EFFECTS OF SOURCE OF DIETARY LIPID ON THE FATTY ACID COMPOSITION AND CHOLESTEROL CONTENT OF TISSUES, SEMEN CRYOPRESERVATION AND EMBRYO SURVIVAL IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Cory V. Pustowka

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ABSTRACT

The objectives of this research were to evaluate the effect of source of dietary lipid on the fatty acid composition and cholesterol content 1) of muscle and liver tissue, 2) of sperm and spermatozoal plasma membranes, 3) and its effect on sperm viability after cryopreservation and 4) and its effect on the fatty acid composition of eggs and developing embryos.

One hundred two-year-old rainbow trout (*Oncorhynchus mykiss*) were fed one of the four treatment diets for 16 months. The diets differed only in the additional source of lipid which comprised 12% of the total diet. Herring oil (adequate n-3 fatty acids), menhaden oil (high in n-3 fatty acids), safflower oil (high in n-6 fatty acids) or tallow (high in saturated fatty acids) were the sources of dietary lipid.

A significantly higher ($P<0.05$) percentage of monounsaturated fatty acids (MUFA) was in the muscle of fish fed the herring oil diet while the highest level ($P<0.05$) of polyunsaturated fatty acids (PUFA) was in the muscle of the safflower group. This information would be of interest to consumers who need to regulate their dietary lipid consumption.

Sperm from fish fed the tallow diet had significantly ($P<0.05$) higher cholesterol (CHOL) levels than sperm from the fish fed the other diets. The spermatozoal plasma membranes from fish fed the tallow diet had significantly ($P<0.05$) higher CHOL levels than those from fish fed the menhaden or safflower oil diets, and tended to have higher levels than those from the fish fed the herring oil diet. Cryopreserved sperm from fish fed the tallow diet exhibited the least membrane damage ($P<0.05$) and produced the highest percentage ($P<0.05$) of eyed embryos as compared with sperm from the other groups. Therefore, it would appear that high levels of CHOL provided the sperm with increased resistance to cryopreservation damage.

The fatty acid compositions of unfertilized and fertilized eggs, hatched and swim-up larvae were influenced by maternal dietary lipid; however, there were no differences in either gross morphology or fertility among eggs from fish fed the experimental diets. As embryo and larval development progressed, the order of the predominant fatty acids converged so at the swim-up stage of development, the patterns of predominant fatty acids were consistent among the treatment groups.

The following fatty acids: C16:0, C18:1 and C22:6n-3, were major components of all the tissues examined regardless of dietary treatment. Muscle, liver, sperm and spermatozoal plasma membrane fatty acids were affected by source of dietary lipid. However, there were some limitations as there appeared to be set physiological levels for some fatty acids. The source of dietary lipid was able to increase the viability of sperm after cryopreservation as well as affect the fatty acid composition and CHOL content of eggs and developing embryos.

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1. GENERAL INTRODUCTION

The field of aquaculture is expanding rapidly as more and more wildstock fisheries collapse. Aquaculture production, encompassing fish, molluscs, crustaceans and aquatic plants, was 19.3 million metric tons worldwide as compared to the 85 million tonnes of wildstock fish caught in 1992, contributing 18.5% of the total fisheries harvest (FAO, 1994). In contrast, the aquaculture production in 1967 was only 1 million tonnes (President's Science Advisors Committee, 1967). In Atlantic Canada, 3 000 tonnes of trout, with an estimated $17 million market value, were produced in 1993 demonstrating the importance of the industry (Dubé and Mason, 1995). Fish and fish products are important protein sources for both human and animal populations, contributing 5% of total protein consumed directly by humans. Due to increasing global human population and decreasing wildstock fish populations, aquaculture will have to fill the gap between supply and demand in coming years.

Research is required in all aspects of aquaculture as it is a relatively new field in modern food production. Aquacultural studies provide valuable information for fish producers who are usually not able to conduct independent research. Despite the recent arrival of aquaculture, there has already been much work done on salmonid species so that reproduction and nutrition are now specialized fields.

Nutritional research is necessary to maintain the health of the fish and can be used to enhance specific attributes of the fish such as muscle quality and fertility (Woodhead, 1961; Watanabe et al., 1984; Leray et al., 1985; Geurden et al., 1995). The fatty acid composition of fish muscle has become increasingly important as more links

are established between dietary fat and human health. In addition, as fish feed costs account for 40-50% of the total costs in an aquaculture operation, nutritional studies are also valuable for the economic consideration of different feed sources (Boghen, 1995). Advances in husbandry methods, such as tailoring broodstock diets for optimal embryo and larval nutrition and semen cryopreservation, are necessary for the success of the industry, as in any farming operation. Semen cryopreservation could be a very useful technique that would enable fish farmers to control the quality, fertility and breeding seasons of male broodstock as well as to facilitate gamete transportation. Cryopreservation could also reduce operating costs by reducing the need for maintaining male broodstock.

The objectives of this research were to evaluate the effect of source of dietary lipid on the fatty acid composition and cholesterol content 1) of muscle and liver tissue, 2) of spermatozoa and sperm plasma membranes, 3) on sperm viability after cryopreservation and 4) on the fatty acid composition of eggs and the developing embryos. This investigation of rainbow trout (*Oncorhynchus mykiss*) nutrition and reproduction may also benefit the rearing of other salmonids such as Atlantic salmon (*Salmo salar*) or Arctic charr (*Salvelinus alpinus*).

1.1 Nutrition

There has been considerable evidence linking dietary fat, or fatty acids, to human health within the past couple of decades. It has been shown that people can reduce the risk of heart diseases by reducing the consumption of total and saturated fat (Carlier et al., 1991). More recently, the type of unsaturated fat consumed is also thought to be an

important health factor (Jonnalagadda et al., 1996). The n-6 (omega 6; denoting 6 carbon atoms between the last double bond and the methyl group in the fatty acid) unsaturated fatty acids give rise to eicosanoids, which are hormone-like substances that include leukotrienes, thromboxane and prostaglandins which are all required in a variety of physiological functions including blood clotting, inflammation and immune response. However, eicosanoids can also cause cellular imbalances leading to such diseases as cardiovascular disease, arthritis and certain types of cancer. The n-3 (omega 3) fatty acids are thought to regulate the effects of the n-6 fatty acids (Skjervold, 1992). In response to this research, there is growing public concern regarding the lipid composition of food and increased public interest in fish and fish products, which are excellent sources of n-3 fatty acids (Ackman, 1989).

Researchers have shown that the source of dietary fat influences the fatty acid composition of tissues in many organisms and that this composition can affect tissue function (Ajuyah et al., 1991; Muriana and Ruiz-Gutierrez, 1992; Jiang and Sim, 1992; Otten et al., 1993; Merican and Shim, 1994; Røsjø et al., 1994; Santos et al., 1995). In this way, the source of dietary lipid may affect cryopreservation, fertilization and embryo development.

1.1.1 Lipids

There are many types of lipids which are generally known as fats and oils. Commonly, lipids can be either esters of fatty acids and an alcohol, such as glycerol, or amides of fatty acids and sphingoids. They also may contain more complex components such as phosphoric acid and sugars. Fatty acids are chains ranging from 2 to 24 carbon

atoms (C) with a terminal carboxyl group (COOH). Unsaturated fatty acids have one or more double bonds within the carbon chain while saturated fatty acids do not contain any double bonds. Fatty acids are often represented by the number of carbon atoms in the chain and by the number of double bonds (e.g. C16:0 has 16 C atoms and no double bonds while linoleic acid C18:2n-6 has 18 C atoms and 2 double bonds). The 6, in the n-6 notation, indicates the number of carbon atoms between the last double bond and the methyl group in the fatty acid.

The family of sterols is another major lipid group. Cholesterol, a type of sterol, is necessary for bile production, hormone synthesis and membrane function. Plasma membranes usually have a much higher concentration of cholesterol than intracellular membranes (Yeagle, 1985). In addition, the presence of cholesterol in membranes alters physical characteristics such as fluidity and permeability (Yeagle, 1985). Fish, like mammals, are able to synthesize sufficient amounts of cholesterol (Stryer, 1988).

Lipids serve as an excellent energy source (39 kJ/g) as well as enhancing the palatability and consistency of feeds. Certain fatty acids, which are required in the diet and cannot be produced in sufficient quantity in the body, are known as the essential fatty acids (EFA). Most freshwater fish species, including rainbow trout, have an EFA requirement for C18:2n-6 and C18:3n-3 fatty acids (National Research Council, 1993). In addition to C18:2n-6 and C18:3n-3, marine fish also require dietary C20:5n-3 and C22:6n-3 for proper growth and development. Lipid biosynthesis, *de novo*, is suppressed when fatty acids compose more than 10% of the total diet (Sargent et al., 1988). Ranges between 10-20% of dietary lipid have been cited to allow optimum protein efficiency in

fish (Cowey and Sargent, 1979). In this study, a basal diet was supplemented with 12% lipid by weight from four sources.

In the digestive tract, dietary lipids are broken down with the aid of bile and lipases into free fatty acids and monoglycerides, which are then absorbed through the anterior intestine and pyloric caeca in rainbow trout. In general, the components are reconstituted into lipoproteins in the endoplasmic reticulum which are then stored or used for structural components or for energy (Henderson and Tocher, 1987). One of the important structural uses for lipid is the synthesis of membranes which can contain up to 80% lipid.

1.2 Membranes

Membranes are composed of two lipid layers forming a bilayer with the hydrophobic ends of the fatty acids of each layer pointing towards each other. They are dynamic, fluid structures composed of both lipids and proteins and were characterized by Singer and Nicholson (1972) in their classic fluid mosaic membrane model. Lipids and some of the protein complexes move freely in the lateral plane while only lipids are capable of rotational diffusion and flip-flop between the two membrane layers (Stryer, 1988). Lateral and rotational movement is possible as most of the membrane components do not have covalent bonds and are propelled by the natural thermal motion of molecules (Stryer, 1988). Membranes average 6-19 nm in thickness (Stryer, 1988).

Membrane components are constantly being produced and replaced. The turnover rate for components ranges from a few hours to several days. Lipases and proteases degrade the membrane while new membrane lipids and proteins are synthesized in the

endoplasmic reticulum (Henderson and Tocher, 1987). Typically, studies dealing with the effect of dietary fatty acid composition on tissues run for several months to ensure adequate time for the membrane fatty acids to be replaced by dietary fatty acids. However, the focus of this study was the effect of dietary fatty acids on gametes, so the length of the trial needed to be at least 12 months to allow for the fatty acid incorporation into gametes.

1.3 Reproduction

Wild rainbow trout spawn either in the spring or in the fall depending on the strain or population of fish. Changing daylength and temperature are strong environmental cues which activate reproductive hormones such as gonadotropin-releasing hormone, gonadotropin and estradiol-17β (Billard and Breton, 1978; Crim and Glebe, 1990; Billard, 1992). Rainbow trout, like all salmonids, do not spawn naturally in aquaculture conditions (Crim and Glebe, 1990). Therefore, the gametes of both sexes must be manually stripped from the fish and mixed for fertilization. The spawning time of cultured female fish can be altered by artificial light cues as well as with injections of gonadotropin or synthetic gonadotropin-releasing hormone (Henderson and Tocher, 1987; Crim and Glebe, 1990; Billard, 1992; Bromage and Cumaranatunga, 1996). Since male fish have a longer spawning time, the hormonal manipulation of female spawning takes precedence. Compared with ova cryopreservation, sperm are relatively easily cryopreserved which can provide a constant supply of semen.

Rainbow trout sperm cells are approximately 30 μm in length and are comprised of a head, mitochondrial collar and flagellum, all of which is enclosed by the plasma

membrane (Furieri, 1962 in Billard, 1983). Unlike mammalian sperm, fish sperm do not possess an acrosome. Instead, fish eggs contain a small pore, or micropyle, through which the sperm can enter (Fribourgh and Soloff, 1976). Rainbow trout are oviparous (external fertilization) and a typical reproductive female weighs 2-6 kg and can spawn approximately 1800 eggs per kg body weight (National Oceanic and Atmospheric Administration, 1992). Rainbow trout sperm concentrations are in the order of billions of cells per ml of semen while one ml of human semen typically contains millions of sperm cells.

During oogenesis, vitellogenin is synthesized in the liver and transported to the developing oocyte to form the yolk and oil droplets. Vitellogenin contains roughly 80% protein and 20% lipid; the lipid is used primarily for energy while both the lipid and protein are incorporated into structural components of the developing larvae. The maternal diet is reflected in the composition of the yolk and oil droplets since they are formed during oogenesis (Mourente and Odriozola, 1990; Rainuzzo, 1993; Harel et al., 1994). After fertilization, the developing embryo relies solely on the yolk sac and oil droplets for energy and nutrients (Henderson and Tocher, 1987). In this way, maternal nutrition may affect embryo and larval development and survival.

1.4 Cryopreservation

Individual cells, such as sperm cells, have been cryopreserved in many species such as bulls, boars, rams, fowl and fish (Ansah and Buckland, 1982; Graham and Foote, 1987; Billard, 1992; Holt and North, 1994; Gilmore et al., 1996). This technique is fundamental in cattle breeding programs as sperm from a quality bull can be frozen, sold

and shipped anywhere in the world, allowing farmers access to a much greater genetic pool than would be normally available. This type of selective breeding enables farmers to produce herds with desirable traits such as high milk production. Similar programs can be applied to other species such as fish, in order to improve breeding lines and facilitate year-round fertilization.

Attempts to cryopreserve fish sperm dates back to the 1950's (Blaxter, 1953; as reported by Holtz, 1993) but it has been only in the past two decades that there has been any reasonable amount of success (Legendre and Billard, 1980; Stoss and Holtz, 1983; Holtz, 1993). Low post-cryopreservation fertility may be related to differences in management practices such as feeding.

1.4.1 Freezing and Thawing

Cells usually freeze when they are below 0°C, the freezing point of water. However, certain conditions, such as the presence of cryoprotectants, allow cells to be supercooled and freezing occurs at a lower temperature than in seminal fluid (Leibo and Mazur, 1971). Usually, damage does not occur to such supercooled cells. Cryopreservation involves temperatures as low as -196°C; however, most cellular freezing damage occurs between the critical temperature range of -15°C and -60°C (Parks and Graham, 1992).

Damage to cells, resulting in low survival rates, is the major problem with cryopreservation. One type of damage, due to slow freeze rates, excessively dehydrates the cells by differences in osmotic pressure which occur in the following way. Since only pure water forms ice, any salts or impurities in the solution are excluded from the

growing ice crystals. Therefore, as the solution outside the cell freezes, the salt concentration also increases and water moves out of the cells to equilibrate intercellular and extracellular solute concentrations (Meryman et al., 1977). The second type of damage is caused by fast freeze rates which induce the formation of intracellular ice. Both intercellular and extracellular ice can lethally rupture cell membranes (Mazur, 1977).

Thaw rates can either rescue or injure cells that have intracellular ice. Fast freezing produces intracellular ice crystals which will aggregate if the warming rate is too slow. The rate of thawing should be consistent with the rate of freezing: e.g. if a cell has been frozen quickly, then it must also be thawed quickly to prevent ice recrystallization and if a cell has been frozen slowly, the thaw rate should also be slow to allow cells time to rehydrate (Mazur, 1984, 1985). Thawing can also cause premature activation of fish sperm. Activation occurs after a change in membrane potential with a decrease of potassium ion concentration and an influx of calcium ions resulting in motile sperm for usually less than 30 seconds (Billard, 1992). Therefore thawing and the concurrent change in membrane potential can prematurely activate the sperm. This activation is thought to be caused by liberated cellular electrolytes.

Freeze and thaw rates can be controlled either by regulating the parameters in an automated cryopreservation unit or by adjusting chemical factors such as the type of cryoprotectant or by physical factors such as the surface area and volume of semen or of the liquid nitrogen which is used to freeze cells. It was thought that most damage occurs during freezing, not thawing (Stoss and Holtz, 1981a), but recently there has been some

research indicating that thawing may cause more damage than freezing (Holt et al., 1992; Holt and North, 1994). Ideally, optimal freeze and thaw rates, as well as membranes that are resistant to cryopreservation damage, are desired. However, there is always some damage from cryopreservation so an increased amount of sperm is required per quantity of eggs in order to obtain adequate fertility levels.

In addition to the damage from ice crystals and changes in osmotic pressure, cryopreservation can dramatically alter the function and fluidity of cellular membranes (Silvius et al., 1980; Røsjø et al., 1994). Membrane fluidity is determined, for the most part, by the lipid composition. Lipids pack closer together and form more covalent bonds when the temperature is below the phase transition temperature. The phase transition temperature (Tc) is the temperature above which the lipids exist in a fluid state and below which the lipids are in a gel or crystalline state. This temperature is unique for each lipid (eg. phosphatidylcholine with C18:1 as both fatty acid groups has a Tc= -22°C whereas phosphatidlycholine with C18:0 as both acyl groups has a Tc= 54°C, (Pringle and Chapman, 1981); therefore, changes in membrane fatty acid composition can affect the temperature at which the membrane maintains fluidity and function.

1.5 Summary

In general, this research examines the relationship between fish nutrition and reproduction. More specifically, the manner and extent to which the source of dietary lipid influenced the fatty acid composition of several tissues were examined. The muscle and liver tissues were evaluated, for the most part, in terms of effects on human health, whereas fatty acid manipulation in the sperm was focused toward improving semen cryopreservation success rates. Finally, the effects of maternal dietary lipid on egg

composition and embryo utilization were evaluated.

2. THE INFLUENCE OF SEX AND DIETARY LIPID ON THE FATTY ACID COMPOSITION OF MUSCLE AND LIVER IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

2.1 Introduction

The fatty acid composition of fish tissues can be altered by dietary lipids (Sargent et al., 1988; Greene and Selivonchick, 1990; Lie et al., 1993; Bell et al., 1994; Ibeas et al., 1994) as evident in other organisms such as birds and mammals (Ajuyah et al., 1991; Jiang and Sim, 1992; Otten et al., 1993; Merican and Shim, 1994; Santos et al., 1995). Fish oils, such as those from Atlantic herring (*Clupea harengus*) and Atlantic menhaden (*Brevoortia tyrannus*), are commonly used in fish feeds, however, the availability and price of fish oils have enticed producers to find alternative sources of dietary lipid, often plant-based, for fish feeds (Greene and Selivonchick, 1990, Lie et al., 1993; Shikata et al., 1994; Fracalossi and Lovell, 1995). The lower quantity of n-3 fatty acids in plant lipid may affect the levels of the desirable n-3 fatty acids in the final fish product. Due to human health concerns, the market may demand more unsaturated fatty acids, especially more n-3 unsaturated fatty acids, in fish muscle.

The type of fat that people consume and the related health issues have been under investigation for several decades. Jonnalagadda et al. (1996) recently reviewed the effects of dietary fat on chronic diseases. This review outlines that high levels of total dietary fat lead to cancer and obesity-related conditions such as hypertension and diabetes and that the consumption of high levels of saturated fat is linked to heart disease, certain cancers and lowered immune function. High cholesterol (CHOL) levels

in serum lipoproteins are also used to indicate the risk of heart disease.

In the past decade, there has also been a lot of research into the n-6 and n-3 types of fatty acids (Kromhout et al., 1985; Kinsella, 1987; Simpoulos, 1991; Skjervold, 1992). Both n-3 and n-6 fatty acids can form eicosanoids (prostaglandins, leukotrienes, etc.) which modulate a variety of physiological responses. However, a powerful feedback reaction makes the n-6 eicosanoid production faster and preferential over the n-3 eicosaniod production, especially when the conversion enzyme, oxygenase, is rate limiting (Higgs, 1985; Lands, 1992; Fracalossi et al., 1994). In addition to preferential concentration, n-6 eicosanoids elicit a more intense receptor response than n-3 eicosanoids. Excess n-6 eicosanoids are believed to result in a variety of diseases including cardiovascular disease, arthritis and certain types of cancer (Kinsella, 1987; Simopoulos, 1991; Skjervold, 1992; Jonnalagadda et al., 1996).

The negative effects of n-6 fatty acids, and thus n-6 eicosanoids, are thought to be balanced by n-3 fatty acids and eicosanoids (Fischer and Black, 1991; Lands, 1992; Li et al., 1994). For example, increased dietary n-3 fatty acids have been shown to lower plasma cholesterol levels (Stangl et al., 1994) and prevent cardiovascular disease (Dyerberg et al., 1975, 1978; Bang et al., 1976; Kromann and Green, 1980; Goodnight et al., 1982). This effect may be due to the fact that the presence of n-3 fatty acids may decrease the production of n-6 eicosanoids. For example, the n-6 eicosanoid, thromboxane A2, activates the detrimental blood clotting of thrombosis and can be lowered by n-3 fatty acids (Lands, 1992). Therefore, the n-3/n-6 ratio is an important determinant of the types and levels of eicosanoids that are formed (Bell et al., 1993).

Consequently, the dietary n-3/n-6 ratio is also an important factor in preventing disease. Skjervold (1992) suggested that the optimal dietary n-3/n-6 ratio is 1/2 since it is thought to be the ratio found in our ancestral diet. Modern Western diets often fall short of this recommended level, with ratios ranging from 1/10-15, due to the increased consumption of plant-based lipids which are generally high in n-6 fatty acids. In the past couple of decades, the substitution of plant-based foods for animal products has been advocated due to the large amount of saturated fatty acids in animal products which has been linked to cardiovascular and inflammatory diseases (Carlier et al., 1991; Jonnalagadda et al., 1996). This advice, however, did not take the type of omega unsaturated fatty acids into account. One direct way to balance the n-3/n-6 fatty acid ratio is to consume more n-3 fatty acids. Fish is an excellent source of n-3 unsaturated fatty acids (Gunstone, 1984; Ackman, 1989; Skjervold, 1992). In spite of this, the per capita consumption of fish in Canada and the U.S. is approximately 7 kg per year which is lower than per capita consumption in Japan, the Netherlands, the United Kingdom, France and Spain (National Institute of Nutrition, 1991).

Dietary lipids have been shown to influence the immune reaction in fish and fowl as well as mammals; however, the relationship between dietary fatty acids, eicosanoids and this response is not clear. It has been shown that n-3 fatty acids have an immunosuppressive effect in mammals (Otto et al., 1990; Meydani et al., 1991) but that lower C18:2n-6/C18:3n-3 dietary levels increased antibody production in fowl (Friedman and Sklan, 1995). In Atlantic salmon (*Salmo salar*), Erdal et al. (1991) found the increased dietary n-3 fatty acids had a negative influence on the immune system resulting

in lower antibody titres and increased mortality when challenged with pathogens. However, Fracalossi and Lovell (1994) demonstrated that both n-3 and n-6 dietary fatty acids reduced immune response in channel catfish (*Ictalurus punctatus*). Fracalossi et al. (1994) found that increased dietary levels of n-6 fatty acids increased leukotrine B levels for improved immune response when there was a lack of n-3 fatty acids. They also stated that this relationship is more complicated when the levels of n-3 and n-6 are similar in that n-3 fatty acids can act as both eicosanoid precursors and inhibitors. These findings suggest that the n-3/n-6 ratio is also important in immune response.

Careful attention must also be paid to the lipid composition of fish broodstock diets in order to avoid nutrient deficiencies and to balance the protein/lipid ratios to ensure protein-sparing while avoiding excessive fat deposition as protein is the most expensive feed ingredient (Sargent et al., 1988). In general, diets with 10-20% lipid provide adequate energy without excessive fat deposition (Cowey and Sargent, 1979). Since dietary lipid influences the fatty acid composition of some tissues differently than others (Bell at al., 1991), we examined both muscle and liver tissue. The fatty acid composition of the liver influences lipid metabolism (Hatano et al., 1989) as the liver is main site of fatty acid synthesis (Henderson and Tocher, 1987; Sprecher, 1991), and muscle and liver lipids are the major fatty acid depots that are mobilized during spawning (Takashima et al., 1971). Vitellogenin, or yolk material, is produced using the liver lipids before transport to the developing oocyte (Henderson and Tocher, 1987). Therefore, the maternal liver fatty acid composition is possibly an important factor in larval survival and development (see Chapter 5).

The essential fatty acid (EFA) requirement for rainbow trout was originally stated by Castell et al. (1972) as C18:3n-3 comprising 1% of the total diet. Less is known about the requirement for C18:2n-6, as this fatty acid can be utilized by fish in an otherwise polyunsaturated (PUFA) free diet; however, C18:3n-3 results in greater growth than does C18:2n-6. It has been reported that dietary lipid level ranging from 5 to 15% may require higher percentages of C18:3n-3 and it has also been shown that 1% of n-3 highly unsaturated fatty acids (HUFA; C22:6n-3 and C20:5n-3) can replace the 1% EFA of C18:3n-3 (Takeuchi and Watanabe, 1982). The National Research Council (1993) most recently recommended that rainbow trout require 1% of diet of both total n-3 and n-6 fatty acids.

Fish, like mammals, have the ability to elongate and desaturate fatty acids. Except for the EFA, fish can also synthesize fatty acids de novo (Henderson and Tocher, 1987). Desaturases add double bonds to fatty acids and can use certain fatty acids, within any omega family, as substrate. However, the enzymes use the substrate in a hierarchical order; the Δ 6 desaturase preferentially uses C18:3n-3>C18:2n-6>C18:1n-9 as substrate. The level of certain fatty acids regulates enzyme activity in a feedback loop as C22:6n-3 can inhibit the Δ 6 desaturase to regulate fatty acid levels (Leray et al., 1985; Henderson and Tocher, 1987). In fatty acid metabolism, desaturation is usually rate-limited while elongation is not (Bell et al., 1993). Fish and mammals can not convert fatty acids between the omega families; for example, C18:2n-6 can be elongated and desaturated to form C20:2n-6, C20:3n-6, C22:5n-6 etc. but cannot be formed into any of the n-3 fatty acids. Although some organisms are able to convert long chain, unsaturated

fatty acids to shorter chain fatty acids, there is evidence that rainbow trout lack this ability (Yu and Sinnhuber, 1972).

In this chapter the incorporation of dietary fatty acids into skeletal muscle and liver is examined to determine the ease and the degree to which these tissues are influenced by dietary lipid. The effect of sex on the alteration of tissue fatty acids due to dietary lipid source was also investigated. Herring oil (adequate n-3 fatty acids), menhaden oil (high in n-3 fatty acids), safflower oil (high in n-6 fatty acids) and tallow (high in saturated fatty acids) were chosen as the sources of lipid.

2.2 Materials and Methods

2.2.1 Experimental Design

The experiment was conducted at the Department of Fisheries and Oceans Cardigan Salmonid Enhancement Centre in Cardigan, Prince Edward Island. One hundred two-year-old rainbow trout (*Oncorhynchus mykiss*) were weighed, measured (fork length) and tagged with visual implants in the transparent tissue adjacent to the eyes, on both sides of each fish while under anaesthetic (benzocaine 100 mg/L). The fish were blocked by weight (mean initial weight=725 g) and randomly assigned to treatments within the block. The fish were held indoors and the photoperiod was set to mimic the natural photoperiod . The 1.2 m^3 tanks had a flow rate of approximately 15 L/min of fresh water which reflected seasonal temperatures except during the winter months when the temperature was maintained at 7°C to ensure continued feeding.

2.2.2 Diets

The experimental diets (Table I) were formulated to meet all dietary requirements (NRC, 1993) and were processed by steam compression into 0.5x1.0 cm

TABLE I. Composition of the experimental diets

Ingredients	Total Diet (%)
Herring meal	20.0
Corn gluten meal	25.0
Casein, vitamin free	10.0
Wheat middlings	23.6
Dried whey	7.0
Vitamin premix[1]	1.0
DL-methionine	0.2
Mineral premix[2]	1.0
Choline chloride	0.2
Lipid source[3]	12.0

[1] Vitamin premix contained (mg/kg mix): 8000 IU vitamin A, 5000 IU vitamin D3, 300 IU vitamin E, 45 vitamin K, 50 thiamin, 60 riboflavin, 200 d-Calcium pantothenate, 0.8 biotin, 20 folic acid, 0.1 vitamin B12, 250 niacin, 40 pyridoxine, 150 inositol, 300 ascorbic acid, 40 astaxanthin, 100 ethoxyquin.

[2] Mineral premix contained (g/kg mix): 7.8 manganous sulfate, 10.0 ferrous sulfate, 22.0 zinc sulfate, 1.0 copper sulfate, 0.4 potassium iodide, 17.5 magnesium sulfate, 0.5 sodium flouride, 0.6 sodium selenite, 125 sodium chloride.

[3] Lipid sources were herring oil, menhaden oil, safflower oil or tallow.

dry, sinking pellets using a Laboratory Pellet Mill (California Pellet Mill Co., Crawsfordsville, Indiana). The diets differed only in the additional source of lipid which comprised 12% of the total diet. Herring oil, menhaden oil, safflower oil or tallow were the sources of dietary lipid. The fish were fed at a rate of 2% of body weight daily, for 16 months. The diets were stored in -4°C until required for feeding.

2.2.3 Tissue Sampling

After 12 months of the feeding trial, 5 males and 5 females were randomly selected from each of the four treatments, weighed, measured, and samples of muscle (1.5x1.0 cm laterodorsal strip) and liver tissue were taken. The fish were killed using a Canadian Council of Animal Care approved method (CCAC, 1993). The samples were placed in plastic tissue sample bags filled with nitrogen vapour and stored at -80°C until analysis.

2.2.4 Lipid Extraction and Transesterification

Approximately 400 mg of tissue was freeze-dried (Lyph-Lock 6, Labconco, Kansas City, Missouri) overnight at -25°C. The diets were ground into a powder and processed without lyophilization. One millilitre of hexane and 2.4 mL of methanol/acetyl chloride (10/1) were added to the freeze-dried samples which were then capped tightly with Teflon-lined caps and heated to 100°C for 1 hour in a block heater and cooled to room temperature. Four millilitres of 6% (w/v) potassium carbonate and 2 mL of hexane were then added. The samples were vortexed and centrifuged at 350 g for 5 minutes and the top hexane portion was removed for analysis by gas-liquid chromatography. The method for the production of fatty acid methyl esters was based on the procedures of

Ulberth and Henninger (1992, 1995) and Lepage and Roy (1986, 1988).

2.2.5 Gas-Liquid Chromatography

The fatty acid profiles of the tissues were determined by analyzing the fatty acid methyl esters by gas-liquid chromatography. A Hewlett Packard 5890 Series II Gas Chromatograph was used with a fused silica capillary column (Supelco: SP-2380 30 m length, 0.25 mm ID, 0.20 μm film thickness with a guard column of the same packing 1-10 m in length, 0.53 mm ID, 0.20 μm film thickness) and controlled by a Zenith Data Systems Z-386SX computer with ChemStation software (Hewlett Packard, Vancouver, Washington). One microlitre samples were injected on-column by a Hewlett Packard 7673 automated injector. Helium was used as the carrier gas at a pressure of 30 psi. The signal was detected by a flame ionization detector. The program was set for 20 minutes at 180°C with a 4.0°C/minute rise to a final temperature of 250°C which was held for 37.5 minutes for a total run of 75 minutes per sample. Individual fatty acid weights were calculated using an internal standard of C15:0 and were reported as percentage of total fatty acids in the sample.

2.2.6 Statistical Analysis

Dietary treatment was the independent variable in the one-way analysis of variance that was used on the levels of fatty acids found in muscle and liver tissue and on the growth parameter data using pooled data from both sexes (SAS, 1982). In addition, data analysis was also performed using two-way analysis of variance using treatment and sex as the independent variables. The probability differences between treatments were based on least square means with $P<0.05$ indicating significance.

2.3 Results and Discussion

2.3.1 Diets

The fatty acid compositions of the diets are presented in Table II. The diets were found to have high levels of the desired fatty acids: the herring oil diet had 11.8% of n-3, the menhaden oil diet had the highest n-3 (24.7%), the safflower diet contained the most n-6 (57.3%) and the tallow diet had 35.3% saturated fatty acids. All of the diets were in excess of the 1% essential fatty acid (EFA) minimum of n-3 and n-6 fatty acids. Values of unsaturated fatty acids over saturated fatty acids (UFA/SFA) and n-3/n-6 fatty acids for the diets were 5.2, 1.4 (herring oil), 3.3, 2.7 (menhaden oil), 6.8, 0.1 (safflower oil) and 1.8, 0.3 (tallow), respectively.

The diets also varied among other major fatty acid constituents. The herring oil diet contained 25.7% of C22:1 and at least 10% of each C16:0, C18:1 and C20:1. The menhaden oil diet contained C16:0, C18:1, C20:5n-3 and C22:6n-3 as major (>10%) fatty acid constituents. Other than C18:2n-6 being a major fatty acid (57.3%), the safflower oil diet had 16.4% of C18:1. The tallow diet contained 35.2% of C18:1 and 20.1% of C16:0 of the total fatty acids; C18:0 and C18:2n-6 were both around 13% of total fatty acids.

2.3.2 Growth Trials

These were not designed as growth trials according to the strict definition as feed intake was not measured. However, since the fish readily accepted all of the diets, the results are still informative. The fish fed the diets supplemented with fish oil, either herring or menhaden, had the greatest increases in weight and length which were

TABLE II. Fatty acid composition of the diets (g/100 g FA) n=2.

Fatty Acid	Herring Oil Diet	Menhaden Oil Diet	Safflower Oil Diet	Tallow Diet
C14:0	3.6	4.4	1.0	1.9
C16:0	10.8	16.3	9.1	20.1
C16:1	5.7	7.8	1.2	3.2
C18:0	1.7	2.9	2.8	13.3
C18:1	11.5	15.2	16.4	35.2
C18:2n-6	8.7	9.2	57.3	13.7
C20:1	17.6	6.5	3.5	4.3
C20:5n-3	6.0	12.5	2.0	1.6
C22:1	25.7	6.3	3.6	3.6
C22:5n-3	0.8	1.9	0	0
C22:6n-3	5.0	10.3	2.6	2.6
Other	2.9	6.8	0.5	0.5
n-3	11.8	24.7	4.6	4.2
n-6	8.7	9.2	57.3	13.7
n-3/n-6	1.4	2.7	0.1	0.3
n-3HUFA[1]	11.0	22.8	4.6	4.2
UFA[1]	82.5	72.8	87.1	64.7
SFA[1]	16.1	23.6	12.9	35.3
UFA/SFA	5.2	3.3	6.8	1.8
MUFA[1]	60.5	35.8	24.7	46.3
PUFA[1]	20.5	33.9	61.9	17.9

[1] Omega-3 highly unsaturated fatty acids (n-3HUFA; C20:5n-3 and C22:6n-3), unsaturated fatty acids (UFA), saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA).

significantly higher than the changes in fish fed the tallow diets (Table III). The fish fed oil group. These findings, however, are contrary to the results of Stickney and Andrews (1972) who found that catfish fed diets containing menhaden or beef tallow grew faster than fish fed a diet containing safflower oil or linseed oil. It has also been shown that rainbow trout fed higher levels of polyunsaturated fatty acids (PUFA) exhibited an increased weight gain (Yang and Dick, 1994); the safflower oil diet in this study had the highest PUFA content at 61.9% but produced only an intermediate weight gain.

In contrast to the preceding results, studies on a variety of fish species including rainbow trout, brown trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*) and developmental stages (smolts to adults) fed vegetable, animal or marine lipids in quantities ranging from 6-13% for an average of 4 months did not find any significant differences in weight gain among treatments (Bell et al., 1989; Greene and Selivonchick, 1990; Arzel et al., 1994). The conflicting results of studies examining how the source of dietary lipid affects growth parameters may be due to variations among species (Sigurgisladóttir and Pálmadóttir, 1993; Ahlgren et al., 1994), life stages (Ahlgren et al., 1994), amount of dietary lipid and factors such as palatability (Kiessling et al., 1989; Cacho, 1990; Fair et al., 1993), water temperature (Malejac et al., 1990) and length of feeding trials (Castell et al., 1972).

Overall, our results show that the use of either herring or menhaden oil in rainbow trout diets produced larger fish in 16 months as compared to fish fed the safflower oil or tallow diets. Therefore, producers desiring maximum increase in growth parameters should use either of these fish oils as the source of dietary lipid. However,

TABLE III. Change in weight and length of fish in the treatment diets n=20.

	Length (cm)	SE	Weight (g)	SE
Herring Oil Diet	15.3[a]	0.7	1161.5[a]	72.7
Menhaden Oil Diet	15.2[a]	0.6	1118.5[a]	65.5
Safflower Oil Diet	13.9[ab]	0.5	998.3[ab]	58.6
Tallow Diet	13.2[b]	0.5	927.9[b]	60.1

[abc] Values in the same column, with different superscript letters, are significantly different ($P<0.05$).

the safflower oil group, while tending to have smaller increases, did not significantly differ in final weight or length from the other groups. Thus, safflower oil could be used without great loss in growth if the price of fish oil was prohibitive. One reason for the greater growth performance of the fish oil groups may be that long chain fatty acids and fats with lower melting points, as in the fish and safflower oils, are more easily digested by rainbow trout (Austreng et al., 1980). In broodstock fish, however, slow growth is desired while the focus is on gamete quality and fertility, which will be examined in subsequent chapters.

2.3.3 Fatty Acid Composition of Muscle

The fatty acid profiles of muscle tissue are shown in Table IV. The main fatty acid found in muscle was C18:1 for all groups except the safflower group. In decreasing order of abundance, C18:2n-6, C18:1 and C16:0 were the main fatty acids found in muscle of the fish fed the safflower oil diet. The muscle from the herring group contained C20:1 and C16:0 for the second and third major fatty acids. The second and third most abundant fatty acids were C16:0 and C22:6n-3 for both the menhaden and tallow groups. Similarly, a review by Ahlgren et al. (1994) revealed that C16:0, C18:1 and C22:6n-3 were consistently among the dominant fatty acids in fish muscle. In a study in which the quantity of feed varied, Kiessling et al. (1989) also found that the muscle content of C16:0, C18:1 and C22:6n-3 was high in relation to the dietary levels. Therefore, despite the dietary differences in fatty acid composition and quantity, there seems to be a necessary level of C16:0, C18:1 and C22:6n-3 in muscle. This was also evident in that there were no significant differences in muscle C16:0 and C22:6n-3 concentrations among the treatments. Contrary to the above results, Olsen and Skjervold

TABLE IV. Fatty acid composition of muscle tissue (g/100 g FA) n=10.

Fatty Acids	Herring Oil Diet	SE	Menhaden Oil Diet	SE	Safflower Oil Diet	SE	Tallow Diet	SE
C14:0	3.3[a]	0.2	3.4[a]	0.2	2.0[b]	0.2	2.4[b]	0.2
C16:0	13.1	1.7	17.5	1.6	16.8	1.7	17.2	1.6
C16:1	8.0[a]	0.7	7.6[a]	0.7	3.8[b]	0.7	6.2[a]	0.7
C18:0	2.8[c]	0.3	3.7[b]	0.3	3.7[b]	0.3	4.8[a]	0.3
C18:1	21.6[b]	1.9	20.1[b]	1.8	17.9[b]	1.9	30.9[a]	1.8
C18:2n-6	6.0[b]	2.1	6.9[b]	2.0	20.7[a]	2.1	7.3[b]	2.0
C18:3n-3	0.1	0.3	0.7	0.3	0.5	0.3	0.2	0.3
C20:1	14.8[a]	0.9	5.5[b]	0.9	5.6[b]	0.9	6.1[b]	0.9
C20:2	0.6	0.3	1.3	0.3	0.6	0.3	0.6	0.3
C20:3	0.5[b]	0.3	0.6[ab]	0.2	1.3[a]	0.3	0.6[ab]	0.2
C20:5n-3	3.8	1.5	7.0	1.4	5.3	1.5	5.6	1.4
C22:1	11.9[a]	0.9	5.6[bc]	0.8	6.8[b]	0.9	4.2[c]	0.8
C22:3	0.5	0.1	0.7	0.1	0.4	0.1	0.5	0.1
C22:4n-6	0.5	0.1	0.5	0.1	0.3	0.1	0.3	0.1
C22:5n-3	1.4[b]	0.3	2.6[a]	0.2	0.8[b]	0.3	1.2[b]	0.2
C22:6n-3	10.8	2.0	16.1	1.9	13.4	2.0	12.0	1.9
n-3	16.2[b]	3.2	26.6[a]	3.0	20.0[ab]	3.2	17.8[ab]	3.0
n-6	6.5[b]	2.1	7.4[b]	2.0	21.0[a]	2.1	7.5[b]	2.0
n-3/n-6	2.6	1.4	4.5	1.4	4.1	1.4	3.7	1.4
n-3HUFA[1]	14.6	3.2	23.1	3.1	18.7	3.2	17.5	3.1
SFA[1]	19.3[b]	1.8	24.7[a]	1.7	22.5[ab]	1.8	24.4[a]	1.7
UFA[1]	80.7[a]	1.8	75.3[b]	1.7	77.5[ab]	1.8	75.6[b]	1.7
MUFA[1]	56.4[a]	3.3	38.8[bc]	3.1	34.1[c]	3.3	47.3[b]	3.1
PUFA[1]	34.4[c]	2.0	36.0[b]	1.9	43.4[a]	2.0	28.6[c]	1.9

[abc] Values in the same row, with different superscript letters, are significantly different ($P<0.05$).

[1] Omega-3 highly unsaturated fatty acids (n-3HUFA; C20:5n-3 and C22:6n-3), saturated fatty acids (SFA), unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA).

(1995) found that C22:6n-3 showed greater variation in salmon than the other n-3 fatty acids. They also found that the C22:6n-3 content decreased with age, which they saw as evidence of a genetic factor. The levels of C22:5n-3 and C22:6n-3 were, in all cases, higher in muscle tissue than in the diets, this was either due to: 1. preferentially selective incorporation of these fatty acids over other fatty acids in the diet, 2. metabolism products: elongation and desaturation products formed from other fatty acids, or 3. fatty acids that were stored in other tissues (liver, adipose, mesentery, etc.).

The fatty acid composition of muscle tissue is important in terms of human health, as the muscle is usually considered the edible portion of the fish. The fish that were fed the menhaden oil diet had significantly higher amounts of the desirable n-3 fatty acids than fish fed the herring oil diet, reflecting the high n-3 content of the menhaden oil diets. The content of n-3 fatty acids in the muscle of fish fed the herring, safflower and tallow diets were similar despite the low dietary levels of n-3 fatty acids in the safflower and tallow groups. The muscle levels of n-3 fatty acids in the safflower and tallow groups were due to the increases in muscle C22:6n-3 as compared to the low levels (0.5%) of C22:6n-3 in the safflower oil and tallow diets. However, contrary to our results, Bell et al. (1989) found no differences in total n-3 fatty acids or C22:6n-3 in muscle and liver of Atlantic salmon after 4 months of feeding a 10% fish oil or an 8% corn oil and lard diet, although the corn oil/lard-fed fish had significantly higher levels of C20:2n-6, C20:3n-6 and C20:4n-6. The fish fed the herring oil or tallow diet had a n-3/n-6 ratio in muscle that was the closest to the 1/2 ratio recommended dietary level for optimal human health. In relation to the current high consumption of n-6 fatty acids in

Western diets, the muscle of fish fed the menhaden oil diet would provide increased n-3 fatty acids to help balance the ratio.

The n-3 highly unsaturated fatty acids, consisting of C20:5n-3 and C22:6n-3 (n-3HUFA), in muscle were not significantly affected by dietary lipid even though this value varied widely in the diets (4.2%-22.8%), suggesting that muscle tissue not only has a required level of C22:6n-3, but also of n-3HUFA. Xu and Hung (1996) concluded that, in white sturgeon (*Acipenser transmontanus*), muscle levels of n-3HUFA are best increased by high dietary n-3HUFA levels, which is contrary to our results. It is interesting to note that Sigurgisladóttir and Pálmadóttir (1993) found that the fat content of two individual wild salmon varied 18.6% in muscle. Based on 35 species of fish, they also found an inverse relationship between n-3 fatty acid content and overall lipid content.

Yu et al. (1978) reported that the SFA level in rainbow trout muscle cente red around 24%, despite dietary levels. Our data supports this finding in that the SFA content of muscle ranged from 19.3% to 24.7% over all treatment groups while the dietary values were more varied (12.9%-35.3%). Despite the smaller SFA range in muscle there were still significant differences between the groups. The lowest concentration of saturated fatty acids in muscle was found in the herring oil group; significantly lower than those of the menhaden or tallow groups ($P<0.05$) while tending to be lower than that of the safflower group. This information would be of interest to consumers who need to be careful of their dietary saturated fatty acid intake. The highest percentage of monounsaturated fatty acids (MUFA) was in the muscle of fish fed the

herring oil ($P<0.05$) while the highest level of PUFA was in the muscle of the safflower group ($P<0.05$). There is some evidence that diets high in MUFA can reduce serum cholesterol (CHOL), low density lipoproteins and serum apoliporoteins to the same degree as a high PUFA diet in humans (Gustafsson et al., 1992); however, Heyden (1994) argued that this has not yet been proven conclusively.

The sex of the fish had an effect on the fatty acid composition of muscle (Table V). Sex, but not diet, was a significant factor affecting the levels of C16:0 and C20:5n-3. Both diet and the sex of the fish sex were significant ($P<0.05$) in influencing fatty acid incorporation in the muscle for the total of the 18 carbon chain fatty acids and the 20 carbon chain fatty acids (sum 18, sum 20). It was also found that there were significant interactions between sex of the fish and diet for C18:1, total 16 carbon chains (sum 16), SFA, UFA and MUFA. This indicates that fatty acid incorporation in muscle varied with sex differently among treatment groups. As muscle is a fatty acid storage depot, differences in fatty acid composition based on sex may be explained by hormonal differences and different fatty acid requirements for sperm and oocyte development. Our results support Garcia-Garrido et al. (1990) who found that male spiny dogfish (*Squalus acanthias*) had significantly higher ($P<0.01$) levels of serum cholesterol than did female dogfish which was thought to be due to the differences in gametogenesis. Sigurgisladóttir and Pálmadóttir (1993) however, did not find fatty acid composition differences between the sexes in capelin (*Mallotus villosus*). However, they analyzed the composition of the whole body whereas our results indicate that the influence of sex on fatty acid deposition appears to be tissue specific as sex influenced fatty acid composition differently for liver than it did for muscle.

TABLE V. Sex differences of percentages of fatty acids between treatments in muscle (g/100 g FA) n=5.

	Females				Males			
	Herring Oil Diet	Menhaden Oil Diet	Safflower Oil Diet	Tallow Diet	Herring Oil Diet	Menhaden Oil Diet	Safflower Oil Diet	Tallow Diet
C16:0[1]	14.5[a]	15.6[a]	12.4[b]	14.4[a]	11.8[b]	19.9[ab]	21.2[a]	20.6[ab]
C18:1[2]	20.2[b]	20.3[b]	20.2[b]	36.3[a]	23.0	20.0	15.5	24.4
C20:5[1]	3.8[b]	6.2[a]	2.0[c]	1.9[c]	3.8	8.1	8.7	10.0
Sum 16[2,4]	22.2[ab]	24.0[a]	17.2[bc]	21.4[b]	20.0[b]	26.4[a]	23.9[ab]	25.8[ab]
Sum 18[3,4]	29.2[c]	31.5[b]	49.3[a]	49.7[a]	32.1	31.3	36.1	35.5
Sum 20[3,4]	30.4[a]	18.6[b]	18.8[b]	16.3[b]	33.0[a]	21.9[b]	20.6[b]	18.0[b]
SFA[2,4]	21.0[b]	22.8[b]	17.7[c]	21.3[ab]	17.6[b]	26.8[a]	27.3[a]	28.2[a]
UFA[2,4]	79.0[b]	77.2[c]	82.3[a]	78.7[bc]	82.4[a]	73.2[b]	72.7[b]	71.8[b]
MUFA[2,4]	41.8[b]	34.3[c]	32.7[c]	51.0[a]	47.1[a]	31.8[ab]	21.9[b]	33.7[ab]

[abc] Values in the same row, with different superscript letters, are significantly different (P<0.05) under each sex.

[1] Sex was significant for fatty acids (P<0.05).

[2] Interactions between sex and diet were significant (P<0.05) for fatty acids or fatty acid groups.

[3] Sex and diet were significant for fatty acid groups (P<0.05).

[4] Total 16:x fatty acids (Sum 16), total 18:x (Sum 18), total 20:x fatty acids (Sum 20), saturated fatty acids (SFA), unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA).

2.3.4 Fatty Acid Composition of Liver

There was much less treatment variation among the major fatty acid constituents in the liver samples (Table VI) as compared to the muscle samples. The two main liver fatty acids were C22:6n-3 and 16:0 for all the treatment groups, whereas C18:1 was the third most abundant fatty acid in the livers of all treatment groups except for the safflower oil group which exhibited more C18:2n-6. The C18:1 liver levels decreased from the dietary levels for all treatment groups except for the herring oil group which had higher levels in the liver than in the diet. The fish oil diets resulted in significantly ($P<0.05$) more C20:5n-3 accumulated in the liver tissue than did the safflower and tallow diets. Similar to our results, Ibeas et al. (1996) found that C16:0, C18:1 and n-3HUFA were the major fatty acids found in all tissues sampled, concluding that these fatty acids were important in terms of energy sources and structural lipids. In the present study, the levels of C22:6n-3 in liver tissue were similar for all groups; albeit a higher value as compared to that of muscle tissue. The fatty acid, C22:6n-3, is important physiologically. Farkas et al. (1980) concluded that C22:6n-3 was especially important in membrane fluidity. High concentrations of C22:6n-3 were found in brain tissue in fish (Greene and Selivonchick, 1987) and in mammals (Crawford et al., 1976; Sprecher, 1991) and are thought to be crucial in the neural and retinal development in rats, primates and humans (Sinclair and Crawford, 1972; Sinclair, 1975; Neuringer et al., 1988; Clandinin et al., 1994).

The percentages of n-3 fatty acids reflected the dietary lipid in that the livers of the herring and menhaden oil groups had percentages significantly higher ($P<0.05$) than

TABLE VI. Fatty acid composition of liver tissue (g/100 g FA) n=10.

Fatty Acids	Herring Oil Diet	SE	Menhaden Oil Diet	SE	Safflower Oil Diet	SE	Tallow Diet	SE
C14:0	2.0[a]	0.3	1.2[ab]	0.3	0.9[b]	0.3	0.9[b]	0.3
C16:0	18.4	1.0	19.1	1.0	17.3	1.0	19.2	0.9
C16:1	3.4	0.4	3.0	0.4	2.4	0.4	2.4	0.4
C18:0	4.6[b]	0.5	6.1[a]	0.5	6.2[a]	0.5	6.1[a]	0.4
C18:1	13.5[b]	0.7	11.6[b]	0.7	11.5[b]	0.7	16.0[a]	0.6
C18:2n-6	4.4[b]	1.1	3.8[b]	1.1	12.8[a]	1.1	6.7[b]	1.0
C18:3n-3	0[b]	0.1	0[b]	0.1	0.3[a]	0.1	0[b]	0
C20:1	4.5[a]	0.2	2.1[b]	0.2	1.7[b]	0.2	2.0[b]	0.2
C20:2	0.6	0.2	0.1	0.2	0.5	0.2	0.1	0.2
C20:3	0.8[b]	0.2	0.1[c]	0.2	2.2[a]	0.2	2.0[a]	0.2
C20:4n-6	3.8[c]	0.7	4.6[bc]	0.7	6.6[a]	0.7	6.3[ab]	0.6
C20:5n-3	8.3[a]	0.7	9.2[a]	0.7	4.2[b]	0.7	4.8[b]	0.6
C22:3	1.7	0.5	1.1	0.5	1.4	0.5	1.7	0.4
C22:4n-6	0.8	0.4	1.5	0.4	1.1	0.4	0.8	0.3
C22:5n-3	2.1[b]	0.2	3.0[a]	0.2	1.9[b]	0.2	1.5[b]	0.2
C22:6n-3	31.1	2.1	33.4	2.1	29.1	2.1	29.6	1.9
n-3	41.5[a]	1.9	45.6[a]	1.9	35.4[b]	1.9	36.0[b]	1.7
n-6	9.0[b]	1.8	10.0[b]	1.8	20.5[a]	1.8	13.8[b]	1.6
n-3/n-6	5.6[a]	0.6	5.0[a]	0.6	2.2[b]	0.6	3.0[b]	0.5
n-3HUFA[1]	39.4[a]	1.8	42.6[a]	1.8	33.3[b]	1.8	34.4[b]	1.6
SFA[1]	25.0	0.8	26.4	0.8	24.4	0.8	26.1	0.8
UFA[1]	75.0	0.8	73.6	0.8	75.6	0.8	73.9	0.8
MUFA[1]	21.4[a]	1.2	16.7[b]	1.2	15.6[b]	1.2	20.4[a]	1.1
PUFA[1]	53.6[b]	1.3	56.9[ab]	1.3	60.0[a]	1.3	53.4[b]	1.2

[abc] Values in the same row, with different superscript letters, are significantly different ($P<0.05$).

[1] Omega-3 highly unsaturated fatty acids (n-3HUFA; C20:5n-3 and C22:6n-3), saturated fatty acids (SFA), unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA).

those of the other groups. Also influenced by diet, the n-6 content was significantly higher ($P<0.05$) in the livers of the safflower diet fed fish. There were no significant differences among the groups in saturated or unsaturated fatty acid levels in liver tissue despite large differences in dietary fatty acid saturation ranging from 13-35%. Monounsaturated fatty acids were incorporated to a greater extent ($P<0.05$) into the livers of the herring oil and tallow groups reflecting the high dietary MUFA levels of these two diets. The safflower oil group had significantly ($P<0.05$) higher levels of liver PUFA than did the herring or tallow groups while tending to have higher levels than those of the menhaden group. The fish fed the fish-based diets, herring or menhaden oil, had significantly higher amounts ($P<0.05$) of n-3HUFA in liver tissue than did the fish fed the other diets but were not significantly different from each other in spite of the fact that the menhaden oil diet had more than twice as much (22.8%) n-3HUFA than the herring oil diet (11.0%).

Skuladottir et al. (1990) found that environmental temperature affected the fatty acid composition in muscle and heart tissue but not in liver tissue. This finding supports the notion that the fatty acid composition of the liver is less variable than that of other tissues. However, Ibeas et al. (1994) found that liver tissue fatty acid composition was influenced by dietary lipid to a greater extent than muscle tissue in juvenile gilthead seabream (*Sparus aurata*), although some of the diets were EFA deficient. Increased fatty acid variation was also found in the liver compared to abdominal fat in Atlantic salmon when the source and/or the quantity of dietary fat varied (Viga and Grahl-Nielsen, 1990).

For most of the major fatty acids and fatty acid groups, diet and sex of the fish were significant factors in the level of incorporation in liver tissue (Table VII). The following fatty acids and fatty acid groups were higher in male fish than in female fish: C18:2n-6, C20:5n-3, n-6 fatty acids and the total of 16 and 18 carbon fatty acids. The female liver tissue was generally higher in C18:0, C20:1, C20:3, C22:5n-3, n-3, n-3HUFA and PUFA. Since none of the interactions between sex and treatment were significant, the differences between the sexes were consistent across all dietary treatments. Since vitellogenesis occurs in the liver, these differences are most likely due to oocyte production.

2.3.5 General Discussion

Overall, the content of C16:0, C18:1 and C22:6n-3 remained consistently high in muscle and liver tissue regardless of dietary lipid. However, the n-3 fatty acid content as well as levels of C18:2n-6 of muscle and liver tissue were influenced by dietary lipid. Tissues incorporate dietary fatty acids to a certain extent but require, and will maintain, a certain composition presumably to ensure function. This phenomenon has been observed in various fish species and tissues (Yu et al., 1978; Suzuki et al., 1986; Greene and Selivonchick, 1990, Arzel et al. 1994, Guillou et a. 1995). Viga and Grahl-Nielsen (1990) observed that while the fatty acid composition of tissues can be influenced by dietary lipid, modest changes in dietary lipid do not affect the fatty acid composition of tissues. They maintained that under natural conditions, genetics have a greater effect than environmental influences in regulating fatty acid composition and that there is a set composition required. Olsen and Skjervold (1995) concluded that 80% of n-3 fatty acid

TABLE VII. Sex and treatment differences of fatty acids in liver tissue (g/100 g FA) n=5.

	Females				Males			
	Herring Oil Diet	Menhaden Oil Diet	Safflower Oil Diet	Tallow Diet	Herring Oil Diet	Menhaden Oil Diet	Safflower Oil Diet	Tallow Diet
C18:0[1]	5.4^{b}	7.7^{a}	7.6^{a}	7.1^{a}	3.6^{b}	4.9^{a}	5.1^{a}	4.9^{a}
C18:2n-6[1]	3.1^{b}	3.0^{b}	11.2^{a}	4.8^{b}	6.1^{b}	4.4^{b}	14.1^{a}	9.0^{ab}
C20:1[1]	4.7^{a}	2.3^{b}	1.9^{b}	2.4^{b}	4.3^{a}	2.0^{b}	1.6^{b}	1.6^{b}
C20:3[1]	1.0^{c}	0.2^{b}	2.6^{a}	2.2^{a}	0.5^{b}	0	1.9^{a}	1.7^{a}
C20:4n-6[1]	3.0^{c}	3.7^{bc}	6.3^{a}	5.3^{ab}	4.3^{b}	5.4^{ab}	6.8^{ab}	7.4^{a}
C20:5n-3[1]	6.8^{b}	7.8^{a}	3.7^{b}	4.5^{b}	10.1^{a}	10.3^{a}	4.6^{b}	5.2^{b}
C22:5n-3[1]	2.6^{b}	3.8^{a}	2.1^{bc}	1.7^{c}	1.6^{ab}	2.3^{a}	1.7^{ab}	1.3^{b}
n-3[1]	45.9^{a}	47.6^{a}	38.0^{b}	39.3^{b}	36.1^{ab}	44.0^{a}	33.4^{b}	32.0^{b}
n-6[1]	6.4^{c}	7.4^{bc}	17.8^{a}	10.1^{b}	12.1^{b}	12.0^{b}	22.6^{a}	18.2^{ab}
n-3HUFA[1,2]	43.3^{a}	43.8^{a}	35.7^{b}	37.5^{b}	34.6^{ab}	41.7^{a}	31.3^{b}	30.7^{b}
PUFA[1,2]	56.8^{b}	57.5^{b}	62.2^{a}	54.7^{b}	49.5^{c}	56.4^{ab}	58.3^{a}	52.0^{bc}
Sum 16[1,2]	19.2^{a}	19.8^{a}	17.2^{b}	19.4^{a}	25.2^{a}	23.9^{b}	21.6^{b}	24.2^{b}
Sum 18[1,2]	20.9^{b}	22.0^{b}	29.5^{a}	27.4^{a}	24.5^{b}	21.1^{b}	31.8^{a}	30.3^{a}

^{abc} Values in the same row, with different superscript letters, are significantly different ($P<0.05$) under each sex.

[1] Sex and diet were significant for fatty acids ($P<0.05$).

[2] Omega-3 highly unsaturated fatty acids (n-3HUFA; C20:5n-3 and C22:6n-3), polyunsaturated fatty acids (PUFA), total 16: x fatty acids (Sum 16), total 18:x (Sum 18).

variation in farmed Atlantic salmon was due to body weight (age) and total lipid content of muscle with 20% due to other non-genetic factors. Genetic factors ensure that the fatty acids necessary for proper physiological function are incorporated into tissues while environmental factors such as dietary lipid can alter the less essential fatty acids. The manipulation of these fatty acids in food fish can be exploited for the benefit of human health.

2.4 Conclusions

1. The fish fed either the herring oil or the menhaden oil diet achieved significantly greater gains in weight and length than the fish fed the tallow diet. These growth parameters also tended to be higher for fish fed the fish oil diets than for fish fed the safflower oil diet.
2. Muscle tissue had a greater variation of fatty acids than liver tissue. In general however, both muscle and liver tissues were high in C16:0, C18:1 and C22:6n-3 despite the differences in dietary lipids. However, the high C18:2n-6 from the safflower oil diet did displace some of these major fatty acids to a certain extent.
3. The lowest concentrations of saturated fatty acids in muscle was found in the fish fed the herring oil diet. The highest percentage of monounsaturated fatty acids was in the muscle of fish fed the herring oil while the highest level of PUFA was in the muscle of the safflower group. This information would be of interest to consumers who need to reduce their dietary saturated fatty acid intake and increase their consumption of monounsaturated and polyunsaturated fatty acids.

4. Dietary lipid high in n-3 fatty acids, such as menhaden oil, tended to increase the n-3 fatty acid content in muscle which has positive implications for human health by providing a food product with even more n-3 fatty acids than is normally found in fish.
5. Sex of fish had an effect on the incorporation of some fatty acids and fatty acid groups primarily in the liver but also in muscle tissue. Differences in lipid requirements for gametogenesis between the sexes is the most probable explanation for this finding.

3. FATTY ACID COMPOSITION AND CHOLESTEROL CONTENT OF SPERMATOZOA AND SPERMATOZOAL PLASMA MEMBRANES FROM RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) FED DIETS DIFFERING IN SOURCE OF LIPID

3.1 Introduction

Membranes as diverse as the milk fat globule membrane in cows (Palmquist and Schanbacher, 1991), mouse cardiac sarcoplasmic reticulum (Swanson et al., 1989) and fish spermatozoal plasma membranes (Labbé et al., 1992, 1995) can be modified by dietary means. However, not all membranes change to the same extent; Izpisúa et al. (1989) found that mitochondrial membranes resisted dietary influence to a greater extent than liver plasma membranes.

There have been many advances on the original fluid mosaic membrane model as proposed by Singer and Nicholson in 1972. It has been demonstrated that membranes are asymmetrical in that the two halves of the bilayer have different components. It has also been discovered that lipids can form a hexagonal arrangement which is thought to occur during fusion events such as when a sperm cell attaches to an egg (Parks and Graham, 1992). In addition, membranes can have distinct domains of fatty acids which are attributed to phase transition temperatures of fatty acids despite the lateral diffusion of these components (Parks and Graham, 1992). Liposomes are useful in the investigation of membrane properties as they are aqueous compartments enclosed by a lipid bilayer that can be manufactured spontaneously from a lipid solution in water;

however, the conclusions are often limited as biomembranes are a very complex heterogenous mix of lipids, proteins and carbohydrates (Cossins, 1981; Quinn, 1985; Hazel et al., 1991). Biomembranes also exhibit homeoviscous adaptation to changes in environmental temperature (Sinesky, 1974).

Homeoviscous adaptation is a natural membrane response to temperature, the fatty acid composition of membranes is altered over time to maintain fluidity and function. If the temperature change is slow enough, membranes can be altered to function normally within a range of temperatures. However, if this temperature change is too fast or too great the membrane will not be successfully altered by the organism. The temperatures and temperature changes that some fish and hibernating organisms are likely to encounter (- 1°C to - 4°C in months or days) are not as extreme or as rapid as those that occur in the cryopreservation process (-196°C in minutes). However, examination of the ability of these organisms to adapt to cold temperatures may provide insight into the cryopreservation process (See Chapter 4).

In general, an increase in membrane unsaturated fatty acids (UFA) are found in organisms acclimated to the lowest temperatures in their temperature range. A high degree of lipid unsaturation enables fish to osmoregulate better in colder water temperatures (Finstad and Thomassen, 1991). More specifically, the concentration of polyunsaturated fatty acids (PUFA) in the liver was found to be elevated at low temperatures in channel catfish (*Ictalurus punctatus*) (Fracalossi and Lovell, 1995). In addition, fish acclimated to cold water temperatures had more n-3 fatty acids in their membranes than did fish acclimated to warmer water temperatures (Hazel, 1984).

Schwalme (1994) found that overwintering northern pike (*Esox lucius*) had considerable increases in C22:6n-3 in ovarian lipids, and rainbow trout plasma high-density lipoproteins demonstrated homeoviscous adaptation to seasonal temperature variations by increasing C22:6n-3 and decreasing C18:0, C18:1n-9 and C20:1n-9 fatty acids in colder conditions (Wallaert and Babin, 1993, 1994). In contrast, Skuladottir et al. (1990) reported that Atlantic salmon with increased amounts of C18:1n-9 and decreased amounts of C22:6n-3 fatty acids in liver and heart tissue were better able to tolerate -1.7°C water temperatures; however, they did not report saturated fatty acids (SFA), UFA, PUFA or n-3 fatty acids.

The discovery of homeoviscous adaptation led to studies attempting to increase the cold tolerance of fish by manipulating tissue fatty acid composition by means of a high PUFA diet. High dietary PUFA enabled carp (*Cyprinus carpio*) to survive at colder than normal temperatures (Farkas et al., 1980; Viola et al., 1988) while having little effect on tilapia (*Oreochromis mossambica*) (Viola et al., 1988) or Atlantic salmon (Skuladottir et al., 1990). Dietary modification of spermatozoal plasma membranes could be applied in order to enable sperm to better withstand cryopreservation damage.

This study determines the extent to which the fatty acid composition and cholesterol (CHOL) content of whole sperm and spermatozoal plasma membranes can be altered by source of dietary lipid. The diets, and thus lipid sources, used were the same as in Chapter 2: herring oil (adequate n-3 fatty acids), menhaden oil (high n-3 fatty acids), safflower oil (high n-6 fatty acids) and tallow (high saturated fatty acids).

3.2 Materials and Methods

The experimental design, diets, lipid extraction and gas-liquid chromatography were as described in 2.2. However, the semen was collected in the period between 12-15 months into the experiment. The fish were usually taken off feed for several days before samples were taken to reduce fecal contamination of semen. Lipids of whole spermatozoa as well as spermatozoal plasma membranes were extracted and analyzed by gas-liquid chromatography.

3.2.1 Sample Collection

Upon sexual maturation, semen was collected by hand stripping the experimental fish. Semen collection was conducted on 4 days spaced throughout the spawning season because the fish matured at different times. The pelvic regions of the fish were dried and care was taken to avoid water and fecal contamination of the semen samples. Semen was microcentrifuged at 1000 g for 5 minutes in Eppendorf tubes and the seminal plasma was removed. The spermatozoa in the residue were flushed with nitrogen vapour and stored at -80°C until use for whole sperm lipid analysis or to isolate spermatozoal membranes for membrane lipid analysis.

3.2.2 Membrane Isolation

The method of sperm membrane isolation was based on the method described by Lou et al. (1990). The sperm were resuspended in distilled water, frozen at -20°C for a minimum of 2 hours and then thawed at 37°C for 15 minutes. This freeze-thaw cycle was performed twice in order to rupture the spermatozoal plasma membranes. The samples were centrifuged at 8000 g for 15 minutes after which the supernatant was drawn off and

spun again according to the same procedure in order to pellet the nuclei and mitochondria. The resulting supernatant was then centrifuged at 140 000 g for 1 hour to pellet the plasma membranes. The membrane pellet was frozen to facilitate the transfer into a glass test tube to be freeze-dried. The purity of the isolated plasma membrane fraction was assessed by measuring various parameters such as alkaline phosphatase activity (Ivanov and Profirov, 1981), cytochrome-c-reductase activity (Ivanov and Profirov, 1981) and protein content (Ivanov and Profirov, 1981; Lou et al., 1990; Labbé and Loir, 1991). Fluorometry (McNiven et al., 1992), as described in section 4.2.3, and electron microscopy (Labbé and Loir, 1991) were also used to assess the purity of the membrane pellets.

3.2.3 Data Analysis

Data analysis was performed using one-way analysis of variance with respect to treatment (SAS, 1982). Significance was indicated by $P<0.05$ on the probability differences of the least mean squares between treatments.

3.3 Results

Dietary levels (Table II, Chapter 2) of C18:1 were similar, or greater than, the levels found in either the sperm (Table VIII) or the spermatozoal plasma membranes (Table IX) while the sperm and membrane levels of C16:0 and C22:6n-3 were greater than in the diets. The fatty acid, C20:5n-3, was a major component in both the sperm and the plasma membranes from the fish oil groups. For all treatments, the sperm and membrane levels of C20:5n-3 were higher than dietary levels; however, the values of C20:5n-3 in sperm and spermatozoal membranes for both of the fish oil groups were

TABLE VIII. Fatty acid composition and cholesterol content of sperm from experimental fish (g/100 g FA).

Fatty Acid	Herring Oil Diet n=7	SE	Menhaden Oil Diet n=10	SE	Safflower Oil Diet n=12	SE	Tallow Diet N=7	SE
C14:0	2.1[a]	0.1	2.2[a]	0.1	1.6[b]	0.1	1.5[b]	0.1
C16:0	25.4	0.4	26.3	0.4	25.9	0.3	25.6	0.4
C16:1	2.0[a]	0.1	2.0[a]	0.1	1.1[c]	0.1	1.6[b]	0.1
C18:0	3.6[c]	0.1	4.4[b]	0.1	4.8[a]	0.1	4.4[ab]	0.1
C18:1	13.4[b]	0.3	14.0[b]	0.2	10.2[c]	0.2	15.3[a]	0.3
C18:2n-6	2.7[c]	0.2	2.7[c]	0.1	9.8[a]	0.1	3.5[b]	0.2
C20:1	2.6[a]	0.1	1.0[b]	0.1	0.7[c]	0.1	1.1[b]	0.1
C20:4n-6	3.1[c]	0.5	3.9[c]	0.4	13.7[a]	0.4	6.2[b]	0.5
C20:5n-3	15.7[a]	1.3	15.4[a]	1.1	6.5[b]	1.0	9.0[b]	1.3
C22:4n-6	0.8[b]	0.2	1.6[a]	0.2	0.6[b]	0.2	1.0[ab]	0.2
C22:5n-3	1.0[a]	0.2	0.8	0.1	0.6	0.1	0.5	0.2
C22:6n-3	27.2[ab]	0.8	25.4[b]	0.7	22.2[c]	0.6	29.3[a]	0.8
OTHER	0.4[c]	0.2	0.4[c]	0.2	2.4[a]	0.2	1.1[b]	0.2
CHOL[1]	21.0[b]	0.6	21.2[b]	0.5	21.1[b]	0.5	23.0[a]	0.6
n-3	43.9[a]	0.9	41.6[a]	0.8	29.3[c]	0.7	38.8[b]	0.9
n-6	6.6[d]	0.5	8.1[c]	0.4	24.1[a]	0.4	10.6[b]	0.5
n-3/n-6	6.8[a]	0.2	5.1[b]	0.2	1.2[d]	0.2	3.7[c]	0.2
n-3HUFA[2]	42.9[a]	1.0	40.6[a]	0.9	28.7[c]	0.8	38.5[b]	1.0
UFA[2]	68.5[a]	0.6	66.7[b]	0.5	65.4[c]	0.4	67.4[ab]	0.6
SFA[2]	31.1[b]	0.6	32.9[a]	0.5	32.3[ab]	0.4	31.6[ab]	0.6
UFA/SFA	2.2[a]	0	2.0[b]	0	2.0[b]	0	2.1[ab]	0
MUFA[2]	18.0[a]	0.3	17.0[b]	0.2	12.0[c]	0.2	18.0[a]	0.3
PUFA[2]	50.5[b]	0.7	49.7[b]	0.6	53.4[a]	0.6	49.4[b]	0.7

[abc] Values in the same row, with different superscript letters, are significantly different ($P<0.05$).

[1] Cholesterol (g/100 g dry matter).

[2] Omega-3 highly unsaturated fatty acids (n-3HUFA; C20:5n-3 and C22:6n-3), unsaturated fatty acids (UFA), saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA). herring, menhaden, safflower and tallow groups were 2.2, 2.0, 2.0 and 2.1, respectively.

TABLE IX. Fatty acid composition and cholesterol content of spermatozoal plasma membranes from experimental fish (g/100 g FA).

Fatty Acid	Herring Oil Diet n=4	SE	Menhaden Oil Diet n=10	SE	Safflower Oil Diet n=11	SE	Tallow Diet N=6	SE
C14:0	2.0^{a}	0.1	2.2^{a}	0.1	1.3^{b}	0.1	1.3^{b}	0.1
C16:0	24.9^{a}	0.4	25.1^{a}	0.3	23.9^{b}	0.2	24.5^{ab}	0.3
C16:1	1.9^{a}	0.1	1.9^{a}	0.1	1.1^{b}	0.1	1.7^{a}	0.1
C18:0	3.9^{b}	0.2	4.5^{a}	0.1	4.7^{a}	0.1	4.6^{a}	0.2
C18:1	13.5^{b}	0.5	13.3^{b}	0.3	10.3^{c}	0.3	15.3^{a}	0.4
C18:2n-6	2.5^{b}	0.6	3.3^{b}	0.4	9.6^{a}	0.4	3.3^{b}	0.5
C20:1	2.4^{a}	0.1	0.9^{b}	0.1	0.6^{c}	0.1	1.1^{b}	0.1
C20:4n-6	3.5^{c}	0.9	4.6^{bc}	0.6	13.2^{a}	0.6	6.1^{b}	0.8
C20:5n-3	15.0^{a}	1.3	16.1^{a}	0.8	6.6^{b}	0.8	9.3^{b}	1.1
C22:4n-6	0.2^{b}	0.2	0.5^{b}	0.1	1.3^{a}	0.1	0.6^{b}	0.2
C22:5n-3	1.5^{ab}	0.2	1.8^{a}	0.1	0.7^{c}	0.1	1.1^{b}	0.1
C22:6n-3	27.1^{a}	0.8	24.5^{b}	0.5	21.6^{c}	0.5	28.8^{a}	0.7
OTHER	1.5^{b}	0.5	1.2^{b}	0.3	5.0^{a}	0.3	2.2^{b}	0.4
CHOL[1]	67.2^{ab}	11.2	50.4^{b}	7.9	56.6^{b}	7.1	81.1^{a}	10.0
n-3	43.6^{a}	1.5	42.4^{a}	0.9	28.9^{c}	0.9	39.2^{b}	1.2
n-6	6.3^{c}	1.5	8.5^{b}	1.0	24.2^{a}	0.9	10.0^{b}	1.2
n-3/n-6	7.3^{a}	0.6	5.9^{a}	0.4	1.2^{c}	0.4	4.0^{b}	0.5
n-3HUFA[2]	42.1^{a}	1.4	40.6^{ab}	0.9	28.3^{c}	0.8	38.1^{b}	1.1
UFA	67.7	0.6	67.0	0.4	65.1	0.4	67.3	0.5
SFA[2]	30.8^{ab}	0.6	31.7^{a}	0.4	29.9^{b}	0.3	30.4^{b}	0.5
UFA/SFA	2.2^{ab}	0.1	2.2^{b}	0	2.3^{a}	0	2.3^{a}	0.1
MUFA[2]	17.8^{a}	0.6	16.2^{b}	0.4	12.1^{c}	0.3	18.1^{a}	0.5
PUFA[2]	49.9^{b}	0.6	50.9^{b}	0.4	53.1^{a}	0.4	49.2^{c}	0.5

^{abc} Values in the same row, with different superscript letters, are significantly different ($P<0.05$).

[1] Cholesterol (g/100 g dry matter).

[2] Omega-3 highly unsaturated fatty acids (n-3HUFA; C20:5n-3 and C22:6n-3), unsaturated fatty acids (UFA), saturated fatty acids (SFA), monounsaturated fatty acids (MUFA); polyunsaturated fatty acids (PUFA).

between 15-16% of total fatty acids while the level (12.5% of fatty acids) in the menhaden oil diet was twice that of the herring oil diet (6.0% of fatty acids).

The fatty acid composition of the whole sperm was significantly ($P<0.05$) affected by source of dietary lipid (Table VIII). Similar to the muscle and liver samples (Chapter 2), C16:0, C18:1 and C22:6n-3 were major lipid components (>10 g/100 g) in the sperm and spermatozoal plasma membranes regardless of the range of dietary levels in the treatment diets. Sperm from fish fed the safflower oil diet had significantly higher ($P<0.05$) levels of n-6 fatty acids while sperm from fish fed the fish oil diets had significantly higher ($P<0.05$) levels of n-3 fatty acids as compared to the other diets. The values of n-3/n-6 for the sperm were 6.8, 5.1, 1.2 and 3.7 for the herring, menhaden, safflower and tallow groups, respectively. The UFA/SFA values for the sperm from the herring, menhaden,safflower and tallow groups were 2.2, 2.0, 2.0,2.1, respectively.

Table IX contains the fatty acid composition of spermatozoal membranes from fish fed the experimental diets. Similar to the fatty acid composition of the whole sperm, the fish fed the safflower oil diet, which was chosen for the high n-6 content, had significantly ($P<0.05$) higher levels of n-6 levels in the spermatozoal plasma membranes than did fish fed the other diets whereas the herring and the menhaden oil diets, which were chosen for the moderate and high levels of n-3 fatty acids respectively, had significantly higher ($P<0.05$) levels of n-3 fatty acids in spermatozoal plasma membranes in relation to the other diets. The UFA/SFA and n-3/n-6 values for the spermatozoal membranes were 2.2, 7.3 for the fish fed the herring oil diet, 2.2, 5.9 for the fish fed the menhaden oil diet, 2.3, 1.2 for the fish fed the safflower oil diet and 2.3, 4.0 for the fish

fed the tallow diet.

The monounsaturated fatty acids (MUFA) levels in the whole sperm and the spermatozoal plasma membranes of the tallow and herring oil groups were significantly ($P<0.05$) greater than those of the menhaden oil group which in turn were significantly ($P<0.05$) greater than those of the safflower oil group. Polyunsaturated fatty acids levels in whole sperm were significantly higher ($P<0.05$) in the safflower oil group compared tothose of the other groups while the PUFA levels in the spermatozoal plasma membranes were significantly higher in the safflower oil group and significantly lower in the tallow group in relation to those of the herring and menhaden oil groups

The cholesterol (CHOL) content was significantly higher ($P<0.05$) in sperm from the tallow group than in sperm from the other groups. The spermatozoal membrane CHOL content of the tallow group was significantly higher ($P<0.05$) than those of membranes from the menhaden or safflower oil groups while tending to be higher than that of the membranes from the herring group.

3.4 Discussion

Dietary levels of C18:1 were similar, or greater than, the levels found in either the sperm or the spermatozoal plasma membranes while the sperm and membrane levels of C16:0 and C22:6n-3 were greater than in the diets and therefore most likely the products of metabolism. The fatty acid, C20:5n-3, was a major component (>10 g/100 g) in both the sperm and the plasma membranes from the fish oil groups. For all treatments, the sperm and membrane levels of C20:5n-3 were higher than dietary levels, which also is most likely due to *in vivo* synthesis. However, the amount of C20:5n-3 in sperm and

spermatozoal plasma membrane for both of the fish oil groups was between 15-16% of total fatty acids while the level (12.5% of fatty acids) in the menhaden oil diet was twice that of the herring oil diet (6.0% of fatty acids).

Similar results showing the major fatty acids, C16:0, C18:1 and C22:6n-3, were also found in rainbow trout (*Oncorhynchus mykiss*) sperm by Labbé et al. (1995) and in blood lipoproteins in spermiating rainbow trout by Fremont and Marion (1982). Furthermore, Darin-Bennett et al. (1974) found that dog sperm contained C16:0, C18:0, C18:1 and C22:5 as major fatty acid components (>10 g/100 g) while the major fatty acids in fowl sperm were C16:0, C18:0, C18:1, C20:4 and C22:4, although diets were not specified. Poulos et al. (1973) found that the only major fatty acids in bull and boar sperm were C16:0 and C22:6 while those for human sperm were C16:0, C18:0 and C22:6, and C16:0, C18:0 and C22:5 for rabbit sperm. Similarly, the diets were not mentioned.

The high standard error associated with the CHOL levels in the spermatozoal plasma membranes was thought to be due to the cumulative variation from a multi-step process such as membrane extraction (Lou et al., 1990). The CHOL values for the sperm cells generally reflected those of the plasma membranes.

Hazel (1972) reported that in response to changes in environmental temperature, the lipid composition of membranes change so that the fluidity of the membranes will remain within functional parameters. Many theories connect membrane fatty acid composition to membrane fluidity; however, the relationship has not been fully revealed. It is generally thought that increased membrane UFA will increase membrane fluidity

(Hazel, 1984; Hazel et al., 1991) but Loo et al. (1991) found that rabbits fed coconut oil (high SFA) had the most fluid membranes in liver tissue compared to rabbits fed corn oil (high n-6 fatty acids) or menhaden oil diets (high n-3 fatty acids). Furthermore, an increase in lipoprotein fluidity is more dependent on lowering lipoprotein CHOL content than on increasing fatty acid unsaturation as demonstrated by Loo et al. (1991) in rabbits and Berlin et al. (1991) in humans. Hazel and Williams (1990) found that cold acclimation of fish, which usually requires that membrane fluidity increase, resulted in decreased cholesterol content. Therefore, at these temperatures, membrane fluidity is inversely related with membrane CHOL content (Yeagle, 1985; see 4.1 for further discussion). According to the previously mentioned literature, it is not clear if high levels of UFA result in increased membrane fluidity. Due to these conflicting reports, it may be warranted that the actual membrane fluidities should be measured in future research.

Labbé et al. (1995) found that several individual fatty acids in rainbow trout spermatozoal plasma membranes were altered after the fish were fed diets supplemented with 6% corn oil (high n-6 fatty acids) or fish oil (high n-3 fatty acids). However, the C22:6n-3 levels in the membranes remained constant despite differences in dietary levels and rearing temperatures. Our results showed that spermatozoal plasma membrane levels of C22:6n-3 varied according to source of dietary lipid; the membranes from the herring oil group and tallow group contained significantly higher ($P<0.05$) amounts of C22:6n-3 than those from the menhaden oil group which contained significantly more ($P<0.05$) C22:6n-3 than those from the safflower oil group. This was also the case with the whole

sperm except that the herring oil group had sperm levels of C22:6n-3 between those of the tallow and menhaden groups. This discrepancy in findings may be a result of our diets containing twice as much additional lipid as the diets in the Labbe et al. (1995) study. Also, contrary to our results, they did not find any differences in CHOL content of the spermatozoal plasma membranes. However, Labbe et al. (1995) acknowledged that the high standard error within treatment groups for cholesterol may have masked the between-group differences. Similar to our results, the spermatozoal plasma membranes from the fish fed the fish oil diet had a greater n-3/n-6 ratio than those from fish fed the plant oil diet.

3.5 Conclusions

1. The spermatozoal membranes from the herring oil group and tallow group contained significantly higher ($P<0.05$) amounts of C22:6n-3 than those from the menhaden oil group which in turn contained significantly more ($P<0.05$) C22:6n-3 than those from the safflower oil group. This was also the case with the whole sperm except that the herring oil group had sperm levels of C22:6n-3 between those of the tallow and menhaden groups.
2. The fish fed the safflower oil diet had significantly ($P<0.05$) higher levels of n-6 levels in whole sperm and spermatozoal plasma membranes than did fish fed the other diets whereas the herring and the menhaden oil diets, which were chosen for their moderate and high n-3 fatty acid levels respectively, had significantly higher ($P<0.05$) levels of n-3 fatty acids in sperm plasma membranes in relation to the other diets.

3. The MUFA levels in the whole sperm and the spermatozoal plasma membranes of the tallow and herring oil groups were significantly ($P<0.05$) greater than those of the menhaden oil group which in turn were significantly ($P<0.05$) greater than those of the safflower oil group.
4. Polyunsaturated fatty acid levels in whole sperm were significantly higher ($P<0.05$) in the safflower oil group compared to those of the other groups while the PUFA levels in the spermatozoal plasma membranes were significantly higher in the safflower oil group and significantly lower in the tallow group in relation to those of the herring and menhaden oil groups
5. Similar to what was reported in Chapter 2 for rainbow trout muscle and liver tissues, C16:0, C18:1 and C22:6n-3 were the major fatty acids found in both rainbow trout sperm cells and the spermatozoal plasma membranes, regardless of source of dietary lipid.
6. Despite the wide range of UFA/SFA values in the experimental diets (1.8-6.8%), the UFA/SFA values for sperm and spermatozoal plasma membranes centered around 2.1 suggesting that this value is of physiological importance.
7. Sperm from fish fed the tallow diet had significantly ($P<0.05$) higher CHOL levels than sperm from fish fed the other diets. The spermatozoal plasma membranes from fish fed the tallow diet had significantly ($P<0.05$) higher CHOL levels than those from fish fed the menhaden or safflower oil diets, and tended to have higher levels than those from fish fed the herring oil diet.

4. SOURCE OF DIETARY LIPID AFFECTS SPERMATOZOAL PLASMA MEMBRANE INTEGRITY AND FERTILITY IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) AFTER CRYOPRESERVATION

4.1 Introduction

Structural damage due to ice crystal formation and changes in osmotic pressure are major causes of spermatozoal damage during cryopreservation (Mazur, 1977; Fujikawa, 1981; Hammerstedt et al., 1990). It is the spermatozoal plasma membrane that is vital in withstanding these stresses (Steponkus et al., 1981, 1990; Steponkus and Lynch, 1989; Lindemann et al., 1982). This is evident in that membranes of frozen-thawed sperm were "weaker" than those of fresh sperm as 30% less sodium dodecyl sulfate was required to lyse frozen-thawed sperm compared to fresh sperm (Schweisguth and Hammerstedt, 1992). Cryopreservation damage, including chemical, mechanical, thermal and osmotic stresses, alters the structure and function of spermatozoal plasma membranes (Lindemann et al., 1982; Hammerstedt, 1979; Steponkus et al., 1981, 1989, 1990; Malejac et al., 1990; Parks and Graham 1992).

Semen from marine fish is more resistant to cryopreservation damage than semen from freshwater fish. This generalization was confirmed by Stoss (1986) in a review that compared the general aspects of freezing semen from marine and freshwater fish. This idea is also supported by a comparison of fine structural changes in frozen sperm from rainbow trout (*Oncorhynchus mykiss*), carp (*Cyprinus carpio*) and marine puffer (*Fugu niphobles*) (Gwo et al., 1993). The reason for this difference in cryopreservation success

has been attributed to the fatty acid composition of the two groups of fish (Drokin and Kopeika, 1992; Drokin 1993).

An important physical property of membranes is fluidity which is determined for the most part by the membrane fatty acids and environmental temperature. Parameters such as the degree of unsaturation, position of double bonds, position of fatty acids on the glycerol backbone of phospholipids, cholesterol levels and lipid-protein interactions all affect membrane fluidity. In general, membrane fluidity increases with decreasing fatty acid chain length and with increasing fatty acid unsaturation. (Cossins, 1981; Quinn, 1985; Hazel et al., 1991). The ability of the spermatozoal plasma membrane to resist structural damage during cryopreservation may be related to the type of spermatozoal plasma membrane fatty acids and the strength of the bonds between membrane components (Hammerstedt et al., 1990).

Membrane fluidity is also influenced by temperature which affects the state or phase of the membrane lipids. The phase transition temperature is a point above which the membrane lipids exist in a fluid state and below which the membrane lipids are in a gelled state. Lipids with short or unsaturated fatty acids have lower phase transition temperatures than those with longer chain or saturated fatty acids (Lee, 1985). Phase transitions have been measured in intact cells such as human, goat and shrimp sperm cells and were found to be correlated with susceptibility to cooling and freezing damage (Crowe et al., 1989). A unique property of cholesterol (CHOL) is that above the phase transition temperature, CHOL decreases membrane fluidity while the presence of CHOL below the transition temperature increases the fluidity of membranes (Pringle and

Chapman, 1981; Shinitzky, 1984; Yeagle 1985). Phase transitions occur over a broader range of temperatures when CHOL is present and are also more difficult to measure in complex biomembranes than in simple membranes composed of only a few lipid and fatty acid types (Pringle and Chapman, 1981).

The state of membrane fluidity is an essential factor in determining membrane function. Loss of function is associated with membranes in the gelled state. Therefore, the fatty acid composition of membranes is fundamental in determining membrane function as well as fluidity (Aloia and Boggs, 1985; Muriana and Ruiz- Gutierrez, 1992; Røsjø et al., 1994). The maintenance of essential membrane functions such as fluidity and permeability are critical for the post-freeze survival of spermatozoa (Steponkus, 1989). A change in the fatty acid composition can influence membrane functions such as permeability and active transport (Hazel, 1972; Silvius et al., 1980; Swanson et al., 1989; Finstad and Thomassen, 1991; Røsjø et. al., 1994). It has even been suggested that as an amino acid sequence determines protein tertiary structure, lipids and fatty acids may determine the structure and function of membranes (Parks and Graham, 1992). The level of membrane CHOL can also affect membrane function; Madden et al. (1979) have shown that CHOL can regulate the activity of intrinsic membrane proteins such as in calcium dependent ATPase of the sarcoplasmic reticulum. Loss of membrane fluidity is thought to be one of the causes of cryopreservation damage (Holt and North, 1984).

As demonstrated in the literature and in the previous chapter, dietary sources of lipid can influence the fatty acid composition and CHOL content of tissues, including spermatozoal plasma membranes (Greene and Selvonchick, 1990; Labbé et. al., 1991;

Lie et al., 1993; Bell et al., 1994). In Chapter 3.0, we found that the monounsaturated fatty acids (MUFA) levels in the whole sperm and the spermatozoal plasma membranes of the tallow and herring oil groups were significantly ($P<0.05$) greater than those of the menhaden oil group which in turn were significantly ($P<0.05$) greater than those of the safflower oil group. Polyunsaturated fatty acid (PUFA) levels in whole sperm were significantly higher ($P<0.05$) in the safflower oil group compared to those of the other groups while the PUFA levels in the spermatozoal plasma membranes were significantly higher in the safflower oil group and significantly lower in the tallow group in relation to those of the herring and menhaden oil groups. The fish fed the tallow diet had a significantly higher ($P<0.05$) amount of CHOL in sperm as compared to those of the other groups and the fish fed the tallow diet also had a significantly higher ($P<0.05$) amount of CHOL in spermatozoal plasma membranes than did the menhaden or safflower oil groups while tending to have higher amounts of CHOL in spermatozoal plasma membranes than did the herring group.

Cryopreservation damage of spermatozoa was assessed by two methods: fertility tests and fluorometry. Fertility tests measure cryopreserved sperm viability. Two parameters, percentage of fertilized eggs and the percentage of eyed embryos, both adjusted to the control groups, were used to measure fertilization success. Fluorometry assesses the integrity of spermatozoal plasma membranes and has been used in human, bull, chicken and fish semen (Peterson et al., 1974; Halangk and Bohnensack, 1982; Bilgili and Renden, 1984; McNiven et al., 1992). This method uses ethidium bromide as a stain that binds to double-stranded, intact DNA and fluoresces. Entry of the stain into

sperm cells is prevented by intact spermatozoal plasma membranes so the fluorescent reading of sperm mixed with ethidium bromide represents damaged sperm membranes. Digitonin renders all the spermatozoal plasma membranes permeable to ethidium bromide so that the fluorescent reading of sperm treated with digitonin and ethidium bromide represents the total amount of sperm. It is the ratio of these two fluorescent readings (damaged/total sperm) that signifies the percentage of damaged sperm.

The objective of this study was to determine if the source of dietary fat in rainbow trout diets could influence the viability of sperm after cryopreservation. The integrity of post-cryopreserved spermatozoal plasma membranes was tested by fluorometry while post-cryopreserved sperm viability was assessed by fertility. As in previous chapters, herring oil (adequate n-3 fatty acids), menhaden oil (high in n-3 fatty acids), safflower oil (high in n-6 fatty acids) and tallow (high in saturated fatty acids) were the sources of lipid in the diets.

4.2 Materials and Methods

The experimental design, diets and semen collection were as described in previous chapters.

4.2.1 Cryopreservation

In each of 4 trials, based on collection days, semen from an average of 8 fish per treatment was cryopreserved. The semen was diluted 1:3 in a 0.3 M glucose, 10% dimethyl-acetamide extender and then aspirated into 0.5 mL straws, plugged with sealant, and cryopreserved in liquid nitrogen vapour. Ten straws were frozen per fish and were stored in liquid nitrogen (-196°C).

4.2.2 Fertilization

Rainbow trout eggs were collected from four non-experimental fish, pooled and volumetrically measured in glass beakers to either 100 or 200 eggs per lot. Fertility tests were conducted with the cryopreserved semen thawed for 30 seconds in 3°C water. (see Table X; n= number of fish per treatment from which semen was cryopreserved. Semen was collected and cryopreserved from each fish approximately 2-3 times. Each sample of cryopreserved semen was used to fertilized eggs, regardless if it was from the same fish. The fertility parameters, % fertilized egg and % eyed embryos are based on 2 lots of eggs averaged from 1 sample of cryopreserved semen.) In the first two trials, 200 eggs were fertilized in duplicate with $3x10^6$ frozen-thawed sperm cells/egg, the minimum ratio to obtain acceptable fertilization rates with frozen-thawed semen (Stoss and Holtz, 1981). In the third and fourth trials, 100 eggs were fertilized, in duplicate, using cryopreserved sperm at $6x10^6$ sperm cells/egg. Fresh semen was used as a control in all four trials at $2.0x10^5$ cells/egg, the minimum ratio for acceptable fertilization rates with fresh semen (Billard et al., 1974). Ten seconds after fertilization, 5 mL of water were added to the fertilized eggs and they were gently swirled for 1 minute. The eggs were then rinsed twice and the batches of eggs were placed randomly into divided sections of Heath incubator trays with 7°C water. Dead eggs were picked every week, cleared in egg clearing solution (47.4 mL formalin (37% w/v), 37.9 mL glacial acetic acid, 56.8 mL glycerin, 804.4 mL distilled water), and the presence of an embryo was determined using a dissecting microscope. Fertility was quantified in two ways, the percentage of fertilized eggs and the percentage of eyed embryos (eggs reaching the eyed stage of

embryo development; approx. 210° days). Both of these parameters were expressed as percentage of control eggs (fertilized control = 75%; eyed control = 64%).

4.2.3 Fluorometry

Sperm membrane integrity of cryopreserved semen was fluorometrically assessed using DNA-staining ethidium bromide (McNiven et al., 1992). The fluorometric equipment consisted of a MPF-66 Fluorescence Spectrophotometer (Perkin-Elmer, Oakbrook, Illinois) with a xenon bulb. The excitatory wavelength was set at 360 nm while the emission wavelength was set at 575 nm (Gallant, 1990). Three straws of frozen semen from each fish were analyzed in duplicate for each freezing trial. The semen was thawed for 30 seconds in 3°C water, as for the fertility trials. Ten microlitres of the frozen-thawed semen were added to 3.99 ml of the ethidium bromide solution for the first reading of the number of damaged sperm. Twenty-five microlitres of digitonin solution were then added to the samples to obtain the second reading of the total number of cells. The two fluorescent readings were then compared to estimate the percentage of damaged sperm. (see Table X; n= the number of fish/treatment, semen was collected from each fish approximately 2-3 times and 3 straws from each cryopreserved semen sample, regardless if it was from the same fish, were analyzed in duplicate and reported as the average of these duplicates.)

4.2.4 Data Analysis

The data was analyzed by one-way analysis of variance with respect to diet (SAS, 1982). Significance was indicated by $P<0.05$ on the probability differences of the least mean squares between treatments.

4.3 Results

The percentages of damaged sperm in the cryopreserved semen from each treatment are presented in Table X. Fish fed the menhaden oil diet had a significantly higher ($p<0.05$) proportion of damaged sperm after cryopreservation compared with those of the other groups, while the sperm from fish fed the tallow diet had significantly less damage ($p<0.05$) than sperm from fish fed the menhaden or safflower oil diets and tended to have less damage than sperm from the herring oil group. The cryopreserved semen of fish fed the herring oil diet or the safflower oil diet sustained intermediate levels of spermatozoal damage.

The mean fertilization rates of eggs fertilized with cryopreserved semen from the treatments were not statistically different (Table X). However, the eggs fertilized with frozen-thawed semen from the tallow group tended to have higher fertilization success than semen from the other groups, while the eggs fertilized with frozen-thawed semen from the safflower group tended to have lower fertilization rates.

This trend was reinforced by the results of the fertility parameter of eyed embryos. Cryopreserved semen from fish that were fed the tallow diet achieved a significantly higher ($p<0.05$) percentage of eyed embryos than semen from fish fed the menhaden or the safflower oil diets and tended to result in more eyed embryos than eggs fertilized with cryopreserved semen from the herring oil group (Table X). The percentages of eyed embryos from eggs fertilized with cryopreserved semen from fish fed the herring, safflower or menhaden oil diets were not statistically significant from each other.

TABLE X. Comparison of mean percentages of damaged sperm and fertility parameters after semen cryopreservation from fish fed diets containing 12% lipid from four sources.

	Herring Oil Diet n=9	SE	Menhaden Oil Diet n=12	SE	Safflower Oil Diet n=10	SE	Tallow Diet n=9	SE
Damaged Sperm (%)	26.5[bc]	0.8	30.7[a]	0.8	27.4[b]	0.8	24.3[c]	0.8
Fertilized Eggs (%)	43.8	4.2	43.3	3.7	42.7	4.1	45.0	3.9
Eyed Embryos (%)	22.8[ab]	3.2	19.0[b]	2.9	19.1[b]	3.1	27.2[a]	3.0

[abc] Values in the same row, with different superscript letters, are significantly different ($P<0.05$).

4.4 Discussion

In order to better demonstrate the treatment effect, the minimum amount of sperm was used to fertilize the eggs which is reflected in the relatively low fertility parameters. The number of sperm per egg that was used to fertilize was doubled for the last two trials in the attempt to increase fertilization rates but without much success. The reason for the low fertility rates is unknown as the same cryopreservation and fertilization procedure has resulted in higher fertility rates when conducted by the same laboratory (Miller, 1996). The low eyed-embryo rates (19-27%) were possibly due to the minimum sperm concentration used to fertilize the eggs. There may also have been interference as several incubator sections were contaminated by fungus (*Saprolegnia* sp.) which may have obscured the fertility results. In future research, fertilized eggs should be treated to prevent fungal growth.

An accurate estimate of fertility requires observing the developing embryo at the earliest moment after semen is applied to the eggs. In fish, this method has practical limitations in that it is labour intensive and the correct identification of early embryos is difficult, even with the aid of a dissecting microscope. The eggs that turn opaque (due to protein coagulation indicating either unfertilized germinal disks or dead embryos (Potts and Wootton, 1984) before reaching the eyed-embryo stage also need to be evaluated to determine whether or not the egg had been fertilized. Assessing the eyed embryo fertility parameter reduces the amount of labour required but may be a conservative estimate of fertilization. Fluorometric assessment is a more direct and sensitive way of measuring cryopreservation damage with respect to spermatozoal plasma membrane integrity than

are fertility parameters.

Labbé et al. (1991) found that diets supplemented with 6% corn oil (high n-6 fatty acids) or fish oil (high n-3 fatty acids) altered the spermatozoal membrane fatty acid composition but did not affect fertility (% eyed) after cryopreservation. In contrast, our results showed that cryopreserved sperm from fish fed the tallow diet (high in saturated fatty acids) achieved a higher percentage of eyed embryos than cryopreserved sperm from the menhaden (high n-3 fatty acids) and safflower (high n-6 fatty acids) groups and tended to have a higher percentage than those of the herring oil group (adequate n-3 fatty acids). This discrepancy may be due to the fact that Labbé et al. (1991) did not test a diet that was high in saturated fatty acids and the level of dietary lipid supplement was half of that tested in this study.

The MUFA levels in the whole sperm and spermatozoal plasma membranes of the tallow and herring oil groups were significantly ($P<0.05$) greater than those of the menhaden oil group which in turn were significantly ($P<0.05$) greater than those of the safflower oil group (Table VIII & IX, Chapter 3). The sperm from the tallow and the herring oil groups were the most resistant to cryopreservation damage although it was the menhaden oil group, and not the safflower oil group, that was the most susceptible to cryopreservation damage as shown by membrane integrity results (Table X). It would appear that high levels of MUFA may also provide sperm with increased resistance to cryopreservation damage.

Unsaturated fatty acids are more susceptible to oxidization than saturated fatty acids due to the presence of double bonds. Therefore, diets that contain high levels of

PUFA may oxidize and become rancid, decreasing palatability as well as incorporating by-products into tissues. Cowey et al. (1984) found that rainbow trout raised in colder water (6°C vs. 12°C) required additional dietary vitamin E in order to prevent erythrocyte peroxidation. At lower temperatures, biomembranes incorporate more PUFA which are more easily oxidized thus requiring higher levels of vitamin E. Greene and Selivonchick (1990) found increased hemolysis with a salmon oil diet compared to soybean oil, linseed oil, chicken fat, pork lard or tallow diets despite high vitamin E levels. The increased hemolysis may be due to runaway peroxidation or membrane fragility due to long chain unsaturated fatty acids. Swine fed safflower oil diets had a higher incidence of oxidation in subcutaneous fat and muscle tissue than did swine fed tallow diets (Larick et al., 1992). In addition, Friedman and Sklan (1995) suggested that high dietary PUFA inhibition of immune function in broiler chicks may be due to membrane fluidity or oxidative damage and not related to eicosanoids. The results of this thesis show that spermatozoal membranes from the tallow group, which had the lowest levels of PUFA, were the most resistant to cryopreservation damage. However, the sperm and spermatozoal plasma membranes from the fish fed the safflower oil diet contained the highest PUFA levels yet were more resistant to damage than the sperm from the menhaden oil group which had the most cryopreservation damage. Nevertheless, membrane fatty acid oxidation is a possible factor in membrane integrity. All of the diets used in this study were stored at -4°C for a maximum duration of 6 months and contained sufficient ethyoxyquin (100mg/kg) to prevent oxidation of the diets and sufficient amount of vitamin E (300 IU/kg) to prevent tissue oxidation.

High levels of CHOL in plasma membranes appear to offer spermatozoa resistance to cryopreservation damage. The fish fed the tallow diet produced sperm which were the most resistant to cryopreservation damage and which also had the highest sperm and spermatozoal plasma membrane levels of CHOL compared to sperm from fish fed the other diets (Tables VIII, IX in Chapter 3 & Table X). This finding is supported in that Darin-Bennett and White (1977) found that rabbit and human sperm sustained considerably less cold shock damage and contained approximately twice as much CHOL than did ram and bull sperm. As mentioned earlier, a unique property of CHOL is that above the phase transition temperature, CHOL decreases membrane fluidity while the presence of CHOL below the transition temperature increases the fluidity of membranes (Pringle and Chapman, 1981; Shinitzky, 1984; Yeagle, 1985). Phase transitions also occur over a broader range of temperatures when CHOL is present. High levels of CHOL in spermatozoal plasma membranes would therefore decrease cryopreservation damage by allowing the membrane to maintain fluidity and function for a longer period of time through the cryopreservation process.

Upon examining phase transitions, proteins are seen to be excluded from the gelling membrane phospholipids and are concentrated into distinct regions (Pringle and Chapman, 1981). These particle aggregations are thought to be due to lateral phase separations of the lipids. This accumulation of intramembranous particles has been observed in the head and tail regions of ram spermatozoa upon slow cooling to 5°C (Holt and North, 1984) and in the head region of bull and boar spermatozoa upon fast freezing to 0°C (De Leeuw et al., 1990). De Leeuw et al. (1990) found that boar sperm exhibited

more extensive particle clustering than bull sperm which they concluded may be attributed to a high level of membrane CHOL in bull sperm. Therefore, the CHOL content and fatty acid composition of the spermatozoal plasma membrane may affect this particle clustering. The clustering did not disperse entirely upon thawing which may contribute to the immotility of the thawed sperm (Holt and North, 1984). This type of intramembranous particle clustering may also partly explain cryopreservation damage as packing faults may occur in the redistribution of the membrane components causing permeability problems. Proteins that are not distributed properly cause lateral stress in the membrane resulting in packing faults that surround the protein (Pringle and Chapman, 1981). The rate of cooling and the presence of cryoprotectant did not have an effect on this particle distribution (De Leeuw et al., 1990).

The semen of marine fish species tend to undergo cryopreservation with less damage than the semen of freshwater fish species. There are many possible factors influencing this difference in cryopreservation success such as temperature, water salinity, metabolic state, diet and perhaps the fatty acid composition of spermatozoal membranes. Drokin (1993) compared sperm from marine and fresh water fish and found that, similar to our results, C16:0, C18:1 and C22:6n-3 are major fatty acids (>10 g/100 g) in most species, regardless of environment. An important fatty acid, C18:2n-6, as well as the UFA/SFA ratio, and the phospholipid groups phosphatidylethanolamine and phosphatidylcholine were found to be higher in marine sperm than in sperm from freshwater fish (Drokin, 1993). Phosphatidylcholine has a great affinity for membranes and is known to protect sperm from differences in osmotic pressure and cold shock

(Drokin, 1993). Drokin and Kopeika (1992) found that fish sperm CHOL/phospholipid levels were positively correlated with cryopreservation success. They also found that marine fish had cholesterol/phospholipid levels that were two to three times higher than for freshwater fish. It is thought that environmental water salinity partially determines membrane properties which control osmoregulation (Drokin 1993).

4.5 Conclusions

These results show that the fatty acid composition and CHOL content of the diet can influence the integrity and viability of sperm membranes and fertility after cryopreservation. The sperm from fish fed the tallow or herring oil diets had the least cryopreservation damage and the greatest post freeze-thaw percentage of eyed embryos as well as the highest spermatozoal plasma membrane CHOL levels. It would appear that high levels of CHOL provide sperm with increased resistance to cryopreservation damage. Low membrane levels of MUFA in addition to high membrane levels of PUFA may also increase cryopreservation damage. High membrane levels of PUFA may result in oxidation which may be a factor in cryopreservation damage. The conclusion of this research is that by selecting the appropriate source of lipid for trout broodstock diets, such as tallow, sperm damage due to cryopreservation can be minimized. While high fertility is the desired outcome for aquaculturists, fluorometric assessment of membrane integrity was confirmed to be a more sensitive method for estimating cryopreservation damage to spermatozoal plasma membranes than were fertility parameters.

5. MATERNAL DIETARY LIPIDS AFFECT EGG AND LARVAL LIPIDS IN RAINBOW TROUT *(ONCORHYNCHUS MYKISS)*

5.1 Introduction

Oogenesis begins soon after hatch in fish and is divided into two growth phases followed by a final maturation phase. Essentially, in the first phase nuclear material is produced and stored. In the second growth phase, which occurs between 12 and 24 months post hatch, egg components such as the cortical alveoli, zona radiata and chorion, are developed. Vitellogenesis begins in this second phase and is influenced by gonadotropins. The final phase includes oocyte maturation culminating in ovulation.

Vitellogenesis is the process by which maternal lipids are transformed to form the yolks of the developing oocytes. The lipids are transferred in the form of a lipophosphoprotein known as vitellogenin. This macro molecule is influenced by estradiol, and is synthesized in the liver and transported in the blood stream (Garcia-Garrido et al., 1990; Billard, 1992). Consequently, the lipid content in fish plasma is related to the physiological and nutritional state of the fish (Babin, 1987a,b).

Developing embryos and larvae are dependent solely on the egg yolk for nutrients. Similar to other tissues, the maternal diet can affect the fatty acid composition of eggs (Mourente and Odriozola, 1990; Corraze et al., 1991; Ashton et al., 1993; Harel et al., 1994). Therefore, the maternal diet must meet or exceed nutrient requirements and essential fatty acid (EFA) levels to ensure that the embryo is not deficient. However, it has also been shown that n-3 fatty acids in northern pike (*Esox lucius*) can be mobilized

from somatic cells in the case of a dietary deficiency and used to increase ovarian n-3 fatty acids (Schwalme, 1994). In addition, EFA requirements may not be the same for early hatched larvae as for older juvenile fish.

Yolk lipid is the most important energy source for developing rainbow trout embryos. Approximately 45-65% of egg lipid is utilized during the development of fish embryos (Corraze et al., 1991). However, the timing, degree and type of lipid that is utilized during embryogenesis varies widely among species (reviewed by Sargent et al., 1988). Rainbow trout (*Oncorhynchus mykiss*) and loach (*Misgurnus fossilis*) are thought to use lipid as an energy source throughout embryogenesis especially in the later stages while other fish tend to use the lipid as an emergency energy source. Atlantic halibut (*Hippoglossus hippoglossus*) embryos are thought to utilize saturated fatty acids (SFA) at the time of, and shortly after, fertilization while unsaturated fatty acids (UFA) are more important as structural components later on in development. Therefore, lipids are not only an energy substrate as they also play an important role in the formation of membranes during embryo organogenesis (Corraze et al., 1991).

Egg quality, including fatty acid composition, is known to influence fertility as measured by hatchability and larval development in fish (Watanabe et al., 1984; Leray et al., 1985; Henderson and Tocher, 1987). For example, Srivastava and Brown (1991) found that wild Atlantic salmon (*Salmo salar*) eggs were significantly ($P<0.05$) larger and contained more protein, lipid and carbohydrate compared with cultured salmon eggs. The eggs from wild Atlantic salmon in turn had greater fertilization and hatching rates as well as greater size and survival of embryos and yolk-sac fry than did the cultured

salmon eggs.

The objective of this research was to determine the extent to which the source of maternal dietary lipid affects the fatty acid composition and cholesterol content of the eggs and developing larvae and whether or not this change affects fertilization success rates. The diets, and thus lipid sources, used were the same as in previous chapters: herring oil (adequate n-3 fatty acids), menhaden oil (high n-3 fatty acids), safflower oil (high n-6 fatty acids) and tallow (high saturated fatty acids).

5.2 Materials and Methods

The experimental design, diets, lipid extraction and gas-liquid chromatography were as described in previous chapters. The maternal fish were fed the experimental diets for 13 months prior to spawning.

5.2.1 Fertilization

Female rainbow trout were anaesthetized 10% (v/v) benzocaine (USP, Wiler) and eggs were expressed into stainless steel bowls. Several batches of 300 eggs were counted and the volume recorded until an average volume for 300 eggs was obtained. Subsequently, the batches of eggs were determined volumetrically. Two lots of 300 eggs were collected from a minimum of 4 fish per treatment (see Table XVII; n=number of fish/treatment) and were fertilized in glass beakers with pooled, fresh semen from several non-experimental male fish. Eggs were fertilized with 2.0×10^5 sperm cells per egg. The rest of the procedure was the same as described in the fertilization section of Chapter 4 (4.2.2).

5.2.2 Tissue Collection

Egg size was recorded by counting the number of eggs that fit into a 15.4 cm egg trough. Approximately 20 unfertilized eggs from each fish (see Tables X1-XVI; n= number of fish/treatment) were collected in glass, screw-top test tubes and stored under nitrogen vapour at -80°C. Ten embryos from each lot were collected at the eyed-embryo stage (approx. 210° days), at hatch (approx. 360° days), and just prior to the swim-up stage of development (after depletion of the yolk sac; approx. 460° days). The yolk-sac larvae collected at hatch and just prior to swim-up were frozen in order facilitate the separation of the yolk from the larvae with the use of a scalpel. All samples were subsequently freeze-dried and the lipids were extracted and methylated and analyzed with gas-liquid chromatography as described in Chapter 2.

5.2.3 Data Analysis

Data analysis was done using one-way analysis of variance with respect to treatment (SAS, 1982). Significance was indicated by $P<0.05$ on the probability differences of the least mean squares among treatments.

5.3 Results

The fatty acid composition and CHOL content of unfertilized eggs from fish fed the experimental diets were influenced by treatment (Table XI). Similar to the results of the tissues examined in previous chapters, the major fatty acids (>10 g/100 g) were C16:0, C18:1 and C22:6n-3 with the addition of C18:2n-6 for the eggs of safflower oil group. However, these major fatty acids varied in percentage among the groups. The tallow group eggs contained C18:1, C22:6n-3 and C16:0 in descending percentages while the eggs from the other groups contained descending levels of C22:6n-3, C18:1 and

TABLE XI. Fatty acid composition and cholesterol content of unfertilized eggs from experimental fish (g/100 g FA).

Fatty Acid	Herring Oil Diet n=5	SE	Menhaden Oil Diet n=7	SE	Safflower Oil Diet n=4	SE	Tallow Diet n=7	SE
C14:0	3.7[a]	0.1	3.5[a]	0.1	1.3[c]	0.1	1.9[b]	0.1
C16:0	15.2[b]	0.3	16.2[a]	0.2	13.4[c]	0.3	15.3[b]	0.2
C16:1	7.2[a]	0.3	6.9[a]	0.2	3.2[c]	0.3	6.2[b]	0.2
C18:0	3.8[b]	0.2	5.5[a]	0.2	5.5[a]	0.3	5.8[a]	0.2
C18:1	23.4[b]	0.5	17.1[c]	0.5	16.6[c]	0.6	30.3[a]	0.5
C18:2n-6	4.7[c]	0.4	4.2[c]	0.3	24.3[a]	0.4	6.3[b]	0.3
C18:3n-3	0.1[b]	0.1	0.1[b]	0.1	1.1[a]	0.1	0.3[b]	0.1
C20:1	7.3[a]	0.3	2.7[c]	0.2	2.3[c]	0.3	4.2[b]	0.2
C20:2	0.9[a]	0.1	1.0[a]	0.1	0.4[b]	0.1	0.9[a]	0.1
C20:3	1.1[c]	0.2	0.5[d]	0.1	3.7[a]	0.2	2.1[b]	0.1
C20:4n-6	1.9[c]	0.2	2.1[c]	0.2	6.1[a]	0.3	2.7[b]	0.2
C20:5n-3	4.5[b]	0.2	9.4[a]	0.2	1.8[d]	0.3	2.7[c]	0.2
C22:3	0.8	0.1	0.8	0.1	0.8	0.1	0.7	0.1
C22:4n-6	0.2[b]	0.1	0.2[b]	0.1	0.8[a]	0.1	0.3[b]	0.1
C22:5n-3	1.7[b]	0.1	3.6[a]	0.1	0.9[c]	0.2	1.0[c]	0.1
C22:6n-3	23.6[b]	0.6	26.2[a]	0.5	17.9[c]	0.6	19.4[c]	0.5
CHOL[1]	9.0[a]	0.6	7.1[b]	0.5	8.9[a]	0.7	9.1[a]	0.5

[abc] Values in the same row, with different superscript letters, are significantly different ($P<0.05$).

[1] Cholesterol (g/100 g dry matter).

C16:0 in descending order with the exception that the highest ($P<0.05$) fatty acid level for eggs from fish fed the safflower oil diet was C18:2n-6 at 24%. The unfertilized eggs from the safflower group tended to have lower levels of any of the major fatty acids compared to unfertilized eggs from the other groups while those of the herring group had intermediate levels of these fatty acids. The CHOL content of eggs from the fish fed the menhaden oil diet were significantly lower ($P<0.05$) than those of the eggs from the other groups.

Table XII lists the fatty acid composition of the fertilized eggs at the eyed-embryo stage of development (approx. 210° days). The pattern of the top three predominant fatty acids did not change within treatment groups between the unfertilized eggs and the eyed embryos. In other words, the eyed embryos from the tallow group had descending order of C18:1, C22:6n-3 and C16:0 for the main fatty acids while the descending order of major fatty acids for eyed embryos of the other groups was C22:6n-3, C18:1 and C16:0 with the exception that those from the safflower oil group contained roughly 24% of C18:2n-6 as the most predominant fatty acid.

The fatty acid distribution of the hatched larval bodies, excluding the yolk, is listed in Table XIII. The order of the main three fatty acids in the larvae from the herring and menhaden groups was similar to that found in both the unfertilized eggs and the eyed embryos; i.e. C22:6n-3, C18:1 and C16:0 in descending order. In addition, in this stage of development (360° days), the larvae from tallow-fed fish exhibited the same fatty acid pattern as the herring and menhaden oil groups (C22:6n-3, C18:1 and C16:0) while the predominant fatty acids of the larvae of the safflower oil group was closer to this order

TABLE XII. Fatty acid composition and cholesterol content of fertilized eggs at the eyed-embryo stage of development (210° days) from experimental fish (g/100 g FA).

Fatty Acid	Herring Oil Diet n=5	SE	Menhaden Oil Diet n=7	SE	Safflower Oil Diet n=4	SE	Tallow Diet n=7	SE
C14:0	3.5^{a}	0.3	3.2^{a}	0.3	1.3^{b}	0.3	2.0^{b}	0.3
C16:0	15.5^{a}	0.6	15.4^{a}	0.5	13.2^{b}	0.6	15.0^{ab}	0.6
C16:1	7.8^{a}	0.3	7.1^{a}	0.3	3.3^{c}	0.3	6.0^{b}	0.3
C18:0	3.9^{b}	0.3	5.5^{a}	0.2	5.4^{a}	0.3	5.7^{a}	0.3
C18:1	16.8^{b}	2.6	17.4^{b}	2.2	16.5^{b}	2.6	28.3^{a}	2.6
C18:2n-6	4.9^{bc}	0.6	3.9^{c}	0.5	23.6^{a}	0.6	6.0^{b}	0.6
C18:3n-3	0.1^{b}	0.1	0.6^{a}	0.1	0.7^{a}	0.1	0.1^{b}	0.1
C20:1	8.1^{a}	0.5	3.1^{c}	0.4	2.4^{c}	0.5	4.5^{b}	0.5
C20:2	1.1	0.4	1.0	0.3	1.7	0.4	1.1	0.4
C20:3	1.2^{c}	0.2	0.5^{d}	0.2	3.5^{a}	0.2	2.1^{b}	0.2
C20:4n-6	2.6^{b}	0.3	2.2^{b}	0.2	6.2^{a}	0.3	2.8^{b}	0.3
C20:5n-3	4.9^{b}	0.3	9.2^{a}	0.2	1.8^{d}	0.3	3.1^{c}	0.3
C22:3	0.6	0.1	0.6	0.1	0.7	0.1	0.6	0.1
C22:4n-6	0.3^{b}	0.1	0.3^{b}	0.1	0.8^{a}	0.1	0.2^{b}	0.1
C22:5n-3	1.9^{b}	0.2	3.7^{a}	0.1	0.8^{c}	0.2	1.1^{c}.	0.2
C22:6n-3	26.4^{a}	0.8	25.9^{a}	0.7	18.1^{c}	0.8	21.2^{b}	0.8
CHOL[1]	6.8	0.9	6.2	0.8	5.2	0.9	6.7	0.9

[abc] Values in the same row, with different superscript letters, are significantly different ($P<0.05$).

[1] Cholesterol (g/100 g dry matter).

TABLE XIII. Fatty acid composition and cholesterol content of larval bodies at hatch (360° days) from experimental fish (g/100 g FA).

Fatty Acid	Herring Oil Diet n=5	SE	Menhaden Oil Diet n=7	SE	Safflower Oil Diet n=4	SE	Tallow Diet n=7	SE
C14:0	2.2^{a}	0.2	2.1^{a}	0.2	0.6^{c}	0.2	1.3^{b}	0.2
C16:0	15.1^{ab}	0.4	15.2^{a}	0.3	14.2^{b}	0.4	15.3^{a}	0.3
C16:1	6.0^{a}	0.4	5.6^{a}	0.3	3.0^{b}	0.4	5.2^{a}	0.4
C18:0	4.3^{b}	0.2	5.7^{a}	0.2	6.0^{a}	0	5.9^{a}	0.2
C18:1	20.1^{b}	1.0	16.3^{c}	0.7	14.9^{c}	1.0	24.1^{a}	0.8
C18:2n-6	4.1^{b}	1.7	6.4^{b}	1.3	18.4^{a}	1.7	5.1^{b}	1.4
C18:3n-3	0^{b}	0.1	0.4^{a}	0.1	0.4^{a}	0.1	0.1^{b}	0.1
C20:1	6.4^{a}	0.3	2.3^{bc}	0.2	2.3^{c}	0.3	3.6^{b}	0.3
C20:2	1.1^{b}	0.3	1.4^{b}	0.2	3.2^{a}	0.3	1.1^{b}	0.3
C20:3	1.2^{b}	0.4	0.9^{b}	0.3	3.3^{a}	0.4	1.7^{b}	0.3
C20:4n-6	2.6^{b}	0.5	3.0^{b}	0.4	7.0^{a}	0.5	3.1^{b}	0.4
C20:5n-3	5.2^{b}	1.0	8.1^{a}	0.8	2.4^{b}	1.0	4.5^{b}	0.8
C22:3	0.4	0.2	0.4	0.1	0.5	0.2	0.6	0.2
C22:4n-6	0	0.3	0.1	0.2	0.5	0.3	0.4	0.2
C22:5n-3	1.7^{b}	0.4	3.1^{a}	0.3	0.7^{b}	0.4	1.8^{b}	0.3
C22:6n-3	29.9^{a}	1.3	28.3^{a}	1.0	22.6^{b}	1.3	26.4^{a}	1.0
CHOL[1]	8.7	4.6	9.4	3.5	18.2	4.6	10.3	3.8

[abc] Values in the same row, with different superscript letters, are significantly different ($P<0.05$).

[1] Cholesterol (g/100 g dry matter).

with C22:6n-3 being the predominant fatty acid followed by C18:2n-6 and C18:1.

The pattern of predominant fatty acids in the swim-up larval bodies for all of the treatment groups were similar (Table XIV). At this stage of development, the larvae from the fish fed any one of the treatment diets contained C22:6n-3 in the highest levels followed by C18:1 and C16:0. However, the larvae from the safflower oil group still tended to have the lowest amounts of these main fatty acids as compared to those of larvae in the other groups but the overall pattern was identical.

The fatty acid composition of the yolk from the hatched larvae is listed in Table XV. The distribution of the predominant fatty acids within each treatment group followed the same order as for the unfertilized eggs and the eyed embryos. Table XVI lists the fatty acid composition of the yolk from the swim-up embryos. The order of the predominant three fatty acids of the yolk from the swim-up embryos from the tallow group was similar in order to those of yolk from the tallow group hatched larvae as well as to those of the unfertilized eggs and eyed embryos of the tallow group: C18:1, C22:6n-3 and C16:0. The swim-up yolk compositions for the herring and menhaden groups were similar to each other for the main fatty acids, C18:1, C22:6n-3 and C16:0 in descending order whereas those of the hatched larval yolk for the herring and menhaden groups were C22:6n-3, C18:1 and C16:0. In the safflower group, the order of the predominant fatty acids for the hatched larval yolk was C18:2n-6, C18:1 and C22:6n-3, differing from the C18:2n-6, C22:6n-3, C18:1 order of the larval yolk fatty acids prior to swim-up.

Maternal dietary lipid did not statistically affect the gross morphology of the eggs nor any of the fertility parameters (Table XVII). There were no significant differences in

TABLE XIV. Fatty acid composition and cholesterol content of the larval bodies prior to swim-up (460° days) from experimental fish (g/100 g FA).

Fatty Acid	Herring Oil Diet n=5	SE	Menhaden Oil Diet n=7	SE	Safflower Oil Diet n=4	SE	Tallow Diet n=7	SE
C14:0	1.8^{a}	0.2	1.4^{a}	0.1	1.0^{b}	0.2	1.5^{a}	0.1
C16:0	15.4	04.	15.8	0.3	14.8	0.4	15.7	0.3
C16:1	5.1^{a}	0.4	4.8^{a}	0.3	3.2^{b}	0.4	4.8^{a}	0.3
C18:0	4.8^{b}	0.2	5.5^{a}	0.2	5.9^{a}	0.2	5.7^{a}	0.2
C18:1	18.9^{ab}	1.1	17.6^{bc}	0.9	15.4^{c}	1.2	20.5^{a}	0.9
C18:2n-6	6.2^{b}	1.7	5.6^{b}	1.4	14.6^{a}	1.9	6.4^{b}	1.4
C18:3n-3	0.1^{a}	0.1	0.2^{a}	0.1	0.4^{a}	0.1	0.1^{b}	0.1
C20:1	4.7^{a}	0.4	3.3^{b}	0.4	2.2^{b}	0.5	3.0^{b}	0.4
C20:2	1.4^{b}	0.3	1.2^{b}	0.2	2.9^{a}	0.3	1.3^{b}	0.2
C20:3	1.4^{b}	0.3	1.2^{b}	0.2	2.6^{a}	0.3	1.6^{b}	0.2
C20:4n-6	3.4^{b}	0.5	3.2^{b}	0.4	6.1^{a}	0.5	3.5^{b}	0.4
C20:5n-3	4.5^{ab}	0.8	6.1^{a}	0.7	2.9^{b}	0.9	4.8^{ab}	0.7
C22:3	0.8	0.1	0.8	0.1	0.9	0.1	0.8	0.1
C22:4n-6	0.2^{b}	0.1	0.2^{b}	0.1	0.7^{a}	0.1	0.1^{b}	0.1
C22:5n-3	1.5	0.3	2.0	0.3	1.1	0.4	1.6.	0.3
C22:6n-3	29.9^{a}	1.4	31.1^{a}	1.2	25.4^{b}	1.6	28.6^{ab}	1.2
CHOL[1]	14.3	2.2	11.5	1.9	12.5	2.5	12.9	1.9

[abc] Values in the same row, with different superscript letters, are significantly different ($P<0.05$).

[1] Cholesterol (g/100 g dry matter).

TABLE XV. Fatty acid composition and cholesterol content of the larval yolk at hatch (360° days) from experimental fish (g/100 g FA).

Fatty Acid	Herring Oil Diet n=5	SE	Menhaden Oil Diet n=7	SE	Safflower Oil Diet n=4	SE	Tallow Diet n=7	SE
C14:0	3.2^{a}	0.2	3.0^{a}	0.2	1.2^{c}	0.2	1.9^{b}	0.2
C16:0	14.1^{a}	0.3	14.6^{a}	0.3	12.5^{b}	0.3	14.5^{a}	0.3
C16:1	7.6^{a}	0.3	7.2^{a}	0.2	3.5^{c}	0.3	6.3^{b}	0.3
C18:0	3.7^{b}	0.2	5.1^{a}	0.1	5.3^{a}	0.2	5.5^{a}	0.2
C18:1	23.2^{b}	0.5	18.1^{c}	0.4	16.7^{d}	0.5	29.2^{a}	0.4
C18:2n-6	5.2^{b}	1.1	6.5^{b}	0.8	23.9^{a}	1.1	6.5^{b}	0.9
C18:3n-3	0.1^{b}	0.1	0.5^{a}	0.1	0.3^{a}	0.1	0^{b}	0.1
C20:1	7.2^{a}	0.3	3.3^{c}	0.3	2.3^{d}	0.3	4.3^{b}	0.3
C20:2	1.3^{b}	0.3	1.3^{b}	0.2	3.4^{a}	0.3	1.3^{b}	0.2
C20:3	1.1^{bc}	0.3	0.9^{c}	0.2	3.4^{a}	0.3	1.9^{b}	0.2
C20:4n-6	2.6^{b}	0.5	2.8^{b}	0.4	6.0^{a}	0.5	2.7^{b}	0.4
C20:5n-3	4.5^{b}	0.8	8.3^{a}	0.6	1.8^{c}	0.8	3.4^{bc}	0.7
C22:3	0.5^{ab}	0.1	0.5^{b}	0.1	0.8^{a}	0.1	0.4^{b}	0.1
C22:4n-6	0.3^{ab}	0.1	0.4^{ab}	0.1	0.5^{a}	0.1	0.2^{b}	0.1
C22:5n-3	1.7^{b}	0.3	3.3^{a}	0.3	0.8^{b}	0.3	1.3^{b}	0.3
C22:6n-3	23.7^{a}	0.6	24.2^{a}	0.5	17.6^{c}	0.6	20.7^{b}	0.5
CHOL[1]	6.2	1.0	7.1	1.0	6.4	1.0	6.2	1.0

[abc] Values in the same row, with different superscript letters, are significantly different ($P<0.05$).

[1] Cholesterol (g/100 g dry matter).

TABLE XVI. Fatty acid composition and cholesterol content of the larval yolk prior to swim-up (460° days) from experimental fish (g/100 g FA).

Fatty Acid	Herring Oil Diet n=3	SE	Menhaden Oil Diet n=3	SE	Safflower Oil Diet n=3	SE	Tallow Diet n=3	SE
C14:0	2.4	7.6	2.5	7.6	16.2	7.6	2.2	7.6
C16:0	13.5[a]	1.0	14.0[a]	1.0	10.0[b]	1.0	13.8[a]	1.0
C16:1	6.8[a]	0.6	7.2[a]	0.6	3.5[b]	0.6	6.9[a]	0.6
C18:0	3.4	0.5	4.5	0.5	4.0	0.5	4.7	0.5
C18:1	24.2[a]	1.9	26.0[a]	1.9	17.1[b]	1.9	29.1[a]	1.9
C18:2n-6	10.4[ab]	3.3	7.2[b]	3.3	20.2[a]	3.3	7.3[b]	3.3
C18:3n-3	0.1[b]	0.1	0.1[b]	0.1	0.5[a]	0.1	0.1[b]	0.1
C20:1	6.2[a]	0.7	6.1[a]	0.7	2.3[b]	0.7	5.3[a]	0.7
C20:2	2.1[ab]	0.4	1.6[ab]	0.4	2.9[a]	0.4	1.5[b]	0.4
C20:3	1.7	0.4	1.3	0.4	2.6	0.4	1.6	0.4
C20:4n-6	3.5	0.6	3.2	0.6	4.1	0.6	2.9	0.6
C20:5n-3	3.4[a]	0.3	3.8[a]	0.3	1.6[b]	0.3	3.2[a]	0.3
C22:3	0.5	0.1	0.5	0.1	0.5	0.1	0.6	0.1
C22:4n-6	0.1[b]	0.1	0.2[b]	0.1	0.4[a]	0.1	0.2[ab]	0.1
C22:5n-3	1.1[ab]	0.2	1.4[a]	0.2	0.6[b]	0.2	1.2[ab]	0.2
C22:6n-3	20.8[a]	1.4	20.6[a]	1.4	13.6[b]	1.4	19.8[a]	1.4
CHOL[1]	5.9	1.2	6.2	1.2	7.2	1.2	8.3	1.2

[abc] Values in the same row, with different superscript letters, are significantly different ($P<0.05$).

[1] Cholesterol (g/100 g dry matter).

TABLE XVII. Comparison of mean percentages of egg size and fertility parameters of eggs from fish fed diets containing lipid from four sources.

	Herring Oil Diet n=5	SE	Menhaden Oil Diet n=7	SE	Safflower Oil Diet n=4	SE	Tallow Diet n=7	SE
Egg Size[1,2]	42.0	0.7	41.3	0.6	41.0	0.8	40.6	0.6
Fertilized [1] Eggs (%)	91.6	7.2	86.4	6.1	94.3	8.1	86.1	6.1
Eyed [1] Embryos (%)	73.0	15.4	70.0	13.0	82.5	17.2	56.4	13.0

[1] Values in each row are not significantly different.

[2] Values are number of eggs in a 15.4 cm egg tray.

egg size among the treatments. There were also no significant differences in fertilization success as evaluated by the percentage of fertilized eggs and while the number of embryos reaching the eyed-egg stage of development was not significantly different between treatments, the percentage of eggs from the tallow-fed fish that reached the eyed-embryo stage was lower than those of the other groups. In addition, no differences in mortality or gross morphological development were observed.

5.4 Discussion

The distribution of C16:0, C18:1 and C22:6n-3 among the treatment groups of eggs resembled the pattern of distribution of these fatty acids found in the diets even though the percentages per se were not similar (Table II, Chapter 2). However, there were some exceptions; the C18:1 levels in the herring oil diet were lower than in the menhaden and safflower diets while the C18:1 levels were higher in the eggs from the herring group than in the eggs from the menhaden and safflower oil groups. The percentages of C18:1, C16:0 and C22:6n-3 descended in that order in the tallow diet while the decreasing order was C18:1, C22:6n-3 and C16:0 in the eggs.

The fatty acid compositions of eggs, yolk and larvae in other fish species have been identified and are consistent with the results of this study. Rønnestad et al. (1995) found that the main fatty acids in Atlantic halibut yolk lipids were C22:6n-3, C16:0 and C20:5n-3. They found minor differences in specific fatty acid utilization by the embryos. Only C22:6n-3 and C20:5n-3 seemed to be used for both energy metabolism and incorporation into the larval body. Parrish et al. (1994) found that C22:6n-3 was the predominant fatty acid in both unfertilized and fertilized eggs of Atlantic halibut.

Vázquez et al. (1994) also found C16:0, C18:1 and C22:6 to be the predominant fatty acids, decreasing in that order in yolk-sac larvae of Senegal sole (*Solea senegalensis*). Ashton et al. (1993) found that C18:1 was the predominant fatty acid in wild and cultured Chinook salmon (*Oncorhynchus tshawytscha*) eggs, while the levels in the cultured salmon were significantly higher than in the wild salmon and the second most abundant fatty acid in both groups was C22:6n-3. However, Ulvund and Grahl-Nielsen (1988) found a high degree of variability in the fatty acid composition of Atlantic cod eggs (*Gadus morhua*) as the differences in fatty acid composition were greater between successive batches of eggs than among females.

In the larval bodies, the order of the predominant fatty acids converged amongst the treatment groups between the hatch and swim-up stages of development so that in the swim-up larvae for all of the treatment groups, the order of the main fatty acids was C22:6n-3, C18:1 and C16:0, in descending order. The safflower group larvae was the only group in which this order changed between these two stages going from C22:6n-3, C18:1, C18:2n-6 to C22:6n-3, C18:1, C16:0, in descending order. Neither the main fatty acids nor the order of predominant fatty acids changed between any of the development stages (ie. eggs, eyed-eggs, larval bodies at hatch and larval bodies at swim-up) in the menhaden or herring oil groups. The order of predominant fatty acids for the tallow group was the same for the eggs and eyed-eggs as well as for the larval bodies between the hatch and swim-up stages of development but changed between the eyed-egg and hatch stages of development. These main fatty acids, C22:6n-3, C18:1 and C16:0, found in all of the treatment groups of swim-up larvae were the same predominant fatty acids

that were found in the liver tissue, however the order was different. The swim-up larvae contained C22:6n-3, C18:1, C16:0 in descending order while the liver tissue consisted of C22:6n-3, C16:0, C18:1 in descnding order for all treatment groups except for the safflower group which contained C22:6n-3, C16:0 and C18:2 in descending order as the predominant fatty acids.

Female fish can mobilize storage lipids from the liver for oogenesis; therefore, it is important to note that as this study lasted 13 months, both the storage and yolk lipids would have been modified by the diet. In chapter 1.0, liver tissue from female fish was found to have significantly higher levels of C18:0, C20:1, C20:3, C22:5n-3, n-3 fatty acids, n-3HUFA (C20:5n-3 and C22:6n-3) and polyunsaturated fatty acids (PUFA) as compared to male liver tissue whereas male liver tissue was higher in C18:2n-6, C20:4n-6, C20:5n-3 and n-6 fatty acids as compared to female liver tissue. This fatty acid sexual dimorphism is most likely due to gametogenesis. However, in contrast, Ackman and Burgher (1964) found that female Atlantic cod livers had a higher content of C18:1 and C20:5 than did the male livers which was reflected in the roe by high levels of C18:1 and C20:5.

In the present study, the pattern of predominant yolk fatty acids from the hatched larvae and the swim-up larvae did not resemble the fatty acid composition of the liver. It would therefore seem that the yolk lipids, and presumably the vitellogenin lipids, did not reflect the liver lipid composition. This is despite the fact that the liver lipids are transferred to lipoproteins as seen in chum salmon (*Oncorhynchus keta*) during spawning (Hatano et al., 1989) and that Lie et al. (1993) showed that the fatty acid

composition of lipoproteins in fish changed in response to diet.

The pattern of fatty acid distribution of the hatched larval yolk was similar to the fatty acid composition of both the unfertilized and fertilized eggs for each of the treatment groups. This was likely due to the fact that the eggs are predominantly yolk and, up until the hatch stage of development, only a small percentage of the yolk would have been utilized. The order of fatty acids in the yolks from the hatch and swim-up stages varied amongst the treatment groups. In the menhaden and herring oil groups, the yolk from the hatched larvae contained C22:6n-3 as the most abundant fatty acid while it ranked the second most abundant in the swim-up larval yolk. A similar decrease in yolk C22:6n-3 was also seen in the safflower group. This may be due to the utilization of the yolk C22:6n-3 for the growing larvae.

The fatty acid, C22:6n-3, was the most predominant fatty acid in the larval bodies at swim-up for all of the treatment groups; this supports the importance of C22:6n-3 in development. Frémont et al. (1984) found that rainbow trout fed a n-3 fatty acid deficient diet during the last 3 months of vitellogenesis showed only slightly lower levels of egg C22:6n-3 than did the control fish while C20:5n-3 egg levels decreased by half in the dietary n-3 deficient fish. The conservation of C22:6n-3 levels over the depletion of C20:5n-3 levels during a dietary deficiency of n-3 fatty acids is evidence of the physiological importance of C22:6n-3. As mentioned previously (Section 2.3.4), C22:6n-3 has been found in high levels in tissues, such as brain and muscle, of fish and mammals (Crawford et al., 1976; Greene and Selivonchick, 1987; Sprecher, 1991) and was shown to be important for neural and retinal development in rats, primates and

humans (Sinclair and Crawford, 1972; Sinclair, 1975; Neuringer et al., 1988; Clandinin et al., 1994). Considering this evidence, high C22:6n-3 levels in fish eggs, regardless of maternal diet might be storage for future structural use in the eye and brain.

Despite the lowest percentage of the three main fatty acids (C16:0, C18:1 and C22:6n-3), as compared to the other groups, the eggs from the safflower group did not exhibit compromised fertility as assessed by fertilization and eyed-embryo rates. Similarly, Corraze et al. (1991) found that corn oil (high in n-6 fatty acids) or cod liver oil (high in n-3 fatty acids) as source of dietary lipid at 6%, affected egg fatty acid composition but did not affect fecundity nor egg viability in rainbow trout; neither maternal dietary lipid nor rearing temperature affected the percentage of eyed embryos, hatchability or survival of fry. However, Harel et al. (1994) studied the effect of maternal diet high in either n-3 or n-6 fatty acids on egg composition and egg quality in gilthead seabream (*Sparus aurata*) and found that egg quality, in terms of egg viability and hatching rate, decreased significantly for fish fed n-3 fatty acid deficient diet.

The fatty acid composition of the swim-up embryo was not similar to the composition of muscle (Table IV, chapter 2). This finding suggests that the fatty acid composition will change further between the swim-up larval state of development and that of the mature, adult fish. Hove and Grahl-Nielsen (1991) found that the fatty acid composition of Atlantic salmon larvae fed starter diets with different lipid sources changed over time but did not reflect the composition of the diets. The authors concluded that genetics had a stronger influence over fatty acid composition than diet. The present study supports this finding that the influence of dietary factors on tissue fatty

acid composition varies with stage of development and that, over time, the fatty acid composition in larval development is regulated by physiology to a greater extent than diet.

The fatty acid composition of the total egg lipid was measured in this study, whereas in future research, the fatty acids, and especially the PUFA of the phospholipid portion of the eggs should be evaluated among the four diets.

5.5 Conclusions

1. The tallow group eggs contained C18:1, C22:6n-3 and C16:0 in descending percentages while the eggs from the fish oil groups contained descending levels of C22:6n-3, C18:1 and C16:0 and the safflower group eggs contained descending percentages of C18::2n-6, C22:6n-3 and C18:1. By hatch (360° days), the larval bodies from tallow-fed fish exhibited the same fatty acid pattern as the herring and menhaden oil groups (C22:6n-3, C18:1 and C16:0).
2. Tissues from the safflower oil group were high in C18:2n-6. In this group, the fatty acid, C18:2n-6, was the most predominant fatty ($P<0.05$) acid in the unfertilized and eyed embryos as well as in the yolk from hatch and swim-up stages of development. The percentage of C18:2n-6 declined in the larval bodies to the second most abundant fatty acid at hatch and the fourth most abundant fatty acid just prior to the swim-up stage of development. The order of the predominant fatty acids in the larval bodies just prior to the swim-up stage of development of the safflower group became consistent with the order of the predominant fatty acids in the larval bodies of the other groups (C22:6n-3, C18:1, C16:0).

3. Egg fatty acid composition was influenced by the fatty acid composition of the maternal diet but did not reflect the pattern seen in the liver tissue. Between hatch and swim-up, the fatty acid composition, but not order of predominant fatty acids, of the developing larvae increasingly resembled that of the liver. Therefore, over time, the influence that dietary factors exert on tissue fatty acid composition varied with stage of development and the fatty acid composition in larval development was regulated by physiology to a greater extent than diet.
4. There were no differences in either gross morphology or fertility among the eggs from fish fed the experimental diets, despite the differences in fatty acid composition.

6. DISCUSSION

Nutrition as a tool to influence physiology has been underutilized in aquaculture. The quality of fish for human consumption can be improved by manipulating the fatty acid composition of fish muscle through diet. In addition, the fatty acid composition of other tissues, such as sperm, can be manipulated through diet in order to influence function. As membranes typically contain a relatively large portion of lipid, the fatty acid composition of membranes can also be modified by dietary means. In general, tissues reflect the fatty acid composition of the diet, although there appears to be levels of certain fatty acids which remain more or less unaffected by dietary modification, presumably due to physiological importance.

Semen cryopreservation offers great advantages to the aquaculturist. Frozen semen allows greater flexability in the timing of fertilization and facilitates the transport and export of genetic material. This procedure also protects against loss of genetic stock due to disease and allows for a reduction of male broodstock. However, fish semen cryopreservation remains in the experimental stages due to the lack of repeatable, high quality results. Fish nutrition appears to be able to increase the success rate of semen cryopreservation.

This thesis combines the disciplines of nutrition, cryopreservation and reproduction. Dietary lipid affected tissues that have implications for human health. In order to improve cryopreservation success, fish sperm were modified by the dietary source of lipid in an attempt to minimize the percentage of sperm damaged from cryopreservation and to maximize fertility rates. The area of membrane biology was also explored as the

spermatozoal plasma membrane is typically involved in cryopreservation damage. The effect of maternal nutrition on the fatty acid composition of eggs and the subsequent fertilization success was also examined.

6.1 Summary

Despite the source of dietary lipid, C16:0, C18:1 and C22:6n-3 were major (>10 g/100 g) fatty acids in all the tissues examined: fish muscle, liver, sperm, spermatozoal plasma membranes, eggs, larvae and yolk. It would appear that these fatty acids are physiologically and structurally necessary in tissues. However, high levels of C18:2n-6 from the safflower oil diet were also found consistently in all of the aforementioned tissues. Similarly, the menhaden oil diet, which is high in n-3 fatty acids, increased the n-3 fatty acid content in the aforementioned tissues. The increase of n-3 fatty acids in muscle tissue has positive implications for human health by providing a food product with increased levels of n-3 fatty acids. The lowest concentrations of saturated fatty acids in muscle was found in the fish fed the herring oil diet. The highest percentage of monounsaturated fatty acids (MUFA) was in the muscle of fish fed the herring oil diets while the highest level of polyunsaturated (PUFA) was in the muscle of the safflower group. This information would be of interest to consumers who need to reduce their dietary saturated fatty acid intake and increase their consumption of monounsaturated and polyunsaturated fatty acids.

Sperm from fish fed the tallow diet had significantly ($P<0.05$) higher cholesterol (CHOL) levels than the sperm from the fish fed the other diets. Similarly, the spermatozoal plasma membranes from fish fed the tallow diet had significantly ($P<0.05$)

higher CHOL levels than did fish fed the menhaden or safflower oil diets and tended to have higher levels than did fish fed the herring oil diet. In addition, the sperm from fish fed the tallow or herring oil diets had the least cryopreservation damage and the greatest post-cryopreservation percentage of eyed embryos. It would appear that high levels of CHOL provide sperm with increased resistance to cryopreservation damage.

Low membrane levels of MUFA in addition to high membrane levels of PUFA, such as in the sperm and spermatozoal plasma membranes of the fish fed the safflower oil diet, may indicate sensitivity to cryopreservation damage. High membrane levels of PUFA may result in oxidation which may be a factor in cryopreservation damage. Despite the wide range of levels of unsaturated/saturated fatty acid (UFA/SFA) in the experimental diets (1.8-6.8%), the UFA/SFA values centered around 2.1 suggesting that this value is of physiological importance.

The sex of the fish was found to have an effect on the incorporation of some fatty acids primarily in the liver but also in muscle tissue. Differences in gametogenesis between the sexes is the most probable explanation for this finding although the fatty acid composition of the eggs did not reflect the fatty acids that were high in the livers of female fish.

The fatty acid, C22:6n-3, was found in high levels in the eggs. Many tissues, in numerous species have high levels of C22:6n-3; this fatty acid has been conserved through evolution due to its physiological importance in neural and retinal development (Sinclair and Crawford, 1972; Sinclair, 1975; Neuringer et al., 1988; Clandinin et al.,1994). Fertility was not affected by source of maternal dietary lipid as there were no

differences in either gross morphology or fertility among the eggs from fish fed the experimental diets, despite the differences in dietary fatty acid composition.

Egg fatty acid composition was influenced by the fatty acid composition of the maternal diet but did not reflect the pattern seen in the liver tissue. However, the fatty acid composition of developing larvae increasingly resembled the liver fatty acid distribution by the swim-up stage of development. Therefore, the degree of influence that dietary factors exerted on tissue fatty acid composition varied with stage of development and the fatty acid composition in larval development was regulated by physiological parameters to a greater extent than diet.

6.2 Further Research

Further research could include the corresponding phospholipid groups in addition to individual fatty acids. Membrane fluidity should also be directly measured with the use of membrane markers and electron spin resonance. In order to determine the effective embryo utilization of fatty acids over time, individual fatty acids should be measured in μg/individual.

6.3 Conclusions

The source of dietary lipid can affect the fatty acid composition of muscle, liver, sperm, spermatozoal plasma membranes, eggs, larvae and yolk. The following fatty acids: C16:0, C18:1, C22:6n-3, were major components of all the tissues examined regardless of dietary treatment. Dietary lipid can modify the fatty acid composition in tissues, such as muscle tissue, sufficiently for practical uses such as increased n-3 fatty acids in edible muscle for the benefit of human health. The fatty acid composition of sperm and spermatozoal plasma membranes can be altered by dietary lipid which in turn

can increase the success of semen cryopreservation as assessed by fluorometry and fertility. Similarly, the fatty acid compositions of unfertilized and fertilized eggs, hatched and swim-up larvae were influenced by maternal dietary lipid, however, there were no differences in either gross morphology or fertility among the eggs from fish fed the experimental diets. As embryo and larval development progressed, the order of the predominant fatty acids converged so that at swim-up, the patterns of predominant fatty acids were consistent among the treatment groups. Therefore, the influence of maternal source of dietary lipid on tissue fatty acid composition varied with the stage of development and was regulated by physiology to a greater extent than diet at the time of the swim-up stage of development. Tissue fatty acid compositions can be altered by dietary means, however, there are some limitations as there appears to be set physiological levels for some fatty acids.

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IMAGE EVALUATION
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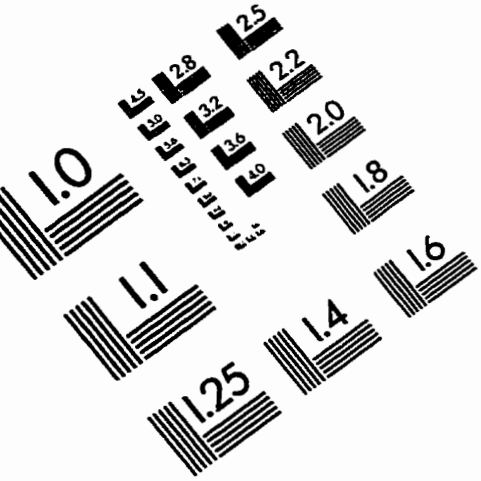

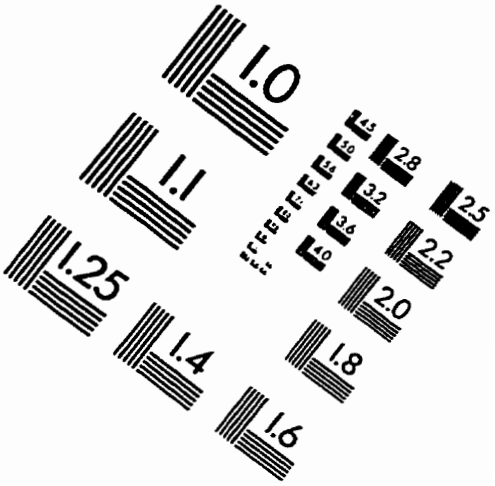

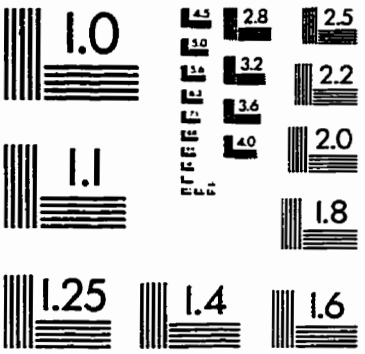

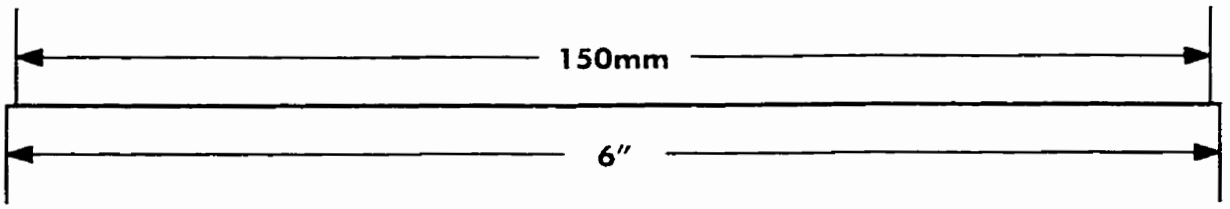

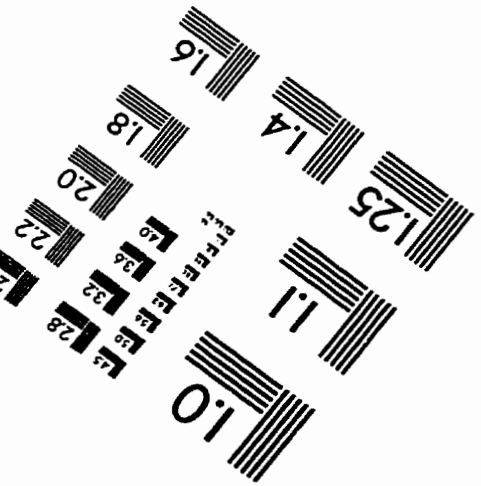

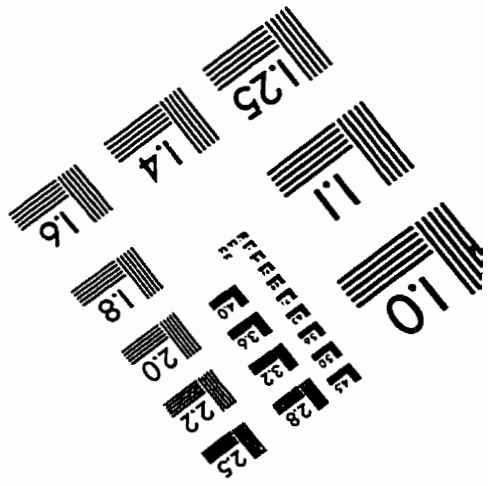

APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989