EFFECT OF WATER TEMPERATURE AND DIET ON CELL MEMBRANE FLUIDITY AND FATTY ACID COMPOSITION OF MUSCLE, LIVER, GILL AND INTESTINE MUCOSA OF ADULT AND JUVENILE STEELHEAD TROUT, *Oncorhynchus mykiss*

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Effect of water temperature and diet on cell membrane fluidity and fatty acid composition of muscle, liver, gill and intestine mucosa of adult and juvenile steelhead trout, *Oncorhynchus mykiss*

by

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Abstract

Chapter 1 highlights the significant role of lipids in fish as a source of metabolic energy for growth, reproduction, movement and health. Dietary lipids also influence the fatty acid composition in various tissues including cellular membranes altering composition and function. Fish oil (FO) derived from capture fisheries is the main source of lipid in aquaculture feed to provide the essential fatty acids for maricultured fish. However, limitations in FO availability have prompted the investigation of the use of vegetable oil and animal fat as substitutes for fish oil and are investigated in this study.

Chapter 2 reports the results of investigation of the effect of FO substituted commercial and experimental diets on muscle lipid class and fatty acid composition in adult and juvenile steelhead trout (*Oncorhynchus mykiss)*. Study (i) compared herring oil (HE) diets with those containing medium levels of omega 3 fatty acids (M-ω3) and lower ω3 levels (L-ω3), and study (ii) compared HE with sunflower oil (SF) and flax seed oil (FLX). The results of both studies indicate the possibility of substitution of FO without compromising growth, feed efficiency or health once the minimal essential fatty acid requirements are met in the diet. HE diets with higher ω3 levels (H-ω3) in both studies significantly increased the ω3 highly unsaturated fatty acid composition in muscle tissue with a corresponding decrease in plant terrestrial fatty acid composition ($p \leq 0.045$). However, fish fed M-ω3, L-ω3, SF and FLX diets selectively accumulated DHA (docosahexaenoic acid) in muscle tissue minimizing the dietary differences in both adult and juvenile fish. All M-ω3, L-ω3 and SF diets were rich in linoleic acid and increased the ω6 composition in muscle tissue altering the ω6:ω3 ratio. In contrast, both HE and FLX diets had similar Σω3 and ω6:ω3 ratios due to the high availability of linolenic acid

in FLX feed. Triacylglycerols were the main neutral lipid stored in muscle tissue (above 80 - 90% total lipid). Changes in water temperature had minimal effects on muscle lipid composition in both adult and juvenile fish.

Chapter 3 investigated the effect of H-ω3, M-ω3 and L-ω3 diets and changes in water temperature on liver membrane fluidity, lipid class and fatty acid composition in adult fish. Fish were sampled from the initial (13.5°C), middle (18.0°C) and final (13.5°C) experimental temperatures used in study (i). The membrane fluidity was determined by measuring the CH_2 symmetric stretching bands in Raman spectroscopy at 2850 cm^{-1}. The stretching frequencies were obtained by subjecting the liver lipid to a temperature range of 2 to 30°C in increments of 4°C. The physical properties of the liver membrane lipid in fish fed H-ω3 diets was the least influenced by quickly adapting to either an increase or decrease in water temperature. The membrane fluidity of fish fed M-ω3 diets at 18.0°C, was similar to fish fed H-ω3. The liver membranes of L-ω3 diet fed fish sampled at both 18.0 and 13.5°C became increasingly more fluid than both H-ω3 and M-ω3 fed fish during the Raman trial. The fatty acid proportion of 20:3ω6 and 20:4ω6 at 18.0°C and 20:3ω6 at 13.5°C (final) was significantly higher in fish fed L-ω3 diets and may have influenced the increased fluidity in liver cell membranes. Both M-ω3 and H-ω3 fed fish have the capability to withstand a broader temperature range without undergoing complete phase separation of membrane lipids.

Chapter 4 evaluated the lipid composition of gills, intestine mucosa and liver tissue of juvenile steelhead trout using HE, SF and FLX diets, during a water temperature increase from 8 to 18.0°C. The lipid class composition of gill tissue was not affected by

diet or temperature. Both SF and FLX diets significantly increased the terrestrial fatty acid composition and ω6:ω3 ratio in gills of juvenile steelhead trout. The variation in diets also influenced the storage and selection of fatty acid substrates for β-oxidation in juvenile steelhead trout. Fish fed HE diets deposited a higher amount of saturated fatty acids in the liver while monounsaturated fatty acids were significantly higher in both SF and FLX diet fed fish. Both SF and FLX diets stimulated chain elongation and desaturation in steelhead juveniles. The SF diet stimulated the production of arachidonic acid while eicosapentaenoic acid production was promoted by the FLX diet.

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List of Abbreviations

ω: omega

AF: animal fat

ALA: α-linolenic acid

ATR-FT-IR: attenuated total reflection-Fourier transform infrared spectroscopy

AA: arachidonic acid

AMPL: acetone mobile polar lipids

ANOVA: analysis of variance

BF_3: boron trifluoride

$CHCl_3$: chloroform

DHA: docosahexaenoic acid

DPA: docosapentaenoic acid

DPPC: dipalmitoylphosphatidylcholine

EFA: essential fatty acids

EPA: eicosapentaenoic acid

ESR: electronic spin resonance

FA: Fatty acids

FAME: fatty acid methyl esters

FAO: Food and Agriculture organisation

FFA: free fatty acids

FLX: flax seed oil

FO: fish oil

FCE: food conversion efficiency

GC: gas chromatograph

GLM: General Linear Model

H_{II} : hexagonal

H-ω3: higher level of ω3 fatty acid

HE: herring oil

HPV: homeophasic adaptation

HUFA: highly polyunsaturated fatty acid

HVA: homeoviscous adaptation

L_{α}: liquid-crystalline

L_{β}: gel state

L-ω3: lower level of ω3 fatty acid

LA: linoleic acid

M-ω3: medium level of ω3 fatty acid

MeOH: methanol

MUFA: monounsaturated fatty acid

MUN: Memorial University of Newfoundland

N_2: nitrogen

NL: neutral lipid

O_2: oxygen

OSC: Ocean Sciences Centre

PC; phosphatidylcholine

PE: phosphatidylethanolamine

PI: phosphatidylinositol

PL: phospholipid

P:S: polyunsaturated to saturate ratio

PS: phosphatidyleserine

PUFA: polyunsaturated fatty acid

SFA: saturated fatty acids,

SF: sunflower oil

SD: standard deviation

SGR: specific growth rate

ST: sterol

TAG: triacylglycerol

TL: total lipid

TLC/FID: thin layer chromatography with flame ionization detection

ν_s: symmetric stretching band

VO: vegetable oils

ww: wet weight

Chapter 1

1.1. Natural life history of steelhead trout (*Oncorhynchus mykiss*)

Steelhead trout belong to the family Salmonidae, which includes all salmon, trout, freshwater whitefish, graylings and char. It is native to the west coast of North America and the Pacific coast of Asia (Burgner et al., 1992). It is the anadromous form of rainbow trout, which is not native to eastern Canada but has been widely introduced throughout central Canada and to the east coast. Records indicate that steelhead trout was first introduced to Newfoundland and Labrador, the most eastern province in Canada, in the late 1800's (Scott and Crossman, 1974).

Steelhead and rainbow trout (*Oncorhynchus mykiss*; Walbaum, 1792) are essentially the same species with two distinctive life history patterns (Burgner et al., 1992). Rainbow trout has a resident life history with all life stages occurring in freshwater. In contrast steelhead trout has an anadromous life history pattern, where fish migrate to the ocean after a year or more (Quinn, 2005). Upon hatching the young spend their first couple of years typically in freshwater followed by oceanic migration where most of their growth occurs. Adult steelhead return to the natal freshwater streams to spawn, after spending one to four growing seasons in the ocean. Both anadromous and resident forms of rainbow trout may inhabit the same stream and show no morphological or genetic differentiation between them, indicating that they belong to a single evolutionary line with two distinctive forms of life history (Behnke, 1992; Quinn, 2005).

Ecological requirements and lifecycles of steelhead trout are similar to that of other Pacific salmon. However, unlike the semelparous Pacific salmon, steelhead trout juveniles typically stay longer in freshwater before migrating to the ocean. Both adults

and juveniles of steelhead trout spend variable times in fresh and sea water, and the time spent could vary depending on the stream in which they originate. Steelhead trout are able to spawn more than once and they do not necessarily die after spawning like the semelparous Pacific salmon. However, post spawning survival rate is very low (McEwan and Jackson, 1996; Fleming, 1998).

Habitat requirement for steelhead trout varies with life stage. Adults need access to streams that are free of barriers for spawning migration. The majority of spawning occurs in the upper tributaries of their natal streams (Leitritz and Lewis, 1980). The females select an area with cool clear water with good inter-gravel flow, free of heavy sedimentation. Well oxygenated water free of excessive silt is ideal for hatching steelhead trout eggs (Leitritz and Lewis, 1980). Temperature plays a vital role in determining the length of the incubation period and survival of steelhead trout in all stages of their life cycle. Incubation of steelhead trout eggs in hatcheries takes about 30 days at 10.5^{o}C (Leitritz and Lewis, 1980). Newly hatched fry emerge from gravel in about six weeks. However, this duration could vary due to factors such as redd depth (specific location selected by the female to lay eggs), gravel size, temperature, and siltation (Shapovalov and Taft, 1954). Elevated temperatures over 21^{o}C could greatly impair the growth of steelhead trout juveniles (Shapovalov and Taft, 1954).

Steelhead trout are carnivorous relying on a mixed diet of fish and invertebrates. However, the diet of steelhead trout varies with life history. During the early freshwater phase they feed primarily on aquatic insects followed by small fish once they grow. Compared to other salmon that spend more time in coastal waters, steelhead trout juveniles spend little time in coastal waters and migrate offshore to the ocean. In coastal

waters steelhead trout feed on small fish and crustaceans. During the maturing phase in the open ocean the primary target is larger nekton such as fish and squid; however, steelhead trout also feed on euphausiids, amphipods and pelagic polychaetes (Brodeur, 1990).

1.2. Key elements in fish nutrition

The specific nutritional requirement of fish varies with species and life stage. Physiologically demanding stages (such as reproduction) require specific nutrients and energy requirements. Environmental factors such as water temperature, turbidity, depth, flow rates, prey density and photoperiod also alter feeding and nutritional requirements.

Fish are efficient converters of energy and protein in their diets. Energy and protein conversion efficiency in salmonids ranges are 30-40% and 20-25%, respectively (Rerat and Kaushik, 1995). Therefore unlike homoeothermic land animals, the major portion of food eaten by fish is available for growth (Rerat and Kaushik, 1995). Their ectothermic nature helps them spend minimal energy on excretion of nitrogenous waste and they expend no energy on maintaining body temperature. However, they loose a portion of their metabolic energy to the environment as heat and through feces, urine, gill excretions and a small portion from their external body surface (De Silva and Anderson, 1995).

Lipids and their constituent fatty acids play a major role as a source of metabolic energy for fish. Lipid being a high energy nutrient, provides about twice the energy (38.5 kJ g^{-1}) compared to other major constituents in fish feed: protein (23.6 kJ g^{-1}) and carbohydrate (17.3 kJ g^{-1}) (Bureau et al., 2002). Lipids are used in aquaculture feed to

partially spare the most expensive ingredient, protein, while providing energy, essential fatty acids and a transporter for fat soluble vitamins. Although carbohydrates are the most economical source of energy of all ingredients used in fish feed formulation, they are quantitatively and qualitatively less important.

Knowledge on fish nutrition has advanced considerably with the advancements of fish culture. Research so far has led to development of species and life stage specific diets. However, larval production in hatcheries still depends on the supply of live prey. In spite of extensive research, development of larval feed has been challenging due to limited knowledge of different species' early nutrient requirements.

1.2.1. Proteins and carbohydrates in fish diets

Protein is one of the most important and expensive elements in fish diets. Dietary protein provides essential amino acids as well as nitrogen for the synthesis of dispensable amino acids. Both are vital for maintenance, reproduction and growth. There are ten essential amino acids required by fish that need to be provided via the feed. Among the 10 essential amino acids (methionine, arginine, threonine, tryptophan, histidine, isoleucine, lysine, leucine, valine and phenylalanine), methionine and lysine are the first limiting amino acids. Both the amino acid pattern of dietary proteins and the protein:energy ratio in diet influence the utilization of dietary protein by fish (Jobling, 1993). Reduced food intake and protein utilization could result from imbalances in the amino acid pattern in fish diets (Berge et al., 2002, Green and Hardy, 2002, Gómez-Requeni et al., 2003).

Protein requirements of fish are influenced by the rearing environment and also vary with species, growth stage and genetic composition. Herbivorous and omnivorous fish have a relatively lower requirement of fish meal supplied essential amino acids than carnivorous fish (Naylor et al., 2000). Smaller, fast growing fish have a higher protein requirement than their adult counterparts, and the protein requirement gradually decreases as the fish grow larger (Tucker, 1998). Protein is efficiently utilized for growth only if adequate amounts of lipids and carbohydrates are present in the diet. Increased proportion of dietary lipids and digestible carbohydrates in diet provide greater proportion of energy, leaving dietary proteins to be utilized for growth (Kaushik and Oliva Teles, 1985, Tibbetts et al., 2005). Research has shown that increasing dietary lipid levels could spare substantial portions of dietary protein while improving the feed efficiency (Bureau et al., 2008). Fish diets with excess amounts of protein or amino acids are invariably used to produce energy. Similarly diets lacking adequate amounts of lipids and carbohydrates could result in use of protein for energy and life support rather than growth. Therefore a balanced diet with an optimum amino acid pattern is vital to best utilize dietary proteins (Green and Hardy, 2002; Rollin et al., 2003). A diet with a best possible dietary amino acid pattern would contain both essential amino acids as well as the optimum dietary essential:non-essential amino acid ratio (Green and Hardy, 2002; Green et al., 2002). Several species including rainbow trout and Atlantic salmon (*Salmo salar*) have shown poor protein utilization due to unbalanced amino acid proportions in the diet (Berge et al., 2002; Green and Hardy, 2002, Gómez-Requeni et al., 2003; Peres and Oliva-Teles, 2006).

Formulated fish diets may be either complete or supplemental. A complete diet provides all necessary ingredients (proteins, fats, carbohydrates, vitamins and minerals)

for optimal growth and health of fish. The formulation varies based on species and life stage. Intensive aquaculture systems commonly use complete diets to maintain high productivity. In contrast supplemental diets are used for fish reared in ponds or outdoor raceways that have access to natural food such as insects, algae and small fish. Such diets are supplemented with extra protein, lipid and carbohydrate, without including the full compliment of vitamins and minerals.

Proteins in commercial feeds are mainly provided by fish meal. The limited supply of fish meal has resulted in use of alternative plant derived ingredients with a sub-optimal amino acid pattern which needs to be enhanced with deficient amino acids in the ingredients. Malt protein flour, canola, soybean meal and sunflower seed protein concentrates and soybean meal are some of the amino acid augmented diets that are being used by commercial feed manufacturers (Yamamoto et al., 1994; Stickney et al., 1996; Cheng et al., 2003).

Carbohydrates are the most abundant, economical and inexpensive source of energy available for feed formulation. Glucose the end product of carbohydrate digestion is of primary importance to the metabolism of all vertebrates. However, the role of carbohydrates in fish metabolism is secondary to the role of lipids and proteins (Hemre et al., 2002). Carbohydrates are used in fish feed as an energy source, to reduce feed manufacturing cost and for their binding properties (Krogdahl et al., 2005), and to reduce effluent nitrogen production by reduction in protein catabolism (Hillestad et al., 2001). Pellet binding properties of carbohydrates, especially starches, pectins and hemicelluloses is a useful asset for feed manufacturers. Also, carbohydrates are useful in extrusion

manufacture of floating feed and during the process of extrusion the dietary starch becomes more biologically available for the fish.

Use of dietary carbohydrate by fish varies considerably depending on species, stage of growth and availability of ingredients (Wilson, 1994). Both herbivorous and omnivorous fish have a greater capability of digesting carbohydrate containing feed than carnivorous species, with the enzymatic capacity to hydrolyse and absorb simple and even complex carbohydrates (Krogdahl et al., 2005). The structure and function of the digestive system and feeding habits also effect carbohydrate utilization in fish (Xiquin et al., 1984). Other factors that affect carbohydrate utilization are degree of gelatinization (Kumar et al., 2006), and its interactions with other nutrients (Yengkokpam et al., 2006; Misra, 2006). Rapid development of the aquaculture industry and the advancement in knowledge of feed technology and fish nutrition has led to greater use of less expensive, carbohydrate rich, ingredients to manufacture fish feed. However, far more research effort is needed to understand the mechanisms of the digestive processes in all cultured species in order to gain the maximum nutritive and economic benefits of carbohydrates in fish feed.

1.2.2. Lipid nutrition

Dietary lipids play a significant role in fish as a source of metabolic energy for growth, reproduction and movement. Fish have also received much attention in human nutrition as an excellent source of protein, selenium, iodine, vitamin E and as an important food source of omega-3 (ω3) highly polyunsaturated fatty acids (HUFA; C_{20} – C_{22} with 2 or more double bonds). The importance of mainly ω3 and ω6 (to some extent)

HUFA, specifically docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic (AA) acid in human nutrition is well understood (Simopoulos, 1991). Incorporation of dietary or supplemental ω3 HUFA in human nutrition is known to positively influence many physiological processes and disease conditions including cardiovascular diseases, metabolic disorders such as obesity, diabetes and neurological disorders (Steffens, 1997).

Lipids are hydrophobic and soluble in organic solvents. They can be broadly separated into polar and neutral lipids. Polar lipids are mainly composed of phospholipid (PL) while triacylglycerol (TAG) are the principal component of neutral lipids. Fatty acids (FA) are carboxylic acids with a long hydrocarbon chain. FA esterify to alcohol groups to form TAG which are commonly called fats. TAG represents a major class of neutral lipids in fish and is a primary deposit of energy. Wax esters are another molecular form of storing energy and are rich in marine zooplankton forming a key natural resource of food for marine fish (Tocher, 2003). They are fatty acid esters of long chain alcohols and constitute another class of neutral lipids.

FA esterification to phosphatidic acid forms PL. PLs are the major class of polar lipids in animals. Phosphatidic acid, which is glycerol-3-phosphate, esterifies with two other fatty acids to form a PL. The 3 carbon atoms in the glycerol backbones are termed *sn*-1, *sn*-2 (middle position) and *sn*-3, where a phosphate group is attached to *sn*-3 in phosphatidic acid. In PL, the *sn*-1 position is usually occupied by a saturated or monounsaturated fatty acid, whereas the *sn*-2 position is occupied by a PUFA in general. The phosphate group of PLs esterifies with a base forming the major classes of PLs in fish tissue: phosphatidylcholine (PC), phosphatidylethanolamine (PE),

phosphatidyleserine (PS) and phosphatidylinositol (PI). PL is a key component in cellular membranes and is important in maintaining cell membrane fluidity. It also acts as a precursor (together with TAG and FFA) for various biologically active mediators such as eicosanoids, diacylglycerols, platelet activating factors and inositol phosphate (Tocher, 2003).

Sphingolipids are another group of polar lipids. Ceramide is an important complex sphingolipid. It is formed by esterification of a fatty acid to the alcohol group of the amino alcohol sphingosine. Esterification of various subgroups to the alcohol group of sphingosine forms various biologically important compounds. For example, sphingomyelin, a principal component in myelin sheath of nerve cell axons, is formed when ceramide is esterified to phosphocholine. Similarly, cerebroside another sphingolipid is the principal component in brain tissue and myelin sheath, it is formed through esterification of ceramide to a sugar, glucose or galactose forming glucocerebrocide or galactocerebrocide respectively (Tocher 2003). A review by Hannon and Obeid (2008) detailed the importance of sphingolipid in cell signalling together with the other bioactive lipid compounds such as eicosanoids, diacylglycerol and inositol triphosphate. Sphingolipid derivatives are known to play an important role in regulating the actin cytoskeleton, the cell cycle, apoptosis and endocytosis (Hannon and Obeid, 2008).

Sterols, specifically cholesterol in all animals play an important structural role together with phospholipids. Sterols are grouped with TAG as neutral lipids based on their polarity. Cholesterol, a tetracyclic alcohol compound, is a principal component of cell membranes that helps to maintain membrane order. The steroid nucleus of cholesterol

lays parallel and buried in between acyl chains of the membrane and helps to maintain the order between gel phase, with tightly packed acyl chains in PLs, and a highly disordered liquid crystalline phase (Hazel and Williams, 1990). Cholesterol together with sphingolipids form lipid rafts in membranes. Lipid rafts are ordered microdomains rich in cholesterol and sphingolipids that aid cellular processes such as signalling, influencing membrane fluidity and transport of material across membrane (Brown and London, 2000).

1.2.3. Dietary lipids

Lipid digestion, absorption and transport in fish are similar to what occurs in mammals, although species-specific differences exist due to structural differences of the digestive tracts (Sargent et al., 1989). Lypolysis is facilitated by bile salts due to its emulsifying capability and by lypolytic enzymes mainly in the proximal part of the digestive tract (stomach, pyloric caeca) and absorbed through pyloric caeca and mid intestine (Tocher 2003, Denstadli et al., 2004). Principal components in fish diet are TAG, PL, ST, FFA, waxes and other pigments. Marine fish have access to prey (e.g. zooplankton, capelin), which largely contain oil in the form of TAG (Tocher 2003). Various factors are known to affect the digestion of lipids. The capacity is mainly influenced by both inter and intra-species variation and by the water temperature (Morais et al., 2005; Bogevik et al., 2008). Digestion of lipid in fish is known to improve with both degree of unsaturation and by including C_8 to C_{10} fatty acids (Glencross et al., 2002); however, inclusion of such highly digestible fatty acids could reduce the digestibility of essential fatty acids such as DHA (Rosjo et al., 2000). Similarly, fish prefer hydrophilic

PL as the digestive substrate over hydrophobic TAG in situations where rapid assimilation of lipid is required (Turchini et al., 2009). Among larval fish in general, omnivorous and herbivorous fish have comparatively lower ability to digest or utilize high dietary lipid levels compared to carnivorous fish (Chou and Shiau, 1996, Sargent et al., 1997, Denstadli et al., 2004, Morais et al., 2005).

1.3. Lipogenesis in marine and freshwater fish

Fatty acids often have a long unbranched aliphatic hydrocarbon chain attached to the carboxyl group, which is either saturated or unsaturated. Fatty acids are identified based on the length of the hydrocarbon chain (the number of carbon atoms (C): up to C_6 – short chain; C_6 to C_{12} – medium chain; above C_{12} as long chain) and degree of unsaturation. Saturated fatty acids have no ethylenic or double bonds along the aliphatic chain with all carbon atoms saturated with hydrogen atoms. In contrast, unsaturated fatty acids contain one (monounsaturated fatty acid, MUFA) or more (polyunsaturated) double bonds in the hydrocarbon chain. For example, palmitic (16 carbon) and stearic (18 carbon) are commonly found saturated fatty acids in fish, and are designated 16:0 and 18:0, respectively, indicating the number of carbon atoms and zero double bonds. In naming unsaturated fatty acids, the double bonds present in the hydrocarbon chain are indicated by the number of double bonds present from the last carbon of the chain (the omega (ω) end) which contains a methyl group ($-CH_3$), and by the position. Addition of one double bond to 18:0 would make it 18:1, and depending on its position, it can be written as 18:1ω9 or 18:1ω7, where ω9 and ω7 indicate the single double bond of the respective hydrocarbon chains are attached to the 9^{th} or 7^{th} carbon from the methyl or

omega end. Fatty acids containing 2 or more double bonds are considered PUFA. In designating PUFA, the nomenclature contains the total number of carbon atoms followed by the number of double bonds and the position of the 1st double bond from the methyl end of the hydrocarbon chain. For example linolenic acid (18:3ω3) has 3 double bonds with the first situated at the 3rd carbon from the methyl end, hence it is termed as ω3 fatty acid.

Liver is the primary site of lipogenesis followed by adipose tissues in trout (Henderson and Sargent, 1985). The two main fatty acids biosynthesized *de novo* by fish are palmitic (16:0) and stearic (18:0) acid (Sargent et al., 1989). Amino acids from protein and carbohydrates provide the carbon source for the endogenous synthesis of lipids. Dietary amino acids are preferred over carbohydrates since natural prey of both marine and freshwater fish are richer in protein than carbohydrates. On the other hand, Brauge et al. (1995) found an increase in lipogenesis in trout with a high carbohydrate to lipid ratio in the diet. Similarly, high lipid diets as well as high lipid to protein ratios are known to decrease the lipogenic capacity of fish (Shimeno et al., 1995). However, both environments as well as different nutritional and dietary factors influence the lipogenesis of fish.

Sargent et al. (1989) demonstrated the cytosolic enzyme system "fatty acid synthetase multienzyme complex" in fish. Fish, like other organisms, have the capability (*de novo*) of desaturating 16:0 and 18:0 to 16:1ω7 (palmitoleic) and 18:1ω9 (oleic), respectively, by removal of 2 carbon acetyl units (Sargent et al., 1976). Although synthesis of fatty acids up to 16 carbons long occurs in cytosol with the cytosolic enzyme system, synthesis of fatty acids longer than 16:0 and desaturation to their respective

monenes occurs utilizing both the microsomal enzyme system and mitochondria (Rawn 1989). The microsomal system is also involved in further chain elongation of C_{18} to C_{24}, making it physiologically more significant (Rawn 1989, Smith et al., 1983).

The capacity and requirement for chain elongation may vary between freshwater and marine fish with the availability of PUFA in their diets (Tocher, 2003). Together with 16:1ω7 and 18:1ω9, marine fish TAG is also rich in 20:1ω9 and 22:1ω11. The latter 2 fatty acids are derived from zooplankton wax esters, limiting the need for chain elongation (Tocher, 2003). Marine food webs are rich in ω3 and ω6 series of PUFAs (e.g. from algae) (Sargent et al., 1995), which further reduces the need for chain elongation and desaturation in marine fish. Lipogenic capacity of marine predatory fish is even further reduced due to accumulation of long chain PUFA from natural diets (Regost et al., 2001).

Fish, as in all other vertebrate species, have an absolute requirement for ω3 and ω6 polyunsaturated fatty acids in their diet such as linoleic (LA) (18:2ω6) and linolenic (ALA) (18:3ω3) acids (Henderson and Tocher, 1987). All biologically active essential fatty acids of C_{20} and C_{22} metabolites are derived from dietary LA and ALA acid. All vertebrates lack the important desaturase enzymes (Δ12 and Δ15) that are required to convert 18:1ω9 to LA and ALA acid (Tocher et al., 2006), which are the precursors for the physiologically essential C_{20} and C_{22} PUFA. Fish have the capability of desaturation and chain elongation, but are incapable of *de novo* synthesis of C_{20} and C_{22} PUFAs. The ability to desaturate and elongate dietary LA and ALA to essential C_{20} and C_{22} metabolites depends on species and availability of preformed long chain PUFA in the diet (Tocher, 2003). Since body fatty acid composition generally mimics the composition in

the diet (Ruyter et al., 2000), EFA deficiency in the diets leads to various pathological conditions such as poor growth rate, increased mortality (Sargent et al, 1995), fin erosion, myocarditis and shock (Watanabe 1982).

The mechanisms of formation of PUFA have been studied in detail in mammalian systems (Garg et al., 1998). Buzzi *et al.* (1996) first demonstrated the complete pathway of chain elongation from 18:3ω3 to 22:6ω3 in fish using rainbow trout hepatocytes. Desaturation and elongation of C_{20} and C_{22} metabolites to their end products, 20:4ω6, 20:5ω3 and 22:6ω3 depends on two crucial desaturation enzymes (Δ5 and Δ6). Expression of these enzymes in tissues depends on the amount of highly unsaturated PUFA in the diet. Sargent et al. (1993) suggested that the regulation of the Δ5 and Δ6 enzyme system occurs through competitive substrate inhibition, with greater affinity for higher chain length and degree of unsaturation. The influence of dietary availability of HUFA on marine and freshwater fish was shown by Sargent et al. (1995), where conversion of 18:3ω3 to 20:5ω3 and 22:6ω3 occurs poorly in marine fish compared to freshwater fish, highlighting the availability of long chain PUFA in the marine environment. Since the discovery by Buzzi et al. (1996), several scientists have shown evidence of the same pathway occurring in other species: Atlantic salmon (Tocher et al., 1997), brown trout and Arctic char (Tocher et al., 2001).

1.4. Alternative lipids in fish nutrition

Expansion of the aquaculture industry has challenged the role of lipids in fish nutrition. Increasing consumer demand has raised production targets that require energy rich diets with higher lipid content to improve growth, feed conversion efficiency and

decrease grow out periods for cultured fish (Sargent, 2002). Fish oil of marine origin, mainly from capture fisheries has been the solitary supplier of lipids for commercial aquaculture feed for quite some time. Fish oil is the best source of lipid for commercial diets, as it contains plenty of essential fatty acids. According to Tacon et al. (2006), 87% of the fish oil produced from capture fisheries is used in aquaculture feed. At present, fish farming provides approximately 50% of the global consumer demand of sea food, once dominated by capture fisheries. However, according to FAO (2007) if the current trend in global seafood demand persists, the capture fishery alone will not be able to meet the fish oil requirement. Mittaine (2005) predicted a decline in fish oil production, resulting from the drop in marine pelagic fish stocks, the main source of fish oil. Therefore it is important for the aquaculture industry to seek alternative lipid sources for aquaculture feed that will provide the same benefits as fish oil for the consumer and the fish. A recent article by Turchini et al. (2009) reviews several alternative lipids from both plant and animal origin currently being investigated by researchers.

Vegetable oil and terrestrial animal fats (lard, tallow and chicken fat) are the two most popular contenders to replace or reduce the burden on fish oil in aquaculture feed. Among the two, animal fats are the cheaper alternative. However, vegetable oils are more readily available than animal fats and they are more affordable than fish oil for the feed manufacturer. Research to date has shown that vegetable oil performs equally well as a source of energy for fish growth (Bell et al., 2001; Ng et al., 2007; Stubhaug et al., 2007). However, the level of vegetable oil incorporation in feed has to be elucidated as its fatty acid composition is quite different to fish oil. Unlike fish oil, vegetable oil lacks ω3 HUFA, which are essential for the health and well-being of fish. Vegetable oils are rich in

ω6 and ω9 fatty acids and they are a good source of short chain ω3 fatty acids as well. The right combination of vegetable oil to be used as a substitute for fish oil and the effects due to lack of HUFA on optimal health and growth of fish are yet to be fully determined. As discussed earlier, fish have varying capabilities of converting short chain ω3 fatty acids to ω3 HUFAs. However, studies regarding marine carnivorous species have not shown enough evidence to support the argument above (Mourente et al., 2005). Research has shown the possibility of partial substitution of fish oil with vegetable oil without affecting growth of fish (Torstensen et al., 2005, Francis et al., 2007). Further investigation is necessary to understand the effects of such substitution on feed conversion and physiology of fish.

Terrestrial animal fats are a good source of energy for aquaculture feeds as they contain high levels of saturated fatty acids (SFA), as well as MUFAs. Animal fats could contain some PUFA (mainly LA) as well as trace levels of ω3 HUFAs. Whereas vegetable oil completely lacks the latter (Morreti and Corino, 2008). The level of HUFA in animal fats depends mainly on dietary history, species and age of the animals used for rendering.

Fish oil and fish meal produced from by-products of seafood processing plants have shown great potential for use in aquaculture feeds (Mondal et al., 2006; Ng et al., 2007). Oil extracted of marine by-products is an excellent source of HUFA and is high in nutritional value for aquaculture feed (Tacon et al., 2006). Other alternatives, such as unicellular algae and benthic invertebrates, containing high amounts of ω3 HUFA also have been tested in aquaculture feeds (Carter et al., 2003; Olsen et al., 2004; Atalah et al.,

2007; Ganuza et al., 2008). However, sustainability of such products at a global scale is questionable due to a high production cost and an unpredictable supply.

1.5. Effect of temperature and constituent lipids on cell membrane structure and function

Lipids, mainly PLs, are the key structural elements of cellular membranes. Structurally diverse PLs are heterogeneous in nature. PL has a polar head group (PC, PS, PE or PI), which determines the particular PL class and fatty acyl chains, the hydrophobic domain, determine the molecular species. Primary constituents of biological membranes are PLs and proteins that interact with each other through weak electrostatic and hydrophobic interactions (Hazel, 1998). PLs in cellular membranes display complex phase behaviours and physical properties and are extremely sensitive to alterations in the external environment. Temperature is the most important environmental variable affecting the metabolic activities of ectothermic organisms. Fish usually respond to such changes through altering their physiological functions in an attempt to maintain their metabolic functions at a relatively constant rate. At the cellular level, biological membranes respond by restructuring their constituent PL molecules.

Sinenski (1974) first observed that viscosity of *Escherichia coli* membranes is preserved with temperature, and termed it homeoviscous adaptation (HVA). Interest in this subject has grown ever since, with the aim of understanding the effects of temperature on the dynamic state of the membrane (Hazel and Williams, 1990). As mentioned earlier, temperature has received the most attention due to its direct influence on the biomolecular assembly of membranes (Hazel and Williams, 1990). Lack of HVA

in certain fish tissues, such as erythrocyte membranes of Arctic charr (*Salvelinus alpinus*) (Lecklin and Nikinmaa, 1999), sperm plasma membranes in trout (Labbe et al., 1995), and rete mirabile (complex of arteries and veins lying close to each other to facilitate counter current exchange of gasses, ions or heat) of tuna (Fudge et al., 1998) has led to the idea of homeophasic adaptation (HPV: Crockett and Londraville, 2006). HPV refers to maintaining membranes in a relatively constant phase state (discussed below). The general understanding is that fish can counteract the effects of environmental temperature on membranes by maintaining a similar fluidity across a wide range of habitat temperatures. The fluidity of a membrane refers to the viscosity of the lipid bilayers or a measure of its resistance to flow. This may be achieved by membrane accumulation of PUFA or MUFA (Farkas et al., 2001), by lowering the phosphatidylcholine:phosphatydylethanolamine (PC:PE) ratio at lower temperatures (Hazel and Carpenter, 1985), and by altering the cholesterol:phospholipid ratio (Robertson and Hazel, 1995).

The predominantly phospholipid matrices of all bio-membranes are in a liquid-crystalline (L_α) lamellar state at physiological temperatures (Hazel and William, 1990). The L_α state is vital in maintaining the elasticity and the function of membranes and membrane associated proteins (Atilgan and Sun, 2007). Any further reduction in temperature results in restricted acyl chain motion, L_β (gel state). In contrast, an increase in temperature above the physiological limit results in a H_{II} (hexagonal) non-lamellar phase (Hazel and Williams, 1990). The diversity of the PL molecule, with different head groups and different molecular species at positions *sn*-1 and *sn*-2, appears to be the major contributor to its dynamic nature. Farkas et al. (2001) reported the accumulation of

specific MUFA (e.g. 18:1ω9), at the *sn*-1 and PUFA at *sn*-2 positions in cold adapted carp liver tissue.

The quality of lipids in marine aquaculture diets is crucial in many ways. Dietary restrictions on essential fatty acids in marine species can lead to growth and health retardation as well as limit supply of the most needed molecular species to maintain the dynamic nature of the membrane (Tocher, 2003). The ratio of PC:PE in the fish membrane is positively correlated with temperature, where cold adapted fish have a higher proportion of PE compared to PC (Hazel, 1995). Incorporation of a single double bond at the *sn*-1 position significantly increases membrane fluidity. Hager and Hazel (1985) found a strong correlation between Δ9 desaturase activity and monoene content in cold adapted (seen between 3-14 days) trout liver microsomal membranes. They suggested that the activity of phospholipases and acyltransferases may be responsible for the immediate changes. All fish have the ability to desaturate 16:0 and 18:0 to their respective monoenes (16:1ω7, 18:1ω9: Tocher, 2003). Such ability may prevent the immediate ordering effect of membranes exposed to cold temperature.

Cholesterol is one of the main components contributing to the dynamic nature of the membrane (Tocher, 2003). Lipid rafts are micro-domains enriched in cholesterol and sphingolipid that form a moving platform and selectively bind proteins (Simon and Ikonan, 1997). The levels of membrane cholesterol in ectothermic organisms generally increase with increasing temperature (Zehmer and Hazel, 2005) as well as contribute to preserving the function of membrane proteins (Fernandez-Ballester et al., 1994). At low temperatures, the rigid fused ring system of the cholesterol molecule interferes with the packing order of the acyl chains increasing the average motional order of the membrane

(Yeagle, 2005). However, with increasing temperature cholesterol increases the order of the acyl chain portion close to the PL head group preventing the formation of loosely arranged bilayers (Zehmer and Hazel, 2005).

1.6. Objectives

It is clear that dietary lipids play a crucial role in fish as an energy source, but they are also fundamental to the supply of adequate amounts of essential fatty acids in the diet for better health and nutrition as well as physiological functioning of cellular membranes. As discussed earlier, fish have a varying capacity to synthesise fatty acids that are lacking in the diet, but the ability to do so depends solely on feeding pattern, type of food available (herbivore, omnivore or carnivore) and ready access to supplies of ω3 HUFA as with fish in the ocean. It is also speculated that EFA requirements could vary by species, age and physiological state of the animal.

The main focus of this study was to investigate the effects of temperature and dietary lipids on lipid class and fatty acid composition and cell membrane fluidity of steelhead trout. There was also interest in understanding the dietary effects on both adult and juvenile steelhead, as well as the influence of fluctuating external environmental temperature on lipid class and fatty acid composition in physiologically important organs. Feeds used in this study include commercial diets that are significantly different in total ω3 PUFA levels, as well as formulated diets which replaced fish oil with either flax or sunflower seed oil. The specific questions that were sought to find answers to through this work include:

1. What is the effect of seasonal temperature changes on muscle lipid class and fatty acid composition in adult steelhead trout (*Oncorhynchus mykiss*) fed three commercial diets containing H- ω3 (high), M- ω3 (medium) and L- ω3 (low) long chain PUFA levels?

2. What is the effect of dietary replacement of fish oil, with sunflower or flaxseed oil on growth, muscle lipid class and fatty acid composition in juvenile steelhead trout (*Oncorhynchus mykiss*) reared under increasing temperature?
3. What are the responses of liver, gill and intestine tissue in juvenile steelhead trout (*Oncorhynchus mykiss*) to temperature and dietary long and short chain PUFA that originates from fish and vegetable oil?
4. What is the effect of diet and temperature on liver cell membrane fluidity, and lipid class and fatty acid composition in adult steelhead trout (*Oncorhynchus mykiss*)?

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Chapter 2

Effect of temperature on muscle lipid class and fatty acid composition in adult and juvenile steelhead trout (*Oncorhynchus mykiss*) fed commercial (Study i) and formulated diets (Study ii).

2.1 Introduction

Benefits of long chain omega-3 (ω3) polyunsaturated fatty acids (PUFA) (e.g. EPA - eicosapentaenoic acid and DHA – docosahexaenoic acid) for humans are well known (Simopoulos, 1991, Ruxton, 2004, FAO, 2008). Deficiency of such fatty acids can result in varied disease symptoms ranging from hypertension, cardiovascular (Lee, 1994), neurological (Conner et al., 1992), inflammatory and immune disorders (Simopoulos, 1991). Fish have always been the primary source of ω3 essential fatty acids (EFA) for the human consumer and species with high body fat content, such as salmon, trout, sardines (*Sardinella* species) and herring (*Clupea* species) have become an important source of such fatty acids (Tocher, 2003).

Dietary lipids contribute significantly to the health of fish. Arachidonic acid (AA) and EPA in particular are responsible for producing highly active eicosanoids, which play an important role in the inflammatory response and proper functioning of the renal and neural systems (Schmitz and Ecket, 2008). Further, lipids also influence fatty acid composition in various tissues, including cell membranes, altering composition and function (Hazel and Williams 1990), swimming performance and cardio-respiratory physiology, visual development and from the perspective of aquaculture, the flesh quality (Hazel and Williams, 1990). Inadequate EFAs in diets could result in growth retardation

and various pathological symptoms in fish, ranging from fin erosion to myocarditis to problems related to reproductive performance (Sargent et al., 2002; Glencross, 2009).

Fish and other vertebrates are unable to synthesize two important fatty acids, namely α-linolenic (ALA) and linoleic (LA) (18:3ω3, 18:2ω6), which need to be provided in the diet (Turchini et al., 2009). Although EFA requirements could vary among different fish species, a significant distinction is seen between marine and freshwater fish. Most freshwater fish studied so far have the ability to convert dietary ALA and LA to longer chain (C_{20} - C_{22}) highly unsaturated fatty acids (HUFA) of the same series. ALA will be transformed into EPA (20:5ω3), DHA (22:6ω3) as well as to 3 and 5 series eicosanoid derivatives, while the LA will be converted to AA (20:4ω6) and 2 and 4 series eicosanoids (Balfry and Higgs, 2001). Freshwater species studied so far (e.g. juveniles and adult stages prior to migration in diadromous fish) are satisfied with 1% of both ALA and LA as dry weight of the diet (Tocher, 2010). However, the EFA requirement could vary depending on the temperature, where salmonids require more ALA as opposed to LA when warm (Sargent et al., 1989).

In contrast, marine fish lack the chain elongation and desaturation ability in general (Teshima et al., 1992, Nakamura and Nara, 2004), and need supplementation of dietary HUFA such as AA, EPA and DHA (Bell et al., 2004). Sargent et al. (1999, 2002) suggested that marine carnivorous species have lost this advantage due to their adaptation to HUFA rich marine environments and consequently show a direct dietary requirement for HUFA. While anadromous fish feed on a range of marine plants and small fish that are rich in HUFA after migration, maricultured conspecifics need to be supplemented with ω3 and ω6 HUFA in their diet (Bell et al., 2004). The quantitative requirement of

EFA varies among marine fish and depends on dietary lipid levels and the DHA:EPA ratio (Ibeas et al., 1997). Studies have shown that a higher ratio of DHA:EPA is more favourable, indicating a higher EFA value for DHA (Ibeas et al., 1994). However, the extent of the loss in HUFA synthesizing ability of cultured diadromous fish such as salmonids with no access to HUFA rich marine food webs has not been fully elucidated.

Rapid expansion of the aquaculture industry has demanded feeds that improve fish growth, feed utilization and reduce grow out periods (Turchini et al., 2009). This has resulted in utilization of high energy, lipid rich diets, further increasing the importance of lipids in commercial aquaculture (Sargent et al., 2002). Furthermore, the industry has also been challenged to meet future global seafood demands by the increasing human population. So far it is responding positively by expanding at an unprecedented rate of 8.8% annually since 1950, compared to 3.0% annual increases in capture fishery landings (FAO, 2007, 2010). Currently, 46% of the ever increasing global total per capita fish consumption is provided by farmed fish, mainly through intensive culture systems (FAO, 2007, 2010). Such intensification has also increased the use of manufactured aquaculture feeds, which currently utilize 87% of global supply of fish oils (FO) (mainly from capture fisheries) as the key lipid source (Tacon et al., 2006, 2010). A significant portion (over 87%) of world fish oil production is used to manufacture feeds for marine carnivorous species, Atlantic salmon (*Salmo salar*, Linnaeus, 1758) and rainbow trout (*Oncorhynchus mykiss*, Walbaum, 1792) (Tacon et al., 2006, Olsen, 2011). If the current trend persists, the capture fishery will not be able to cope with the FO demand of the aquaculture industry (Pike and Barlow, 2003). Prospects of reviving the capture fishery have become slim with the current exploitation trends and a collapse of all wild seafood species by the

year 2050 has even been projected (Worm et al., 2006). Therefore, it is vital to utilize alternative lipid sources in aquaculture feeds for the sustainability of the aquaculture industry.

A number of studies have investigated the limitations and cost benefits of replacing FO with alternative lipid sources on muscle fatty acid composition. Alternative oil sources that have been tested in aquaculture feeds are vegetable oils (VO) (Bell et al., 2001; Carbellero et al., 2002; Francis et al., 2006; Ng et al., 2007; Stubhaug et al., 2007), rendered animal fat (AF) from beef, pork and poultry industries (Watanabe 2002; Turchini et al., 2003; Bureau and Gibson, 2004), aquatic by-products (Mondal et al., 2006, Ng et al., 2007), unicellular algae and certain pelagic or benthic invertebrates (Olsen et al., 2004, Gaunza et al., 2008). Research of this nature has focused primarily on growth and feed utilization of the fish, with less attention to environmental stresses and its effect on fatty acid composition of the final product. However, further investigation of this is critical to developing a long term sustainable strategy as most feeding trials are conducted in a relatively short period under strictly controlled environments.

Although several alternative oils have been researched for their suitability to reduce the burden on FO, both VO and AF have created the most interest among researchers due to availability and low cost. Studies conducted in the recent past have suggested FO can be fully or partially replaced by single oils or blends of VO and/or AF without hindering the growth, health or condition in salmonids (Turchini et al., 2003, Bell et al., 2004, Bureau and Gibson, 2004, Tortensen et al., 2004, Tortensen et al., 2005, Stubhaug et al., 2007). Studies have also focused on fatty acid composition (Bell et al., 2003, Regost et al., 2004, Tortenson et al., 2005), physical characteristics of the fillet

(Regost et al., 2003, Mørkøre 2006, Nanton et al., 2007), metabolic disorders (Vegusdal et al., 2005) and disease resistance (Thompson et al., 1996, Bransdon et al., 2003).

The key difference between FO and alternative lipids commonly tested in aquaculture feeds is the fatty acid composition. FO is generally rich in ω3 HUFA (C_{20} or higher) such as DHA (22:6ω3), EPA (20:5ω3) and AA (20:4ω6) (Menoyo et al., 2007). In contrast, VO is rich in fatty acids containing 18 carbon ω6, ω9 and ω3 PUFA, mainly LA (18:2ω6), oleic (OL; 18:1ω9) with the exception of linseed or flax oil (FLX) containing α-linolenic acid (ALA; 18:3ω3). The other alternative, AFs are rich in saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA). VO of terrestrial origin lacks mainly AA, EPA and DHA. In contrast, AF may contain trace levels of ω3 HUFA depending on dietary history, species and age, but is not considered a significant source for FO replacement (Turchini et al., 2003, Bureau and Gibson, 2004).

The striking difference in biosynthetic ability of ω3 HUFA between marine and freshwater fish has made FO replacement in aquaculture feeds a difficult task. Several growth studies have shown the benefit of ALA in the diet of rainbow trout due to its ability to desaturate and elongate to produce EPA and DHA (Castel et al., 1972, Ruyter et al., 2000, Menoyo et al., 2007). Although LA had no effect on growth in the studies mentioned above, Cho and Cowey (1991) highlighted the importance of having LA in fish diet as a precursor in prostaglandin and leukotriene production. However, Sargent et al. (1999) suggested that EFA ratios (ω3:ω6 as well as EPA:DHA:AA) in fish diets play a much bigger role in fish physiology and health rather than a minimum EFA content. Limited synthetic capacity of HUFA in marine carnivores and the resultant high dietary requirement for HUFA could be explained by the significant role played by DHA and

EPA in maintaining the structure and function of cell membranes (Sargent et al., 1999). In contrast AA, a minor compound in membranes, produces eicosanoids that have a higher potency than the eicosanoids produced from EPA (Balfry and Higgs, 2001). Eicosanoids are 20 carbon compounds that are involved in various pathophysiological functions including immune and inflammatory processes (Lall, 2000). Dietary FO replacement with VO or AF with a lower ω3:ω6 ratio will directly change the tissue EPA:AA and DHA.

The fatty acid composition of fish lipids is also influenced by fluctuating water temperature. A drop in water temperature usually increases the proportion of unsaturated fatty acids with a corresponding decrease in SFA (Farkas et al., 2001). The extent of change in fatty acid composition depends on species, diet and type of tissue (Farkas et al., 2001, Hochachka and Somero, 2002). Although non-polar neutral lipid (NL) is primarily affected by dietary lipids, polar lipid is most influenced by changes in water temperature (Hazel and Williams, 1990, Fodor et al., 1995, Farkas et al., 2001, Hochachka and Somero, 2002). Studies conducted to determine the dietary influence of fatty acid composition on tissues with corresponding changes in water temperature has usually kept the temperatures at two distinct levels. A study conducted on rainbow trout acclimated to 20°C had a higher composition of NL, triacylglycerol (TAG) and free fatty acids (FFA) than the one at 5°C (Hazel 1979). A similar study on Atlantic salmon parr raised at 2°C and 8°C on FO or VO based diets until the fish doubled in mass, showed significant changes in the polar and NL fractions (Jobling and Bendikson, 2003). In the same study, feed had a pronounced effect on NL while the polar fraction was markedly influenced by the temperature with only a moderate change due to diet. In another study involving European sea bass (*Dicentrarchus labrax*) juveniles, fish were held at 22°C or 29°C and

given a diet composed of ω3 HUFA either below or over the minimal required level for growth. The EFA deficient diet showed a marked drop in the NL fraction with a moderate influence on polar lipid of muscle, liver and gills and with a very low influence on eyes and brain polar lipids at 29°C (Skalli et al., 2006). Studies conducted to determine the tissue fatty acid composition with simultaneous changes in water temperature are limited and have given inconsistent results (Craig et al., 1995, Fracalossi and Lovell, 1995, Kelly and Kohler, 1999, Grisdale-Hellend et al., 2002).

Intensive sea cage aquaculture operations in the North East Atlantic have always been challenging due to uncontrollable and unexpected environmental changes that can be stressful for caged trout, compromising growth and survival. This may also reflect on the timing of harvest and the quality of the final product delivered to the consumer. Physical damage to fish cages from wind and ocean currents is partially addressed by locating cage sites in bay areas overwinter. Steelhead trout (*Oncorhynchus mykiss*) is one of the species cultured in Bay d'Espoir region, Newfoundland, which experiences such stressful conditions. The present study was conducted to examine the effect of temperature fluctuations on muscle lipid and fatty acid composition in juvenile and adult steelhead trout.

In study (i) the adult steelhead trout were fed with three available commercial diets that are in use by the aquaculture industry in Canada. These diets were manufactured with either FO as the primary source of lipids or partially substituted with AF or VO. The type of AF or VO used by the feed manufacturer is not known. As opposed to strictly experimental diets, commercial diets were used due to their relevance to the aquaculture industry.

In study (ii) juvenile steelhead trout were fed with an experimental diet with either FO (herring oil; HE) as the primary source of lipid or completely substituted by either flax seed oil (FLX; also known as linseed oil) or sunflower oil (SF). FLX is known for its high natural content of ω3 PUFA, with a greater level of ALA than LA in contrast to SF.

In both studies (i & ii), the water temperature was also manipulated to mirror the common environmental changes occurring in the region. Therefore, these studies were conducted to understand the effect of dietary lipid composition on muscle lipid and fatty acid composition of juvenile and adult steelhead trout at varying environmental temperatures naturally occurring in the region.

2.2. Materials and methods – Study (i)

2.2.1. Experimental fish

This study was conducted at the Ocean Sciences Centre (OSC), Memorial University, St. John's, Newfoundland (MUN) with the approval of the Animal Care Committee of MUN. Three hundred and seventy steelhead trout, 1.6 kg average body weight, were transported to the OSC from a Bay d'Espoir aquaculture site (ambient temperature 5 ± 1°C) using a specialized truck for fish transportation. The condition of the fish was visually observed during transportation by qualified technicians and dissolved oxygen (O_2) and water temperature were measured and were within acceptable limits.

At the OSC, fish were moved to a 45 m^3 holding tank at ambient temperature (6 ± 1°C). Non abrasive nets (2-3 fish at a time) were used to carefully transfer the fish from

the truck into the holding tank. Fish were examined by a veterinarian at the end of the 1st week to determine their condition. Samples obtained during the diagnostic visit did not reveal any significant infectious disease in the population. Additional O_2 was also provided during the first week at a rate of 0.25 – 0.5 L min^{-1} to minimize transport stress. Dissolved O_2 and temperature (YSI 55 handheld dissolved O_2 meter, YSI Incorporated, Yellow Springs, OH, USA) were monitored twice daily and dead fish were promptly removed and examined for external lesions (e.g. ulcers, fin erosions). All waste accumulated at the bottom of the tank was removed daily by pulling the stand pipe.

Feed was introduced gradually (1% body weight per day) to alleviate transportation stress. The first feeding was on the second day after arrival and then once every other day. Appetite did improve after 1 - 2 weeks, after which they were fed twice a day, morning and evening (2% of body weight per day). During the first month, fish were given the same feed type as used at the aquaculture site (Corey feed). It was switched to the experimental base diet (section 2.2.3) at the end of first month. Fish were held in the holding tank for approximately 3-4 months prior to the experiment.

2.2.2. Experimental tanks

Six identical 6000 L tanks were used for the feeding experiment. Fifty five fish were haphazardly picked and distributed to each of the experimental tanks. For the acclimation period, fish were held at ambient seawater temperature (12.0 ± 1.0°C) with a water flow of 6 -7 L min^{-1}. Lighting was automatically controlled to follow the ambient daily light and dark periods.

2.2.3 Experimental feed

Three different commercially available diets were used to feed the fish in this experiment. Diets were picked based on the information given on raw materials used by the respective feed manufacturers. All three diets were analyzed to determine the lipid class and fatty acid compositions. Based on the lipid analysis, the three diets were named according to the relative proportions of ω3 as H-ω3 (higher level of ω3); M-ω3 (medium level of ω3); L-ω3 (lower level of ω3 fatty acids: Table 2.1). According to the feed manufacturers (personal communication), the protein and fat content of the H-ω3 diet originated mainly from fish meal and FO. In contrast, in the M-ω3 and L-ω3 diets an amount of fish meal and FO were partially replaced by a combination of terrestrial AF and VOs. All feed was stored in a -20°C freezer to minimize lipid oxidation. A portion of each feed type was kept separately in a walk-in cooler for daily use. All fish were fed with the experimental base diet (M-ω3), which contained a mixture of FO, VO and AF during the acclimation period.

Two tanks were randomly assigned for each feed type by drawing ballots. Fish were fed twice daily to satiation and left over feed was measured to calculate the amount consumed. The point of satiation was determined as follows: a known amount of feed (usually 1- 2% of body weight) was measured in advance for each tank. A handful of feed pellets was thrown into each assigned tank from the respective feed type and observed for consumption. This was continued until more than half the pellets thrown in sank to the bottom of the tank uneaten. Once this point was reached another handful of feed was thrown into the tank after 5-10 min. If left uneaten, this was considered the satiation point.

2.2.4. Experimental temperature

Fish were left for almost 2 weeks at 12.0 ± 1.0°C to acclimatize to the experimental tanks. The average temperature was increased following seasonal temperature, in a stepwise manner from 13.5°C (1st sampling) to 16.5°C (2nd sampling) to a maximum of 18.0°C (3rd sampling) but with plateaus (Figure 2.1). Thereafter, the temperature was dropped from 18.0°C back to 13.5°C (5th sample) with the same in-between step at 16.5°C (4th sample). At each step, the temperature was increased or decreased gradually to the next level over a ~1 - 2 day period and left stable thereafter for 14 ± 1 days. Ambient water was heated and adjusted through an automatically controlled system at the header tank to obtain the required temperatures in the experimental tanks.

2.2.5. Sampling protocol

Baseline sampling was obtained at the end of the acclimation period prior to the feeding trial. Thereafter, the fish were fed with the previously assigned feed type (H-ω3, M-ω3 or L-ω3) for a 12 week period. Feed was withdrawn 2 days prior to the day of sampling. A minimum of 6 fish per treatment were picked haphazardly at the end of each thermal period. Fish were euthanized with an overdose of anaesthetic TMS (MS-222; tricane methane sulfonate, Syndel Laboratories, British Columbia, Canada). Muscle samples for lipid class and fatty acid analysis were obtained from the left epaxial region of the fish caudo-dorsal to the pectoral fin and ventral to the anterior base of the dorsal fin. Samples of muscle weighing approximately 1 g were collected in 50 ml glass vials previously cleaned for lipid residues by rinsing 3 times with methanol (MeOH) and

chloroform ($CHCl_3$). Then each vial was flushed with nitrogen (N_2) after filling with 4 ml $CHCl_3$ and sealed with Teflon lined caps and Teflon tape and stored at -20°C.

2.2.6. Lipid extraction and analysis

2.2.6.1 Lipid extraction

All material coming into contact with lipid samples was made of either glass or was Teflon coated. All glassware was heated in a muffle furnace at 450°C for 12 h and lipid cleaned by rinsing three times with MeOH followed by three times with $CHCl_3$. All solvents used were of analytical or chromatographic grade and standards used for calibration and verification were supplied by Sigma (St. Louis, MO, USA). Distilled water used for experimental analysis was lipid cleaned with $CHCl_3$ in a separatory funnel to remove lipid residues and then stored in a lipid clean glass bottle for future use.

Total lipids were extracted from triplicate samples from each tank sampled at each temperature in $CHCl_3$:MeOH following Parrish (1999) using a modified Folch procedure (Folch et al. 1957). Fish muscle samples were quickly homogenized on ice (Brinkman Polytron blender, NY, USA) to a pulp and the metal rod of the blender was washed in 2:1 $CHCl_3$:MeOH (v/v) and then with $CHCl_3$ extracted water to a final dilution ratio of 2:1:1 (8:4:4 ml) of solvent and water. Then glass tubes containing the ground mixture were recapped and sonicated in ice for 4 min. The sonicated mixture was then centrifuged for 2 - 3 min at 3000 rpm. The bottom organic layer was then removed using 2 lipid cleaned ashed pipettes. A short tip (2 ml) pipette was first inserted into the organic layer while bubbling air to prevent the entry of upper aqueous layer into the pipette. Once the tip of

the pipette was carefully placed in the organic layer, the rubber bulb on top was carefully removed and a long tip of another baked pipette (2 ml) was carefully placed inside the previous pipette. The entire organic layer was then removed using the second pipette into a pre-cleaned round bottom flask on ice. At the end of each double pipetting, the outside pipette was washed three times with 4 ml of $CHCl_3$ and this was repeated three times. At the end of each wash, the sample was re-sonicated, centrifuged and the organic layers were pooled using the same technique. The sample was then concentrated down using first a rotary evaporator and then with a gentle stream of N_2 to near dryness. The sample was then dissolved in 10 ml of $CHCl_3$ and stored at -20°C in a 15 ml glass vial pre-cleaned for lipid residues, flushed with N_2 and sealed with a Teflon cap and tape.

2.2.6.2 Lipid class separation

Lipid classes were separated using thin layer chromatography with flame ionization detection (TLC/FID) by means of a MARK V Iatroscan (Iatroscan Laboratories, Tokyo, Japan). Specific amounts of lipid classes contained in each sample were determined as described by Parrish (1987). The flow of the FID combustion gasses, air and hydrogen were set to 200 ml min^{-1} and 20 ml min^{-1}, respectively. Extracts were spotted on silica gel-coated Chromarods and a three-stage development system was used to separate lipid classes. Chromarods consist of silica bonded to thin quartz rods and 10 rods were aligned in one rack. At the end of each development system with different polarities, the rods were partially scanned to detect lipid classes. The resulting three chromatograms were combined using T-data scan software (RSS, Bemis, TN, USA).

Lipid classes were identified using known standards (Sigma-Aldrich Corp., Oakville, ON, Canada).

2.2.6.3 Fatty acid analysis

The same crude lipid extracts were also subjected to transesterification using 14% boron trifluoride (BF_3) in MeOH to produce fatty acid methyl esters (FAME) following a procedure based on Morrison and Smith (1964) as outlined in Parrish (1999). Analysis of the resultant FAME derivatives were carried out using an HP 6890 model gas chromatograph (GC) equipped with an HP 7683 autosampler (Agilent Technologies Canada Inc., Mississauga, ON, Canada). Fatty acid peaks were integrated using HP Enhanced Chemstation G 1701BA Version B.OO.OO (Agilent Technologies Canada Inc., Mississauga, ON, Canada) and identified against known standards (PUFA 1, PUFA 3, BAME, and a Supelco 37-component FAME mix, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada).

2.2.7. Specific growth rate and food conversion efficiency

Specific growth rate (SGR) and food conversion efficiency (FCE) were calculated for adult steelhead trout fed the three diets for each sampling period. Total wet weight of 5 fish per tank (10 per treatment) was measured to the nearest 1 g in order to calculate the growth rate. Using sample wet weight, the weight specific growth rate (SGR) of each tank was determined using the following relationship:

$$SGR = ((\ln(W_f) - \ln(W_i))t^{-1}) \times 100$$

where W_f is the mean final sample wet weight(g), W_i is the mean initial wet weight (g), and t is the duration between initial and final sampling (days).

Food conversion efficiency (FCE) was determined using the following relationship:

$$FCE = W_g W_{fe}^{-1}$$

Where W_g is the weight gain of fish in each tank which is calculated from the total sample wet weight and W_{fe} is the weight of food eaten by fish in each tank over a specific time period.

2.2.8. Statistical analysis

All data sets were examined to verify normality, independence and homogeneity of variance before further analysis was undertaken. Muscle and feed lipid class and fatty acid data were subjected to analysis of variance using the General Linear Model procedure of the Statistical analysis System (GLM procedure, SPSS 13.0 for Windows). The test for muscle lipid class and fatty acid was performed with temperature and feed type as explanatory variables (two-way ANOVA) with interactions (feed x temperature) to determine the effect of temperature and feed type on lipid class and fatty acid composition. This test was performed when no effects were found due to individual tanks used in the experiment. If significant interactions were present, the effect of feed type was examined at each sampling temperature using only feed type as the explanatory variable. Multiple comparisons of means for lipid class and fatty acids were made using Tukey corrections. The significance level was set at $\alpha = 0.05$ for all the tests.

2.3. Materials and methods - Study (ii)

2.3.1. Experimental fish

This study was conducted at the Ocean sciences Centre (OSC), Memorial University, St John's, NL. Four hundred and eighty juvenile steelhead trout (transported from a Bay d'Espoir aquaculture site), 120 g average body weight were moved to a 45 m^3 holding tank at ambient temperature (5 ± 1°C). Fish were transported and handled following the same procedure described earlier (section 2.2.1).

During the first month, fish were given the same feed type as used at the aquaculture site (Corey feed, Fredericton, NB, Canada) and then switched to the experimental base diet (section 2.3.3) at the end of first month. Fish were held in the holding tank for approximately a month prior to the experiment.

2.3.2 Experimental tanks

Six identical 6000 L tanks were used for the feeding experiment. Fifty-five fish were haphazardly picked and distributed to each experimental tank. Fish were held at 8.0 ± 1.0 °C water temperature with a flow of 6 -7 L min^{-1} for approximately 2 weeks to acclimate to the experimental tanks. Fish were fed with experimental base diet (HE diet) and reared following the same protocol described earlier (section 2.2.1).

2.3.3. Experimental feed

Three experimental diets used in this experiment were formulated at the Marine Research Station, Sandy Cove, Halifax. The basal diet was formulated using the ingredients listed in Table 2.5. The origin of lipids was different among the three diets

(Herring oil, HE; flax seed oil, FLX, and sunflower oil, SF). The three diets were stored at -20°C and fed following the same procedure as in section 2.2.3.

2.3.4. Experimental temperature

The temperature was increased to 10.0 ± 1.0°C after the initial acclimation period (8.0 ± 1.0°C) in 1 to 2 days. Thereafter the fish were fed with the three experimental diets in duplicate and left for 14 ± 1 days. The average temperature was increased in a stepwise manner, following seasonal temperature changes, from 10.0°C (1st sampling) to a maximum of 18°C (6th sampling) following ~2°C incremental steps but with plateaus (Figure 2.2). At each step, the temperature was increased or decreased gradually to the next level over a ~1 - 2 day period and left stable thereafter for 14 ± 1 days. Ambient water was heated and adjusted as in study (i).

2.3.5. Sampling protocol

Sampling was done following the same procedure described in study (i).

2.3.6. Lipid analysis

Lipid extraction, lipid class separation and fatty acid analyses were done following the procedures described in study (i), section 2.2.6.

2.3.7. Statistical analysis

Statistical analysis was performed as described in section 2.2.7.

2.4. Results - Study (i)

2.4.1 Feed lipid class and fatty acid composition

Label information of the three feeds used in the experiment is given in Table 2.1. To preserve anonymity the feeds were identified by the level of ω3 as H-ω3, M-ω3 or L-ω3. Total lipid, neutral lipid and polar lipids (ww^{-1}%) were not significantly different among the 3 feed types (Table 2.2). The most abundant classes of lipid present in the 3 feed types were triacylglycerol (TAG), sterol (ST), and phospholipid (PL). The H-ω3 feed had a significantly lower ST over both M-ω3 and L-ω3 feed ($p \leq 0.031$). TAG had the highest % lipid class composition and it was not significantly different among the 3 feed types. Similarly, PL was not significantly different among feeds (Table 2.2).

The total MUFA, SFA, PUFA and ω3 fatty acids present in the 3 feeds are given in Table 2.2. Both ΣPUFA (%) and Σω3 (%) levels were different ($p = 0.0001$) among the 3 feeds, with H-ω3 diet having the highest and the L-ω3 diet having the lowest percent composition (Table 2.2). SFA (%) level was lowest in fish oil rich H-ω3 feed ($p = 0.0001$), and the MUFA (%) was highest in vegetable oil rich L-ω3 feed ($p = 0.0001$) (Table 2.2). The ratios of P:S was highest in H-ω3 feed and lowest in the L-ω3 feed ($p = 0.0001$). Fish oil rich H-ω3 feed had the lowest ω6:ω3 ratio and vegetable oil rich L-ω3 the highest (Table 2.2). Further EFA and fatty acids above 1% ww^{-1} (except for 18:3ω3 and AA) are given in Table 2.2. All fatty acids were present in different proportions in each feed ($p \leq 0.026$), except 22:5ω3 in M-ω3 and L-ω3. Mainly terrestrial (18:3ω3, 18:2ω6) and marine originating ω3 fatty acids (EPA, DPA and DHA) were different among the three feed types as shown in Table 2.2. L-ω3 diet had significantly higher 18:3ω3 while both H-ω3 and M-ω3 had similar levels. Marine originating lipids were

highest in H-ω3 and lowest in L-ω3 diets. Conversely terrestrial plant lipids were highest in L-ω3 and lowest in H-ω3 diets.

2.4.2 Tissue lipid class composition

Table 2.3 shows the effect of diet and temperature on lipid class composition (%) in muscle tissue of steelhead trout fed three diets. Both TL and NL (ww^{-1}%) in muscle tissue were affected by the increase in temperature with a significantly higher level in L-ω3 fed fish ($p \leq 0.03$) at 18.0°C over H-ω3 fed fish. Diet, temperature and temperature rearing history had significant ($p \leq 0.027$) effects on major lipid class composition in muscle tissue of steelhead trout (Table 2.3). The significant differences occurred in muscle TAG, ST and polar lipid, both at the end of the first temperature increment (2^{nd} sampling point) and after the final drop in temperature at 13.5°C (5^{th} sampling point). TAG was the most abundant lipid class (>85%) present in muscle tissue. The PL level was only affected at the end of the temperature trial (13.5°C), where H-ω3 fed fish had a higher level of PL over the other 2 diet types ($p = 0.0001$). Sterol levels were higher in L-ω3 fed fish at 2^{nd} sampling over both H-ω3 and M-ω3 and at 3^{rd} and 4^{th} sampling points over H-ω3 fed fish. The relative composition of ST in H-ω3 fed fish was at a comparatively lower level compared to the other 2 diets and was not affected by the change in temperature. ST of both M-ω3 and L-ω3 fed fish increased with the increase in temperature from 16.5 to 18°C ($p \leq 0.029$).

The TAG composition of M-ω3 fed fish decreased with the drop in temperature from 18.0°C to 16.5°C and increased with the further drop in temperature to 13.5°C ($p \leq$

0.039). TAG composition of L-ω3 fed fish had a similar pattern to M-ω3 fed fish without the significant drop in composition with the drop in temperature from 18.0°C to 16.5°C.

2.4.3. Tissue fatty acid composition

Fatty acid composition of muscle tissue for each diet at each temperature is given in Table 2.4. The total PUFA, SFA and MUFA (%) present in muscle tissue was not significantly different in fish fed the 3 diets at any given temperature.

The dietary influence in muscle tissue was mostly seen in H-ω3 and L-ω3 fed fish with comparatively less change occurring in muscle tissues of M-ω3 fed fish at any given temperature. Significant changes in fatty acid percentages or ratios frequently occurred with drops in temperature (from 18.0 to 16.5 and then to 13.5°C) as opposed to an increase in temperature (Table 2.4). Increase in temperature from 16.5°C to 18.0°C decreased both terrestrial fatty acids and ω6:ω3 ratios in H-ω3 fed fish compared to the L-ω3 diet ($p \leq 0.011$). There was a consistent pattern in muscle fatty acid composition after decreasing temperature from 18.0 to 16.5°C in H-ω3 and L-ω3 fed fish. The tissue composition of total marine fatty acid, ω3, polyunsaturate:saturate fatty acid ratio (P:S), DHA and EPA became higher in H-ω3 fed fish than fish fed L-ω3 diet ($p \leq 0.045$) at 16.5°C. In contrast, terrestrial fatty acid and ω6:ω3 ratio in L-ω3 fed fish significantly increased above H-ω3 fed fish once the temperature was decreased to 16.5°C.

The final decline in temperature to 13.5°C resulted in significant muscle fatty acid changes in fish fed all 3 diets. However, a pattern occurred in the change of fatty acid composition in the final temperature (13.5°C), as in the penultimate step (Table 2.4). The marine origin fatty acids, EPA:AA ratio, Σω3, EPA and 14:0 was higher in H-ω3 fed fish

than L-ω3 fed fish ($p \leq 0.033$). In contrast terrestrial fatty acids, ω6:ω3 ratio and 18:1ω9 was higher in L-ω3 fed fish than the fish fed H-ω3 diet ($p \leq 0.030$). The fatty acid composition of 18:1ω9, 18:2ω6 and ω6:ω3 ratio of M-ω3 fed fish was higher, and EPA was lower than H-ω3 fed fish at the final temperature (13.5°C; $p \leq 0.029$).

Significant changes in muscle fatty acid composition of M-ω3 fed fish occurred with increasing temperature to 18.0°C from the initial (13.5°C) and with the final sampling upon decreasing the temperature back to 13.5°C (Table 2.4). The content of 18:0 increased with temperature increase to 18.0°C with a corresponding decrease in 18:2ω6 ($p \leq 0.024$). Subsequent drops in temperature to 13.5°C, decreased the total SFA composition by decreasing 16:0 in muscle tissue ($p = 0.018$). The total MUFA percentage was not affected significantly by the temperature change, but the composition of 18:1ω9 was increased with the decrease in temperature to 13.5°C ($p = 0.027$). Similarly, the total PUFA in muscle tissue was not affected by diet at any temperature, but the amounts of both 18:2ω6 and ω6:ω3 ratios increased with the final decline in water temperature to 13.5°C ($p \leq 0.03$).

The change in water temperature influenced the HUFA composition in H-ω3 fed fish (Table 2.4). Both Σω3 and marine origin fatty acid (EPA+DPA+DHA) composition in muscle tissue were not affected by the increase in temperature but increased when the temperature was decreased from 18.0°C to 16.5°C ($p \leq 0.002$), followed by a decrease with decreasing temperature to 13.5°C ($p \leq 0.013$) (Table 2.4). The P:S ratio followed a similar pattern with an increase at decreasing temperature of 16.5°C ($p \leq 0.044$), followed by a non-significant decline at 13.5°C.

The proportions of 16:0 and 18:1ω9 were affected by temperature in L-ω3 fed fish (Table 2.4). As in the other two diets, the changes occurred with decreasing temperature from 18.0 to 13.5°C ($p \leq 0.037$). The composition of 18:1ω9 increased with decreasing temperature while the composition of 16:0 decreased.

2.4.4 Specific growth rate, mortality and food conversion efficiency

Temperature and feed had no significant affect on growth of fish or the mortality (%) during the experimental period. The average wet weight of the fish increased 30 – 40% during the experimental period in fish fed all three feed types (initial mean wet weight of fish assigned H-ω3: 1662 ± 319 g, M-ω3: 1741 ± 302 g, L-ω3: 1945 ± 721 g; final mean wet weight of fish fed H-ω3: 2626 ± 197.9, M-ω3: 2268.5 ± 314.6, L-ω3: 2372 ± 245.98).

2.5. Results – Study (ii)

2.5.1 Feed lipid class and fatty acid composition

The ingredients and composition of the 3 experimental diets are given in Table 2.5. The basal diet was supplemented with herring oil (as indicated in Table 2.5) or replaced with either flax or sunflower seed oil. The most abundant lipid classes and fatty acid in the 3 diets are given in Table 2.6. Total and neutral lipid contents in the HE diet was higher than in FLX ($p \leq 0.015$) and polar lipid was higher than in both SF and FLX diets ($p \leq 0.043$). TAG was the most abundant lipid class present and both TAG and PL proportions were not significantly different among the 3 diets.

Fatty acid compositions of the 3 diets are given in Table 2.6. The HE diet had the highest total marine fatty acid (EPA + DPA + DHA) proportions as expected ($p = 0.0001$) and also the highest SFA (%) and lowest ω6:ω3 ratio. In contrast FLX diet had the highest terrestrial fatty acid (18:3ω3 + 18:2ω6) proportions, PUFA, Σω3, P:S and lowest SFA and MUFA proportions ($p \leq 0.003$). However, the proportion of 18:2ω6 fatty acid was highest in SF ($p = 0.0001$), with FLX having the highest 18:3ω3 ($p = 0.0001$) proportion among the 3 diets. The DHA:EPA ratio was not significantly different among the 3 feeds.

2.5.2. Muscle lipid class and fatty acid composition

The variations in lipid class composition of muscle tissues of juvenile steelhead trout fed the 3 diets were compared for the effect of temperature and diet. The lipid class composition of TAG, ST, PL, NL and polar lipid ($ww^{-1}\%$) were not significantly affected by diet or temperature and was not included in the results.

The dietary effects of SFA (%) was observed in HE fed fish (Table 2.7). A significant change in muscle total SFA (%) was first observed in HE fed fish at 12.5°C ($p = 0.012$), with the composition of both SF and FLX fed fish being lower. The difference in total SFA (%) in HE diet fish continued among the 3 fish groups with each sampling point from 12.5 to 18.0°C ($p \leq 0.012$), corresponding to the high SFA (%) in the HE diet. The dietary effects on muscle SFA compositions were mostly seen with increasing temperature from 12.5 to 14.5°C followed by 16.5 and 18.0°C (Table 2.7). The two SFA responding to temperature increases were 14.0 and 16.0. Both SF and FLX fish had similar SFA composition in muscle during the same incremental steps, but unlike HE fed

fish, the proportion of both 14:0 (in SF fed fish) and 16:0 (in FXL fed fish) significantly decreased with increasing temperature.

The muscle MUFA and ω6:ω3 ratio was higher in SF fed fish with increasing temperature from 14.5 to 18.0°C ($p \leq 0.008$), with a corresponding lower proportion in PUFA and ω3 ($p = 0.04$) compared to HE and FLX fish (Table 2.7). The significantly higher MUFA in SF fed fish was due to 18:1ω9, which stayed the same over the temperature increment while the composition of the same fatty acid declined in both HE ($p = 0.000$) and FLX ($p = 0.000$) fed fish with increasing temperature from 14.5 to 18.0°C (Table 2.7). The proportion of 18:1ω7 was the least affected by the temperature while the proportion of 16:1ω7 gradually declined in all 3 groups.

Muscle terrestrial and marine fatty acid composition was directly affected by the change in diet (Table 2.7). Muscle marine lipid (%) was higher in fish fed HE diet compared to SF and FLX fed juveniles ($p \leq 0.018$), and change in temperature had no effect on the differences among the 3 groups except at 12.5°C. Similarly, the muscle terrestrial lipid composition increased steadily in FLX fed fish ($p \leq 0.015$) with a gradual decline seen in fish fed HE diet ($p = 0.000$). The terrestrial fatty acid of FLX fish was higher than HE fish at 10.0°C and stayed the same over the temperature increments ($p \leq 0.015$). Both HE and SF fish had similar terrestrial composition until the 14.5°C increment, but terrestrial fatty acid proportions in SF fish were higher during the remaining increments ($p \leq 0.008$). However, fish fed FLX had the highest terrestrial lipid composition followed by SF ($p \leq 0.004$), with the lowest being in the juveniles fed HE diet. Muscle P:S ratio was higher in FLX fed fish than HE fed fish and first seen at 12.5°C and remained the same for the rest of the experimental period ($p \leq 0.009$). Both HE and

SF fish had similar P:S ratios until 14.5°C, however SF fed fish had a higher ratio thereafter ($p \leq 0.049$). The EPA:AA ratio of fish fed HE was higher compared to both SF and FLX fed juveniles at any given temperature ($p \leq 0.028$). Both SF and FLX fish had similar EPA:AA ratio until 14.5°C, followed by a higher ratio in FLX fish during the final two temperature increments, 16.5°C and 18.0°C ($p \leq 0.011$). The EPA:DHA ratio of muscle tissue had a similar pattern, with the HE diet fed fish having a higher value over SF and FLX fish during the last four and three temperature increments, respectively ($p \leq 0.033$) (Table 2.7).

Table 2.1. Label information of the 3 commercial diets used in the experiment. The three diets were identified by the level of ω3 polyunsaturated fatty acids (PUFA) present in the diet (Higher, H-ω3; Medium, M-ω3; Lower, L-ω3).

Composition	H-ω3	M-ω3	L-ω3
Crude protein (min) %	41.0	42.0	42.0
Crude fat (min) %	24.0	27.0	23.0
Crude fibre (max) %	1.5	4.0	4.0
Calcium (actual) %	1.6	1.1	0.9
Phosphorus (actual) %	1.3	1.0	0.8
Sodium (actual) %	0.5	0.4	0.54
Vitamin A (min) IU/kg	5000	5000	5000
Vitamin D (min) IU/kg	2400	4000	4000
Vitamin E (min) IU/kg	200	250	250

Table 2.2. Selected lipid class (%), total lipid, polar lipid, neutral lipid (% ww^{-1}) and fatty acid (%) average composition and ratios (mean ± SD) of experimental feed containing medium ω3 polyunsaturated fatty acids (M-ω3), lower ω3 polyunsaturated fatty acids (L-ω3) and higher ω3 polyunsaturated fatty acids (H-ω3).

	H-ω3	M-ω3	L-ω3
Total lipid	34.0 ± 6.4	33.8 ± 1.5	29.5 ± 5.3
Neutral lipid	31.7 ± 6.3	32.0 ± 1.3	27.8 ± 5.0
Polar lipid	2.3 ± 0.1	1.8 ± 0.3	1.7 ± 0.3
Lipid class (% total lipid)[1]			
Triacylglycerol	91.6 ± 0.9	88.9 ± 3.1	89.9 ± 0.7
Sterol	0.8 ± 0.2[a]	5.6 ± 2.8[b]	3.77 ± 1.1[b]
Phospholipid	3.9 ± 0.5	3.6 ± 0.4	3.98 ± 0.6
Fatty acid (% total fatty acid)			
14:0	6.0 ± 0.1[a]	4.6 ± 0.1[b]	3.6 ± 0.03[c]
16:0	15.5 ± 0.1[a]	19.0 ± 0.3[b]	19.7 ± 0.1[c]
18:0	2. 7 ± 0.02[a]	6.2 ± 0.2[b]	6.9 ± 0.1[c]
18:1ω9	8.8 ± 0.1[a]	21.0 ± 0.4[b]	23.3 ± 0.2[c]
20:1ω9	4.8 ± 0.3[a]	1. 5 ± 0.04[b]	2.1 ± 0.03[c]
22:1ω11(13)	7.6 ± 0.5[a]	1.7 ± 0.1[b]	2.5 ± 0.1[c]
18:2ω6	2.5 ± 0.1[a]	8.1 ± 0.2[b]	9.8 ± 0.2[c]
18:3ω3	0.7 ± 0.0[a]	0.8 ± 0.04[a]	0.9 ± 0.03[b]
20:4ω6 (AA)	0.9 ± 0.01[a]	0.9 ± 0.01[a]	0.8 ± 0.0[b]
20:5ω3 (EPA)	15.7 ± 0.3[a]	10.5 ± 0.5[b]	8.3 ± 0.1[c]
22:5ω3 (DPA)	2.4 ± 0.01[a]	1.3 ± 0.1[b]	1.2 ± 0.03[b]
22:6ω3 (DHA)	9.3 ± 0.3[a]	6.3 ± 0.2[b]	4.4 ± 0.1[c]
ΣSFA[2]	25.4 ± 0.1[a]	31.0 ± 0.4[b]	31.3 ± 0.1[b]
ΣMUFA[3]	33.4 ± 0.8[a]	34.0 ± 0.2[a]	37.4 ± 0.3[b]
ΣPUFA[4]	40.0 ± 0.7[a]	34.3 ± 0.8[b]	30.5 ± 0.4[c]
P:S	1.6 ± 0.02[a]	1.1 ± 0.03[b]	1.0 ± 0.01[c]
Σω3	31.1 ± 0. 6[a]	21.0 ± 0. 7[b]	16.5 ± 0.2[c]
ω6:ω3	0.13 ± 0.01[a]	0.5 ± 0.02[b]	0.7 ± 0.01[c]
ΣTerrestrial[5]	3.2 ± 0.1[a]	8.8 ± 0.2[b]	10.7 ± 0.2[c]
ΣMarine[6]	27.3 ± 0.5[a]	18.1 ± 0.7[b]	13.9 ± 0.2[c]

Significant differences in each row are indicated by superscript letters.
Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), polyunsaturated to saturate ratio (P:S), Omega (ω), arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA).
[1] Lipid class (% total lipid), which also includes: hydrocarbon, steryl/wax ester, acetone mobile polar lipids at <2% each.
[2]Sum of saturated fatty acids (SFA), which also includes: *i*15:0, 15:0, *ai*16:0, *i*17:0, *ai*17:0, 20:0, 22:0 and 23:0 at < 1.0% each.
[3] Sum of monounsaturated fatty acids (MUFA), which also includes: 14:1, 15:1, 16:1ω7, 16:1ω9, 16:1ω5, 17:1, 18:1ω7, 20:1ω11, 22:1ω11, 22:1ω9 and 24:1.
[4] Sum of polyunsaturated fatty acids (PUFA), which also includes: 16:2ω4, 16:3ω4, 16:4ω3, 16:4ω1, 18:1ω7, 18:2ω4, 18:3ω6, 18:4ω1, 18:4ω3, 20:1ω9, 20:2a, 20:2ω6, 20:3ω3, 21:5 ω3 and 22:4 ω6 at < 1.0% each.
[5] Terrestrial fatty acid includes: 18:2ω6 and 18:3ω3.
[6] Marine fatty acids includes: EPA, DPA and DHA.

Table 2.3. Effect of H-ω3, M-ω3 and L-ω3 diet on total lipid, neutral lipid, polar lipid (ww^{-1}%) and percent lipid class composition of muscle tissue of steelhead trout (*Oncorhynchus mykiss*) measured at 4 sampling periods and rearing temperatures of 13.5 - 18.0°C.

Sample	2			3			4			5		
Feed	H-ω3	M-ω3	L-ω3	H-ω3	M-ω3	L-ω3	H-ω3	M-ω3	L-ω3	H-ω3	M-ω3	L-ω3
Temp	16.5°C			18.0°C			16.5°C			13.5°C		
TAG	$91.3 \pm 0.8^{a+}$	$93.3 \pm 2^{a*}$	$88.2 \pm 1.6^{b\S}$	$94.4 \pm 1.5^{+}$	$92.8 \pm 2.3^{*}$	$91.9 \pm 3.3^{\S}$	$93.8 \pm 2.9^{+}$	$89.6 \pm 3.2^{**}$	$91.5 \pm 1.7^{\S}$	$84.9 \pm 2.8^{a++}$	$93.4 \pm 0.7^{b*}$	$93.4 \pm 2.0^{b\S\S}$
ST	0.4 ± 0.1^{a}	$0.4 \pm 0.2^{a*}$	$0.8 \pm 0.1^{b\S}$	0.7 ± 0.3^{a}	$1.7 \pm 1.0^{**}$	$2.8 \pm 1.7^{b\S\S}$	0.5 ± 0.1^{a}	$1.08 \pm 0.5^{**}$	$1.2 \pm 0.3^{b\S}$	0.6 ± 0.3^{a}	$1.4 \pm 0.7^{b**}$	$0.9 \pm 0.4^{\S}$
PL	4.6 ± 0.3	3.7 ± 1.0	4.0 ± 1.0	2.1 ± 0.7	3.5 ± 2.2	3.1 ± 1.4	3.1 ± 1.8	6.6 ± 4.8	6.1 ± 2.4	12.1 ± 2.4^{a}	3.6 ± 0.5^{b}	2.8 ± 1.7^{b}
TL	20.4 ± 4.9	18.9 ± 10.0	14.1 ± 5.0	13.7 ± 7.7^{a}	22.8 ± 9.2	23.2 ± 2.9^{b}	17.3 ± 6.9^{a}	18.2 ± 4.9	20.3 ± 3.0^{b}	15.4 ± 6.1	13.5 ± 3.0	15.7 ± 9.1
NL	19.2 ± 4.7	18.2 ± 10.0	13.3 ± 5.0	13.2 ± 7.7^{a}	22.1 ± 8.8	22.3 ± 3.0^{b}	16.9 ± 7.0	17.0 ± 4.4	18.9 ± 2.7	13.4 ± 5.8	12.9 ± 3.0	15.1 ± 9.1
Polar lipid	1.6 ± 0.4^{a}	0.7 ± 0.2^{b}	$0.78 \pm 0.1b^{c}$	0.5 ± 0.1	0.8 ± 0.4	0.9 ± 0.3	0.5 ± 0.2	1.2 ± 0.9	1.41 ± 0.5	2.01 ± 0.5^{a}	0.6 ± 0.1^{b}	0.5 ± 0.2^{b}

Values are mean ± SD.
Significant differences among diets at each temperature are indicated by superscript letters and among temperatures for each diet are indicated by symbols respectively.
Diets labelled as: H-ω3 (Higher ω3 fatty acids), M-ω3 (Medium ω3 fatty acids) and L-ω3 (Lower ω3 fatty acids).
Temperature (Temp), triacylglycerol (TAG), sterol (ST), phospholipid (PL), total lipid (TL), neutral lipid (NL).

Table 2.4. Effect of H-ω3, M-ω3 and L-ω3 diet on muscle fatty acid composition of adult steelhead trout (*Oncorhynchus mykiss*) at different temperatures.

Sample	1	2			3			4			5		
Temperature	13.5 °C	16.5°C			18.0°C			16.5°C			13.5°C		
Feed	M-ω3	H-ω3	M-ω3	L-ω3	H-ω3	M-ω3	L-ω3	H-ω3	M-ω3	L-ω3	H-ω3	M-ω3	L-ω3
14:0	$3.6 \pm 0.4^{**}$	3.2 ± 0.4	3.0 ± 0.2	3.2 ± 0.3	3.5 ± 0.4	3.2 ± 0.3	2.9 ± 0.3	2.8 ± 0.5	$2.8 \pm 0.3^{*}$	3.0 ± 0.3	3.6 ± 0.3^{a}	$2.7 \pm 0.2^{b*}$	2.8 ± 0.1^{b}
16:0	15.4 ± 0.8	14.8 ± 1.1	15.1 ± 0.5	$15.1 \pm 0.6^{\S}$	15.8 ± 0.6	$17.3 \pm 0.6^{**}$	$16.8 \pm 1.7^{\S}$	15.3 ± 1.6	15.5 ± 1.9	$15.6 \pm 1.2^{\S}$	14.7 ± 1.0	$14.1 \pm 2.7^{*}$	$14.5 \pm 0.8^{\S\S}$
18:0	$3.6 \pm 0.3^{**}$	3.8 ± 0.3	3.9 ± 0.1	3.8 ± 0.1	3.9 ± 0.1^{a}	$4.8 \pm 0.7^{b*}$	4.4 ± 0.4^{ab}	3.3 ± 1.7	4.1 ± 0.4	4.4 ± 0.8	3.7 ± 0.2	4.0 ± 1.0	3.8 ± 0.2
ΣSFA[1]	23.5 ± 1.3	22.6 ± 1.6	22.9 ± 0.8	23.0 ± 0.9	24.1 ± 0.9	$26.6 \pm 1.6^{**}$	24.8 ± 2.3	22.2 ± 2.2	23.3 ± 2.4	23.9 ± 2.0	22.6 ± 1.5	$21.0 \pm 3.1^{*}$	21.7 ± 1.0
18:1ω9	28.0 ± 1.6	27.9 ± 1.6	27.9 ± 1.1	$27.7 \pm 1.3^{\S}$	24.5 ± 1.5	$25.2 \pm 2.8^{**}$	$26.3 \pm 2.8^{\S}$	23.0 ± 4.2	26.3 ± 3.6	$26.1 \pm 1.5^{\S}$	25.7 ± 2.7^{a}	$29.9 \pm 3.4^{b*}$	$29.7 \pm 1.5^{b\S\S}$
22:1ω11(13)	1.1 ± 0.2	1.4 ± 0.3	1.1 ± 0.1	1.1 ± 0.1	1.58 ± 0.5^{a}	0.9 ± 0.3^{b}	1.0 ± 0.2^{b}	1.0 ± 0.4	0.9 ± 0.2	0.9 ± 0.2	1.5 ± 0.6^{a}	0.9 ± 0.1^{b}	1.0 ± 0.1
Σ MUFA[2]	43.6 ± 1.9	43.6 ± 1.7	43.0 ± 1.4	43.2 ± 1.6	42.4 ± 2.5	40.4 ± 2.3	42.4 ± 3.2	39.2 ± 6.01	41.3 ± 5.2	43.1 ± 0.8	43.1 ± 2.2	45.1 ± 3.5	46.0 ± 1.5
18:2ω6	$9.5 \pm 0.6^{**}$	9.1 ± 0.6	9.4 ± 0.1	9.4 ± 0.5	7.03 ± 0.8^{a}	$8.1 \pm 0.7^{*}$	8.9 ± 0.8^{b}	8.0 ± 0.8	9.1 ± 0.9	9.3 ± 0.6	8.2 ± 1.0^{a}	$9.5 \pm 1.1^{b**}$	9.7 ± 0.2^{b}
20:4ω6	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.1	1.1 ± 1.3	0.6 ± 0.07	0.6 ± 0.06	0.7 ± 0.07	0.6 ± 0.08	0.7 ± 0.07	0.5 ± 0.2	0.6 ± 0.1
20:5ω3	4.4 ± 0.7	4.5 ± 0.6	4.4 ± 0.4	4.9 ± 0.6	5.2 ± 0.3	4.0 ± 1.8	4.2 ± 0.5	5.6 ± 0.9^{a}	4.7 ± 0.6	4.3 ± 0.4^{b}	5.1 ± 0.5^{a}	4.1 ± 0.4^{b}	4.1 ± 0.5^{b}
22:5ω3	1.8 ± 0.1	2.1 ± 0.1	2.0 ± 0.3^{a}	2.4 ± 0.1^{b}	2.3 ± 0.2	1.7 ± 0.7	2.1 ± 0.6	2.6 ± 0.2^{a}	2.2 ± 0.2^{b}	2.2 ± 0.2	2.5 ± 0.1	2.2 ± 0.4	2.2 ± 0.1
22:6ω3	9.8 ± 1.0	11.0 ± 0.8	$11.1 \pm 1.$	9.9 ± 0.6	11.8 ± 0.4	11.8 ± 2.4	10.7 ± 2.7	16.2 ± 4.2^{a}	12.9 ± 3.3	10.7 ± 1.8^{b}	11.3 ± 1.6	10.5 ± 1.4	9.5 ± 1.5
ΣPUFA[3]	32.1 ± 1.8	33.3 ± 1.5	33.5 ± 1.5	33.2 ± 1.3	32.9 ± 1.8	32.4 ± 2.7	32.2 ± 2.3	38.1 ± 4.2	34.9 ± 3.6	32.4 ± 2.5	33.8 ± 2.0	32.2 ± 2.3	31.7 ± 2.2
P:S	1.4 ± 0.1	$1.5 \pm 0.1^{+}$	1.5 ± 0.1	1.4 ± 0.1	$1.4 \pm 0.06^{+}$	1.2 ± 0.1	1.3 ± 0.1	$1.7 \pm 0.1^{a++}$	1.5 ± 0.1	1.4 ± 0.2^{b}	1.5 ± 0.1	1.5 ± 0.3	1.5 ± 0.1
Σω3	18.6 ± 1.8	$19.9 \pm 1.0^{+}$	19.9 ± 1.3	19.6 ± 1.3	$21.7 \pm 0.6^{+}$	20.4 ± 2.6	19.5 ± 2.8	$26.3 \pm 4.8^{a++}$	21.9 ± 4.0	19.3 ± 2.3^{b}	$21.3 \pm 2.2^{a+}$	18.8 ± 1.7	18.0 ± 2.1^{b}
ω6:ω3	0.6 ± 0.4	$0.6 \pm 0.0^{+}$	0.6 ± 0.0	0.6 ± 0.0	$0.4 \pm 0.04^{a++}$	$0.5 \pm 0.1^{**}$	0.6 ± 0.1^{b}	0.4 ± 0.09^{a}	0.5 ± 0.1	0.6 ± 0.1^{b}	0.5 ± 0.07^{a}	$0.6 \pm 0.1^{b*}$	0.7 ± 0.1^{b}
EPA:AA	6.2 ± 0.7	5.98 ± 1.2	5.7 ± 0.7	6.6 ± 0.6	6.9 ± 0.9	5.2 ± 2.3	5.7 ± 0.8	5.9 ± 0.6	5.8 ± 0.7	5.7 ± 0.4	6.5 ± 0.9^{a}	4.9 ± 0.6^{b}	5.4 ± 0.5^{b}
ΣTerrestrial[4]	$10.4 \pm 0.6^{**}$	$9.9 \pm 0.6^{+}$	10.2 ± 0.2	10.2 ± 0.6	$7.7 \pm 0.9^{a++}$	$8.9 \pm 0.8^{*}$	9.7 ± 0.9^{b}	8.5 ± 0.9^{a}	9.8 ± 1.0	10.0 ± 0.7^{b}	9.0 ± 1.0^{a}	10.2 ± 1.2	10.5 ± 0.3^{b}
ΣMarine[5]	15.9 ± 1.6	$17.6 \pm 0.8^{+}$	17.4 ± 1.4	17.1 ± 1.0	$19.4 \pm 0.5^{+}$	17.5 ± 2.8	17.0 ± 3.0	$24.4 \pm 5.1^{a++}$	19.8 ± 4.1	17.2 ± 2.4^{b}	$18.9 \pm 2.2^{a+}$	16.8 ± 1.7	15.7 ± 2.1^{b}

Values are mean ± SD.
Significant differences among diets at each temperature are indicated by superscript letters and among temperatures for each diet are indicated by symbols.

[1]Sum of saturated fatty acids (SFA), which also includes: *i*15:0, 15:0, *ai*16:0, *i*17:0, *ai*17:0, 20:0, 22:0 and 23:0 at < 1.0% each.
[2] Sum of monounsaturated fatty acids (MUFA), which also includes: 14:1, 15:1, 16:1ω7, 16:1ω9, 16:1ω5, 17:1, 18:1ω7, 20:1ω11, 22:1ω11, 22:1ω9 and 24:1 at < 1.0% each.
[3] Sum of polyunsaturated fatty acids (PUFA), which also includes: 16:2ω4, 16:3ω4, 16:4ω3, 16:4ω1, 18:2ω4, 18:3ω6, 18:4ω1, 20:1ω9, 20:2a, 20:2ω6, 20:3ω3, 21:5 ω3 and 22:4 ω6 at < 1.0% each.
[4] Terrestrial fatty acid: 18:2ω6 and 18:3ω3.
[5] Marine fatty acids: EPA, DPA and DHA.
Polyunsaturated to saturate ratio (P:S), Omega (ω), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), arachidonic acid (AA).

Table 2.5. Feed ingredients and composition of the three experimental diets in study (ii); HE (Herring oil), FLX (flax seed oil) or SF (sunflower oil).

Feed Ingredient	Composition (%)
Herring meal	37
Soybean meal	15
Corn gluten meal	10
Dried whey	7
Wheat middling's	13.6
Vitamin premix	1.5
Choline chloride	0.4
Mineral premix	1.5
Lipid supplement *	14

* Herring, flax or sunflower oil was used as the lipid supplement to prepare the three experimental diets.

Table 2.6. Selected lipid class (%), total lipid, neutral lipid, polar lipid (ww^{-1}%) and fatty acid (%) average composition and ratios (mean ± SD) in three experimental diets, HE (Herring oil), FLX (flax seed oil) or SF (sunflower oil).

Lipid composition	**HE**	**SF**	**FLX**
Total lipid (ww^{-1} %)	29.3 ± 3.7^{a}	25.8 ± 0.4	21.9 ± 0.2^{b}
Neutral lipid (ww^{-1} %)	29.1 ± 3.7^{a}	25.7 ± 0.4	21.8 ± 0.1^{b}
Polar lipid (ww^{-1} %)	0.2 ± 0.04^{a}	0.07 ± 0.05^{b}	0.08 ± 0.07^{b}
Lipid class (% total lipid)[1]			
TAG	99.2 ± 0.2	99.6 ± 0.1	99.5 ± 0.4
PL	0.8 ± 0.2	0.3 ± 0.18	0.4 ± 0.3
Fatty acid (% total fatty acid)			
14:0	6.5 ± 0.1^{a}	2.3 ± 0.03^{b}	1.9 ± 0.2^{c}
16:0	15.9 ± 0.04^{a}	9.5 ± 0.4^{b}	9.3 ± 0.3^{b}
16:1ω7	6.9 ± 0.12^{a}	2.5 ± 0.06^{b}	2.2 ± 0.1^{c}
18:0	2.8 ± 0.01^{a}	3.1 ± 0.03^{b}	2.5 ± 0.05^{c}
18:1ω9	8.1 ± 0.7^{a}	32.4 ± 1.6^{b}	11.3 ± 0.6^{c}
18:1ω7	2.9 ± 0.6	4.5 ± 1.3^{a}	1.6 ± 0.4^{b}
18:2ω6	7.2 ± 0.2^{a}	23.0 ± 0.3^{b}	15.0 ± 0.2^{c}
18:3ω3	1.1 ± 0.06^{a}	1.6 ± 0.2^{a}	37.4 ± 0.7^{b}
18:4ω3	2.0 ± 0.03^{a}	0.6 ± 0.01^{b}	0.6 ± 0.02^{c}
20:1ω9	2.0 ± 0.1^{a}	$1.8 \pm 0.1a$	1.6 ± 0.1^{b}
20:4ω6	1.1 ± 0.01^{a}	0.3 ± 0.03^{b}	0.3 ± 0.01^{b}
20:5ω3	16.8 ± 0.2^{a}	5.0 ± 0.1^{b}	4.5 ± 0.1^{c}
22:1ω11(13)	2.6 ± 0.01	2.59 ± 0.1	2.4 ± 0.2
22:5ω3	1.8 ± 0.0^{a}	0.5 ± 0.1^{b}	0.5 ± 0.02^{b}
22:6ω3	8.8 ± 0.11^{a}	4.4 ± 0.5^{b}	4.2 ± 0.3^{b}
ΣSFA[2]	26.6 ± 0.15^{a}	16.0 ± 0.47^{b}	14.3 ± 0.4^{c}
ΣMUFA[3]	25.4 ± 0.1^{a}	45.9 ± 0.8^{b}	20.8 ± 0.1^{c}
ΣPUFA[4]	46.8 ± 0.2^{a}	37.6 ± 0.4^{b}	64.4 ± 0.4^{c}
P:S	1.8 ± 0.02^{a}	2.4 ± 0.6^{b}	4.5 ± 0.1^{c}
Σω3	31.7 ± 0.2^{a}	12.5 ± 0.7^{b}	47.5 ± 0.5^{c}
ω6:ω3	0.3 ± 0.01^{a}	1.9 ± 0.13^{b}	0.3 ± 0.01^{c}
EPA:AA	15.8 ± 0.1^{a}	15.5 ± 1.1^{b}	15.4 ± 0.8^{a}
DHA:EPA:	0.5 ± 0.01	0.9 ± 0.1	0.9 ± 0.1
ΣTerrestrial[5]	8.3 ± 0.2^{a}	24.6 ± 0.4^{b}	52.4 ± 0.6^{c}
ΣMarine[6]	27.4 ± 0.2^{a}	9.9 ± 0.7^{b}	9.1 ± 0.3^{b}

Significant differences in each row are indicated by superscript letters.
Triacylglycerol (TAG), phospholipid (PL), saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), polyunsaturate to saturate ratio (P:S), omega (ω), arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA).
[1] Lipid class (% total lipid), which also includes: hydrocarbon, steryl/wax ester, acetone mobile polar lipids at <2% each.
[2]Sum of saturated fatty acids (SFA), which also includes: *i*15:0, 15:0, *ai*16:0, *i*17:0, *ai*17:0, 20:0, 22:0 and 23:0 at < 1.0% each.
[3] Sum of monounsaturated fatty acids (MUFA), which also includes: 14:1, 15:1, 16:1ω9, 16:1ω5, 17:1, 22:1ω9 and 24:1.
[4] Sum of polyunsaturated fatty acids (PUFA), which also includes: 16:2ω4, 16:3ω4, 16:4ω3, 16:4ω1, 18:2ω4, 18:3ω6, 18:4ω1, 20:2a, 20:2ω6, 20:3ω3, 21:5ω3 at < 1.0% each.
[5] Terrestrial fatty acid: 18:2ω6 and 18:3ω3.
[6] Marine fatty acids: EPA, DPA and DHA.

Table 2.7. Effect of HE, SF and FLX diet on muscle fatty acid composition of steelhead trout juveniles (*Oncorhynchus mykiss*) at different temperatures.

Feed	HE	SF	FLX	HE	SF	FLX
Sampling temperature		10.0			12.5	
14:0	2.41 ± 0.4	2.3 ± 0.3	$2.7 \pm 0.2^{+}$	2.6 ± 0.4^{a}	1.6 ± 0.1^{b}	1.9 ± 0.2^{b}
16:0	16.4 ± 0.4	$16.5 \pm 0.9^{\$}$	16.4 ± 0.3	18.2 ± 1.8^{a}	$16.5 \pm 1.1^{\$}$	15.9 ± 0.7^{b}
18:0	4.0 ± 0.4	4.0 ± 0.4	3.8 ± 0.2	4.1 ± 0.4	4.2 ± 0.1	4.0 ± 0.3
16:1ω7	4.6 ± 0.7	$4.5 \pm 0.6^{a\$\$}$	$5.5 \pm 0.3^{b++}$	3.9 ± 0.4^{a}	$2.6 \pm 0.4^{b\$}$	$2.8 \pm 0.3^{b+}$
18:1ω9	$15.3 \pm 2.6^{a**}$	18.6 ± 1.8^{b}	$20.5 \pm 1.5^{b++}$	13.2 ± 1.5^{a}	16.8 ± 2.8^{b}	$13.3 \pm 1.2^{a+}$
18:1ω7	2.4 ± 0.3	2.2 ± 0.1	2.4 ± 0.17	2.2 ± 0.4	2.0 ± 0.2	1.8 ± 0.3
18:3ω3	$0.8 \pm 0.1^{a**}$	2.2 ± 0.9^{b}	$2.04 \pm 1.1^{b++}$	0.6 ± 0.1^{a}	1.0 ± 0.4^{a}	$7.3 \pm 2.1^{b+}$
18:2ω6	$8.2 \pm 1.3^{a**}$	$9.2 \pm 0.9^{\$\$}$	10.0 ± 0.8^{b}	$6.3 \pm 0.9^{a*}$	$8.3 \pm 1.3^{b\$\$}$	7.9 ± 1.3
20:4ω6	1.3 ± 0.2	1.3 ± 0.1	1.0 ± 0.4	1.4 ± 0.2	1.5 ± 0.1	1.2 ± 0.1
20:5ω3	8.4 ± 1.2^{a}	$6.8 \pm 0.6^{b\$\$}$	6.2 ± 0.3^{b}	8.7 ± 1.8	$7.2 \pm 0.8^{\$\$}$	6.9 ± 0.7
22:5ω3	2.5 ± 0.2^{a}	$2.2 \pm 0.2^{b\$\$}$	1.9 ± 0.1^{c}	2.7 ± 0.4^{a}	2.1 ± 0.2^{b}	1.9 ± 0.2^{b}
22:6ω3	22.8 ± 4.1**	20.8 ± 2.71	$18.0 \pm 3.1^{++}$	26.1 ± 2.9	27.9 ± 2.7	$25.8 \pm 4.4^{+}$
ΣSFA	23.6 ± 0.6**	$23.5 \pm 1.0^{\$\$}$	$23.4 \pm 0.2^{++}$	25.6 ± 2.4^{a}	$22.8 \pm 1.3^{b\$\$}$	22.4 ± 0.8^{b}
ΣMUFA	$26.2 \pm 3.8^{a**}$	28.4 ± 2.4	$31.5 \pm 1.9^{b++}$	22.7 ± 2.4	24.4 ± 2.9	$21.0 \pm 2.1^{+}$
ΣPUFA	49.6 ± 3.9^{a}	47.6 ± 2.3	$44.6 \pm 2.07^{b++}$	51.1 ± 4.3	52.3 ± 1.9	$56.1 \pm 1.3^{+}$
P:S	2.1 ± 0.1	$2.0 \pm 0.1^{\$\$}$	$1.9 \pm 0.1^{++}$	2.0 ± 0.3^{a}	2.3 ± 0.1	$2.5 \pm 0.1^{b+}$
Σω3	$36.3 \pm 5.0^{a**}$	33.7 ± 2. 9	$29.6 \pm 2.3^{b++}$	39.9 ± 4.9	39.5 ± 3.0	$44.3 \pm 2.6^{+}$
ΣTerrestrial	$8.95 \pm 1.4^{a**}$	11.3 ± 1.7	$12.0 \pm 1.9^{b++}$	$6.9 \pm 0.9^{a*}$	9.3 ± 1.6^{a}	15.2 ± 3.4^{b}
ω6:ω3	0.3 ± 0.07**	0.4 ± 0.05^{a}	$0.4 \pm 0.05^{b++}$	0.2 ± 0.05*	0.3 ± 0.06	$0.2 \pm 0.04^{+}$
EPA:AA	6.3 ± 0.37^{a}	$5.3 \pm 0.83^{b\$\$}$	5.4 ± 0.47^{b}	6.3 ± 0.64^{a}	5.0 ± 0.2^{b}	5.6 ± 0.68
EPA:DHA	0.4 ± 0.0.04	$0.33 \pm 0.06^{\$\$}$	0.35 ± 0.06	0.3 ± 0.05^{a}	$0.26 \pm 0.03^{b\$}$	0.27 ± 0.05
ΣMarine	33.6 ± 5.5^{a}	29.8 ± 3.5	26.0 ± 3.5^{b}	37.5 ± 5.3	37.2 ± 3.8	34.6 ± 5.4

Table 2.7 Cont.

	HE	SF	FLX	HE	SF	FLX	HE	SF	FLX
Sampling point		14.5			16.5			18.0	
14:0	2.8 ± 0.2	2.6 ± 1.2$^{\$\$}$	2.3 ± 0.9	3.1 ± 0.2^{a}	1.6 ± 0.2$^{b\$}$	1.7 ± 0.5^{b+}	3.0 ± 0.6^{a}	1.4 ± 0.2$^{b\$}$	1.0 ± 0.4^{b+}
16:0	18.3 ± 0.9^{a}	14.4 ± 0.8$^{b\$\$}$	14.4 ± 2.2^{b}	17.8 ± 0.4^{a}	15.2 ± 0.7^{b}	15.9 ± 1.4^{b}	17.5 ± 0.8^{a}	14.6 ± 0.5$^{b\$\$}$	11.7 ± 5.5^{b}
18:0	4.3 ± 0.3	4.02 ± 0.2	4.1 ± 0.3	3.9 ± 0.1^{a}	4.1 ± 0.2	4.4 ± 0.4^{b}	4.1 ± 0.3^{a}	4.2 ± 0.3^{a}	3.9 ± 1.9^{b}
16:1ω7	4.3 ± 0.4^{a}	3.0 ± 0.6$^{b\$}$	2.3 ± 0.9^{b+}	4.2 ± 0.4^{a}	2.3 ± 0.4$^{b\$}$	2.6 ± 0.7^{b+}	4.1 ± 0.7^{a}	2.0 ± 0.2$^{b\$}$	1.6 ± 0.8^{b+}
18:1ω9	11.9 ± 1.4^{a*}	22.1 ± 3.8^{b}	16.0 ± 4.3^{a+}	10.1 ± 1.2^{a*}	18.2 ± 2.3^{b}	11.8 ± 2.0^{a+}	10.0 ± 1.3^{a*}	19.2 ± 2.6^{b}	10.8 ± 4.3^{a+}
18:1ω7	2.3 ± 0.4	2.3 ± 0.9	1.7 ± 0.2	2.3 ± 0.1	3.2 ± 0.7^{a}	2.1 ± 0.7^{b}	2.2 ± 0.2	3.9 ± 1.2	2.0 ± 0.5
18:3ω3	0.5 ± 0.1^{a*}	2.4 ± 0.4^{b}	8.9 ± 1.8^{c+}	0.6 ± 0.04^{a}	1.8 ± 0.6^{b}	12.3 ± 2.9^{c+}	0.5 ± 0.1^{a*}	2.2 ± 1.3^{b}	11.3 ± 5.0^{c+}
18:2ω6	5.6 ± 0.4^{a*}	11.4 ± 1.7$^{b\$}$	9.4 ± 2.2^{b}	5.0 ± 0.5^{a*}	10.0 ± 1.2^{b}	8.8 ± 2.2^{b}	4.8 ± 0.6^{a*}	11.3 ± 0.7$^{b\$}$	7.8 ± 4.1^{b}
20:4ω6	1.4 ± 0.1^{a}	1.6 ± 0.2^{b}	1.1 ± 0.1^{b}	1.4 ± 0.04^{a}	1.3 ± 0.1^{a}	1.1 ± 0.2^{b}	1.4 ± 0.1	1.2 ± 0.1	1.0 ± 0.5
20:5ω3	9.6 ± 1.0^{a}	4.9 ± 1.4$^{b\$}$	6.1 ± 1.0^{b}	9.4 ± 0.3^{a}	5.7 ± 0.6^{b}	6.0 ± 0.9^{b}	9.6 ± 0.4^{a}	5.0 ± 0.3$^{b\$}$	5.3 ± 2.8^{b}
22:5ω3	2.6 ± 0.3^{a}	1.8 ± 0.1$^{b\$}$	1.7 ± 0.3^{b}	2.9 ± 0.1^{a}	1.7 ± 0.2$^{b\$}$	1.8 ± 0.2^{b}	2.9 ± 0.2^{a}	1.5 ± 0.1$^{b\$}$	1.6 ± 0.8^{b}
22:6ω3	27.4 ± 1.7^{a}	20.1 ± 4.6^{b}	23.2 ± 4.1	28.1 ± 3.1	26.2 ± 3.7	22.8 ± 6.0	28.6 ± 4.0^{a}	25.0 ± 1.3	19.3 ± 8.9^{b*}
ΣSFA[1]	26.1 ± 0.7^{a*}	21.6 ± 1.4^{b}	21.3 ± 1.2^{b+}	25.6 ± 0.4^{a}	21.6 ± 0.7^{a}	22.6 ± 1.6^{b}	25.4 ± 0.7^{a}	20.7 ± 1.0$^{b\$}$	22.1 ± 1.7^{b}
ΣMUFA[2]	21.5 ± 2.0^{a*}	31.6 ± 4.3^{b}	23.0 ± 3.6^{a+}	20.9 ± 2.0^{a*}	27.1 ± 3.2^{b}	19.7 ± 3.8^{a+}	20.8 ± 2.7^{a*}	28.8 ± 2.0^{b}	21.3 ± 2.1^{a+}
ΣPUFA[3]	52.0 ± 1.6a	46.3 ± 4.2^{b}	55.3 ± 2.7^{a+}	52.9 ± 2.1^{a}	50.9 ± 2.6^{b}	57.4 ± 3.6^{a+}	53.2 ± 2.5^{a}	50.0 ± 2.2^{b}	56.2 ± 3.3^{a+}
P:S	2.0 ± 0.1^{a}	2.2 ± 0.2^{a}	2.6 ± 0.1^{b+}	2.07 ± 0.10^{a}	2.4 ± 0.1$^{b\$}$	2.6 ± 0.2^{b+}	2.1 ± 0.1^{a}	2.4 ± 0.19$^{b\$}$	2.6 ± 0.3^{b+}
ω3	41.6 ± 2.1a	30.7 ± 5.5^{b}	42.4 ± 4.5^{a+}	42.8 ± 2.7^{a}	36.7 ± 3.9^{b}	45.5 ± 4.8^{a+}	43.4 ± 3.4^{a*}	34.8 ± 2.1^{b}	45.3 ± 2.7^{a+}
ΣTerrestrial[4]	6.3 ± 0.4^{a*}	13.8 ± 2.0^{b}	18.2 ± 2.6^{c+}	5.6 ± 0.58^{a*}	11.9 ± 1.6^{b}	21.1 ± 5.0^{c+}	5.3 ± 0.7^{a*}	13.5 ± 1.5^{b}	19.7 ± 3.6^{c+}
ω6:ω3	0.2 ± 0.02^{a*}	0.5 ± 0.15^{b}	0.3 ± 0.08^{a+}	0.2 ± 0.03^{a*}	0.4 ± 0.07^{b}	0.3 ± 0.07^{a+}	0.2 ± 0.03^{a*}	0.4 ± 0.03^{b}	0.2 ± 0.06^{a+}
EPA:AA	6.7 ± 0.95^{a}	4.3 ± 0.87$^{b\$}$	5.4 ± 0.49^{b}	6.8 ± 0.39^{a}	4.3 ± 0.44$^{b\$}$	5.6 ± 0.91^{c}	6.7 ± 0.35^{a}	4.1 ± 0.23$^{b\$}$	5.1 ± 0.71^{c}
EPA:DHA	0.4 ± 0.03^{a}	0.24 ± 0.05$^{b\$}$	0.26 ± 0.03^{b}	0.34 ± 0.06^{a}	0.22 ± 0.02$^{b\$}$	0.27 ± 0.05^{b}	0.34 ± 0.06^{a}	0.2 ± 0.01$^{b\$}$	0.3 ± 0.09
ΣMARINE[5]	39.6 ± 3.0^{a}	26.8 ± 6.1^{b}	31.0 ± 5.5^{b}	40.4 ± 3.6^{a}	33.6 ± 4.6	30.6 ± 7.3^{b}	41.1 ± 4.7^{a}	31.6 ± 1.8^{b}	31.1 ± 2.2^{b}

Values are mean ± SD.

Significant differences among diets at each temperature are indicated by superscript letters and among temperatures for each diet are indicated by symbols.

[1]Sum of saturated fatty acids (SFA), which also includes: *i*15:0, 15:0, *ai*16:0, *i*17:0, *ai*17:0, 20:0, 22:0 and 23:0 at < 1.0% each.

[2] Sum of monounsaturated fatty acids (MUFA), which also includes: 14:1, 15:1, 16:1ω9, 16:1ω5, 17:1, 20:1ω11, 22:1ω11, 22:1ω9 and 24:1 at < 1.0% each.

[3] Sum of polyunsaturated fatty acids (PUFA), which also includes: 16:2ω4, 16:3ω4, 16:4ω3, 16:4ω1, 18:1ω7, 18:2ω4, 18:3ω6, 18:4ω1, 20:1ω9, 20:2a, 20:2ω6, 20:3ω3, 21:5 ω3 and 22:4 ω6 at < 1.0% each.

[4] Terrestrial fatty acid: 18:2ω6 and 18:3ω3.

[5] Marine fatty acids: EPA, DPA and DHA.

Polyunsaturated to saturate ratio (P:S), Omega (ω), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), arachidonic acid (AA).

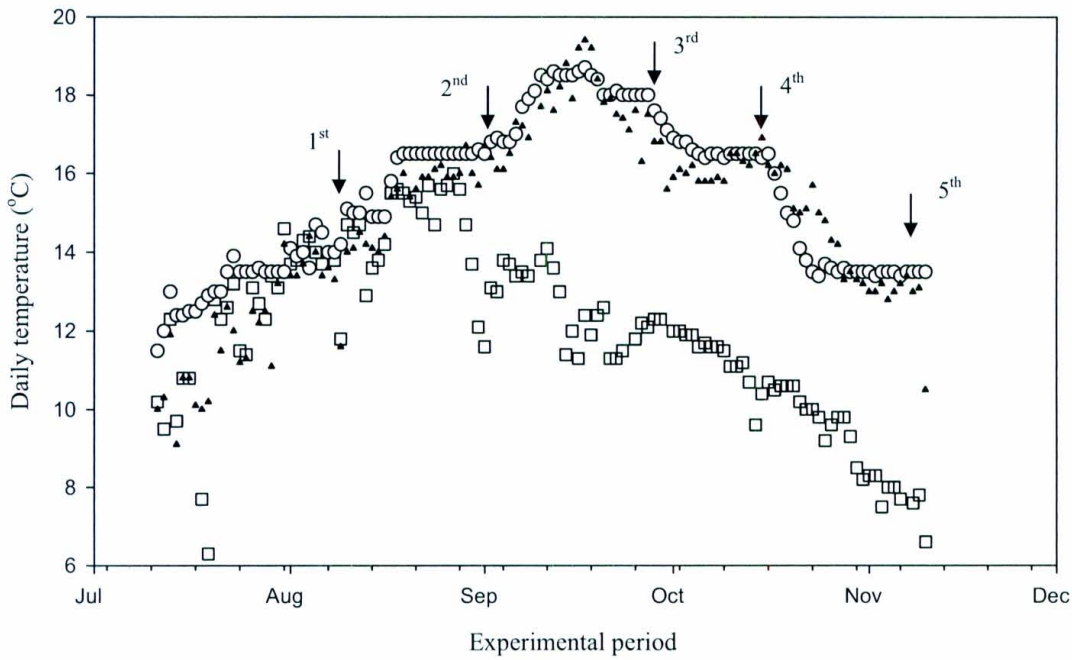

Figure 2.1. Daily recorded temperatures of ambient water (□), header (▲) and experimental tanks (○) mean during the experimental period (Study i). Sampling points are indicated by an arrow and the sampling number; 1st (13.5°C), 2nd (16.5°C), 3rd (18.0°C), 4th (16.5°C) and 5th (13.5°C).

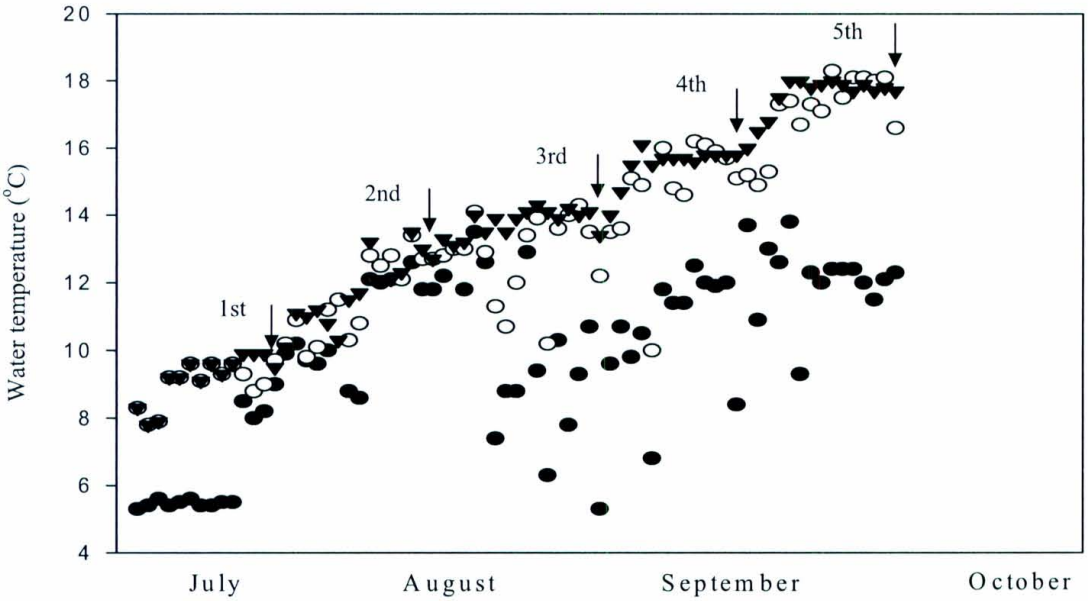

Figure 2.2. Daily recorded temperatures of ambient water (●), header (○) and experimental tanks (▼) during the experimental period (Study ii). Sampling points are indicated by an arrow and the sampling number; 1st (10.0°C), 2nd (12.0°C), 3rd (14.0°C), 4th (16.0°C) and 5th (18.0°C).

2.6. Discussion

2.6.1. Study (i)

Lipid class and fatty acid analyses indicated the qualitative difference between the three commercial diets used in this study. The percent total lipid was higher than the crude fat (minimum) percentage reported on the label information for the three feeds. Parrish (1987) found a 10 – 15% decrease in the total lipid values obtained for freshwater lipid samples through Chromarod-Iatroscan analysis as opposed to gravimetric analysis. The difference in Iatroscan system was attributed to the measuring of only non-volatile lipids and the possibility of non-lipid compound inclusion in the gravimetric method. Although the method of crude fat analysis by the respective manufacturer is unknown, the above difference may be due to reporting of the minimum crude fat content in respective diets by the manufacturer. The different levels of marine lipids (EPA, DPA, DHA) in the H-ω3 diet shows the origin of lipids were primarily from FO (Tocher 2003). The H-ω3 diet had half as much again, and nearly twice as much Σω3 fatty acids as the M-ω3 and L-ω3 diets, respectively, and the M-ω3 diet had a third more Σω3 than L-ω3 diet. The high composition of Σmarine fatty acids suggests Σω3 in H-ω3 feed is primarily from FO. The Σmarine fatty acid composition in H-ω3 feed was half as much again, and nearly twice as much higher than M-ω3 and L-ω3 diets respectively, which accounts for the major difference in Σω3 fatty acids among the three feed types. Incorporation of AF and VO (saturated, 18:2ω6 and 18:3ω3 fatty acids, respectively) in H-ω3 diet was at a significantly lower level than the other two diet types. Marine origin oils were partially replaced in the L-ω3 diet resulting in higher MUFA and lower P:S ratios. VO is rich in ω6 and ω9 fatty acids (Turchini et al., 2009). Fatty acid analysis of the M-ω3 diet shows

partial substitution of fish oil with a combination of animal and plant originating lipids. The Σω3 composition of M-ω3 feed was significantly higher than L-ω3 feed and it also had more marine origin HUFA than L-ω3 feed. Animal fats in particular are rich in SFA and MUFA. The composition of SFA would vary with species of origin. Beef tallow has approximately twice as much SFA as poultry fat. Animal fat might also contain certain amounts of C_{18} PUFA based on dietary history of the source animal. Moretty and Corino (2008) reported 28.5% SFA fatty acids in poultry fat with 20% PUFA (primarily LA) and 47.5% SFA fatty acid in beef tallow with less than 4% PUFA. Full or partial replacement of fish oil in marine aquaculture could also lead to the risk of net deficiency in EFAs. Therefore, supplementation of aquaculture feeds with some fish meal is necessary to fulfill the EFA requirement in most cultured species. Turchini et al. (2009) suggested that a minimum supplementation of 300 g kg^{-1} of fish meal in feed was sufficient to fulfill the EFA requirement of most farmed finfish.

The diet had no effect on growth of adult steelhead trout. Similar findings were reported in previous studies in Atlantic salmon, where growth was not affected by replacing a portion of fish oil with vegetable oil in diets containing 45 to 50% crude protein primarily from fish meal (Torstenson et al., 2000, Bell et al., 2003).

TAG was the predominant lipid class found in muscle tissue with all 3 diets in this study (diacylglycerol and monoacylglycerol was not detected in measurable quantities). Salmonids are known to deposit TAG as the predominant lipid class in muscles (Bell et al., 2003). Fish are known to store excess dietary lipids in the form of TAG in mesenteric adipose tissues and in red and white muscles (Henderson and Tocher, 1987). The majority of TAG in red muscles are stored as fine oil droplets within muscle fibres (Sheriden,

1994). In species like Atlantic cod, the primary storage site is the liver (Sargent et al., 1989), unlike trout. Lipid class composition in fish muscle is usually a reflection of dietary lipid intake (Higgs and Dong, 2000; Sargent et al., 2002), environmental temperature (Skalli et al., 2006), lipid digestibility (Tortensen et al., 2000) and metabolism (Tortensen et al., 2000; Bell et al., 2002). Utilization of storage lipids results in changes of muscle lipid composition particularly during periods of high energy demand, such as food deprivation, reproduction and smoltification (Tocher, 2003; Turchini et al., 2009).

Neutral lipids are known to respond more readily to changes in dietary lipid than polar lipids (Sargent et al., 1989; Xu et al., 1996; Higgs and Dong, 2002). The muscle lipid composition in this study was affected both by diet and the rearing temperature history. Vegetable oil rich L-ω3 fed fish at 18.0°C had more stored neutral lipids and sterols compared to fish fed H-ω3 diet contributing to the higher total lipid composition. The difference in total lipid was also seen with decreasing the temperature to 16.5°C. However, total and neutral lipid levels did not differ between L-ω3 and H-ω3 fish with a further decrease in water temperature to 13.5°C. The sequence of temperature changes affected TAG and was prominent at the final temperature of 13.5°C, with H-ω3 diet fed fish storing less TAG in muscle compared to both M-ω3 and L-ω3 diets. The decreasing temperature also increased the polar lipid fraction of H-ω3 fed fish in comparison to fish fed the other two diets.

Environment temperature is known to affect the fatty acid composition in fish (Hazel and William, 1990, Fordor et al., 1995; Farkas et al., 2001; Hochachka and Somero, 2002). However in this study, in addition to the rearing temperature, the

sequence of temperature experienced by the fish also influenced the lipid composition. The differences in lipid composition seen in muscle tissues of fish sampled at the same temperature, but at different sampling points (during temperature increase and decrease) were mainly due to the temperature rearing history of the fish. Exposure to lower temperatures results in deposition of unsaturated fatty acids in body tissues, with a greater effect on polar lipids than neutral lipids (Hazel and Williams, 1990). The reduction in temperature is also known to accumulate MUFA in the *sn*-1 position of PLs (Hazel and William, 1990; Bell et al., 2002; Farkas et al., 2002; Hochachka and Somero, 2002). The behaviour of acyl moiety attached to TAG in fish lipids (e.g. *sn*-1 and *sn*-3 occupied by SFA and MUFA, *sn*-2 commonly occupied by PUFA) is not well elucidated. However, Arts et al. (2001) found that the MUFA molecular species attached to *sn*-3 of TAG are primarily derived from diet, making the NL fatty acid composition readily responsive to dietary lipids as well as polar lipids.

Cholesterol is the most common ST in animal lipid (Tocher, 2003). Cholesterol is a major component of cell membranes, while some is stored together with neutral lipids in muscle tissue. Fish fed the L-ω3 diet had a greater ST composition until the temperature reached 13.5^{o}C. Plant oil rich feeds are known to contain higher levels of sterols (mainly phytosterols: Pickova and Morkore, 2007). Both M-ω3 and L-ω3 fed fish had a greater proportion of ST in muscle tissue as opposed to those fed on the fish oil rich diet, and L-ω3 fed fish responded to change in temperature during the latter half of the experiment.

Replacing the base diet (M-ω3) with a fish oil rich H-ω3 diet resulted in significant changes in the composition of Σmarine lipids of H-ω3 fed fish. Changes in the

composition of EPA and DHA significantly increased the ω3 fatty acid and the P:S ratio, with a corresponding decrease in terrestrial lipids and the ω6:ω3 ratio. The finding for HUFA levels in H-ω3 fed fish was in line with previous studies (Fodor et al., 1995; Farkas et al., 2001). The decline in temperature and the dietary influence on H-ω3 fed fish was very clear once the temperature was decreased from 18.0°C (the highest) to 16.5°C, which caused H-ω3 to be significantly different to L-ω3 fed fish. The Σmarine lipid, Σω3, and P:S ratio of L-ω3 fed fish were unaffected by the vegetable oil rich diet or temperature.

Exposure to low temperature is known to reduce the proportion of SFA with a corresponding increase in unsaturated fatty acids. The types of unsaturated fatty acid that accumulate may be MUFA or PUFAs (Jobling et al., 1995; Logue et al., 2000). The total SFA composition in muscle tissue in this study was neither affected by feed nor decreasing temperature. However, L-ω3 and M-ω3 fed fish accumulated 18:1ω9 in muscle with decreasing temperature and M-ω3 fed fish accumulated both 18:1ω9 and 18:2ω6. Studies conducted to date are primarily focused on determining the effects of diet and temperature on the polar lipid fraction due to its significance in membranes (Hazel and Williams, 1990; Fodor et al., 1995; Logue et al., 2000; Farkas et al., 2001; Hochachka and Somero, 2002). Similar compositional changes in polar lipids were also seen in this study with the final drop in temperature to 13.5°C. However, one needs to be cautious in attributing findings in the polar lipid fraction to muscle tissue because over 80% of lipids present in muscle are stored as TAG which falls under neutral lipids.

Studies examining simultaneous influence of diet and temperature on muscle tissues of trout are very scarce (Jobling and Bendickson, 2003). Most studies are

conducted by acclimating fish to two distinct temperatures with a primary focus on determining fatty acid changes in PLs (Skuladottir et al., 1990; Labbe et al., 1995; Grisdale-Helland et al., 2002). Studies with simultaneous manipulation of temperature and diet have given inconsistent results for various reasons such as species differences, types of oils and fat used in feeds and types of lipid classes and fatty acids investigated (Craige et al., 1995; Fracalossi and Lovell, 1995, Labbe et al. 1995, Grisdale-Helland et al., 1995).

Both M-ω3 and L-ω3 fed fish responded to decreasing temperature by manipulating 16:0, 18:1ω9 and 18:2ω6 in contrast to H-ω3 fed fish, which changed P:S, ω3 and marine fatty acids. Temperature had no significant influence on marine lipids, ω3 or P:S ratio in M-ω3 or L-ω3 fed fish. Stubhaug et al. (2007) showed the capability of Atlantic salmon for selective storage of long chain PUFA with low dietary intake. In that study the fish fed a FO based diet stored a lower percentage of EPA (20%) and DHA (30%) compared to VO containing diet, 70% EPA and 80% DHA. Therefore, Stubhaug et al. (2007) suggested that fish primarily place EPA and DHA in membranes when they are found in low amounts in the diet, but that they use these for β-oxidation when they are overly present in the diet. This reinforces the fact that cold water fish use surplus fatty acids for energy while storing the essential fatty acids that are limiting (Bell et al., 2003; Stubhaug et al., 2007), hence highlighting the lack of response in HUFAs in both L-ω3 and M-ω3 fed fish. Fish are known to selectively mobilize or incorporate fatty acids in response to physiological demand or in response to environmental perturbation (Webber et al., 2002, Webber et al., 2003). Webber et al. (2003) reported that oleic acid (18:1ω9), the most abundant MUFA in fish tissues are readily incorporated into NLs in rainbow

trout signifying its role in energy metabolism. Previous studies have also shown preferential mobilization and oxidation of oleic acid from TAG reserves (Ballantyne, 1996) over the most abundant saturated fatty acid, palmitic acid (Sidell et al., 1995).

Dietary requirements vary with species, stage of development and environment (Turchini et al., 2009) and muscle fatty acid composition is dependent upon digestibility (Toerstensen et al., 2000), uptake, elongation, desaturation (Bell et al., 2002) and β-oxidation (Toerstensen et al., 2000). In fish, ω3 fatty acids are absorbed at faster rates than ω6s (Francis et al., 2007). Similarly, relative absorption increases with increasing degree of unsaturation, although there is a tendency for decreased absorption with increasing chain length (Morasis et al., 2005; Francis et al., 2007). Chen et al. (2006) found that increasing dietary FO content in rainbow trout significantly increased the fat and ω3 fatty acid content in muscle tissue. Studies have also indicated that Atlantic salmon give preference to certain fatty acids (readily oxidizing MUFA and C_{18} PUFA when present in high concentrations over HUFA). However, both EPA and DHA are highly oxidized when in dietary surplus (Stubhaug et al., 2007). Therefore, increased accumulation of long chain HUFA relative to terrestrial fatty acids (LA and ALA) in H-ω3 fed fish is primarily due to high PUFA levels in the diet.

This is the first study to evaluate the effect of fluctuating temperature on muscle lipid and fatty acid composition in steelhead trout using commercially available feeds. Research conducted to date has primarily focused on using strict experimental diets or comparing such diets with a commercially available diet. Therefore, this study provides more relevant information for the aquaculture industry and the consumer.

Although substituting FO with plant or animal originated lipids did not affect the total SFA, MUFA or PUFA levels in muscle tissue, fish fed FO rich diet accumulated significantly higher levels ω3 rich marine lipids compared to the other feeds. Replacing the base diet (mixture of both animal fat and vegetable oil) with fish oil rich H-ω3 diet resulted in significant differences in muscle fatty acid composition between H-ω3 and L-ω3 fed fish. However, such a difference was not seen between L-ω3 and M-ω3 fed fish. Fish fed the HUFA rich diet actively utilized the most abundant HUFAs to counteract the temperature perturbation as opposed to fish fed vegetable oil or animal fat rich diets.

2.6.2. Study (ii)

The aim of this study was to evaluate the effect of dietary fatty acids in juvenile steelhead trout by replacing herring oil with either SF or FLX dominated diets. In addition, the water temperature was simultaneously increased to understand the effects of fluctuating environmental temperature on muscle fatty acid composition. Inclusion of SF or FLX oil in place of HE had no significant effect on growth of juvenile steelhead trout. These results are in line with previous studies done on salmonid fish, where substantial amounts of FO were replaced by vegetable oil mixes without any detrimental effect on fish growth (Bell et al., 1991, 1996; Tocher et al., 2000; Bransden et al., 2003; Tortensen et al., 2005; Menoyo et al., 2007).

SF and FLX oils are rich in LA and ALA acid, respectively (Bell et al., 1993). Both LA and ALA are considered essential fatty acids in fish due to their inability to be biosynthesized *de novo* (Tocher, 2003). However, once ω6 and ω3 C_{18} fatty acids are made available in the diet, fish are potentially capable of converting them into

corresponding C_{20-22} ω6 HUFA and C_{20-22} ω3 HUFA, respectively, through a series of alternating steps involving acyl chain desaturation and elongation (Nakamura and Nara, 2004). The enzymatic pathway and the biosynthesizing capability of HUFA in freshwater rainbow trout are well known (Buzzi et al., 1997; Tocher 2003). However, it is also known that marine fish lack or have lost this capability due to adaptation to a HUFA rich marine environment (Sargent et al., 2002). The muscle terrestrial fatty acid composition of both SF and FLX fed fish was higher than the HE fed fish over the experimental period irrespective of the change in temperature, and is thus considered a dietary effect. The total terrestrial fatty acid composition of FLX fed fish (higher level of both 18:2ω6 and 18:3ω3) was significantly higher over both HE and SF fed fish. Previous studies indicated that the muscle fatty composition closely resembles dietary fatty acid conditions in fish (Bell et al., 2004; Menoyo et al., 2005). A similar dietary response was also seen in marine fatty acid composition in HE diet fed fish with increasing marine lipid over the experimental period. The marine lipid of both SF and FLX fed fish was unchanged during the experimental period, supporting findings for fish in previous studies. However, in this experiment, fish fed the base diet (HE) decreased the terrestrial fatty acid composition in muscle tissue over the experimental period compared to the initial composition.

Both SF and FLX fed fish selectively retained essential ω3 HUFAs due to dietary limitations. Several studies have shown selective metabolism of fatty acids. Bell et al. (2003) found that Atlantic salmon postmolts fed with varying concentrations of linseed or rapeseed oil selectively deposited 22:3ω6 in muscle tissue regardless of the dietary concentration. In another study in seabream (*Pagrus auratus*), Glencross et al. (2003) found variability in retention of biologically important fatty acids EPA, DHA, LA and

ALA. Seabream were fed with FO, rapeseed or soybean. The efficiency of retention times of individual fatty acid varied with the diet. Both LA and ALA were selectively retained when fish were fed FO diet but were selectively catabolised when the diet was rich in LA or ALA. Both EPA and DHA retention were unaffected by the change in diet. In another study, Bell et al. (2002) were able to replace 50% of fish oil with vegetable oil in Atlantic salmon diets resulting in minimal changes to muscle 22:6ω3. Similarly, Stubhaug et al. (2007) found that Atlantic salmon exhibited selective storage of ω3 HUFA when fed a diet deficient in HUFA. However, a similar efficiency in retaining ALA and LA was not shown by fish fed the HE diet. Although both ALA and LA are essential precursors for ω3 and ω6 HUFA synthesis, respectively, surplus of dietary HUFA in HE diet fed fish had a negative impact on the retention of ALA and LA in fish muscle.

In steelhead trout, muscle lipid class composition was closely related to diet, especially the TAG composition. TAG was the predominant lipid class present in the NL fraction of fish, which closely resembles the NL fraction of the diet. Lipid is the primary source of energy in fish. The amount and location of lipid deposition in fish could vary depending on the species and physiological status of the fish. Atlantic salmon are known to store lipid mostly in viscera and muscles (Jobling et al., 2003). Jobling et al. (2003) found that Atlantic salmon parr mostly stored lipid in viscera followed by the carcass (skin and bones) and muscle. Although polar lipid composition is known to be influenced by diet (Bell et al., 1999; Jump, 2002; Regost et al., 2003), the polar lipid composition in this experiment was not significantly different among groups. The only difference was seen with SF diet fed fish at 16.5^{o}C over the other 2 feed types, but there was no pattern in change of polar lipids during the experimental period. The fatty acid composition of

muscles in fish more closely resembles the NL composition in the diets than the polar lipids (Jobling and Bendiksen, 2003). Several previous studies with similar results have attributed the lesser dietary response on muscle polar lipid fraction due to the involvement of fewer dominant fatty acids (16:0, 18:1ω9, EPA and DHA) in structural phospholipids (Higgs and Dong, 2000; Sargent et al., 2002; Jump 2002). Deposition of excess dietary neutral lipid, mainly TAG could over shadow the polar lipid in muscle tissue (Tocher, 2003).

Fish are known to selectively incorporate fatty acids to form TAG, mainly by incorporating SFA, MUFA or PUFA to *sn*-1 and *sn*-3 position and a HUFA to *sn*-2 position (Tocher, 2003). Arts et al. (2001) also found a close correlation between the diet and the fatty acid incorporated into *sn*-3 position of neutral lipid, mainly TAG. The total PUFA proportion was significantly higher in FLX diet than both HE and SF. The HE diet was rich in marine fatty acids (mainly HUFA), which was the main contributor towards the total PUFA composition. Terrestrial fatty acids were the main contributors in both SF (18:2ω6) and FLX (18:3ω3 and 18:2ω6) diets. Both SF and FLX diets had significantly lower and similar HUFA content compared with the HE diet. These compositions directly correspond to the dietary intake.

The dietary influence on muscle fatty acid compositions were mainly seen in muscle tissue during the last 3 increments in temperature. The SFA compositions of HE diet fed fish were greater than both SF and FLX fed fish from the 2nd (12.5^{o}C) until the final increment (18.0^{o}C). The higher SFA value in HE diet fed fish directly corresponds with the high composition of SFA in HE diet. Fish are known to increase SFA in their tissues with exposure to higher water temperatures (Fodor et al., 1995; Jobling et al.,

1995; Logue et al., 2000). However, both SF and FLX fed fish were unable to increase the total SFA composition in muscle tissue in response to temperature increase, although changes were seen in some fatty acids: 14:0, 16:0 and 18:0. Decreasing percent composition of a particular muscle tissue fatty acid indicates selective catabolism (Kiessling and Keissling 1993). The concentration of 14:0 in muscle tissue of HE diet fed fish was well below the corresponding concentration in the HE diet. This indicates selective utilization of 14:0 by HE diet fed fish. Both SF and FLX diet fed fish showed a similar trend with increasing temperature although the dietary composition of 14:0 in both SF and FLX diets were significantly lower than HE diet. However, the concentration of both 16:0 and 18:0 in muscle tissue of fish fed all 3 diets were higher than the dietary composition, indicating preferential deposition over 14:0. Selective deposition of 16:0 and 18:0 fatty acids in rainbow trout were also reported by Bell et al. (2003), suggesting their importance in structural involvement in membranes at the *sn*-1 position (Bell et al., 1991).

The response of muscle MUFA, PUFA, ω3 and ω6:ω3 ratio of HE and FLX diet fed fish was similar as opposed to SF diet fed fish over the last 3 temperature increments. Both MUFA and ω6:ω3 ratio of SF fed fish increased with increasing temperature, with a corresponding increase in PUFA and ω3 content of HE and FLX fed fish. Previous studies have indicated a reduction in MUFA content in fish tissues with increasing temperature (Cossins and Lee, 1985; Fodor et al., 1995; Jobling et al., 1995; Logue, 2000), which corresponds to the results for MUFA in both HE and FLX fed fish. Fish fed SF diet had significantly higher 18:1ω9 composition in muscle compared to the other two fish groups at any temperature due the high composition in the SF diet. Preferential

catabolism or deposition of MUFA, mainly 18:1ω9, were seen in fish fed all 3 diets. All 3 fish groups showed a preference in catabolising MUFAs with increasing temperature (especially from 14.5°C onwards) as opposed to selective deposition. However, differences were seen in MUFA metabolism during the initial stages of the experiment with change in diet and subsequent increase in temperature. The tissue composition of MUFA (mainly 16:1ω7, 18:1ω9 and 18:1ω7) at 10.0°C was higher in FLX fed fish compared to the dietary composition. This indicates an initial deposition of MUFA similar to the deposition of SFA at the same temperature. However, with subsequent increase in temperature the tissue MUFA composition, mainly 18:1ω9 and 16:1ω7 decreased, indicating increased preference in catabolism of the fatty acids. Similarly, the 18:1ω9 composition in muscle tissue of HE fed fish decreased with subsequent increase in temperature. This indicates increased utilization of this fatty acid by HE diet fed fish. However, increasing temperature had no effect on the proportion of 18:1ω9 in the fish fed SF diet (Jobling et al., 1995; Logue et al., 2000). The high occurrence of 18:1ω9 in the SF diet is the most likely reason for the above finding in SF diet fed fish. Dietary lipids play a key role in energy production through β-oxidation in salmonids and are also known for their protein-sparing effect (Hemre and Sandnes, 1999). Muscle tissue is a primary site of β-oxidation in fish and it is greatly influenced by dietary factors (Henderson and Tocher, 1987). Therefore, replacing dietary fish oil with vegetable oil could significantly affect the fatty acid composition in muscle tissue as well as the β-oxidation capacity and respective fatty acid preference in trout (Bell et al., 2003; Torstensen et al., 2004; Stubhaug et al., 2007). The findings of MUFA metabolism in steelhead trout juveniles are in line with previous studies conducted on rainbow trout. Another abundant fatty acid

22:1ω11, commonly found in fish TAG, is primarily utilised for β-oxidation. However, the presence of other MUFA, including 22:1ω11 (C_{16} monoenes with ω5, 9 and 11; C_{18} monoenes with ω5 and 6; C_{20} monoenes with ω7 and 9; C_{22} monoenes with ω7, 9) was less than 1% of the total lipid (and consequently were not presented in the results). The C_{20} and C_{22} MUFAs are a major component of TAG in wild fish and are known to derive from wax esters of zooplankton (Tocher, 2003). Bell et al. (2003) found that 18:1ω9 is preferred over 22:1ω11 for β-oxidation when abundant in the diet of rainbow trout.

Replacement of fish oil (HE) with SF or FLX oil in the feed caused significant compositional changes in terrestrial fatty acid content in muscle tissue of fish. Both SF and FLX diet fed fish had greater terrestrial fatty acid content in muscle tissue compared to HE diet fed fish, with FLX fed fish having the highest value at any given temperature. In contrast, a relative decrease in the terrestrial fatty acid composition was seen in fish fed HE diet. The 18:2ω6 in the diet primarily contributed towards the total muscle terrestrial composition in both SF and HE diet fed fish, with 18:3ω3 being the major contributor in FLX fed fish. Bell et al. (2003) found that similar concentrations of 18:1ω9 and 18:3ω3 in the diet results in selective catabolism of 18:3ω3 over 18:1ω9; similarly 18:2ω6 was preferred over 18:1ω9 when the diet was rich in linseed oil. Also, there was an apparent preference for catabolism of 18:3ω3 over 18:2ω6 when both fatty acids were in similar concentrations in the diet. The relative proportion of 18:1ω9 was higher than 18:2ω6 and 18:3ω3 in both HE and SF diets. Both 18:3ω3 and 18:2ω6 were equally preferred for β-oxidation in HE diet fed fish. The tissue fatty acid composition of both 18:1ω9 and 18:2ω6 in HE diet fed fish decrease gradually with increasing temperature, with both FAs demonstrating a relative decrease of over one third between 10.0 and 18.0°C. The relative

concentration of 18:3ω3 in muscle tissue also decreased in HE diet fed from 10.0 to 18.0°C. However, 18:3ω3 was less preferred over 18:2ω6 for β-oxidation due to high relative concentration of the latter in the HE diet over 18:3ω3. The behaviour of 18:1ω9, 18:3ω3 and 18:2ω6 in SF diet fed fish was different than HE diet fed fish. The SF diet is naturally rich in 18:2ω6 due to incorporation of sunflower oil, however, the diet was also rich in 18:1ω9 with relative concentrations higher than 18:2ω6 and 18:3ω3, respectively. In SF diet fed fish, there was neither a significant increase nor a decrease in the relative muscle fatty acid content of 18:1ω9 or 18:3ω3 over the thermal gradient. However, the tissue composition of 18:1ω9 in SF diet fed fish was considerably less than the diet, indicating selective catabolism of 18:1ω9 with increasing temperature. Similarly, the tissue content of 18:2ω6 in SF diet fed fish was considerably less than the diet. This indicates selective catabolism of the fatty acid; however, there was a net gain in the concentration of 18:2ω6 in muscle tissue of SF diet fed fish at the end of the experiment due to high proportions of 18:2ω6 in the diet. Both C_{18} MUFA and C_{18} PUFA are well known to be readily oxidized in salmon when present in high concentrations in the diet (Tortensen et al., 2004; Stubhaug et al., 2007). However, oxidation of C_{18} PUFA over C_{18} MUFA and ALA over LA, as suggested by Bell et al. (2003), was not seen in this study presumably due to high dietary concentration of C_{18} MUFA and LA in both HE and SF diets, and the concentration of ALA was significantly lower in both diets.

The relative preference and fatty acid catabolism of C_{18} MUFA and C_{18} PUFA (18:1ω9, 18:3ω3 and 18:2ω6) in FLX diet fed fish was different to the pattern suggested by Bell et al. (2003). Previous studies suggested selective preference in catabolism of 18:3ω3 and 18:2ω6 over 18:1ω9, and 18:3ω3 over 18:2ω6 (Bell and Dick, 1991; Bell et

al., 2001, 2003). The relative concentration of both 18:3ω3 and 18:2ω6 was higher than 18:1ω9 in FLX diet and also the relative proportion of 18:3ω3 was over half as much again higher than 18:2ω6 in the diet. However, at the end of this study the relative proportions of 18:1ω9 in muscle tissue was similar to that of it in the diet, with 18:1ω9 at 18.0°C being significantly lower than that at 10.0°C. The final amounts of both 18:3ω3 and 18:2ω6 in muscle tissue were less than their dietary content, but 18:3ω3 showed a gradual accumulation over the experimental period with increasing temperature. Accumulation of SFA and MUFA in tissues at higher temperature as opposed to colder temperatures has previously being shown (Cossins and Lee, 1985; Hazel and Williams, 1990; Fodor et al., 1995; Logue et al., 2000; Farkas et al., 2001; Hochachka and Somero, 2002). However, tissues investigated (e.g. liver) in the above studies were rich in PL as opposed to muscle tissue that usually has a high TAG content. The composition of muscle tissue is a reflection of fatty acid oxidation or deposition. The decreased content of both 18:1ω9 and 18:2ω6 in muscle tissue at 18.0°C in comparison to the initial composition at 10.0°C indicates oxidation with increasing temperature. However, selective catabolism of 18:3ω3 over 18:2ω6 was not seen in this study due to high dietary availability of 18:3ω3 in flax seed, which resulted in accumulation of this fatty acid.

Marine fatty acids (HUFA) significantly accumulated in muscle tissues of HE diet fed fish during the experimental period with no significant change in SFA fatty acid composition. Increasing temperature had no effect on muscle accumulation of HUFA. Several other studies have shown the opposing pattern in HUFA deposition in various other tissues, with reduction in the HUFA content accompanied by a rise in SFA level with the rise in water temperature (Cossins and Lee, 1985; Fodor et al., 1995; Jobling et

al., 1995; Logue et al., 2000). As opposed to dietary influences on tissue fatty acid compositions (mainly NL fraction in muscles and other storage locations), the change in environmental temperature primarily affects the PL fraction in tissues (Hazel and Williams, 1990; Fodor et al., 1995; Logue et al., 2000; Farkas et al., 2001; Hochachka and Somero, 2002). Therefore, high concentration of HUFA in muscle tissues of HE diet fed fish at the end of the experiment was due to dietary influence. Low availability of HUFA in both SF and FLX diet prompted the fish fed these diets to maintain the HUFA values over the study period. Stubhaug et al. (2007) reported similar results in Atlantic salmon, where fish fed vegetable oil rich diets retained 70% of EPA and DHA as opposed to fish fed fish oil rich diets which retained only 30%. Fish fed vegetable oil based diets used different fatty acids as the dominant energy substrate by selectively retaining HUFA. Fish fed both HE and FLX diets accumulated PUFA and ω3 fatty acids in muscle tissue towards the end of the experimental period regardless of the thermal increments with a corresponding decrease in MUFAs. In addition to HUFAs, terrestrial fatty acids also contributed towards the final PUFA content of FLX fed fish. In contrast, there were no significant differences among MUFA, PUFA, Σω3 and marine fatty acids of SF diet fed fish between the two temperature extremes. However, a lesser proportion of MUFA and terrestrial fatty acid in tissues compared to the diet indicates oxidation of such fatty acids.

The HE diet had 3 times more marine fatty acids (EPA + DPA + DHA) compared to FLX and SF diets. However, SF and FLX fed fish managed to maintain a muscle marine fatty acid composition of ~ 30% as opposed to fish fed the fish oil enriched diet (40%). The relative narrowing of the difference between the diet and muscle tissue is due to selective deposition of HUFA over oxidation and due to inclusion of fish meal in the

diet. Bimbo (2000) suggested that essential fatty acid deficiency of fish is unlikely if an appropriate amount of fish meal is included in the diet. The residual fat (~ 8-10%) of fish meal naturally contains 20 – 30% of ω3 HUFA, which invariably fulfils the essential fatty acid requirement of most marine fish. Herring meal (37%) was used as the fish meal supplement for the 3 experimental diets used in this study, which accounts for the HUFA content in SF and FLX diets (~ 10%). Bimbo (2000) suggested that a minimum inclusion of fish meal (300 g/kg of feed) would provide 0.5 to 1% of dry dietary ω3 HUFA, which is sufficient to fulfill the essential fatty acid requirement of fish. The amount of herring meal included in formulating the experimental diets in this study was well within this range. Therefore, a difference in specific growth of experimental fish due to net deficiency of essential fatty acids is unlikely. Most fish oil replacement studies have not seen any difference in fish growth due to replacement of the lipid supplement in the diet (Dosanjh et al., 1988; Tocher, 2000, Stubhaug et al., 2007). This finding highlights the capacity of steelhead trout to accumulate HUFA even with a significant dietary difference in HUFA between HE and SF, FLX diets. Bell et al. (2003) were able to replace 66% of fish oil with vegetable oil (rapeseed or linseed), with only a minimal reduction in ω3 HUFA content in Atlantic salmon. The final HUFA content in all 3 fish groups in the present study might have also been affected by temperature, because high temperature is generally known to reduce the HUFA content in tissues.

Sargent et al. (1999) highlighted the importance of dietary and tissue fatty acid ratios in fish oil replacement studies. Different ratios (P:S, ω6:ω3, EPA:AA and EPA:DHA) were calculated in the present study to further understand the effect of fish oil replacement on muscle fatty acid composition of steelhead trout. Inclusion of vegetable

oil invariably affects the ω6:ω3 ratio in diet, which will directly modify the tissue fatty acid composition in fish. The muscle ω6:ω3 ratio of both HE and FLX fed fish was significantly lower than fish fed SF diet. In addition, both HE and FLX fed fish had a decreasing trend of ω6:ω3 ratio with increase in temperature. In contrast, the higher ratio of ω6:ω3 in SF diet fed fish was mainly due to high ratio in the SF diet. Inclusion of excess sunflower oil is known to cause several negative effects on fish: cardiac histopathology, increased arachidonic acid and subsequent reduction in EPA and DHA concentration (Bell et al., 1993), increased susceptibility to diseases (Bransden et al., 2003), and decreased lipid digestibility (Menoyo et al., 2007). In all of the above studies, the tissue fatty acid composition was invariably altered with increased concentration of ω6 fatty acids. The elevation of ω6 fatty acid composition in tissues as a result of feeding vegetable oil rich diets increased the production of AA derived eicosanoids as opposed to EPA derived ones. The increase in AA derived eicosanoids had several detrimental effects on salmonids mainly affecting the stress response (Bell et al., 1996, 1997; Jutfelt et al., 2007). Both ω6 and ω3 derived eicosanoids are also known to have opposing effects with respect to inflammatory reactions (Robinson and Stone, 2006). Mediators derived from EPA and DHA (ω3) are anti-inflammatory, while AA derived eicosanoids (ω6) are pro-inflammatory and show disease propagating effects (Schmitz and Ecker, 2008). EPA, DHA and AA, all act as competitive substrates for eicosanoid producing enzymes (Healy et al., 2000), with AA producing more potent eicosanoids than the ones produce by EPA and DHA. Although both types of eicosanoids are essential for proper mediation of physiological functions, an increased ω6:ω3 ratio leads to production of more pro-inflammatory eicosanoids altering physiological response (Sargent et al., 1999;

Tocher, 2000). The EPA:AA ratio in the 3 diets used in this experiment was more or less similar, with only FLX feed having a slightly lower ratio. Although the EPA:AA ratio in muscle was less than the ratio in the respective diets, there were significant differences among the fish fed the 3 feed types. The fish fed HE diet had the highest EPA:AA ratio while fish fed SF diet had the lowest. The differences between fish oil and vegetable oil (SF and FLX) were very apparent in EPA:AA ratio with increasing temperature from 12.5 to 18.0°C. Fish fed HE diet had a significant accumulation of EPA during the last 3 temperature increments as opposed to the other 2 groups, where muscle EPA concentration of HE diet fed fish were similar to the dietary intake. However, the lower EPA:AA ratio in SF and FLX fed fish was primarily due to increased AA composition in muscle (3 fold increase compared the respective diets). Bell et al. (2003) suggested that substitution of fish oil with alternative lipids should be done with caution as it could result in fatty acid compositions in tissues that are less favourable to fish, as well as to human consumers. Substitution of fish oil with vegetable oil could lead to accumulation of 18:2ω6, which could be detrimental to the health of fish as well as to human consumers (Bell et al., 1997; Simopoulos, 1999; Seierstad et al., 2005). In the same study, Bell et al. (2003) made an attempt to "wash out" the C_{18} PUFA of terrestrial origin by providing previously vegetable oil fed fish with a diet containing 100% fish oil. During the wash out period (20 weeks) the HUFA content was restored to 80% in comparison to fish fed only fish oil, but the 18:2ω6 composition of vegetable oil fed fish was 50% higher than fish oil only fed fish. In a similar wash out study, the organoleptic qualities (appearance, texture and taste) of turbot (*Psetta maxim*) were affected by replacing FO with soybean and linseed oil (Regost et al., 2003). Therefore, any alterations in the

composition of 18:2ω6 could lead to excessive accumulation and change the ω6:ω3 ratio in muscle tissue.

2.7. Conclusion

Both studies show the effects of fish oil substitution on muscle lipid composition in steelhead trout. The dietary fish oil substitution is more marked in study ii. However, inclusion of fish meal and fish oil to fulfill the minimal protein and essential fatty acid requirement in fish for all feed formulas resulted in similar growth among the fish groups in both studies without developing pathological symptoms due to deficiencies. The dietary lipid directly influenced the muscle fatty acid compositions in both adult and juvenile fish during the relatively short experimental period. Both H-ω3 and HE diet fed fish readily accumulated marine origin HUFA in muscle tissue with a corresponding decrease in terrestrial fatty acids. Similarly, fish fed fish oil substituted diets readily accumulated terrestrial fatty acids in correspondence with its dietary availability. However, fish fed fish oil substituted diets in both studies showed selective deposition and accumulation of marine fatty acids minimizing the dietary differences. Inclusion of vegetable oils, mainly SF and FLX seed oils, has a direct influence on Σω6 fatty acids and ω6:ω3 ratio in muscle fatty acids which could be detrimental to the health of the fish, as well as the quality of the end product for human consumption. The effect of temperature on muscle lipid composition was only seen in study i. The temperature effects are mostly seen in tissues that are rich in polar lipid. However, TAG was the predominant lipid class present in muscle tissues of both adult and juvenile steelhead trout used in both of the above studies. Therefore, the thermal influence on the polar lipids in muscle tissue was not prominent, with the dietary influence overpowering the thermal effects.

2.8. References

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Chapter 3

Effect of diet and temperature on liver cell membrane fluidity and lipid class and fatty acid composition in adult steelhead trout (*Oncorhynchus mykiss*)

3.1. Introduction

Fish cellular membranes are capable of reacting to temperature change by altering membrane lipid components (Hazel, 1984). The structural integrity of membranes is partly determined by the ambient water temperature. Successful acclimation of the organism to its surrounding is determined by compensatory restructuring of the membrane lipid composition (Craig et al., 1995; Fracalossi and Lovell, 1995; Labbe et al., 1995; Kelly and Kohler, 1999; Grisdale-Helland et al., 2002).

During the restructuring process the chemical composition of membranes is changed so that the phospholipids and the constituent acyl chains are matched to the existing thermal environment, resulting in a change in physical properties of the membrane (Hazel and Williams, 1990; Fodor et al., 1995; Logue et al., 2000; Farkas et al., 2001). The natural tendency of the membranes with decreasing water temperature is to increase motional freedom of acyl chains and constituent phospholipids, whereas the opposite is true with increasing temperature (Farkas et al., 2001). Fish achieve this by altering chemical composition of the membranes, and maintaining the physical characteristics, unit cell dimensions and order independent of growth temperatures. This well known response of all poikilotherms was first described by Sinenskey (1974) as "homeoviscous adaptation" in *Escherichia coli*.

Among the constituents of cell membranes, lipids are the only known structural components that modify with acclimation temperature, which could alter the function of membrane proteins (Shinitzky, 1984). Such alterations could disrupt various membrane associated functions, such as membrane bound enzyme and associated transport activities, membrane permeability and function (Hazel and Williams, 1990; Stillwell and Wassall, 2003; Wassall, et al., 2004). The membrane response elicited in fish due to change in acclimation temperature is attained by a host of different activities, such as altering the acyl chain composition by unsaturation, modifying the distribution of fatty acids within the phospholipid molecules, altering the phospholipid head group composition and also by altering sterol to phospholipid ratios (Hazel, 1984; Farkas et al., 2001). Membrane alterations and underlying metabolic responses are mainly studied in prokaryotes (Hazel, 1984). However, the metabolic adjustments in response to simultaneous alterations in diet and temperature on chemical and physical properties of membranes in fish are not defined. Several studies have been conducted to determine the effect of temperature on fatty acid and membrane composition in fish (Cossins and Lee, 1985; Henderson and Tocher, 1987; Malak, et al., 1989; Hazel and Williams, 1990; Sørensen, 1993; Wallaert and Babin, 1993, 1994; Delgado et al., 1994; Fodor et al., 1995; Jobling et al., 1995; Logue et al., 2000; Farkas et al., 2001). The long chain HUFA incorporated into cell membranes of marine fish mainly originate in diets due to limited synthetic capacity of marine fish. Therefore, it is important to understand the metabolic response and associated dietary requirements, especially in marine aquaculture.

The diet affects the availability of fatty acids, which influence the molecular configuration of phospholipids (Arts and Kohler, 2009). Even though diet largely

influences non-polar NLs (mainly TAG), the membrane PLs are also known to be influenced by the diet (Bell et al., 1999; Jump, 2002; Regost et al., 2003). The *sn*-1 and *sn*-2 positions of the glycerol backbone of the PL molecule are primarily occupied by SFA or MUFA and HUFA, respectively. Marine fish preferentially incorporate HUFA to the *sn*-2 position, which are mainly derived from the diet. Therefore, dietary perturbations that affect the availability of HUFA for marine fish could adversely influence the fish's ability to maintain membrane fluidity with subsequent change in environmental temperature (Herbert et al., 2008) and other aspects of membrane functionality. There are examples from natural ecosystems where decreased HUFA content in the diet of fish exposed to cold temperatures had adverse effects on the population. For example, decreasing populations of lake white fish (*Coregonus clupeaformis*) in lakes Michigan and Huron were mainly attributed to decreased populations of the amphipod *Diporeia* species, which are rich in both EPA and DHA (Nalepa et al., 2006). The decrease in dietary EPA and DHA, which are important constituents of membranes and EFA in maintaining membrane fluidity, had a significant effect on lake white fish, which spent a significant amount of time feeding at the cold lake bottom (Nalepa et al., 2006; Herbert et al., 2008). Similarly, the underlying cause for disease outbreak in Chinook salmon (*Oncorhynchus kisutch*) in Lake Michigan was thought to be triggered by poor nutrition, mainly due to decreases in ω3 HUFA content in the diet (Benjamin and Bence, 2003; Snyder and Hennessey, 2003; Madenjian et al., 2005). Climate change and global warming also affect the availability of ω3 HUFA in cold water ecosystems. The decreasing populations of ω3 HUFA rich diatoms, cryptophytes and dinoflagellates are being replaced with ω3 HUFA poor chlorophytes and cyanobacteria (Brett and Muller-

Navarra, 1997; Muller-Navarra et al., 2004; Brett et al., 2006), thus decreasing the availability of ω3 HUFA for higher trophic levels. Similarly, algae which are the main source of ω3 HUFA for planktivorous fish low on the food chain decrease the accumulation of ω3 HUFA in warmer temperatures. This could adversely affect fish preying on ω3 HUFA rich fish at lower trophic levels resulting in alterations in the membrane composition (Schlechtriem et al., 2006).

Exposure of fish membranes to low temperature usually results in decreasing the proportion of saturates with a corresponding increase in unsaturated fatty acids. MUFAs or PUFAs mainly accumulate in response to low temperature (Cossins and Lee, 1985; Fodor et al., 1995; Jobling et al., 1995; Logue et al., 2000). The temperature-induced changes in fatty acid composition in membranes also depend on species, tissue and lipid type (Cossins and Lee, 1985; Hazel and Williams, 1990; Farkas et al., 2001, 2002). The temperature induced changes are mostly prevalent in membrane PLs as opposed to tissue NLs. Such adaptations in membrane lipid minimize the thermal influence on fish exposed to different environmental temperatures (Hazel and Williams, 1990; Fodor et al. 1995; Logue et al. 2000; Farkas et al. 2001). The lower melting point of HUFA accumulated in cell membranes greatly influences the fluidity of the membranes at colder temperature as opposed to saturated fatty acids with relatively higher melting points (Arts and Kohler, 2009). Although cell membranes require a certain degree of rigidity, adequate fluidity for lateral movement of constituent lipids and functional proteins is achieved by incorporating HUFA (Eldho et al., 2003). DHA is one of the most important ω3 HUFAs incorporated into cellular membranes due to long acyl chains with six double bonds which increase the chain flexibility (Huber et al., 2002). DHA is one of the main

membrane constituents associated with various functions of the membrane such as permeability, fusion, elasticity and vesicle formation and sustaining membrane associated enzymes (Hazel and Williams, 1990; Stillwell and Wassall, 2003; Wassall, et al., 2004).

The first double bond in "*cis*" conformation greatly influences the physical property of the membrane by decreasing the melting point, but subsequent additions of double bonds to the acyl chain of the PL molecule progressively have a lesser effect (Coolbear et al., 1983; Stubbs and Smith 1984). The other areas of interest that have yet to be fully elucidated are the influence of membrane-bound proteins and their interaction with PL with changes in temperature (Budda et al., 1994). Several studies have shown the effect of PL head groups and different combinations of acyl chains attached to the *sn*-1 and *sn*-2 positions on fluidity of membranes (Dey et al., 1993; Budda et al., 1994; Fodor et al., 1995; Farkas et al., 2000; Brooks, 2002).

One of the other important constituents in determining the physical properties of biological membranes are the sterols, mainly cholesterol. Cholesterol molecules have a close association with membrane PLs, where they interfere both with the motion of acyl chains and the close packing of head groups of the PL molecules to control the fluidity of the membranes at both high and low temperature. Cholesterol is a primary constituent of highly ordered microdomains called "lipid rafts" in seemingly disordered fluidized membranes (Simon and Ikonen, 1997). Lipid rafts are self forming membrane domains enriched in cholesterol and sphingolipids (mainly sphingomyelin). The hydrophobic cholesterol backbone aligns parallel with the acly chains of the sphingolipids in the rafts and stabilizes the raft by forming van der Waals interactions and hydrogen bonds between the –OH group of cholesterol and the –NH- of sphingomyelin. Preferential and

spontaneous movement of cholesterol between mostly saturated acyl chains of sphingolipids increases the overall stability of the microdomain and pharmacological extraction of cholesterol has resulted in destroying the rafts (Gulbin et al., 2006). Local accumulation of such domains results in the transition of cell membrane to a more ordered liquid crystalline phase (Gulbins et al., 2006). There also exists another subset of lipid rafts called caveolae, which are invaginations of the membrane enriched with an integral protein called caveolin (Razani et al., 2002). In addition to reducing the fluidity of the membranes, these lipid rafts contain many signalling molecules, protein receptors and membrane bound enzymes such as protein kinase C and adenylate cyclase (Pike, 2003; Chini and Parenti, 2004).

There are several methods used to quantitatively assess the membrane lipid order at the cellular level. The most commonly used methods include steady-state fluorescence anisotropy, electron spin resonance (ESR) spectroscopy, attenuated total reflectance-Fourier transform infrared (ATR-FT-IR) spectroscopy and Raman spectroscopy. The degree of disordering of the fatty acyl chains is usually measured by Raman spectroscopy (see methods for further details) in terms of stretching of the CH_2 groups in the acyl chains of the PL molecules. Fluid membranes will have a higher C-H stretching frequency as opposed to CH_2 groups with a lower stretching. In steady-state fluorescence anisotropy various synthetic fluorescent probes are used and the degree of polarization of the probe is used to measure fluidity. An inverse relationship exists between the degree of polarization and fluidity of the membrane (Hossain et al., 1999). In ESR, an electron spin label is incorporated to the membrane and the rate of motion of the spin label, which is measured by an electron spin resonance spectrometer, is considered directly proportional

to the fluidity of the membrane (Buda et al., 1994). Finally, the ATR-FT-IR method is used together with fluorescence recovery after photo-bleaching to measure the fluidity of the sample (Hull et al., 2005).

Few studies have simultaneously investigated the influences of dietary lipid sources and temperature on tissue fatty acid compositions giving variable results (Fracalossi and Lovell, 1995; Labbe et al., 1995; Kelly and Kohler, 1999; Grisdale-Helland et al., 2002). These studies were primarily focusing on determining the lipid composition of different tissues and none of the studies measured the cell membrane fluidity. Other studies that made inferences regarding membrane fluidity of various tissues were primarily interested in the lipid compositional changes in membranes due to the effect of temperature alone or by measuring fluidity of various tissues of fish acclimated to two distinct temperatures or by feeding two distinct diets at the same temperature (Behar et al., 1989; Hazel and Williams, 1990; Fodor et al., 1995; Logue et al., 2000; Farkas et al., 2001). The present study simultaneously evaluated the effect of diet and change in temperature on membrane fluidity in fish.

This study measured the fluidity of liver membrane lipid of steelhead trout fed H-ω3 (higher ω3), M-ω3 (medium ω3) and L-ω3 (lower ω3) diets (Tables 2.1 and 2.2). Measurements were done on samples collected at acclimation temperatures of 13.5°C at the beginning and after increasing the temperature to 18.0°C and finally after dropping the temperature to 13.5°C. The objective of the study was to investigate the effect of temperature on liver cell membrane fluidity of steelhead trout fed commercial diets with different levels of HUFA and lipids originating from different sources such as marine, terrestrial and vegetable oil. Liver is a physiologically important organ for growth and

survival of fish that takes part in nutrient metabolism, removing toxins from the body and producing bile

3.2. Materials and methods

3.2.1. Experimental fish and tanks

Fifty five fish averaging 1.6 kg were raised in six 6000 L experimental tanks as described in sections 2.2.1 and 2.2.2.

3.2.2. Experimental feed

Three different commercially available diets were fed during the experimental period. Based on the lipid analysis the 3 diets were named according to the relative proportions of ω3 as H-ω3 – higher level of ω3; M-ω3 – medium level of ω3; L-ω3 - lower level of ω3 as described in section 2.2.3.

3.2.3. Experimental temperature

Fish were left for almost 2 weeks at 13.0 ± 1.0°C to acclimatize to the experimental tanks. The average temperature was increased in a stepwise manner from 13.5°C (1st sampling) to 16.5°C (2nd sampling) to a maximum of 18.0°C (3rd sampling) following ambient environmental temperatures (Figure 2.1), but with plateaus. Thereafter, the temperature was dropped from 18.0°C back to 13.5°C (5th sample) with the same in-between step as described in section 2.2.4.

3.2.4. Sampling protocol

Sampling followed the same procedure as described in section 2.2.5. Samples of liver tissue weighing approximately 1 g were collected in 50 ml glass vials previously cleaned for lipid residues by rinsing 3 times with methanol (MeOH) and chloroform ($CHCl_3$). Then each vial was flushed with nitrogen (N_2) after filling with 4 ml $CHCl_3$ and sealed with Teflon lined caps and Teflon tape and stored at -20°C for later lipid analysis.

3.2.5. Lipid class and fatty acid analysis

Lipid extraction, lipid class separation and fatty acid analysis were done following the procedures described in sections 2.2.6. Total lipids were extracted from triplicate liver samples from each tank sampled at 13.5°C (1st sampling), 18.0°C (middle sampling) and 13.5°C (last sample) in $CHCl_3$/MeOH following Parrish (1999) using a modified Folch procedure (Folch et al. 1957). Lipid classes were separated using thin layer chromatography with flame ionization detection (TLC/FID) by means of a MARK V Iatroscan (Iatroscan Laboratories, Tokyo, Japan: section 2.2.6.2). Fatty acid methyl ester (FAME) derivatives were produced from crude lipid extracts by transesterification using 14% boron trifluoride (BF_3) in MeOH following a procedure based on Morrison and Smith (1964) as outlined in Parrish (1999). Analysis of the resultant FAME derivatives was carried out using an HP 6890 model gas chromatograph (GC) equipped with an HP 7683 auto-sampler (Agilent Technologies Canada Inc., Mississauga, ON, Canada) as described in section 2.2.6.3.

3.2.6. Raman spectroscopy

Raman spectroscopy is a vibrational molecular spectroscopy technique which derives from an inelastic light scattering process. With Raman spectroscopy, a laser photon is scattered by a sample molecule and looses (or gains) energy during the process. The amount of energy lost is seen as a change in energy (frequency in wave numbers) of the irradiating photon. This energy change is characteristic for a particular bond in the molecule. It is a technique which can be used for the analysis of solids, liquids and solutions and can even provide information on physical characteristics such as crystalline phase and orientation, polymorphic forms, and intrinsic stress.

The C-H stretching bands in the 2800 – 3100 cm^{-1} region were chosen as one of the regions of interest as the CH_2 symmetric stretching and asymmetric stretching modes in Raman at 2850 and 2890 cm^{-1}, respectively, are generally the strongest bands in the spectra of lipids. The frequencies of these bands are conformation-sensitive and also respond to changes of the *trans/gauche* ratio in acyl chains. This is also the case, although to a lesser extent, for the vibrational frequency changes due to the terminal CH_3 groups found at 2930 cm^{-1} (symmetric stretch) and 2960 cm^{-1} (asymmetric stretch). In this study, the CH_2 symmetric stretching at 2850 $cm^{-1}$of acyl chains was used to measure the liver cell membrane fluidity in adult steelhead trout in response to temperature and diet.

The Raman spectra were obtained using a LABRAM confocal microscope (Horiba Jobin Yvon Inc, Edison, NJ, USA) with a grating (1800 groves/mm), a Leica microscope equipped with a long-working distance objective with magnification factor of 50X, and a Peltier CCD detector. The spectra were obtained using the 532 nm green laser

(Ar ion laser) line. The filter D_0 (no attenuation) and the acquisition time of 15 sec were used in acquiring the spectra. The short time period was chosen to minimize the drying effects on the sample.

The Raman spectra of liver lipid extracts obtained at the start of the experiment (13.5°C), the middle (18.0°C) and after returning to 13.5°C. All experiments were carried out in NaCl-Trizma-HCl buffer and after mixing the liver lipid sample with an appropriate volume of buffer (see below for details), by placing a few drops of the sample on a concave pit in a microscopic slide and placing the slide on a heating-cooling stage. This heating-cooling stage is where the microscopic slide with the sample is placed on the Raman confocal microscope. A full spectrum of the sample (500 – 3300 cm^{-1}) was obtained at room temperature. Thereafter, temperature was dropped to 2°C and 3 spectrographs were obtained in the 2800 – 3100 cm^{-1} region. The temperature was gradually increased in 4°C steps up to 30°C and 3 spectrographs were taken at each incremental temperature (Figure 3.1).

3.2.7. Sample preparation for Raman spectroscopy

Ten miligrams of lipid were removed from the liver lipid extract (in $CHCl_3$) using a lipid clean glass pipette into a lipid clean glass vial. The volume of lipid extract corresponding to 10 mg lipid was predetermined using the total lipid data obtained through Iatroscan analysis. After pipetting the required volume of the lipid extract, the original sample was flushed with N_2, capped, and sealed with Teflon tape and stored at -20°C for future analysis. The newly obtained sub-samples for Raman analysis were first evaporated under a steady stream of N_2 to remove $CHCl_3$. Upon evaporation, 1 ml of

NaCl-Trizma HCl buffer, pH 7, was added using a lipid clean ashed pipette. Water used in preparation of the buffer solution in this study was double distilled, with the second distillation done from dilute potassium permanganate to remove all organic surface-active impurities (Keough et al., 1988; Nag et al., 1998).

After preparing the 10 mg ml^{-1} with NaCl-Trizma HCl buffer, the sample was capped with a Teflon lined cap and vigorously vortexed for approximately 5 minutes to form multi-lamellar vesicles as discussed previously by others (Chapman and Collin, 1965; Veldhuizen et al., 1998). All samples prepared for Raman analysis were flushed with N_2, capped and sealed with Teflon tape and kept in refrigerator.

3.3. Statistical analysis

Statistical analysis was done as in section 2.2.7 for liver lipid class and fatty acid analysis. The General Linear Model procedure of the Statistical analysis System (GLM procedure, SPSS 13.0 for Windows) was used to analyze the difference in wave number at each incremental temperature (2°C to 30°C) of the Raman study (one-way ANOVA). The effect of feed on shift in wave number of CH_2 symmetric stretching at 2852 $cm^{-1}$of acyl chains in the liver lipid extract of fish reared at initial (13.5 °C), mid (18 °C) and final (13.5 °C) temperatures was analysed as above. Multiple comparisons of means for wave numbers of fish fed the three diets were made using the Tukey test. Significance level was set at 0.05 for all the tests.

3.4. Results

Liver total and polar lipid, sterols, phospholipids and sterol:phospholipid ratios were not affected by diet or temperature. Liver neutral lipid (Figure 3.2), mainly triacylglycerols and sterols, were less than 0.7 ww^{-1} % in fish fed all 3 types of feed at the mid (18.0°C) and end points (13.5°C) of the experiment. Liver sterols (% total lipid) were below 10% at both high and low temperatures (Figure 3.3a). However, the liver lipid extracts of fish fed the 3 diets were rich in phospholipids (Figure 3.3b), averaging over 80% initially with the baseline diet (M-ω3) and a minimum of over 77% and 82% at both 18.0 and 13.5°C, respectively, for all 3 feed types. Both sterols and triacylglycerols were the main neutral lipids and the presence of triacylglycerols alone in the liver lipid extracts was very low compared to phospholipids. The highest triacylglycerol: phospholipid ratio (0.15) was reported for fish fed H-ω3 diet at 18.0°C (Figure 3.4).

The total and neutral lipid contents of the 3 experimental diets were not significantly different (Table 2.2). However, when the temperature was decreased from 18.0 to 13.5°C, the NL of M-ω3 fed fish was lower than that of the fish fed fish oil rich H-ω3 diet ($p = 0.045$) (Figure 3.2). The dietary difference seen in sterol content (Table 2.2) was not reflected in liver lipid class composition of steelhead trout (Figure 3.3a). The triacylglycerol:phospholipid ratio increased with increasing temperature from 13.5 to 18.0°C in fish fed M-ω3 diet ($p = 0.003$) (Figure 3.4), followed by a subsequent decrease with the decrease in temperature to 13.5°C ($p = 0.002$).

Liver ΣSFA, ΣMUFA, Σω3 and marine fatty acid compositions were not different among steelhead fed 3 diets at 18.0 or 13.5°C (Table 3.1). The liver tissue of L-ω3 fed

fish had lower fatty acid content of 14:0 ($p = 0.037$) and a higher content of both 20:3ω6 and 20:4ω6 fatty acid ($p \leq 0.040$) than that of H-ω3 fed fish at 18.0°C (Table 3.1). Similarly, the 18:2ω6 content of M-ω3 and L-ω3 fed fish was higher than that of H-ω3 fish at 18.0°C (Table 3.1). Decreasing temperature to 13.5°C resulted in disappearance of the significant differences seen at 18.0°C in 20:4ω6 and 14:0. However, the significance of 20:3ω6 seen at 18.0 °C remained the same in both M-ω3 and L-ω3 fed fish. The H-ω3 fed fish had higher 20:5ω3, 22:5ω3 and M-ω3 fed fish had higher 22:5ω6 over L-ω3 ($p \leq 0.015$) at the final temperature (13.5°C). Changing temperature had no significant effect on the PUFA content, P:S ratio or the ω3 fatty acid composition of fish fed the H-ω3 diet (Figure 3.5, 3.6 a, b). However, the increase/or and decrease in temperature significantly affected the liver PUFA, P:S ratio and total ω3 fatty acid composition (Figure 3.5 & 3.6 a, b) of L-ω3 and M-ω3 fed fish (Figure 3.5 & 3.6 a, b). Fish fed the M-ω3 diet were able to reduce the P:S ratio and Σω3 fatty acid composition in liver membranes ($p \leq 0.032$) with the increase in temperature to 18°C, but the subsequent drop in temperature to 13.5°C did not significantly increase the P:S ratio and Σω3 fatty acid composition within the same time period. The PUFA content, P:S ratio and Σω3 of fish fed L-ω3 diet decreased when the temperature was decreased from 18.0 to 13.5°C ($p \leq 0.049$) (Figures 3.5 & 3.6 a, b). A difference was also seen in P:S ratio of fish fed M-ω3 and L-ω3 diets at the final temperature of 13.5°C ($p = 0.043$) (Figure 3.6a).

The frequency of the $\nu_s CH_2$ stretching mode of the acyl chains near 2850 cm^{-1} is plotted as a function of Raman temperature for liver lipids of steelhead (Figures 3.7 – 3.9). This energy change is characteristic for a particular bond in the molecule and the

frequency near 2850 cm^{-1} was previously assigned for the $\nu_s CH_2$ stretching mode of the acyl chains of phosphatidylcholine (Spiker and Levin, 1975). Figure 3.7 shows results from trout fed the base diet M-ω3 at the beginning of the experiment. The effect of changing the diets and increasing the temperature to 18.0°C is shown in Figure 3.8 and the effect of the subsequent decrease in temperature to 13.5°C is shown in Figure 3.9. The thermotropic behaviour of liver lipid in steelhead trout fed the M-ω3 diet as monitored through temperature induced alterations in the frequency of $\nu_s CH_2$ stretching bands of the lipid acyl chains near 2850 cm^{-1} is shown in Figure 3.7. The sample was subjected to a temperature increase from 2 to 30.0°C in 4° increments.

The frequency of the average wave numbers corresponding to $\nu_s CH_2$ stretching shifted from ~ 2849 to 2855cm^{-1} with the increase in Raman temperature from 2 to 30°C for fish sampled at the beginning of the experiment (13.5°C, Figure 3.7). A phase transition of membrane lipids from gel to liquid phase occurred between 10.0 and 26.0°C. All fish were fed M-ω3 diet at this stage. Thereafter M-ω3 feed was replaced for 2 out of the 3 experimental groups and all 3 groups were examined using the Raman spectroscopy as described earlier.

L-ω3 fed fish had higher wave numbers than both M-ω3 and H-ω3 fed fish for the $\nu_s CH_2$ stretching frequency at 18.0°C at each Raman temperature (2 to 30°C) ($p \leq 0.044$, Figure 3.8). The $\nu_s CH_2$ stretch wave numbers for liver membrane lipid of M-ω3 and H-ω3 fed fish were not different ($p > 0.05$) and ranged between 2850 and 2856cm^{-1}. The numbers observed were in the same range as the initial sampling with M-ω3 feed.

A clear phase transition was observed in fish fed the L-ω3 diet between 6 and 26°C, with an onset temperature around 14 - 16°C and a completion temperature of about 26°C (Figure 3.8). The sigmoid pattern of the regression line for L-ω3 indicates the transfer of membrane lipid from gel to liquid crystalline state. Increases in wave number with the increase in temperature are indicative of progressively increasing fluidity and lower wave numbers are indicative of higher number of *trans* bonds or condensations at low temperature (Mendelsohn and Mantsch, 1986). The relatively higher wave numbers of L-ω3 fed fish for $\nu_s CH_2$ stretch reflects the lower melting point of constituent liver membrane lipid compared to M-ω3 and H-ω3 fed fish sampled at 18°C. Accumulating a higher percentage of polyunsaturated fatty acids and a lower percentage of saturated fatty acids could lower the melting point of liver membranes in L-ω3 diet fed fish at 18.0°C.

The phase transition of liver membrane lipid from gel to liquid crystalline of fish fed M-ω3 and H-ω3 diet was less prominent compared to L-ω3 fed fish. However, a gradual increase in wave number for $\nu_s CH_2$ stretch was observed for both M-ω3 and H-ω3 fed fish, but both having a lower wave number compared to L-ω3 fed fish throughout the Raman temperature increase (Figure 3.8). This indicates a comparatively higher melting point of membrane lipids accumulated at 18.0°C in both M-ω3 and H-ω3 fed fish than L-ω3 fed fish.

Finally, the temperature was decreased to 13.5°C and fish acclimated for 2 weeks prior to obtaining the last samples (Figure 3.9). The frequency of the average wave numbers corresponding $\nu_s CH_2$ stretching shifted within the range of 2849 to 2859 cm^{-1} for all 3 feeds with the increase in Raman temperature from 2 to 30°C (Figure 3.9). The

$\nu_s CH_2$ stretching frequency of L-ω3 fed fish was significantly higher than numbers observed with the other two feed types, indicating accumulation of membrane lipid with a lower melting point. The $\nu_s CH_2$ stretching frequency at Raman temperature of 2.0°C for L-ω3 fed fish in the final sample (13.5°C) (Figure 3.9) was higher than the corresponding measurement at 18.0°C (Figure 3.8), indicating an increase in fluidity of membrane lipids due to the drop in temperature from 18.0 to 13.5°C.

Fish fed M-ω3 feed had a significantly lower vibration frequency than L-ω3 feed (Figure 3.9) and unlike at 18.0°C, the $\nu_s CH_2$ stretching frequency was even significantly lower than H-ω3 fed fish at Raman temperatures of 14.0 to 26.0°C. The behaviour of membrane lipids of M-ω3 fed fish at final sampling (13.5°C) were different from the initial sampling (13.5°C) (Figure 3.7 and 3.9) indicating the effect of temperature and diet. However, the liver membranes of fish fed the H-ω3 diet were not affected by change in experimental temperature (18.0°C or 13.5°C) and the $\nu_s CH_2$ stretching frequency remained within the same range during the Raman trial.

Table 3.1. Effect of diet and temperature on liver fatty acid (% total) composition of adult steelhead trout.

Fatty acid	Initial	Middle			Final		
Temperature	13.5°C	18.0°C			13.5°C		
Feed	M-ω3	H-ω3	M-ω3	L-ω3	H-ω3	M-ω3	L-ω3
14:0	0.9 ± 0.1	1.8 ± 0.3[a]	1.0 ± 0.6[ab]	0.9 ± 0.1[b]	0.9 ± 0.1	0.9 ± 0.2	0. 9 ± 0.4
16:0	15.6 ± 3.6	17.8 ± 2.1	18.5 ± 2.9	17.4 ± 3.3	19.1 ± 4.9	18.0 ± 3.1	20.9 ± 2.5
18:0	9.2 ± 1.6	11.9 ± 3.3	11.9 ± 1.5	11.0 ± 2. 8	13.5 ± 5.0	8.5 ± 1.8	13.2 ± 4.8
18:1ω9	9.5 ± 1.9	10. 7 ± 1.7	11.0 ± 1.2	10.5 ± 2.4	8.6 ± 2.8	12.3 ± 2. 6	10.3 ± 2.9
18:2ω6	2.3 ± 1.2	2.0 ± 0.5[a]	2.8 ± 0.5[b]	2.8 ± 0.6[b]	2.3 ± 0.9[a]	4.0 ± 0.5[b]	3.0 ± 0.4[ab]
18:3ω3	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
20:3ω6	0.8 ± 0.3	0.4 ± 0.1[a]	0.7 ± 0.2[b]	0.8 ± 0.2[b]	0.4 ± 0.6[a]	0.8 ± 0.3[ab]	0.8 ± 0.5[b]
20:4ω6	4.8 ± 0.2	3.6 ± 0.3[a]	4.2 ± 0.5[ab]	4.4 ± 0.4[b]	3.8 ± 0.7	4.8 ± 0.8	4.0 ± 0.4
20:5ω3	7.0 ± 1.0	7.1 ± 0.5	6.9 ± 0.8	6.2 ± 0.7	9.2 ± 2.2a	6.9 ± 1.9[ab]	6.0 ± 1.3[b]
22:5ω6	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.7	0.2 ± 0.03[ab]	0.3 ± 0.1[a]	0.2 ± 0.1[b]
22:5ω3	3.1 ± 0.4	2.9 ± 0.4	2.7 ± 0.6	2.9 ± 0.7	3.3 ± 0.5[a]	2.9 ± 0.3[ab]	2.2 ± 0.6[b]
22:6ω3	35.4 ± 2.3	29.6 ± 2.3	28.2 ± 5.4	31.0 ± 2.5	27.0 ± 3.4	28. 5 ± 5.5	26.8 ± 1.8
ΣSFA[1]	26.3 ± 3.2	31.8 ± 3. 8	32.8 ± 4.1	30.0 ± 6.1	34.5 ± 9.6	28.1 ± 4.6	35.9 ± 6.4
ΣMUFA[2]	17.1 ± 3.4	19.0 ± 3.1	18.8 ± 2.4	18.5 ± 3.8	16.5 ± 4.2	20.5 ± 4.0	18.1 ± 5.4
ΣPUFA[3]	56.4 ± 2.1	48.8 ± 2.7	48.7 ± 5.0	51.2 ± 3.6	48.8 ± 6.3	51.1 ± 6.5	45.8 ± 1.5
Terrestrial[4]	2.5 ± 1.2	2.2 ± 0.6	3.1 ± 0.5	3.1 ± 0.7	2.5 ± 1.0[a]	4.2 ± 0.6[b]	3.2 ± 0.4[ab]
Marine[5]	45.4 ± 1.8	39.6 ± 2.7	37.8 ± 5.5	40.1 ± 2.4	39.5 ± 5.2	38.3 ± 6.0	35.0 ± 1.6

Values are mean ± SD.
Significant differences among diets at each temperature are indicated by superscript letters.
[1]Sum of saturated fatty acids (SFA), which also includes: *i*15:0, 15:0, *ai*16:0, *i*17:0, *ai*17:0, 20:0, 22:0 and 23:0 at < 1.0% each.
[2] Sum of monounsaturated fatty acids (MUFA), which also includes: 14:1, 15:1, 16:1ω7, 16:1ω9, 16:1ω5, 17:1, 18:1ω7, 20:1ω9, 20:1ω7, 20:1ω11, 22:1ω11, 22:1ω9 and 24:1 at < 1.0% each.
[3] Sum of polyunsaturated fatty acids (PUFA), which also includes: 16:2ω4, 16:3ω4, 16:4ω3, 16:4ω1, 18:2ω4, 18:3ω6, 18:4ω1, 20:1ω9, 20:2a, 20:2ω6, 20:3ω3, 20:4ω3, 21:5 ω3 and 22:4 ω6 at < 1.0% each.
[4] Terrestrial fatty acid: 18:2ω6 and 18:3ω3.
[5] Marine fatty acids: EPA, DPA and DHA.
Polyunsaturated to saturate ratio (P:S), Omega (ω).

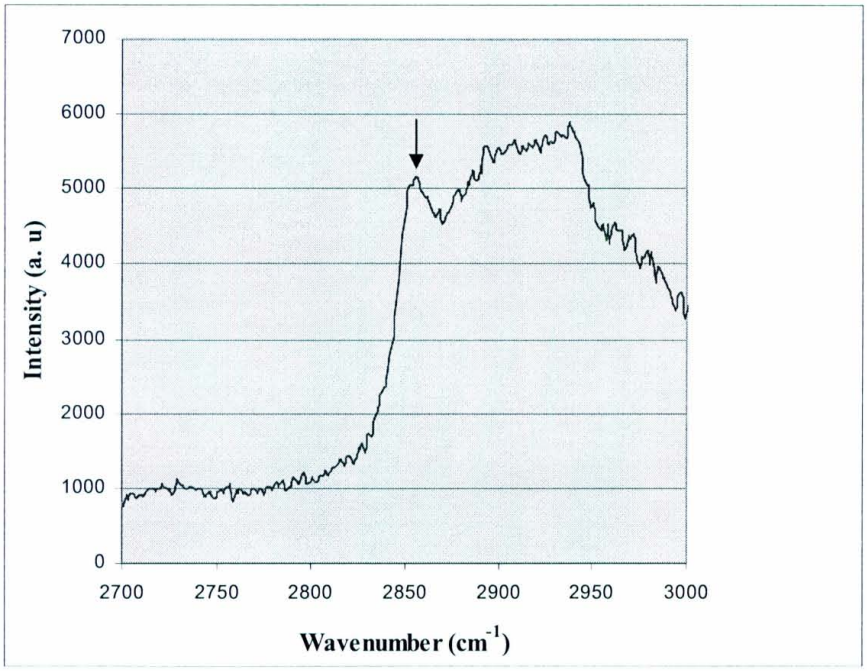

Figure 3.1. Raman spectra of liver tissue showing the CH_2 symmetric stretching band (ν_s) at 2852 cm. Raman bands have been assigned previously by others (Lippert and Peticolas, 1971; Spiker and Levin, 1975)].

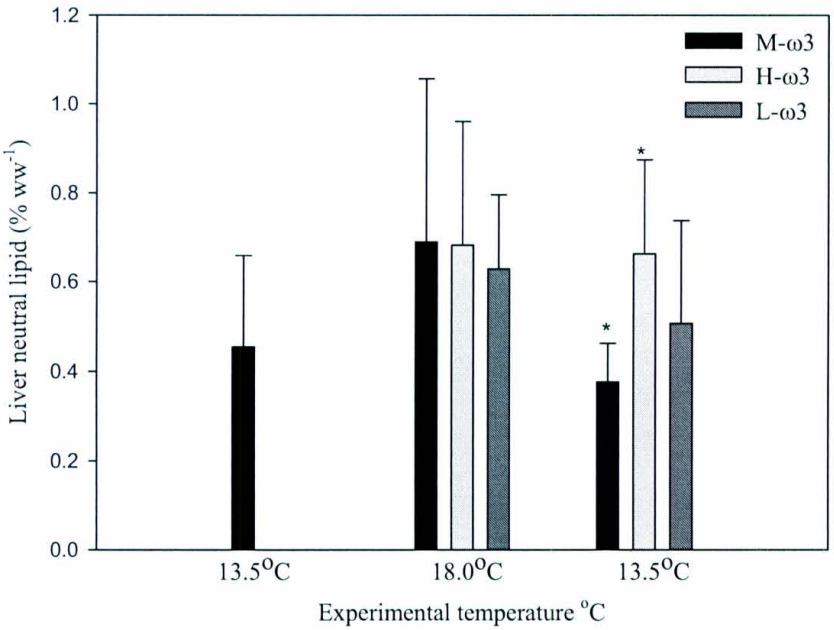

Figure 3.2. Effect of temperature and diet on liver neutral lipid (% ww^{-1}) in adult steelhead trout fed M-ω3, H-ω3 and L-ω3 diets. Asterisks indicate significant difference ($P < 0.05$) among the three feeds at a given experimental temperature.

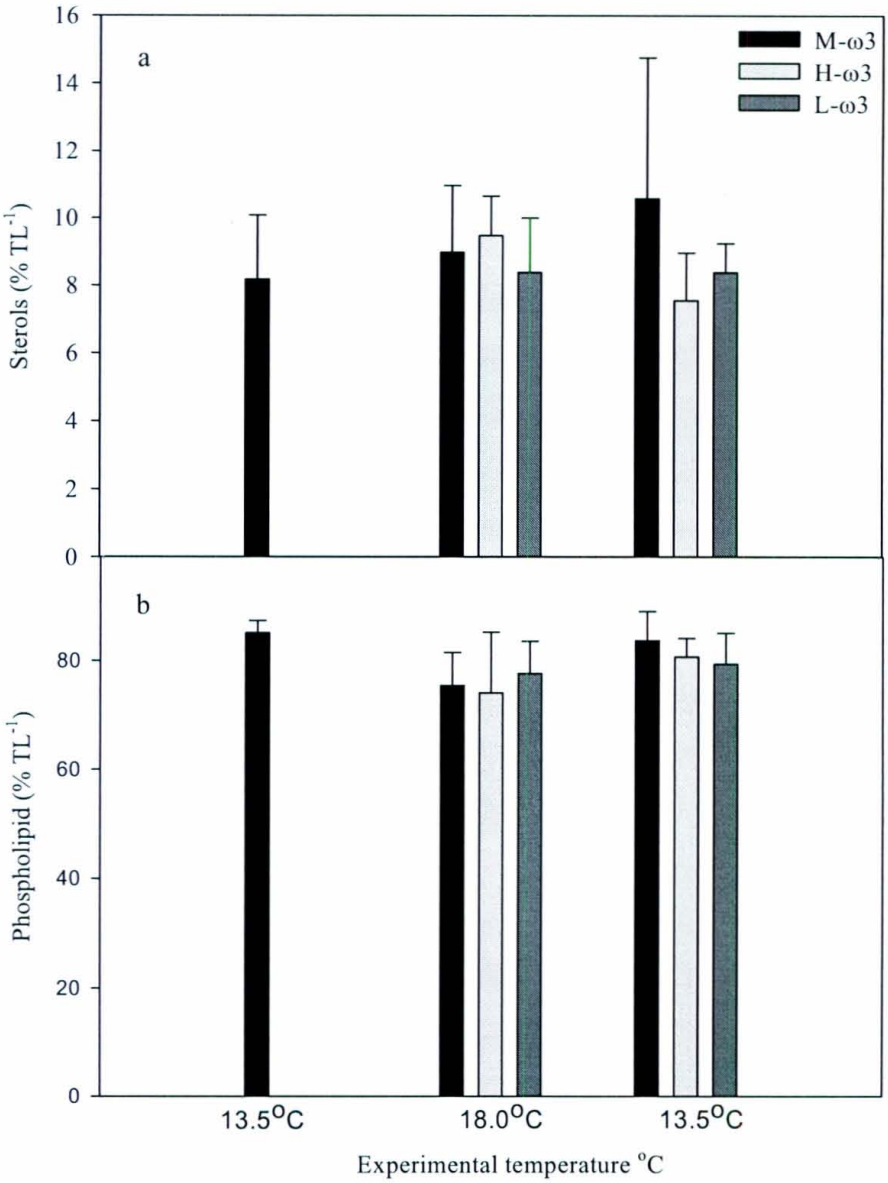

Figure 3.3. Effect of temperature and diet on liver sterol (a) and phospholipid (b) as % total lipid (TL) in adult steelhead trout fed M-ω3, H-ω3 and L-ω3 diets.

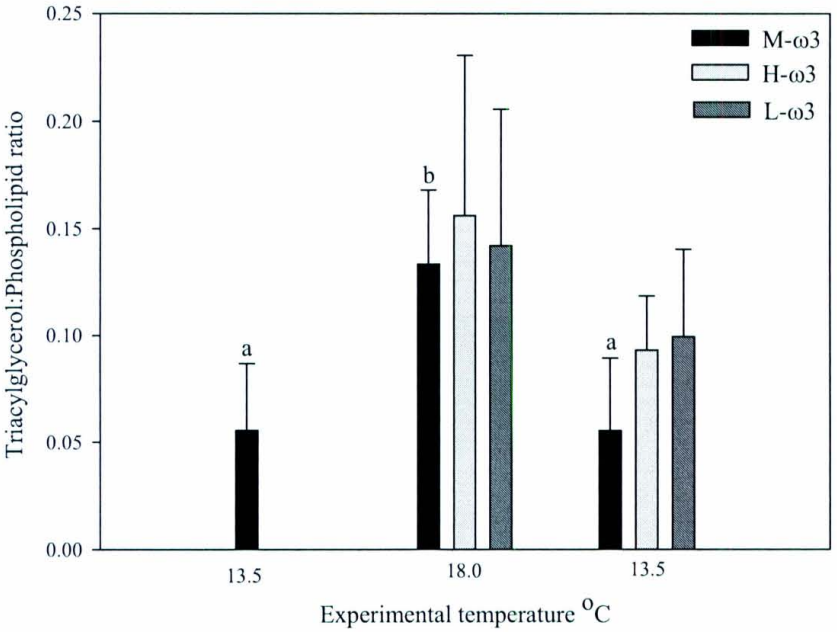

Figure 3.4. Effect of temperature and diet on liver triacylglycerol:phospholipid ratio in adult steelhead trout fed M-ω3, H-ω3 and L-ω3 diets. Letters indicate significant differences ($p < 0.05$) over the experimental temperatures for a given feed type.

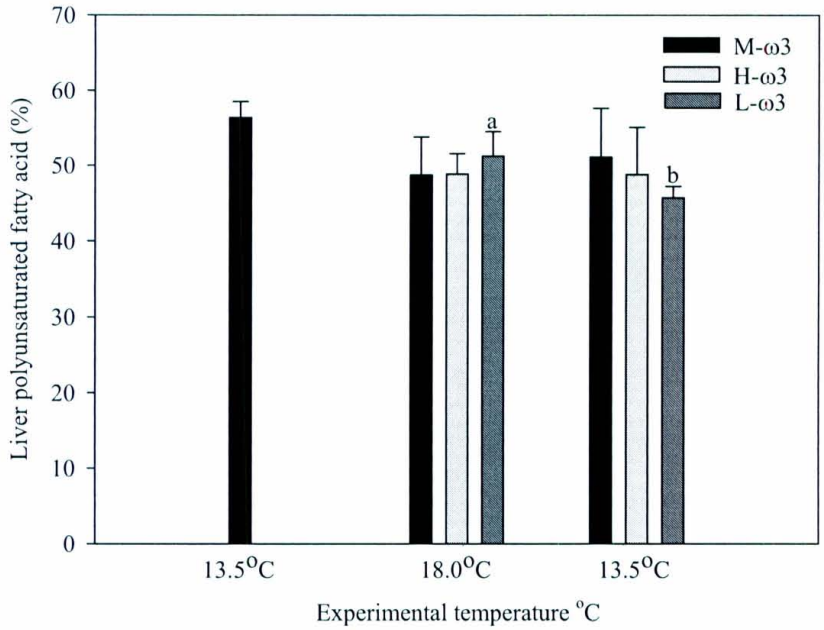

Figure 3.5. Effect of temperature and diet on liver polyunsaturated fatty acid (%) composition in adult steelhead trout fed M-ω3, H-ω3 and L-ω3 diets. Letters indicate significant differences ($p < 0.05$) among experimental temperatures for a given feed type.

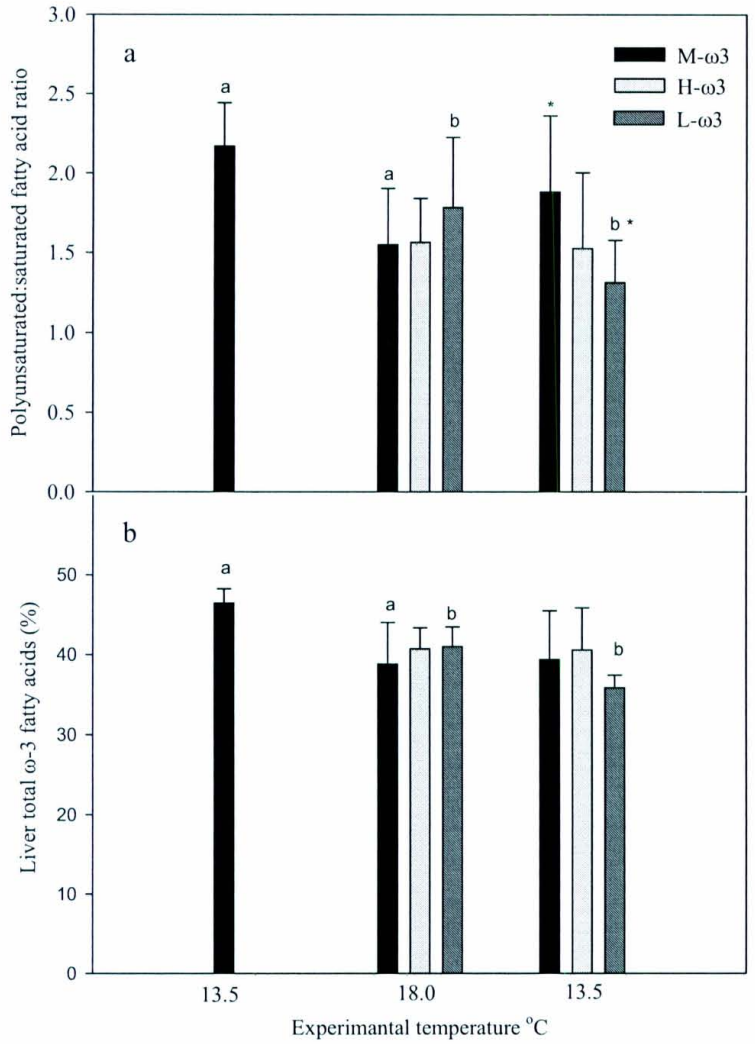

Figure 3.6. Effect of temperature and diet on liver polyunsaturate:saturate ratio (a) and total ω3 fatty acid (b) % composition in adult steelhead trout fed M-ω3, H-ω3 and L-ω3 diets. Asterisks indicate significant difference ($p < 0.05$) among the three feeds at each experimental temperature and similar letters indicate significant differences among individual feed groups between temperatures.

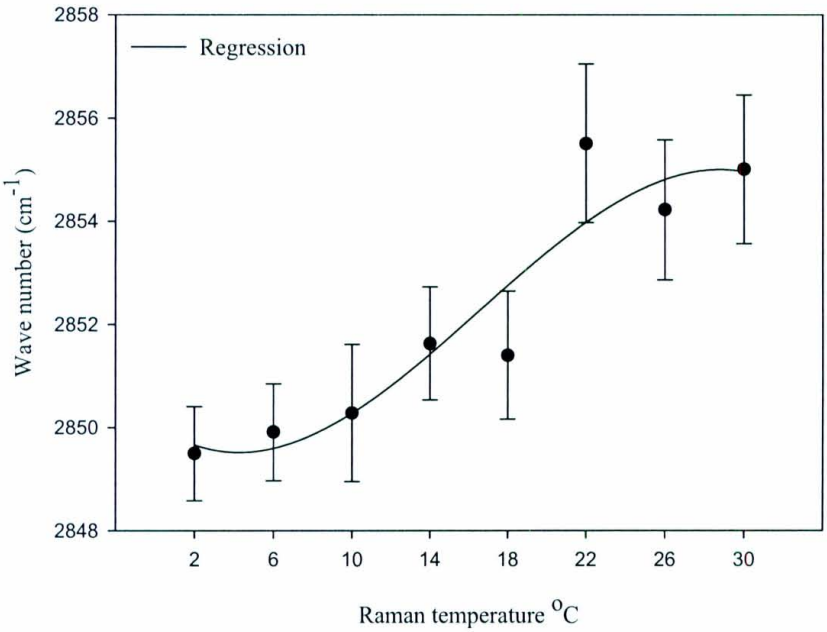

Figure 3. 7. Thermotropic behaviours in vibrational shift of Raman frequency for total liver lipid extract of adult steelhead trout fed M-ω3 feed at the beginning of the experiment 13.5°C. The parameter monitored is the frequency of the CH_2 symmetric stretching mode of the acyl chains near 2850 cm^{-1}.

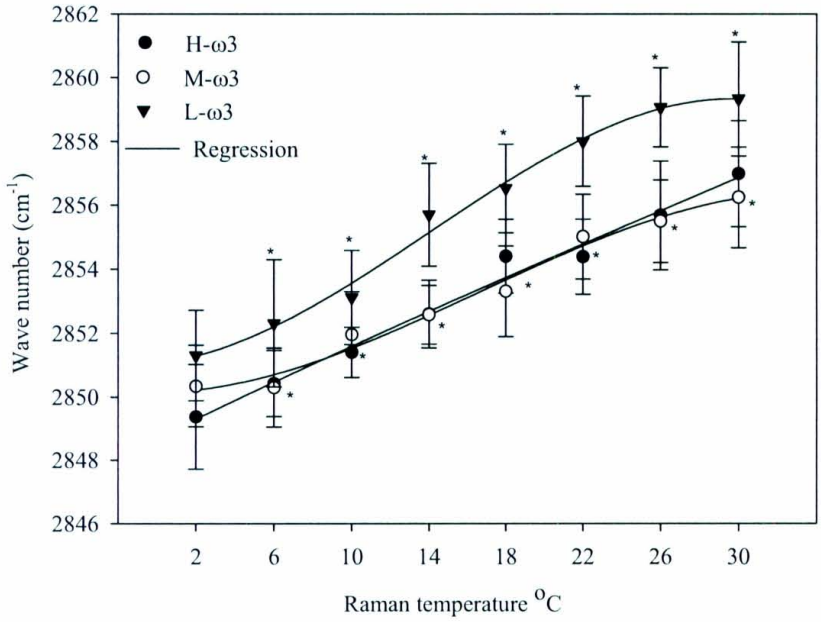

Figure 3.8. Comparison of thermotropic behaviours in vibrational shift of Raman frequency for total liver lipid extract of adult steelhead trout fed H-ω3, M-ω3 and L-ω3 feed at 18.0°C. The parameter monitored is the frequency of the CH_2 symmetric stretching mode of the acyl chains near 2850 cm^{-1}. Asterisks indicate significant differences ($p < 0.05$) in CH_2 symmetric stretch at each Raman temperature among the three feeds. Significant differences between L-ω3 and both M-ω3 and H-ω3 are indicated by "*". The wave numbers were not significantly different between H-ω3 and M-ω3.

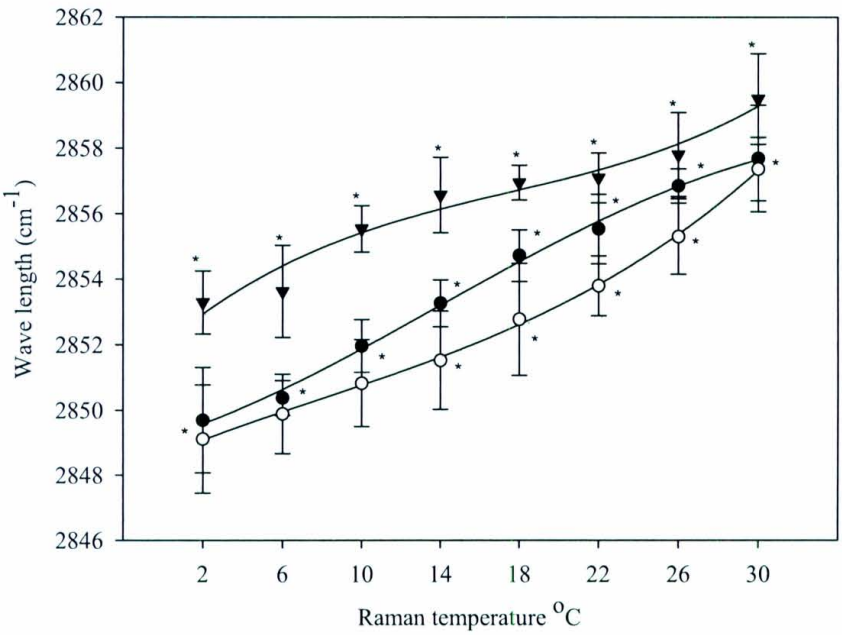

Figure 3.9. Comparison of thermotropic behaviours in vibrational shift of Raman frequency for total liver lipid extract of adult steelhead trout fed H-ω3, M-ω3 and L-ω3 feed at the end of the experimental period (13.5°C). The parameter monitored is the frequency of the CH_2 symmetric stretching mode of the acyl chains near 2850 cm^{-1}. Symbols indicate significant differences ($p < 0.05$) in CH_2 symmetric stretch at each Raman temperature among 3 feeds. Significant differences between L-ω3, H-ω3 and M-ω3 are indicated by "*" .

3.5. Discussion

Phospholipids were the main lipid class present in the liver lipid extracts of steelhead trout. Phospholipids are the main structural components of cell membranes and therefore any alterations in the fatty acid composition primarily reflect the effect of diet and temperature on steelhead trout liver cell membrane fatty acids. The close resemblance of diet and tissue lipid composition is more readily seen in non-polar neutral lipids, such as muscle tissue (Chapter 2) rather than the polar, structural phospholipids in the membranes. There is a contrasting difference in both the way diet and temperature influence fatty acid composition in fish. Dietary lipid primarily affects the muscle and visceral tissues, fatty acids altering the neutral lipid composition and in contrast, the environmental temperature markedly influences the membrane phospholipids causing adaptive changes in cell membrane characteristics (Hazel and Williams, 1990; Fodor et al., 1995; Logue et al., 2000; Farkas et al., 2001).

In this study, both diet and temperature had minimal effects on liver lipid class composition. The only significant differences observed in major lipid groups were the neutral lipid composition between H-ω3 and M-ω3 fed fish at the end of the experiment (Figure 3.2). However, membrane lipids responded to the change in temperature in a similar fashion to studies conducted in the past, where increasing temperature tended to increase neutral lipid, sterol and triacylglycerol:phospholipid ratio and decrease PL in liver tissue and reversal of the trend with the subsequent drop in temperature though neither was significant (Fodor et al., 1995; Jobling et al., 1995; Logue et al., 2000; Jobling et al., 2003).

Cholesterol is the most abundant sterol present in fish lipid which affects membrane physical properties, including fluidity and phase behaviour (Yeagle 1985; Jemiola-Rzeminska et al., 1996; Stillwell et al., 1996). Although polar phospholipids are the key constituent in cell membranes, cholesterol is one of the neutral lipids known to influence physical properties of a membrane during variations in environmental temperature (Crockett and Hazel, 1995; Robertson and Hazel, 1995; Zehmer and Hazel, 2003). However, in this experiment cholesterol composition was not significantly affected by diet or change in temperature and the dietary differences (Table 2.2) were not reflected in liver tissues. Cholesterol is known to order the fluid-phase membranes at high temperatures, particularly those that are rich in phospholipids containing highly unsaturated acyl chains. Cholesterol concentration increases to counter the effects of higher temperatures and decreases at low temperatures (Hazel and Williams, 1990; Crockett 1998). In this study, liver membrane cholesterol of steelhead trout fed H-ω3 diet closely followed the manner expected when perturbed with temperature (Figure 3.3a) resembling studies conducted in the past (Robertson and Hazel 1995; Zehmer and Hazel, 2003). However, there are reports where low body temperature resulted in no modification of cholesterol content (Crockett and Hazel, 1995; Labbe et al., 1995; Robertson and Hazel, 1995) or even elevated cholesterol levels (e.g., Crockett and Hazel, 1995).

Biological membranes are dynamic in nature and the term membrane "fluidity" defines the dynamic motions of membrane constituents (Mouritsen and Bloom, 1993). Dynamism of membranes is preserved by structural motion of various lipid molecules e.g.

bond rotations, bond isomerisation from *trans* to *gauche*, oscillation, lateral diffusion and flip flop type motions. Although various spectroscopic techniques are used to measure motion of lipids, no one technique can measure all motional parameters of membrane lipids. However, these techniques are capable of detecting the gel to liquid crystalline transition temperature (Mouritsen and Bloom, 1993). Here, Raman spectroscopy was used to study the conformational order of acyl chains attached to liver membrane phospholipids. Unlike most techniques used in investigating membranes, Raman spectroscopy is a non-perturbing technique that provides a snapshot of membrane structure on the order of 10^{-14} seconds (Ingel and Stanly, 1988).

The parameter examined in this study, the CH_2 symmetric stretching frequency, provides information on conformational order of constituent lipids (% total) in membranes. The temperature acutely influences molecular motion and rate of membrane constituents and phase state and order of membrane lipids in poikilotherms (Hazel and Williams, 1990). Studies are generally conducted on homogenous suspensions of phospholipids illustrating possible phase states of membrane lipids in a wide range of temperatures (Hazel and Williams, 1990). Such studies were interested in identifying specific influences of selected fatty acids, PL or PL head group, whereas the present study uses the total lipid content in liver. At low temperatures acyl chains of phospholipids become fully extended and closely packed with all *trans* configuration. This highly ordered close packing of acyl chains restricts chain mobility and lateral diffusion within the plane of bilayers and is considered a "gel" phase or state of the membrane. At higher temperatures, acyl chains tend to melt and become fluid in nature forming a state called "liquid crystalline phase". This phase is characterized by introduction of several gauche

rotamers per acyl chain, effectively reducing the length of acyl chains and thereby significantly increasing the motional rate and freedom. Consequently, the fluid nature of the membrane also loses membrane order resulting in lateral expansion with a reduction in bilayers thickness (reviewed by Housely and Stanley, 1982; Hazel and Williams, 1990).

In the present study, the *vs*CH_2 stretching frequency obtained by subjecting the liver lipids of steelhead trout to a temperature range of 2 to 30°C (during the Raman trial) provides an understanding of the degree of *trans* and *gauche* isomerisation of the membrane lipids. Also, it provides information on the influence of diet at respective rearing temperature on the phase state of the membrane.

The results show the stretching frequencies for fish fed M-ω3 diet at 3 stages of the study (Figure 3.7) at the beginning (13.5°C), middle (18.0°C) and once the temperature was back to 13.5°C. For H-ω3 and the L-ω3 fed fish, the frequencies were recorded only at 18.0°C and upon decreasing to 13.5°C. A comparison of the spread in wave numbers for the 3 fish groups in Figures 3.7 to 3.9, illustrates how the liver membrane fluidity changed with the alteration in the diet and temperature. Fish fed all 3 diets responded to increasing Raman temperature (2 to 30°C) as seen by the increase in wave number for CH_2 symmetric stretch. Such increase was expected due to the influence of temperature on acyl chains of the phospholipid molecules. However, what is important to consider is the difference in wave number at individual sampling temperatures, which highlights the effect of diet on the physical properties of the liver membrane. Changes in physical attributes of the membrane, besides fluidity, such as lipid composition,

membrane phase behaviour, membrane thickness, and membrane permeability (Hazel et al., 1998: Lee 2004; Hulbert and Else, 2005) could affect the membrane-associated proteins and their function (Wu et al., 2001; Starke-Peterkovic et al., 2005; Lee 2003).

Fish fed the H-ω3 diet had the least variation in fluidity when compared both at 18.0 and 13.5°C. At both temperatures, H-ω3 fed fish responded similarly to the increase in Raman temperature. This indicates that H-ω3 fed fish can quickly adapt to an increase or decrease in environmental temperature with the least effect on liver membrane physical properties. The H-ω3 diet is rich in fish oil and the substituent HUFA make it the closest to the natural diet of fish. This indicated H-ω3 fed fish had all the necessary elements to counteract thermal influences and adapt to the changing environment without changing the physical properties of the membrane. For example, docosahexaenoic acid with 6 double bonds has an extremely high degree of acyl chain flexibility (Feller et al., 2002; Huber et al., 2002) and affects several membrane properties including membrane permeability, membrane fusion, and elasticity and vesicle formation (Stillwell and Wassall, 2003; Wassall et al., 2004). H-ω3 fed fish may have benefited from the significantly higher polyunsaturate:saturate ratio of the diet (Table 2.2) in counteracting the rigidifying influence of low temperature.

The behaviour of liver membrane lipids of fish fed both M-ω3 and H-ω3 diets were very similar in response to the increase in temperature during Raman experiments. The symmetric stretching frequency of CH_2 bonds for both fish groups ranged from 2850 to 2856 cm^{-1} at 18.0°C and 2849 to 2857 cm^{-1} after the temperature was decreased to 13.5°C. However, there was one exception in M-ω3 fed fish sampled at 13.5°C. The

membranes of M-ω3 fed fish remained relatively rigid in comparison to H-ω3 fed fish during the Raman temperature increase from 14.0 to 26.0°C. This variation in membrane fluidity may have been influenced by the FO based diet, where the HUFA rich H-ω3 diet results in a more fluid membrane. However, both M-ω3 and H-ω3 fed fish had a similar fatty acid composition at 13.5°C, with the exception of 18:2ω6, which was higher than H-ω3 fed fish. The gradual increase in wave number for both H-ω3 and M-ω3 groups indicates fluidization of the membrane in response to increased environmental temperature. However, absence of phase transition in response to temperature in both of these fish groups indicates lack of evidence for conformational transformation of lipids from ordered state (all *trans*) to complete disordered state (all *gauche*). Snyder (1982) in a model study defined the change in conformational order from all *trans* to *gauche* with an increase in frequency for CH_2 symmetric stretch by 6 cm^{-1}. However, unlike model membrane studies, the diversity of lipid species in biological membranes leads to complex phase behaviours resulting in broader phase transitions (Hazel and Williams, 1990), where both gel and fluid phases coexist in different regions (Ohvo-Rekila et al., 2002; Wassall et al., 2004).

Sterols, mainly cholesterol (most abundant sterol in fish lipid) is also known to play a role in phase separation in biological membranes, although sterol concentration was not significantly different between rearing temperatures in this study. Biological membranes are known to form micro-domains "rich in cholesterol with poor presence of PUFA" and *vice versa* prompting phase separation (Ohvo-Rekila et al., 2002; Wassall et al., 2004), resulting in lateral patches of PUFA-rich domains and cholesterol-rich

membrane rafts (Wassall et al., 2004). Lack of clear phase separation and co-existence of both gel and fluid phases are known to aid in preserving the function of the membrane and its associated proteins (Illsley et al., 1987, 1988). This provides evidence that the liver membranes of both M-ω3 and H-ω3 fed fish have the capability of withstanding a broader temperature range without undergoing complete phase separation.

The Raman spectroscopic analysis suggests liver membranes of L-ω3 fed fish were more significantly fluid in comparison to the other two feed groups at both rearing temperatures (18.0 and 13.5°C). The CH_2 symmetric stretching frequency and related wave numbers were significantly higher throughout all Raman temperature increments for L-ω3 fed fish. In addition, the results also suggest a change in phase behaviour at both rearing temperatures. Changes in conformational order of membrane lipid are evident in fish reared at 18.0°C between the increases in Raman temperature from 10 to 26.0°C. The sigmoid spread in wave numbers (Figure 3.8) suggests a clear shift in wave numbers from a mean of 2852 to 2859 cm^{-1}, indicating a change in conformational order of constituent lipids. In contrast, both H-ω3 and M-ω3 fed fish had a gradual increase in wave numbers in response to the increase in temperature without a clear phase separation. The change in phase behaviour at 13.5°C for L-ω3 fed fish is not as clear as at 18.0°C. However, the spread (Figure 3.9) in wave numbers suggests a conformational change in constituent lipid at a much lower temperature (between 6 and 18.0°C). The phase behaviours of L-ω3 fed fish membranes were much different to what was expected. The liver membranes of M-ω3 and H-ω3 fed fish were expected to have lesser membrane order or higher fluidity compared to L-ω3 fed fish. H-ω3 diet followed by M-ω3 are rich in PUFA, mainly fatty

acids of marine origin (Table 2.2), which are known to increase membrane fluidity due to the length and flexibility of HUFA and due to the higher number of double bonds (Eldho et al., 2003; Feller et al., 2002; Huber et al., 2002).

The results suggest that increased fluidity in membranes of L-ω3 fed fish are influenced by the dietary fatty acid composition. L-ω3 feed is rich in monounsaturated and terrestrial fatty acids compared to the other two diets (Table 2.2). L-ω3 feed also had the highest 18:1ω9 content and may have affected the fluidity, since addition of the 1st double bond is known to have the biggest influence on membrane fluidity. Farkas et al. (2001) compared diverse groups of fish from different geographic regions that were adapted to a wide range of temperatures and concluded that dietary fatty acids did not determine the membrane composition or fluidity. Liver SFA, MUFA and PUFA composition of L-ω3 fed fish was not significantly different from the other 2 fish groups, which supports the argument made by Farkas et al. (2001). Nevertheless, the authors also suggested that marine fish are more likely to rely on the diet to obtain HUFA for phospholipid formation in contrast to freshwater fish (Farkas et al., 1980; Olsen et al., 1990; Gnoni 1992). However, fish collected in the Farkas et al. (2001) study were from their natural habitats, adapted to the respective temperature and available food sources as opposed to the steelhead trout used in this experiment.

A well known response to cold exposure is reduction in saturated fatty acids with a proportional increase in unsaturated fatty acids (Crockett and Hazel, 1995; Feller et al., 2002; Huber et al., 2002; Eldho et al., 2003). However, opposing results have also been reported in response to cold exposure in the non-raft portion of the rainbow trout liver

plasma membrane (Zehmer and Hazel, 2005) and the sarcoplasmic reticulum of skeletal muscle (Vornanen et al., 1999). In addition to acyl chain compositions, the specificity of phospholipid head groups also influences the fluidity of membranes. Restructuring of phospholipids occurs more frequently in PE than in PC followed by PI (Farkas et al., 2000; Logue et al., 2000; Trueman et al., 2000; Brooks et al., 2002). Cold exposure of membranes is known to accumulate higher proportions of conical shape PE compared to PC (Farkas et al., 1984; Hazel and Landrey, 1988). Similarly replacing a saturated fatty acid at the *sn*-1 position of PE with an unsaturated fatty acid is known to make the PE molecule more conical as well as influence close packing of phospholipid molecules (Zabelinski et al., 1995; Farkas et al., 2001).

The presence of polyunsaturated fatty acids in membrane phospholipids and its effect on fluidity, compressibility and permeability is well known. The presence of HUFA like DHA has sometimes been assumed to provide maximum fluidity due to the long acyl chain and six double bonds. However, membrane fluidity does not appear to be directly correlated to the number of double bonds, displaying a steady increase with increase unsaturation. Studies on artificial membranes containing phosphatidylecholine and different fatty acids in the *sn*-2 position have shown increases in membrane fluidity and decreases in melting points only with the addition of first and second double bonds. Further addition of double bonds failed to produce additive effects, in fact producing changes in the opposite direction, e.g. with substitution of 20:4ω6 or DHA for 18:2ω6 (Coolbear et al., 1983; Dratz et al., 1986). The number and the position of double bonds also play an important role in membrane fluidity (Hazel and Williams, 1990), with varying impacts on the physical properties of phospholipids (Bell et al., 1986). For

example, in a model study conducted by Coolbear et al. (1983), a double bond (such as 18:1) was introduced at the *sn*-2 position of dipalmitoylphosphatidylcholine (DPPC) replacing 16:0 to form 16:0/18:1-PC, which reduced the gel to fluid transition temperature by 50°C. Addition of the second double bond to form 16:0/18:2-PC further reduced the melting point by 22°C. However, addition of the third double bond (16:0/18:3-PC) increased the melting point by 3°C. The location of a double bond also has an impact on the physical property of the lipid molecule, where a double bond at C_{9-10} (the centre) of a C_{18} fatty acid gives a lower melting point than when the first double bond is at $C_{12,}$ (Brenner, 1984). Smith et al. (1984) found that the molecular area occupied by 16:0/18:3-PC in a monolayer film is greater than 16:0/22:4-PC which could hinder close packing of phospholipid molecules. Theoretically, an increase in the number of double bonds should increase the area occupied by the respective molecule and thereby, decrease the packing order. However, Cossin et al. (1985) suggested that although double bonds occupy a relatively larger area, PUFAs also restrict the freedom of rotation of single bonds between carbons thereby offsetting the two effects. Similarly, an increase in acyl chain unsaturation did not have a significant effect on gel to fluid transition temperatures, e.g. the difference in the gel to fluid transition temperature between 16:0/16:1-PC and 16:0/22:6-PC was only 2°C (-12 and -10°C respectively).

Much of the predictions made on membrane fluidity, acyl chain restructuring and behaviour and alterations in phospholipid head groups in fish are based on model membrane studies or by exposing live animals to two distinct temperatures (Dey et al., 1993; Buda et al., 1994; Fodor et al., 1995; Farkas et al., 2000; Brooks et al., 2002). Adjustments in membrane lipid composition may occur at different times during the

acclimation process and certain responses evident during that period may not be evident once acclimated (Hazel and Williams, 1990). Unlike freshwater fish that have the capacity for chain elongation and desaturation, marine fish primarily rely on fatty acids of dietary origin to manipulate the fatty acid composition in membranes. However, the enzymatic processors involved in both of these situations will be affected by change in environmental temperature. Previous studies indicate slower changes in PUFA composition in membranes in response to decreases in temperature, e.g. a lag period of 3 – 6 days prior to change in PUFA composition (Sellner and Hazel, 1982); unchanged desaturation activity of hepatic Δ5 desaturase for 6 days upon transfer of fish from 20 to 5°C (Hager and Hazel, 1985). Longer acclimation times or sudden changes in temperature could result in different fatty acid compositions and behaviours in membrane phospholipids. Only a few fatty acids seem to be influencing the increased fluidity in L-ω3 fed fish at both high and low temperatures. The composition of 18:2ω6, 20:3ω6 and 20:4ω6 at 18.0°C and 20:3ω6 at 13.5°C (final) was significantly higher and may have influenced the fluidity of L-ω3 liver tissue. Although not significantly, different the collective influence of Σω3, DHA, PUFA, terrestrial and marine fatty acids and P:S ratio at 18.0°C also contribute to the higher fluidity in L-ω3 fed fish. However, lowering of both the ratio and the Σω3 composition did not correspond to the higher fluidity in L-ω3 fed fish at 13.5°C (final). Further, a significant change in a lipid class or a fatty acid that directly correlated with increased fluidity of L-ω3 fed fish at the final temperature was not evident.

This is the first study to measure both the effect of diet and environmental change in temperature on liver membrane fluidity of steelhead trout. Increased membrane fluidity of L-ω3 fed fish at both temperatures shows the influence of terrestrial fatty acids on the physical properties of the membrane. Such changes could influence membrane functions sensitive to phase state or fluidity hampering numerous membrane associated processes vital to cell function and physiological activity of fish in general. In contrast, liver membranes of fish oil rich H-ω3 and M-ω3 (fish oil partially substituted with animal fat) fed fish were least influenced by changing environmental temperature.

3.6. References

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Chapter 4

Effect of temperature and diet on liver, gill and intestine mucosa lipid composition in juvenile steelhead trout (*Oncorhynchus mykiss*)

4.1 Introduction

Steelhead trout (*Oncorhynchus mykiss)* is the anadromous form of rainbow trout. Although rainbow trout appears to have the capability to elongate and desaturate short chain (C_{18}) PUFA to long chain (C_{20-22}) HUFA, marine fish have lost this capacity (Henderson and Tocher, 1987). Marine fish reportedly have a low Δ5 desaturase activity due to the high availability of HUFAs, including EPA and DHA in marine food chain. Therefore, HUFA, mainly EPA (20:5ω3) and DHA (22:6ω3), which are essential for all cell membranes and organs, need to be supplied in the diet for species cultured in salt water (Sargent et al., 1989). The activation of various endocrine processes during smoltification is known to create profound behavioural, morphological and physiological changes in anadromous fish in addition to altering their fatty acid profile prior to seaward migration (Hoar 1976; Wedermeyer et al., 1980). Studies conducted on steelhead trout and Atlantic salmon have shown that tissue fatty acid composition of juvenile fish pre-adapt to undergo parr-smolt transformation by changing fatty acid to a higher composition of long-chain PUFA prior to seawater entry (Li and Yamada, 1992). However, unlike wild populations, the hatchery reared steelhead trout juveniles in commercial operations may have limitations in the timing of the smoltification process, as they are transferred to seawater net pens after reaching a certain size within a short period of time. Following the transfer, the juvenile fish are usually fed with a diet rich in fish oil

that suits the marine environment. Therefore, information available on changes occurring in fatty acid desaturation and elongation capacity of these fish during the period of sea water transfer is limited.

Salmonids and marine fish culture generally use feeds rich in fish meal and fish oil (FO) to supply essential fatty acids. This is to compensate for the lost capacity for chain elongations and desaturation of anadromous fish (Sargent et al., 1989). Increased production of feed grade fisheries is one possible way to address the demand for FO and fish meal in mariculture. However, depleting stocks and ethical issues have pressured the aquaculture industry to look for alternative lipid sources to be used in feed (Sargent and Tacon, 1999; Barlow 2000; Tidwell and Allan, 2002). Use of vegetable oils (VOs), which are rich in α-linolenic (LNA; 18:3ω3) and linoleic (LA; 18:2ω6) acids, is identified as a potential source of replacement for FO and a way forward in salmonid culture (Sargent et al., 2002). Several studies conducted in the past, testing different VOs in fish feed, highlight the interest and urgency of finding an alternative lipid source to replace FO, e.g. soybean (Hardy et al., 1987; Lie et al., 1993), sunflower (Bell et al., 1993), borage (Tocher et al., 1997), rapeseed (Tocher et al., 2000; Bell et al., 2001), linseed (Tocher et al., 2000, 2002a) and palm oil (Torstensen et al., 2000; Bell et al., 2002). Some studies have examined mixtures of different vegetable oils (Bell et al., 2003; Rosenlund et al., 2001; Jobling et al., 2002a,b; Tocher et al., 2003) and also different levels of dietary oils (Jobling 2001, Jobling et al., 2002a,b).

Although the capacity for fatty acid elongation and desaturation is limited in marine fish owing to increase availability of HUFA in seawater food chains, the activity of the crucial enzymes (Δ5 and Δ6 desaturases) involved in fatty acid conversion

processes are not completely lost (Hastings et al., 2001; Agba et al., 2004; Zheng et al., 2004). It is commonly accepted that salmon fed VO supplemented diets are unable to compensate for the dietary reduction in EPA and DHA by *in vivo* synthesis of EPA and DHA using ω3 precursors (Bell et al., 2001; Tocher et al., 2003; Zheng et al., 2005). However, several studies have shown significantly higher desaturase and elongase activity in hepatocytes and enterocytes of salmonids (Trocher et al., 2001; 2002; 2003; Oxley et al., 2005; Stubhaug et al., 2005). Several other studies have shown increased hepatic desaturase and elongase activety in fish fed VO containing diet during the seawater rearing stage (Henderson et al., 1994; Ruyter et al., 2000; Bell et al., 2001; Tocher et al., 2001, 2003; Zheng 2005). Further research is essential to identify the use of VO in aquafeed, which could potentially replace FO. Therefore, it is crucial to understand the possible types and optimal blends of VO as replacements, and more importantly the biochemical and physiological influence of such replacement on specific tissues.

Several studies have successfully replaced FO with VO without any influence on growth of marine fish (reviewed by Turchini et al., 2009). Nonetheless, VO, depending on the type chosen, could contain a higher level of ω6, ω9 and short chain ω3 fatty acids, which could not ideally fulfill the HUFA requirement of cultured marine fish (Tortensen et al., 2000; Sargent et al., 2002; Ng et al., 2004; Tortensen et al., 2005; Mourente et al., 2005; Francis et al., 2007). Therefore, excessive use of VO in place of FO in marine diet could cause deleterious effects in fish affecting their metabolic and physiological functions. Studies have shown various lipid and metabolic disorders in fish fed with diets containing high levels of VO and poor in HUFA, decreased flesh quality due to increased oil deposition and low pigment visualization (Sheehan et al., 1996; Johansen and Jobling,

1998), low disease resistance in salmon to *Aeromonas salmoncida* and *Vibrio anguillarum* (Thompson et al., 1996; Bransden et al., 2003), increased stress and cortisol levels (Jutfelt et al., 2007) and modified fatty acid composition in flesh (Bell et al., 2003; Regost et al., 2004; Tortensen et al., 2005).

In the present study, juvenile steelhead trout were fed in duplicate tanks, with a diet rich in either herring oil (HE), sunflower (SF) or flax seed oil (FLX), while simultaneously increasing the water temperature. The fish oil containing diet is rich in HUFA and it provides the EFA required by marine reared fish, while the other two are rich in either 18:3ω3 (FLX) or 18:2ω6 (SF) and closely resemble a freshwater diet. The effect of water temperature and FO replacement on lipid class and fatty acid composition in physiologically important organs such as liver, gills and intestine were investigated.

4.2. Materials and methods

4.2.1. Experimental fish

Four hundred and eighty juvenile steelhead trout averaging 120 g body weight were moved to a 45 m^3 holding tank at ambient temperature (5 ± 1°C). Fish were transported and handled following the same procedure described earlier (section 2.1.1).

During the first month fish were given the same feed type used at the aquaculture site (Corey feed) and then switched to the experimental base diet (section 2.3.3) at the end of first month. Fish were held in the holding tank for approximately a month prior to the experiment.

4.2.2. Experimental tanks

Six identical 6000 L tanks were used in duplicate for the feeding experiment. Fifty five fish in total were placed in each experimental tank. Fish were held at 10.0 ± 1.0 ^{o}C water temperature with a flow of 6 -7 L min^{-1} for approximately 2 weeks to acclimate to the experimental tanks. Fish were reared following the same protocol described earlier (section 2.3.2).

4.2.3. Experimental feed

Three experimental diets were formulated at the Marine Research Station, Sandy Cove, Halifax, NS, Canada (section 2.3.3). The basal diet was formulated using the ingredients listed in Table 2.6. Origin of lipid was different among the 3 diets (Herring oil, HE; flax seed oil, FLX, and sunflower oil, SF). They were stored at -20^{o}C and fed following the same procedure as in section 2.2.3. All fish were fed with the experimental base diet (HE) during the acclimation period.

4.2.4. Experimental temperature

Fish were left for 2 weeks at 8.0 ± 1.0^{o}C to acclimatize to the experimental tanks. The average temperature was increased in a stepwise manner from ~ 10.0^{o}C (1st sampling) to maximum of 18^{o}C (6th sampling) following ~ 2^{o}C incremental steps (Figure 2.10) but with plateaus. At each step, the temperature was increased gradually to the next level over a ~2 day period and left stable thereafter for 12 – 14 days. Ambient water was heated and adjusted as in section 2.3.4.

4.2.5. Sampling protocol

Sampling was done following the same procedure described in section 2.2.5. Gill samples were collected from the left side. Soon after the fish was anesthetised, two gill arches on the left side were incised from one end in order to drain blood for couple of minutes and thereafter both gill arches were completely removed from the fish and placed on a paper towel to swab the remaining blood from the gill filaments. Thereafter, the filaments were excised from the arch, weighed (approximately 1 g) and transferred into a lipid clean glass vial. Then each vial was flushed with N_2 after filling with 4 ml $CHCl_3$ and sealed with Teflon lined caps and Teflon tape, and stored at -20°C.

The complete liver was removed and weighed, and then approximately 1 g of liver tissue was weighed and transferred to a lipid clean glass vial. Upon transfer each vial was flushed with N_2 after filling with 4 ml $CHCl_3$ and sealed with Teflon lined caps and Teflon tape and stored at -20°C.

The anterior intestine was separated below the attachments of pyloric caeca. Any material remaining in the anterior part of the intestine was squeezed out from this opening. Thereafter, 5-7 cm long portion of the intestine was completely removed, and sectioned to expose the lumen. The luminal side was then thoroughly washed with distilled water and the top mucus layer was carefully scraped off with a scalpel blade without damaging the underlying mucosa. Once cleaned, approximately 1 g of mucosa was scraped, weighed and placed in a lipid clean glass vial and each vial was then flushed with N_2 after filling with 4 ml $CHCl_3$ and sealed with Teflon lined caps and Teflon tape and stored at -20°C.

4.2.6. Lipid class and fatty acid analysis of liver, gill and intestine mucosa

Lipid extraction, lipid class separation and fatty acid analysis were done according to the procedures described in sections 2.2.6 following Parrish (1999) using a modified Folch procedure (Folch et al. 1957).

4.2.7. Statistical analysis

Statistical analysis was done as in section 2.2.8 using the General Linear Model procedure of the Statistical Analysis System (GLM procedure, SPSS 13.0 for Windows). The analysis of muscle lipid class and fatty acid was performed with temperature and feed type as explanatory variables (two-way ANOVA) (feed x temperature) with interactions to determine the effect of temperature and feed type on lipid class and fatty acid composition between initial and final sampling for each tissue type. If significant interactions were present the effect of feed type was examined at each sampling temperature using only feed type as the explanatory variable. Multiple comparisons of means for lipid class and fatty acids were made using Tukey corrections. The significance level was set at $\alpha = 0.05$ for all the tests.

4.3. Results

Tables 2.5 and 2.6 provide information on feed ingredients, lipid class and fatty acid composition of the 3 experimental diets. Results of this study show the effect of replacing HE with SF or FLX on gill, proximal intestine mucosa and liver tissue lipid class and fatty acid composition of juvenile steelhead trout. Further, the results for fish fed basal diet containing primarily herring oil were compared between those of the initial

and final sampling to understand the effect of increased temperature on gill, intestine mucosa and liver tissue of juvenile steelhead trout (Tables 4.1, 4.2 and 4.3). Similarly, the tissue lipid class changes in fish fed all diets were compared at 18.0°C. .

4.3.1. Lipid class composition of gill, intestine mucosa and liver tissue

Although the HE diet had significantly higher TL and NL (%ww^{-1}) than FLX diet and higher polar lipid (% ww^{-1}) than both FLX and SF diets, there was no significant effect on lipid class composition of gill tissues of juvenile steelhead trout (Table 4.1). Similarly, the TL (%ww^{-1}), NL (%ww^{-1}) and polar lipid (%ww^{-1}) composition of intestine mucosa were not affected by the diet (Table 4.2). The ST composition of the intestinal mucosa of fish fed HE diet increased with temperature (Table 4.2). The final ST composition of the intestine mucosa of HE fed fish was higher than the initial level and twice as high as both SF and FLX fed fish at the end of the experiment ($p \leq 0.05$). A similar increase in ST composition with temperature was observed in liver tissue ($p = 0.00$) (Table 4.3). However, the final ST compositions of the liver tissue among the three feed groups were not significantly different (Table 4.3). Although the dietary TAG composition was similar in all 3 diets (Table 2.6), the intestine TAG content in SF fed fish (Table 4.2) was higher than in HE fed fish ($p = 0.018$) and the liver TAG content in FLX fed fish was lower ($p = 0.021$) than in HE fed fish at 18°C. Increased temperature decreased the TAG content in the liver and intestine tissue of fish fed HE diet compared to the initial temperature (Tables 4.2 and 4.3). At the initial temperature the TAG content of both gill and liver of HE fed fish was similar (Table 4.4), but it was twice as high compared to liver tissue at 18°C (Table 4.5: $p = 0.000$). Although diet and temperature

had significant effects on the final lipid composition of intestine mucosa and liver tissues of HE diet fed fish, the gill lipid composition was not affected by either diet or temperature (Table 4.1). In contrast to the HE diet, TAG deposition in gill and intestinal mucosa was 3 times higher than that in liver tissue for both SF and FLX (Tables 4.6 and 4.7: $p \leq 0.000$).

The lipid class composition of gill, intestine and liver tissue were compared at the beginning of the experiment (Table 4.4). All juveniles at this stage were fed with HE diet and were at 8°C. Major lipid classes, TAG, ST and PL were compared among the tissues. The TAG composition of intestinal mucosa was significantly higher than gill ($p = 0.002$) and liver ($p = 0.001$), while the PL composition was significantly lower than both gill ($p = 0.005$) and liver ($p = 0.000$) (Table 4.4). The gills had a significantly higher ST content than both intestinal mucosa ($p = 0.002$) and liver ($p= 0.005$). Polar lipid (%ww^{-1}) was highest in liver tissue ($p = 0.000$) and TL and NL (%ww^{-1}) was highest in intestinal mucosa ($p < 0.004$) (Table 4.4).

The ST content in gill tissue of HE diet fed fish was significantly higher than both intestine and liver tissues at 8°C (Table 4.5; $p = 0.000$). However, all 3 tissues had similar ST levels after increasing the temperature to 18°C (Table 4.5). PL composition of both intestinal mucosa and liver increased with increasing temperature in HE diet fed fish ($p \leq 0.037$) (Tables 4.2 and 4.3). The final PL composition of liver tissue was twice as high as both gill and intestinal mucosa for all 3 feeds (Tables 4.5, 4.6 and 4.7) ($p = 0.000$). The increase in temperature had no significant effect on the ST:PL ratio of gill and intestinal mucosa of HE diet fed fish, although the temperature increase doubled the ratio in liver tissue (Tables 4.1, 4.2 and 4.3: $p = 0.000$). Similarly, the liver ST:PL ratio of HE fed fish

was twice as high as that for the fish fed the other two feed types ($p \leq 0.009$) at 18°C (Table 4.3). The ST:PL ratio in gill tissue was not affected by either temperature or diet (Table 4.1). The fish fed all 3 diets had a higher ST:PL ratio in both gills and intestine mucosa than liver tissue at the end of the study (Tables 4.5, 4.6 and 4.7).

4.3.2. Tissue fatty acid composition of liver, gill and intestine mucosa

The 3 diets were significantly different in their fatty acid composition (Table 2.6). The HE diet with crude lipid supplied primarily from herring oil, was significantly higher in both SFA (mainly 14:0 and 16:0) and marine (20:5ω3, 22:5ω3 and 22:6ω3) origin fatty acids, and had the lowest terrestrial plant fatty acids (18:3ω3, 18:2ω6), P:S ratio and ω6:ω3 ratio. The SF diet was rich in 18:1ω9 and 18:2ω6 due to inclusion of sunflower oil and the FLX diet was rich in 18:3ω3 and 18:2ω6 as a result of flax seed oil.

Dietary influences on fatty acid composition of liver, gill and intestinal mucosa are shown in Tables 4.8, 4.9 and 4.10, respectively. The results also include the influence of temperature on the same tissues in fish fed the HE diet at both 10 and 18°C. SFA composition in the HE diet was significantly higher than in the other 2 feeds, which was reflected in liver SFA composition ($p = 0.000$). In contrast, FLX diet had significantly less SFA in the diet compared to SF diet (Table 2.6), but the liver SFA composition in FLX fed fish was higher than SF fed fish, possibly due to the slightly higher 14:0 and 16:0 levels in liver tissue (Table 4.9; $p \leq 0.014$). Higher MUFA in liver tissue of SF diet fed fish over those fed HE and FLX ($p = 0.000$) was primarily due to 18:1ω9 in sunflower oil (Table 2.6). The 18:1ω9 proportion in liver tissue of SF fed fish was twice as high as that of the other two diets ($p = 0.000$). The high proportion of 18:2ω6 in SF diet increased

the Σω6 content (%) in the liver ($p = 0.000$; Table 4.8). The SF diet also produced significantly higher concentrations of 18:2ω6, 20:2ω6, 20:3ω6 and total ω6 and the ω6:ω3 ratio than the other two diets.

Terrestrial fatty acids (18:3ω3, 18:2ω6) were significantly higher in liver tissue of fish fed FLX diet ($p \leq 0.00$). As with the SF diet, FLX diet incorporated the most abundant dietary fatty acids available in the feed to liver tissue, mainly 18:3ω3. As a result, liver tissue of FLX fed fish had a significantly higher ω3 over the rest and also a lower ω6 and ω6:ω3 ratio than SF diet ($p \leq 0.022$). The total PUFA of FLX fed fish was significantly higher than the other two groups ($p \leq 0.042$) due to increased ω3 levels. Although HE diet had twice as much DHA as the other 2 diets (Table 2.6), the final tissue compositions were similar among the 3 feeds. Both HE and FLX fed fish had similar DHA:EPA, EPA:AA, ω6:ω3, average double bonds and chain length in liver tissue.

The SFA content of gill tissue was not affected by diet (Table 4.9), unlike liver tissue and intestinal mucosa (Tables 4.8 and 4.10, respectively). However, both 16:0 and 18:0 were the dominant SFA present in gill and intestinal mucosa as in liver tissue. The significantly higher 18:1ω9 composition of the SF diet increased MUFA content in all tissues of SF diet fed fish ($p \leq 0.045$; Tables 4.9 and 4.10). The 3 diets had no significant effect on the PUFA level in the gill tissue (Table 4.9). DHA composition (%) of gills in HE fed fish was similar to that of both SF and FLX fed fish at the end of the study, despite the fact that HE diet had twice as much DHA. However, EPA composition (%) of gills was significantly higher in HE fed fish compared to those fed on the other 2 diets ($p = 0.00$), which also corresponds to the dietary composition. The difference in EPA levels in gill tissue with the respective diets resulted in significantly different DHA:EPA ratios

($p = 0.000$: Table 4.9). However, dietary differences did not affect average fatty acid chain lengths and double bonds of gill and intestinal mucosal lipids of steelhead trout juveniles.

The influence of terrestrial plant fatty acids in the diet (18:2ω6 and 18:3ω3) was reflected in all 3 tissues, with FLX fed fish having the highest terrestrial fatty acid composition, followed by SF and HE fed fish (Table 4.8, 4.9 and 4.10). The highly available 18:2ω6 in SF feed elevated the proportion of 18:2ω6, resulting significantly higher total ω6 and ω6:ω3 ratios in all 3 tissues. However, ω6:ω3 ratios of both HE and FLX fed fish remained lower among all 3 tissue types ($p \leq 0.017$) due to the increased amounts of ω3 fatty acids in the respective diets (ω3 from marine and terrestrial sources, respectively).

The main fatty acid groups and ratios in liver tissue closely corresponded with their respective dietary compositions. However, both HE and FLX fed fish had similar fatty acid chain lengths, MUFA levels, ω6:ω3, EPA:AA, DHA:EPA ratios. Fatty acid composition of gill tissue was the least affected by the 3 feed types. SF diet significantly affected the fatty acid composition of all 3 tissues, while both HE and FLX fed fish closely resembled each other in gills (Σω3, ω6:ω3, MUFA) and intestinal mucosa (PUFA, Σω3, ω6:ω3, EPA:AA, DHA:EPA, SFA and MUFA) (Tables 4.9 and 4.10).

Fish oil rich HE diet was used as the base diet for all experimental groups until the initial sampling and continued for one group (HE) until the end of the study. Fatty acid compositions for SF and FLX fed fish that closely resembles (insignificant differences) values to HE diet fed fish at 18°C indicates a similar behaviour to temperature increase.

The fatty acids of intestinal mucosa in HE diet fed fish were the least affected by the change in temperature. HE diet fed fish did show a significant decrease in Σω6, ω6:ω3 and DHA:EPA ratio ($p \leq 0.049$) in intestinal mucosa with increased temperature and the fish fed FLX diet also had the same response to temperature increase (Table 4.10). Liver tissue of HE diet fed fish on the other hand reacted similarly to intestinal mucosa with a decrease in Σω6, ω6:ω3 and DHA:EPA ratio ($p \leq 0.023$; Table 4.8). In addition, the P:S ratio and terrestrial fatty acid composition also decreased with an increase in liver Σω3 and SFA ($p \leq 0.042$). The fatty acid composition of gill tissue had a similar response to increased temperature with a decrease in Σω6, ω6:ω3, terrestrial fatty acids and increased Σω3 level ($p \leq 0.015$; Table 4.9). In addition, the PUFA and the double bond number increased in gill tissue in response to temperature ($p \leq 0.042$: Table 4.9). The gill Σω3 and ω6:ω3 ratio of FLX fed fish had the same response to temperature as HE diet fed fish and PUFA and average double bond number of both SF and FLX fed fish followed the same pattern as HE fed fish (Table 4.9).

Table 4.1. Effect of diet and temperature on gill tissue of juvenile steelhead trout (there were no significant differences). Selected lipid classes (%), total, polar and neutral lipid (% ww^{-1}) composition in juvenile steelhead trout are shown.

Experimental data	Initial	Final		
Temperature °C	8	18		
Feed	HE	HE	SF	FLX
TAG	55.6 ± 23.0	70.0 ± 13.3	67.3 ± 8.3	66.2 ± 9.5
ST	7.3 ± 3.0	5.9 ± 2.5	6.31 ± 1.6	5.8 ± 1.4
PL	32.6 ± 19.6	21.8 ± 11.0	23.6 ± 6.2	24.8 ± 8.1
TL	3.7 ± 1.6	4.1 ± 1.6	3.4 ± 1.5	3.6 ± 2.4
NL	2.6 ± 1.5	3.2 ± 1.6	2.7 ± 1.3	2.8 ± 2.1
Polar lipid	1.1 ± 0.3	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.4
ST:PL	0.24 ± 0.09	0.3 ± 0.07	0.3 ± 0.05	0.2 ± 0.06

Values are mean ± SD.
Diets are HE (Herring oil), SF (Sunflower oil), FLX (Flax seed oil).
Triacylglycerol (TAG), sterol (ST), phospholipids (PL), total lipid (TL), neutral lipid (NL), sterol:phospholipid ratio (ST:PL).

Table 4.2. Effect of diet and temperature on intestine mucosa tissue selected lipid class (%), total, polar and neutral lipid (% ww^{-1}) composition in juvenile steelhead trout.

Experimental data	Initial	Final		
Temperature °C	8	18		
Feed	HE	HE	SF	FLX
TAG	89.8 ± 3.1*	64.1 ± 8.8*a	78.6 ± 9.2b	69.6 ± 5.6
ST	2.2 ± 1.8*	8.1 ± 1.9*a	4.3 ± 2.1b	4.3 ± 0.7b
PL	5.8 ± 2.4*	23.8 ± 7.1*	15.8 ± 7.2	24.8 ± 6.24
TL	13.8 ± 4.9	8.0 ± 6.8	13.2 ± 5.9	10.6 ± 3.8
NL	13.1 ± 4.8	6.3 ± 6.2	11.3 ± 5.7	8.1 ± 3.1
Polar lipid	0. 8 ± 0.3*	1.7 ± 0.7*	1.9 ± 0.9	2.5 ± 0.8
ST:PL	0.4 ± 0.2	0.4 ± 0.2a	0.3 ± 0.1	0.2 ± 0.1b

Values are mean ± SD.
Significant differences among diets at 18°C are indicated by superscript letters. Asterisks indicate significant differences for HE diet between initial and final temperatures.
Diets are HE (Herring oil), SF (Sunflower oil), FLX (Flax seed oil).
Triacylglycerol (TAG), sterol (ST), phospholipids (PL), total lipid (TL), neutral lipid (NL), sterol:phospholipid ratio (ST: PL).

Table 4.3. Effect of diet and temperature on liver selected lipid class (%), total, polar and neutral lipid (% ww^{-1}) composition in juvenile steelhead trout.

Experimental data	Initial	Final		
Temperature °C	8	18		
Feed	HE	HE	SF	FLX
TAG	$49.3 \pm 8.7^{*}$	$34.1 \pm 7.5^{*a}$	22.7 ± 11.1	17.1 ± 10.0^{b}
ST	$2.8 \pm 1.4^{*}$	$8.5 \pm 2.2^{*}$	6.7 ± 1.3	7.0 ± 1.7
PL	$44.4 \pm 8.7^{*}$	$56.0 \pm 7.9^{*a}$	67.2 ± 8.8^{b}	73.3 ± 7.5^{b}
TL	$7.2 \pm 0.8^{*}$	$5.6 \pm 0.8^{*}$	4.7 ± 0.8	5.7 ± 1.4
NL	$4.0 \pm 1.0^{*}$	$2.5 \pm 0.8^{*a}$	1.4 ± 0.6^{b}	1.5 ± 0.7
Polar lipid	3.1 ± 0.5	3.1 ± 0.1^{a}	3.2 ± 0.5^{a}	4.2 ± 1.0^{b}
ST:PL	$0.1 \pm 0.04^{*}$	$0.16 \pm 0.05^{*a}$	0.1 ± 0.01^{b}	0.1 ± 0.02^{b}

Values are mean ± SD.
Significant differences among diets at 18°C are indicated by superscript letters. Asterisks indicate significant differences for HE diet between initial and final temperatures.
Diets are HE (Herring oil), SF (Sunflower oil), FLX (Flax seed oil).
Triacylglycerol (TAG), sterol (ST), phospholipids (PL), total lipid (TL), neutral lipid (NL), sterol:phospholipid ratio (ST: PL).

Table 4.4. Lipid class (%), total, polar and neutral lipid (% ww^{-1}) comparison of gill, intestine mucosa and liver tissue of steelhead trout juveniles fed herring oil based (HE) diet at 8°C.

Feed	Herring oil		
Tissue	Gill	Intestine	Liver
TAG	55.6 ± 23.0^{b}	89.8 ± 3.1^{a}	49.3 ± 8.7^{b}
ST	7.3 ± 3.0^{a}	2.2 ± 1.8^{b}	2.8 ± 1.4^{b}
PL	32.6 ± 19.6^{b}	5.8 ± 2.4^{a}	44.4 ± 8.7^{b}
TL	3.7 ± 1.6^{b}	13.8 ± 4.9^{a}	7.2 ± 0.8^{b}
NL	2.6 ± 1.5^{b}	13.1 ± 4.8^{a}	4.0 ± 1.0^{b}
Polar lipid	1.1 ± 0.3^{b}	0.8 ± 0.3^{b}	3.1 ± 0.5^{a}
ST:PL	0.24 ± 0.09^{b}	0.4 ± 0.2^{b}	0.1 ± 0.04^{a}

Values are mean ± SD.
Tiacylglycerol (TAG), free fatty acids (FFA), sterol (ST), phospholipids (PL), total lipid (TL), neutral lipid (NL), sterol:phospholipid ratio (ST:PL).

Table 4.5. Lipid class (%), total, polar and neutral lipid (% ww^{-1}) comparisons of gill, intestine mucosa and liver tissue of steelhead trout juveniles fed herring oil based (HE) diet at 18°C.

Feed	Herring oil		
Tissue	Gill	Intestine	Liver
TAG	70.0 ± 13.3^{a}	64.1 ± 8.8^{a}	34.1 ± 7.5^{b}
ST	5.9 ± 2.5	8.1 ± 1.9	8.5 ± 2.2
PL	21.8 ± 10.7^{a}	23.8 ± 7.1^{a}	56.0 ± 7.9^{b}
TL	4.1 ± 1.6	8.0 ± 6.8	5.6 ± 0.8
NL	3.2 ± 1.6	6.3 ± 6.2	2.5 ± 0.8
Polar lipid	0.8 ± 0.2^{a}	1.7 ± 0.7^{b}	3.1 ± 0.1^{c}
ST:PL	0.3 ± 0.07^{a}	0.4 ± 0.2^{a}	0.16 ± 0.05^{b}

Values are mean ± SD and significant differences among tissue types are indicated by superscript letters.
Triacylglycerol (TAG), free fatty acids (FFA), sterol (ST), phospholipids (PL), total lipid (TL), neutral lipid (NL), sterol:phospholipid ratio (ST:PL).

Table 4.6. Lipid class (%), total, polar and neutral lipid (% ww^{-1}) comparisons of gill, intestine mucosa and liver tissue of steelhead trout juveniles fed sunflower oil based (SF) diet at 18°C.

Feed	Sunflower oil		
Tissue	Gill	Intestine	Liver
TAG	67.3 ± 8.3^{a}	78.6 ± 9.2^{a}	22.7 ± 11.1^{b}
ST	6.31 ± 1.6	4.3 ± 2.1	6.7 ± 1.3
PL	23.6 ± 6.2^{a}	15.8 ± 7.2^{a}	67.2 ± 8.8^{b}
TL	3.4 ± 1.5^{a}	13.2 ± 5.9^{b}	4.7 ± 0.8^{a}
NL	2.7 ± 1.3^{a}	11.3 ± 5.7^{b}	1.4 ± 0.6^{a}
Polar lipid	0.8 ± 0.2^{a}	1.9 ± 0.9^{b}	3.2 ± 0.5^{c}
ST:PL	0.3 ± 0.05^{a}	0.3 ± 0.1^{a}	0.1 ± 0.01^{b}

Values are mean ± SD and significant differences among tissue types are indicated by superscript letters.
Triacylglycerol (TAG), free fatty acids (FFA), sterol (ST), phospholipids (PL), total lipid (TL), neutral lipid (NL), sterol:phospholipid ratio (ST:PL).

Table 4.7. Lipid class (%), total, polar and neutral lipid (% ww^{-1}) comparisons of gill, intestine mucosa and liver tissue of steelhead trout juveniles fed flax seed oil based (FLX) diet at 18°C.

Feed	Flax seed oil		
Tissue	Gill	Intestine	Liver
TAG	66.2 ± 9.5^{a}	69.6 ± 5.6^{a}	17.1 ± 10.0^{b}
ST	5.8 ± 1.4	4.3 ± 0.7^{a}	7.0 ± 1.7^{b}
PL	24.8 ± 8.1^{a}	24.8 ± 6.24^{a}	73.3 ± 7.5^{b}
TL	3.6 ± 2.4^{a}	10.6 ± 3.8^{b}	5.7 ± 1.4^{a}
NL	2.8 ± 2.1^{a}	8.1 ± 3.1^{b}	1.5 ± 0.7^{a}
Polar lipid	0.8 ± 0.4^{a}	2.5 ± 0.8^{b}	4.2 ± 1.0^{c}
ST:PL	0.2 ± 0.05^{a}	0.2 ± 0.04^{a}	0.1 ± 0.02^{b}

Values are mean ± SD and significant differences among tissue types are indicated by superscript letters.
Triacylglycerol (TAG), free fatty acids (FFA), sterol (ST), phospholipids (PL), sterol:phospholipid ratio (ST:PL).

Table 4.8. Effect of diet and temperature on liver fatty acid (% total) composition of steelhead trout juveniles.

Fatty acid	Initial	Final		
Temperature °C	8	18		
Feed	HE	HE	SF	FLX
14:0	1.3 ± 0.3	1.4 ± 0.2[a]	0.6 ± 0.2[b]	0.8 ± 0.1[b]
16:0	12.2 ± 2.1[*]	16.8 ± 0.5[*a]	13.1 ± 0.4[b]	14.2 ± 0.8[c]
18:0	7.4 ± 2.8	7.8 ± 0.5[a]	7.2 ± 0.4[b]	7.2 ± 0.3
ΣSFA[1]	**21.4 ± 3.8[*]**	**26.8 ± 0.6[*a]**	**21.7 ± 0.6[b]**	**23.1 ± 0.7[c]**
16:1ω7	3.4 ± 0.9[*]	1.5 ± 0.7[*]	1.0 ± 0.09	1.1 ± 0.3
18:1ω9	16.6 ± 6.7[*]	7.8 ± 0.7[*a]	18.3 ± 1.9[b]	9.5 ± 1.0[a]
18:1ω7	2.9 ± 1.0	2.6 ± 0.3[a]	1.7 ± 0.5[b]	1. 6 ± 0.2[b]
20:1w9	1.4 ± 1.0	1.3 ± 0.4	1.5 ± 0.56	1.14 ± 0.4
ΣMUFA[2]	**26.4 ± 9.7**	**17.0 ± 1.8[a]**	**25.2 ± 1.2[b]**	**16.4 ± 2.8[a]**
18:2ω6	5.8 ± 1.7[*]	2.9 ± 0.3[*a]	8.7 ± 0.7[b]	5.4 ± 0.4[c]
18:3ω3	0.3 ± 0.1[*]	0.2 ± 0.04[*a]	0.4 ± 0.1[a]	7.0 ± 1.0[b]
20:2ω6	0.9 ± 0.4[*]	0.5 ± 0.06[*a]	1. 6 ± 0. 8[b]	1.2 ± 0.3
20:3ω6	0.8 ± 0.2[*]	0.2 ± 0.06[*a]	1.4 ± 0.3[b]	0.6 ± 0.07[c]
20:4ω6	3.5 ± 0.9[*]	4.5 ± 0.5[*a]	4.2 ± 0.7[a]	3.2 ± 0.5[b]
20:3ω3	0.1 ± 0.04[*]	0.05 ± 0.01[*a]	0.05 ± 0.02[a]	1.5 ± 0.3[b]
20:4ω3	0.5 ± 0.2[*]	0.3 ± 0.1[*a]	0.14 ± 0.03[b]	0.9 ± 0.1[c]
20:5ω3	5.7 ± 1.3[*]	8.4 ± 0.7[*a]	4.7 ± 0.8[b]	6.6 ± 1.5[c]
22:5ω6	0.3 ± 0.1	0.4 ± 0.1[a]	0.3 ± 0.05[a]	0.2 ± 0.02[b]
22:5ω3	2.3 ± 0.3[*]	3.1 ± 0.3[*a]	1.57 ± 0.3[b]	1.77 ± 0.3[b]
22:6ω3	29.8 ± 7.9	33.1 ± 1.2[a]	28.7 ± 2.3[b]	30.6 ± 2.0
ΣPUFA[3]	**51.7 ± 7.8**	**55.7 ± 1.3[a]**	**53.0 ± 1.5[b]**	**60.2 ± 2.5[c]**
P:S	**2.5 ± 0.6[*]**	**2.1 ± 0.03[*a]**	**2.4 ± 0.1[b]**	**2.6 ± 0.1[c]**
Σω3	**39.1 ± 8.8[*]**	**45.7 ± 1.4[*a]**	**36.0 ± 1.9[b]**	**49.1 ± 2.6[c]**
Σω6	**11.6 ± 1.4[*]**	**8.7 ± 0.4[*a]**	**16.5 ± 0.4[b]**	**10.6 ± 0.6[c]**
ω6:ω3	**0.3 ± 0.1[*]**	**0.2 ± 0.01[*a]**	**0.4 ± 0.1[b]**	**0.2 ± 0.02[a]**
Terrestrial[4]	**6.1 ± 1.8[*]**	**3.1 ± 0.3[*a]**	**9.1 ± 0.7[b]**	**12.3 ± 1.3[c]**
Average Chain Length	**19.0 ± 0.3**	**19.2 ± 0.1[a]**	**19.1 ± 0.1[b]**	**19.1 ± 0.1[a]**
Average Double Bonds	**2.8 ± 0.4**	**2.9 ± 0.1[a]**	**2.6 ± 0.1[b]**	**2.96 ± 0.1[a]**
EPA:AA	**1.8 ± 0.3**	**1.9 ± 0.1[a]**	**1.1 ± 0.3[b]**	**2.1 ± 0.3[a]**
DHA:EPA	**5.3 ± 1.0[*]**	**4.0 ± 0.3[*a]**	**6.4 ± 1.7[b]**	**4.8 ± 0.9[a]**

Values are mean ± SD.
Significant differences among diets at 18°C are indicated by superscript letters. Asterisks indicate significant differences for HE diet between initial and final temperatures.
[1]Sum of saturated fatty acids (SFA), which also includes: *i*15:0, 15:0, *ai*16:0, *i*17:0, *ai*17:0, 20:0, 22:0 and 23:0 at < 1.0% each.
[2] Sum of monounsaturated fatty acids (MUFA), which also includes: 14:1, 15:1, 16:1ω9, 16:1ω5, 17:1, 20:1ω11, 22:1ω11, 22:1ω9 and 24:1 at < 1.0% each.
[3] Sum of polyunsaturated fatty acids (PUFA), which also includes: 16:2ω4, 16:3ω4, 16:4ω3, 16:4ω1, 18:2ω4, 18:3ω6, 18:4ω1, 20:2a, 21:5ω3 and 22:4ω6 at < 1.0% each.
[4] Terrestrial fatty acid includes: 18:2ω6 and 18:3ω3.
Polyunsaturate to saturate ratio (P:S), Omega (ω), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), arachidonic acid (AA).

Table 4.9. Effect of diet and temperature on gill fatty acid (% total) composition of steelhead trout juveniles.

Fatty acid	Initial	Final		
Temperature °C	8	18		
Feed	HE	HE	SF	FLX
14:0	1.5 ± 0.2	2.04 ± 0.7[a]	1.6 ± 0.3[b]	1.2 ± 0.2[b]
16:0	20.0 ± 1.3	19.0 ± 2.3	16.7 ± 1.4	16.7 ± 1.3
18:0	6.8 ± 0.5	6.3 ± 0.8	6.2 ± 0.5	6.9 ± 1.3
ΣSFA[1]	**29.4 ± 2.0**	**28.3 ± 2.8**	**25.1 ± 2.3**	**25.8 ± 1.6**
16:1ω7	3.9 ± 0.6	3.51 ± 1.2[a]	1.9 ± 0.6[b]	2.1 ± 0.4[b]
18:1ω9	16.5 ± 2.5[*]	12.0 ± 1.6[*a]	18.2 ± 1.9[b]	14.6 ± 1.5[c]
18:1ω7	2.4 ± 0.6	2.4 ± 1.03	2.02 ± 0.3	1.6 ± 0.2
20:1ω7	0.5 ± 0.1	0.5 ± 0.2[a]	0.3 ± 0.02[b]	0.3 ± 0.03[b]
ΣMUFA[2]	**27.0 ± 3.1**	**22.1 ± 4.5[a]**	**26.6 ± 2.1[b]**	**22.6 ± 1.5[a]**
18:2ω6	5.4 ± 0.9[*]	3.4 ± 1.4[*a]	8.9 ± 2.0[b]	6.3 ± 1.1[c]
18:3ω3	0.3 ± 0.1	0.3 ± 0.1[a]	0.6 ± 0.3[a]	7.8 ± 1.6[b]
18:4ω3	0.2 ± 0.1	0.3 ± 0.2	0.2 ± 0.08[a]	0.6 ± 0.5[b]
18:4ω1	0.2 ± 0.03	0.3 ± 0.1[a]	0.1 ± 0.03[b]	0.1 ± 0.1[b]
20:2ω6	0.5 ± 0.1[*]	0.3 ± 0.1[*a]	0.9 ± 0.2[b]	0.5 ± 0.1[c]
20:3ω6	0.4 ± 0.2[*]	0.2 ± 0.08[*a]	0.9 ± 0.2[b]	0.4 ± 0.1[c]
20:4ω6	5.3 ± 1.4	5.0 ± 0.8[a]	4.9 ± 0.5[a]	3.7 ± 0.3[b]
20:3ω3	1.7 ± 2.65	0.13 ± 0.1[a]	0.2 ± 0.1[a]	1.0 ± 0.1[b]
20:4ω3	0.3 ± 0.04	0.53 ± 0.5	0.2 ± 0.04[a]	0.7 ± 0.1[b]
20:5ω3	6.6 ± 1.8[*]	9.4 ± 1.5[*a]	4.8 ± 0.5[b]	6.1 ± 0.3[b]
22:5ω6	0.3 ± 0.1	0.32 ± 0.1[a]	0.3 ± 0.04[a]	0.2 ± 0.01[b]
22:5ω3	1.3 ± 0.7[*]	2.7 ± 0.8[*a]	1.10 ± 0.1[b]	1.1 ± 0.1[b]
22:6ω3	18.0 ± 5.6	23.5 ± 4.2	23.5 ± 2.5	21.2 ± 1.0
ΣPUFA[3]	**43.1 ± 5.0[*]**	**49.2 ± 4.0[*]**	**47.8 ± 2.6**	**51.1 ± 2.7**
P:S	**1.5 ± 0.3**	**1.8 ± 0.3**	**1.9 ± 0.3**	**2.0 ± 0.2**
Σω3	**29.7 ± 5.7[*]**	**38.0 ± 4.7[*a]**	**31.2 ± 2.3[b]**	**39.0 ± 2.1[a]**
Σω6	**12.6 ± 1.4[*]**	**9.7 ± 0.8[*a]**	**16.5 ± 1.5[b]**	**11.8 ± 0.6[c]**
ω6:ω3	**0.5 ± 0.1[*]**	**0.26 ± 0.04[*a]**	**0.5 ± 0.1[b]**	**0.3 ± 0.02[a]**
Terrestrial[4]	**5.8 ± 0.9[*]**	**3.7 ± 1.4[*a]**	**9.5 ± 2.1[b]**	**14.1 ± 2.7[c]**
Avg. chain length	**18.3 ± 0.3**	**18.6 ± 0.2**	**18.6 ± 0.1**	**18.5 ± 0.1**
Avg. double bond	**2.1 ± 0.3[*]**	**2.5 ± 0.2[*]**	**2.3 ± 0.1**	**2.4 ± 0.1**
EPA:AA	**1.3 ± 0.5[*]**	**1.9 ± 0.2[*a]**	**1.0 ± 0.1[b]**	**1.7 ± 0.1[c]**
DHA:EPA	**2.72 ± 0.7**	**2.5 ± 0.2[a]**	**5.0 ± 0.9[b]**	**3.5 ± 0.2[c]**

Values are mean ± SD.
Significant differences among diets at 18°C are indicated by superscript letters. Asterisks indicate significant differences for HE diet between initial and final temperatures.
[1]Sum of saturated fatty acids (SFA), which also includes: *i*15:0, 15:0, *ai*16:0, *i*17:0, *ai*17:0, 20:0, 22:0 and 23:0 at < 1.0% each.
[2] Sum of monounsaturated fatty acids (MUFA), which also includes: 14:1, 15:1, 16:1ω9, 16:1ω5, 17:1, 20:1ω9, 20:1ω11, 22:1ω11, 22:1ω9 and 24:1 at < 1.0% each.
[3] Sum of polyunsaturated fatty acids (PUFA), which also includes: 16:2ω4, 16:3ω4, 16:4ω1, 18:3ω6, 18:2ω4, 20:2a, 21:5ω3 and 22:4ω6 at < 1.0% each.
[4] Terrestrial fatty acid includes: 18:2ω6 and 18:3ω3.
Polyunsaturate to saturate ratio (P:S), omega (ω), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), arachidonic acid (AA).

Table 4.10. Effect of diet and temperature on intestine mucosal fatty acid (% total) composition of steelhead trout juveniles.

Fatty acid	Initial	Final		
Temperature °C	8	18		
Feed	HE	HE	SF	FLX
14:0	1.7 ± 0.5*	2.6 ± 0.5*a	1.34 ± 0.5b	1.4 ± 0.4b
16:0	16.2 ± 0.8	17.0 ± 2.4a	14.9 ± 1.6b	15.1 ± 1.2c
18:0	7.1 ± 1.2	7.05 ± 0.9	6.64 ± 1.3	6.8 ± 1.7
ΣSFA[1]	**26.0 ± 1.8**	**27.9 ± 2.2a**	**23.6 ± 2.4b**	**24.0 ± 3.4**
16:1ω7	3.5 ± 1.4	2.9 ± 0.6	1.8 ± 1.0	2.0 ± 0.9
18:1ω9	14.2 ± 4.9*	9.0 ± 2.8*a	20.0 ± 4.5b	12.0 ± 3.5a
18:1ω7	2.5 ± 0.3	2.8 ± 0.4	2.3 ± 0.8	2.1 ± 0.2
20:1ω9	1.1 ± 0.3	1.2 ± 0.1a	2.2 ± 0.2b	1.3 ± 0.4a
ΣMUFA[2]	**25.2 ± 6.07**	**20.4 ± 2.9a**	**29.8 ± 6.0b**	**21.0 ± 4.6a**
16:2ω4	0.2 ± 0.1	0.4 ± 0.3a	0.12 ± 0.1b	0.1 ± 0.1b
18:2ω6	5.7 ± 2.3	3.7 ± 1.07a	8.5 ± 3.3b	7.2 ± 3.0
18:2ω4	0.2 ± 0.04	0.43 ± 0.3a	0.1 ± 0.03b	0.1 ± 0.07b
18:3ω3	0.4 ± 0.1	0.3 ± 0.07a	0.9 ± 0.6a	10.3 ± 4.4b
18:4ω3	0.3 ± 0.2	0.5 ± 0.2	0.2 ± 0.15a	0.7 ± 0.35b
20:2ω6	0.8 ± 0.1	0.6 ± 0.3a	1.7 ± 0.4b	1.0 ± 0.16a
20:3ω6	0.5 ± 0.1	0.4 ± 0.1a	1.0 ± 0.2b	0.4 ± 0.1a
20:4ω6	2.3 ± 0.5	2.7 ± 0.8a	1.9 ± 0.4	1.4 ± 0.5b
20:3ω3	0.4 ± 0.3	0.3 ± 0.2a	0.4 ± 0.3	2.2 ± 0.5b
20:4ω3	0.4 ± 0.12	0.5 ± 0.1a	0.3 ± 0.1b	1.0 ± 0.6c
20:5ω3	5.2 ± 0.6*	7.9 ± 0.3*a	4.0 ± 0.5b	5.0 ± 1.0c
22:5ω6	0.3 ± 0.04	0.4 ± 0.2a	0.3 ± 0.07	0.15 ± 0.03b
22:5ω3	2.2 ± 0.2*	3.3 ± 0.56*a	1. 5 ± 0.2b	1.5 ± 0.1b
22:6ω3	28.1 ± 6.7	26.8 ± 4.5	24.4 ± 6.8	22.6 ± 6.9
ΣPUFA[3]	**48.4 ± 4.4**	**51.0 ± 3.8a**	**46.2 ± 3.8b**	**54.7 ± 1.8a**
P:S	**1.9 ± 0.1**	**1.9 ± 0.3a**	**2.0 ± 0.1a**	**2.3 ± 0.3b**
Σω3	**37.2 ± 6.7**	**40.1 ± 4.1a**	**31.8 ± 6.5b**	**43.6 ± 4.5a**
Σω6	**11.5 ± 1.6***	**8.9 ± 1.5*a**	**14.2 ± 2.9b**	**11.0 ± 2.5**
ω6:ω3	**0.3 ± 0.1***	**0.2 ± 0.1*a**	**0.5 ± 0.2b**	**0.3 ± 0.1a**
Terrestrial [4]	**6.2 ± 2.4**	**4.1 ± 1.1a**	**9.4 ± 3.9a**	**17.5 ± 7.0b**
Average Chain Length	**18.7 ± 0.3**	**18.7 ± 0.2**	**18.8 ± 0.3**	**18.7 ± 0.3**
Average Double Bonds	**2.5 ± 0.3**	**2.6 ± 0.2**	**2.3 ± 0.3**	**2.5 ± 0.2**

Table 4.10 continued;

EPA:AA	**2.4 ± 0.5**	**3.1 ± 0.8[a]**	**2.1 ± 0.3[b]**	**3.8 ± 0.7[a]**
DHA:EPA	**5.5 ± 1.4[*]**	**3.4 ± 0.5[*a]**	**6.2 ± 1.4[b]**	**4.5 ± 0.6[a]**

Values are mean ± SD.
Significant differences among diets at 18°C are indicated by superscript letters. Asterisks indicate significant differences for HE diet between initial and final temperatures.
[1]Sum of saturated fatty acids (SFA), which also includes: *i*15:0, 15:0, *ai*16:0, *i*17:0, *ai*17:0, 20:0, 22:0 and 23:0 at < 1.0% each.
[2] Sum of monounsaturated fatty acids (MUFA), which also includes: 14:1, 15:1, 16:1ω9, 16:1ω5, 17:1, 20:1ω11, 22:1ω11, 22:1ω9 and 24:1 at < 1.0% each.
[3] Sum of polyunsaturated fatty acids (PUFA), which also includes: 16:4ω1, 18:3ω6, 20:2a, 21:5ω3 and 22:4ω6 at < 1.0% each.
[4] Terrestrial fatty acids: 18:2ω6 and 18:3ω3.
Polyunsaturate to saturate ratio (P:S), omega (ω), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), arachidonic acid (AA).

4.4. Discussion

This study examined the effect of elevated water temperature on lipid composition of gills, intestine mucosa and liver tissue of juvenile steelhead trout fed FO substituted diets. Many studies examined the effect of low temperature on lipid class and fatty acid metabolism in fish (Hazel 1990; Fodor et al., 1995; Jobling et al., 1995; Logue et al., 2000; Tocher et al., 2000; Bell et al., 2001), with limited studies evaluating both dietary and thermal effects (Craig et al., 1995; Fracalossi and Lovell, 1995; Labbe et al., 1995; Kelly and Kohler, 1999; Grisdale-Helland et al., 2002). The results of the latter group are inconsistent due to differences in species and type of tissues examined and type of lipids and oils used in the experimental diets. The main focus of these studies was growth, muscle, body and liver lipid composition and some studies also examined the intestine (Olsen et al., 2003; Jutfelt et al., 2007) and gill lipids (Sellner and Hazel, 1982; Cornelius, 2001; Bystriansky and Ballantyne, 2007; Grant et al., 2008). Miller et al. (2006) examined the lipid profile of gill, white and red muscle in Atlantic salmon reared at 19°C, in contrast the temperature in this study was elevated from 10 to 18°C. Although, 18°C may not be the average temperature prevailing during late summer/fall in the Bay d'Espoir region every year, the change in water temperature closely mimics the ambient water temperature fluctuations in the region.

The differences in TL, NL and polar lipid (%ww^{-1}) (Table 2.6) content in the diets had no effect on lipid class composition of gill and intestinal mucosa. Also, diet had no influence on all other major classes of lipid (TAG, ST, and PL) in the gill tissue (Table 4.1) either. The change in temperature from 10 to 18°C did not affect major lipid classes, or TL, NL or polar lipid (%ww^{-1}), or ST:PL ratios of gills, in fish fed HE diet. The effect

of temperature was statistically compared only for fish fed HE diet because these were the only fish fed with the same diet at both temperatures (8 and 18 ^{o}C). Comparable lipid class composition for both SF and FLX diet fed fish and fish fed HE diet at 18^{o}C (Table 4.1) indicates minimal effect of temperature on gills of both SF and FLX fed fish as well. Although fatty acid composition of tissues in fish is widely believed to reflect dietary lipids (Henderson and Tocher, 1987; Sargent et al., 1989; Higgs and Dong, 2000; Sargent et al., 2002), the above results on gills suggest that incorporation of lipids to specific tissues could vary depending on the physiological role of the tissue. Gills are the primary site of gas exchange in fish. Gill filaments are the basic functional unit and each filament is composed of many lamellae evenly distributed along the filament length. Each lamella is essentially two layers of epithelial tissue held together by a series of individual cells called pillar cells. The space between the two epithelial layers is perfused with blood, while the exterior of lamellae are in touch with water (Evans et al., 2005). Gills are not structurally oriented towards storing lipids; rather the direct interface of the gill membrane with the external environment mediates exchange reactions essential to the regulation of the internal environment (Sellner and Hazel, 1982). Therefore, factors affecting the membrane composition of gills could have adverse affects on function.

Diet can influence gill membrane structure through changes in fatty acid composition. Such change could alter the structural integrity of the membrane compromising the performance of associated proteins (Lee, 2003). Membrane fatty acids are known to limit or stimulate activities of membrane proteins by specific interactions of carbon atoms in fatty acyl chains with proteins and through membrane phase changes (Cornelius, 2001; Lee, 2003; Esmann and Marsh, 2006). Membrane fatty acid

composition has been shown to have substantial direct effects on Na^+/K^+-ATP*ase* enzyme activity (Else and Wu, 1999; Wu et al., 2004). In a study involving Arctic char (*Salvelinus alpinus*), Bystriansky and Ballantyne (2006) found a correlation between membrane fluidity and Na^+/K^+-ATP*ase* activity in seawater reared fish, but not in freshwater reared fish suggesting such influence could vary by species and environment. Grant et al. (2008) suggested the influence of certain dietary fatty acids (of terrestrial and marine origin) on Na^+/K^+-ATP*ase* isoform that could potentially play a role in salinity tolerance in juvenile Chinook salmon (*Oncorhynchus tshawytscha)* and other *Oncorhynchus* species. The enzyme Na^+/K^+-ATP*ase* α1a is predominantly a freshwater isoform, while Na^+/K^+-ATPase α1b is predominant in seawater. Diets rich in terrestrial fatty acids (e.g. SF and FLX) closely resemble natural food sources of freshwater fish (Higgs et al. 1995). These diets may selectively decrease the expression of the main gill isoform suited for seawater, Na^+/K^+-ATP*ase* α1b, since less of this isoform may be required in a freshwater dietary regime. Conversely, HE diet resembles the natural marine food source (Higgs et al., 1995), which may elevate the gill Na^+/K^+-ATP*ase* α1b mRNA expression in seawater (Grant et al., 2008). The HE diet will also increase tissue concentration of EPA, DHA and EPA:AA ratios resulting in increased production of EPA derived eicosanoid compounds (Higgs and Dong, 2000). Eicosanoids are found in both freshwater and marine fish and play a critical role in immune response in fish (discussed below). The enzymes responsible for production of eicosanoids, cyclooxygenases and lipoxygenases (substrate specificity towards AA and EPA, respectively) are known to be most active in gill tissue (Tocher 2002a). The gill terrestrial fatty acid composition of fish fed both SF and FLX diets were significantly higher compared to those fed HE diet,

which may affect the activity of corresponding eicosanoid producing enzymes in gills. Further, SF diet fed fish in particular had significantly lower EPA:AA ratios in gills compared to the other two groups, which also may influence the action of respective gill enzymes (Table 4.9).

The total SFA, PUFA, P:S ratio, average chain length and number of double bonds were unaffected in gills by the dietary lipid composition even though the respective feeds were significantly different in fatty acid composition (Table 2.6). However, certain dietary fatty acids that were present in high concentrations did affect the fatty acid composition in gill tissue (Table 4.9). The high composition of MUFA, mainly 18:1ω9 was reflected in gill tissues of fish fed SF diet. Although total PUFA levels were unchanged by the dietary differences, the lower Σω3 and higher ω6:ω3 ratio in gills of SF diet fed fish was directly influenced by the corresponding polyunsaturated fatty acids (mainly 18:2ω6) in the SF diet. A similar influence was also seen in total terrestrial plant fatty acid composition (18:2ω6 and 18:3ω3) in gill tissues, with HE diet fed fish having the lowest terrestrial fatty acid composition, followed by SF and FLX fed fish. In general, the individual terrestrial fatty acid compositions (%) in gill tissue of fish fed each diet was 2 to 3 times lower than in the respective diets. Similarly, the high concentration of EPA and DPA in HE diet fed fish reflects the higher occurrence of these fatty acids in the HE diet. However, dietary differences in DHA were not reflected in the gill tissue. Values obtained for gill DHA composition in this study are comparable to those previously reported values in rainbow trout (Sellner and Hazel, 1982). The gill DHA composition was not affected by change in temperature or diet (Skalli et al., 2006). The physiological importance of DHA to maintain membrane structure and function may lead to selective

deposition of DHA in gill membranes irrespective of the dietary influence (Stubhaug et al., 2007).

However, some gill fatty acids in this study behaved differently to those previously reported (Sellner and Hazel, 1982). In a study conducted by Sellner and Hazel (1982), rainbow trouts acclimated at 5°C were gradually re-acclimated to 20°C in order to examine the time course alterations in fatty acids. They found an increase in SFA and MUFA and a decrease in PUFA, Σω3, chain length, unsaturated:saturated ratio and unsaturation index (average double bonds). It is difficult to make a direct comparison with the results reported by Sellner and Hazel (1982), due to differences in the rearing environments (marine *vs* freshwater) and the diets used. A single commercial trout diet (Hazel 1979) was used by Sellnar and Hazel (1982) with a relatively lower composition of marine origin lipids and in contrast the HE diet used in this study was rich in marine origin lipids. In the present study both total SFA and PUFA in fish fed HE diet were not significantly influenced by the change in temperature. The high gill PUFA levels of HE fed fish with increasing temperature is mainly due to the concentration of EPA and DPA in the HE diet. However, the other polyunsaturated fatty acids (e.g. 18:2ω6, 20:2ω6, 20:3ω6) decreased with increasing temperature similar to that reported by Sellner and Hazel (1982). The comparatively lower proportions of EPA and DPA in gills of SF and FLX fed fish compared to the HE treatment at both temperatures highlights the influence of diet over temperature. In contrast, marine HUFA (EPA and DHA) and the ω3 level in gills of HE diet fed fish increased with the rise in temperature. However, comparable Σω3 levels in HE and FLX fed fish at 18°C was mainly due to high dietary input of 18:3ω3 in

FLX diet. The P:S ratio, average chain length and the double bond number did not change among gill tissues due to diet or temperature.

The TAG and PL composition of both liver and intestine mucosa was significantly influenced by the experimental diets. The proportion of TAG deposition between intestine and liver was approximately 3:1 with both SF and FLX diets (Tables 4.6 and 4.7, respectively). However, the TAG content in the intestinal mucosa was as twice as high compared to the liver with HE diet (Table 4.5). This suggests that a higher proportion of TAG assimilated in the intestine mucosa from HE diet was deposited in liver. Conversely, both SF and FLX fed fish incorporated higher proportions of PLs into liver tissue. TAG and PL are the major lipid components in cells. PLs are the major structural components of membranes and TAG is stored in lipid droplets in the cytoplasm and mobilised to other tissues, or used for β-oxidation (Gibbons et al., 2000 and 2004).

In salmonids, both protein and dietary lipids provide energy (Van den Thillart 1986) and a high dietary lipid is known to have a sparing effect on proteins (Hemre and Sandnes, 1999). Both liver and red muscles act as primary sites of β-oxidation in salmonids and both diet and physiological status could impact capacity (Henderson and Tocher, 1987, Stubhaug et al., 2006). Dietary lipid composition is also known to influence the storage and selection of fatty acid substrates for β-oxidation (Glencross 2009). Fish fed HE diet tend to store more saturated fatty acids in liver tissues, while fish fed SF diet stored more MUFA (Table 4.8). This directly corresponds to the fatty acid composition of the respective diets (Table 2.6). Previous studies have suggested preference in SFA and MUFA over PUFA for β-oxidation (Kiessling and Kiessling, 1993; Henderson 1996). In contrast, the level of PUFA was highest with the FLX feed.

Both HE and FLX fed fish had similar DHA levels, but the FLX diet was rich in LA and LNA which contributed to a significantly higher PUFA level. While both FLX and HE had similar DHA levels in liver tissue, the dietary composition of DHA in HE feed was twice as high in this fatty acid compared to both FLX and SF diets. However, the compositional difference in the diet was not reflected in liver tissue to the same magnitude. Although DHA composition of SF diet fed fish was significantly lower than HE diet fed fish, the final liver DHA compositions were not far off from each other. These findings suggest selective storage of DHA in liver tissue of both FLX and SF fed fish. Studies conducted by Bell et al. (2003a) and Stubhaug et al. (2007) have shown that fish are capable of selective storage of ω3 HUFA, mainly EPA and DHA, when fed diets deficient of such fatty acids. Further, fish were capable of utilizing surplus dietary fatty acids for β-oxidation and energy production while preserving essential ω3 HUFAs in membranes. However, the preference of one fatty acid over the other could be curtailed with a surplus of dietary fatty acids.

Atlantic salmon can readily oxidize MUFAs (18:1ω9 and 22:1ω11) and C_{18} PUFA (18:2ω6 and 18:3ω3) when present at high concentrations (Bell et al., 2003a,b; Torstensen et al., 2004; Stubhaug et al., 2007). Surplus EPA and DHA in the diet was preferred during high growth periods (Stubhaug et al., 2007). Such an ability to switch between energy substrates enhances the capacity of steelhead trout to withstand dietary fatty acid alterations while preserving the liver membrane composition. However, excess accumulation of C_{18} PUFAs (mainly LNA and LA) in membranes with both SF and FLX diet could result in undesirable effects on membrane function.

Lipid accumulation in specific tissues and liver β-oxidation capacity could vary depending on the species and physiological status (Turchini et al., 2009). Stored fat is known to provide energy during periods of high energy demand such as low feed availability, smoltification and migration. Both liver and red muscle β-oxidation is known to contribute significantly during par smolt transformation in Atlantic salmon (Stubhaug et al., 2007). However, the preferred site of fat accumulation in growing salmonids is known to be in the abdomen and flesh, while Atlantic cod accumulates significant quantities of fat in the liver (Lie et al., 1986).

Studies conducted in the past to evaluate deposition of lipids with dietary FO substituted with VO have given contradicting results. An increase in hepatic lipid storage was reported when FO was replaced with sunflower oil and a canola/rapeseed oil combination (Torstensen et al., 2000; Bell et al., 2001). This was quite opposite to the findings of the present study with no such difference in liver total lipid ($\%ww^{-1}$) among HE, SF or FLX fed fish. Similarly total lipids were not significantly affected when fish oil was replaced with palm oil (Torstensen et al., 2000; Bell et al., 2002) or an equal mixture of canola/rapeseed and linseed oil (Tocher et al., 2001). The different results in hepatic lipid storage may be due to differences in water temperature, level of dietary lipid inclusion and growth stage of the fish. Jordal et al. (2007) found increased hepatic lipid storage with VO blends in salmon diets at colder temperatures. Ruyter et al. (2006) found that Atlantic salmon tend to increase liver lipid at 5°C but not at 12°C when fish oil is replaced with soya bean oil. The effect of temperature on lipid deposition in the 3 tissues examined in this study will be discussed later.

The lack of significant differences among TL, NL and polar lipid (%ww^{-1}) compositions in the intestinal mucosa suggests no dietary effects on lipid digestion and absorption in steelhead trout juveniles. The dietary lipids are generally well digested and absorbed by fish, although addition of VO to the diet could potentially modify the characteristics and quality of the flesh (Sigurgisladottir et al., 1992; Olsen and Ringø, 1997). The TL (%ww^{-1}) composition of intestinal mucosa was significantly higher than both gills and liver at 10°C (HE feed) and at 18°C with all 3 diet types. TAG was the main component present followed by PL and sterols. Increased TAG composition in the intestinal mucosa is primarily due to re-esterification of assimilated dietary fatty acids with glycerol to form TAG in the intestinal mucosa (Sheridan 1988). Dietary TAGs are hydrolysed by a non-specific lipase in fish to release fatty acids from the glycerol backbone and are absorbed by the intestinal mucosa (Patton et al., 1975). However, absorption of individual fatty acids is known to occur at different rates depending on the degree of unsaturation, location of the first double bond, fatty acid chain length and melting point (Røsjø et al., 2000; Morais et al., 2005; Francis et al., 2007). Steelhead trout juveniles readily absorbed SFA fatty acids, mainly 16:0 and 18:0. The HE diet had a significantly higher percentage of 16:0 above the other 2 feeds, which was reflected in the intestinal mucosa of HE diet fed fish. Intestinal mucosa fatty acid composition was closely correlated to the dietary availability of fatty acids. The main MUFA absorbed by all 3 fish groups was 18:1ω9 and it was significantly higher in SF fed fish due to its increase availability in the feed. A similar dietary response was also seen in some PUFA levels in fish fed SF (increased 18:2ω6) and FLX diets (both 18:2ω6 and 18:3ω3). The accumulation of EPA, DPA and DHA in intestinal mucosa of fish fed the HE diet was

significantly higher, which corresponds to the fatty acid availability of the HE diet. However, the intestine DHA composition of juvenile steelhead trout fed all 3 diets was similar, which suggests preferential absorption and deposition of DHA by the intestinal mucosa irrespective of dietary differences. The fatty acid digestibility of fish is known to be highest with HUFA, which has a higher degree of unsaturation with lower melting points. The melting point of fatty acids decreases with increasing unsaturation and higher melting points are known to reduce digestibility and energy availability of fatty acids (Ng et al., 2007). Individual dietary fatty acids that are easily digested and absorbed are EPA and DHA (Jutfelt et al., 2007), which explains its high availability in the intestinal mucosa. The SF and FLX diets abundant in C_{18} PUFAs, mainly 18:2ω6 and 18:3ω3 are quickly digested and absorbed after HUFAs. The digestibility of C_{18} PUFA is higher than both MUFA and SFA (Jutfelt et al., 2007). The substitution of fish oil with SF and FLX oil significantly modified the intestinal mucosal fatty acid composition in steelhead trout juveniles. Similar modifications in Atlantic salmon were reported by Caballero et al. (2003), Oxley (2005a) and Ruyter et al. (2006), where such modofications could alter absorption of fatty acids in Atlantic salmon (Caballero et al., 2003; Martins et al., 2006).

The diet had no influence on the PL composition of the intestinal mucosa (Table 4.2), but the higher sterol composition in HE fed fish than the fish fed SF and FXL feed could be due to the influence of both diet and temperature. The intestinal sterol composition of both SF and FLX fed fish was similar, but approximately half of that of fish fed the HE diet. Similar results were reported in two other studies conducted on the effect of a vegetable base diet on Atlantic salmon (Jordal et al., 2007) and rainbow trout plasma (Richard et al., 2006) and the lowering of cholesterol was attributed to low

availability of cholesterol in vegetable oil based diets. Further alterations in fatty acid composition of the intestinal mucosa could influence the membrane function and fluidity. Jutfelt et al. (2007) replaced FO with SF oil and found a decrease in the ratios of EPA:AA, thus influencing membrane bound enzymes and epithelial barrier function. A decrease in EPA:AA ratio was also seen in the present study in fish fed SF diet in all 3 tissues examined.

Inclusion of SF and FLX oil in place of FO brought significant qualitative changes to the diet used in this study. The HE diet is rich in HUFA, mainly of the ω3 series, while FLX and SF diets are rich in LNA (ω3) and both LA (ω6) and LNA (ω3), respectively. Marine fish have a requirement for ω3 HUFA, and LA and LNA are considered absolutely essential fatty acids for all vertebrates including fish (Sargent et al., 2002). Although all fish potentially have the capability of converting the two basic C_{18} ω3 and ω6 PUFA into corresponding C_{20} and C_{22} ω3 and ω6 HUFA, respectively (Nakamura and Nara, 2004), many marine species have lost this capability as a result of adapting to HUFA rich environments (Sargent et al., 2002). Therefore, marine fish have developed a net dietary requirement for ω6 and ω3 HUFA, such as 20:4ω6 (AA), 20:5ω3 (EPA) and 22:6ω3 (DHA) (Sargent et al., 1999). Further, the ω3 HUFA requirement is known to increase with salinity and decrease with the trophic level occupied due to variability in food that are available for fish (Turchini et al., 2009). Therefore, fatty acid desaturation and elongation capacity of juvenile steelhead trout deprived of HUFA by substituting FO with SF and FLX oil in their diet was evaluated. Fish oil replacement with SF oil increased the LA proportion in liver tissue by 1.8 and 3 times over fish fed FLX and HE diet, respectively (Table 4.8). As a result, the SF diet fed fish produced one third more

AA than fish fed FLX feed. Feeding fish SF diet produced comparable results in both gill and intestinal tissue. The SF diet fed fish had 1.4 and 2.5 times more LA in gill tissue than FLX and HE diet fed fish (Table 4.9) and 1.2 and 2.2 times more in intestinal mucosa, respectively (Table 4.10). As a result, the AA production in SF diet fed fish was increased by one third more over FLX diet fed fish in gill and intestinal mucosa. In contrast, the FLX diet was significantly higher in ALA (over 20 to 30 times that of SF and HE, respectively; Table 2.6). The influence of dietary ALA from FLX diet was seen in all 3 tissue types examined. The liver ALA composition of FLX fed fish was significantly higher (~7%) as opposed to less than 1% in both SF and HE diet fed fish (Table 4.8). This resulted in an increase ($p = 0.017$) in EPA in the liver tissue of FLX diet over SF diet fed fish. The ALA composition in both gill and intestinal mucosa was several fold higher in FLX fed fish. This resulted in an increase in EPA composition in both gills and intestinal mucosa by a quarter more in FLX fed fish compared to the other two.

Various enzymatic and molecular studies have been done in the past to evaluate the fatty acid desaturation and elongation pathways in fish (Henderson et al., 1994; Tocher et al., 1997, 2001, 2002a,b, 2003, 2006; Ruyter et al., 2000; Bell et al., 2001; Hastings et al., 2001; Agaba et al., 2004; Zheng et al., 2005; Oxley et al., 2005b; Stubhaug et al., 2005a,b). The two desaturase ($\Delta 5$ & $\Delta 6$) and elongase enzymes responsible for desaturation and elongation of $\omega 3$ and $\omega 6$ C_{18} PUFA into respective HUFA have been identified and cloned from Atlantic salmon (Henderson and Trocher, 1987; Hasting et al., 2004; Zheng et al., 2004). Sargent et al. (1993) suggested that these enzyme systems are regulated through competitive substrate inhibition with preference

for longer chains and higher levels of unsaturation, which would prefer ALA over LA. Further, ω3 fatty acids have been shown to have an affinity towards the Δ6 desaturase enzyme system. Therefore, ALA has a higher affinity than LA towards Δ6 (Sargent et al., 1993).

The FLX feed had both LA and ALA in considerable amounts compared to the other 2 feed types and it was reflected in all 3 tissue types. However, it is difficult to determine the affinity of ALA towards Δ6 due to the higher concentration of ALA in feed and tissue to begin with. The activity of desaturase and elongase enzymes are known to be higher in hepatocytes than enterocytes in Atlantic salmon (Tocher et al., 2001, 2002a,b, 2003; Oxley et al., 2005b; Stubhaug et al., 2005a,b). Results of the present study support the above finding, where fish fed FLX diet had higher EPA proportions than SF fed fish in the liver and intestine, but not in gill tissue. The conversion of LA into AA may have resulted in a significantly higher proportion of AA in liver and gills of fish fed SF diet than those fed FLX diet. Similarly, the higher proportion of EPA in liver and intestinal mucosa of fish fed FLX diet may have resulted from conversion of ALA to EPA by the respective tissues. Although conversion capability of C_{18} PUFA to ω3 and ω6 HUFA is known to be minimal in marine and anadromous species (Tocher et al., 2006b), the SF and FLX fed steelhead juveniles in the present study showed a significant difference between chain elongation and extension capability. Previous dietary studies involving marine reared Atlantic salmon have shown enhanced desaturation and elongation activity once fish oil was replaced with vegetable oil (Henderson et al., 1994; Ruyter et al., 2000b; Bell et al., 2001; Tocher et al, 2001, 2002a,b, 2003; Stubhaug et al., 2005a,b; Zheng et al., 2005). The results indicate that desaturation and elongation activity

was stimulated only up to a certain step in the conversion process, the conversion of LA into AA and ALA into EPA. The compositional difference between fatty acids produced beyond AA (mainly 24:5ω6) and EPA (mainly DHA) between SF and FLX diets are considerably less. The main end product of LA desaturation and elongation is AA, although AA could be further converted to 22:5ω6 (Tocher 2003). DHA on the other hand, the main end product of ALA desaturation and elongation (Tocher 2003), was not significantly affected by diet in all 3 individual tissues examined. DHA, which may have selectively accumulated in these tissues due to its essential nature of the fatty acid irrespective of dietary availability (Glencros et al., 2003), could potentially have a negative impact on *de novo* synthesis. Further, Tocher (2002) suggested differences in specificities of the enzymes involved in the desaturation process. The Δ6 enzyme acting on LA and ALA fatty acids earlier on in the desaturation process may not be the same as the one acting on the final desaturation step converting LA and ALA to 22:5ω6 and DHA, respectively. The substrate affinity of enzymes in the desaturation and elongation process favours ω3 over ω6 fatty acids, with a relative decrease in the activity of the enzyme with subsequent increase in the length of the fatty acid. Therefore, all the above mentioned factors could lead to a higher concentration of EPA and AA in tissues. However, studies conducted on Atlantic salmon and brown trout suggest that dietary vegetable oil alone cannot adequately compensate for the EPA and DHA requirement in fish fed diets deficient in these fatty acids (Bell et al., 2001; Tocher et al., 2001, 2002a,b, 2003; Zheng et al., 2005b). Fish deprived of dietary EPA and DHA have developed various deficiency symptoms, such as poor growth rate and increased mortality (Watanabe 1982; Sargent et al., 1995b; Ryter et al., 2000), caudal fin erosion, myocarditis and shock syndrome in

rainbow trout (Castell et al., 1972) and stress associated mortalities (Watanabe 1982). However, clinical signs associated with essential fatty acid deficiencies are unlikely in the present study for fish fed SF and FLX diet due to inclusion of a relatively large amount of fish meal in the feed formula.

Herring meal (~37%) was used in formulating the experimental feed with a residual fat content of ~8 - 10%, which could comprise up to 20 – 35% ω3 HUFA (Bimbo 2000). Turchini et al. (2009) pointed out that inclusion of fish meal at a rate as low as 300 g kg^{-1} (~ 30%) would provide 0.5 to 1% of dry diet ω3 HUFA, which is sufficient to fulfill essential fatty acid requirements of most farmed fish. However, inclusion of SF and FLX oil instead of fish oil leads to alterations in various fatty acids and important lipid ratios, such as ω6:ω3, DHA:EPA and EPA:AA, in the diet as well as in the tissues (Sargent et al., 1999). The diet directly modified the Σω6 fatty acid composition in all 3 tissues examined with fish fed SF diet having the highest proportion followed by those fed FLX and then those fed HE. However, the ω6:ω3 ratios were influenced only by SF diet in all 3 tissues types. The same ratio in HE and FLX fed groups was similar, but lower than in SF diet fed fish, which also directly influenced the EPA:AA ratios. Both EPA and AA are responsible for producing bioactive compounds called eicosanoids that are involved in both immune and inflammatory responses (Tocher 2003). The activity level of the two eicosanoids varies, with the AA derived eicosanoids being more biologically active than ones derived from EPA. The ratio EPA:AA determines the eicosanoid activity in cell membranes, with AA being considered the preferred substrate. EPA is found in abundance in fish tissue. Therefore, dietary changes in ω6:ω3 could lead to detrimental modifications in the immune and inflammatory response in fish (Bell et al.,

1997; Sargent et al., 1999; Tocher 2003). Okuyuma et al. (1997) reported high AA as a result of increase dietary ω6:ω3 ratio could lead to pathological inflammatory conditions in the cardiovascular system. Similar results were also reported by Terano et al. (1986) and Calder (2001). In Atlantic salmon post smolts, elevated ω6:ω3 ratio increased incidence of atherosclerotic lesions, increased susceptibility to bacterial diseases and altered liver detoxifying ability of xenobiotics (Thompson et al., 1996). In contrast, dietary supplementation of ω3 HUFA reduced the pathological effects of ω6 by reducing the excessive production of AA. Studies conducted on the causes of increased eicosanoid production in fish are scarce; however, enzymes responsible for eiconasoid production are known to be highest in gills (Tocher, 2002). The EPA:AA ratio in the gill tissue (as in liver and intestine) of fish fed SF diet was significantly lower than in those fed FLX diet, mainly due to increased proportions of AA in gill tissue. This may lead to increased availability of AA for eicosanoid production in gills of SF diet fed fish compared to the other two groups.

The steelhead trout in this study were also exposed to increasing temperature from 8 to 18°C. The comparison of lipid classes and fatty acids among the 3 tissue types (gill, intestinal mucosa and liver) at both 8 and 18°C shows the distribution of lipids among tissues of physiological importance. At 8°C with the HE diet intestine mucosa had a high affinity towards TAG as opposed to gill and liver (Table 4.4). Increased TAG concentration in intestinal mucosa is due absorption of lipid digestion products, mainly in the form of free fatty acids which are known to undergo re-esterification with glycerol to form TAG (Sargent et al., 1989). In contrast, phospholipids were richer in gills and liver as opposed to intestinal mucosa. Colder temperatures tend to increase the PL composition

in membranes and lower the SFA fatty acid composition (Farkas et al., 2001). Sterols are known to accumulate in membranes at low temperature (Yeagle et al., 1988). The tendency of gills to accumulate more sterols compared to intestine mucosa and liver may be due to its direct exposure to the external environment. Sterols are known to interfere with close packing of acyl chains in phospholipid molecules of the membrane bilayers, thereby increasing the fluidity of the membrane at cold temperatures (Hazel and Williams, 1990). At low temperatures, sterols effectively broaden the phase transitions of membrane lipids by reducing the cooperative size of the units involved in gel to fluid transition (Hazel and Williams, 1990). Broader phase transition may help preserve the structural integrity of the membrane and the functionality of membrane bound proteins in gills. Sterol composition of both intestine mucosa and liver was influenced by increasing temperature irrespective of dietary differences (Tables 4.2 and 4.3). At 18^{o}C, all 3 tissues had similar sterol levels (Tables 4.5, 4.6 and 4.7), but the composition was not altered in gill tissue in response to change in temperature (Table 4.1). This further signifies the effort to minimize the influence of temperature on gill membrane structure and functions. Sterols are also known to counteract the increasing fluidity of membranes at high temperature by ordering the fluid phase lipids, particularly in the region of acyl chains closest to the head groups (Yeagle 1985).

The influence of temperature was clearly seen in the fatty acid profile of liver tissue in steelhead trout. The adaptive changes in response to environmental temperature are mainly seen in the structural phospholipids due to involvement in membranes (Hazel and Williams, 1990; Fodor et al., 1995; Farkas et al., 2001). In the present study, steelhead trout at the lower temperature tended to accumulate SFA, 18:1ω9 (the main

MUFA) and all PUFA and HUFA examined, except for EPA, DHA, AA and 22:5ω6, Σω6 fatty acids and terrestrial plant fatty acids. Increased water temperature resulted in an opposite trend in the above mentioned fatty acid groups. Several other studies have shown similar results in response to change in environmental temperature (Fodor et al., 1995; Jobling et al., 1995; Logue et al., 2000). The increase in EPA, DHA, AA and 22:5ω6 was unusual, as previously reported data suggest a decrease in average chain length and unsaturation index (average number of double bonds) (Sellnar and Hazel, 1982). However, the P:S ratio decreased in liver tissue with increasing temperature due to increase SFA, which agrees with data reported by Sellnar and Hazel (1982). Research done in the past to evaluate the effect of temperature on membrane lipids of ectotherms are mainly focused on changes associated with decreasing temperature. The general trends found so far with exposure of fish to low temperature are increased incorporation of unsaturated fatty acids into the body lipids (Cossins and Lee, 1985; Hazel and Williams, 1990), increased MUFA at the expense of SFA accompanied by an increase in EPA and DHA (Henderson and Tocher, 1987; Sargent et al. 1989, 2002; Hazel and Williams, 1990; Logue *et al.* 2000; Hochachka and Somero, 2002), and an increase in P:S ratio and average double bonds (Cossins and Lee, 1985; Malak et al. 1989; Sørensen 1993; Wallaert and Babin, 1994; Fodor et al. 1995; Logue *et al.* 2000). Therefore, the general assumption is a reversal of the above mentioned parameters with increase in environmental temperature. Although some of the parameters examined in the present study (EPA, DHA, AA and 22:5ω6) are at odds with the general understanding of dietary influence, the composition of sterol, ST:PL ratio, SFA, P:S ratio and terrestrial fatty acids played a critical role in counteracting the effects of increasing temperature. The ST

composition in liver tissue increased by 3 fold and the ST:PL ratio increased by 2 fold in response to increased temperature. Cholesterol is the dominant sterol in fish (Napolitano et al., 1993) and is known to increase in animals acclimated to high temperatures (Crocket 1998; Robertson and Hazel, 1995; Wodtke 1978). Therefore, increased sterols and ST:PL ratios together with decreased P:S ratios counteracted the increasing fluidity of the membrane due to temperature and dietary effects of FO (increased EPA, DHA, AA and 22:5ω6 at 18°C) (Tables 4.3 and 4.8). The behaviour of intestinal mucosal lipids in response to temperature was similar to liver tissue in steelhead trout juveniles. The ST composition increased in response to increasing temperature accompanied by a decrease in 18:1ω9, the main monoenic fatty acid in membranes (Farkas et al., 2001). However, the parameters usually expected to react to a change in temperature (ST:PL and P:S ratio, SFA) did not.

In summary, dietary fatty acids influenced the fatty acid composition in the 3 tissues examined. The dietary influence was seen most in liver lipids followed by intestinal mucosa, with minimum effects in gill tissue. The key fatty acids, HUFA, LA and ALA in the HE, SF and FLX diets, respectively, changed the fatty acid composition of all three tissues regardless of the thermal influence. Both SF and FLX diets stimulated fatty acid elongation and desaturation processes, altering the substrate available for eicosanoid production in individual tissues. Steelhead trout juveniles were able to manipulate the ST composition in gills in response to temperature change to overcome the possible effects on membrane fluidity with increased HUFA levels when fed HE diet.

4.5. References

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Chapter 5

5.1. Summary and conclusions

Dietary lipids play an important role in fish as a source of metabolic energy for growth, reproduction, movement and health. Fish have always been the primary source of ω3 HUFA that are essential for the human consumers. Fish oil from capture fisheries is increasingly utilized with the expansion of the aquaculture industry. Therefore, it is important to find and implement sustainable alternatives to FO and investigate the potential physiological and metabolic influences on fish.

FO substituted feeds with vegetable oil and animal fat were used in this study. Both study (i) and (ii) explored the effect of FO substitution and change in water temperature on muscle lipid composition in steelhead trout adults and juveniles using commercially available and experimental diets, respectively. The results indicated that fish oil can be substituted with either AF or VO without affecting growth, feed efficiency or health once essential fatty acid requirements are met in the diet. One of the potential drawbacks of FO replacement in aquaculture feed is the resultant modification of the fatty acid composition in various tissues, mainly ω3 HUFA in fish fillets. FO rich diets in both study i and ii significantly increased the marine fatty acid composition in muscle tissue. In both studies, the ω3 HUFA content in muscle tissue increased significantly with a corresponding decrease in terrestrial fatty acid composition in muscle tissue. However, fish fed FO substituted feed in both studies showed selective accumulation of marine origin fatty acids in muscle tissue minimizing the dietary differences. Such capacity of steelhead trout could potentially benefit the aquaculture feed industry by significantly

reducing the amount of FO necessarily incorporated in diets while still avoiding essential fatty acid deficiencies in fish.

The use of vegetable oils rich in LA as FO substitute invariably increased the ω6 composition in muscle tissue altering the ω6:ω3 ratio in both studies. The tissue ω6 PUFA levels could potentially increase the eicosanoids compared to those produced from AA compared to ones produced from EPA, which are known to be more active. Therefore, diets rich in ω6 could modify the type and quantity of eicosanoid production, which could possibly alter their physiological activities, including immune and inflammatory responses that could be detrimental for the fish. Further, increase in ω6 PUFA and higher ω6:ω3 ratio reduces the quality of the fish fillet for the human consumers since fish with high concentration of EPA and DHA with a lower ω6:ω3 ratio is known to be beneficial for human health. Change in water temperature primarily affects the membrane associated polar lipids in fish tissues. Muscle tissue in both study (i) and (ii) had TAG as the main neutral lipid stored (above 80 - 90 % total lipid) by both adults and juveniles, with comparatively little polar lipid. Resultant effects of temperature on muscle tissue were minimal.

In Chapter 3, the effect of temperature and diet on liver membrane fluidity, lipid class and fatty acid composition were examined. Phospholipids (80 – 90 % total lipid) were the main lipid class present in liver lipid extract. Phospholipids are the primary structural components in cell membranes and alterations in fatty acid composition reflect the diet and temperature effects on liver cell membranes. The effect of diet and temperature on the liver lipid class composition was minimal, with no significant effect on ST and PL content. The Raman spectroscopy study showed the degree of *trans* and

gauche isomerisation of the liver membrane lipids. When liver lipid extracts were subjected to a temperature range of 2 to 30°C, the wave numbers for CH_2 stretching frequency for fish fed all three feed types increased. This illustrates the influence of temperature on increasing the motion of acyl chains in phospholipid molecules of the membrane.

The fish fed H-ω3 diet had the least variation in fluidity at both 18.0 and 13.5°C. The H-ω3 diet was the closest to the natural food source of marine fish and H-ω3 fed fish showed the ability to quickly adapt to an increase or decrease in environmental temperature, with the least effect on liver membrane physical properties. The CH_2 stretching response of the acyl chains in liver membrane PLs in fish fed both H-ω3 and M-ω3 diets were similar at 18.0°C. The fish fed H-ω3 diet were able to maintain the fluidity of liver membranes following the drop in temperature to 13.5°C. However, the liver membranes of fish fed M-ω3 diet were less fluid compared to H-ω3 fed fish. Fatty acid composition of M-ω3 and H-ω3 fish were not significantly different at 13.5°C, except for 18:ω6 which was higher in M-ω3 and had no significant influence in fluidizing the membrane. Both M-ω3 and H-ω3 fed fish lack evidence of conformational transformation of lipids from ordered state (all *trans*) to complete disordered state (all *gauche*) in response to increase in temperature. This finding shows that liver membranes of both M-ω3 and H-ω3 fed fish have the capability to withstand a broader temperature range without undergoing complete phase separation.

The liver membranes of L-ω3 fed fish were significantly more fluid in comparison to the other two feed groups at both rearing temperatures (18.0 and 13.5°C). The results

also suggest change in phase behaviour in liver membrane lipids of L-ω3 fed fish at both rearing temperatures. The fatty acid composition of 20:3ω6 and 20:4ω6 at 18.0°C and 20:3ω6 at 13.5°C (final) was significantly higher and may have influenced the fluidity in liver cell membranes of fish fed L-ω3 diet. In addition, several other lipid fractions that were not significantly different, but had relatively higher proportion (Σ ω3, DHA, PUFA, P:S, terrestrial and marine) at 18.0°C in L-ω3 fed fish, may have collectively influenced the fluidity of liver membranes. However, a significant change in a lipid class or a fatty acid that directly correlates with increased fluidity of L-ω3 fed fish at the final temperature was not evident.

Manipulation of cell membrane fluidity in response to thermal perturbation is a complex process that may not be fully understood simply by tissue fatty acid composition alone. The lower melting point of liver membrane lipids in L-ω3 diet fed fish may also have been influenced by C_{18} PUFA as seen in other experimental studies (Chapter 3). Although cell membrane fluidity is influenced by various factors, both H-ω3 and M-ω3 fed fish were least influenced by the change in diet and temperature. Therefore, it is necessary to further investigate the physiological effects of dietary vegetable oil incorporation in fish feed as the main substitute for fish oil.

Chapter 4 examines the effect of elevated water temperature on lipid composition of gills, intestine mucosa and liver tissue of juvenile steelhead trout fed FO substituted diets. The diets were the same as in study ii, which evaluated the muscle lipid composition of steelhead juveniles by replacing herring oil with either SF or FLX oil. The SF and FLX oils are rich in LA (ω6) and ALA (ω3) acid, respectively, with FLX diet

having a substantial amount of LA as well. The lipid class composition of gill tissue was not affected by diet or temperature. This suggests tissue specific incorporation of lipids in gills to minimize the environmental influence from diet or temperature. Both SF and FLX diets closely resemble natural food source of freshwater fish and may have to be used cautiously in diets of marine fish. Both SF and FLX diets significantly increased the terrestrial fatty acid composition and ω6:ω3 ratio in gills of juvenile steelhead trout. Such alterations could change the enzyme activity and eicosanoid production in gills which may be detrimental to the fish. However, DHA composition of gill tissue was not affected by diet or temperature, highlighting both selective deposition and the importance of DHA in maintaining gill membrane structure and function.

The diet and temperature significantly influenced the lipid class and fatty acid composition in both liver and intestine mucosa of juvenile steelhead trout. Deposition of TAG and PL differ between HE and the two FO substituted diets. The liver TAG (mainly SFA) deposition in HE diet fed fish was relatively higher compared to the other two diets. SF and FLX diet fed fish had a relatively higher proportion of PL, mainly MUFA deposited in liver compared to fish fed HE diet. Therefore, dietary lipid composition can directly influence the storage and selection of fatty acid substrates for β-oxidation in fish. Further, increased deposition of PL, mainly C_{18} PUFA, in liver tissue of SF and FLX fed fish could result in undesirable effects on membrane function.

The fatty acid composition of intestine mucosa reflected the most abundant fatty acids available in each diet. Absorption of 18:1ω9 and 18:2ω6 was highest with SF diet, while both 18:3ω3 and 18:2ω6 were readily absorbed by fish fed FLX diet. Similarly,

marine fatty acid (EPA, DPA and DHA) was richer in intestine mucosa of HE diet fed fish compared to other 2 groups.

Marine fish are usually exposed to a HUFA rich marine environment and are known to lose the capacity of desaturation and elongation of ω3 and ω6 C_{18} fatty acids into the corresponding HUFAs. Therefore marine fish have developed a net dietary requirement for ω6 and ω3 HUFA such as 20:4ω6 (AA), 20:5ω3 (EPA) and 22:6ω3 (DHA) respectively. However, results of this study indicate retention of some capacity for chain elongation desaturation by steelhead juveniles. The production of AA was stimulated in fish fed SF diets due to the high availability of LA in the diet, while the production of EPA was promoted by the higher availability of LNA in their diet. The results further indicate that desaturation and elongation activity between SF and FLX feed was stimulated only up to a certain step in the conversion process, the conversion of LA into AA and LNA into EPA. The effect of temperature was predominantly evident in the liver tissue of steelhead trout followed by intestine mucosa and gill tissue. Decreasing temperature increased the accumulation of PL in gill tissue of HE diet fed fish. However, steelhead trout juveniles were able to manipulate the ST composition in gills in response to temperature change to overcome the possible effects on membrane fluidity with increased HUFA levels in HE diets.