Assessment of growth performance and liver lipid accumulation in Atlantic salmon (*Salmo salar*) fed an insect-based diet

Master's degree in fish health biology

Petter Gjesdal

Department of biology

University of Bergen, Norway

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Abstract

Increase in price and reduced availability of fish meal (FM) has caused the aquaculture industry to look at alternative sources for nutrients for feed production. Vegetable protein sources can replace a substantial part of FM and has been doing so in recent years. Vegetable sources do, however, have limitations due to an unbalanced amino acid (AA) profile, being high in fibre, antinutritional factors and competition with human consumption. Alternative sources for protein must be considered for increased flexibilities in future fish feed production. In this study, insect meal (IM) made from Black soldier fly (*Hermetia illucens)* pre pupae fed on brown algae (*Ascophyllum nodosum)* enriched medium was used to substitute 100% of the fishmeal in a diet for Atlantic salmon *(Salmo salar)* in seawater. Commercially available feed was used as control. The diets were balanced so no nutrient deficiencies were expected to have an impact on growth and other health parameters. After 16 weeks, fish length and weight were recorded and hepatic- and visceral somatic indices measured. No differences in growth and survival were discovered. The liver lipid accumulation was evaluated through histology to assess the size of hepatic lipid droplets and by lipid class analysis. Histology did not reveal any difference between the dietary groups in the size distribution of lipid droplets, and lipid class analysis did not show any significant differences in lipid class composition. In all, the IM based diet used in this trial showed great potential as feed in future Atlantic salmon farming.

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Abbreviations

AA – Amino acid

ANOVA – Analysis of variance

BSF – Black soldier fly

CF – Condition factor

DHA - Docosahexaenoic acid

EPA - Eicosapentaenoic acid

FA – Fatty acid

FFA – Free fatty acid

FCR – Feed conversion rate

FM – Fish meal

HSI – Hepatosomatic index

HPTLC – High performance thin-layer chromatography

IM – Insect meal

LP – Lipoprotein

MUFA – Mono unsaturated fatty acid

NL – Neutral lipid

ORO – Oil red o

PBS – Phosphate buffered saline

PL – Polar lipids

SEM – Standard error mean

SD – Standard deviation

SPG – Specific growth rate

TAG – Triacylglycerols

TBARS – Thiobarbituric acid reactive substances

VSI – Viceralsomatic index

WW – Wet weight

1 Introduction

1.1 Aquaculture in Norway and history of feed

Harvesting food from the ocean has a long history in Norway. Aquaculture in Norway started as a small industry in the 1970s and today it is a big industry, which in 2016 produced 1.23 million tons of Atlantic salmon (*Salmo salar)* and 87.9 thousand tons of rainbow trout (*Oncorhynchus mykiss)* (SSB, 2017). The first stage of the cultured salmon's life happens indoors in tanks at the hatcheries. Since Atlantic salmon is an anadromous fish, it is kept in freshwater through fertilization, hatching, the yolk sack stage, parr stage, and through smoltification until it is ready to be put to seawater. The salmon is then kept in fish farms located all over the Norwegian coastline. At around 5 kg the salmon is transported by well-boats to the slaughterhouses. From hatching to slaughter takes around 2-3 years depending on water temperature, water quality and feeding regimes (Handeland et al., 2008; Havforskningsinstitutet, 2009). Diseases and other factors like stress can also affect the growth rate and time (McCormick et al., 1998).

Salmon has a very efficient feed conversion efficiency compared to terrestrial livestock's and can convert around ~1.15 kg of feed into 1 kg of bodyweight (Ytrestøyl et al., 2015). This makes for a feed conversion ratio (FCR) of 1.15. FCR for other animals for food production is 2.5 for chicken, 3.9 for swine and 33 for sheep (Åsgård and Austreng, 1995). In addition, the salmon have a high specific growth rate (% bodyweight growth/day, SGR), which makes it an effective animal for human food production. This is however, dependent on having a good feeding regime and stable access to feed in order to exploit these biological properties. The fish is fed pellets containing all the needed macronutrients, mostly fat and proteins in addition to some carbohydrate for technical properties of the pellet (table 1.1). The feed also contains a small percentage of other elements like added minerals, vitamins, pigments and amino acids. Previously, FM from marine fish was the main source for protein used in fish feed. FM is an excellent source of protein for fish feed, as it is easily digested and has an exceptional amino acid profile. FM also contains some lipids containing the essential fatty acids; eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). It is also a good source of vitamin A and D, riboflavin, niacin and minerals such as calcium, phosphorus, iron, zinc, selenium and iodine

(Olsen and Hasan, 2012). Increased demand for FM over the years has led to limited supplies and increased cost of fishmeal, in addition to consumer concerns regarding sustainability (Sørensen, 2011). It is estimated that the production of Atlantic salmon in Norway has the potential to increase by 500 % between 2010 and 2050 (DKNVS/NTVA, 2012). The issues regarding supply, price and sustainability will increase if these estimates are to become reality.

In recent years, vegetable sources have replaced many of the marine-based sources in fish feed. Price- and sustainability concerns forced feed producers over to vegetable sources, like soya, sunflower, rapeseed, corn, vegetable beans and wheat (table 1.1). The amino acid profile in the vegetable sources differ from the marine sources, and is not optimal for Atlantic salmon (Olli et al., 1994), which is the reason why some additional amino acids must be added to the feed when using high levels of vegetable ingredients. These ingredients make up around 70 % of the feed on average today (Laksefakta, 2016). The other 30 % of the feed is mainly fishmeal and fish oil. Around 25-35 % of the globally produced fish meal and fish oil comes from heads, spines and other parts of the fish not used for human consumption (FAO, 2016). With low expectation of increase in raw materials from whole fish catches, this percentage must increase to meet the increasing demand. Alternatively, different sources of nutrients must be considered for the future of feed production.

Table 1.1: Use of plant ingredients vs marine ingredients over the past 20 years in Norwegian aquaculture (% used of total feed sold from three feed companies) (Sørensen, 2011)*

	1990	2000	2010
Fish meal	63.8	37.5	25.6
Plant protein (various sources)	0	15.4	36.9
Starch (mainly wheat)	10.3	10.9	9.4
Fish oil	23.4	30.7	17.0
Plant oil	0	0.0	12.0

*Microingredients such as vitamins, minerals and amino acids are excluded

1.2 New sources of feed ingredients?

There are several things to think through when considering new sources for feed ingredients. Most importantly, the source needs to provide the correct nutritional value (correct amino acid profile, correct fatty acids etc.). The raw materials are the source of muscle growth and energy storage in addition to having an impact on the immune system. Having the correct macro- and micro nutritional values are essential to maintain growth and avoid malnourishing leading to bad fish health, increased mortality and decreased fish welfare.

The source of nutrients must also be low in unwanted substances that could cause harm to the fish, the end consumer or the environment. These maximum limits of unwanted substances are defined by EC Directive 2002/32. Heavy metals like lead, cadmium, mercury, arsenic, as well as dioxides, polychlorinated biphenyls (PCBs), pesticides, some natural plant or fungal toxins are defined with maximum values in this directive. The potential risks of transferring microorganisms like viruses, bacteria and fungi from the raw materials to the fish and/or the end consumer must also be subject to risk assessment and documentation. Cost of production must also be considered when looking for alternative feed in order to maintain the incentive to produce salmon. Recent years, feeding cost stands for around 50 % of all production cost in salmon farming in Norway (Fiskeridirektoratet, 2017). Small differences in feed prize will therefore have a huge effect on the profitability in salmon farming. Price efficiency when producing new ingredients is therefore also important.

1.3 Insects as a viable nutrient source for future feed production

In freshwater, insects makes up over 50 % of the wild salmon's diet (Gabler and Amundsen, 1999). This fact makes it reasonable to believe that insects could be a viable ingredient in salmon feed. Trials have tested different insect larvae meals as alternative sources of nutrients for both freshwater and postsmolt Atlantic salmon, with promising results on growth performance and quality (Belghit et al., 2018; Henry et al., 2015; Lock et al., 2016; Sánchez-Muros et al., 2014). Insects also reproduce quickly, have an efficient feed conversion and can

be fed on bi-products and bio-waste (Van Huis et al., 2013). A variety of insects also have amino acid profiles suitable for fish feed (Henry et al., 2015). On average, 1 kg of insect biomass can be produced from 2 kg of feed biomass, making it an efficient use of resources (Collavo et al., 2005). Insects' fat content vary widely from 7 to 77 g/100g of dry weight (Ramos-Elorduy et al., 1997). A lot of insect species have a very high protein content, some containing over 60 % protein of dry weight (Belluco et al., 2013). The amino acid (AA) profile of insects is usually satisfactory for fish feed. It also has good nutritional qualities regarding minerals and vitamins (Henry et al., 2015; Rumpold and Schlüter, 2013; Sánchez-Muros et al., 2014). However, most insects have low content of or do not contain the typical marine polyunsaturated fatty acids like DHA and EPA (Sánchez-Muros et al., 2014). However, there have been studies proving enriching insects with marine nutrients can be done if the insect are fed on a marine-based medium (Liland et al., 2017; Sealey et al., 2011; St-Hilaire et al., 2007). In conclusion, insects have potential to be a good source of both protein and lipids for the fish feed production of the future.

1.3.1 Black soldier fly pre-pupae

Black soldier fly (*Hermetia illucens*, BSF) is common widespread fly in the Stratiomyidae family and is found all over the western hemisphere (Diclaro and Kaufman, 2009). It is common around livestock, usually around decaying organic matter such as food- or animal waste. The BSF larvae have a great ability to utilize organic waste from fruit, meat and vegetables (Čičková et al., 2015). Depending on the feeding medium, it can contain high concentrations of both lipids (>30% of dry weight) and protein (40 % of dry weight) (Diener et al., 2009). Adult female BSF lay between 320-620 eggs with egg eclosion occurring after approximately 4 days (Tomberlin et al., 2002). The larvae can reach 27 mm in length and 6 mm in width. It goes through six instars, which takes approximately 14 days before reaching the pre-pupal stage (Tomberlin et al., 2002). The life cycle of BSF is described in figure 1.1.

The interest for using BSF pre-pupae in fish feed has increased because of its potential as a high-quality protein source (Kroeckel et al., 2012). The nutritional composition of the BSF pre-pupae can partly be affected by the composition of the medium the larvae feeds on. Trials with brown algae (*Ascophyllum nodosum*) as feeding medium for the larvae has shown the

possibility to add valuable nutrients, such as EPA, vitamin E and iodine, to the insects by varying the feeding media (Liland et al., 2017). (St-Hilaire et al., 2007) also discovered that feeding the larvae on fish offal significantly increased the amount of marine PUFA in the insects. This ability makes the larvae a candidate for tailoring nutrient profiles for specific feed purposes.

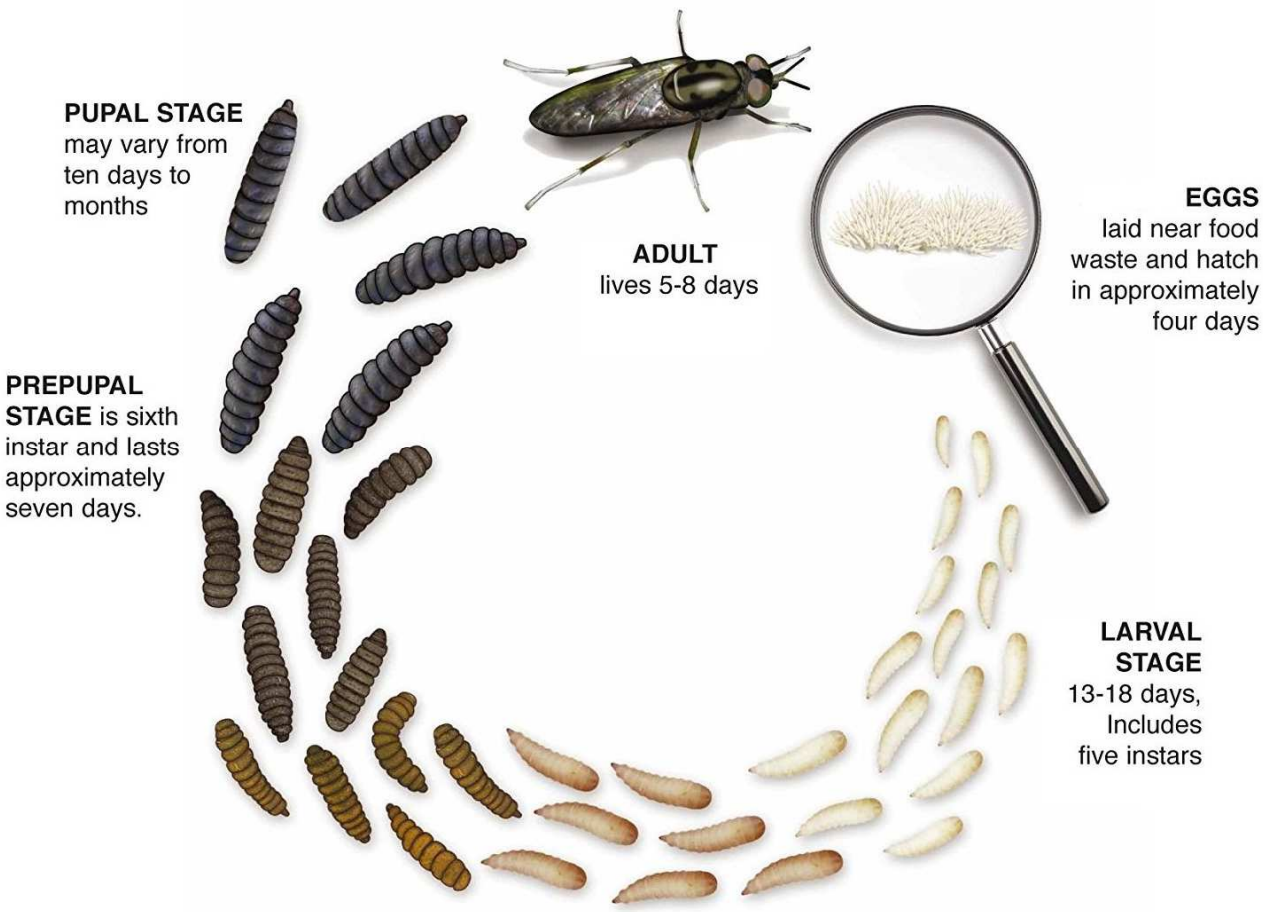

Figure 1.1: Life cycle of the BSF (Source: kopernik.info)

1.4 Liver, lipids and digestion

1.4.1 Liver

The liver is an organ located anterior in the abdominal cavity and has a variety of biochemical functions related to amino acid-, carbohydrate- and fatty acid metabolism (Kryvi and Poppe, 2016). β-oxidation, triacylglycerol- (TAG), lipoprotein-, cholesterol- and bile synthesis makes it an important organ for the digestion process of lipids (Kjaer et al., 2008; Kortner et al., 2014; Moya-Falcon et al., 2006; Stubhaug et al., 2007; Stubhaug et al., 2005). Storage of vitamins, fat and glycogen also happens in the liver, but vary a lot from specie to specie. The liver of Atlantic salmon contains very little fat compared to for example Atlantic cod (*Gadus morhua),* in which the liver is a vital fat storing organ. The organ also plays a vital role in detoxification of environmental toxins (Kryvi and Poppe, 2016). Imbalances in the metabolic processing of fatty acids (FA) can lead to accumulation of lipids in the hepatocytes and changes in the secretion of cholesterol- and TAG in VLDL (Lie et al., 1993; Vegusdal et al., 2005). Metabolic imbalances in the liver can therefore potentially affect the metabolism in the other parts of the fish through the circulatory system. Increased storage of fat in the liver have been reported as indicator of suboptimal levels of essential fatty acids (Owen et al., 1975). Newer studies have also drawn similar conclusions in Atlantic salmon, were low EPA and DHA levels has been associated with increased lipid accumulation in the liver (Berge et al., 2015; Ruyter et al., 2015).

1.4.2 Definition and structure of lipids

Lipids are defined as group of organic molecules, which is insolvable in water, but solvable in non-polar organic solvent (Fahy et al., 2011). Lipids play important roles as structural components in cell membranes, precursors for signal molecules, carriers for other non-polar molecules and as energy storage (Waagbø, 2001). Lipids are classified by their function and structure. *Triacylglycerols* function as energy storage and transportation. They are structured as three fatty acids attached to a glycerol molecule. *Glycerophospholipids* are two fatty acids bound to a glycerol molecule, and a phosphate group on the third carbon atom. The phosphate is then bond to a nitrogen base, amino acid or alcohol. These have a variety of purposes, like

structural functions in the cell membrane and creation of micelles during digestion. *Phosphatidyl-choline* (PC) is the most common lipid in cell membranes, followed by *Phosphatidyl-Ethanolamine* (PE), *Phosphatidyl-serine* (PS) and *Phosphatidyl-inositol* (PI). *Cholesterol* is structured as a steroid skeleton, and is the pre-state of a range of hormones, vitamin D and bile acid. Cholesterol is also found in cell membranes helping maintaining integrity, avoiding the membrane to become too fluid (Waagbø, 2001). A schematic overview over the different lipid classes is given in figure 1.2.

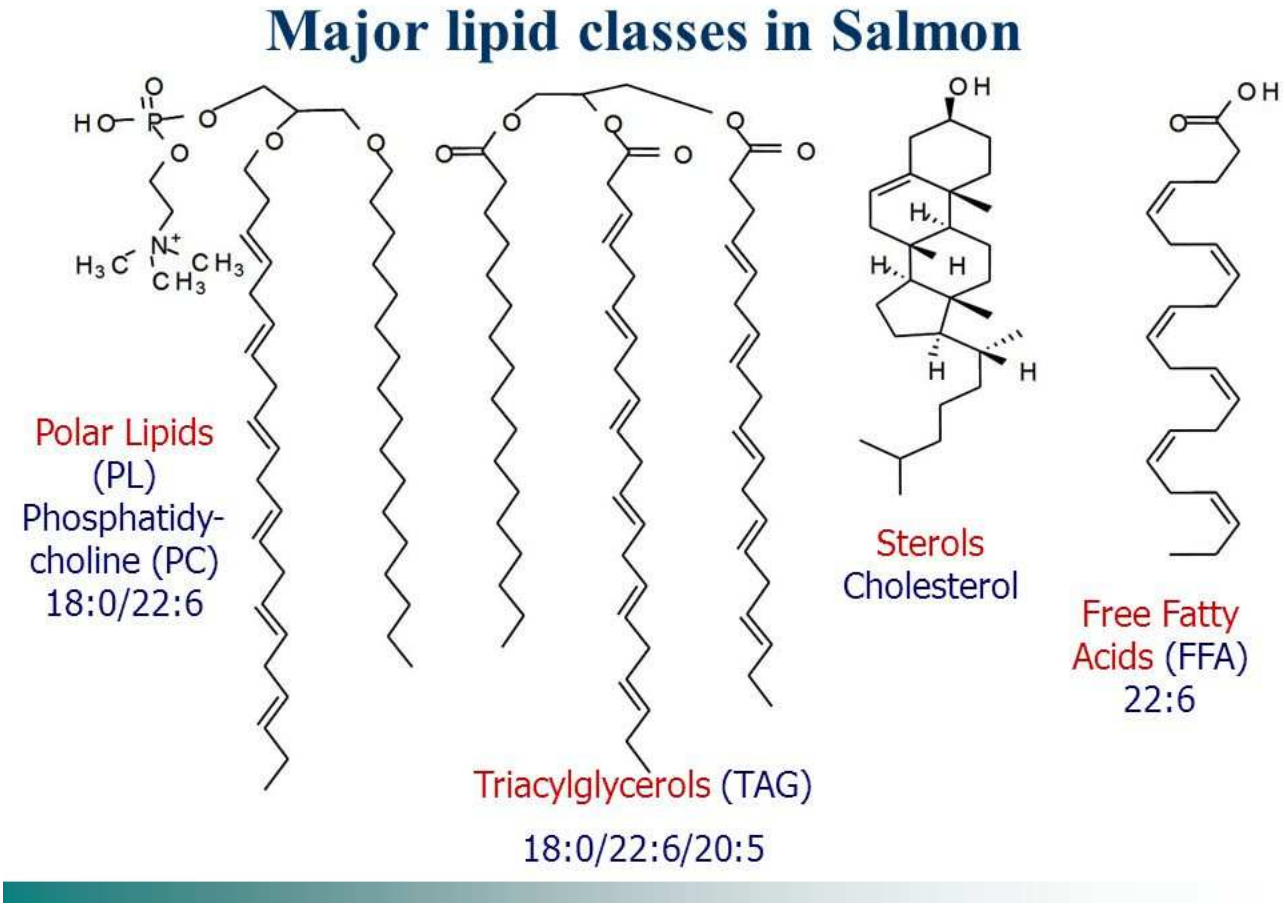

Figure 1.2: Structures of PC, TAG, Cholesterol and free fatty acids. (Miller et al., 2004)

Some of the lipid classes, like TAG and phospholipids contain FAs. FAs have the general formula of $CH_3(CH_2)_nCOOH$. It is important to differentiate between the three main types of FAs: saturated fatty acids, monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Saturated fatty acids consist solely of single bonds between the carbon atoms.

MUFA has one double bond between two carbon atoms and PUFA has two or more double bonds between carbons. Some well-known PUFAs like docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (figure 1.3), commonly known as long chained omega-3 fatty acids, play an important role in the fish metabolism and to maintain structural and functional integrity of cell membranes (Tocher, 2015). EPA is also an important precursor to a group of biological active paracrine hormones called eicosanoids (Sargent et al., 1999). Both EPA and DHA are widespread in the marine environment (Valentine and Valentine, 2004). Farmed Atlantic salmon today contains less EPA and DHA than it used to due to the use of alternatives oil sources of terrestrial origin (Sprague et al., 2016).

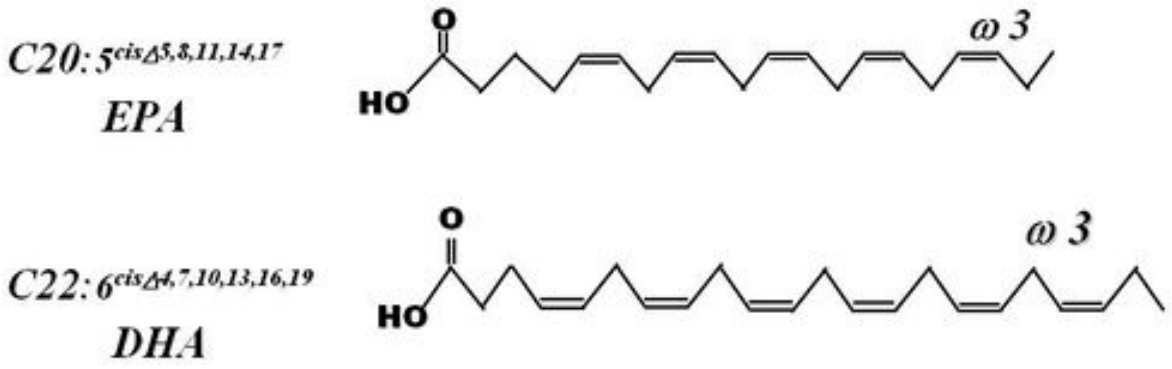

Figure 1.3: Structure of EPA and DHA.

Atlantic salmon require EPA and DHA in their feed to avoid reduced growth. In freshwater, a minimum of 1 % of the total feed should be EPA and DHA before showing reduced growth (Ruyter et al., 2000). In seawater trials, 1% EPA and DHA has seemed to be insufficient to maintain good health under difficult environmental conditions. Salmon feed 0.2 % and 1 % EPA and DHA had a much higher morality compared to fish feed on 1.7 % of these PUFA when exposed to stress (Ruyter et al., 2015). Another study with Atlantic salmon has also shown that increasing DHA between 0.1-2% of total feed, lead to an increase in survival in groups fed more than 1 % DHA of feed (Glencross et al., 2014). (Sissener et al., 2016b) has also found changes in vital organs such as brain and retina with EPA and DHA levels lower than 0.9 % of total feed.

1.4.3 Digestion of lipids

Digestion is the process that turns eaten feed into molecules that can be absorbed through the intestinal wall (figure 1.4). When feed is eaten, the pellets are exposed to the acid environment in the stomach and broken down by hydrolysis and solubilization. Fat however, is not broken down before reaching the pylorus area of the intestine. When chyme from the stomach enters the intestine, a drop in pH and the increase in fatty acids will lead to the secretion of cholecystokinin (CCK) into the blood stream. This hormone will stimulate secretion from the pancreas and cause contraction in the gallbladder. The bile acid will emulsify fatty acids, producing micelles consisting of polar lipids and bile salts on the outer layer, making the micelles water solvable. This will even the distribution of lipids in the chyme, increasing the total area exposed to the digestion enzymes. Lipase will break the ester bonds in TAGs, phospholipids, wax esters and cholesterol-esters. Next, after the breakage of the ester bonds, the micelles will consist of free fatty acids, diacylglycerols, monoacylglycerols, phospholipids, cholesterols etc. When in contact with the unstirred water layer, the micelles will dissolve due to lower pH, and the free fatty acids, monoacylglycerols, cholesterol and fatty alcohols from wax esters will travel through the intestinal wall, through both passive diffusion and active transport (Tocher, 2003; Waagbø, 2001). Most of the fatty acid diffusion happens in the front end of the midgut and around the pylorus, but some lipid uptake occurs also in the hindgut (Tocher, 2003; Waagbø, 2001).

After having passed through the intestinal wall, the lipids are transported in the blood. Due to the nonpolar nature of the molecules, the lipids are packed in a polar apolipoprotein and become lipoproteins (LP). LP are thus lipids packed in a cape of polar proteins making the particle water-soluble. The protein-coated core consists of TAGs and/or cholesterol esters. The outer layer consists of the proteins and the polar side of the phospholipids and cholesterols. Lipoproteins (LP) are categorized by density and will reduce in size with increasing density; Chylomicrons (also known as ultra low density LP), very low density LP (VLDL), intermediate density LP (IDL), low density LP (LDL), high density LP (HDL), very high density LP (VHDL). Some of the lipids are taken directly to the muscle and fatty tissues, while some are transported to the liver for a temporary storage and/or metabolism. From the liver, VLDL is secreted to the bloodstream where lipoprotein lipase will metabolize TAGs to free fatty acids

and monoacylglycerols that cells in different tissue in the body will take up. Inside the cells, if not used directly as an energy source, the monoacylglycerols and free fatty acids will be bound to TAGs, and stored as lipid droplets (Waagbø, 2001).

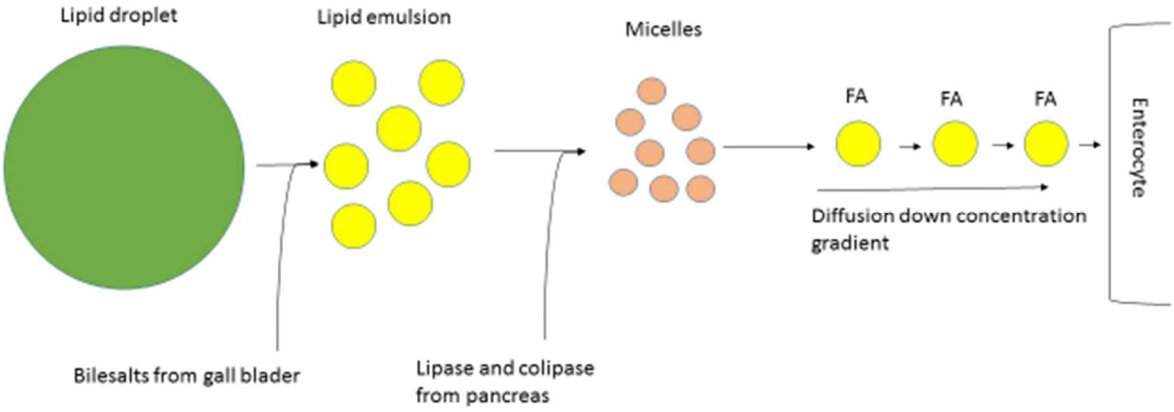

Figure 1.4: Digestion of lipids. First the lipid droplets get exposed to bile and emulsificated before enzymes and bile salts pack them in micelles. Micelles then get exposed to the unstirred water layer, dissolve and the lipids get transported through the intestinal wall. Source: Adapted from (Waagbø, 2001)

1.5 AquaFly

Aquafly is a project lead by the Institute of Marine Research, IMR funded by the Norwegian Research Council. It started in July 2014 and has an end date in December 2018. The main purpose of the project is to explore the potential of using insects as a converter of under-used marine organic material into high value nutrient sources for sustainable salmon production. Secondary, the goal is to design insect meal based diets to Atlantic salmon with correct micro- and macro nutritional values in order to maintain good fish health and welfare.

In the first part of the project, BSF larvae were grown on substrate with different percentage of marine algae in order to look at the potential of modifying the nutrient composition of the insect larvae. The ability to add marine omega-3 fatty acids to the insect was of particular interest here. Lipid and protein meal made from the marine algae-fed BSF larvae was used to formulate diets for use in feeding trials in both fresh- and salt water.

AquaFly has looked at all aspects of using insects as fish feed and the end goal is to be able to determine if using insects as a nutrient source is a viable option for future feed production. Unwanted substances, including pathogens is monitored through the chain from insect production, to the feed and finally to the fish filet, in order to make sure it is safe for humans to consume the final product. Economical, sustainability, social and ethical aspect of producing salmon on an insect-based diet is also researched in the project.

1.6 Aim of this thesis

In the last stage of AquaFly, Atlantic salmon were kept in seawater from the time they were put to sea (mean weight ~1.4 kg) to a final weight of ~4 kg. The goal of this thesis was to look at the effect of replacing 100% of the dietary fishmeal with insect meal made from black soldier fly pre-pupae fed on brown algae (*Ascophyllum nodosum)* enriched medium. IM diet (IM100) fed fish and the control fed fish were compared for hepatic lipid composition and lipid droplet accumulation in the liver. This was done with cryo-cutting liver histology colored with oil red o, and lipid class analysis using high performance thin-layer chromatography. Differences in growth, specific growth rate (SGR), mortality, condition factor (CF), hematosomatic- and viceralsomatic index (HSI and VSI) and whole-fish fatty acid composition were also examined. These results will be subject to discussion where fish health will be the main concern.

2 Materials and Methods

2.1. Experimental design

In this trial, 100 % of the fishmeal was replaced with insect meal made from BSF pre-pupae fed on brown algae enriched medium (table 2.1). This was fed to Atlantic salmon in seawater in triplicate cages. Both diets were designed so no limiting nutritional factors such as essential amino acid-, mineral- or trace element deficiency were expected to have an effect on the performance of the fish. Additional fish oil was added to the IM100 diet to balance EPA and DHA. The control diet was a commercial high-performance feed by EWOS.

Table 2.1: Diet formulations of the diets used in the trial.

%	**Control**	**IM-100%**
Fishmeal LT94	10	0
BSF meal	-	14.74
Soy protein concentrate	25	25
Corn gluten meal	7.5	7.5
Wheat gluten meal	3.35	6.88
PPC 55	8.8	2.84
Fish oil	10.18	14.76
Rapeseed oil	20.95	14.73
Binder	12.32	11.24
Additives	1.89	2.29
Sum:	**100**	**100**

2.2 Feeding trial

The salmon used in the trial were produced at Gildeskål Forskningsstasjon AS (GIFAS) in Inndyr, Nordland. At GIFAS, the fish were kept in sea cages measuring 125 m^3 (5x5x5m) holding 90 fishes per cage. All the fish shared the same background and had not been subject to previous trials. Individuals deviating >15 % from target weight, having abnormalities or showing signs of early maturation were removed from the stock in order to have a homogenous group of fish. The fish were placed randomly in the cages. Sea ice skirt was surrounding the outer perimeter of the cages. There was no use of artificial light. The feeding trial started the 7th of august 2017.

The feed was stored at the site location, indoors at ambient temperature. All feeding was done by hand. The fish were fed two times a day with a minimum of 4 hours between each feeding as long as light conditioning allowed this. A pump collected uneaten and floating feed, in order to make it possible to measure and calculate true feed-intake.

2.3 Sampling

The final sampling took place between 5th-7th of December 2017. From each cage, six fish were brought to a tub near the measuring and tissue collection site. The fish were anaesthetised with a sublethal dose of metacaine (7 g/L, MS 222; Alpharma Animal Health, Hampshire, UK) and killed by a blow to the head. The fish were killed one at the time in order to minimize the time between death and tissue sampling. Individual measurements of length and weight were done for all fish. The fish was gutted by knife and the liver was carefully removed and weighed. For histological analyses, a sample of around 0.5x0-5x1.0cm was cut from the midsection of each liver (Figure 2.1), put in a tissue processing/embedding cassette (Simport, Quebec, Canada) and put in a bottle containing 4% formaldehyde in 1xPBS. Here the samples were fixated for 24 hours before being moved to a 10% sucrose in 1xPBS solution. After 24 hours, they were moved to 20 % sucrose in 1xPBS, and after another 24 hours to 30 % sucrose in 1xPBS. All the sucrose solutions were autoclaved in advance. Tissue was cut out from the lower-mid section of the liver for lipid class analysis (Figure 2.1). These samples were put in Eppendorf tubes and flash-frozen in liquid nitrogen. The samples were later kept at -80^{o}C.

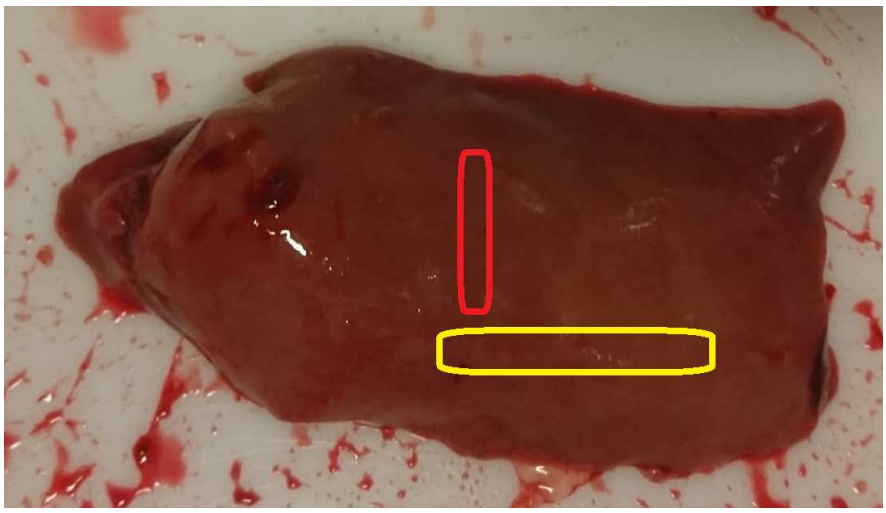

Figure 2.1: Illustration of sampling liver. Histology from area marked in red. Lipid class analysis from area marked in yellow.

2.4 Histology and lipid droplet quantification

2.4.1 Making histology slides

Following the four-step tissue fixation and sucrose infiltration described in 2.3, the fixed tissue samples were washed in PELCO® Cryo-Embedding Compound (Ted Pella, Altadena, USA) before being frozen in 22x22x 20 mm Peel-a-way embedding molds (Ted Pella, Altadena, USA) using the same medium. An ice bath made of dry ice mixed with 70 % ethanol was used for the initial freezing. The frozen blocks were then put in a freezer at -22 ^{o}C for a minimum of 24 hours.

The sections were cut with a Cryostat Leica CM19500 (Leica, Wetzlar, Germany). Prior to cutting, the machine was turned on a minimum of 3 hours in advance in order to obtain a stable temperature of -25 ^{o}C. The frozen blocks were mounted on the cutting plate using the same embedding medium the cubes were made from. After cutting the cube down to where the tissue was located, slices of the liver were made and put on a Superfrost® PLUS microscope slide (Thermo Scientific, Waltham, USA). Each sample was cut in 10 µm slices. After cutting, the slides air-dried at room temperature in a protected environment for 24 hours.

2.4.2 Coloring structures and lipid drops

Oil red O (ORO) is a fat-soluble dye used for staining lipid. As it is powdered in pure form, it has to be prepared and liquefied. This was done by first making an ORO stock stain. 2.5 g of Oil red O (Sigma 0625) (Sigma Aldrich, St. Louis, USA) was mixed with 500 ml of isopropanol. After making sure the mixture was properly homogenized, it rested for a minimum of 24 hours before becoming a finished stock stain. This stock stain was made into a working solution by mixing 3 parts of stock stain with 2 parts of distilled water (Milli-Q). After 10 minutes, the solution was filtered through a Whatman #1 filter paper (Sigma Aldrich, St. Louis, USA). After filtering, the solution was ready for use.

As ORO for most parts only stain lipids, hematoxylin solution, gill no. 1 (Sigma Aldrich, St. Louis, USA) was used in order to add color to the general structure of the liver. In addition to the dyes, a bath containing 60 % isopropanol and a bath of distilled water were set up prior to staining (figure 2.2). These were used to rinse the slides.

Figure 2.2: Histology staining in process. Left to right: ORO working solution, 60 % isopropanol, distilled water, hematoxylin.

First, the slides were put in ORO working solution (as in figure 2.2) for 15 minutes. After rinsing in isopropanol, they were dipped 5 times in hematoxylin. A final rinsing in distilled water was done before the slides were set to air-dry at room temperature before being ready to be put under a microscope.

2.4.3 Quantifying lipid

The finished slides were photographed with Visiopharm Integrator System (Version 3.6.5.0) to randomly select 20 frames covering the outlined area of interest. An Axioskop microscope (Zeiss, Oberkochen, Germany) equipped with an Olympus DP72 camera (Olympus, Tokyo, Japan) was used for the microscopy. The pictures were taken at 20x magnification.

The pictures were processed with the java-based image processing and analysis tool ImageJ (Version: 1.50i). By actively adjusting the threshold colors on a red-, green-, blue (RGB) scale, one could select a certain range of hues, which in this case were the lipid drops colored red (figure 2.3). When having the lipid properly selected, the particles were quantified using the "analyze particles"-function, giving data containing size and number of lipid droplets and % of area covered by lipids. Settings were adjusted to only count particles with a minimum of 15 pixels to avoid irrelevant/non-lipid selections.

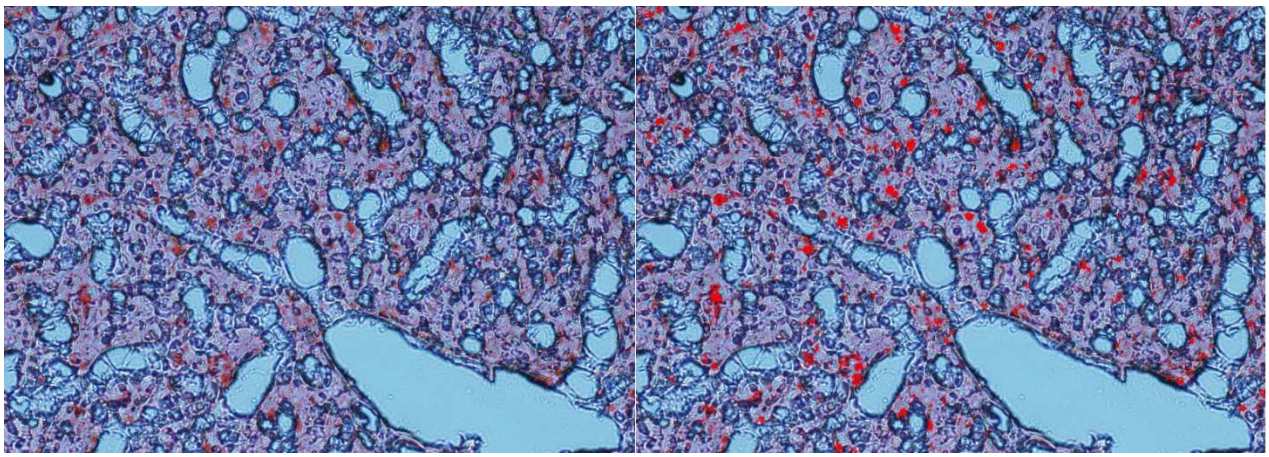

Figure 2.3: Illustration of particle selection, with lipid droplets marked red by the Image J particle counter in the picture on the right. This liver is from a fish from the IM100-diet group.

2.5 Lipid class analysis

To analyze the lipid class profile of the liver, a high-performance thin-layer chromatography system (HPTLC-system) was used. This was done by isolation of the lipids, followed by a series of steps to make a silica plate with the samples, which was developed, scanned and processed in order to get an estimated distribution and concentration of the different lipid classes.

2.5.1 Lipid extraction

From each sample, 0.2 grams of sample was weighed with 4 decimals with a XS204 scale (Mettler Toledo, Collombus, USA) before being mixed in 10 ml glass test tubes with 4 ml of chloroform/methanol (2:1) with 0.01 % BHT. Next, these tubes were mixed for 10 seconds using a whirl mixer in order to mix the samples properly with the chloroform/methanol. The samples were then put in a freezer at -20^{o}C for 24 hours.

After 24 hours, the tubes were exposed to room temperature for 10 minutes before being mixed using a whirl mixer again. This process was performed to dissolve a maximum of the lipids from the liver sample. The samples were then filtered using a RV3 vacuum pump (Edwards, Burgess Hill, UK). A Chromabond © filter, ref 730163 (Macherey-Nagel, Düren, Germany), placed in 6 mL empty reservoirs (Telos, Vernon Hill, USA) and placed on the vacuum block. Just before the samples were removed from the freezer, 20 ml tubes with caps were weighed, as these were to be put in the vacuum pump and receive the liquefied samples after the vacuuming. The samples were poured into the filtered reservoir and the tubes were washed two times using 2 ml of the chloroform/methanol solution each time to make sure nothing was left in the tubes. The reservoirs were washed with the chloroform/methanol solution along the edges using a pasteur pipette. The vacuum pressure was adjusted to make sure all the liquid went through the filter and into the 20 ml tubes. This vacuuming process was done in order to isolate the lipids in the liver from the rest of the tissue.

The next step was to remove the chloroform/methanol. This was done by placing the tubes with cork off in a RapidVap © Vacuum N_2 & N_2/48 Evaporation System model 7900003 (Labconco Corporation, Kansas City, USA). This is a system that increases temperature and decreases pressure and by doing so, makes liquids evaporate efficiently. Before turning the RapidVap on, the vacuum and the cooling system were switched on. The tubes were placed inside and the chamber sealed. Temperature was set to 40 ^{o}C, rotatory to 50 %, and vacuum at 300 mbar. When the temperature reached 40 ^{o}C, the vacuum was adjusted to 80 mbar. The RapidVap was running on these setting until all of the solvent was evaporated and the tubes were left with just lipids. At this point, the sample tubes with caps were weighed. Since the weight of the tubes and caps were measured, the mass of lipids in the samples could now be measured.

After weighing, the samples were mixed with chloroform with 0.005 % BHT to make a solution of approximately 5 mg lipids/ml. The exact volume of solvent added to each sample was noted. The samples were frozen at -20 ^{o}C while preparing the next steps of this method.

2.5.2 Elution, develop and scanning

10 ml of chloroform was transferred to a glass tank. An HPTLC-plate 20x10 cm Silica 60 (Merck, Kenilworth, USA) was prepared by checking it for damages and wiping the backside for dust. The upper, right corner of the plate was marked with a pencil. The marked side was then put upwards as the plate was put in the glass tank with the polar solution. The tank was covered and the plate was left to elute the chloroform for one hour, which is the time the plate needs to be completely eluted. The plate was then removed and put in a fume hood for 10 minutes followed by heating at 110 ^{o}C for 30 minutes. All of this was done to wash, prepare and activate the plate. The plate was then cooled at room temperature in a covered container in order to keep dust away. An automatic sample applicator, ATS4 (Camag, Muttenz, Switzerland) was used to apply samples to the plate. Samples were transferred to 2 ml glass tubes (Thermo Scientific, Waltham, USA) and placed in the ATS4 at the correct location according to settings selected. The applicator was set to make two parallels with one standard as reference for a total of 21 tracks each run (10 samples of 2 parallels and the standard, 1 µl of sample at each point). Settings were set to start at 20 mm in order to not get to close to the

edge of the plate. Each sample was smeared over 6 mm, and the distance between the tracks were set to 8 mm.

After the sample applicator was finished, the next step was to elute the plate in polar and neutral solutions in order to separate the lipids from each other. This was done in an automatic exchange chamber called AMD2 (Camag, Muttenz, Switzerland). The plate was put in the AMD2 and closed. The machine was set to go up to 48 mm with polar solution, which was a mixture of 9 ml KCL, 10 ml methanol, 25 ml chloroform, 25 ml isopropanol and 25 ml methyl acetate. This will cause the different polar lipids to go up on the plate following the polar solution (figure 2.4). Due to different chemical properties, the lipids will spread out on different locations on the plate. After the elution, the plate vacuum dried in the AMD2 for 30 minutes before being eluted up to 88 mm by a neutral solution made of 80 ml isohexane, 20 ml diethyl ether and 1.5 ml vinegar acid. This will do the same with the non-polar lipids. Both the polar and the neutral solutions was mixed in advance and attached in a bottle to the AMD2. After the neutral elution, the plate vacuum dried for 30 minutes.

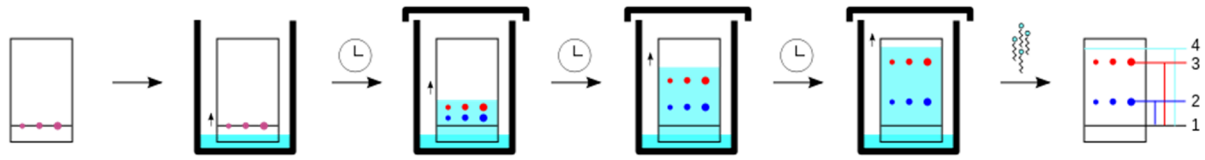

Figure 2.4: Illustration on how the lipids separate through elution in the AMD2 chamber. The red and blue dots represent lipids separating in the chamber. The numbers 1 and 4 indicates top and bottom of the eluting, 2 and 3 is the separated lipids.

After finishing in AMD2, the plate was put in a copper solution made of 3 % CuAc and 8 % H_3PO_4 in distilled water. The plate was dipped in the solution for 10 seconds before being dried with nitrogen gas. The plate was then heated at 160 ^oC for 15 minutes. After burning the development was complete and it was put in a fume hood for 10 minutes for cooling before being placed in Densitometer TLC Scanner3 (Camag, Muttenz, Switzerland) with D_2-lamp at 350 nm. The results would then appear as a graph in WinCats (version 1.3.3). This software is also were the settings were adjusted for the ATS4, AMD2 and TLC Scanner.

2.5.3 Data management and estimating results

After having the lipids graphed, some adjustment had to be made in order to make sure that the different peaks of the graph showed the correct lipid. After these adjustments, the graph was integrated in order to quantify the amount of different lipids. All the different lipids had to be within a given range in order to fit this model of estimating. Lipids with integrated values exceeding the linear range were diluted and re-done.

The raw data from Wincats was exported to Microsoft Excel (Version 2013) and sorted in order to make the numbers useable for premade models made in excel. The results are estimated using the standard curve y=ax+b axis for each lipid, the starting sampling weight, the weight of lipid after isolation, the dilution after isolation and the area of the integrated graph of each lipid. All the information were put in to a excel sheet calculating the quantity of each lipid in order to get the results in mg lipid/g sample.

2.6 Calculations and statistics

2.6.1 Formulas

Specific growth rate % = $\frac{\ln BW2 - \ln BW1}{Days\ of\ feeding} * 100$

Growth % = $\frac{BW2 - BW1}{BW1} * 100$

CF = $\frac{Total\ weight}{Length\text{^}3} * 100$

HSI = $\frac{Liver\ weight}{Total\ weight} * 100$

VSI = $\frac{Intestine\ weight}{Total\ weight} *100$

*BW2 = weight at last sampling, BW1 = weight at beginning of trial

2.6.2 Statistics

For the statistical evaluation, the free software environment R was used (R Development Core Team, 2011). The data was analyzed for homogeneity of variance using a Levene's test and for normality using a Shapiro Wilk's test, as well as being evaluated graphically by QQ-plots before utilizing parametric tests. For all results except for data from measurements of lipid droplet size, nested one-way ANOVA was used to determine statistically significant differences due to dietary treatment (random effect factor: tank) using the *nlme* package (Pinheiro et al., 2010). For data from histological assessment of area covered by lipid in liver,

also picture was added as a random factor. For significantly different data according to ANOVA, Tukey's post hoc test was performed using the *multcomp* package (Hothorn et al., 2008) to detect differences between the groups of interest. For lipid droplet size, generalized linear models (*glmer*) was used due to the gamma distribution of the data. Picture and tank were added as random factors.

All data are given as mean ± SD, unless otherwise is stated. A significance level of 5 % ($P < 0.05$) was used.

2.7 Methodical considerations

2.7.1 Experimental design and sampling

The seawater dietary trial was on an experimental scale, thus smaller compared to commercial size fish farms. Although the conditions were the same for all the fish, we do not know if poorer environmental factors such as water quality or higher infection pressure due to higher density of fish will magnify trends seen in this thesis if repeated on a commercial scale.

2.7.2 HPTLC

The principle behind the HPTLC method is based on chemical visualization of double bonds in the different lipids. When dipping the plate in CuAc (as described in 2.5.2) the double bonds get saturated and visualized after the following heating process. Due to the nature of this process, samples containing more unsaturated FAs will show higher values than samples with more saturated FAs since unsaturated FAs are the once containing double bonds. The FA composition will therefore affect the results if the saturated/unsaturated ratio varies. Quantitative determination for each lipid is based on standard equations for each lipid class, with a given range for each lipid, matched with a standard to determine the quantity of each lipid. The numerous times of weighing, dilutions and pipetting steps in this method opens for

many potential sources of error. However, this method is commonly used and accepted (Bell et al., 1993; Jordal et al., 2007; Sanden et al., 2016; Torstensen et al., 2011).

2.7.3 Lipid droplet quantification

Some of the frames selected by the VIS microscope were of such bad quality that they were not included. Examples of unusable frames were if the area of interest were small to non-exciting in the frame, big folds in the sliced tissue, leading to false positive coloring. Pictures with large discoloration (most likely caused by dust/damage) were also not used for lipid droplet estimation. This lead to some samples with less than the planned 20 frames. Lipid droplets covering less than 15 pixels were not included, as a lot of smaller non-lipid fractions within the same color threshold were in the size range of 5-14 pixels.

3 Results

3.1. Dietary composition

3.1.1 Formulation and proximate composition

Proximate analysis showed little to no differences in crude protein, lipids and carbohydrates between the diets. Slightly higher concentrations of thiobarbituric acid reacting substances (TBARS) were found in the IM-100 diet compared to the FM control diet (Table 3.1).

Table 3.1: Formulation and proximate composition of the diets.

	Ctl	**IM-100**
Proximate analysis		
Dry matter (%)	93	95
Crude Protein (%)	38	39
Crude Lipid (%)	29	29
Ash (%)	4.6	4.5
Carbohydrates (%)	11.6	11.4
Gross energy (MJ/kg DM)	24.6	25.0
TBARS (nmol g^{-1})	3.0	4.9

3.1.2 Amino acid composition

The levels of glutamic acid (GLU), proline (PRO), tyrosine (TYR) and Valine (VAL) were slightly higher in IM100 than in the control diet. Aspartic acid (ASP) and hydroxyproline (HYP) had somewhat lower levels in the insect diet than in the control. Taurine (TAU) was below the limit of quantification in IM100, whilst having 0.5 g/kg wet weight (ww) in the control diet (Table 3.2).

Table 3.2: Amino acid composition (g kg^{-1} wet weight) of the diets.

	Ctl	IM-100%
ALA	19.3	19.7
ARG	22.5	20.6
ASP	36.0	34.6
GLU	73.0	79.0
GLY	16.6	15.6
HIS	8.4	8.8
HYP	1.0	0.3
ILE	14.2	14.7
LEU	33.5	33.7
LYS	20.6	20.2
MET	10.0	10.0
PHE	20.3	20.0
PRO	23.4	27.5
SER	20.0	20.3
TAU	0.5	0.0
THR	14.4	14.3
TYR	13.7	16.2
VAL	16.5	18.0

3.1.3 Fatty acid composition

The dominating FA in both diets was oleic acid (18:1n-9), which made up 40 g /100g FA in the control diet and 30 g/100g FA in the IM100 diet. IM100 contained a slightly higher amount of total saturated FA. There were more of the medium chained (12:0, 14:0, 16:0) FAs in IM100, while the levels of 18:0 and 20:0 were identical. In total, IM100 had 4 g more saturated FA compared to the control. There were a total of 2.6 g more EPA and DHA/100 g FA in IM100, even though the total amount of PUFA was the same in the two diets (table 3.3).

Table 3.3: Fatty acid composition (g/100 g FA) in the diets.

	Ctl	**IM-100%**
12:0	n.d	2.3
14:0	2.2	3.6
16:0	8.5	9.1
16:1n-7	2.0	2.8
18:0	2.9	2.9
18:1n-9	40	30
18:1n-7	2.5	2.0
18:2n-6	14	11
20:0	0.4	0.4
18:3n-3	6.5	5.1
20:1n-9	4.5	5.9
18:4n-3	1.4	2.0
20:4n-6 ARA	0.2	0.3
22:1n-11	5.2	7.4
20:5n-3 EPA	3.0	4.4
22:5n-3 DPA	0.3	0.4
22:6n-3 DHA	2.9	4.1
Saturated FA	15	19
Sum 16:1	2.0	3.0
Sum 18:1	42	32
Sum 20:1	4.7	6.2
Sum 22:1	5.8	8.0
Sum MUFA	55	50
Sum EPA+DHA	5.9	8.5
Sum n-3	14.8	17
Sum n-6	13.8	11.6
Sum PUFA	29	29
n-3/n-6	1.1	1.4
n-6/n-3	0.9	0.7

3.1 Growth and performance

The average weights at the initial sampling were 1398±31g (n=90) for the control fish and 1409±23 g (n=90) for the IM100 fish. At the final sampling, the mean weights were 3702±165 g for the control fed fish and 3668±160 g for the IM100 fed fish (figure 3.1). This makes for a growth increase of 165±14 % for the control fed fish and 160±8 % for the IM100 fed fish. SGR in the same period were 0.86±0.04 for control and 0.85±0.02 for IM100. There were no significant differences between the dietary groups in any of the growth parameters as mentioned above. There were exactly 10 dead fish from each diet during the trial. This counts for 3.7 %.

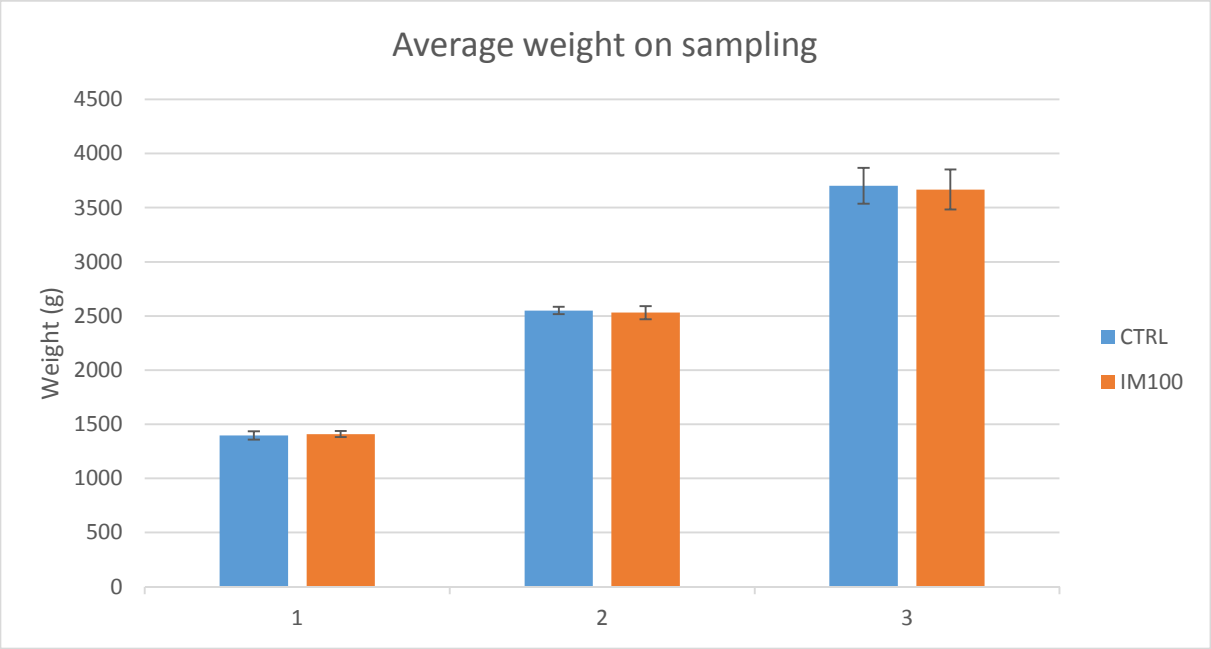

Figure 3.1: Average weight on sampling. X-axis is sample number. First sampling took place at 07. Aug. 2017, second at 3rd of October 2017 and third/final sampling at 5th December 2017. CTRL is control fed fish, IM100 is insect meal fed fish.

The average condition factor was 1.46±0.08 in the control group and 1.44±0.12 in the IM100 group (figure 3.1). There were no significant difference in the condition factor between the two diets (nested ANOVA, p = 0.51).

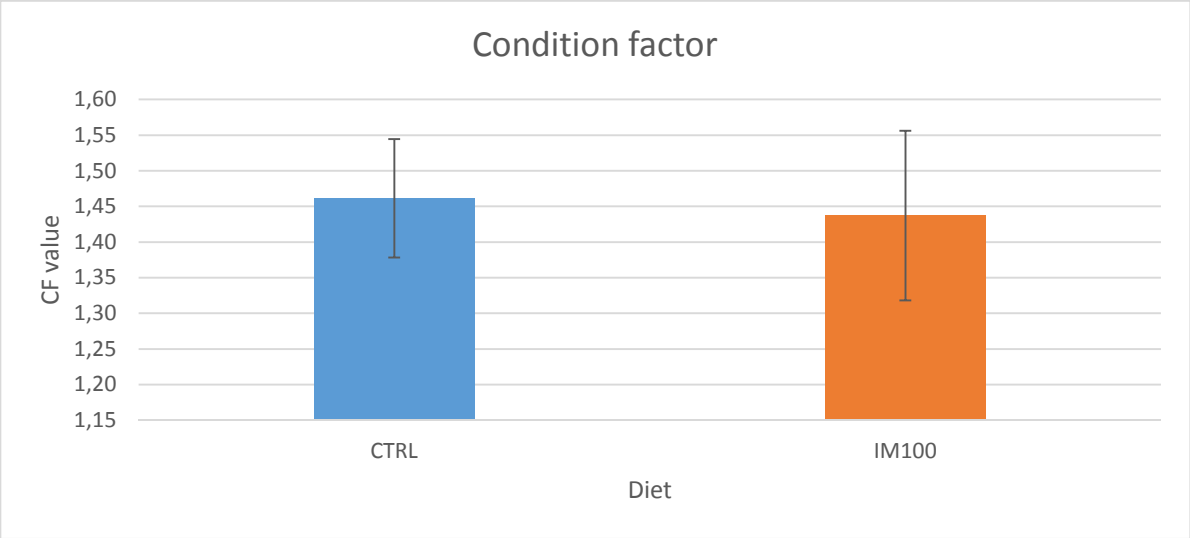

Figure 3.2: Condition factor for the two dietary groups. CTRL is control fed fish, IM100 is insect meal fed fish.

The average hepatosomatic index was 1.11±0.11 in the control group and 1.16±0.13 in the IM100 group (figure 3.2). There was no significant difference in the hepatosomatic index between the two diets (nested ANOVA, p = 0.77).

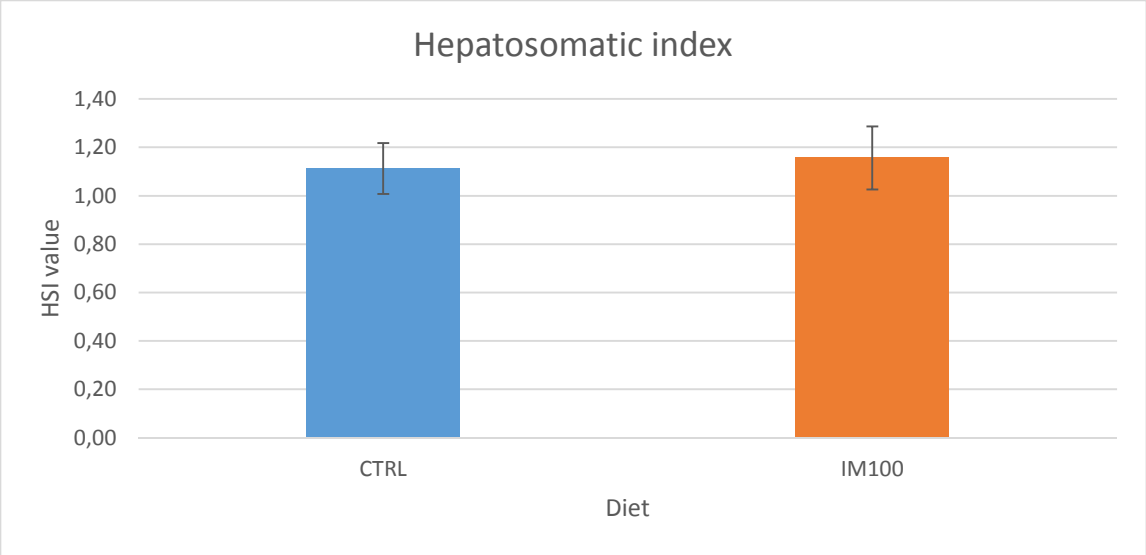

Figure 3.3: Hematosomatic index of the two dietary groups. CTRL is control fed fish, IM100 is insect meal fed fish.

The average visceral somatic index (VSI) was 11.93±1.56 for control and 11.76±1.9 for IM100 (figure 3.4). No significant differences were observed between the two dietary groups (nested ANOVA, p = 0.69).

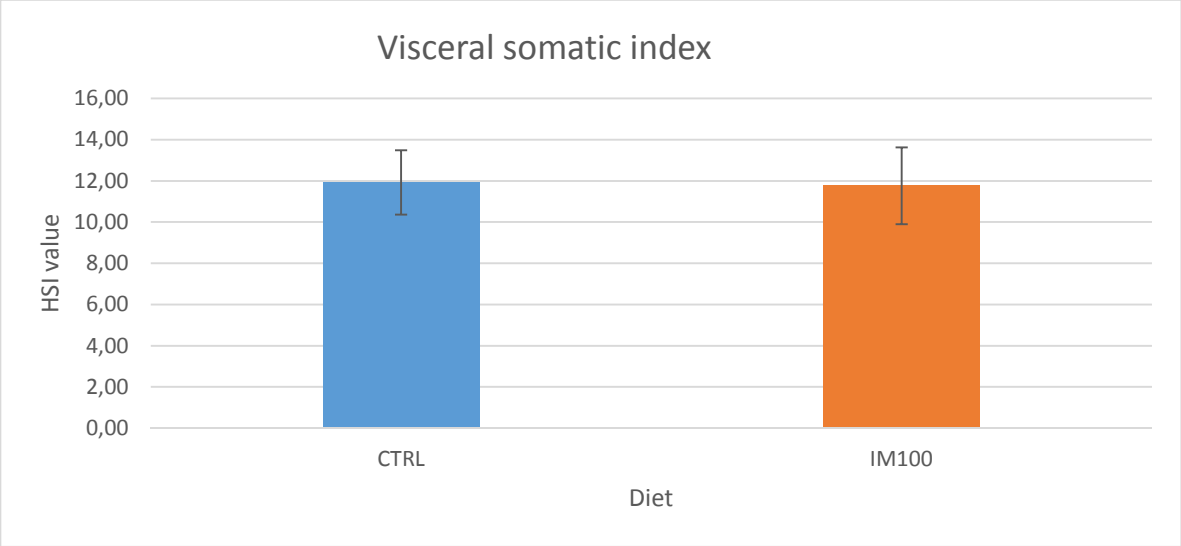

Figure 3.4: Visceral somatic index for the two dietary groups. CTRL is control fed fish, IM100 is insect meal fed fish.

3.2 Composition of whole fish

3.2.1 Proximate composition

Around 60 % of the proximate composition of the whole fish came from dry matter, making an average of ~39 g/100 g ww in both dietary groups. Total fat made for a total of ~20.5 g / 100 g ww for both groups. Protein made up approximately 17 g / 100g ww in both groups. There was approximately 1.6 g ash per 100 g ww (figure 3.5). There were no significant differences in the proximate composition between the two dietary groups.

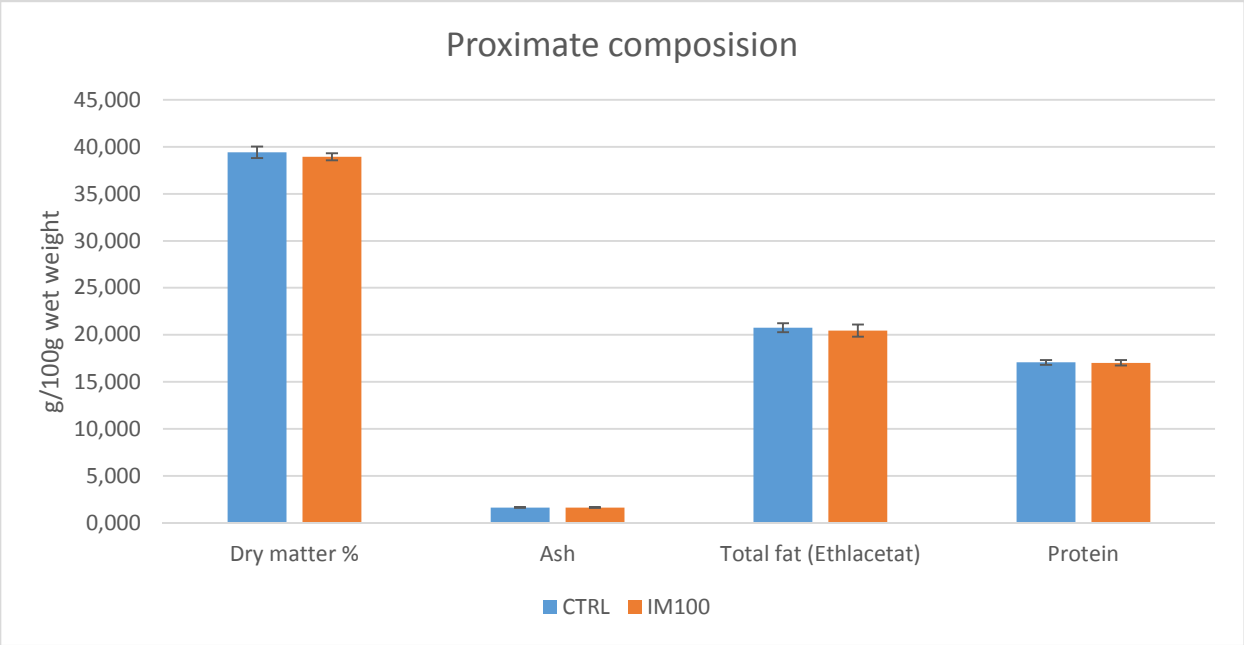

Figure 3.5: Proximate composition of whole fish. CTRL is control fed fish, IM100 is insect meal fed fish.

3.2.1 Fatty acid composition

Oleic acid, 18:1n-9, was the single biggest fatty acid, followed by linoleic acid, 18:2n-6, and palmitic acid, 16:0, in both dietary groups. In both dietary groups, there was around three times as much total MUFA and two times as much PUFA compared to saturated FAs. The control fish contained approximately 15 % more MUFA compared to the IM100 fish. Total saturated acid was 15% higher in the IM100 fed fish compared to the control fed fish. The control fish had a little more, but only borderline significantly different, PUFA compared to the IM100 fish (P = 0.051). The control group contained approximately 30 % more oleic acid compared to the fish fed the IM100 diet. The linoleic acid level was also 20 % higher in control fish. Control fish also had around 20% higher levels of 18:1n-7, 20:0 and 18:3n-3. IM100 fish had 3 mg/g sample of levels of lauric acid, 12:0, while control fed fish had <0.1 mg/g sample. IM100 fish also had 35% more 14:0, 20 % higher 16:1n-7, and 15% higher DHA levels than control fed fish. There was also 11% higher total of EPA+DHA in IM100 fish (figure 3.6).

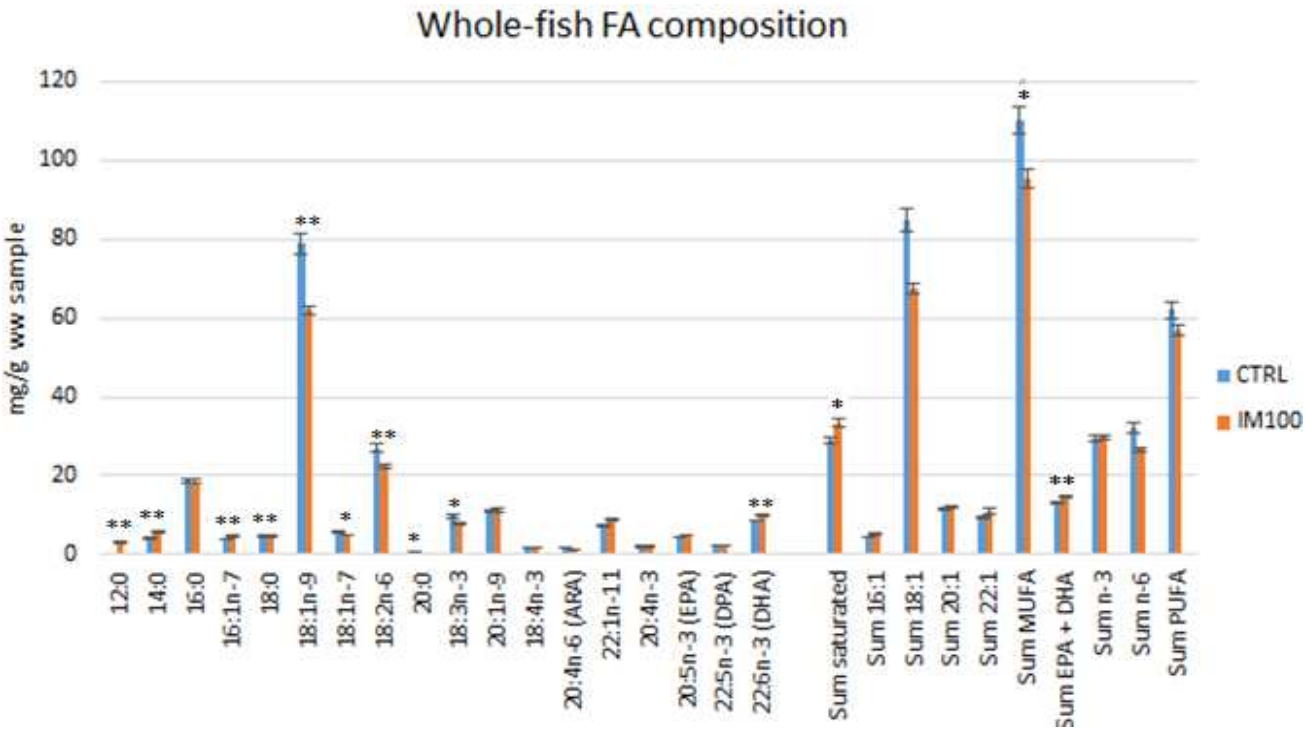

*Figure 3.6: Fatty acid composition of whole fish. ** = significant difference (p<0.01), * = significant difference (p<0.05)*

3.3 Liver lipid class distribution

TAG and PC were the two largest groups of lipid classes in the liver. The other lipids were as following (from highest to lowest concentration): PE, SM, CHOL, PI, PS, FFA, DAG and CL. The average of total polar lipids (PL) were 36.45±7.23 mg/g liver for control and 34.57±9.76 mg/g liver for IM100. For total non-polar lipids (NL), control had 36.29±19.15 and IM100 had 29.97±18.81. The total lipids in the liver was 72.7±20.8 mg/g sample for control fish and 64.5±20.2 mg/g for IM100 (figure 3.7). There were no significant differences in total amount of polar and non-polar lipids between the two groups (nested ANOVA, p (PL) = 0.81) and p (NL) = 0.50).

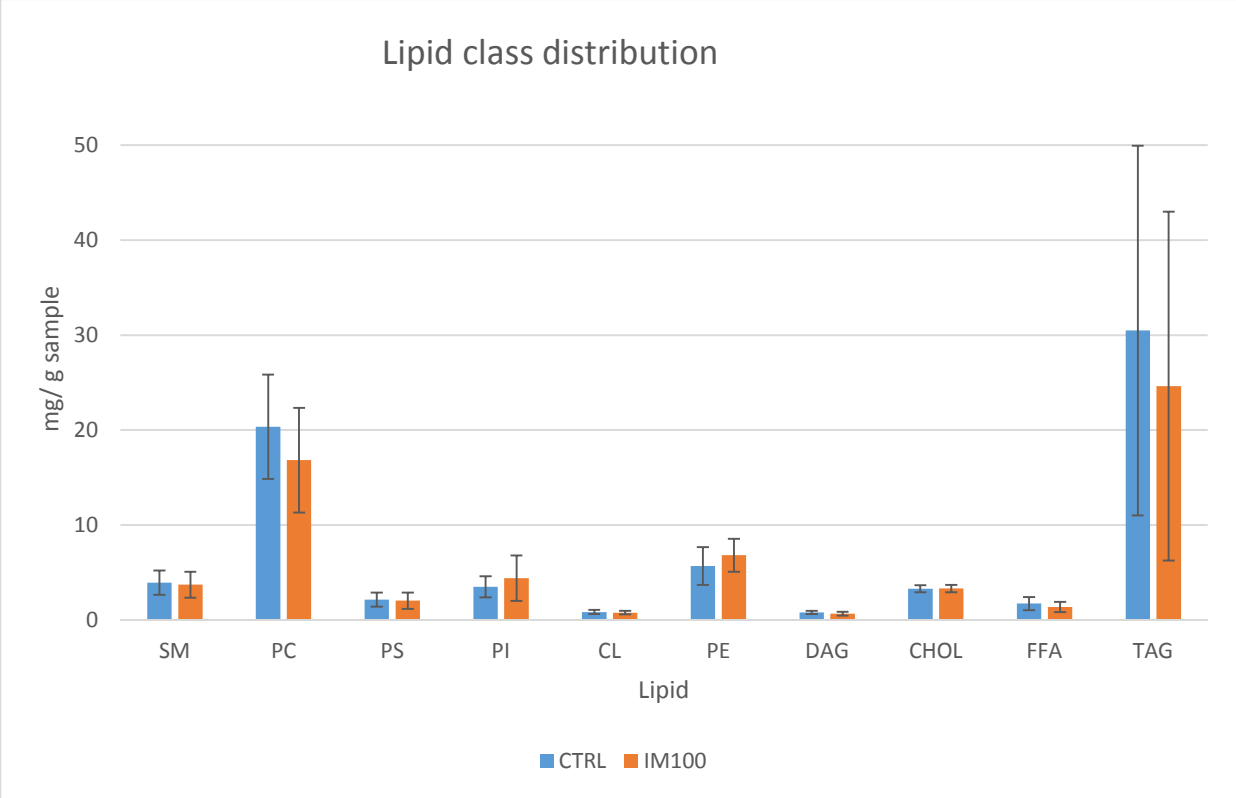

Figure 3.7: Lipid class distribution in the liver. CTRL is control fed fish and orange bar is IM100 fed fish.

3.4 Histological assessment of liver lipid droplets

In the liver of the control fed fish, 76 % of the droplets were in the range between 15-30 pixels. IM100 fed fish had 71 % of the droplets in the 15-30 pixel range (figure 3.8). The liver of the IM100 fish had 3 % more of their droplets in the 50-200 pixel range compared to control fish (figure 3.9).

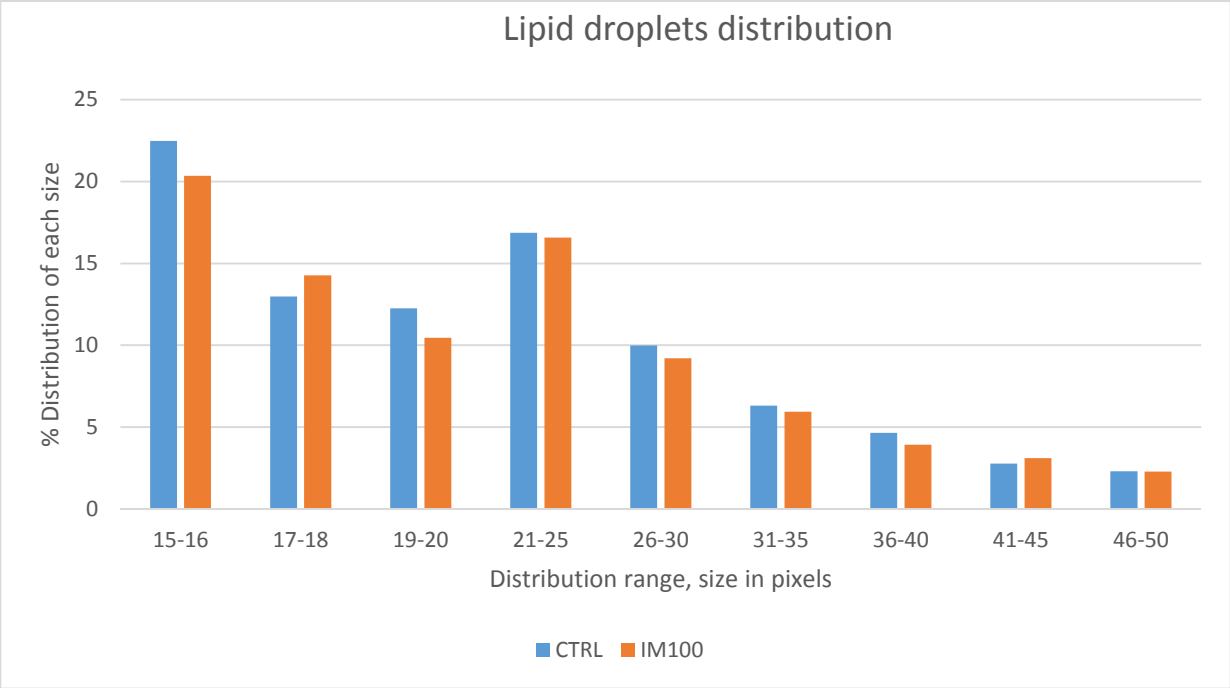

Figure 3.8: Distribution (%) of lipid droplet size in the liver. Graph shows >85 % of measured droplets. Note the increase in distribution interval from 2 pixels to 5 pixels after 19-20.

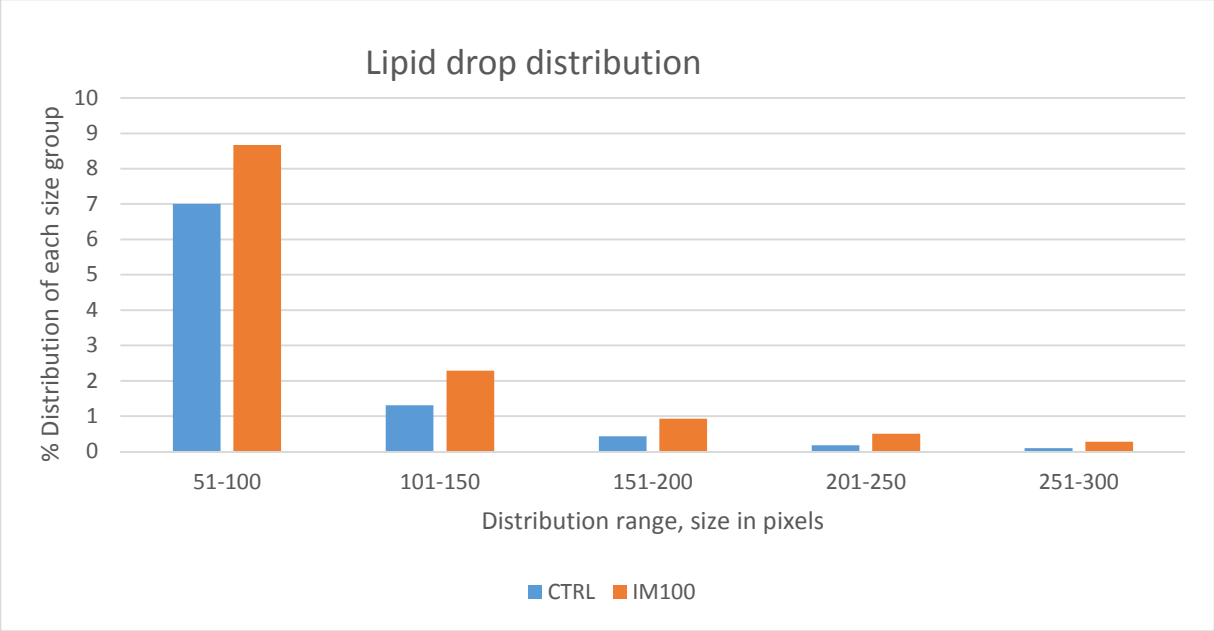

Figure 3.9: Distribution (%) of lipid droplet size in the liver. Graph shows 9 % of liver lipid droplets of the control fed fish, and 12.6% of liver lipid droplets of the IM100 fed fish.

The mean size of the liver lipid droplets was 31±0.42 (SEM, n = 29045) pixels for control and 40.9±0.49 (SEM, n = 51675) pixels for IM100 (figure 3.10). Median values were 21 for control and 22 for IM100. There was no significant difference in the average size of the droplets (nested ANOVA, p = 0.48). There were no significant difference in total lipid covering area between the two dietary group (nested ANOVA, p = 0.20).

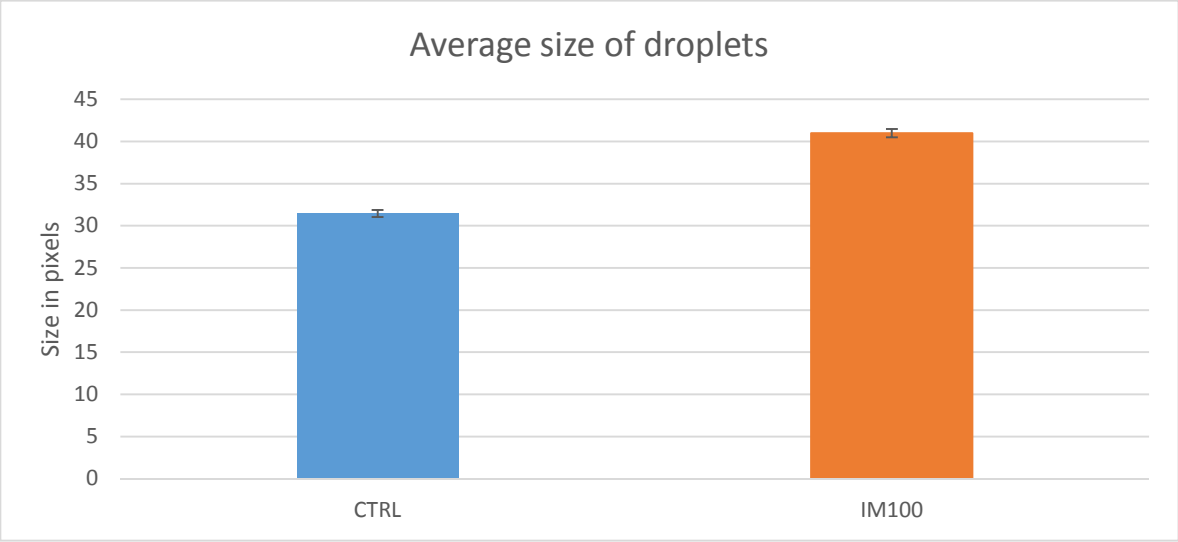

Table 3.10: Average size (pixels) of lipid droplets in the two diets. Error bars in standard error of mean (SEM).

4. Discussion

4.1 Growth and survival

Except for the obvious difference of FM and IM, there were few differences in the diet composition except that IM100 had slightly higher levels of TBARS. There was a close relation between the composition of the feed, and the composition of the whole fish. Comparing the nearly identical proximate composition in the dry matter, protein, lipid and ash in the diets, a similar pattern can be seen in the whole fish. The little to no difference between the diets is also reflected in the whole fish proximate composition. This is an indicator for similar metabolic properties for both diets and should lead to similar growth given that both diets meet the nutritional requirements for Atlantic salmon.

There was no difference in growth, condition factor, HSI or VSI between the two dietary groups. There was low mortality, good growth and no signs of poor health registered on any of the diets through the trial. This indicates that the IM100 diet was a well-balanced diet with satisfactory nutrient requirements for growth and survival of Atlantic salmon. Other studies using IM in fish feed have showed ambiguous results in regard to growth and health parameters in fish. Growth performance in juvenile turbot (*Psetta maxima)* was significantly reduced when incorporating any amount of BSF pre-pupae meal to the feed (Kroeckel et al., 2012). However, this was explained as a result of decreased diet acceptability and feed intake, which was not a problem in the current study. A study with Atlantic salmon in freshwater replaced 80 % of fish meal with BSF pre-pupae meal without finding reduced growth performances (Belghit et al., 2018). The study by Belghit et al. (2018) was part of the same project as this thesis using the same producer of BSF IM. Studies with BSF pre-pupae meal in feed for European seabass juveniles (*Dicentrarchus labrax)* has also been done, with up to 45 % FM replacement. No negative effects on growth performance were observed (Magalhães et al., 2017). Like in this study, some additives were added in order to avoid deficiencies, which could explain why Magalhães et al.'s (2017) study also didn't show reduced performance. Feeding trials with rainbow trout (*Oncorhynchus mykiss)* have been done with two different kinds of BSF meals as fish meal replacement (Sealey et al., 2011). One with BSF pre-pupae fed a vegetable-based media, and the other with BSF pre-pupae that had been grown on fish-offal. The normal BSF

meal had significantly lower growth compared to the fishmeal control, while the enriched had no significant difference compared to control. Sealey et al.'s (2011) study also showed a significantly lower HSI in BSF fed fish compared to control, but insignificant between the control fed fish and enriched BSF fed fish. Large differences in fatty acid composition was pointed out as a possible cause of the decreased HSI in the BSF fed fish. The enriched BSF meal contained significantly higher levels of EPA and DHA. The IM diet in this thesis was enriched with marine algae, with similar levels of EPA and DHA as the enriched BSF meal from Sealey et al. (2011), which could be an explanation for the similarity found in growth performances. Lock et al. (2016) also tested two different types of BSF-based diets on Atlantic salmon. The difference in the diets were caused by testing two different nutrient isolation and processing techniques. The two types did not perform equally well as only one of the diets resulted in same gross weight gain as the control. The other diet lead to considerably lower growth at all levels of replacement. The difference was explained by higher levels of TBARS in the diet for the group with reduced growth, leading to decreased palatability. The TBARS levels in the diets of the current study were much lower than any of the diets in Lock et al. (2016), and there shouldn't have been any reason to expect reduced feed intake as a result of the TBARS levels.

As there were little to no differences in the amino acid composition in the two diets in this study, any deficit problems caused by amino acids deficiency were not expected. All of the studies mentioned in the paragraph above had amino acid profiles above the known minimum requirements for the given specie. Essential amino acid deficiency should therefore not be the reason for reduced growth in any of the mentioned studies. Amino acid levels can therefore not the explanation for the successful growth in IM fed fish in this study compared to reduced growth seen in the other studies. Although the above mentioned studies were on several different species, they give pointers on what may cause reduced growth when using BSF meal in fish diets. Lower feed intake caused by reduction in palatability is frequently discussed when replacing fishmeal with alternative protein sources (Espe et al., 2007; Fournier et al., 2004; Nagel et al., 2012). The results from the current feeding trial suggest that the IM based diet used in this trial was of satisfactory quality and taste to maintain growth and survivability at the same level as commercial feed used in aquaculture in Norway today.

4.2 Composition of the fish

4.2.1 Whole fish composition

Several similarities between the fatty acid composition in the feed and the whole fish can be seen as expected according to literature (Sissener et al., 2016a). Increased levels of saturated FAs in the diet does little to none difference in the amount of saturated FAs in the fillet of the fish (Bell et al., 2002; Torstensen, 2000). The IM100 diet contained higher levels of some of the medium length saturated FAs like lauric acid (12:0), myristic acid (14:0) and palmitic acid (16:0), which added up for slightly higher levels of total saturated FAs compared to the control fed fish. This is because of the fatty composition of the BSF, which has higher levels of saturated FAs compared to FM. The IM in this study contained 17.7 % fat, so increased levels of these fatty acids were expected in the whole fish composition. As for MUFA, there were several noticeable similarities between diet and whole-fish FA composition. This has also been seen in other studies (Rosenlund et al., 2001). Control diet had around 33% more oleic acid compared to IM100 diet. The same difference is also reflected in the whole-fish FA composition. Oleic acid is common in a lot of vegetable sources, and the higher levels of vegetable oil in the control diet explains the considerably higher level of oleic acid in the control diet. Studies in other salmonids have previously seen similar correlation between dietary, and whole-fish composition of oleic acid (Skonberg et al., 1994). Other notable similarities between dietary and whole fish FAs was EPA+DHA, and the n-3/n-6 ratio, which were a little higher in both IM100 diet and whole fish compared to in the control diet and in the control fed fish. The total amount of EPA and DHA was slightly in the IM100 diet, which can be explained by the higher level of fish oil added to the IM100 in order to compensate for the low levels of these fatty acids in the IM. Although not significant, the total amount of PUFA was slightly higher in the control fish, despite having exactly the same levels in the diet. It is difficult to determine the cause of this, and Lock et al. (2016) had opposite results, with somewhat higher PUFA in IM fish compared to control, despite have equal amounts of PUFA in the diets. Overall, there is strong similarity between diet and whole fish FA composition, confirming there is a relationship between consumed FA and whole fish FA composition.

4.2.2 Liver lipid class composition

The lipid class composition was not significantly affected by inclusion of dietary IM. Although not significant, TAG trended towards being a little higher in control fed fish, having a mean value 24% higher than the IM100 fed fish. *In vitro* experiments with rats have shown increased liver TAG synthesis with increased oleic acid levels (Berge et al., 1999; Nossen et al., 1986; Ranheim et al., 1994). Studies in Atlantic salmon have also shown increased total hepatic lipid storage with increasing levels of oleic acid in the diet (Bell et al., 2001; Torstensen, 2000). A positive correlation has also been found between oleic acid levels in diet and liver TAG (Ruyter et al., 2006). This was, however, only at lower water temperatures (5^{o}C) and not at higher temperatures (12^{o}C). In this trial, the fish was sampled during the winter in Northern Norway when the water temperature was low (6^{o}C). This, in addition to the fact that the control diet contained more oleic acid than IM100, suggest that there could be ground to connect the higher dietary oleic acid to the trend of higher liver TAG levels in the control fish. The total lipids in the liver was 72.7±20.8 mg/g sample for control fish and 64.5±20.2 mg/g for IM100, but without being significantly different. More than 90 % of the difference in hepatic lipid between the groups was caused by higher TAG levels in the control group, and not by differences in any of the other lipid classes. An increase in total liver fatty acid where TAG has stood for most of the increase has been reported in other studies as well (Liland et al., 2015; Sissener et al., 2017). In Sissener et al. (2017)'s study, higher levels of oleic acid in the feed correlated with higher total liver lipids, where TAG stood for a big majority of the increased total lipid. Further analysis in that study showed an increase in oleic acid in the liver lipids. This increase in liver TAG, only happened in cold water (6^{o} C) and not in warmer (12^{o} C), similar to the findings in (Ruyter et al., 2006). There were other factors in Sisseners et. al (2017) which could lead to increased TAG levels in the liver, such as a negative correlation between TAG levels in the liver and dietary EPA+DHA as well as varying n-3/n-6 ratios in the diets. However, the fact that increased oleic acid levels in liver lipids were detected could indicate a correlation between dietary oleic acid and liver TAG levels. This could explain the trend in this study towards higher TAG levels as a result as higher dietary oleic acid levels.

4.2.3 Liver lipid droplet accumulation

There were no significant differences between the liver lipid droplets accumulation in neither the size distribution of lipid droplets nor the area of liver covered with lipid droplets. Increased lipid droplet accumulation in the liver is often associated with nutrient imbalance in the diet and studies have found that fish feed diets with <1 % of EPA and DHA of total feed composition had higher occurrences of hepatic lipid accumulation (Berge et al., 2015; Ruyter et al., 2016). Other studies have linked high intake of linoleic acid (Alvheim et al., 2013), increased use of vegetable sources (Jordal et al., 2007; Torstensen et al., 2011), lack of methionine (Espe et al., 2010) and low water temperature (Ruyter et al., 2006; Sissener et al., 2017) to increased liver TAG storage. In a report from (Ruyter et al., 2016) , fish breeding towards increased EPA and DHA self-production (high desaturate-families) led to fish containing more DHA and significantly lower hepatic lipid accumulation compared to families not bred for this purpose. This points toward genetic background as another important factor in lipid accumulation in the liver of Atlantic salmon. There is little knowledge of health issues linked to fatty liver in Atlantic salmon, but the studies of Berge et al. (2015) and Ruyter et al. (2016), where Atlantic salmon were fed suboptimal levels of EPA and DHA showed an increase in fatty liver, visceral fat and mortality (Berge et al., 2015; Ruyter et al., 2016; Sissener et al., 2016a). The mechanisms behind TAG accumulation in the liver is not clearly known in Atlantic salmon, but Gu et al. (2014) discuss that it is most probable as a result of reduced lipid export rate caused be lack of major polar LP, such as PC, cholesterol as well as reduced production of apolipoproteins. Sanden et al., (2016) also suggested that suboptimal levels of cholesterol in the diet combined with low levels of EPA and DHA could have caused the increased lipid droplet accumulation they observed in their trial. There are probably several different factors, which by them self or combined will cause increased TAG accumulation in the liver. The findings in this trial showed no indications that lipid accumulation in the liver is a problem using this insect meal.

4.3 Conclusions

There were overall no significant differences in growth, performance, liver lipid class composition or hepatic lipid droplet accumulation between the two dietary groups in this thesis. This conclude that using BSF pre-pupae grown on brown algae enriched medium as a protein source ensures good growth and hepatic health in Atlantic salmon, and has a great potential for use in fish feed production of the future.

5. Future perspectives

Based on the conclusion of this study it is clear that insect meal has a big potential for being used as protein source in future feed production. A study on a commercial scale should be done in order to determine if there are any differences in performance when exposed to environmental conditions associated with commercial scale fish farms, such as higher density of fish, reduced water quality, increased sea lice pressure and increased stress from handling. Studies on immune response should also be done in order to find if this IM replacement has an effect on disease resistance and prevention.

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