CHOLESTEROL SUPPLEMENTATION IN PLANT-BASED DIETS FOR RAINBOW TROUT (*ONCORHYNCHUS MYKISS)*

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In the Department of Animal and Poultry Science

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ABSTRACT

Total replacement of marine oils and proteins with plant proteins and oils in rainbow trout diets reduces growth performance and feed intake. It has been suggested that this is due to the presence of growth factors present in marine products but absent in plant ingredients. One putative factor is cholesterol. Thus, the effect of supplementing cholesterol with and without dietary polyunsaturated fatty acids in plant-based diets fed to rainbow trout was investigated in two experiments. In both experiments, the experimental period was 12 weeks and fish were weighed on day 0 and 84. Fish were fed to satiation twice daily. All diets were formulated to contain 43.0% crude protein (as is basis) and 92.0% digestible energy (as is basis). In Experiment 1, there were 8 experimental diets arranged in a 2 x 4 factorial design, with 2 levels of PUFA (olive oil or olive oil + linseed oil) and 4 levels of cholesterol (0.00, 0.05, 0.10 and 0.15%) added using synthetic cholesterol. Rainbow trout (n=13/tank; initial body weight 13.4 g and 3 tanks/treatment) were randomly assigned to one of the 8 diets. Between days 28 – 35, there was an outbreak of bacterial cold water disease (BCWD) (*Flavobacterium psychrophilum*). Fish fed the olive oil-based diets had significantly higher mortalities than fish fed the olive/linseed oil diets. The interaction between PUFA level and cholesterol was significant for average daily feed intake (ADFI) and near significant for average daily gain (ADG) (P = 0.085) and specific growth rate (SGR) (P = 0.082). The experiment was therefore reanalyzed using regression analysis. Fish fed the olive oil-based diets had significantly increased ADG, SGR and feed intake when cholesterol levels were increased, while there was no significant effect of cholesterol level on growth parameters of fish fed the olive oil + linseed oil-based diets. In Experiment 2, there were 6 experimental diets arranged in a 2 x 3 factorial design, with 2 levels of PUFA (olive oil or olive oil + linseed oil) and 3 levels of cholesterol (0.00, 0.075 and 0.15%) added using synthetic

cholesterol. Rainbow trout (*n*=20/tank; initial body weight 27.7 g and 4 tanks/treatment) were randomly assigned to one of 6 diets and fed to satiety 2x daily for 84 days. Growth and feed intake were measured over the entire experimental period and at the end of the trial, blood and liver samples were taken from 4 fish per tank for measurement of cholesterol. There was a trend towards increased ADG ($P = 0.089$) and SGR ($P = 0.082$) with increasing cholesterol levels. There were no clinical signs of disease and both main effects and interactions were not significant for mortality. There were no significant effects of cholesterol, effects of PUFA level or an effect of the interaction between the two factors on serum or hepatic cholesterol levels in the fish. However, values for cholesterol content in both blood serum and hepatic tissue samples were found to be lower in the high PUFA diets, than those in the low PUFA diets (1.80 mg/g vs. 2.00 mg/g in hepatic tissue and 2.39 mg/ml vs. 2.66 mg/ml in serum). The results of the two experiments suggest that cholesterol is a conditionally limiting nutrient in rainbow trout and one constituent of marine products that improves growth when supplemented in a completely plant-based diet.

Key words: rainbow trout, cholesterol, growth, polyunsaturated fatty acids, flax oil, linseed oil, olive oil

DEDICATION

This thesis is dedicated to:

Bunny – the bite is on.

Mom and Dad – I finished it.

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TABLE OF CONTENTS

LIST OF TABLES viii

LIST OF FIGURES ix

LIST OF ABBREVIATIONS x

1. INTRODUCTION 1

2. LITERATURE REVIEW 4

2.1 World aquaculture production 4

2.1.1 The state of marine fish stocks 4
2.1.2 Overfishing 4
2.1.3 Bycatch (Discarding) 6
2.1.4 Environmental degradation 7
2.1.5 The development of salmonid aquaculture 7
2.1.6 The use of marine ingredients in aquafeeds 10
2.1.7 The use of plant ingredients in aquafeeds 11
2.1.8 Pronutritional factors in marine ingredients 12
2.1.8.1 Taurine *13*
2.1.8.2 Nucleotides *14*

2.2 Polyunsaturated fatty acid metabolism in salmonids 15

2.2.1 Biosynthesis of highly unsaturated fatty acids (HUFAs) 15
2.2.2 Role of HUFAs in inflammatory responses in salmonids 17
2.2.3 Health benefits of HUFAs in the human diet 18

2.3 Cholesterol 21

2.3.1 Introduction 21
2.3.2 Biosynthesis of cholesterol 22
2.3.3 Regulation of biosynthesis of cholesterol 23
2.3.4 Effects of PUFA on cholesterol biosynthesis 25
2.3.5 The role of cholesterol in the biosynthesis of steroid hormones 26

2.4 The role of biological membranes and cholesterol 27

2.4.1 The effects of cholesterol on lipid phase behavior and raft formation 27
2.4.2 Cholesterol-protein interactions in membranes 30

2.5 Biological roles of cholesterol in fish 31

2.5.1 Buoyancy....31
2.5.2 Disease resistance....32
2.6 Cholesterol research on finfish....33
2.6.1 Cholesterol effects on organ indices and fatty acid composition....33
2.6.2 Cholesterol effects on growth parameters....34
2.7 Hypothesis....35
3. CHOLESTEROL SUPPLEMENTATION IN PLANT-BASED DIETS FOR RAINBOW TROUT (*Oncorhynchus mykiss)*....36
3.1 Introduction....36
3.2 Materials and methods....37
3.2.1 Experiment 1....37
3.2.1.1 Environmental conditions and fish management....37
3.2.1.2 Diets....38
3.2.1.3 Statistical analysis....38
3.2.2 Experiment 2....39
3.2.2.1 Environmental conditions and fish management....39
3.2.2.2 Diets....41
3.2.2.3 Sample collection....41
3.2.2.4 Analytical methods....41
3.2.2.5 Statistical analysis....42
3.3 Results....44
3.3.1 Experiment 1....44
3.3.2 Experiment 2....47
3.4 Discussion....50
4. CONCLUSIONS....55
5. LITERATURE CITED....57

LIST OF TABLES

Table 2.1 The fatty acid composition of common fish and vegetable oils used in aquaculture diets……...20

Table 2.2 Effect of replacing fish oil with vegetable oils on the DHA and EPA contents of rainbow trout and Atlantic salmon. Values are % of total lipid……...21

Table 3.1 Diet formulations and calculated nutrient analysis (n=2) for Experiment 1…….41

Table 3.2 Diet formulations and calculated nutrient analysis (n=2) for Experiment 2…….45

Table 3.3 Performance of rainbow trout fed cholesterol supplemented diets with varying levels of PUFA for days 0 – 84 of Experiment 1…….....47

Table 3.4 Performance of rainbow trout fed cholesterol supplemented diets with varying levels of PUFA for days 0 – 84 of Experiment 2…….....50

Table 3.5 Hepatic and serum cholesterol levels in rainbow trout fed cholesterol supplemented with varying levels of PUFA in Experiment 2……..51

LIST OF FIGURES

Figure 2.1 The exploitation and depletion of world fish supplies in 2008...……..5

Figure 2.2 Total production of capture fisheries 1970-2009.....6

Figure 2.3 Trends in world aquaculture production by major species groups……..10

Figure 2.4 Pathways of HUFA synthesis from LA and ALA ……..……....17

Figure 2.5 The link between dietary PUFA, tissue HUFA and eicosanoid production.……19

Figure 2.6 Structure of cholesterol.……22

Figure 2.7 The biosynthetic pathway to cholesterol.…….24

Figure 3.1 The effect of dietary cholesterol level combined with olive oil or a mixture of olive and linseed oil on the growth performance of rainbow trout in Experiment 1...48

LIST OF ABBREVIATIONS

ACAT	Acyl-CoA acyltransferase
ADFI	Average daily feed intake
ADG	Average daily gain
ALA	Alpha-linolenic acid
ANF	Antinutritional factors
ARA	Arachidonic acid
BCWD	Bacterial cold water disease
CDO	Cysteine dioxygenase
CSD	Cysteine-sulfinate decarboxylase
DNA	Deoxyribonucleic acid
DHA	Docosahexaenoic acid
EPA	Eicosapentanoic acid
ER	Endoplasmic reticulum
FAO	Food and Agriculture Organization
FPP	Farnesyl diphosphate synthase
HMGR	HMG-CoA reductase
HUFA	Highly unsaturated fatty acids
IFFO	International Fishmeal and Fish Oil Organization
LA	Linoleic acid
LDL	Low density lipoprotein
LDLr	Low density lipoprotein receptor
mRNA	Messenger ribonucleic acid

n-3	Linolenic acid (18:3)
n-6	Linoleic acid (18:2)
NADH	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
PNF	Pronutritional factors
PUFA	Polyunsaturated fatty acids
RNA	Ribonucleic acid
S1P	Site-1 protease
S2P	Site-2 protease
SCAP	SREBP cleavage-activating protein
SCFA	Short chain fatty acids
SE	Standard error
SEM	Standard error of the mean
SGR	Specific growth rate
SRE	Sterol regulatory element
SREBP	Sterol regulatory element binding protein
SSD	Sterol-sensing domain
StAR	Steroidogenic acute regulatory
T_c	Transition temperature
UN	United Nations
US	United States

1. INTRODUCTION

Fish meal and fish oil are commonly used ingredients in commercial fish diets because of their high palatability and nutritive value. In recent decades, global aquaculture has seen consistent rises in the price of these commodities as a direct result of stable or decreasing stocks of fish meal and oil and increasing demand by aquaculture. In 1999, 2.1 million tons of fish meal and close to 0.7 million tons of fish oil were used in the global aquafeed industry (New and Wijkstrom, 2002). New and Wijkstrom (2002) also predict that the global demand for fish meal in aquafeeds will exceed total available supplies by 2020 and the demand for fish oil has already exceeded available supplies (Naylor et al., 2009). There are many factors that will affect the actual utilization of these products within the next twenty years, including resource supply, resource competition, economics, public image, environmental and ethical issues, safety, and quality. Ultimately, the growing scarcity of these products means that their replacement in aquaculture diets is essential, if the economic sustainability of the industry is to be maintained. As part of the solution to this problem, aquaculture research in the past decade has focused primarily on replacement of marine ingredients with plant oil and protein sources.

Although there have been successes in replacing fish meal and oil with vegetable products, the growth performance of finfish is significantly reduced when they are fed diets devoid of marine products. While a number of antinutritional factors have been touted as causal factors, the exact mechanisms that bring about this effect are not well understood and are probably multifactorial (Francis et al., 2001). Thus further investigation is required before plant sources can completely replace marine products. Studies conducted at the University of Saskatchewan and elsewhere by Thiessen et al. (2004), Mundheim, Aksnes and Hope (2004) and Sener and Yildiz (2003) demonstrate that when fish performance is not significantly reduced

with plant oil replacement, fish meal or other animal products have been included in the diet. This suggests that there are compounds present in marine or animal products, which may contribute to growth, and that are not present in plant products.

Twibell and Wilson (2004) reported that supplementation of plant-based catfish diets with cholesterol significantly improved growth and feed intake. Since plant proteins and oils are devoid of cholesterol, this suggests that cholesterol may be a growth factor in catfish diets. Although teleost fish have the ability to synthesize cholesterol, the rate of synthesis may not be sufficient to support maximal growth. Furthermore, high levels of omega-3 polyunsaturated fatty acids (n-3 PUFA) have been shown to suppress expression of HMG-CoA reductase (HMGR); the rate-limiting enzyme in cholesterol synthesis (Horton et al., 2003; Le-Jossic Corcos et al., 2005). While most plant oils are devoid of or low in PUFA, two commonly used plant oils for aquafeed, canola and linseed oil, are exceptions. Canola oil contains approximately 12% alpha linolenic acid (similar to fish oil), and linseed oil contains approximately 53% (NRC, 1994). Taken together with the study conducted by Twibell and Wilson, this suggests that cholesterol may be one constituent of marine products which contributes to growth, particularly with diets high in n-3 PUFA.

Fish oil contains 4-10 mg/g of cholesterol and fish meal contains large, but variable amounts as well, depending on the source of the meal. Cholesterol is an essential molecule synthesized in all animals and required for many important biological processes. It is one of the key structural components of cell membranes and an important precursor of essential vitamins and hormones. However, most experiments where marine ingredients are replaced with plant ingredients have fed diets containing significant sources of cholesterol. Therefore, cholesterol levels may have remained high enough to meet the requirements of the fish. However, in studies

where only plant protein and oil sources have been used in diets growth rate has been significantly reduced. This suggests that there are unknown growth factors present in fish meal and oil and one of these putative factors might be cholesterol.

2. LITERATURE REVIEW

2.1 World aquaculture production

2.1.1 The state of marine fish stocks

In recent decades, concern related to the rapid decline and sustainability of marine fish stocks worldwide has become increasingly pronounced. Trends demonstrate an increase in the percentage of overexploited, depleted and recovering stocks, and a decrease in under- and moderately exploited stocks (FAO, 2010). It has been reported that slightly more than half (53%) of marine fish stocks are estimated to be fully exploited and the remaining 32% are estimated to be overexploited (28%), depleted (3%) or recovering from depletion (3%) (FAO, 2010; Figure 2.1). Even more alarming, most of the stocks of the top ten species, which account in total for about 30% of the world marine capture fisheries production in terms of quantity, are fully exploited (Tacon and Mettian, 2008; Naylor et al., 2009). Worm et al., (2006) predict a total collapse of all major fisheries by the year 2048 if current capture levels are maintained. The practice of overfishing, discarding of bycatch and environmental degradation caused by the fisheries sector threaten many of the world's major marine fish stocks, and thus the ocean's ability to provide food for a growing world population.

2.1.2 Overfishing

The major decline in world fish stocks is largely due to the act of overfishing by capture fisheries. For two decades after 1950, the annual growth in world capture averaged 6%, from 18 million tons in 1950 to 56 million tons in 1969 (FAO, 2011). However, these increases were not

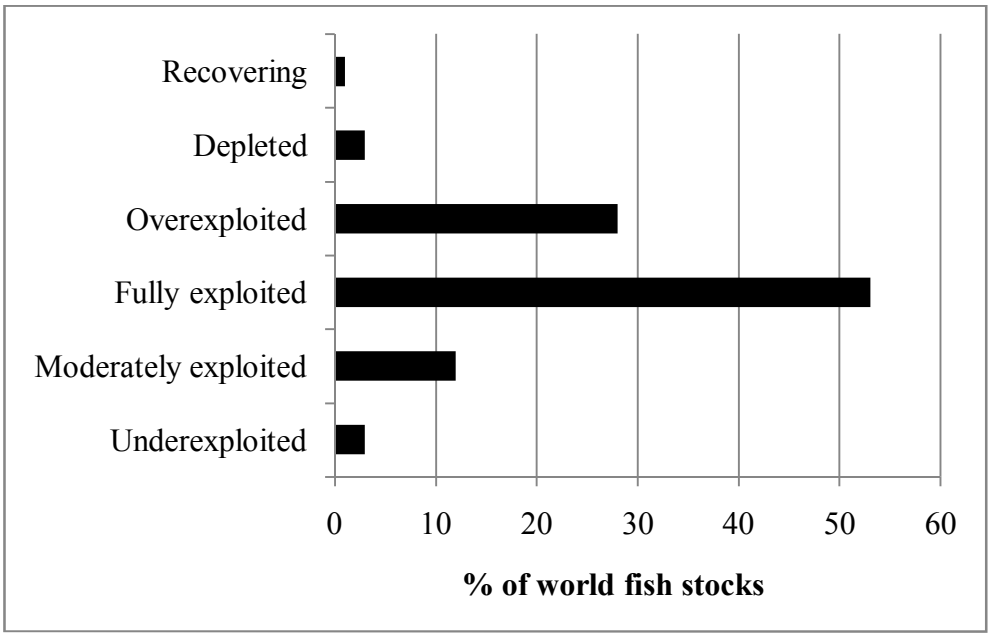

Figure 2.1 The exploitation and depletion of world fish supplies in 2008

sustainable, and by the late 1980s, capture fisheries had plateaued (Figure 2.2; Worm et al., 2006). Worldwide, every commercial species had been fully exploited, overexploited or depleted. The Committee on Fisheries (1996) states that as far back as 1977, strict regulations had been adopted by Canada and other major fishing nations to manage fish stocks off their shores. This attempt to install regulatory regimes, that would reduce foreign fishing, resulted in a large increase of domestic catches after 1977. Canada was forced to close one of the most productive fisheries in the world in 1992 – the Grand Banks cod fishery of Newfoundland and Labrador. This was the typical scenario for many other fishing nations at this time. Too much fishing pressure by commercial industries, on already stressed stocks, had accelerated the decline of capture fisheries production. In the 1990's, the average annual growth of world capture fisheries had fallen to almost zero (FAO, 2011). This levelling off of total catch trends for world fisheries continues into present times, as most of the world's major fishing areas have reached

their maximum potential and the majority have stocks have been fully exploited, as previously stated.

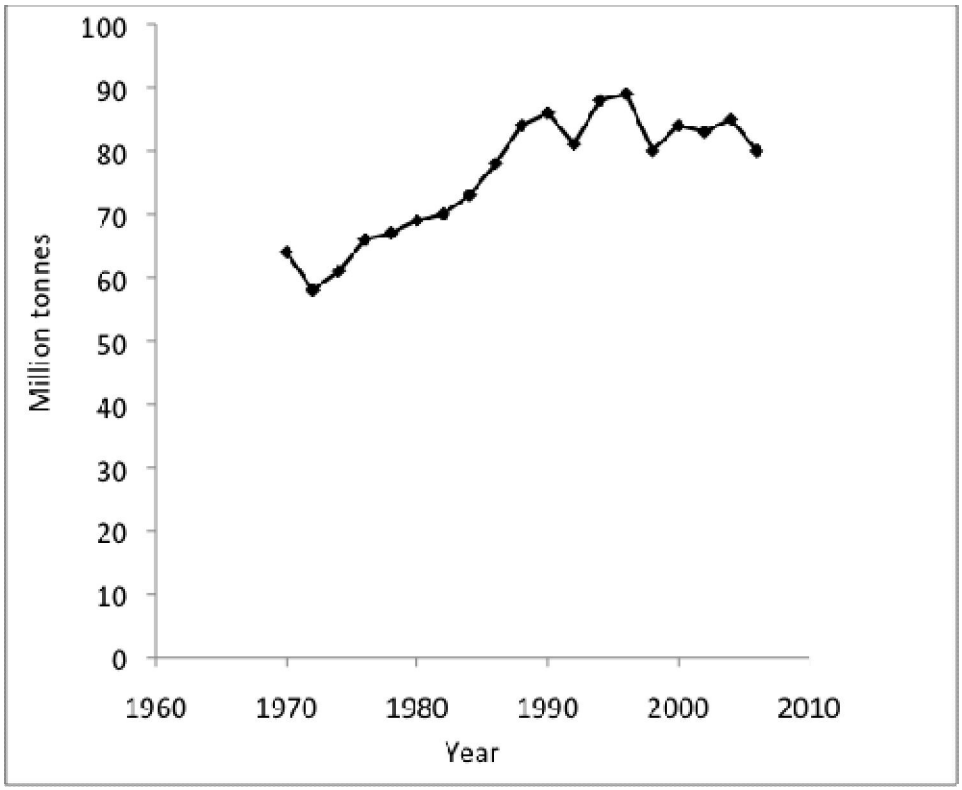

Figure 2.2 Total production of capture fisheries 1970-2009 (FAO, 2010)

2.1.3 Bycatch (Discarding)

Because most capture fishing gear is not entirely selective of target species, the incidental catch of non-target species is often the result. Aquatic organisms that are not meant to be captured or harvested in commercial fishing are thrown back into the oceans, as they are often of incorrect size or are identified as an endangered/protected species (Read et al., 2006). These organisms are referred to as bycatch and their removal or displacement from marine ecosystems has significant biological impacts. Generating no revenue, discarding also produces added costs to the fisheries industry, such as the cost of sorting and dumping of the discards (Read et al.,

2006). Although efforts to reduce the direct biological and economic impacts of discarding (such as gear selectivity, discard regulations, and use of bycatch for animal or human food through improved processing technologies) are gaining acceptance, the most important action to reduce the negative impacts of discarding will be to reduce limits for capture fisheries (Kelleher, 2005). This reduction will undoubtedly provide the greatest improvement to the bycatch and discard problem.

2.1.4 Environmental degradation

A third major threat to world fish supplies, which stems from the capture fishery industry, is the ongoing destruction of marine ecosystems, including coastal habitat and spawning grounds. The fishing methods and type of equipment used in capture fisheries is often responsible for this environmental damage (Nixon, 1997). Significant alteration to sea beds, turning over of sediments, and cutting off coral heads through the use of heavy trawl nets can disturb or kill off much of the bottom-dwelling fauna (Nixon, 1997). Dredging, long-hauling, and the use of explosives have also contributed to the destruction of important organisms in these areas (Nixon, 1997). Marine ecosystems provide essential environments required for the reproduction and growth of a large number of important species. The loss of biodiversity in these areas is increasingly damaging the ocean's ability to provide food, maintain water quality and recover from these agitations (Worm et al., 2006; 2009).

2.1.5 The development of salmonid aquaculture

As traditional methods of supplying the world with fish face serious issues of sustainability, the only current alternative has seen increasing expansion within the past few decades. This alternative is aquaculture production. It is defined as the farming of aquatic organisms in salt or freshwater, with an intervention of sorts in either the rearing or growing

process or both, in order to enhance production. It also implies individual or corporate ownership and the manufacture of compound feeds (aquafeeds).

The current world population is nearly 7 billion people, and is projected to reach 9.3 billion by the middle of this century (United Nations, 2010). As a direct result of this phenomenon, fish consumption trends have increased and will continue to do so, especially with the emergence of certain health trends in more developed countries. Globally, fish provide more than 1.5 billion people with almost 20% of their average per capita intake of animal protein, and 3.0 billion people with at least 15% of such protein (FAO, 2010). The nutritional value of fish has seen increased recognition due to its levels of two important fatty acids: eicosahexanoic acid (EPA) and docosahexaenoic acid (DHA); highly unsaturated omega-3 (n-3) fatty acids. Excluding aquatic plants, the value of the world aquaculture harvest was estimated at US$98.4 billion in 2008 and aquaculture farmers made up one quarter of the total number of workers in the fisheries sector (FAO, 2010).

Globally, freshwater finfish, molluscs, crustaceans, and diadromous fish (salmonids) comprise the top four groups of cultured aquatic life, respectively, in terms of production and value (FAO, 2010). Figure 2.3 shows the growth of world aquaculture production by major species groups. The diadromous group, which includes Atlantic salmon and rainbow trout, reached a production output of 3.3 million tons in 2008, led by Norway and Chile, which are responsible for 36.4% and 28% of world production, respectively (FAO, 2010). Aquaculture contributes 68.2% to the total global production of diadromous species. In Western countries, such as the U.S., salmon consumption trends showed a dramatic increase from 130, 000 tons in 1989, to 300,000 tons in 2004 (FAO, 2011).

Although helping to alleviate the pressure of capture fisheries on world fish stocks, aquaculture faces its own issues of sustainability. Problems related to pollution and environmental damage, wild seedstock collection, the diminishing supply of marine products in aquafeeds, disease, and food quality have forced aquaculture research efforts to focus on these issues. Of particular significance to the present studies are issues surrounding the changing composition of aquafeeds and its subsequent effects on salmonid aquaculture production.

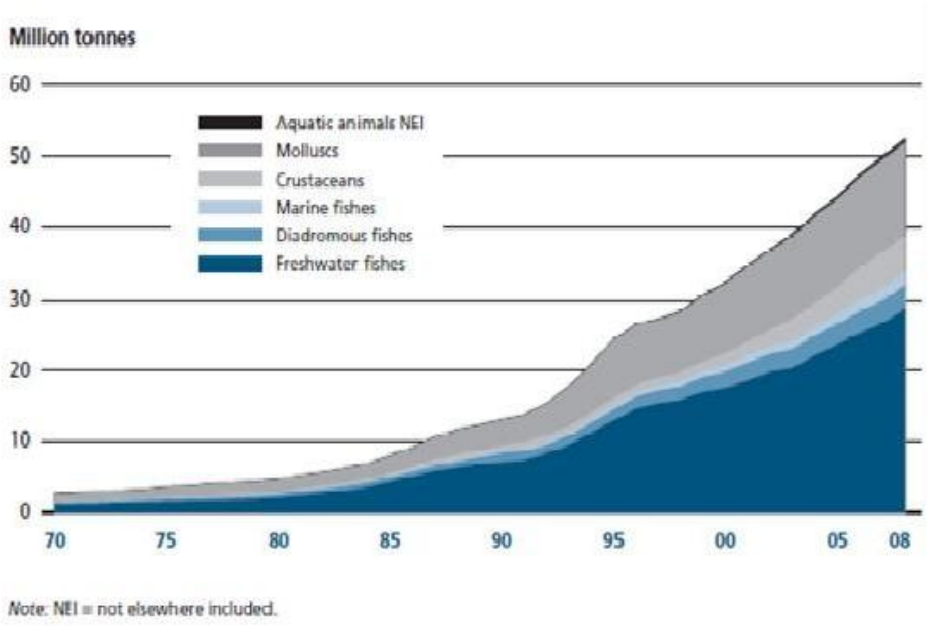

Figure 2.3 Trends in world aquaculture production by major species groups

Source: FAO. The State of World Fisheries and Aquaculture, 2010

2.1.6 The use of marine ingredients in aquafeeds

Aquafeeds for salmonids and many other marine species have traditionally contained high proportions of fish meal and fish oil to satisfy protein and fatty acid requirements. Of the world fish production used for non-human food purposes, 76% was reduced to fish meal and fish oil in 2008 (FAO, 2010). Cooking, pressed drying and milling of either fresh, raw fish and/or food fish trimmings results in a brown powder (fish meal) and fish oil is normally a clear brown or yellow liquid pressed from the cooked fish and refined (IFFO, 2006). Fish species captured or farmed to produce fish meal and fish oil are typically fast-growing and short-lived and are normally unwanted for human consumption (IFFO, 2006). Pelagic species, such as anchovy, horse mackerel, menhaden, capelin, and sand eel are small, bony and oily fish typically used in the production of these products (IFFO, 2006). Fish meal and fish oil are very desirable ingredients in salmonid aquafeed, due to their high energy content and palatability, balance of amino acids and high quality and concentration of essential nutrients, including the omega-3 PUFA, EPA and DHA. However, the overexploitation of world fish stocks has forced the aquaculture industry to decrease its reliance on fish meal and oil as dietary ingredients. Static supplies of a finite resource combined with increasing demand mean that prices of these commodities will inevitably continue to rise. A larger proportion of fish oil, as opposed to fish meal, is used in aquaculture production, with 85% of production being used in feed for fish and shrimp (FAO, 2010). In 2009, a decline of 100,000 tons of fish oil production from 2008 resulted in a 50% increase in its price the following year, reaching US$950/ton in March 2010 (FAO, 2010). Aside from high prices, the use of fish meal in aquaculture also competes with its use in animal production, as it is a primary protein source in the diets of cattle, poultry and pigs.

The future economic sustainability of the aquaculture industry depends on its ability to seek and establish alternative feed ingredients to fish meal and fish oil, without jeopardizing production efficiency. Research aimed at finding new ingredients of marine origin has found several benefits of using plankton. These marine organisms, including copepods, euphausiids, amphipods, and krill, contain bioactive compounds like omega-3, bound phospholipids and axastanthin, and feed in low trophic levels (Rana et al., 2009). They have the potential to provide an alternative source of protein, oil, attractant, and pigment (Hardy, 2004). There is research to indicate that Atlantic krill may be suitable for inclusion in fish diets. A 56-day study conducted by Anderson et al. (1997) found that juvenile salmon (*Oncorhynchus tshawytscha)* increased weight gain when the inclusion of air-dried krill was increased from 0 – 25% of the diet by dry weight. There was also no effect on feed intake or feed conversion. In a shorter study (12 weeks) by Julshamn et al. (2004), there was no effect on growth rate when krill were included as up to 30% of the diet (dry matter). Results of a study by Olsen et al. (2006) also show that moderate amounts of krill (20-60% of krill protein) fed to Atlantic salmon during the first 71 days of feeding increased growth, compared with a fish meal control. Olsen et al. (2006) suggest that a feeding attractant function of krill meal may explain this growth difference between the beginning (first 71 days) and end (last 69 days) of the experiment, where there was no growth observed.

2.1.7 The use of plant ingredients in aquafeeds

In the search to find a viable alternative to marine ingredients in aquafeeds, much research in recent decades has focused on the inclusion of plant ingredients. However, most of the potential plant-derived sources contain a wide variety of antinutritional factors (ANFs). These are substances within plant products which interfere with the nutrient utilization, growth

and health of fish, particularly carnivorous species, such as salmonids. Studies which examine the effects of the inclusion of plant ingredients in cultured fish have been published in the hundreds. Francis et al. (2001) divides the effects of ANFs into four main groups: 1) factors which affect the digestion and utilization of protein, such as protease inhibitors, tannins and lectins; 2) factors which affect mineral utilization, including phytates, gossypol pigments, oxalates, and glucosinolates; (3) antivitamins; (4) miscellaneous substances, such as mycotoxins, mimosine, cyanogens, nitrate, alkaloids, photosensitizing agents, phytoestrogens, and saponins. Another method of classification used for ANFs takes into account their ability to withstand thermal processing, which is the most common method of destroying them (Francis et al., 2001). Research in this area continues to focus on methods which can be used to reduce or eliminate the amount of ANFs in plant-based products for carnivorous fish. Investigations into the use of micro-organisms, such as yeast and bacterial and fungal fermentations, have shown potential in their ability to reduce the effects of antinutrients and add protein and essential amino acids (Mukhopadhyay and Ray, 1999; Bairagi et al., 2004). Genetic alterations which lower levels of phytic acid (therefore increasing available phosphorous) (Guttieri et al., 2004), increase lysine (Gibbon and Larkins, 2005), levels of oil (Laurie et al., 2004) and antioxidants (Capell and Christou, 2004), have also been examined in research activity.

2.1.8 Pronutritional factors in marine ingredients

ANFs may not be the only cause of decreased growth when marine products are replaced. There also appears to be pronutritional factors in marine ingredients that may affect fish growth, and growth is reduced when they are absent. Plant ingredients may have negative effects on the growth of salmonid fish due to antinutritional factors. However, it appears that there are compounds present in fish meal and oil which may enhance the growth and immune function of

salmonids (Refstie et al, 2004; Aksnes et al., 2006a; b). Aksnes et al. (2006 a; b) found that small molecular weight solubles found in fish hydrolysate, when included in rainbow trout diets, had a significant increase in growth performance, the higher level of hydrolysate inclusion resulting in the best growth. This suggests that unknown pronutritional factors present in fish meal and oil improve growth performance greater than predicted from their nutrient composition alone. Thus, part of the decreased performance in the growth of salmonids, when fed plant protein diets, may be due to the lack of these low molecular weight compounds. These putative pronutritional factors include taurine (Stapleton et al., 1997; Gaylord et al., 2006), nucleotides (Burrels et al., 2001) and cholesterol (Twibell and Wilson, 2004).

2.1.8.1 Taurine

Taurine (2-aminoethanesulfonic acid) appears to be a pronutritional growth factor in fish meal. Plant ingredients are devoid of taurine (Spitze et al., 2003), however, most animal species can synthesize taurine and have no requirement for dietary taurine (Morris, 2002). Taurine is synthesized from the amino acid precursor cysteine and requires two important enzymes in its biosynthesis, cysteine dioxygenase (CDO) and cysteine-sulfinate decarboxylase (CSD). However, obligate carnivores such as cats and salmonids (Sturman et al., 1987; Pion et al., 1987) cannot synthesize sufficient taurine to meet their metabolic requirements, due to very low levels of the CSD enzyme (Schuller-Levis and Park, 2003). Taurine's functions include: osmoregulation, membrane stabilization, calcium homeostasis, antioxidation, glycolysis and glycogenesis stimulation (Stapleton et al.1997). Taurine has been shown to be an essential nutrient in salmonid fish (Gaylord et al., 2006). Gaylord et al. (2006) reported that the addition of taurine to plant-based diets improved the growth of rainbow trout up to an inclusion level of 100

mg/kg. This supports the role of taurine as a pronutritional factor present in fish meal and other animal-derived ingredients.

2.1.8.2 Nucleotides

Nucleotides are precursors for nucleic acids, which are fundamental constituents of all organisms. They are also important in energy metabolism (adenosine tri-phosphate), components of coenzymes (NAD, NADP, FAD, coenzyme A), and as second messengers mediating cellular processes (cyclic AMP, cyclic GMP). While nucleotides can be synthesized de novo, dietary nucleotides appear to be important for maximizing cell synthesis in tissues undergoing rapid cell division. Thus the requirement for exogenous nucleotides is important for maximizing growth in young animals, including rainbow trout (Li et al., 2006). Burrells et al., (2001a) reported that rainbow trout fed diets containing nucleotides grew significantly faster than trout fed nucleotide deficient diets. They also had lower levels of plasma cortisol after being subjected to stress (handling, environmental changes). Furthermore, nucleotides have also been shown to increase disease resistance in rainbow trout to both viral and bacterial diseases (Burrells et al. 2001b; Li et al., 2006). Plant ingredients are poor sources of nucleotides compared to fish meal. Mateo et al., (2004) reported that soybean meal contained 38 ug/g total nucleotides compared to 75 ug/g for fish meal. This supports the notion that nucleotides may be a pronutritional factor present in fish meal.

While the evidence that taurine and nucleotides are water-soluble pronutritional factors present in fish meal, there also appear to be lipid soluble pronutritional factors present in fish oil. However, these factors have not been conclusively identified. Thus, an understanding of the lipid metabolism of salmonids and the chemical constituents of fish oil is critical to identifying these unknown factors.

2.2 Polyunsaturated fatty acid metabolism in salmonids

2.2.1 Biosynthesis of highly unsaturated fatty acids (HUFAs)

Linoleic 18:2(n-6) and alpha linolenic 18:3(n-3) acids (LA and ALA) are essential nutrients as teleost fish cannot synthesize these fatty acids and therefore, they must be provided in the diet. Biosynthesis of highly-unsaturated fatty acids (HUFA) from plant oils requires desaturation and elongation of these 18 carbon precursors (Tocher, 2003). Fresh water fish, including salmonids, possess all of the enzymes to accomplish these conversions and produce the primary HUFAs ARA (arachidonic acid) (n-6) and EPA and DHA (n-3) (Ruyter et al., 1999; Ruyter et al., 2000; Tocher, 2003; Figure 2.4). Synthesis of EPA requires Δ6 desaturation of 18:3(n-3) to produce 18:4(n-3), that is elongated to 20:4(n-3), followed by Δ5 desaturation. DHA synthesis requires two further elongation steps, a second Δ6 desaturation and a chain-shortening β-oxidation step (Tocher, 2003). Teleost fish have considerable differences in their ability to convert 18 carbon n-3 and n-6 fatty acids to longer chain HUFA. Species differences are a major determinant of the ability of fish to synthesize HUFA. Freshwater fish require only 18:2(n-6) and 18:3(n-3) and can produce HUFA from these precursors. In contrast, most marine fish require dietary 20:5(n-3) and 22:6(n-3), because of low or non-existent activities of one or more of the enzymes required for HUFA synthesis (Ghioni et al., 1999; Tocher and Ghioni, 1999, Hansen et al., 2008). Salmonids possess all of the enzymes required to synthesize HUFA, although the efficiency of this pathway is relatively low (Ruyter et. al., 1999; 2000; Tocher, 2003). Without proper supplementation of essential fatty acids in the diet, fish will begin to demonstrate fin rot, reduced growth rate, reduced feed efficiency, and increased mortality (Castell et al., 1972; Takeuchi and Watanabe, 1977a, b; Takeuchi et al., 1980; Satoh et al., 1989; NRC, 1994). However, the requirements for LA and ALA in salmonid diets are not well defined and are

reported as ranging from 1-2% of the diet (NRC, 1994).

These essential fatty acids also function as components of phospholipids in all biological membranes and the balance of unsaturated and saturated fatty acids within phospholipids of biomembranes will determine membrane fluidity (Tocher, 2003). Dietary n-3 PUFA, especially, play an important role in homeoviscous regulation. This is the process whereby fish will change the phospholipid composition of their biomembranes in response to changes in their environmental temperature (Hazel, 1984). In Hazel's (1984) research, he explains that the total amount of phospholipid in the fish biomembrane does not change when fish acclimate to cold-water temperatures, but the relative proportion of individual phospholipids does, as well as the distribution of fatty acids within the phospholipids.

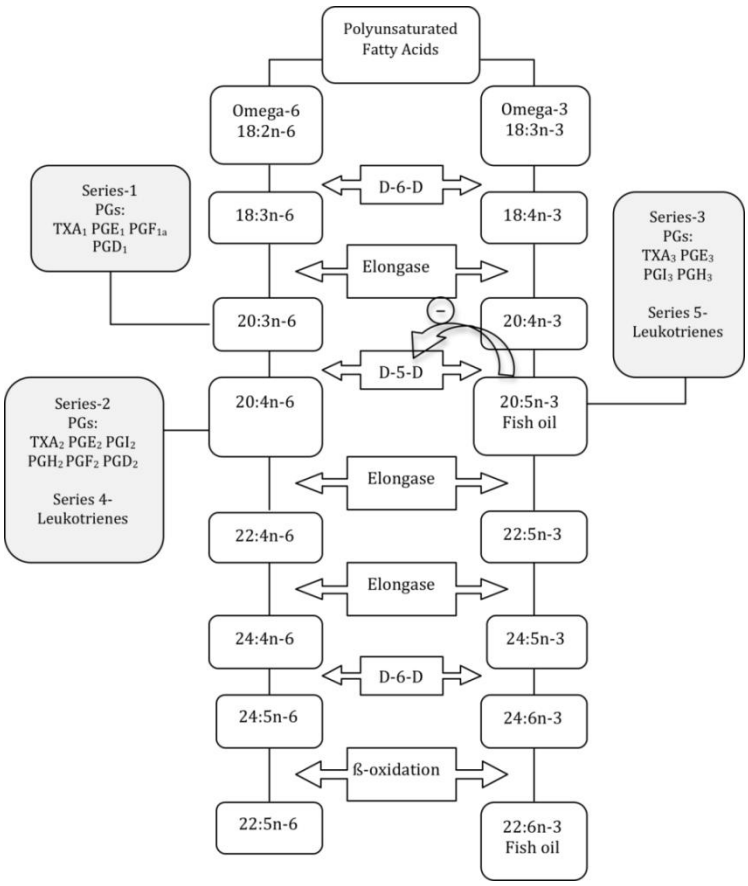

Figure 2.4 Pathways of HUFA synthesis from LA and ALA

2.2.2 Role of HUFAs in inflammatory responses in salmonids

HUFAs also play an important metabolic role as precursors of eicosanoids. Eicosanoids are hormone-like molecules involved in numerous biological activities, including regulation of inflammation and immune response, and are derived from the 20 carbon fatty acids ARA and EPA. However, the formation of eicosanoids from ARA is normally more inflammatory than those formed by EPA, and EPA-derived eicosanoids are also less biologically active. Furthermore, EPA-derived eicosanoids competitively inhibit the actions of ARA-derived eicosanoids (Figure 2.5). This means that the ratio of the n-6 and n-3 fatty acids can have significant effects on the related activities of eicosanoids and associated changes in inflammatory responses. Schmitz and Ecker (2008) have shown that salmonids have significantly lower n-6:n-3 ratios than mammals, meaning that the high n-6:n-3 ratios in most vegetable oils can negatively alter the activity of eicosanoids in the fish. In a study by Oxley et al., (2010), it was reported that feeding diets high in vegetable oils significantly altered the ratio of n-6:n-3 eicosanoids in Atlantic salmon, in contrast with fish fed fish oil. They suggested that this could lead to increased inflammation in the gut, which in turn would affect osmoregulation and absorption of nutrients. These findings demonstrate that the ratio of dietary LA:ALA is not only important for the fatty acid composition of fish products, it also has a significant effect on lipid metabolism and inflammation. Therefore, these issues should be considered when replacing fish oil with vegetable oils.

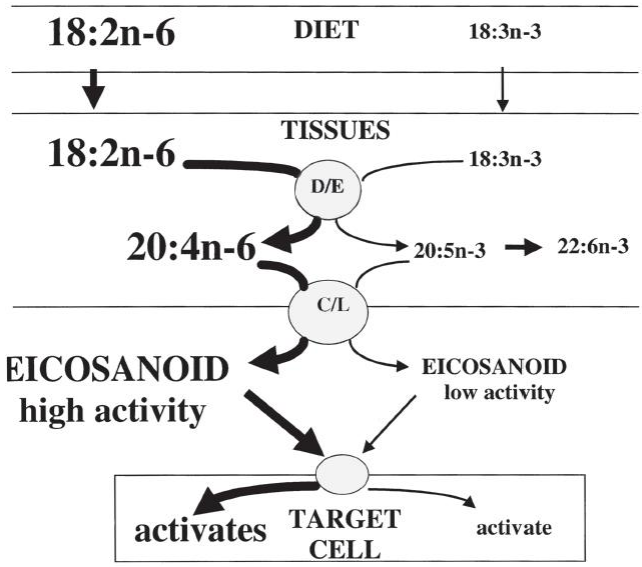

Figure 2.5 The link between dietary PUFA, tissue HUFA and eicosanoid production (After Tocher, 2003)

2.2.3 Health benefits of HUFAs in the human diet

The replacement of fish oil in salmonid diets raises concerns. Although vegetable oils are the most readily available alternative sources of lipids for aquafeeds, they are generally poor sources of n-3 fatty acids and are devoid of HUFAs, such as EPA and DHA (Table 2.1). When fish oil is replaced with vegetable oil in salmonid diets, the effect is a significant change in the fatty acid composition of fish tissues, and a reduced nutritional quality of fish products for human consumption (Caballero et al., 2002; Grisdale-Helland et al. 2002; Rørå et al., 2005; Rinchard et al., 2007; Berge et al., 2009; Østbye et al., 2010). When fed vegetable oil-based diets, salmonids experience significantly reduced levels of EPA and DHA, compared to the levels found in fish fed fish oil (Table 2.2).

The health benefits of consuming highly unsaturated n-3 fatty acids (n-3 HUFA) are well known (review: Van Horn et al., 2008), and include reduced risk of cardiovascular disease,

stroke and autoimmune and inflammatory diseases. Research also shows some evidence that they may reduce the risk of cancer, dementia and Alzheimer's disease (Kris-Etherton et al., 2003; Hooper et al., 2006; Fernandez et al., 1999; Singer et al., 2008; Nestel, 2000). It is recommended by The Food and Nutrition Board, USA (2002) that the adult males consume 1.6 g/d and adult females 1.1 g/d of EPA and DHA. In the human diet, the primary source of these lipids are cold water fish, such as salmon, trout, sardines, mackerel, and tuna. Alpha linolenic acid (ALA) can be found in flax and canola/rapeseed oils, and humans have the ability to convert this short chain n-3 fatty acid to EPA and DHA. However, this conversion is inefficient and only 2-5% of ALA is converted to longer chain n-fatty acids (Goyens et al., 2005; Hussein et al., 2005).

Table 2.1 The fatty acid composition of common fish and vegetable oils used in aquaculture diets (%; NRC, 1994)

Oil	18:1n-9	18:2n-6 (LA)	18:3n-3 (ALA)	20:5n-3 (EPA)	22:6n-3 (DHA)
Fish	12.7-22.7	0.6 – 10.5	0.3 – 1.0	0.4 – 17.0	1.3 – 13.8
Canola	60.0	20.2	12.0	-	-
Corn	58.0	58.0	0.7	-	-
Flax	20.2	12.7	53.3	-	-
Sunflower	19.5	65.7	-	-	-
Soybean	22.8	51.0	6.8	-	-

Table 2.2 Effect of replacing fish oil with vegetable oils on the DHA and EPA contents of rainbow trout and Atlantic salmon. Values are % of total lipid.

Study	DHA+EPA fish oil-fed fish	Fish oil replacement used in test group	DHA+EPA test group
Rainbow trout			
Caballero et al., 2002	6.9	20% FO + 80% canola/palm oil	3.1
Bell and Dick, 2004	5.3	Linseed oil/Soybean oil/Sunflower	2.4
Rinchard et al., 2006	9.2	Linseed oil	1.9
Drew et al., 2007	21.2	Linseed/Canola	4.4
Nilson, 2007	14.5	Linseed oil	8.9
Ng et al., 2010	19.9	25% FO + 75% palm oil	5.1
Atlantic salmon-fresh water			
WingKeong et al., 2007	22.0	Palm oil	8.3
Bell et al., 2001	20.6	Canola oil	8.3
Tocher et al., 2002	28.1	Linseed oil	6.4
Brandsen et al., 2003	23.8	Sunflower oil	10.8
Grisdale-Helland et al., 2002	15.0	Soybean oil	9.2
Østbye et al., 2009	48.7	Rapeseed oil	43.2
Atlantic salmon- seawater			
Bell et al., 2003a	21.7	Canola oil	10.4
Bell et al., 2003b	18.4	Linseed oil	5.8
Bell et al., 2004a	12.4	Linseed oil	4.4
Bell et al., 2004b	12.4	Canola oil	6.6
Berge et al., 2009	14.4	Soybean oil	5.7
Rørå et al., 2005	21.0	Soybean oil	4.5

2.3 Cholesterol

2.3.1 Introduction

A member of the neutral lipid family called sterols, cholesterol is an essential, natural product of all animal cells. Its role in membrane structure, function and dynamics, as well as being a precursor for the synthesis of steroid hormones and bile acid, mark cholesterol as an extremely important biological molecule. This critical role in cell biology, as well as its potential in the pathogenesis of disease in humans, has resulted in an intense research focus on the role of cholesterol within cell membranes. Cholesterol is the energy-costly end product of many enzyme-catalyzed steps, and its pathway of synthesis also delivers many other important sterols along the way (King, 2005). Its unique structure (Figure 2.6) has the ability to stimulate or inhibit the function of membrane proteins vital to cell life through sterol-protein interactions and modulation of the internal lipid bilayer (Bastiaanse et al., 1997; Brown and London, 2000; Flemming, 2001; Simons and Vaz; 2004). Although much research has focused on the mammalian aspects of cholesterol behavior, few papers and studies have given concern to the role of cholesterol in fish.

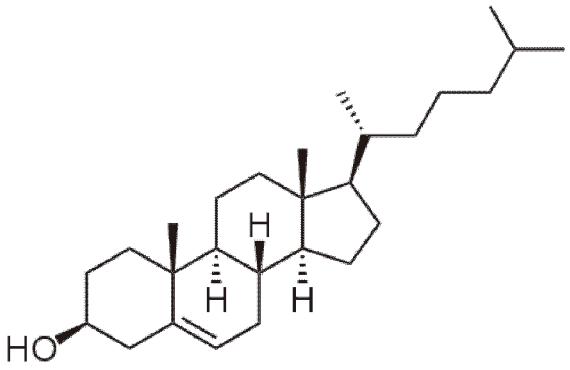

Figure 2.6 Structure of cholesterol

2.3.2 Biosynthesis of cholesterol

Cholesterol biosynthesis is an energy-costly process, requiring an extensive use of endogenous reducing agents, such as NADH and NADPH, enzymes, cofactors, and molecular oxygen. More specifically, the process uses 18 moles of ATP and 16 moles of NADPH to produce 1 mole of cholesterol (Coffee and Coffee, 2004). Acetyl-CoA is the primary source of sterol carbon in animal tissues; however acetate is also an important source (King, 2005). Figure 2.7 depicts the steps of the biosynthetic pathway to cholesterol synthesis.

In a study conducted by Kayama et al., (1971) the liver of rainbow trout, carp and mice was homogenized and anaylzed to determine the role of cholesterogenesis of aquatic animals (compared to that of mammalians) using acetate-1-^{14}C incorporation into the total lipids. Both molecular oxygen requirements and coenzymes were examined under aerobic conditions. The results of the first experiment with carp showed that sterol synthesis was high (5.8% radioactivity) when the cofactors ATP, NAD and NADP were incorporated under aerobic conditions. These results also demonstrate that oxygen is required for the transformation of squalene to sterols, although the formation of squalene is possible under anaerobic conditions. In the second experiment with rainbow trout, it was found that the percentage of sterol activity increased from 16.9% in 4 hours of incubation, to 22.7% in 8 hours and to 27.2% in 16 hours. The total activity of the non-saponifiable fraction was 40.6%. However, although rainbow trout may synthesize fatty acids in amounts that exceed sterol formation, the majority of radioactivity was observed within the saponifiable fraction of the lipids, as opposed to the non-saponifiable fraction which contains squalene and cholesterol. Thus in rainbow trout, it would appear that lipogenesis surpasses cholesterolgenesis. The main conclusion of one of the only studies to examine cholesterol biosynthesis in fish, is that the cofactor requirement for cholesterol synthesis

in fish is not significantly different from those required of mammalians.

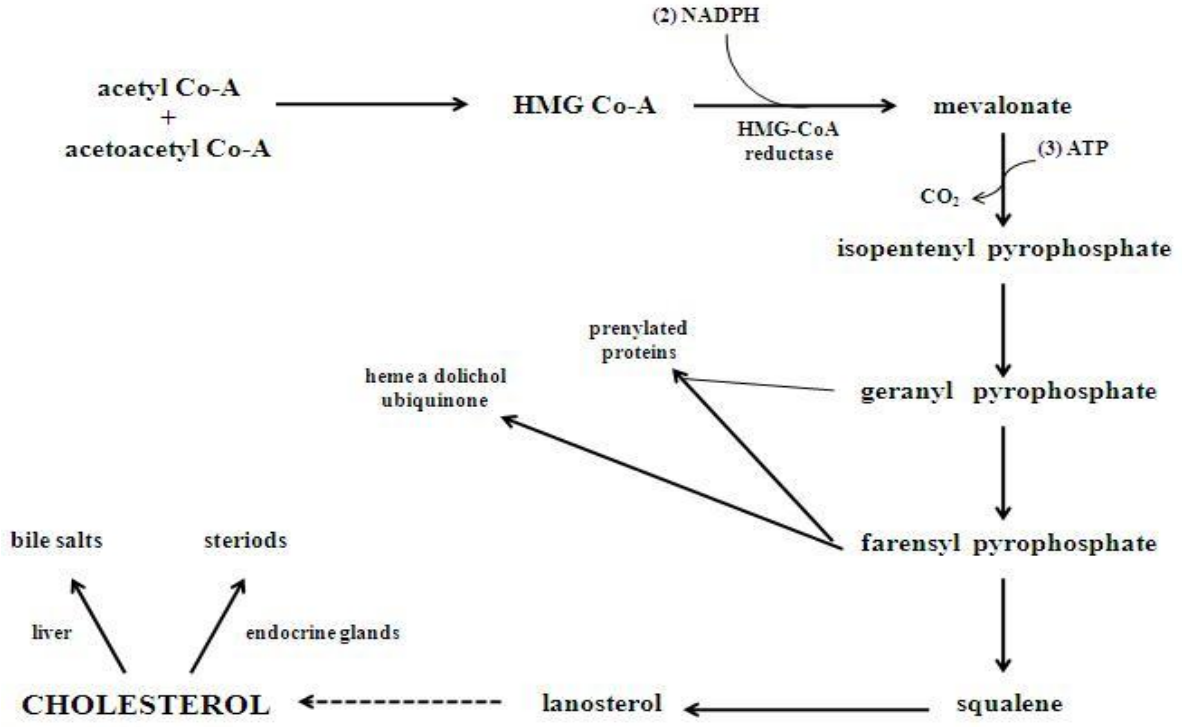

Figure 2.7 The biosynthetic pathway to cholesterol (After King, 2005)

2.3.3 Regulation of biosynthesis of cholesterol

There are three important mechanisms, which help to regulate the cellular supply of cholesterol. The primary control site of cholesterol synthesis is through regulation of the rate-limiting enzyme, HMGR. Four different mechanisms operate to control the enzyme, including its own feedback inhibition, gene expression of the enzyme, rate of its degradation, and phosphorylation-dephosphorylation (King, 2005). The second mechanism of cholesterol regulation is through control of excess intracellular free cholesterol, through the activity of acyl-CoA acyltransferase (ACAT) (Suckling and Stange, 1985). Thirdly, regulation of plasma

cholesterol levels through LDL receptor-mediated uptake of HDL-mediated reverse transport helps to control cholesterol levels (Horton et al., 2002; Brown and Goldstein, 1997).

Cholesterol levels are controlled primarily through the long-term feedback regulation of HMGR. The amount of the enzyme itself present within a cell will determine the cholesterol level of that cell (Brown and Goldstein, 1997). When the levels of mevalonate or LDL-cholesterol fall, the amount of HMGR within a cell will rise significantly, due to an increase in synthesis and a decrease in its degradation (DeBose-Boyd, 2008). The reverse occurs when either mevalonate or LDL-cholesterol is added. Horton et al. (2002), King (2005), Bengoechea-Alonso and Ericsson (2007), and DeBose-Boyd (2008) explain how the sterol-regulated transcription of genes that encode this key rate-limiting enzyme, as well as others, also controls cellular cholesterol content. These genes all contain a DNA sequence called the sterol regulatory element (SRE). A specific transcription factor, the sterol regulatory element binding protein (SREBP) must bind to the SRE in order for transcription to occur. SREBP is synthesized as a membrane protein and when cholesterol concentration is very high, resides in the endoplasmic reticulum (ER) in a complex known as SREBP cleavage-activating protein (SCAP). SCAP contains a sterol-sensing domain (SSD). When cholesterol within the ER is low, SCAP proceeds to change conformation and transports its bound SREBP to the Golgi apparatus. SREBP is then cleaved by two membrane-bound proteases, site-1 protease (S1P) and site-2 protease (S2P). When this occurs, transcription of target genes is activated within the nucleus. Thus sterol content regulates the ability of SCAP to donate SREBP to S1P. When sterols are low, SCAP and SREBP move along to the Golgi and when sterol content is high, the movement of SCAP ceases. This is a classic case of feedback inhibition.

2.3.4 Effects of PUFA on cholesterol biosynthesis

Studies have shown that high levels of PUFA in a diet can regulate the expression of genes involved in cholesterol synthesis (Le Jossic Corcos et al., 2005; Horton et al., 2003; Ntambi and Sampath, 2005). In 2005, Le Jossic Corcos et al. conducted a study to examine the effects of n-3 and n-6 PUFA on the expression of SREBPs (sterol regulatory element-binding proteins), which in turn code for the rate-limiting enzyme in cholesterol synthesis, HMGR and FPP synthase (farnesyl diphosphate synthase), a precursor for sterol compounds. Mice were fed a diet enriched with tuna fish oil (high in n-3 PUFA) for two weeks, or a control consisting of olive oil (low n-3 PUFA diet). The results indicated that mRNA for FPP synthase was decreased by 70% and by 40-60% for HMGR in high n-3 PUFA diets. In another study by Choi et al. (1989), it was found that the activity of HMGR was lower in rats fed diets rich in n-3 PUFA (fish, perilla and linseed oils), than in those fed diets rich in n-6 PUFA (safflower and borage oils).

High PUFA diets may also affect cholesterol synthesis by increasing LDL receptor (LDLr) activity, protein and mRNA abundance. Mustad et al. (1996) conducted a study in which pigs were fed a corn-soybean meal diet with three different levels of cholesterol (0.25%, 0.5% and 1.0%) with either no added fat, or fats rich (30% of calories) in palmitic or linoleic acid. The results of their study showed that with pigs fed the 0.25% cholesterol, linoleic acid had increased hepatic LDLr protein levels by 40%, whereas palmitic acid had reduced it by 40% ($P<0.05$), in comparison to pigs consuming only cholesterol or a low fat/cholesterol-free diet (control pigs). Analogous changes were observed in hepatic LDLr mRNA. Theoretically, animals consuming low fat/cholesterol-free diets should exhibit maximal LDLr expression. The different effects of PUFA on the expression of LDLr were only observed in the low level cholesterol diets, which

suggest that high cholesterol intake may have a suppressive effect on LDL receptor mRNA levels, which cannot be removed by PUFA. It is suggested by Mustad et al. (1996) and also Fernandez and West (2005) that modification of hepatic membrane fluidity may be one way in which high PUFA diets affect LDL receptor activity, after previous studies in rats (Tripoldi et al., 1991) showed significant changes in LDL binding to LDL receptors, due to changes in membrane fluidity.

2.3.5 The role of cholesterol in the biosynthesis of steroid hormones

In a review by Stocco (2001), the importance of cholesterol in steroid hormone synthesis and regulation is discussed. Cholesterol is an important precursor to the synthesis of many important steroid hormones. The major steroid classes include: 1) glucorticoids, responsible for regulation of carbohydrate metabolism and managing stress levels, 2) adrenal mineralcorticoids, which maintain blood pressure and regulate salt balance, 3) ovarian and placental estrogens and progestins, which regulate female secondary sex characteristics and reproductive function, 4) testicular androgens, responsible for male fertility and secondary sex characteristics, and 5) neurosteroids, located in the brain, which stimulate and regulate GABAergic responses. Although diverse in their functions, the steroid hormones are synthesized through a pathway identical in its initial stage. This initial step is the conversion of cholesterol to the first steroid, pregnenolone. In the majority of species, pregnenolone is then converted to progesterone, and then to an assortment of other steroids. The biosynthesis of steroids (steroidogenesis) is regulated primarily by pituitary trophic hormones and requires the synthesis of a protein whose role is to move cholesterol from the outer to the inner mitochondrial membrane in steroidogenic cells. This has recently been identified as the rate-limiting step in steroidogenesis. The protein responsible for the movement of cholesterol is called StAR (steroidogenic acute regulatory), and it is

expressed when steroid hormone synthesis is stimulated. However, the mechanism by which StAR mediates cholesterol transfer in the mitochondrial membrane is unknown. As cholesterol plays a crucial role not only in the synthesis of steroidogenesis, but also in its regulation, changes in the dietary supply or biosynthesis of cholesterol itself would in turn have an effect on the level of steroids within the body and their associated functions. Mondul et al. (2010) examined the effects of serum cholesterol and cholesterol-lowering drug use (statin drugs) on the testosterone and estradiol (an estrogen) levels in men. These drugs have been hypothesized to reduce prostate cancer, but the biological mechanisms associated with this are unknown. One hypothesis is that inhibition of cholesterol synthesis (through cholesterol-lowering drugs) may reduce circulating levels of testosterone and estradiol, thereby reducing the growth and development of prostate cancer. They report that serum cholesterol or cholesterol-lowering drugs did not have an effect on testosterone levels. However, high serum cholesterol did reduce estradiol levels, but cholesterol-lowering drugs had no effect. As cholesterol is a precursor for testosterone and hence, estradiol synthesis, it was expected that the opposite effect would have occurred. Mondul et al. (2010) conclude that their data provides no evidence for the relationship between serum cholesterol and cholesterol-lowering drugs on circulating testosterone levels; however it does suggest the circulating cholesterol concentration may be inversely related to circulating estrogen levels in men.

2.4 The role of biological membranes and cholesterol

2.4.1 The effects of cholesterol on lipid phase behavior and raft formation

As previously mentioned, a large percentage of cholesterol resides within the membranes of a cell, especially the plasma membrane. Therefore, it can be seen that cholesterol exerts its greatest influence on membrane properties and function. Changes in the dietary lipid content, or

the direct addition or removal of pure cholesterol from the diet will determine the extent of alteration on membrane properties (Bjerkeng et al., 1999; Brown and London, 1998; Brown and London, 2000; Sener and Yildiz, 2003; Twibell and Wilson, 2003).

In the past few years, attention has focused on the research related to membrane rafts - a specific type of membrane domain, which are sphingolipid and cholesterol-based. In a minireview by Brown and London (2000) and a full review by Simons and Vaz (2004), the structure and function of sphingolipid and cholesterol-rich membrane rafts is discussed. They note that unlike other biological phospholipids, sphingolipids are able to pack together tightly because they contain long, mainly saturated acyl chains, whereas membrane (glycero)phospholipids usually contain high amounts of kinked unsaturated acyl chains. This also gives sphingolipids higher melting temperatures (T_m). Research has shown that raft lipid formation is dependent upon these tightly packed acyl chains (Brown and London, 1998; Ahmed et al., 1997; Schroeder et al., 1998). The result of this different packing ability of sphingolipids and phospholipids is phase separation in the membrane. This means that sphingolipid-rich rafts can exist together with phospholipid-rich domains, which are in the more typical disordered, loosely packed state (Brown and London, 1998). Of the various physical states and their associated phase separation between lipids, the gel phase, in which acyl chains are highly ordered, is one of the most well-known (Brown and London, 1998).

However, within plasma membranes, where a high concentration of cholesterol can be found, raft lipids do not exist in the gel phase. Cholesterol has significant effects on phase behavior. When cholesterol is added to a phospholipid bilayer, the sharp thermal transition between the gel and l_c phase is normally eliminated, resulting in membrane properties which are between the two phases (Brown and London, 2000; Simons and Vaz, 2004). This effect would

imply that domains in ordered and disordered states cannot co-exist at high cholesterol concentrations. However, Brown and London (2000) suggest that a special kind of phase separation can occur in dual mixtures of individual phospholipids with cholesterol. This is also mentioned in the review by Simons and Vaz (2004). In these mixtures, domains in an l_c-like phase can exist with domains in the liquid-ordered (l_o) phase. In this phase, acyl chains are tightly packed (similar to the gel phase), but have a high amount of sideways mobility (Brown and London, 1998). A second effect of cholesterol on phase behavior involves the similarities between l_o/l_c phase separation and gel/l_c phase separation; highly ordered acyl chains (gel or l_o) separate from a phase in which they are disordered (l_c). This means that in the absence of cholesterol, lipid mixtures can experience gel/l_c phase separation, but in cholesterol's presence, they experience l_o/l_c phase separation (Brown and London, 2000; Simons and Vaz, 2004). Looking at the phase behavior of mixtures with and without cholesterol demonstrates that this sterol is sometimes able to promote phase separation (Ahmed et al., 1997; Silvius et al., 1996), due to the positive packing interactions between saturated lipids and sterols (Xu and London, 2000). This means that in phospholipid/sphingolipid mixtures, when cholesterol is present, less sphingolipid is needed to form the l_o phase. The opposite holds true in the absence of cholesterol to form the gel phase (Ahmed et al., 1997; Schroeder et al., 1998). In cell membranes that contain low levels of sphingolipids, this effect of cholesterol helps to clarify the formation of rafts. Secondly, it helps to explain why removal of cholesterol can disturb rafts and affect the function of rafts (Brown and London, 2000). Lastly, this effect of cholesterol explains how sphingomyelin can be just as effective in promoting raft formation as glycosphingolipids, even though glycosphingolipids have a much higher melting temperature (Ostermeyer et al., 1999).

The raft-stabilizing effect of cholesterol is much stronger compared to any differences in raft stability caused by this difference in melting temperatures (Brown and London, 2000).

2.4.2 Cholesterol-protein interactions in membranes

With such significant effects on the lipid bilayer of membranes, it is likely that cholesterol also modulates membrane proteins, which are embedded within the membrane environment itself. Therefore membrane enzymes, and any process that depends on membrane protein function, such as ion channels, transporters, and receptors, are affected as well. Experiments have demonstrated that in the absence of cholesterol, there is little or no activity of the Na^+K^+ATPase enzyme, which is responsible for the energy-dependent transmembrane transport of Na^+ and K^+ (Bastiaanse et al., 1997; Flemming, 2001). However, at low-to-moderate levels of membrane cholesterol, addition of more cholesterol results in stimulation of the enzyme. The opposite occurs at high membrane cholesterol levels. Stimulation is explained through a direct interaction of cholesterol with the protein. Inhibition at high membrane cholesterol levels is thought to occur as a result of the restriction of conformational changes by the enzyme (Bastiaanse et al., 1997; Yeagle, 1991). These changes, which are required in order for the enzyme to function properly, are the result of a decrease in free volume within the lipid bilayer, due to the presence of cholesterol (Flemming, 2001; Bastiaanse et al., 1997; Yeagle, 1991). Experiments have also shown the effect of cholesterol content on the operation of the Ca^{2+}-dependent K^+-channel. Bastiaanse et al., (1997) discuss experiments that show decreased membrane fluidity after the addition of cholesterol, and a decrease in probability of the calcium channels opening by 50%. This suggests that there is a direct inverse relationship between the conductance of this channel and the membrane cholesterol content. There have also been several studies, which suggest that increasing cholesterol content of the plasma membrane results in an

increase flow of Ca^{2+} through the Ca^{2+} channel (Bastiaanse et al., 1997). Based on these studies, cholesterol plays a critical role in ionic balance within the cell membrane; a critical function in salmonid fish.

2.5 Biological roles of cholesterol in fish

2.5.1 Buoyancy

Cholesterol is found in high amounts in the lipid-rich membranes of the swimbladder of deep sea fishes and it may facilitate gas secretion by combining with oxygen gas (Phleger, 1998). Even though cholesterol has a density greater than sea water (1.067 g/L vs. 1.025 g/L, respectively), it still acts as a major lipid in the swimbladder. Cholesterol is especially important in fish that have a physoclistous swimbladder; there is no connection to the gut and therefore gas secretion and absorption must occur in the swimbladder itself. There are two hypotheses for the function of the cholesterol-rich membrane system in the swimbladder of deep sea fishes (Phleger, 1998). First, it provides a barrier to oxygen diffusion out of the swimbladder. Freshly caught deep-sea fish often have bright, reflective swimbladders, which suggests that lipids are below their phase transition temperatures and have very low oxygen solubility. Second, swimbladder lipids dissolve oxygen gas, reducing oxygen back pressure on the gas gland. Because the phospholipids of the swimbladder membrane are highly unsaturated, this hypothesis suggests that they must be above their phase transition temperature and thus a barrier to oxygen diffusion is not provided, as the other hypothesis suggests. Further measurement on the physical state of lipids at such great depths and pressures is needed to fully understand the role of cholesterol in swimbladder membranes and to solve these questions.

2.5.2 Disease resistance

Recent studies have shown that plasma lipid components are good indicators of disease resistance in fish, including plasma cholesterol. In a study conducted by Maita et al. (1998), mortalities of yellowtail and rainbow trout that were experimentally infected with two different strains of bacteria, had their plasma components compared with those of fish prior to the challenge. It was found that when rainbow trout were experimentally infected with *Vibrio anguillarum*, fish surviving the infection had significantly higher levels of total cholesterol, free cholesterol and phospholipid. In the experimental challenge with rainbow trout, fish with low plasma total cholesterol levels (< 520mg/100ml) had significantly greater mortality than fish with high cholesterol levels (> 560 mg/100ml).

Maita et al. (2006) conducted another study examining the role of cholesterol and taurine in disease resistance and hypocholesterolemia in yellowtail fish. Taurine that is supplemented in non-fishmeal diets plays an important role in cholesterol metabolism in two ways: 1) stimulating bile acid production by increasing production of cholesterol 7α-hydroxylase, a rate limiting enzyme in bile acid synthesis and 2) stimulating cholesterol synthesis by increasing production of HMG-CoA reductase, the rate limiting enzyme of cholesterol biosynthesis. Five experimental groups of fish were involved in the study; a control group fed a fishmeal diet and four other groups (D-1, D-2, D-3 and D-4) fed a non-fishmeal diet, with various supplements of cholesterol and taurine. D-2 included cholesterol at a rate of 14%, D-3 included taurine at a rate of 1% and D-4 included both cholesterol (14%) and taurine (1%). The highest morality rate was observed in the D-1 group, after infection from a natural source (pseudotuberculosis). After 30 days of the experiment, mortality from experimental infection (*Lactococcus garvieae*) was significantly greater in the D-1, D-2 and D-3 groups, as compared to the D-4 and control groups. After 60

days, mortality in the D-1 and D-2 groups was significantly greater, with D-3 mortality comparable to the D-4 and control groups. The results of this experiment suggest that disease resistance of fish fed a non-fishmeal diet was lower than that of fish fed the same diet, but supplemented with cholesterol or taurine. Plasma total cholesterol in group D-4 was similar to the control group, but was still lower. Along with its critical role in cholesterol metabolism, taurine has also been shown to improve the growth of rainbow trout in plant-based diets (Gaylord et al., 2006) and has been identified as a pronutritional growth factor present in fish meal. The results of Maita et al. (2006) would suggest that both dietary cholesterol and taurine, together, play a critical role in disease resistance of finfish. Their study indicates that anemia is the most important indicator of reduced disease resistance, and anemia was improved through dietary supplementation with taurine. De novo synthesis of cholesterol was also active, as results of semiquantitative RT-PCR showed a significant increase in the expressions of HMGR mRNA in fish fed a non-fish meal diet. Through supplementation with both cholesterol and taurine (D-4), levels of plasma cholesterol were comparable to levels in control fish. These results of Maita et al. (2006) would therefore imply that hypocholesterolemia that is often seen in fish fed a non-fishmeal diet, compared to a fishmeal diet, is due to lack of insufficient dietary cholesterol and insufficient production of endogenous cholesterol, which is due to the lack of dietary taurine.

2.6 Cholesterol research on finfish

2.6.1 Cholesterol effects on organ indices and fatty acid composition

The effect of cholesterol and short-chain fatty acids (SCFA) on growth, organ indices, macronutrient digestibility, and fatty acid composition in Atlantic salmon has been examined by Bjerkeng and Storebakken (1999). In this study, salmon were fed one of six diets; diet 1 had no cholesterol/SCFA supplement, diets 2 and 3 contained only a SCFA supplement and diets 4, 5

and 6 contained a 1.0% cholesterol, 1.0% cholesterol/0.5% SCFA, and 1.0% cholesterol/2.0% SCFA supplement, respectively.

Dietary cholesterol did not significantly affect final weight or specific growth rate (SGR) in salmon. However, there were significant effects on the fatty acid composition of the liver and feces, of salmon fed the cholesterol supplemented diets. More mono- and poly-unsaturated fatty acids were excreted in the feces and the relative concentrations of hepatic palmitic acid (C16:0), arachidonic acid (C20:4, n-6) and DHA (C22:6, n-3) were significantly reduced. The concentration of oleic acid (C18:1, n-9) and eicosenoic acid (C20:1, n-9) were significantly increased in fecal excretions. Fecal content of cholesterol was also significantly greater in salmon fed the cholesterol supplemented diets ($P<0.001$). Salmon supplemented with cholesterol had accumulation of cholesterol in the liver and concentrations of hepatic cholesterol were significantly different among treatments ($P<0.001$). This was entirely related to cholesterol supplementation, with no effect credited to dietary SCFA. This accumulation of hepatic cholesterol appeared to significantly alter fatty acid metabolism of the salmon, as was mentioned previously. In studies with rats, SCFA have been shown to affect the hepatic cholesterol pool, however this was not the case with Atlantic salmon. This suggests that the hepatic cholesterol pool may play a less important role in evaluating cholesterol status in salmon, as compared to mammals. The results of this study would also suggest that regulation of cholesterol synthesis in Atlantic salmon differs from that of mammals.

2.6.2 Cholesterol effects on growth parameters

Much of the cholesterol research in recent decades has focused on human aspects, using mice or other mammals as experimental models. Very little has focused on the role of cholesterol in fish. Preliminary research conducted by Twibell and Wilson (2004) looks at the effects of a

soybean meal-based diet, supplemented with cholesterol, in channel catfish. Cholesterol was added to diets containing 0% or 55% soybean meal. The fish fed the supplemented cholesterol and 55% soybean meal gained significantly more weight and ate significantly more than the non-supplemented fish. These results therefore suggest that the lack of cholesterol in diets with little or none marine products may be the cause of depressed growth when these diets are fed to salmonids.

2.7 Hypothesis

The decreased growth performance seen in fish fed plant-based diets versus fish meal/fish oil-based diets has always been attributed to the anti-nutritional factors present in plants. However, even with the use of processing techniques that significantly diminish and eliminate anti-nutritional factors and growth inhibitors in plants, reduced growth performance is still observed (Drew et al., 2007). This suggests that there may be pro-nutritional factors in fish meal/oil that are missing in plant-based diets. Some compounds of interest are the low molecular weight nitrogen compounds, (Gil and Rueda, 2002) such as taurine (Stapleton et al., 1997), HUFA (EPA, DHA) and cholesterol (Aksnes et al., 2006a;b). These pro-nutritional factors would be most important in carnivorous fish, including rainbow trout. Further, aquaculture fish have been selected for high growth rates and this may increase their requirements for these putative pro-nutritional factors. Thus, supplementation of these factors in plant-based diets may ameliorate the depression in growth performance that is generally observed in fish fed such diets.

Based on these observations, we hypothesized that rainbow trout have a requirement for dietary cholesterol and the supplementation of plant-based diets with cholesterol will improve their growth.

3. CHOLESTEROL SUPPLEMENTATION IN PLANT-BASED DIETS FOR RAINBOW TROUT (*Oncorhynchus mykiss)*

3.1 Introduction

The use of plant oils and protein sources as replacements for fish meal and oil is one of the most important problems facing the aquaculture industry. This problem is especially acute for carnivorous fish such as rainbow trout or Atlantic salmon (salmonids). Feeding plant proteins frequently results in reduced growth and feed intake (for reviews see: Drew et al., 2007; Gatlin et al., 2007). Similarly, replacement of fish oil with vegetable oils in diets fed to carnivorous fish have been shown to depress growth (Kissil et al., 2000; Gomez-Requeni et al., 2004; De Francesco et al., 2004). Studies conducted by Thiessen et al. (2003, 2004), Mundheim et al. (2004) and Sener and Yildiz (2003) demonstrate that when fish performance is not significantly reduced with plant oil replacement, fish meal or other animal products have been left in the diet. This suggests that there is a constituent of marine and animal products which may contribute to growth, which is not present in plant oils.

Cholesterol may be one such constituent. Fish oil contains 4-10 mg/g of cholesterol and fish meal contains significant amounts as well, depending on the oil content of the meal. In support of this notion, Twibell and Wilson (2004) performed a study in catfish to examine the effects of a soybean meal diet with cholesterol supplementation. Cholesterol (0% and 1.0%) was added to diets containing 0% or 55% soybean meal. The fish fed the diets supplemented with cholesterol gained significantly more weight and ate significantly more than the non-supplemented fish. This suggests that cholesterol may be required in diets fed to finfish. Furthermore, the fatty acid content of the diet affects endogenous cholesterol synthesis and may affect dietary requirements.

Previous studies in mice reported that feeding high levels of n-3 polyunsaturated fatty acids reduced the activity of hepatic HMG-CoA reductase; the rate-limiting enzyme in the synthesis of cholesterol (Horton et al., 2003; Le Jossic-Corcos et al., 2005). This suggests that n-3 PUFA-rich diets might decrease endogenous cholesterol synthesis and increase the requirement for dietary cholesterol. The majority of fish feeding experiments use marine or animal-source ingredients and so the diets contain cholesterol (Thiessen et al., 2003, 2004; Mundheim et al., 2004; Sener and Yildiz, 2003). However, in studies where only plant ingredients have been used in diet formulation, fish growth rate was significantly reduced (Thiessen et al., 2003, 2004). Based on these observations, we hypothesize that rainbow trout have a conditional requirement for cholesterol and the lack of this nutrient in plant-based diets is partially responsible for the depression in growth performance. The following studies were conducted to determine if rainbow trout have a conditional requirement for dietary cholesterol and the effect of dietary n-3 PUFA on this requirement.

3.2 Materials and Methods

3.2.1 Experiment 1

3.2.1.1 Environmental conditions and fish management

Experiment 1 was conducted at the Fish Nutrition Research Lab at the University of Guelph, Guelph, Ontario. Rainbow trout (*Oncorhynchus mykiss*) were obtained from Alma Aquaculture Research Station (AARS). The fish were housed in 60 L tanks in a recirculating system using a gravel biological filter and each tank was individually aerated. The water temperature of the tanks was maintained at $15^{o}C \pm 1^{o}C$ and water quality (oxygen, pH, nitrate, nitrite ammonia) was monitored daily. Photoperiod was maintained on a 14-h light: 10-h dark cycle. The fish were acclimatized for two weeks during which they were fed a high quality

commercial feed (Martin Mills, Elmira, Ontario). Fish were maintained in accordance with the guidelines of the Canadian Council on Animal Care (Canadian Council on Animal Care, 2005).

All fish in the tanks were equalized for weight the day before the trial start date and 13 fish (average 13.4 g each) were stocked into each of 27 tanks, with 3 tanks per experimental diet (completely randomized design). Each experimental diet was randomly assigned to three tanks and fish were hand fed to apparent satiation twice daily for 56 days. Feed intake was measured weekly and fish weight gain (total tank weight) was measured on day 0 and 84.

3.2.1.2 Diets

The eight experimental diets were formulated as shown in Table 3.1. They were arranged in a 2 x 4 factorial design, with 2 levels of PUFA and 4 levels of cholesterol (0.00, 0.05, 0.10, and 0.15%) added using synthetic cholesterol (Sigma-Aldrich, Oakville, ON). Diets 5 - 8 were PUFA enriched diets, containing a combination of linseed oil and olive oil as shown in Table 3.2. Low PUFA diets (1 – 4) contained only olive oil. All diets were formulated to contain 43.0% digestible protein and 92.0% digestible energy. Feed ingredients were weighed, mixed and cold extruded in a pasta maker, then dried in a forced air oven (55^{o}C, overnight). The diets were stored in plastic bags at -20^{o}C. The calculated nutrient analysis of the diets on a dry matter basis is shown in Table 3.1. Experimental diets also contained soy protein concentrate at 21.0% and corn gluten meal at 16.0%.

3.2.1.3 Statistical analysis

The growth parameters of the experiment were analyzed as a 2 x 4 factorial (2 levels of PUFA x 4 levels of cholesterol) using the General Linear Models procedure of SPSS (SPSS v 13.0, SPSS Inc., Chicago, IL, USA). Differences between dietary treatments were determined using the Ryan – Einot – Gabriel – Welsch *F* test and were considered significantly different

when $P < 0.05$. The interaction between PUFA and cholesterol was significant ($P < 0.05$), so the experiment was reanalyzed using regression analysis. The relationship between cholesterol level for each PUFA level was analyzed by linear regression and regression models were considered significant when $P < 0.05$. . Fish mortalities were weighed out and statistical calculations were adjusted accordingly.

3.2.2 Experiment 2

3.2.2.1 Environmental conditions and fish management

Experiment 2 was conducted at the Prairie Aquaculture Research Centre (PARC) at the University of Saskatchewan, Saskatoon, Saskatchewan. Rainbow trout (*Oncorhynchus mykiss*) were obtained from CanGro Processors Ltd. (Lucky Lake, SK). The fish were housed in 360 L tanks in a recirculating system using biological filtration and each tank was individually aerated. The water temperature of the tanks was maintained at 15^{o}C and water quality (oxygen, pH, nitrate, nitrite ammonia) was monitored daily. Photoperiod was maintained on a 14-h light: 10-h dark cycle. The fish were acclimatized for 2 weeks during which they were fed a high fish meal: fish oil starter diet. Fish were maintained in accordance with the guidelines of the Canadian Council on Animal Care (Canadian Council on Animal Care, 2005).

All fish in the tanks were equalized for weight the day before the trial start date and 20 fish (average 27.7 g each) were stocked into each of 28 tanks, with 4 tanks per experimental diet (completely randomized design). Each experimental diet was randomly assigned to four tanks and fish were hand fed to apparent satiation twice daily for 84 days. Feed intake was measured weekly and fish weight gain (total tank weight) was measured on day 0 and 84.

Table 3.1 Diet formulations and calculated nutrient analysis (n=2) for Experiment 1

Ingredient (g/kg)	**Olive oil**				**Linseed/olive oil**			
Cholesterol (%)	**0.0**	**0.05**	**0.10**	**0.15**	**0.0**	**0.05**	**0.10**	**0.15**
Corn gluten meal	160.0	160.0	160.0	160.0	160.0	160.0	160.0	160.0
Soycomil	213.0	213.0	213.0	213.0	213.0	213.0	213.0	213.0
Wheat gluten	220.0	220.0	220.0	222.0	220.0	220.0	220.0	222.0
Wheat middlings	146.0	146.0	146.0	146.0	146.0	146.0	146.0	146.0
S-type Gold Fat	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Linseed oil	0.0	0.0	0.0	0.0	100.0	100.0	100.0	100.0
Olive oil	150.0	150.0	150.0	150.0	50.0	50.0	50.0	50.0
Vitamin premix[1]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Ascorbic acid	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
BioLysine	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Methionine	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Taurine	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Choline chloride	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Mineral premix[2]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
KH_2PO_4	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
NaCl	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Selenium	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Celite[2]	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Digestible Nutrient (g/kg)								
Crude protein	475.5	477.4	456.6	457.6	460.5	460.2	454.9	463.9
Energy (MJ/kg)	24.2	24.7	24.6	24.4	24.3	24.3	24.3	24.1
Acid ether extract	20.6	20.5	20.9	21.1	20.1	19.9	19.9	20.1
Ash	49.0	50.6	50.6	53.2	53.9	52.3	52.7	51.2
n-6:n-3 ratio	12.9	12.9	12.9	12.9	4.6	4.6	4.6	4.6

[1]Supplied per kilogram of diet: retinyl acetate (vitamin A), 3000 IU; cholecalciferol (vitamin D), 2400 IU; all-raca-tocopheryl acetate (vitamin E), 60 IU; menadione sodium bisulfite (vitamin K), 1.2 mg; ascorbic acid polyphosphate (Stay C25% ascorbic acid), 240 mg; cyanocobalamine (vitamin B12), 0.024 mg; d-biotin, 0.168 mg; choline chloride, 1200 mg; folic acid, 1.2 mg; niacin, 12 mg; d-calcium pantothenate, 26 mg; pyridoxine HCl, 6 mg; riboflavin, 7.2 mg; thiamin HCl, 1.2 mg.

[2] Supplied per kilogram of diet: sodium chloride (NaCl, 39% Na, 61% Cl), 3077 mg; ferrous sulfate ($FeSO_4.7H_2O$, 20% Fe), 65 mg; manganese sulfate ($MnSO_4$, 36% Mn), 89 mg; zinc sulfate ($ZnSO_4.7H_2O$, 40% Zn), 150 mg; copper sulfate ($CuSO_4.5H_2O$, 25% Cu), 28 mg; potassium iodide (KI, 24% K, 76% I), 11 mg.

3.2.2.2 Diets

The six experimental diets were formulated as shown in Table 3.2. They were arranged in a 2 x 3 factorial design, with 2 levels of PUFA and 3 levels of cholesterol (0.00, 0.075, and 0.15%) added using synthetic cholesterol (Sigma-Aldrich, Oakville, ON). Diets 4 - 6 were PUFA enriched diets, containing a combination of linseed oil and olive oil as shown in Table 3.2. Low PUFA diets (1 – 3) contained only olive oil. All diets were formulated to contain 43.0% digestible protein and 92.0% digestible energy. The calculated analysis of the diets on a dry matter basis is shown in Table 3.2. Experimental diets also contained soy protein concentrate at 21.0% and corn gluten meal at 16.0%. Feed ingredients were weighed, mixed and cold extruded in a pasta maker, then dried in a forced air oven (55^{o}C, overnight). The diets were stored in plastic bags at -20^{o}C.

3.2.2.3 Sample collection

At the end of the trial, fish were fasted for 48 hours prior to sampling. Blood and liver samples were taken from 4 fish per tank (16 fish per diet). Each fish was anesthetized using MS - 222 (100 mg per liter of water) and 1 mL of blood was taken from the caudal vein and transferred to tubes containing EDTA as an anticoagulant. The fish was then euthanized with a sharp blow to the top of the head while firmly holding the fish; liver sample was collected and flash frozen in liquid nitrogen and then stored at -80^{o}C until analyzed. Blood samples were centrifuged (1000 *xg*, 10 min) on the day of collection to obtain plasma, which was then separated and stored at -80^{o}C until analyzed.

3.2.2.4 Analytical methods

Serum samples were analyzed using the Amplex Red Cholesterol Assay Kit (A12216) (Invitrogen, Carlsbad, CA). Liver samples were analyzed using the R-Biopharm Cholesterol

colorimetric method for determination of cholesterol in foodstuffs and other materials. Two liver samples per tank were analyzed for liver cholesterol level. The blood cholesterol and liver cholesterol level was measured in duplicate for each sample, and the mean value was determined.

3.2.2.5 Statistical analysis

The growth parameters of the experiment were analyzed as a 2 x 3 factorial (2 levels of PUFA x 3 levels of cholesterol) using the General Linear Models procedure of SPSS (SPSS v 13.0, SPSS Inc., Chicago, IL, USA). Differences between dietary treatments were determined using the Ryan – Einot – Gabriel – Welsch *F* test and were considered significantly different when $P < 0.05$. Regression analysis was used to determine the strength of the relationship between PUFA level and cholesterol. Fish mortalities were weighed out and statistical calculations were adjusted accordingly.

Table 3.2 Diet formulations and calculated nutrient analysis (n=2) for Experiment 2

Ingredient (g/kg)	**Olive oil**			**Linseed/olive oil**		
Cholesterol (%)	**0.0**	**0.075**	**0.15**	**0.0**	**0.075**	**0.15**
Corn gluten meal	160.0	160.0	160.0	160.0	160.0	160.0
Soycomil	210.0	210.0	210.0	210.0	210.0	210.0
Wheat gluten	230.0	230.0	230.0	230.0	230.0	230.0
Wheat middlings	170.0	170.0	170.0	170.0	170.0	170.0
Linseed oil	0.0	0.0	0.0	100.0	100.0	100.0
Olive oil	170.0	169.0	168.5	70.0	69.0	68.5
Vitamin/mineral Premix[1]	6.5	6.5	6.5	6.5	6.5	6.5
Vitamin C	0.9	0.9	0.9	0.9	0.9	0.9
Lysine HCl	20.0	20.0	20.0	20.0	20.0	20.0
DL-Methionine	5.0	5.0	5.0	5.0	5.0	5.0
Taurine	2.0	2.0	2.0	2.0	2.0	2.0
Choline chloride	3.5	3.5	3.5	3.5	3.5	3.5
KH_2PO_4	20.0	20.0	20.0	20.0	20.0	20.0
NaCl	3.0	3.0	3.0	3.0	3.0	3.0
Selenium	0.06	0.06	0.06	0.06	0.06	0.06
Celite[2]	0.1	0.1	0.1	0.1	0.1	0.1
Digestible Nutrient (g/kg)						
Crude protein	482.7	483.6	483.3	481.7	481.6	478.6
Energy (MJ/kg)	22.80	22.98	22.65	22.84	22.59	22.65
Acid ether extract	187.7	196.4	196.0	195.7	191.8	194.6
Ash	36.5	36.3	3.6	36.1	36.5	35.4
n-6:n-3 ratio	12.9	12.9	12.9	5.5	5.5	5.4

[1] Vitamin/mineral premix (mg kg^{-1} dry diet unless otherwise stated), vitamin A (as acetate), 7500 IU kg^{-1} dry diet; vitamin D_3 (as cholecalcipherol), 6000 IU kg^{-1} dry diet; vitamin E (as dl-a-tocopheryl-acetate), 150 IU kg^{-1} dry diet; vitamin K (as menadione Na-bisulfate) 3; vitamin B_{12} (as cyanocobalamin), 0.06; Ascorbic acid (as ascorbyl polyphosphate), 150; d-biotin, 42; choline (as chloride), 3000; folic acid, 3; niacin (as nicotinic acid), 30; pantothenic acid, 60; pyridoxine, 15; riboflavin, 18; thiamin, 3; NaCl, 6.12; ferrous sulfate, 0.13; copper sulfate, 0.06; manganese sulfate, 0.18; potassium iodide, 0.02; zinc sulfate, 0.3; carrier (wheat middling or starch).

[2] Celite 545, <125µg; Celite Corporation, World Minerals Co., Lompoc, CA, USA

3.3 Results

3.3.1 Experiment 1

Table 3.3 provides a summary of the results. During Experiment 1, there was an outbreak of bacterial cold water disease (BCWD) (*Flavobacterium psychrophilum*). At the beginning of the second period of the trial (day 28 – 35), fish began to show clinical signs of BCWD, such as whitish discoloration of the fins, and necrosis and lesions on the peduncle and caudal area. Mortality of fish is shown in Table 3.6. Fish fed the olive oil-based diets had significantly higher incidence of mortality than fish fed the olive/linseed oil diets. However, neither the effect of cholesterol nor the interaction between oil source and cholesterol were significant ($P > 0.05$). In contrast, the interaction between PUFA level and cholesterol was significant for average daily feed intake (ADFI) and near significant for average daily gain (ADG) ($P = 0.085$) and specific growth rate (SGR) ($P = 0.082$) (Table 3.3). The experiment was therefore reanalyzed using regression analysis. Linear regressions of all cholesterol concentrations on growth parameters were performed separately for the olive and olive/linseed oil-fed fish (Figure 3.1). Linear regressions were not significant for any growth parameter in the olive/linseed oil fed fish. However, linear regressions for ADFI, ADG and SGR (Panels A, B and D respectively) were significant for the olive oil-fed fish ($P < 0.05$); increasing cholesterol concentration resulted in increased growth and feed intake.

Table 3.3 Growth performance of rainbow trout fed cholesterol supplemented diets with varying levels of PUFA for days 0 – 84 of Experiment 1

Main Effects	Average Daily Feed Intake (g/d)	Average Daily Gain (g/d)	Feed Conversion (g feed/g gain)	Specific Growth Rate (%/d)	Mortality (%)
PUFA					
Olive oil	0.66	0.81	0.81	2.64	15.38
Lin/Olive oil	0.86	1.01	0.90	2.91	1.34
Pooled SEM	0.037	0.050	0.065	0.094	3.33
Cholesterol (%)					
0.00	0.66	0.83	0.80	2.65	16.24
0.05	0.73	0.93	0.78	2.82	6.62
0.10	0.79	0.88	0.98	2.71	1.28
0.15	0.86	1.00	0.86	2.93	9.30
Pooled SEM	0.052	0.071	0.092	0.132	3.33
***P*-values**					
PUFA	0.010	0.015	0.337	0.054	0.033
Cholesterol	0.072	0.406	0.423	0.451	0.389
Interaction	0.036	0.085	0.565	0.082	0.35

SEM = Standard error of the mean

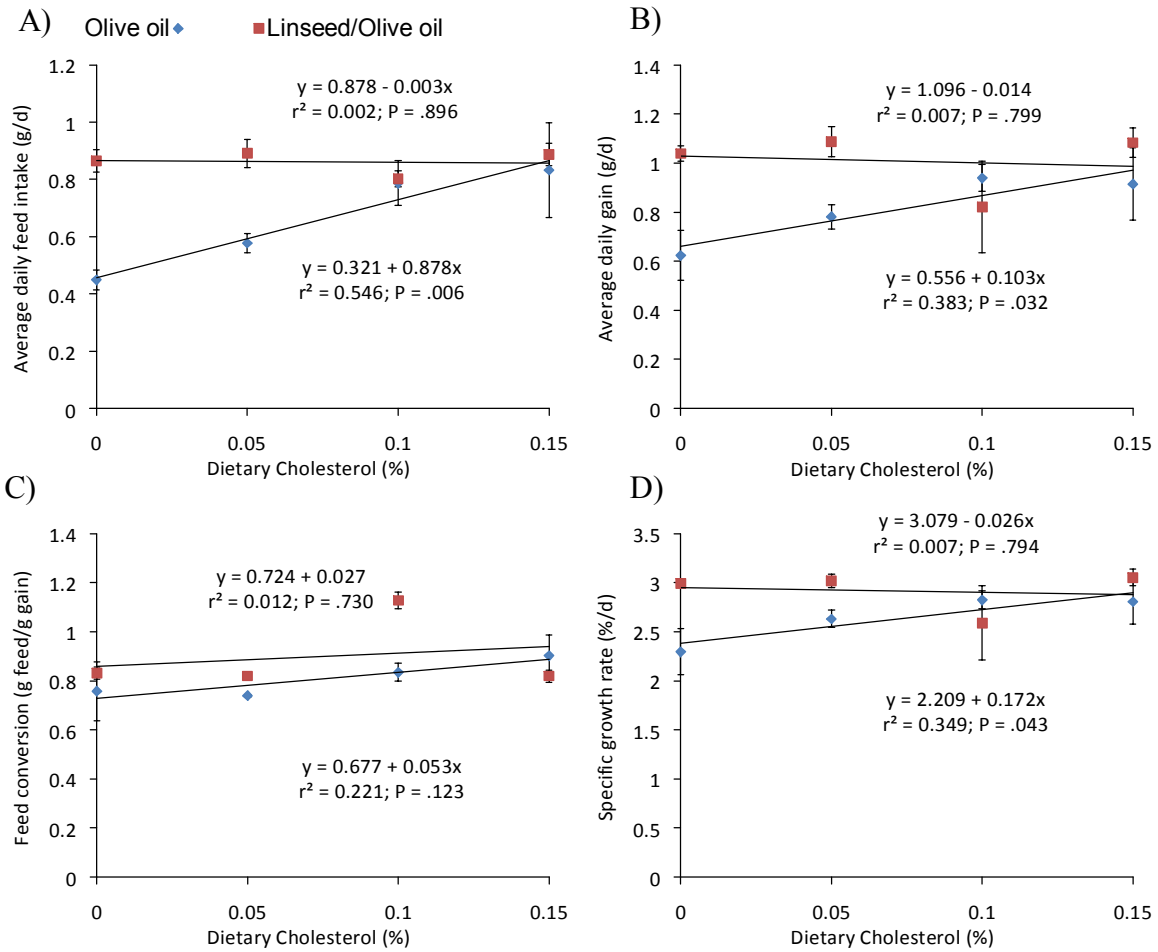

Figure 3.1 The effect of the interaction between dietary cholesterol level and oil source (olive oil or a mixture of olive oil and linseed oil) on the growth performance of rainbow trout in Exp. 1. A) Average daily feed intake (g/d), B) Average daily gain (g/d), C) Feed conversion (g feed/g gain), D) Specific growth rate (%/d)

3.3.2 Experiment 2

During Experiment 2, there were no clinical signs of disease and both main effects and interactions were not significant for mortality (Table 3.4). The main effect of PUFA was not significant for any of the growth parameters (Table 3.4). There was a trend towards a significant effect of cholesterol on ADG ($P = 0.089$) and SGR ($P = 0.082$). However, there was no significant effect of cholesterol concentration on ADFI or feed conversion. Finally, there was no significant effect of cholesterol, PUFA level or the interaction between cholesterol and PUFA level on serum or hepatic cholesterol levels in the fish (Table 3.5).

Table 3.4 Performance of rainbow trout fed cholesterol supplemented diets with varying levels of PUFA for days 0 – 84 of Experiment 2

Main Effects	Average Daily Feed Intake (g/d)	Average Daily Gain (g/d)	Feed Conversion (g feed/g gain)	Specific Growth Rate (%/d)	Mortality (%)
PUFA					
Olive oil	0.83	1.09	0.91	1.65	11.7
Lin/olive oil	0.85	1.05	0.84	1.69	19.5
SEM	0.020	0.115	0.043	0.067	5.36
Cholesterol (%)					
0	0.82	0.89	0.93	1.56	11.4
0.075	0.86	0.95	0.91	1.62	11.2
0.15	0.82	1.37	0.77	1.84	23.1
SEM	0.038	0.059	0.034	0.031	6.57
***P*-values**					
PUFA	0.618	0.210	0.147	0.112	0.376
Cholesterol	0.297	0.089	0.147	0.088	0.378
Interaction	0.353	0.968	0.722	0.855	0.633

SEM = Standard error of the mean

Table 3.5 Hepatic and serum cholesterol levels in rainbow trout fed cholesterol supplemented diets with varying levels of PUFA in Experiment 2

	Cholesterol content	
Main effects	Serum (mg/ml)	Liver (mg/g)
PUFA		
Olive oil	2.66	2.00
Linseed/olive oil	2.39	1.80
SEM	0.10	0.15
Cholesterol %		
0.00	2.38	2.00
0.075	2.52	1.80
0.15	2.69	2.10
SEM	0.08	0.19
***P*-Values**		
PUFA	0.19	0.124
Cholesterol	0.45	0.388
Interaction	0.32	0.628

SEM = Standard error of the mean

3.4 Discussion

The static supply of fish meal and oil, combined with increasing demands on this resource due to the explosive growth of aquaculture, has resulted in rising levels of plant proteins and oils in aquafeeds. However, the performance of salmonids is greatly reduced when plant ingredients comprise a significant portion of their diet (Thiessen et al., 2003, 2004; Mundheim, Aksnes, and Hope 2004; Sener and Yildiz, 2003). Many studies have shown that much of this growth impairment is due to antinutritional factors in plants (Dogan and Bircan, 2009; Iwashita et al., 2008; Hart, 2006; Wang, Bao and Li, 2004; Francis, Makkar and Becker, 2001). However, there is also evidence that fish meal contains pronutritional factors (PNF), which are lacking in plant ingredients (Aksnes et al., 2006a;b). Obvious candidates for these PNF are compounds present in fish meal and oil, but not present in plant ingredients, such as cholesterol. High amounts of polyunsaturated fatty acids, which can be found in many plant-based aquafeeds, have also been shown to suppress endogenous synthesis of cholesterol. This combination of lack of dietary cholesterol and decreased biosynthesis could contribute to the reduced performance of the fish. In the present study, completely plant-based diets, containing either high or low amounts of n-3 PUFA, were supplemented with varying levels of synthetic cholesterol.

Fish of the first experiment were fed diets formulated with S-type Gold Fat, a commercial source of marine algae (*Schizochytrium sp.*), in order to meet DHA requirements of the fish. After feed analysis was conducted, the product was found to contain 3.9 mg/g of cholesterol. Therefore, the calculated cholesterol level in the 0% cholesterol diets was actually 0.0195%. Given the low growth rate of the 0% cholesterol/olive oil-fed fish, this suggests that this level is well below the cholesterol requirement level for rainbow trout. To eliminate this problem, S-type

Gold Fat was not included in the diets in Experiment 2 and thus, the 0% cholesterol diets were devoid of cholesterol.

In the both the first and second experiments, supplementation of cholesterol in plant-based diets for rainbow trout resulted in increased growth performance. The effects of cholesterol in these trials are similar to those observed by Twibell and Wilson (2004), in which channel catfish increased their feed intake and growth in response to supplementation with cholesterol. This suggests that cholesterol is one of the putative growth factors present in marine ingredients (Aksnes et al., 2006a;b). However, the growth performance of fish fed cholesterol-free diets was significantly greater than 0, suggesting that cholesterol is conditionally limiting, and not essential in rainbow trout.

The expression of HMGR, the rate-limiting enzyme in cholesterol synthesis, is suppressed by high levels of n-3 PUFA (Horton et al., 2003; Le Jossic-Corcos et al., 2005). This suggests that supplementation of dietary cholesterol in high n-3 PUFA-diets should show a greater growth response than in low n-3 PUFA diets. However, the results of the first trial had the opposite effect, with fish fed the low n-3 PUFA diets showing a greater response to growth, when supplemented with dietary cholesterol. This suggests that the suppressive effect of dietary n-3 PUFA on cholesterol synthesis is negligible, compared to the overall synthesis rate. Alternatively, greater suppression of de novo cholesterol synthesis in the high n-3 PUFA diets would mean less fatty acids and ATP are being put towards cholesterol synthesis, instead of growth. Another finding from the second trial, which supports this theory, is the hepatic and serum cholesterol values. Although not significant, values for cholesterol content in both serum and hepatic tissue samples were found to be lower in the fish fed the high n-3 PUFA diets, than those fed the low n-3 PUFA diets (1.80 mg/g vs. 2.00 mg/g in hepatic tissue and 2.39 mg/ml vs.

2.66 mg/ml in serum). This would also suggest that there is some suppression of cholesterol biosynthesis taking place in fish fed high PUFA diets.

In Experiment 2, the main effects and the interaction between PUFA and cholesterol level were not significant for any growth parameter. These conflicting results between the first and second experiment might be due to the disease outbreak that occurred during Experiment 1. Higher mortality was observed in fish fed the low PUFA and low cholesterol diets in Experiment 1. Previous studies have reported that higher levels of plasma cholesterol correlate with the ability of fish to resist bacterial infection and are consistent with the present findings. In a study conducted by Maita et al. (1998), rainbow trout (average body weight of 295g) were artificially infected with *Vibrio anguillarum.* Fish with cholesterol levels lower than 520mg/100ml had significantly higher mortality than fish with levels higher than 560mg/100ml ($P < 0.005$). Maita et al. (2006) also found that yellowtail fish had increased susceptibility to infectious disease when fed diets in which fish meal was replaced with alternative protein sources. Those fed the non-fish meal diet also had significantly lower levels of plasma total cholesterol, free cholesterol and phospholipids. Although useful indicators of fish health, it is unknown why fish with low plasma cholesterol are more susceptible to infection and mortality.

The level of PUFA also affected mortality in Experiment 1. Eicosanoids formed from n-3 fatty acids are less active biologically than those formed from n-6 fatty acids, and competitively inhibit the actions of n-6-derived eicosanoids (Tocher, 2003). When supplemented in the diet, n-3 PUFA compete with n-6 PUFA as a precursor for eicosanoid production, and result in a decrease in the production of proinflammatory eicosanoids and cytokines (Hwang, 1989; Stulnig, 2003). Therefore, the ratio of the n-6 and n-3 fatty acids can have profound effects on the relative activities of eicosanoids and concomitant alterations in inflammatory responses. Salmonids have significantly lower n-6:n-3 ratios than mammals (Schmitz and Ecker, 2008) and hence the high n-6:n-3 ratios in

most vegetable oils can negatively alter the activity of eicosanoids in the fish. Oxley et al. (2010) reported that feeding diets high in vegetable oils significantly altered the ratio of n-6:n-3 eicosanoids in Atlantic salmon, compared with fish fed fish oil. They suggested that this could increase inflammation in the gut with concomitant effects on nutrient absorption and osmoregulation. Some of the molecular mechanisms through which PUFA alter immune function include gene expression, cellular signaling, eicosanoid metabolism, and membrane organization (Shaikh and Edidin, 2006; Stulnig, 2003; Nakamura et al., 2004; Benatti et al., 2004; Yaqoob, 2003). Although most research has focused on human beings, a study by Thompson et al. (1996) reported that non-vaccinated fish fed a diet with high n-6 levels experienced faster mortality when challenged with the bacterium *Aeromonas salmonicida* and *Vibrio anguillarum,* than the fish fed high n-3 PUFA. In the present studies, the n-6:n-3 ratio was 12.9 for the olive oil diets and 5.5 for the linseed/olive oil diets. Thus, the eicosanoid activity may have been significantly altered by the two different lipid contents.

Manera and Britti (2006) published an assessment of the normal ranges in blood chemistry in rainbow trout. The rainbow trout used in their experiment had an average body mass of 240.10 g $\pm$ 19.40. Converting the units used by Manera and Britti (2006) into the units used for the present study, they reported an average blood cholesterol mean of 247.38 mg dl^{-1} $\pm$ 10.32 mg dl^{-1} (2.47 mg/ml) and a normal range estimate of 111.73 – 383.02 mg dl^{-1} (1.18 – 3.83 mg/ml). The serum cholesterol levels found in the present study fall within this range. Although not significant, values for cholesterol content in both blood serum and hepatic tissue samples from Experiment 2 were found to be lower in the high PUFA diets, than those in the low PUFA diets (1.80 mg/g vs. 2.00 mg/g in hepatic tissue and 2.39 mg/ml vs. 2.66 mg/ml in serum). This would also support the notion that there is some suppression of cholesterol biosynthesis taking place in high PUFA diets. In contrast to these results, previous research by Bjerkeng et al. (1999)

examined the effect of cholesterol and short-chain fatty acids (SCFA) on growth and organ indices in Atlantic salmon. They found that salmon supplemented with cholesterol had accumulation of cholesterol in the liver and concentrations of hepatic cholesterol were significantly different among treatments ($P < 0.001$). This was entirely related to cholesterol supplementation, with no effect credited to dietary SCFA. However, there was no variation in PUFA level for their study, which means fish were also not experiencing a suppression of de novo cholesterol synthesis or an alteration of their n-6:n-3 PUFA ratio. The results of a study conducted by Zelenka et al. (2003) would agree with those of the present study. Zelenka et al. (2003) fed fish high (linseed oil) and low (sunflower oil) PUFA diets, along with a control, and also found no significant differences in level of cholesterol in fish fillets. Using one year old rainbow trout (average body weight of 257 g), the average cholesterol content was found to be 0.562 – 0.594 g/kg, which is comparable to the cholesterol content of white poultry meat (0.582 g/kg).

These studies support the notion that cholesterol is a conditionally limiting nutrient in growing rainbow trout. The interaction between dietary cholesterol and n-6 and n-3 PUFA levels, and their role in immune function, appears to be an important factor that should be taken into consideration in future studies.

4. CONCLUSIONS

In an industry where fish meal and oil has become scarce and expensive, the use of plant protein and oil sources in aquaculture diets is an obvious solution. However, it is crucial that producers are able to maintain optimal fish growth and feed efficiency when incorporating plant sources into aquafeed. Research has shown that complete removal of marine products from the diet and the sole use of plant protein sources results in a significant reduction in growth. The exact mechanisms that cause this remain a mystery and there are likely more than one, however the presence/absence of cholesterol is one factor that may help to explain such drastic differences in growth between animal and plant-based diets for salmonids.

The data collected from this present study would indicate that cholesterol is indeed one constituent of marine and animal products that may be the missing link between animal and plant feed sources. In both trials, supplementation of cholesterol had positive effects on growth. Information on the effect of cholesterol supplementation in salmonid diets is scarce, and thus information collected from this study contributes to growing knowledge concerning these effects. It should serve as a good starting point to develop future studies in this area, especially when the diets are largely or solely plant based. The results of the present study indicate that the minimal level of synthetic cholesterol inclusion should be 0.15%, as the most pronounced effects on growth were observed at this level. Further research should concentrate on determining an optimum level of cholesterol needed in salmonid diets, taking into account the rate of inclusion of plant-based products. It would also be wise to consider alternative animal and marine products that would be more economically feasible for producers to use, as synthetic cholesterol is quite costly. Research in this area should direct its focus towards innovative and cheaper alternatives, such as rendered land animal ingredients or by-products of the seafood industry.

As the aquafeed industry continues to increase its reliance on alternative protein sources, greater focus needs to be directed towards examining the beneficial properties of marine products. While the present study suggests that cholesterol is one of the putative pro-nutritional factors present in fish meal and fish oil, other unidentified factors still remain. Some nutrients of interest that have shown effects on the growth performance of fish include taurine, anserine, nucleotides, and free amino acids (Aksnes, 2006). Future efforts also need to determine to what extent these properties should be used as replacements for fish meal and oil in the diet.

In future studies which examine cholesterol in aquafeeds, it would be beneficial to measure levels of HMGR, the rate-limiting enzyme in cholesterol biosynthesis, to develop a better understanding of what is occurring at a cellular level during growth. It is well known that the level of PUFA in the diet interacts with cholesterol at the cellular level to exhibit various effects on cellular membrane structure, function and activity. Nevertheless, better knowledge is required of how these effects transform into functional consequences at the cellular level, specifically within fish.

The present studies support the hypothesis that there are not only antinutritional factors present in plant ingredients that depress the growth performance of salmonids, but that there are pronutritional factors present in marine products that improve growth, performance and health as well. The characterization of pronutritional factors, their role in fish nutrition and physiology and required dietary levels, is essential to our ability to formulate plant-based diets for salmonids and other aquaculture species.

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