for dietary ARA in modulating fish health. To the best of our knowledge, this was the first study to investigate the effects of ARA on growth and health response in rainbow trout.

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Table 5.1. Formulation and proximate composition of the experimental diets (as fed).

Ingredients (%)	Diets			
	ARA 0	ARA-L	ARA-M	ARA-H
Fish meal, white fish[a]	10	10	10	10
PBM, feed grade[b]	10	10	10	10
Blood meal, spray dried[b]	5.0	5.0	5.0	5.0
Soy meal, sol ext.[b]	15	15	15	15
Soy protein concentrate[c]	5.0	5.0	5.0	5.0
Wheat gluten meal[b]	5.0	5.0	5.0	5.0
Corn protein conc. 75% CP[d]	15	15	15	15
Wheat flour[b]	14	14	14	14
L-Lysine HCL[e]	1.2	1.2	1.2	1.2
DL-Methionine[e]	1.0	1.0	1.0	1.0
Monocalcium phosphate[b]	0.9	0.9	0.9	0.9
Trace mineral mix, Trouw[f]	0.1	0.1	0.1	0.1
Vitamin Premix, ARS 702[g]	1.0	1.0	1.0	1.0
Choline chloride (60%)[h]	0.6	0.6	0.6	0.6
Stay C (35%) vitamin[h]	0.2	0.2	0.2	0.2
Linseed oil[i]	**16**	**14.78**	**13.53**	**11.03**
ARA oil[j]	**0.00**	**1.22**	**2.47**	**4.97**
Composition (% as-fed basis)				
Dry Matter	95.42	95.15	95.53	95.49
Protein	51.4	51.8	51.6	51.5
Fat	14.9	14.9	14.8	15.5
Ash	4.95	4.96	5.00	5.02
Gross energy (MJ/kg)	22.8	23.2	22.8	23.4

[a] SeaPro 75, BioOregon Protein, Inc. Warrenton, OR, USA.
[b] Rangen Inc., Buhl, ID, USA.
[c] Profine VF, The Solae Company, St. Louis, MO, USA.

[d] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
[e] Sigma Aldrich, St. Louis MO, USA.
[f] US Fish and Wildlife Service Trace Mineral Premix #3 supplied the following (mg kg^{-1} diet): Zn (as $ZnSO_4 \cdot 7H_2O$), 75; Mn (as $MnSO_4$), 20; Cu (as $CuSO_4 \cdot 5H_2O$), 1.54; I (as KIO_3), 10.
[g] Vitamin premix supplied the following per kg diet: vitamin A, 2.4 mg; vitamin D, 0.15 mg; vitamin E, 267 mg; vitamin K as menadione sodium bisulfite, 20 µg; thiamin as thiamin mononitrate, 32 mg; riboflavin, 64 mg; pyridoxine as pyridoxine-HCl, 64 mg; pantothenic acid as Ca-d-pantothenate, 192 mg; niacin as nicotinic acid, 240 mg; biotin, 0.56 mg; folic acid, 12 mg; vitamin B_{12}, 50 µg; and inositol as meso-inositol, 400 mg.
[h] Skretting USA, Tooele, UT, USA.
[i] Barlean's Ferndale, WA, USA
[j] Cabio Biotech, Wuhan, China

Table 5.2. Analyzed fatty acid profile of the experimental diets.

	Diets			
	ARA 0	**ARA-L**	**ARA-M**	**ARA-H**
C14:0	0.19	0.12	0.18	0.15
C16:0	5.24	5.60	5.73	6.07
C18:0	2.84	2.92	2.99	3.35
C18:1n-9	20.7	18.6	17.6	16.5
C18:2n-6	18.4	18.4	18.1	17
C18:3n-3	39.2	36.3	32.4	25.9
C20:4n-6	0.10	3.95	7.67	14.8
C20:5n-3	0.99	1.09	1.09	0.96
C22:6n-3	1.04	0.99	1.04	0.95

Table 5.3. Growth performance and feed utilization of rainbow trout sub-adult fed for 12 weeks[1].

	Diets			
	ARA 0	**ARA-L**	**ARA-M**	**ARA-H**
Initial weight (g)	309 ± 5.13	307 ± 3.81	306 ± 2.53	308 ± 1.90
Final weight (g)	606 ± 39.0	617 ± 50.0	595 ± 23.1	612 ± 18.9
Weight gain (g/fish)	298 ± 43.6	310 ± 46.2	289 ± 23.0	304 ± 19.8
SGR (%/day)[2]	0.80 ± 0.10	0.83 ± 0.08	0.79 ± 0.05	0.82 ± 0.04
Feed intake (g/fish)	501 ± 70.0	479 ± 18.7	489 ± 50.4	498 ± 35.0
FCR[3]	1.69 ± 0.04	1.58 ± 0.25	1.69 ± 0.05	1.64 ± 0.01
Survival (%)	79.7 ± 2.05	78.3 ± 3.55	81.2 ± 7.39	87.0 ± 9.39
Condition factor (%)	1.43 ± 0.08	1.43 ± 0.07	1.34 ± 0.05	1.44 ± 0.04
HSI[4]	0.77 ± 0.05	0.75 ± 0.04	0.72 ± 0.05	0.75 ± 0.05
GSI[5]	0.53 ± 0.06	0.60 ± 0.11	0.64 ± 0.09	0.59 ± 0.03

[1]Values are mean ± SE (n=3). In the same row, absence of superscripts are not statistically different ($P > 0.05$; Completely Randomized Design, One-way ANOVA; Tukey's HSD test).
[2]SGR: specific growth rate (% day^{-1})
[3]FCR: feed conversion ratio
[4]HSI: hepatosomatic index (%)
[5]GSI: gonadosomatic index (%)

Table 5.4. Primers sequences used in real-time qPCR for the determination of gene expression.

Genes	Component	Sequences	Gene Accession NO
il-8	Forward Reverse	CTCGCAACTGGACTGACAAA TGGCTGACATTCTGATGCTC	AJ279069
tnf-α	Forward Reverse	CACACTGGGCTCTTCTTCGT CAAACTGACCTTACCCCGCT	NM_001124357.1
il-10	Forward Reverse	CGACTTTAAATCTCCCATCGAC GCATTGGACGATCTCTTTCTTC	AB118099
tgf-β	Forward Reverse	TCCGCTTCAAAATATCAGGG TGATGGCATTTTCATGGCTA	X99303
cox-2	Forward Reverse	GGGCTTTGACATCCTCAACA CATCGGACAAGAACCCTTGA	NM_001124348.1
lox-5	Forward Reverse	CTGTCCTCCCTCTGTTCTATCT AGCATTCTGTAGGACGCTAATG	NM_001165225.1
arp	Forward Reverse	GAAGGCTGTGGTGCTCAT CAGGGCAGGGTTCTC	XM_021610240.2
elf1α	Forward Reverse	ATGCCCCCAAGTTCCTGAAG AACAGCAACAGTCTGCCTCA	NM_001124339.1

Table 5.5. Whole-body proximate composition and gross energy (wet basis) of rainbow trout fed experimental diets for 12 weeks*.

	Initial**	Diets			
		ARA 0	**ARA-L**	**ARA-M**	**ARA-H**
Proximate composition					
Dry matter (%)	28.6	30.1 ± 2.80	31.0 ± 1.52	28.8 ± 1.06	29.0 ± 1.23
Crude protein (%)	17.4	18.2 ± 0.33	18.5 ± 0.31	18.6 ± 0.23	18.4 ± 0.59
Crude fat (%)	8.47	8.70 ± 1.07	9.07 ± 0.90	8.17 ± 0.82	7.75 ± 0.37
Ash (%)	2.65	1.9 ± 0.17	1.7 ± 0.32	2.1 ± 0.14	2.2 ± 0.37
Gross energy (MJ/kg)	26.1	27.0 ± 1.32	27.8 ± 1.01	26.7 ± 0.05	26.8 ± 0.86

*Mean±SE (n=3) in the same row that share the same superscript are not statistically different ($P > 0.05$; Completely Randomized Design, One-way ANOVA; Tukey's HSD test).

**Initial values were not included for statistical analysis

Table 5.6. Fatty acid composition (% of total fatty acids) of whole-body of rainbow trout sub-adult fed experimental diets for 12 weeks*.

	Diets			
	ARA 0	**ARA-L**	**ARA-M**	**ARA-H**
C14:0	1.54 ± 0.13	1.38 ± 0.30	1.21 ± 0.04	1.76 ± 0.42
C16:0	14.1 ± 0.35	14.2 ± 0.39	14.7 ± 0.21	15.8 ± 1.40
C18:0	4.35 ± 0.79	4.24 ± 0.35	4.83 ± 0.00	5.31 ± 0.40
C18:1n-9	20.6 ± 1.25	19.8 ± 2.90	19.5 ± 0.31	17.6 ± 0.83
C18:2n-6	15.5 ± 0.74	15.6 ± 0.63	16.1 ± 0.10	15.8 ± 1.18
C18:3n-3	29.1 ± 0.29^{a}	28.3 ± 0.43^{ab}	27.4 ± 0.23^{ab}	24.7 ± 0.45^{b}
C20:4n-6	0.43 ± 0.02^{d}	0.59 ± 0.00^{c}	0.66 ± 0.02^{b}	0.85 ± 0.03^{a}
C20:5n-3	0.63 ± 0.10^{a}	0.62 ± 0.06^{a}	0.49 ± 0.02^{ab}	0.36 ± 0.06^{b}
C22:6n-3	4.86 ± 0.41	4.64 ± 0.77	4.31 ± 0.08	4.33 ± 0.10

*Mean±SE (n=3) in the same row that share the same superscript are not statistically different (*P*> 0.05; Completely Randomized Design, One-way ANOVA; Tukey’s HSD test).

Table 5.7. Fatty acid composition (% of total fatty acids) of ovaries of rainbow trout sub-adult fed experimental diets for 12 weeks*.

	Diets			
	ARA 0	**ARA-L**	**ARA-M**	**ARA-H**
C14:0	0.70 ± 0.11	0.65 ± 0.04	0.76 ± 0.06	0.84 ± 0.05
C16:0	18.4 ± 0.11^{c}	19.1 ± 0.64^{bc}	20.9 ± 0.74^{b}	24.0 ± 0.70^{a}
C18:0	2.42 ± 0.09^{b}	2.47 ± 0.07^{b}	2.65 ± 0.10^{ab}	2.73 ± 0.04^{a}
C18:1n-9	22.5 ± 0.72^{a}	21.1 ± 0.74^{ab}	20.7 ± 0.50^{ab}	19.5 ± 0.30^{b}
C18:2n-6	7.63 ± 0.30^{ab}	8.29 ± 0.10^{a}	7.72 ± 0.21^{a}	6.83 ± 0.17^{b}
C18:3n-3	11.8 ± 0.54^{a}	9.79 ± 1.04^{ab}	9.21 ± 0.15^{bc}	7.13 ± 0.22^{c}
C20:4n-6	7.65 ± 0.28^{c}	9.82 ± 0.66^{b}	12.3 ± 0.08^{a}	12.8 ± 0.54^{a}
C20:5n-3	3.03 ± 0.08	2.96 ± 0.00	3.32 ± 0.68	2.90 ± 0.60
C22:6n-3	14.3 ± 1.47	14.3 ± 0.35	13.5 ± 0.36	13.7 ± 0.34

*Mean±SE (n=3) in the same row that share the same superscript are not statistically different (*P*> 0.05; Completely Randomized Design, One-way ANOVA; Tukey's HSD test).

Table 5.8. Plasma non-specific immune responses, prostaglandin E2, and cortisol concentrations of rainbow trout fed for 12 weeks[*].

	SOD[1]	CAT[2]	LYS[3]	PGE_2[4]	COR[5]
Means of main effects					
Stress					
Non-stress group	5.12[b]	53.6[b]	116[b]	168	38.1[b]
Stress group	5.57[a]	61.9[a]	152[a]	183	234[a]
ARA level					
0.10	5.10[b]	50.1[b]	114[b]	101[c]	142
3.95	5.17[b]	57.7[ab]	136[ab]	144[b]	150
7.67	5.57[a]	61.2[a]	145[a]	227[a]	136
14.8	5.55[a]	60.7[a]	140[a]	231[a]	115
Multi factors ANOVA (P Value)					
Stress	< 0.001	< 0.001	< 0.001	0.141	< 0.001
ARA level	< 0.001	< 0.001	< 0.001	< 0.001	0.167
Stress × ARA level	0.93	0.075	0.628	0.497	0.425

[*]Main effect means followed by a different letter are significantly different at $P < .05$.
[1]SOD: superoxide dismutase (%inhibition)
[2]CAT: catalase activity (nmol min^{-1} ml^{-1})
[3]LYS: lysozyme activity (unit ml^{-1} enzyme)
[4]PGE_2: Prostaglandin E_2 (pg/ml)
[5]COR: Cortisol (ng ml^{-1})

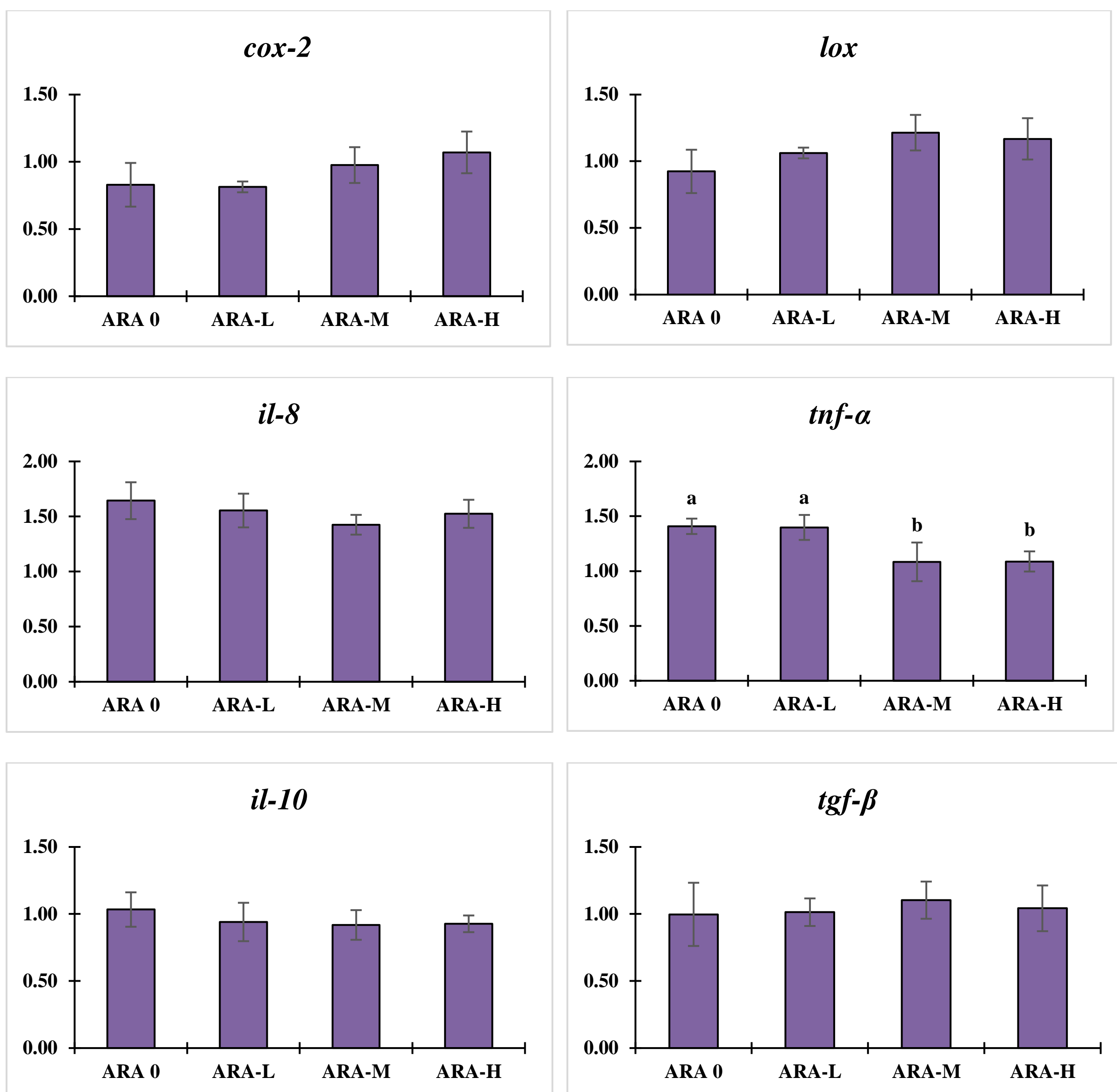

Figure 5.1. Relative mRNA expression of genes involved in conversion of ARA to eicosanoids (*cox-2* and *lox*), and pro- and anti-inflammatory response (*il-8, tnf-α, il-10, and tgf-β*) in distal intestine of rainbow trout fed experimental diets for 12 weeks. Mean ± SE (n = 9 fish per treatment) in the same row that share the same superscript are not statistically different ($P > 0.05$). Three fish from each tank were used for gene expression. Abbreviations: *cox-2*: Cyclooxygenase-2; *lox*: Lipoxygenase; *il-8*: Interleukin-8; *tnf-α*: Tumor necrosis factor alpha; *il-10*: Interleukin-10; *tgf-β*: Transforming growth factor beta.

Chapter 6: Conclusion

Synthesis of Research Findings

Today, we face a problem because the majority of capture fisheries are fully or over exploited in the sea as well as in freshwater, whereas the demand for seafood continues to grow. For this reason, aquaculture has developed rapidly, and is now recognized as an essential food production sector to bridge the gap between supply and demand for food fish. Undoubtably, aquaculture will expand further in response to increasing global population and per capita consumption of seafood. Aquafeed production will also continue to increase, creating higher demand for protein and lipid ingredients. In the early phases of aquaculture development, only fishmeal was thought to be a potential limiting factor, and fish oil became important only in the last decade, with the development of high-fat salmonids diets and the growing significance of n-3 long-chain polyunsaturated fatty acids (LC-PUFA) for human consumption, especially eicosapentaenoic acid (EPA) and docosapentaenoic acid (DHA). As reviewed in Chapter 1, the meta-analysis reported in 2020 has integrated over ten years of experiments and data from aquaculture operations and noted that dropping the use of fish oil in aquafeeds would substantially lower pelagic fish demand more than lowering fishmeal levels since it requires more biomass to produce one ton of fish oil than one ton of fishmeal. In general, 100 kg of fresh-fish raw material yields an average of 10 kg of fish oil (Turchini et al., 2010). Aquaculture uses around 75% of the global fish oil supply, and fish oil costs are expected to continue to rise (Naylor et al., 2021). Therefore, alternative methods are needed to ensure the continuous growth of aquaculture.

Nutritional and Biochemical Approach to Enhance n-3 LC-PUFA

There are several approaches to enhance the n-3 LC-PUFA content in the edible portion of fish, of which using nutritional or biochemical approaches are favored (Tocher, 2015). These approaches are based on utilizing a specific dietary supplement or ingredient that would increase n-3 LC-PUFA biosynthesis by mechanisms such as affecting gene expression and enzyme activity. In our experiment presented in Chapter 2, 1.43 mg/g diet cholesterol was added to the trout diet, based on the findings of Norambuena et al. (2013) that cholesterol appears to stimulate fatty acid β-oxidation and the conversion of α-linolenic

acid (ALA) to DHA. The results of our study showed that cholesterol supplementation numerically increased EPA and DHA contents, but not significantly. In addition, the levels of EPA and DHA in fillet were half that of the group fed fish oil, which is insufficient considering the generally known EPA and DHA recommended intake level (500-1,000 mg per day) announced by multiple health agencies (Aranceta and Pérez-Rodrigo, 2012). One could ask whether a further increase in cholesterol supplementation would lead to an even greater increase in EPA and DHA content, but this does not appear to be a practical option, as this would lead to a sharp rise in feed price. Considering the cholesterol powder price of $60 per gram and the price of fish oil at around $ 2 per kilogram, as well as the non-significant increase in EPA and DHA observed, it would not be cost-effective. Furthermore, increasing the amount of linseed oil in aquafeed is also limited in practical use due to the high price of linseed oil compared with other vegetable oils (USDA, 2018). In the experiment described in Chapter 2, 21% of crude fat was used, the same as the fat content level typical in feed formulations in trout. When more fat is included than the recommended level for high EPA and DHA deposition, the feed price may be reduced by the reduction of crude protein level, but the excessive energy supply not only reduced feed intake but also damaged fish health by increased fat deposition in the liver. Considering the results obtained from this study and the limitations mentioned above, I concluded that it is not a practical strategy to replace fish oil by supplementing cholesterol with linseed in trout diets.

Following the cholesterol supplementation experiment with the main purpose of tapping into the fatty acid biosynthesis capability of trout, a follow-up study was conducted to confirm the efficacy of genetically modified (GMO) oil (Latitude oil™), high in docosapentaenoic acid (DPA) and arachidonic acid (ARA), compared to fish oil (Table 6.1). The hypothesis of this experiment was similar to the previous experiment in that it takes advantage of the biochemical capacity of fish, with the goal being to confirm whether the DPA in Latitude oil can be converted into DHA in trout fillet. Based on the experimental results, it can be concluded that Latitude oil can successfully replace fish oil and provides another approach that enables sustainable fish production, an important goal for global aquaculture. It is well known that alternating crops can help manage soil fertility and prevent the build-up of disease and pests (Ball et al., 2005; Bennett et al., 2012); hence, this new canola crop could be a win-win for both aquaculture and agriculture, serving as a rotational

crop for Montana growers as well as helping relieve pressure on wild-caught fish to produce fish oil. In addition, it is an attractive alternative, if priced competitively, in the current situation where fish oil prices are increasing due to the rapidly growing aquaculture industry and the increased demand by nutraceutical industries.

Although the results show that Latitude oil can be a good alternative for trout, it is necessary to consider whether this oil is a suitable ingredient in feeds for marine fish species. It is generally acknowledged that marine fish, as well as crustaceans such as shrimp, require n-3 LC-PUFA due to their limited ability to convert ALA to EPA or DHA (Lim et al., 1997; Tocher, 2010). Numerous studies have shown that essential fatty acid requirements cannot be met by either LA or ALA (see Chapter 1). Two separate experiments were conducted to evaluate Latitude oil in Atlantic salmon *Salmo salar* (unpublished) and Pacific white shrimp *Litopenaeus vannamei* (Gia Vo et al., 2021). The study with Atlantic salmon was conducted with two size groups of fish, fingerling (10g) in freshwater and juvenile (184g) in seawater, and neither trial showed significant differences in growth, FCR, and survival. In the study with Pacific white shrimp, Latitude oil was able to replace fish oil up to 100% in fishmeal-based diets and up to 75% in poultry meal-based diets without compromising growth, suggesting that Latitude oil may not be a suitable oil source to replace 100% fish oil without fishmeal inclusion. In addition, EPA and DHA contents in Pacific white shrimp decreased as the inclusion level of Latitude oil increased, which is consistent with the generally known fact that Pacific white shrimp have limited capacity to synthesize EPA and DHA from shorter unsaturated fatty acids. Given that the study with Atlantic salmon did not provide the fatty acid analysis data, it would be inappropriate or likely misguided to conclude that Latitude oil may not be a suitable alternative for marine species. However, at least for Pacific white shrimp, there appears to be a limit regarding complete substitute.

In this study, the improved growth of fish fed Latitude oil, which has lower EPA and DHA content than fish oil, was unexpected. Given that this experiment was a one-year study and the growth difference started to separate at 24 weeks, it is presumed that the improved growth is probably due to some subtle positive interactions of the fatty acids that compounded from the beginning the experiment. In a year follow-up study (not reported here), fish fed diets containing Latitude oil also showed improved growth performance. In study presented in Chapter 3, the histology analysis indicated moderate inflammation in the

distal intestines of fish fed the Latitude oil diets. However, there was no significant difference in feed intake, feed utilization, or survival during the entire production cycle. Thus, intestinal inflammation is not considered to be a significant problem with respect to aquaculture production. However, this study was not done on a commercial farm where pathogens and poor water quality might have worsened the health of fish already experiencing an inflammatory response. Taken together with results of the first study, it is challenging to expect a high biochemical fatty acid conversion rate using ALA as a substrate; however, a higher conversion rate can be expected when higher levels of DPA and EPA are used as substrates, such as when feeds contain Latitude oil.

Potential Issues of Fish oil Replacement

We hypothesized that the signs of inflammation in the distal intestine and improved growth performance of trout fed diets containing a high inclusion of Latitude oil were attributed to ARA, thus follow-up studies in two different sizes of trout, swim-up fry (0.17 ± 0.01 g) and sub-adult (307 ± 3.34 g) were conducted to characterize the effects of dietary ARA. According to the gene expression results in the distal intestine in the sub-adult experiment, ARA alone showed an anti-inflammatory effect rather than a pro-inflammatory effect. In mammals, it is known that n-6 fatty acids are converted to ARA in the body and promote pro-inflammatory effects; thus, the importance of the n-3/n-6 ratio has been highlighted (Burghardt et al., 2010). However, at least the results of this experiment suggest that ARA may play the opposite role in trout.

By linking the results of the Latitude oil and ARA sub-adult studies, it can be concluded that ARA in Latitude oil was not responsible for inflammation in the distal intestine. Based on the published literature, ARA/EPA ratio or DPA may have been a factor potentially causing the inflammation. There are very few studies on the integrated effect of EPA and ARA on the inflammatory response in fish. However, one of them confirmed that ARA alone did not have a pro-inflammatory effect, but when combined with EPA, the expression of genes involved in pro-inflammatory pathways was upregulated in Atlantic salmon (Holen et al., 2015). In our ARA experiment, only 10% fish meal was used to minimize EPA and DHA to evaluate the effect of ARA alone; therefore, the residual EPA and DHA content in fishmeal was extremely low, which led to the absence of a combined

effect of EPA and ARA. Commercial feeds, especially for trout and salmon, use a lipid source with an optimal amount of EPA and DHA, and therefore, are not expected to show similar results to our experimental results in production aquaculture settings. However, given the importance of pursuing sustainable aquaculture, it is necessary to investigate the appropriate ARA/EPA ratio to maintain fish health.

The other potential factor that may have caused intestinal inflammation is DPA in Latitude oil (more than twice that of fish oil). DPA has recently attracted much attention in humans, with a few reports that it can significantly lower cholesterol and triglycerides compared to EPA or DHA (Drouin et al., 2019) and inhibit the expression of pro-inflammatory genes (Backes et al., 2016). Usually, eicosanoids are directly involved in the inflammatory response, and since these eicosanoids are derived only from C20, the fact that DPA is involved in the inflammatory response is interesting. Perhaps it can be speculated that DPA is oxidized through beta-oxidation to EPA and is involved in the inflammatory response. Very few trials have investigated the bioavailability of DPA in humans, but none have been studied in fish. The main reason for the scarcity of studies is due to the scarcity of highly purified DPA at a reasonable cost to conduct *in vivo* studies (Drouin et al., 2018).

As fish oil has become an increasingly limited and costly ingredient for fish feeds, a significant amount of research has been conducted to replace fish oil with alternative lipid sources, primarily vegetable oils, which are ARA deficient. ARA was not classified as an essential fatty acid since the content of this fatty acid contained in fishmeal, and fish oil is relatively small compared to EPA and DHA. However, studies demonstrated that selective retention of ARA is generally observed in fish due to physiological requirements in various organs, such as the heart (Om et al., 2003), muscle (Norambuena et al., 2013), brain (Trushenski et al.; 2012), and gonad (Emata et al., 2003), when dietary levels are low (Rivero-Ramirez et al., 2020). Based on the literature showing that ARA is selectively retained in muscle and brain, as referenced above, a follow-up study was performed with swim-up fry rainbow trout under the hypothesis that ARA would play an important role in muscle growth and neural development. Since the results indicated that ARA did not affect growth and survival during the 24-week growth trial, it is suggested that trout can sufficiently synthesize ARA from intermediate fatty acids, mainly from LA. These results indicate that the growth differences in the Latitude oil-fed group (Chapter 3) may not be attributed to

ARA alone. Again, given that there may be an interaction between fatty acids, it is inappropriate to conclude that ARA is not involved in growth performance or survival, but at least it can be concluded that ARA itself does not have an effect on these parameters.

In recent years, intensive aquaculture farming has been facing a series of new issues, including low dissolved oxygen levels and sub-optimal water temperature conditions as the result of climate change, such as is the case for Atlantic Salmon (*Salmo salar*) farming in Australia during the summer season (Norambuena et al., 2016). Given the putative role of ARA in inflammation, I also evaluated the effects of ARA on inflammatory response factors under two extreme environmental factors (hypoxia and hyperthermia), where the results revealed that ARA has an antioxidant effect and plays a role in relieving the inflammatory response caused by these environmental factors. Given that anti-inflammatory pathways were upregulated in two separated ARA experiments, ensuring adequate ARA in diets low in fish oil is very likely beneficial to trout. In particular, the swim-up fry stage is more susceptible to disease than later life stages; thus, ARA supplementation in the starter diet may prove useful to fight against pathogens and unexpected stressors. In general, since a significant amount of fish oil is used in starter diets, it seems unnecessary, currently, to supplement ARA. However, when replacing fish oil with ingredients with low to no ARA, it is recommended to provide ARA, either through other oil sources or supplements.

To date, studies on ARA are remarkably lacking compared to other essential fatty acids and even published literature often shows conflicting results. Current research results on rainbow trout show that ARA is a fatty acid that is particularly important for the immune system and inflammatory response. However, its role and effect are not yet fully understood, as it may be conditionally determined by many factors, including the relationship between other fatty acids and the environment.

Future Research Directions

Seafood is a rich source of omega-3 fatty acids, which have well-known beneficial effects for cardiovascular and inflammatory diseases and neurodevelopment (Tur et al., 2012; Calder, 2013; Tocher, 2015). In particular, feeds for high-trophic species have traditionally consisted of fishmeal and fish oil, for which these ingredients have been very efficient in supplying EPA and DHA. However, continued aquaculture growth cannot be achieved by

relying solely on marine resources to supply protein and oil in fish feeds. Therefore, the steady growth of the aquaculture industry depends on the development of sustainable feeds containing alternative ingredients that supply n-3 LC-PUFA.

As covered in Chapter 1, dietary supplementation has been investigated with several compounds, such as bioactive fatty acids, sesamin, lignin, and cholesterol, with some success, including enhancing n-3 LC-PUFA biosynthesis. However, the increase in biosynthesis levels observed in the above studies was relatively small and insufficient compared to the amount supplied from dietary fish oil. Therefore, fatty acid metabolism in fish is fundamentally limited by biochemical mechanisms, and maintaining high n-3 LC-PUFA levels in fish itself is unlikely in the absence of external supply. This suggests that dietary supply is the only feasible option for maintaining high levels of n-3 LC-PUFA in farmed fish. Several promising oil sources have emerged recently, such as insect oil, single-cell oil, and genetically modified plant (GMO) oils, each of which has drawbacks. In the case of insect oil, raising insects on a substrate rich in n-3 LC-PUFA increases the n-3 LC-PUFA content in insects, but this also has a limitation in that it is necessary to source byproducts with a high contents of LC-PUFA. In general, the production cost of algae oils is exceptionally high due to the small scale of production and apparent technical challenges. One of the most significant challenges in terms of the use of GMO oil, especially in the EU, is to change public perception towards acceptance of GMO products so that these products can be used commercially in the aquaculture sector. There are two potential ways to alleviate reliance on limited resources for aquaculture's future success: the application of a blend of vegetable oils and algae oils or GMO oils and a finishing diet. The idea of an oil blend is already known to be more effective than using the single source alone, and high n-3 LC-PUFA levels can be expected without compromising fish growth by employing a mixture of vegetable oils and the minimal levels of algae or GMO oils containing high n-3 LC-PUFA values. Since various fatty acids in the feed interact with each other, this method requires much research and careful formulation considering the ratio of fatty acids in the feed. Fish fed a diet mainly containing fish oil generally have a lower EPA and DHA retention, indicating that a large proportion of EPA and DHA are used for energy purposes in fish. In order to minimize energic waste, increasing the level of monounsaturated fatty acids (oleic

acid) available as an energy source in the feed will reduce the reliance on limited feed sources without detrimental effects on fish.

The implementation of a finishing diet on algae oils or GMO oils could also be an option. Studies with several species, such as Atlantic salmon, gilthead seabream, and European sea bass, were conducted to identify the efficacy of a finishing diet with fish oil-based after a grow-out period. In all the trials, the fatty acid composition was similar to that of the group supplied with fish oil. Yet, a finishing diet with a fish oil-based diet is considered a mitigation solution to the problem of fish oil replacement in aquafeed since a significant amount of fish oil is still needed. Therefore, replacing fish oil with algal or similar oil during the fishing period could lower the production costs, rather than supplying fish oil or algae oil during the entire production cycle. However, the initial fatty acid concentration in fillet affects the time needed for fatty acid composition to be improved. In general, according to the papers published, it is known to take 8 to 14 weeks to restore the fatty acid contents tissues depending on initial levels (see Chapter 1).

More research is required not only on the efforts to replace fish oil but also on the issues that may arise from replacing fish oil. Fish oil may have unknown interactions between fatty acids and other components that makes fish oil "special," similar to the situation with taurine supplementation, which as not considered to be necessary until fishmeal was removed from marine fish feeds. Unlike the biochemical mechanisms of amino acids, fatty acids involved in n-3 and n-6 pathways interact, making it more complex to identify the specific role of each fatty acid. Therefore, future studies are needed identify an optimal ratio of n-3/n-6 LC-PUFA or monounsaturated fatty acids/polyunsaturated fatty acids to identify the overall impact of each group of fatty acids rather than one specific fatty acid alone.

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Table 6.1. Fatty acid composition of fish oil and Latitude oil used in the study (Chapter 3).

Fatty acid (%)	**Fish oil[a]**	**Latitude oil**
C14:0	2.24	0.07
C15:0	0.26	0.07
C16:0	21.53	4..09
C16:1 n7	7.56	0.15
C18:0	3.40	2.45
C18:1 n9	23.24	24.47
C18:1 n7	6.78	2.95
C18:2 n9	0.00	2.27
C18:2 n6	0.98	27.18
C18:3 n3	0.44	2.52
C18:4 n3	0.00	0.30
C20:0	0.00	0.64
C20:1 n9	0.86	0.69
C20:2 n6	0.00	0.24
C20:3 n6	0.00	5.72
C20:4 n6 (ARA)	**0.80**	**2.25**
C20:4 n3	0.00	2.22
C20:5 n3 (EPA)	**15.98**	**9.13**
C22:0	0.00	0.32
C22:5 n3 (DPA)	**0.98**	**2.28**
C22:6 n3 (DHA)	**8.99**	**0.91**
Sum n3	**26.39**	**17.36**
Sum n6	**1.78**	**36.07**
EPA+DHA	**24.98**	**10.04**
EPA+DHA+DPA	**25.95**	**12.32**

[a] Rangen Inc., Buhl, ID, USA.

[b]Cargill Inc., Minneapolis, Mn, USA.