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The effects of dietary EPA and two production systems (RAS & FT) on growth and lipid class compositions of gills of Atlantic salmon (*Salmo salar*)

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Abbreviations

AGPAT	1-Acyl-Sn-Glycerol-3-Phosphate Acyltransferase
ARA	Arachidonic Acid
DHA	Docosahexaenoic Acid
EPA	Eicosapentaenoic Acid
FT	Flow Through System
GC	Gas Chromarography
GPAT	Glycerolphosphate Acyltransferase
LPAAT	Lysophosphatidic Acid Acyltransferase
LPC	Lysophosphatidylcholine
LPCAT	Lysophosphatidylcholine Acyltransferases
LPLAT	Lyso-Phospholipid Acyltransferase
MBOAT	Membrane Bound O-Acyl Transferase
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine N-Methyltransferase
PI	Phosphatidylinositol
PS	Phosphatidylserine
PUFA	Poly Unsaturated Fatty Acids
RAS	Recirculating Aquaculture Systems
SLC44A2	Choline Transporter Like Proteins 2
SM	Sphingomyelin
TLC	Thin Layer Chromatography

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Abstract

Recirculating aquaculture systems (RAS) are becoming quite common for commercial production of large post smolts, primarily because of their potential for sustainable use of water, better control over culture conditions and greater biosecurity. However, little is known about how dietary lipids affect the salmon's membrane lipids in RAS compared to fish cultivated in flow through system (FT). In the present study, we compared the growth performance, and gill lipid class composition of Atlantic salmon (*Salmo salar)* post smolt in RAS and FT systems. Fish in the two systems were held at identical temperatures (12 °C) and fed on the same two diets, either a control diet with commercial relevant level of eicosapentaenoic acid (EPA) or an EPA diet with higher level of this fatty acid. The weight of the fish at the start of the experiment in both systems was 101g and the fish was followed for 76 days to an average final weight of 403,74g in RAS and 437,47g in FT.

We found significantly higher growth rates in FT than in RAS (SGR 1,93 vs1,84). Investigation of apparent digestibility coefficient (ADC) of minerals such as phosphorous (P), calcium (Ca), magnesium (Mg), and zinc (Zn) indicated accumulation of Mg in water in the RAS units.

We also analyzed the gills of A. salmon from the 4 different experimental groups for the total fat content, total fatty acid compositions and composition of different phospholipid classes, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), Phosphatidylserine (PS), phosphatidylinositol (PI) and a combined fraction of Lysophosphatidylcholine + Sphingomyelin (LPC + SM). Further the ratio between the main PL classes was determined. Furthermore, we examined the expression of genes coding for enzymes involved in phospholipid synthesis and remodeling.

The total fatty acid compositions of the gills primarily reflected the fatty acid composition of the diets. For instance, Fish fed with EPA diet had higher levels of EPA and ARA in their gill total lipids and in all phospholipid classes except PS and PI. The production systems and dietary EPA content did not influence the total fat percentage of gill tissue of Atlantic salmon.

However, the relative composition of several fatty acids in the gills, especially polyunsaturated fatty acids (PUFA) showed significant differences between the production systems. Compared to the gills of fish in FT, fills of fish in RAS had higher arachidonic acid (ARA) (11-15%) in total fatty acid composition. While in phospholipid classes, gills of fish in RAS had lower (6-9%) EPA in PC, lower (11-13%) EPA and lower (5-13%) ARA in PE, PS of fish in RAS had lower (6-12%) DHA and PI had lower (14-29%) ARA. Moreover, a higher PC/ PE ratio was seen in gills of fish from RAS than in gills of fish in FT. The gene known to affect the incorporation of unsaturated fatty acids to the

sn-2 position during *de novo* phospholipid synthesis, 1-acyl-sn-glycerol-3-phosphate acyltransferase -4 (AGPAT4) was down regulated in RAS compared to FT.

The result demonstrated that RAS compared to FT environment used in the study led to down regulation of a gene that is important for unsaturated fatty acids incorporation during the *de novo* phospholipid synthesis, which indicate that this enzyme may be one of the factors causing the significant lower levels of the PUFAs ARA, EPA or DHA in all phospholipid classes of gills of fish in RAS. The seemingly contradiction with higher ARA in total fatty acids of fish in RAS than in FT, probably indicates that these fatty acids are found in higher levels in non-polar lipids such as free fatty acids and /or triacylglycerols.

The change in PL composition of fish in different production systems may indicate a metabolic adaptation of the gill membranes to different environmental conditions.

1. **Introduction**

Over the last 50 years, Atlantic salmon (*Salmo salar*) farming has grown into a major food producing industry in Norway, with 1.3 million tonnes worth 64 billion NOK in 2020 (Directorate of Fisheries, 2021a). This success can be attributed to a variety of factors, including favorable natural conditions, rising seafood demand, and consistent investment accompanied by technological advancement.

However, the rapid growth lasted until around 2013, after which the growth rate became stagnant (figure.1) (Statistics Norway, 2021). The poor growth rate after 2013 and later reflected a multitude of factors like biological and ecological factors, including genetic introgression of escapees on wild salmon strains and the impact of lice infestations and associated treatments (Forseth *et al*., 2017; Karlsson *et al*., 2016; Samuelsen *et al*., 2015; Vollset *et al*., 2018). This has led to the enforcement of regulations in order to minimize the negative effect on the environment, to welfare of the farmed fish, to the society, and to the commercial activities that share the same resources (The Norwegian Government, 2017, 2019).

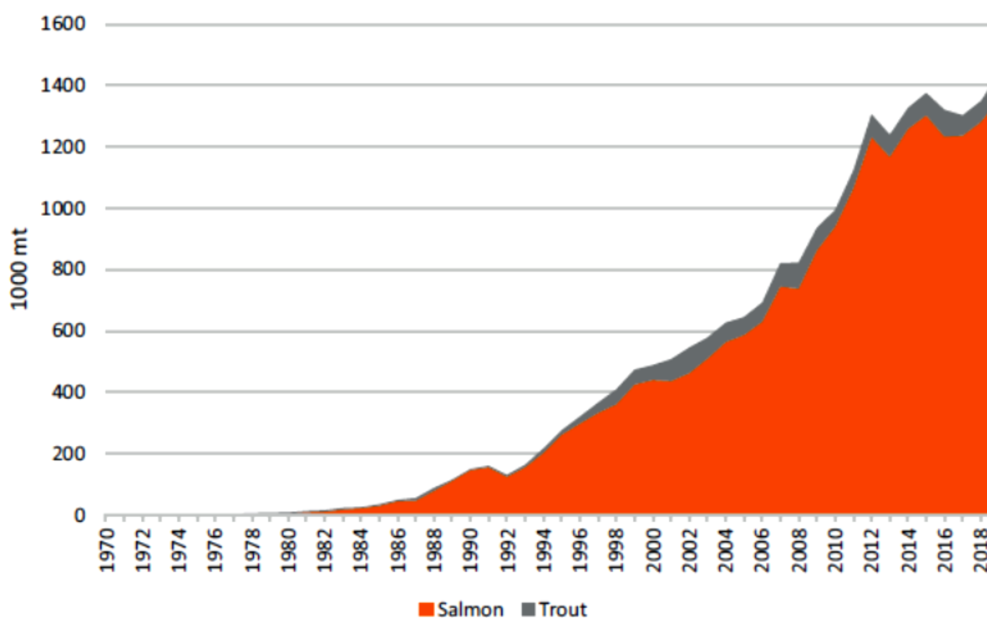


Figure.1 Norwegian production of salmon and trout 1970–2019 Source: (Hersoug, 2021)

Sea lice is one of the major concerns and the most challenging environmental issue for the Norwegian aquaculture industry. This ectoparasite infections, and delousing procedures in farmed salmon results in increased stress, stunted growth, and increased mortality (Oppedal *et al*., 2011; Øverli *et al*., 2014). Additionally, an average of 16% of Atlantic salmon smolts transferred to sea cages in Norway die before they reach harvest size. The two main causes of these deaths are poor smolt quality and associated infections (Bleie & Skrudland, 2014). It may be possible to mitigate

the issues with sea lice and diseases by reducing the amount of time spent in open sea cages by transferring parts of the production cycle into land-based aquaculture systems (RAS or FT) (Ytrestøyl *et al*., 2020). This practice is currently adopted by the industry and has resulted in an increase in average smolt size used in sea cages in recent years especially in the west coast of Norway, where the infection rates are high (Directorate of fisheries, 2021b).

Thus, there is a commercial interest in keeping Atlantic salmon longer periods of time on land in recirculating aquaculture systems (RAS), since these systems are thought to have the potential to provide strict environmental controls and higher biosecurity than flow through (FT) systems. RAS environments on the other hand are extremely complex, and vastly different from FT systems (Crouse *et al*., 2021). Recent research found that despite similar growth of Atlantic salmon smolts produced in a RAS and FT system, several physiological and molecular level differences were noticeable in the fish between these systems. For instance, external morphological welfare indicators like fin damage and operculum shortage suggested that smolts grown in RAS had poorer welfare than those grown in FT (d'Orbcastel *et al*., 2009; Kolarevic *et al*., 2014). Another study comparing RAS and FT showed that early development of skin and skeleton were hindered by RAS environment (Robinson *et al*., 2021). However, to this date no study has compared the lipid class and fatty acid profiles of barrier tissues (skin, intestine and gills) of fish kept in these two systems. It is crucial to gain a deeper understanding of how the production environment and dietary lipid composition affects the composition of membrane phospholipids and levels of PUFAs in Atlantic salmon gills to ensure the production of healthy and resilient smolt prior to their seawater transfer.

2. **Literature review**

2.1. Lipids

The name 'lipids' generally represent a wide variety of heterogenous compounds that are insoluble in aqueous solutions but soluble only in a variety of organic solvents (Gurr *et al*., 1991). Lipids are vital components of all living organisms, especially for aquatic animals. For instance, amphiphilic lipid bilayer of cells acts as a barrier between inner and outer environments which is very important in aquatic animals like fish (Deamer, 2017). Apart from this, lipids play many other vital roles which vary from concentrated energy storage to signaling molecules between cells. The deficiency of carbohydrates in the aquatic environment makes lipids and proteins primary sources of energy. Therefore, lipid nutrition in aquatic environment is very important (Hemre *et al*., 2001; Sargent *et al*., 2002).

Depending on their solubility, lipids can be broadly divided into polar lipids which are soluble in a wide range of solvents; and non-polar lipids or neutral lipids, that are soluble mainly in non-polar solvents like chloroform. Polar lipids are phospholipids, sphingolipids, glycolipids and sulpholipids. While important neutral lipids are triacylglycerols (TAG), wax esters, sterols, steryl esters and free fatty acids (Gurr *et al*., 1991; Sargent *et al*., 2002).

2.2. Fatty acids

All lipid classes except cholesterol contain fatty acids, esterified to alcohols in glycerides and to amino groups in the sphingolipids (Sargent *et al*., 2002). Based on the degree of unsaturation (number of double bond), fatty acids can be divided in to 3 groups: 1) Saturated fatty acids (SFA) without double bond with in the chain (e.g. C16:0), 2) Monosaturated fatty acids (MUFA) with one double bond with in the chain (e.g. C18:1n-9) and 3) Poly unsaturated fatty acids (PUFA) with two or more double bonds with in the chain (e.g. C20:4n-6). PUFA with 18-22 carbon are called long chain poly unsaturated fatty acid (LC-PUFA), which can be further grouped into two different series, omega-6 series (n-6) and omega-3 series (n-3), based on the position of first double bond from the methyl end group of fatty acid (Colombo *et al*., 2017). The main n-6 LC-PUFA are linoleic acid (LA, C18:2n-6) and arachidonic acid (ARA, 20:4n-6) and the main n-3 LC-PUFA are α-linolenic acid (ALA, C18:3n-3), eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3). Among the fatty acid classes, some fatty acids like C16:0 and C18:1n-9 can be synthesized *de novo* in fishes and are called non-essential fatty acids. However, fishes cannot synthesize fatty acids such as 18:2n-6 and 18:3n-3, are called essential fatty acids, which should be supplemented through the diet (Ruyter & Thomassen, 1999).

2.3. Phospholipids

Phospholipids (PL) are the major polar lipids. They are distinguished by their amphiphilic characteristics of being hydrophilic and hydrophobic in different parts of their structure. The basic backbone of PL is a phosphatidic acid (PA), which consists of an L-glycerol-3-phosphate molecule with 2 fatty acids esterified into the sn-1 and sn-2 positions. PA is a metabolically important compound which acts as a precursor of PL and a signaling molecule (Christie, 1996). Phosphatidylcholine (PC) is the most common PL and a major constituent of cellular membrane, which is composed of a PA with choline as head group. Other bases such as ethanolamine, serine and inositol can be incorporated to PA back bone and form other important PL classes such as phosphatidylethanolamine (PE), Phosphatidylserine (PS) and phosphatidylinositol (PI). Another important phosphorous containing lipid is Sphingomyelin which is a complex polar lipid, based on the long-chain amino alcohol sphingosine. All sphingolipids have a long chain, usually saturated or monounsaturated fatty acid, linked to the amino group of sphingosines (creating a ceramide) and diverse polar groups, like phosphocholine in sphingomyelin (SM), bonded to the main alcohol group (Tocher *et al.*, 2008). The presence and abundance of these PL classes are highly variable in different tissues because of their different and specific metabolic functions (Turchini *et al.*, 2010).

2.4. Biosynthesis of phospholipids

Generally, the biosynthesis pathway of PL in fishes is similar to mammals (Tocher, 1995). The enzymes involved in this pathway control the production of phospholipids PC, PE, PS, PI, and SM, and also influence the fatty acid composition in these phospholipid classes (Kennedy & Weiss, 1956). The first step of the phospholipid synthesis pathway is formation of glycerol-3-phosphate (G3P), an intermediate from the glycolysis pathway (figure.2). Firstly, the G3P is acylated to produce 1-acylglycerol-3-phosphate, otherwise known as lysophosphatidic acid (LPA). During this reaction an enzyme called glycerolphosphate acyltransferase (GPAT) transfers one fatty acyl-coenzyme A (CoA) to the *sn-1* position of (G3P). following this, LPA is further acylated by transfer of an acyl-CoA in to sn-2 position of glycerol back bone of LPA and PA is produced. This reaction is catalyzed by an enzyme from 1-acylglycerol-3-phosphate-O-acyltransferase (AGPAT) family, and also known as lysophosphatidic acid acyltransferase (LPAAT) (Kume & Shimizu, 1997). PA forms the basic structure of all membrane phospholipids. There are two main pathways for the biosynthesis of phospholipids (Lykidis, 2007). One utilizes cytidine diphosphate (CDP) activated polar head group and produce PC, PE or PS and other utilizes CDP- diacylglycerol (CDP-DAG) and produce PI, PG, or CL (Carman & Han, 2009; Kennedy & Weiss, 1956).

In order to produce PI, PG, and CL, CDP-DAG is formed by the condensation of cytidine triphosphate (CTP) with PA and this reaction is catalyzed by CDP-DAG synthase (CDS) also called phosphatidate cytidyltransferase. The CDP-DAG can then convert to PI by phosphatidylinositol synthase (PIS) (Paulus & Kennedy, 1960).

In all mammalian cells the PC and PE are synthesized via CDP-choline and CDP-ethanolamine Pathway also known as Kennedy pathway. The DAG derived from dephosphorylation of PA, catalyzed by PA phosphatase (PAP), is reacted is with CDP- choline and CDP-ethanolamine to form PC and PE (Kennedy & Weiss, 1956). PC synthesis is catalyzed by CDP- choline:1,2-DAG choline phosphotransferase (CPT), while PE synthesis is catalyzed by CDP-ethnolamine:1,2-DAG ethanolamine phosphotransferase (CEPT) (Henneberry & Mcmaster, 1999). Formation of PS in the membrane bilayer is by exchange of serine for choline or ethanolamine in PC and PE respectively. During PS synthesis, PS synthase 1 (PSS1) utilizes PC, whereas PS synthase 2 (PSS2) utilizes PE (Kuge & Nishijima, 1997). PS can be converted back to PE (Tocher *et al*., 2008). Sphingomyelin (SM) is synthesized from PC and ceramide by the enzyme SM synthase 1 (SMS1) (Vance, 2015).

PC is the most abundant phospholipid in a eukaryotic cell membrane, which constitutes 30-60% of total phospholipid mass. Any interruption to their synthesis will lead to growth arrest and apoptosis (Anthony *et al*., 1999). Hence the PC synthesis is achieved by CDP-choline pathway and by triple methylation of PE. The triple methylation is catalyzed by PE N-methyltransferase (PEMT) (Ridgway & Vance, 1987). In addition to this, PC synthesis is dependent on choline transport proteins (SLC44A) which regulate the uptake of choline into the cells and choline kinases (CHK), which phosphorylate choline to bioavailable phosphocholine (Aoyama *et al*., 2002; Traiffort *et al*., 2013)

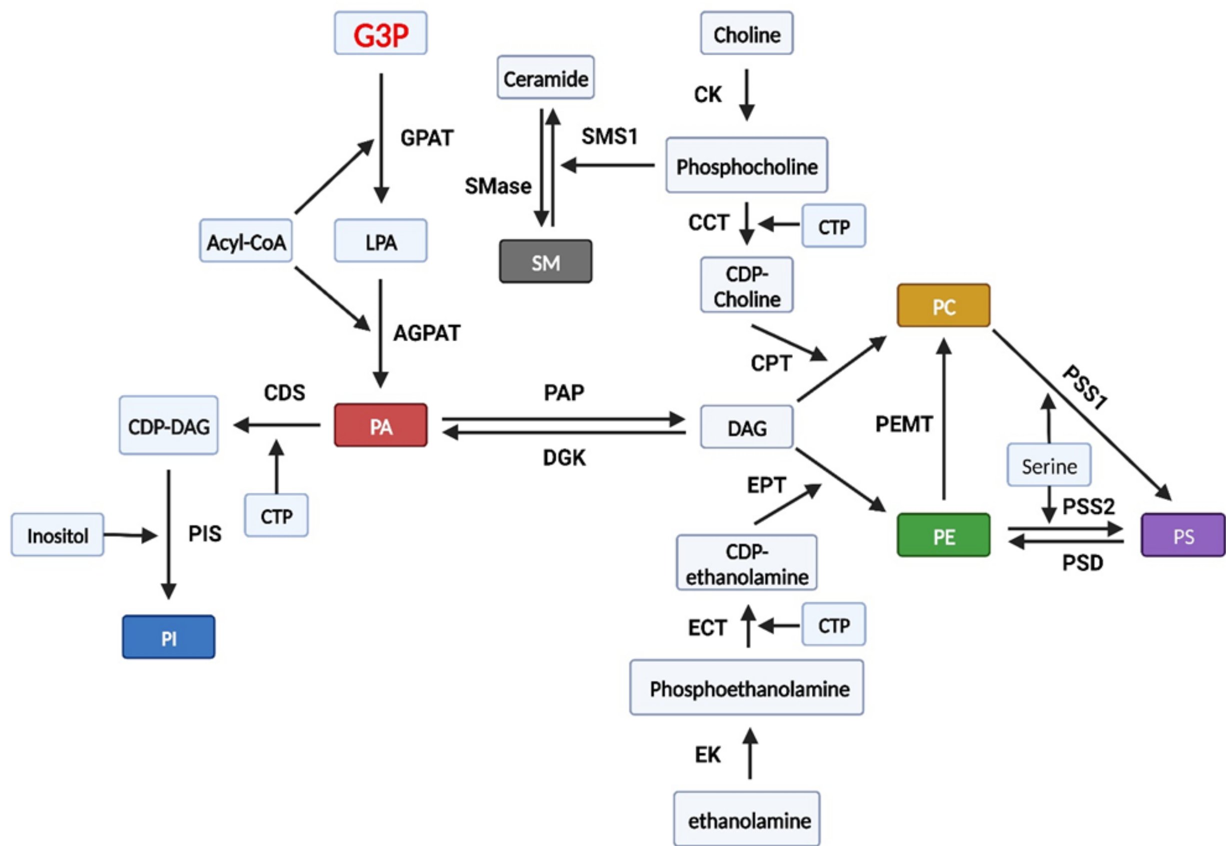


Figure.2 Pathway for biosynthesis of major phospholipid classes (modified figure from Morita & Ikeda (2022) and prepared using Biorender).**G3P**, glycerol-3-phosphate; **GPAT**, glycerophosphate acyltransferase; ; **LPA**, lysophosphatidic acid; **AGPAT**, 1-acylglycerol-3-phosphate-O-acyltransferase; **PA**, phosphatidic acid; **PAP**, phosphatidic acid phosphatase; **DAG**, diacylglycerol; **DGK,** diacylglycerol kinase; **PC,** phosphatidylcholine; **CK,** choline kinase; **CCT**, CTP:phosphocholine cytidylyltransferase; **CPT**, CDP-choline:diacylglycerol cholinephosphotransferase; **EK**, ethanolamine kinase; **ECT**, CTP:phosphoethanolamine cytidylyltransferase; **EPT,** CDPethanolamine:diacylglycerol ethanolaminephosphotransferase; **PE**, phosphatidylethanolamine; **PEMT**, phosphatidylethanolamine N-methyltransferase; **PSS**, phosphatidylserine synthase; **PS**, phosphatidylserine; **PSD**, phosphatidylserine decarboxylase; ; **SM**, sphingomyelin; **SMase**, sphingomyelinase; **SMS**, sphingomyelin synthase; **CDS**, CDP-diacylglycerol synthase; **; PI**, phosphatidylinositol; **PIS**, phosphatidylinositol synthase

2.5. Remodeling of phospholipids

Just like the interconversion of polar head group of phospholipids, the fatty acid composition of individual phospholipids can be changed or remodeled. Once formed, the phospholipids undergo extensive remodeling mainly at the sn-2 position by the combined action of phospholipase A2 (PLA2) and lyso-phospholipid acyltransferase (LPLAT), which is composed of 1-acyl-sn-glycerol-3-phosphate acyltransferase (AGPAT) and membrane bound O-acyl transferase (MBOAT) gene families (Hishikawa *et al*., 2014). This remodeling is also known as the Lands cycle, which increases the asymmetry and diversity of fatty acids in phospholipids. This remodeling mechanism is essential for maintaining the membrane and cellular function. PUFAs such as ARA, EPA or DHA,

that are involved in cell signaling and maintenance of fluidity and permeability of the membrane are introduced into the phospholipid's classes during this remodeling (MacDonald & Sprecher, 1991; Yamashita *et al*., 1997).

In mammals, the LPLAT family except AGPAT4 (only utilize lyso PA) utilizes all the major lyso phospholipids as their substrates and often shows overlapping substrate specificity. This overlapping substrate specificity is making it difficult to understand the specific functions of the individual LPLAT enzyme in *in vivo* studies. However, evidence suggests that these enzymes help to maintain the optimal composition of fatty acid in individual phospholipids for sustaining the membrane function. For instance, LPC acyltransferases 2 (LPCAT2) (AGPAT 11) and LPCAT3 (MBOAT5) utilize lyso-PC (LPC) and polyunsaturated fatty acyl-CoAs as substrates to synthesize PC with ARA and EPA in their sn-2 position (Hishikawa *et al*., 2014; Ridgway, 2021).

2.6. Role of phospholipids

The main role of phospholipids is to form the structural basis of cell membranes. They are arranged in a bilayer with their hydrophilic heads facing the aqueous environment both inside and outside of the cell, while their hydrophobic tails face each other in the interior of the bilayer. This arrangement provides a barrier that separates the interior of the cell from the external environment and helps to maintain the integrity of the cell. The outer leaflet of the cell membrane is concentrated with choline containing phospholipids such as PC and SM, while PE, PS and lesser extent PI are concentrated in the inner leaflet (Tocher *et al*., 2008).

PLs are also important in the transport of lipids and other molecules across cell membranes. Lipid rafts, which are specialized regions of the cell membrane enriched in cholesterol and sphingolipids, contain a high concentration of phospholipids and are involved in the transport of certain molecules into and out of the cell (McMullen *et al*., 2004; Tocher, 1995). They also act as precursors for the synthesis of biologically active compounds such as eicosanoids, DAG, inositol phosphates and platelet activating factors (PAFs). These compounds carry out variety of vital functions such as immune and inflammatory response, regulate the cell signaling, mediate leukocyte functions etc (Tocher *et al*., 2008).

PLs can act as an energy source through β-oxidation of acyl chains. For instance, in the eggs of Atlantic herring (*Clupea harengus*), cod (*Gadus morhua*), halibut (*Hippoglossus hippoglossus*) the main source of energy for embryogenesis and larval development is derived from PC (Tocher *et al*., 2008).

2.7. PC/PE ratio

PC and PE are the two major phospholipids that are distributed asymmetrically in the plasma membrane, where most of the PC is located on the outer leaflet, while PE is enriched in the inner leaflet. Together, PC and PE regulate the membrane integrity. Abnormally high or abnormally low PC/PE molar ratio in various tissues can affect energy metabolism and can lead to various disease conditions in mammals. For instance, inhibition of hepatic PC synthesis impairs the secretion of very low-density lipoproteins and changes the phospholipid composition of hepatic tissue and leads to fatty liver disease. In mitochondria, the altered PC/PE ratio affects energy production (van der Veen *et al.*, 2017). In Endoplasmic reticulum (ER) an abnormally high PC/PE ratio will trigger a signaling pathway called unfolded protein response (UPR). Chronic UPR activation is associated with nonalcoholic fatty liver diseases (NAFLD) in humans (Pagliassotti *et al.*, 2016). Alternatively, in fishes, the plasma membrane achieves the desired fluidity by modulating the PC/PE ratio in response to external stimuli such as temperature, salinity.etc (Hazel & Landrey, 1988).

2.8. Environmental factors and membrane phospholipids

Various environmental factors such as temperature, salinity, light and pressure have largely been seen to affect poikilothermic animals such as fish. Changes in these environmental factors affect membrane fluidity. Maintaining proper cell membrane fluidity is crucial for the health and integrity of the cell and necessary for the movement and performance of embedded proteins and lipids. In order to counter act the change in fluidity, the plasma membrane undergoes restructuring of the phospholipids both in terms of polar head groups and the fatty acyl chains (Farkas *et al.*, 2001; Sinensky, 1974). This type of structural rearrangement in phospholipids, in response to external stimuli is called ‘homeoviscous adaptation’ (Sinensky, 1974).

2.8.1. Temperature

Thermal adaptation is related to regaining the motional freedom of acyl chains of phospholipids. In fish, several methods are followed to retain the fluidity. In general case, phospholipids are remodeled to accumulate equal amount of monoenic acid in *sn-1* position and polyenic in *sn-2* position. Accumulation of conic-shaped phospholipids such as PE is observed in cold adapted fishes. Conic-shaped molecules are shown to contribute stability to the membrane during the cold. This will lead to a low PC/PE ratio (Hazel & Carpenter, 1985; Hazel & Prosser, 1974). Another strategy is to alter the cholesterol to phospholipid ratio (Wodtke, 1978).

2.8.2. Salinity

The salinity adaptation is particularly related with changing the permeability of the membrane. Previous studies in rainbow trout brush boarder membrane showed that seawater adaptation increased the content of n-3 fatty acids, especially DHA, associated with decreased proportions of saturated fatty acids in PC of salt secreting epithelia for increasing the fluidity. The desired fluidity is achieved without affecting the cholesterol content and phospholipid polar head groups (Leray *et al.*, 1984). The n-3 fatty acids such as EPA and DHA are associated with osmoregulation mechanism of marine animals (Borlongan & Benitez, 1992).

2.8.3. Pressure

Variation in hydrostatic pressure is experienced by the fishes that migrate vertically or live in deep water. The hydrostatic pressure will laterally compress the bilayer and create a disturbance in the movement of molecules through it (Cossins & Macdonald, 1989). The pressure stress is more or less similar to the temperature stress since it disturbs the motional freedom of acyl chains of phospholipids. (Cossins & Macdonald, 1986) showed that saturated/unsaturated fatty acid ratio in PC and PE decreases with increase in depth. While Lewis, (1967) showed a higher proportion of 18:1 fatty acid with depth at the expense of saturated and long chain unsaturated fatty acids. However, the role of PUFAs in maintaining structure under pressure is unclear (Cossins & Macdonald, 1989).

2.8.4. Light

Light is an important environmental factor that controls the physiological and biochemical process of the fish. The increasing light intensity from 0-550 lx, shown to have increased the levels of EPA, DPA, DHA and total PUFAs in total fatty acids with reduction in saturated fatty acids (Wang *et al.*, 2013).

3. Aim of the study

The primary goal of this study was to examine the effect of two different production systems, RAS and FT, and two dietary EPA levels on fish performance and gill lipid compositions.

Sub goals were:

1. To examine how two production systems RAS and FT and dietary EPA levels affect growth performance, condition factor and heart and liver organ indexes of A.salmon.
2. To examine the changes in the total fatty acid profile of gills between two different production systems and two different diet groups.
3. To examine the changes in different phospholipid class composition in gill tissue between two different production systems and two different diet groups.
4. To examine the changes in PC/PE ratio in gill tissue between two different production systems and two different diet groups.
5. To compare the expression of genes related to the phospholipid *de novo* synthesis and remodeling in gill tissue between two different production systems and two different diet groups.

4. Materials and methods

The experimental fish trial was carried out in Nofima research station for sustainable aquaculture Sunndalsøra, Norway. The feeding study was performed in compliance with the Norwegian and EU regulation for use of experimental animals.

4.1. Experimental design

The experiment started on 9th August 2022, using salt water adapted Atlantic salmon (*Salmo salar*) post smolts from the same cohort (ER-4-21). The experimental fish were kept in both Recirculating Aquaculture system (RAS) and traditional Flow through system (FT) until 24th and 25th of October 2022 (76 days). All experimental tanks were stocked with 50 individuals with an average body weight of 101 g at the beginning of the experiment. Fish in both RAS and FT tanks were fed either a control diet with standard concentration of EPA or an EPA diet with higher level of this fatty acid (figure.3).

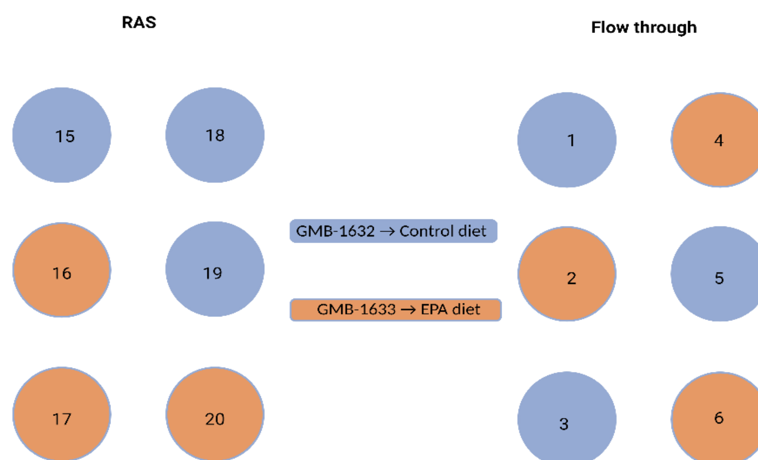


Figure.3 showing the experimental design.

4.2. Experimental systems

The salt water for both RAS and FT were sourced from 40m below the surface in the Sunndal fjord close to the research station. The water was treated by 2 step filtration process, the drum filter removed all the sold particles and a UV filter disinfected for the microbial load. The filtered water was pumped inside an overhead reservoir before supplying it into the FT or RAS tanks. The wastewater from these tanks was filtered for the solid waste before discarding.

All the water quality parameters, feeding regime, light regime physio chemical parameters were kept identical between these two systems during the experiment. The RAS had a water exchange rate of 22% per day (175ml/minute)

Table.1 *showing the water quality parameters (Average) and system specification of both RAS and FT tanks.*

Parameters	**RAS**	**FT**
Temperature	12,03°C	12,00°C
O2 concentration	86,46	87,30
Flow (L/min)	25 L/min	25 L/min
Salinity	32,90 ppm	32,90 ppm
Water level	72,81	74,00
Tank volume	Tank volume- 500l, biofilter volume- 650l	Tank volume- 500l
Photoperiod	Continuous(24hrs)	Continuous (24hrs)
TAN	0,50 ppm	
NO2-N	0,05 - 0,29 ppm	

4.2.1. Single RAS systems

Six individual identical micro-RAS units were used in this experiment (figure.4&5). The tanks were equipped with Cornell dual drain system, emergency oxygen channel with an air stone and a sensor for temperature and oxygen (Oxyguard, Farum, Denmark). The wastewater leaves the tank through the Cornell dual drain to a compact drum filter (Trome TM1, dimension 550 ×310× 410) with a filter screen with effective surface area of 0.11 m^2 and mesh size 40µm. The back washed water with solid residue will be flushed into the septic tank. At the same time, the filtered water flows into a Moving Bed Biofilm Reactor (MBBR) (V =0.2 m^2, 50% filled with bio media). The filter media (RK plast, Denmark) has a surface area of 750 m^2/m^3 with a density of 1kg/l. The high-density polyethylene (HDPE) bubble diffusers (pore size120 microns) inside the media are connected to Aquaforte AP-series air pumps (AP-45), which help to keep the media in motion for effective biofiltration. In the biofilter chamber, *sodium bi carbonate* is being added using a belt feeder to control the pH of the water. The biofilter chamber is connected to reservoir 1 (sump1) with perforated partition hence the water diffused from MBBR to reservoir 1. From reservoir 1, water pumped (pump 1- degassing pump) directly into a degassing chamber where water get stripped for CO2 and falls into reservoir 2 (sump 2).

Figure.4 *Single RAS system: NOFIMA, Sunndalsøra*

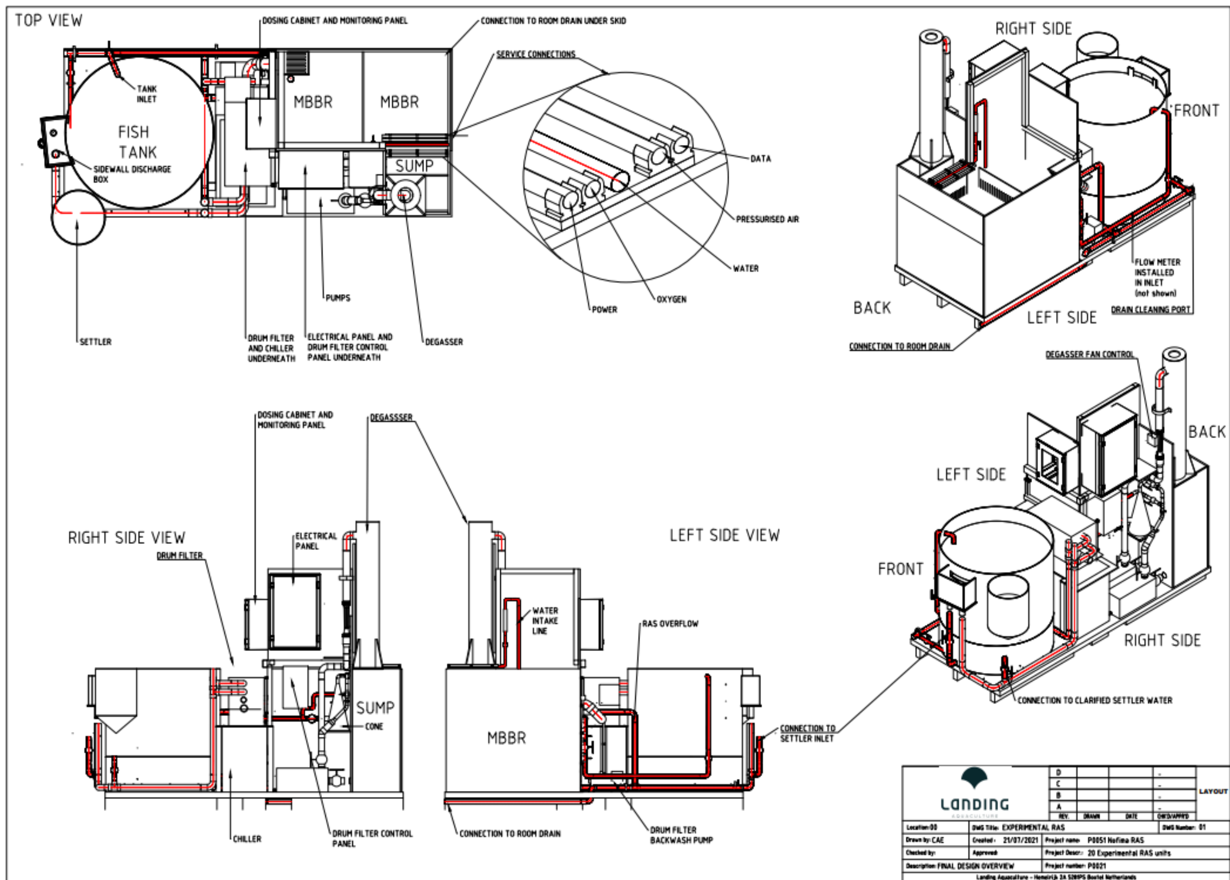


Figure.5 *Single RAS system layout (source: Landing Aquaculture operation manual)*

Reservoir 1 and reservoir 2 are connected so that the pump won't run dry. In reservoir 2 water is agitated with air in order to remove the protein fraction from the water. The protein fraction is then discarded into the septic tank. From reservoir 2 the clean water will be pumped into an oxygen cone using a main pump (pump 2- circulation pump) where the pressure has maintained under 0.3 bar inside, and water will be mixed with pure oxygen. Oxygen enriched water will flow into a chiller/ heater unit for temperature regulation and finally enter back into the fish tank. In case of any clogging inside the oxygen cone, it can be bypassed directly to the chiller/ heater unit. Two pumps (Speck Badu 42) are used to facilitate the movement of water in a single RAS unit. The makeup water (175ml/minute) will be added to the reservoir.

4.2.2. Flow through (FT) system

Flow through tanks were cylindro-conical in shape (figure.6) Water to the FT system is temperature regulated before it enters tanks. Oxygen concentration was checked once every week to ensure the levels above 80% saturation and start oxygenation if the levels below 80%. All FT tanks received water with identical water quality parameters. Similarly, all other water quality parameters such as temperature, salinity etc. in FT are monitored real time.

Figure.6 *FT system: NOFIMA, Sunndalsøra*

4.3. Feed

Two diets for the feeding experiment were produced at Nofima feed technology center, Bergen. The control diet and one test diet with increased level of EPA. The pellet size of the feed was 3,5mm which was suitable for the post smolt size ranging from 100-300g. The control feed and experimental feed had an EPA content of 9.73mg/g and 14.55 mg/g respectively. The diets were

isoenergetic and contained 46% protein, 25% lipids, 6.9% starch, 8.2% ash and 6.5% water; with an energy content of 22.1MJ/Kg. 100mg/kg of Yttrium oxide ($Y2O3$) was added to both control diet and EPA diet as a marker for digestibility estimation of minerals (table.2).

The two diets were fed to three tanks each, in both RAS and FT. All the tanks were fed by using automatic belt feeders and feeding frequency was 72 times per day (in every 15 minutes). The quantity of the feed given was according to feeding tables and expected growth rate and adjusted according to observed appetite. The feed was placed evenly on the feeding belt so that the feed would fall into the tank when the belt moves. The quantity of the feed was eventually increased from 110 gm per day in the beginning of the experiment and up to 270 in FT and 240 in RAS at the end.

Table.2 *Feed formulation*

Feed ingredients	Diet composition (%)	
	Control	**EPA**
Fish meal [1]	30	30
Soy protein concentrate [2]	17	17
Wheat gluten [3]	15	15
Wheat [4]	11,77	11,77
Fish oil [1]	7,52	
Rapeseed oil	10,48	11,74
Rapeseed oil in mixing	2,29	2,29
Anchovy oil		
EPAX6015 [5]		1,46
Choline chloride (70%) [6]	0,5	0,5
Rapeseed lecithin	0,5	0,5
Vitamin premix [7]	0,5	0,5
Monosodium phosphate (26% P)	2,5	2,5
Carophyll pink (10% Astax) [8]	0,05	0,05
Yttrium oxide	0,01	0,01
L-lysine (79%)	0,35	0,35
L-Threonine	0,2	0,2
DL- Methionine	0,04	0,04
L-Histidine	0,25	0,25
Mineral premix [9]	0,5	0,5
Water adjustment	0,54	0,54

[1] Vedde AS (Norway)
[2] Agrokorn (Germany)
[3] Tereos Syral (France)
[4] Norgesmøllene AS (Norway)
[5] Epax Norway AS (Norway)
[6] Vilomix (Norway)

[7] Normin (Norway). Added per 100gms of feed: vitamin D -300mg, vitamin E – 16mg, thiamin – 2mg, riboflavin – 3mg, pyridoxine -HCL – 3mg, vitamin C – 20mg, calcium d-pantothenate – 6mg, biotin – 0,11mg, folic acid – 1mg, niacin – 20,1mg, cobalamin – 0,005mg, vitamin K3 – 2mg.

[8] DSM (The Netherlands)

[9] Normin (Norway). Added per 100g of feed: potassium – 80mg, magnesium – 75mg, zinc – 12mg, iron – 6mg, manganese – 3mg, copper – 0,6 mg, selenium – 0,03mg.

Table.3 *Fatty acid composition of feed (**mg/g** of total fatty acids)*

Fatty acid	Control diet	EPA diet
C 14:0	8,17	5,31
C 16:0	25,36	20,84
C 18:0	5,08	4,62
C 20:0	0,87	0,97
ΣSFA[1]	**41,12**	**33,21**
C 16:1 n-7	5,73	3,58
C 18:1 n-9	81,13	97,26
C 18:1 n-7	5,92	6,04
C 20:1 n-9	11,06	9,76
C 22:1 n-11	13,88	11,42
ΣMUFA[2]	**122,35**	**132,45**
C 18:2 n-6	33,41	40,75
C 18:3 n-6	0,12	0,29
C 20:2 n-6	0,36	0,29
C 20:4 n-6	0,51	0,80
Σn-6	**34,41**	**42,13**
C 18:3 n-3	10,00	12,22
C 20:4 n-3	0,87	0,75
C 20:5 n-3	9,73	14,54
C 22:5 n-3	0,98	0,75
C 22:6 n-3	11,10	9,82
Σn-3	**32,69**	**38,08**
ΣPUFA[3]	**67,09**	**80,21**
EPA+DHA	20,83	24,36
n-3/n-6	0,95	0,90
Others[4]	7,89	6,02

[1]Σ SFA includes 15:0, 17:0, 22:0.

[2]Σ MUFA includes 14:1n-5, 15:1,17:1n-7, 22:1n-7, 22:1n-9, 24:1n-9, 24:1n-9

[3]Σ PUFA is the sum of Σ n-6 and Σ n-3.

[4]Σ Others correspond to the sum of the unidentified GC peaks in mg/g values.

4.4. Sample collection

At the end of the experiment, all the fishes were killed by an overdose* of Finquel (1000mg/g, Nederland) and length and weight were measured. 10 fish per tank were used to collect samples for different analyses. The liver and heart were dissected out to calculate hepatosomatic index (HSI) and heart index (cardiosomatic index,CSI).

Figure.7 *Gill sample*

The gill samples were collected for lipid analysis. In order to standardize the procedure, second gill arch from the left side (upside down position) of the fish were collected immediately after the fish was killed. A 2×2mm section from the gill sample (with gill arch and gill filaments) was collected and placed in a cryotube with RNA later solution for gene expression, a small piece of sample was collected and placed in formalin for histology. The rest of the tissue was placed in another cryotube and quick frozen in liquid nitrogen. The samples in RNA later were kept in refrigerator (10^{0}C) for 24hrs and later stored at-20^{0}C. The quick-frozen samples were stored at -80^{0}C for fat extraction and gene expression studies.

The faecal samples from the rest of the fishes in the trial were collected by stripping the fish as described by Austreng, E. (1978). Pooled samples from each tank were frozen and stored at -20^{0}C. the samples were freeze dried before analysis of mineral content.

Figure.8 *Fish (Atlantic Salmon) sample from our trial*

**Finquel stock solution: 20 gm /l, Anesthetic solution: 25-40ml stock solution to 10 l water*

4.5. Calculation for growth parameters

Condition factor (K- factor) = $\frac{\text{Body weight (g)}}{\text{Length (cm)}^3} \times 100$

Liver index (hepatosomatic index, HSI) = $\frac{\text{Liver weight (g)}}{\text{Round weight (g)}} \times 100$

Cardiac index (cardiosomatic index, CSI) = $\frac{\text{Heart weight (g)}}{\text{Round weight (g)}} \times 100$

Specific growth rate (SGR) = $\left[\left(\frac{W_2}{W_1}\right)^{\frac{1}{d}} - 1\right] \times 100$

Growth factor (Thermal growth coefficient, TGC) = $\left(\frac{\sqrt[3]{W_2} - \sqrt[3]{W_1}}{\text{T}\times\text{d}}\right) \times 1000$

W_2 is weight of fish at the end of the trial (g)
W_1 is weight of fish at the beginning of the trial (g)
d is total number of days
T is average temperature (^{0}C)

4.6. **Fatty acid extraction**

In this experiment, the fatty acids from the gill tissue are extracted by following 5 step procedure :1) Extraction of total fats, 2) Separation of polar and nonpolar lipids using Thin layer liquid chromatography (TLC), 3) Separation of phospholipid (PL) classes using TLC, 4) Separation of Fatty acids from PL classes (methylation), 5) Identification of fatty acids using Gas chromatography (GC).

Table.4 *List of chemicals used for extraction of fatty acids and their origin.*

Chemicals	**Origin**
Chloroform	Merck KGaA, Darmstadt, Germany
Methanol	Merck KGaA, Darmstadt, Germany
Sodium chloride	Merck KGaA, Darmstadt, Germany
Butylated hydroxytoluene (BHT)	Sigma-Aldrich chemie GmbH, Steinheim, Germany
Petroleum ether	Merck KGaA, Darmstadt, Germany
Diethyl ether	VWR international bvba, Leuven, Belgium
Acetic acid	Merck KGaA, Darmstadt, Germany
2-7-Dichlorofluorescin	Merck KGaA, Darmstadt, Germany
Ethanol	VWR international S.A.S, Fontenay-Sou-Bois, France
Benzene	VWR international S.A.S, Rosny-Sous-Bois-Cedex, France
Methanol	Merck KGaA, Darmstadt, Germany
Hydrochloric acid (HCL)	Merck KGaA, Darmstadt, Germany
2,2-Dimethoxypropane	Sigma-Aldrich chemie GmbH, Steinheim, Germany
Hexane	VWR international S.A.S, Rosny-Sous-Bois-Cedex, France
Sodium bicarbonate	Merck KGaA, Darmstadt, Germany
C23	NU-CHEK PREP, INC, Elysian, USA
GLC-85	NU-CHEK PREP, INC, Elysian, USA

4.6.1. Extraction of total fats (Folch extraction)

Gill tissue was used for analysis of fat content. The total lipid was isolated from the gill tissue by extraction method described by Folch *et al*., (1957). In this method, the lipids are separated from biological samples based on their polarity by using a mixture of chloroform and methanol, where chloroform is a non-polar solvent and methanol is a polar solvent. This fat extraction involved 2 consecutive steps: **1)** Separation of lipids from the tissue bases on their polarity by homogenizing it with 2:1 chloroform: methanol (v/v), **2)** liberating the lipids from non-lipid substance by adding at least 5-fold volume of water containing sodium salt (NaCl).

8 gill samples from each tank were pooled before fat extraction. Each pooled sample was weighed (approximately 2gm) and transferred to an Erlenmeyer flask. Each weighed sample was mixed with 12ml of 0.9% 50ml of chloroform-methanol (2:1) with an antioxidant BHT (0,7 mg/l of 2,6-Di-*tert*-butyl-*p*-cresol). This part was done under the hood because of the potential hazard from these chemicals used. The mixture was homogenized with Ultra Turrax homogenizer for 60 seconds. The homogenate was then mixed with 6ml of 0,9% NaCl and homogenize again for 5 seconds. The resulting mixture was allowed to stand in cold until it forms a complete biphasic system. The upper phase is the polar phase with chloroform: methanol: water (3:48:47) while the lower phase is non-polar phase with chloroform: methanol: water (86:14:1) and extracted fat from the tissue. This homogenate filtered through a cotton filter in order to remove all the solid debris. The filtrate was spin in centrifuge and allowed to separate completely under the hood. After two hours the upper water/methanol phase with all the remaining debris was removed by a water-vacuum pump pipette. The rest of the non-polar chloroform phase was used for the calculation of total fat percentage, analysis of total fatty acids and separation of lipid classes. For the calculation of total fat percentage, 20 ml of the chloroform phase was transferred to a pre weighed beaker. 1 ml from each sample (to save more for the lipid class analysis, otherwise 1,5ml) was transferred into separate glass tubes using a Hamilton syringe and stored at -40 °C for later analysis of total fatty acid content. The remaining chloroform extract volume was stored at -40 °C for thin layer chromatography (TLC) to separate different lipid classes.

Calculation of fat percentage: 20 ml chloroform phase was evaporated on a heating plate under the fume hood until all chloroform gets evaporated from the sample. Then incubated inside a hot air oven (105°C) for 20 min to remove all traces of water and chloroform. The weight of the container with fat was measured after the samples were cooled down. The fat content in gram per 100-gram gill tissue (percentage) was calculated by using the formula:

$$\textbf{Percentage of fat} = \frac{g\ fat \times 100}{(I \times U)/37.5}$$

Where **g fat** is the weight of evaporated sample in the beaker, **I** is the weight of gill sample in grams, **U** is the volume of chloroform extract (20ml) used for evaporation, **37.5** is the total volume of solvent and multiplication with 100 to convert the value in to percentage.

Note: In order to form the biphasic separation, the ratio between chloroform: methanol: water should be 8:4:3 (in case of 50 ml chloroform it should be 12ml NaCl). Volume of chloroform in 50ml chloroform: methanol (2:1) solvent used for extraction is 33,3 ml. However, after separation the chloroform concentration in the biphasic system is 89 parts (86 parts from chloroform phase and 3 parts from methanol/water phase) and in chloroform phase, the chloroform and the methanol make 100 parts (86:14:1 and water is removed). Hence the total volume of the solvent will be corrected from 33,3 to 37,5ml (33.3×100/89).

4.6.2. Separation of lipid classes using TLC

Thin Layer chromatography (TLC): TLC is a separation method used to separate different compounds in a mixture depending on their polarity and size. The principle of TLC is based on the differential migration of compounds on a thin layer of absorbent material (silica gel) on a glass plate. The silica gel plate is called a 'stationary phase', whereas the organic solvent use for separation is called 'mobile phase'. When the sample applied on to one end of the plate and vertically placed inside the developing chamber with solvent, the solvent travels vertically upwards along with different compounds from the sample due to capillary action. The upward movement of the sample component is highly dependent on their differential affinities for stationary and mobile phases, leading to their separation.

Preconditioning of the plates: In this experiment 3 TLC plates (Silica gel plate, 60Å (20×20 cm); Merck KGaA, Darmstadt, Germany) were used for separation of lipid classes. These plates were placed inside a chamber containing pure methanol until they became saturated. These plates were marked on the top side according to the migrating direction and were dried inside the incubator (120°C) for 20 minutes. After that, stored inside a desiccator until use.

Sample preparation: The chloroform extract from the freezer was placed inside the hood under room temperature for 10 minutes and evaporated all chloroform on block heater (60°C) under nitrogen flow (prevent oxidation). The dry samples were mixed with 100µl of chloroform + BHT and mixed well before applying on the TLC plate.

Preparation of plates: In order to pre-condition the chamber, 80ml of mobile phase: a mixture of Petroleum ether: Diethyl ether: Acetic acid (113:20:1) was poured into the chamber with one side of the filter paper touches mobile phase and waited for 20 min to condition the

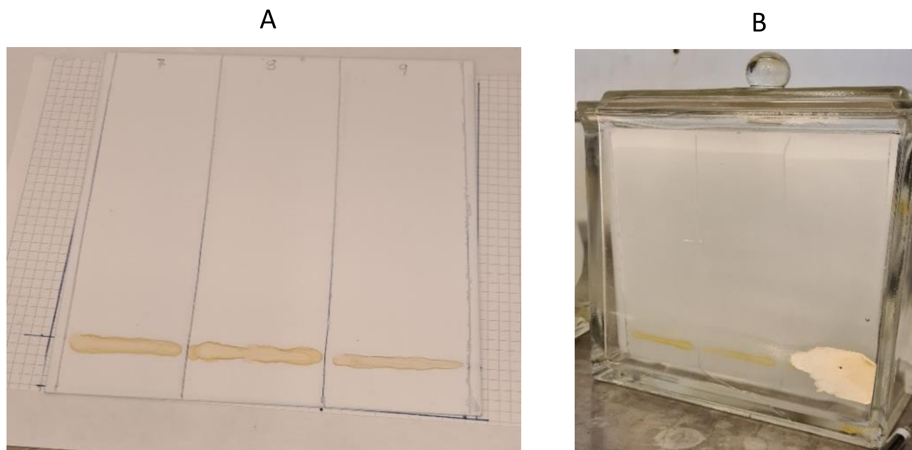


Figure.9 *A. The lipid extract from the samples (yellow) applied inside the respective columns in the TLC plate.*
B. The applicated TLC plates placed inside the closed chamber with mobile phase.

chamber. Meanwhile, the dried silica gel plates were divided into separate columns (Figure. 9A&B when applying samples shouldn't touch the line) vertically from top to bottom (along with migration direction) for separate samples. Each column was marked with respective sample number on the top.

Application of sample: The lipid extract was carefully applied horizontally on each column (ca. 2 cm from the bottom edge to avoid mixing with mobile phase) using a pasture pipette. Each sample

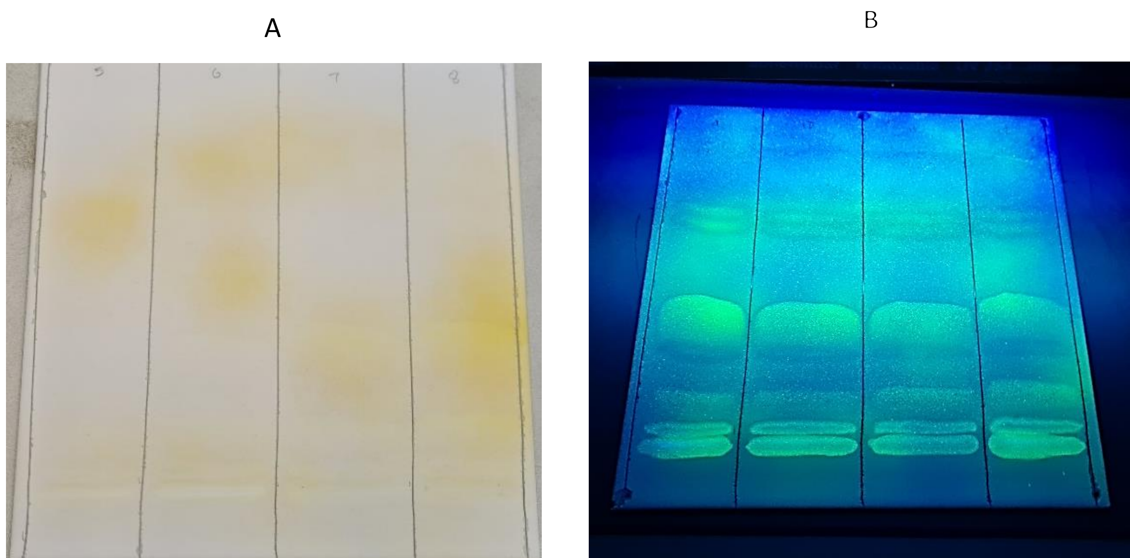


Figure.10 *A. The TLC plates sprayed with 2-7-Dichlorofluorescein dye for detection.*
B. Different lipid classes detected under UV light.

tube was washed 2 times with 5-6 drops of chloroform + BHT and applied on the respective columns as mentioned earlier. The plates were then carefully put inside the preconditioned chamber.

Detection: The plates were removed from the chamber when the mobile phase reached 1 cm below the top edge and kept under the hood until it dried. The lipid classes were then detected by spraying the plates evenly with 2% 2-7-Dichlorofluorescein in 96% ethanol. 2-7-Dichlorofluorescein is a fluorogenic dye that can be detected under UV light (366 nm) (figure.10A&B). The lipid classes will appear as yellow dots and the more double bond the lipid classes have the more yellow it appears. Each lipid class was marked with a pencil under UV light. The lipid classes detected were Phospholipids (PL), mono and di glycerides (MAG & DAG), free fatty acids (FFA), triglycerides

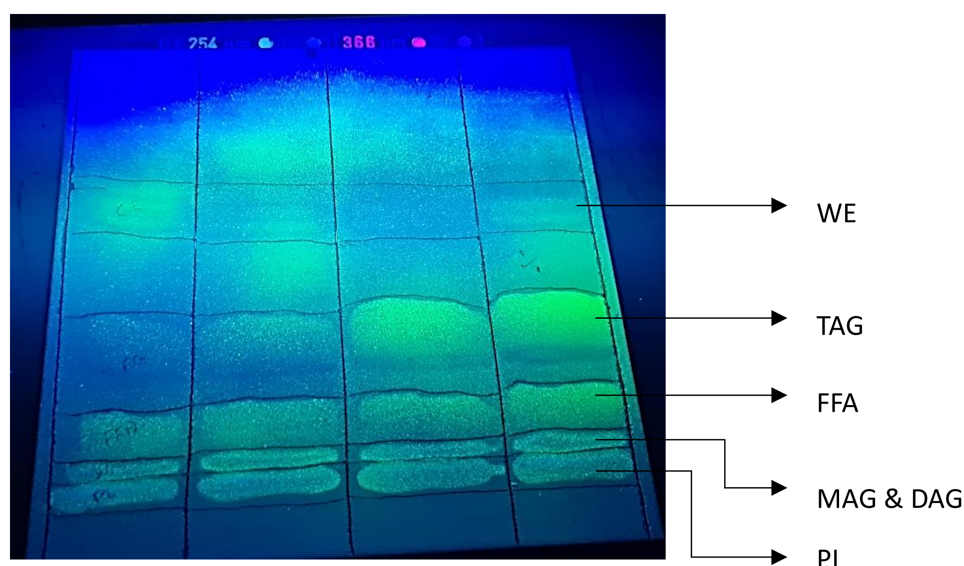


Figure.11 *The bright patches (marked) in the column represent lipid classes PL, MAG&DAG, FFA, TGA and WE for different samples.*

(TAG) and wax esters (WE) (figure.11). After detection, the area corresponding to PL classes were scrapped off from the silica plate and transferred into glass tubes for separation of different PL classes.

4.6.3. Separation of PL classes using TLC

Different phospholipid classes such as Lysophosphatidylcholine (LPC), Sphingomyelin (SM), Phosphatidylcholine (PC), Phosphatidylserine (PS), Phosphatidylinositol (PI), Phosphatidic acid (PA), and Phosphatidylethanolamine (PE) were separated using the TLC extraction principle mentioned above.

Separation of PL from silica: The PL fraction from silica was separated by using polar solvent called Arvidsons solvent (chloroform: methanol: acetic acid: water in a ratio of 50:39:1:10). 2ml of Arvidsons solvent was added to glass tube containing PL fraction, mixed well and

waited 2-3 hours for the reaction to complete. After that, 1ml of 0,9% NaCl was added into these tubes and kept inside -40°C freezer overnight. Two clear phases were formed. The upper phase was with Dichlorofluorescein, methanol, water and silica residue while the lower phase contained chloroform with PL. The lower phase was carefully transferred into a new glass tube using a pasture-

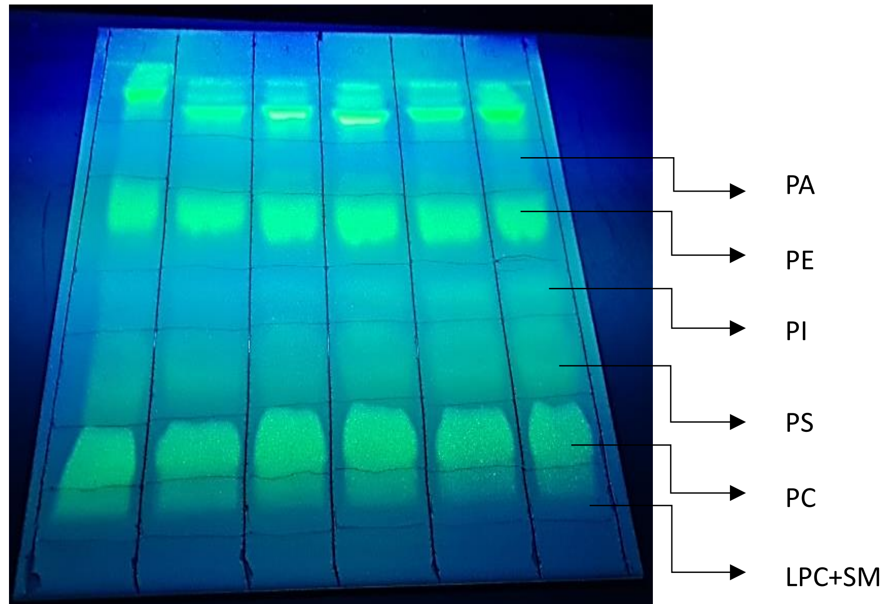


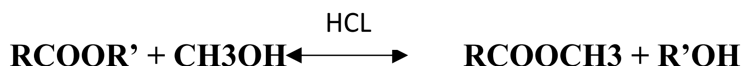
Figure.12 *Bright patches (marked) in the in the column represent PL classes LPC+SM, PC, PS, PI, PE and PA for different samples.*

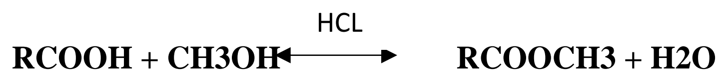
pipette and washed with 2ml of Chloroform + BHT and empty the chloroform to the corresponding glass tubes.

Separation of PL groups: The chloroform extract was dried on a block heater (60°C) under N2 flow. The dried fraction was mixed with 50µl of chloroform + BHT and followed the same TLC procedure mentioned earlier. In this separation, a more polar solvent (chloroform: methanol: acetic acid: water in the ratio 100:75:6:2) was used to separate the PL groups. Two silica gel plates were used for the second separation and each plate was divided into 6 separate columns. The PL groups detected were LPC+SM, PC, PS, PI, PE and PA (from bottom). The areas of these PL groups were marked under the UV light and scrapped off to new glass tubes (figure.12).

4.6.4. Derivatization of fatty acids from PL classes

Trans-esterification (Methylation): In this step, phospholipids are hydrolyzed to produce free fatty acids and then the free fatty acids are derivatized to methyl using an alcohol in the presence of an acid catalyst.





Where R is carbon chain of fatty acid and R' is alkyl group

Trans-esterification procedure described by Mason & Waller, (1964) was used in this experiment for preparing the methyl esters from the total lipids and different phospholipid classes.

Before adding the methylation reagents, 10 µl of Tricosanoic acid (C:23, 1.2303 gm/ml chloroform for total fatty acid and 0.2227 gm/ml chloroform for PL fatty acids) was added to all tubes as an internal standard for calculation of relative concentration of phospholipids. Methylation reagents were added in the following order: 1 ml Benzene, 1 ml 10 % Methanolic HCL and 100 µl of 2,2-dimethoxypropane (DMP) in a ratio 10:10:1. The resulting solution was stored under room temperature until next day (Benzene is an organic solvent help to dissolve the lipids from silica and act as a medium for the reaction, the Methanolic HCL release the fatty acids from the lipid classes as methyl esters and DMP is a strong water scavenger, speed up the reaction by removing excess water).

The methylated solution was mixed with 2ml of Hexane + BHT for extraction of fatty acids and 2ml of 6% Sodium bicarbonate ($NaHCO_3$) for neutralizing the acid. After 2 -3 hours two phases were formed: the upper phase with benzene, hexane +BHT and fatty acid methyl esters (FAME), and lower phase with silica, glycerol, water etc.

Sample preparation for GC: The upper phase was carefully transferred into a new glass tube using a pasture pipette. The upper phase containing FAME, Benzene and Hexane + BHT were dried on block heater under N_2 overflow. The dried samples were mixed with 100 µl of hexane + BHT and transferred into GC glass vials. The vials for total fatty acid content were mixed with 5-6 drops of hexane +BHT (higher fatty acid content), whereas vials for PL fatty acids with 2-3 drops. The vials were stored in side -40°C freezer for Gas chromatography.

4.6.5. Identification of fatty acids using Gas chromatography (GC)

Gas chromatography: GC is an analytical separation technique used to analyze volatile substances in a complex sample. The separation is accomplished with the help of two phases: stationary phase and mobile phase. The FAME were separated and analyzed using a gas chromatograph (Hewlett Packard 6890; HP, Wilmington, DE, USA) with a split injector, using an SEG BPX70 capillary column (length 60 m, internal diameter 0,25 mm, and film thickness 0,25µm; SGE Analytical Science, Milton Keynes, UK), flame ionization detector and HP Chem Station software. The carrier gas used was helium.

In the GC analysis 1µl of analyte is injected into the heated head of the column using a micro syringe. The oven temperature at the start of injection is set to 50°C for 1.2 min then race to a maximum 300°C with 3 stages: in the beginning the temperature raised to 170°C with a rate of 4°C/min, second stage to 200°C at a rate of 0,5°C/min and final stage to 300°C at a rate of 10°C /min. The heat causes pyrolysis (chemical decomposition) of the samples and sample matrix, and the gas sweeps these vapors through the column. The motion of the vaporized analytes is, however, restricted by the absorption towards column walls or the packing materials inside the column. GC modules are equipped with Flame ionization detectors (FID). The pyrolyzed samples with charge are being detected by FID and a high impedance pico-ammeter measures this current and record it on a graph. Since different molecules have different rates of progression, they reach the detector at different time points (retention time). Hence the gas chromatograph consists of peaks of variable height with current on the y-axis and time on the x-axis. With the help of HP Chem Station software, the area of the peaks is calculated by integration. This enables the quantification of each fatty acid by its corresponding retention time.

The 1st slot of GC auto-sampler was loaded with hexane and 2nd slot with a standard called GLC-85 followed by samples. GLC-85 is a standard solution containing a known quantity of different fatty acid classes with standardized retention time.

Calculations for FA content:

Total FA content (g) in the lipid extract ($TFA_{extract}$) = $\frac{W_{C23} \times (PA_{Sum} - PA_{C23})}{PA_{C23}}$

W_{C23} - Amount of C23 (internal standard) in g
PA_{sum} - Sum of peak areas of all detected FA in %
PA_{C23} – Peak area corresponding to C23 in %

Total FA(g) in the sample (TFA_{sample}) = $\frac{TFA_{extract} \times 37,5}{V_{extract} \times W_{sample}}$

37,5 – is the total volume of solvent (chloroform) used for extraction in ml.
$V_{extract}$ - Volume of chloroform extract used for GC analysis in ml.
W_{sample} – Weight of the sample used for extraction in g.

FA content in mg/g = $\frac{TFA_{sample} \times PA_{FA}}{PA_{Sum} - PA_{C23}}$

PA_{FA} – Peak area of the respective FA in %

FA content in % = $\frac{PA_{FA} \times PA_{Sum}}{PA_{sum} - PA_{C23}}$

4.7. Mineral analysis

The mineral analysis was done in order to determine the Apparent Digestibility (AD) of the minerals. The analysis was carried out by Biolab, Nofima, Bergen.

Mineral analysis: The element concentration (P, Mg, Zn, Ca) was determined using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Agilent 5110 VDV, Agilent Technologies, Mulgrave, Australia). For plasma generation, nebulization and auxiliary gas, argon (Linde Gas As, Oslo, Norway) with a purity of 99.996% was used. The ICP-OES element determination conditions were in accordance with the NS: EN 15621:2017 method adapted for OES. An external laboratory (Eu-rofins, Molde, Norway) examined the selenium content of all samples.

Calculations for Apparent Digestibility coefficient (ADC):

ADC (%) = $\left(100 \left[\frac{M_{feed} \times N_{faeces}}{M_{faeces} \times N_{feed}}\right]\right) \times 100$

M_{feed} – Concentration of Marker (Y_2O_3) in the feed in mg/kg.
M_{faeces} – Concentration of Marker (Y_2O_3) in the faeces in mg/kg.
N_{feed} – Concentration of nutrient (mineral) in the feed in mg/kg.
M_{faeces} – Concentration of nutrient (mineral) in the faeces in mg/kg.

4.8. Quantitative analysis of gene expression (qPCR)

The gill tissue was analyzed to quantify the gene expression using quantitative polymerase chain reaction (qPCR). The principle of qPCR is based on the amplification of the target sequence using a specific set of primers and a fluorescent probe, and the measurement of the amount of fluorescence emitted during the amplification process. Two samples per tank were analyzed for quantitative gene expression (total 24 samples).

Total RNA was extracted from gills with the help of Biomek 4000 Automated Workstation (Beckman Coulter, Indianapolis, IN, USA). Agencourt® RNAdvance tissue kit (Agencourt Bioscience Corporation, Beverly, MA, USA) was used for the mRNA extraction by following the manufacture instructions. The purity and concentration of RNA was evaluated by NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Seven hundred twenty-five nanograms of total RNA were reverse transcribed into cDNA in 20 µL reaction volume, using Quantitect® Reverse Transcription kit (Qiagen, Hilden, Germany) according to manufactures protocol. cDNA synthesis was carried out under 42°C for 30 minutes and terminated at 95°C for 3 minutes.

The qPCR master mix consisted of 1 µL forward primer and 1 µL reverse primer (final concentration :0,5 µM, Table.5), 4 µL of 1:10 dilution of cDNA, and 5 µL PowerUP SYBR Green Master Mix (Applied Biosystems, Foster City, California, United States). The primer specificity was confirmed by Sanger sequencing (Eurofins genomics). To assess the effectiveness of the primers, a standard curve was added for each pair of primers. All samples were analyzed in duplicates with non-template and non-enzyme controls for each gene. The qPCR reaction was carried out on a QuantStudio 5 instrument (Thermo Fisher Scientific, MA, USA), where the samples undergo initial denaturation at 95°C for 20 seconds, followed by 40 cycles of amplification at 95°C for 1 second and 60°C for 20 seconds, melting at 95 °C for 1 second and 60 °C for 20 seconds, and dissociation at 95 °C for 1 seconds. DNA-directed RNA polymerase subunit (*rpol2*), eukaryotic translation initiation factor 3 (*eif-3*) and Elongation factor 1-alpha (*ef1a*) were evaluated as reference genes using RefFinder (Xie *et al*., 2012). The calculation of relative gene expression level was done according to ΔΔCt method and efficiency correction was done by using rpol2 as reference gene (Pfaffl, 2004).

Table.5 *Overview of gene bank numbers and primer sequence. Efla, Elongation factor 1-alpha; rpol2, DNA-directed RNA polymerase subunit; eif-3, eukaryotic translation initiation factor 3; LPCAT, Lysophosphatidylcholine Acyltransferases; AGPAT, 1-Acyl-Sn-Glycerol-3-Phosphate Acyltransferase; GPAT, Glycerolphosphate Acyltransferase; chk, Choline kinase; slc44a2, Choline transporter like protein 2.*

Gene	Accession no.	Direction	Primer sequence 5'→3'
ef1a	AF321836	Forward	CACCACCGGCCATCTGATCTACAA
		Reverse	TCAGCAGCCTCCTTCTCGAACTTC
rpol2	CA049789	Forward	TAACGCCTGCCTCTTCACGTTGA
		Reverse	ATGAGGGACCTTGTAGCCAGCAA
eif-3	DW542195	Forward	CAGGATGTTGTTGCTGGATGGG
		Reverse	ACCCAACTGGGCAGGTCAAGA
LPCAT	NM_001141753	Forward	TCGGACTGGTTTTGGCTCTC
		Reverse	CGCTGCAACTATTTGGCGTT
AGPAT3	NM_001140138	Forward	CTTCCAGAACAAGGTCTGTGGT
		Reverse	TATGGTGCATTCTGTCCCCG
GPAT3	NM_001141489	Forward	CTCGCTAAGACAACGAAGAGGT
		Reverse	CAGAGAGGTCTGTCATGCACT
AGPAT4	NM_001141264	Forward	AGAAAGGCCTGCCTAAACTCA
		Reverse	ATGTGTAGCATTTTGTCATTCTGGA
GPAT4	NM_001140176	Forward	GCTGCTATGCAATGGTTGGG
		Reverse	AGACTCGAGCGGACTGACTT
chk	DY706802	Forward	CTCAAGTTTGCCCGTCTGAT
		Reverse	CACAGGGGAATGAGTGGAGT
slc44a2	NM_001140367	Forward	TCGTCATCATTTTGCTGCTC
		Reverse	AGGCGATGACAATGGATAGG
pemt	XM_014158251	Forward	GTTGCTGTCATCGCCATCAT
		Reverse	GAGGAGGATGATGAGGGTGC

4.9. **Statistical analysis**

Tank values were used as experimental units. A two-way analysis of variance (two-way ANOVA) was used to test the effect of production system (RAS vs FT) and on production parameters, digestibility of minerals, total fatty acid composition and fat content, relative levels and ratio of PL classes, fatty acid composition of phospholipid classes and relative gene expression. The difference was considered significant at $P<0,05$. P values were included and interpreted as trends when fall between 0,05 and 0,10. These statistical analyses were performed using the software JMP® Pro version 16.0.0 (SAS institute Inc., Cary, NC, 1989-2007).

Principal component analysis (PCA) was performed for relative composition of PL fatty acids of Atlantic salmon gills tissue using the software Unscrambler, version 11.0 (CAMO Analytics, Corvallis, OR, USA). Score plot obtained from the PCA was used to examine the main trends and grouping of the data, and the respective correlation loadings showed the variables contributing to the sample grouping.

5. Result

5.1. Growth and survival

During the entire period of experiment the survival rate of fish in RAS was 100 %, while 2 dead fish were reported in the flow though system. Table-6 shows different production parameters for fish grown in both RAS and FT. According to this table, there is a clear significant difference between the production groups (RAS and FT) for all production parameters except for final length, liver index and heart index. The final weight of the fishes in RAS was 7-9% lower than their counterparts in FT, which was a result of approximately 5% lower growth rates: SGR and TGC. The condition factor (K-factor) was 3-5% lower of fish in RAS compared to FT Besides that, fish in RAS had a tendency for lower liver index (P=0,06) than in FT. However, no significant differences between the diet groups within the production system or interactions between production systems and diet groups were observed for the production parameters.

Table 6. *Average values of various production parameters of Atlantic salmon in the two production systems, RAS and FT, after feeding with control diet and EPA diet. A 2-way ANOVA is used to test for significant difference between the groups (n=6). The standard error means (SEM) are represented as pooled S.E.M P <,05 is considered as significant.*

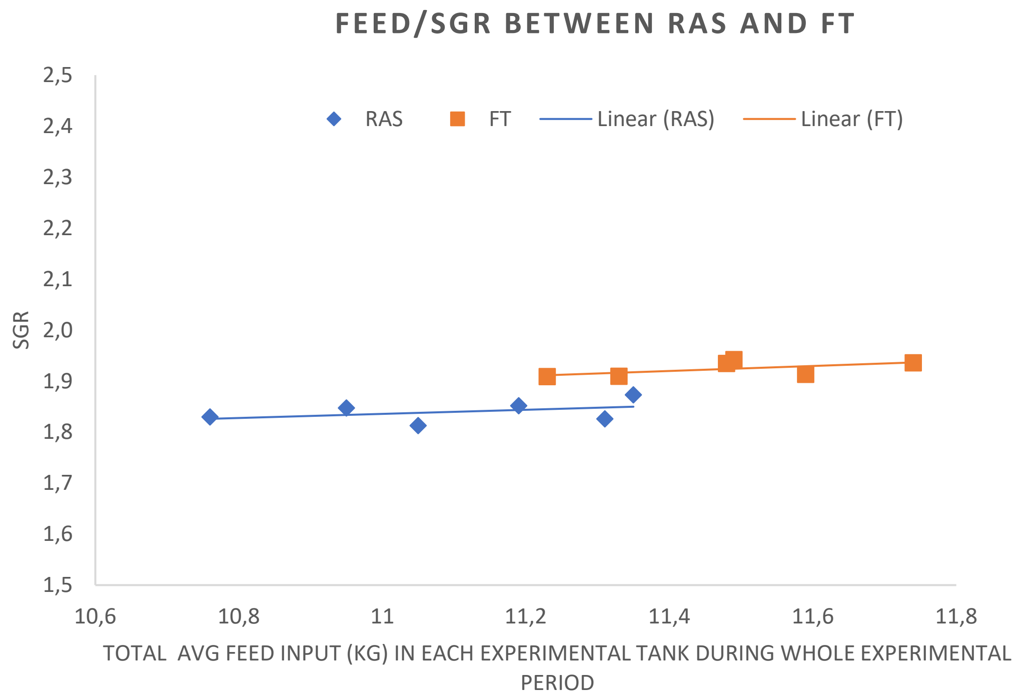
	RAS		Flow-through				Two-way ANOVA P-values			
Production parameters	**Control diet**	**EPA-diet**	**Control diet**	**EPA-diet**	**Pooled S.E.M**	**R^2**	**Model**	**System (FT×RAS)**	**Diet (Con× EPA)**	**Interaction**
Initial Weight (g)	101,00	101,00	101,00	101,00	0,00	0,03	0,97	0,74	0,74	1,00
Final wt (g)	406,58	400,9	435,92	439,02	2,98	0,94	<,0001	<,0001	0,68	0,18
Final length (cm)	30,96	30,92	30,84	31,19	0,11	0,4	0,23	0,51	0,21	0,13
K- factor	1,39	1,39	1,43	1,46	0,01	0,81	0,003	0,0006	0,18	0,28
Hepatosomatic index (HSI)	1,1	1,13	1,2	1,22	0,04	0,39	0,24	0,06	0,54	0,89
Heart index	0,15	0,14	0,15	0,15	0,01	0,07	0,89	0,8	0,61	0,63
SGR	1,85	1,83	1,92	1,93	0,01	0,89	0,0004	<,0001	0,57	0,23
TGC	3,01	2,97	3,17	3,19	0,02	0,91	0,0002	<,0001	0,51	0,17

C-factor: Condition factor
SGR : Specific growth rate
TGC : Thermal growth coefficient

5.2. Feeding vs Growth

In figure-13, the trend lines indicate that the fish grown in FT has eaten more feed during the experimental period than fish in RAS.

Figure .13 *Average specific growth rate (SGR) of the fish in RAS and FT production system (each tank) are plotted against total feed input in each experimental tank during whole experimental period. The trendlines indicate that the fish in RAS production tanks have lower SGR and lower feed average input than fish in FT Production tanks.*



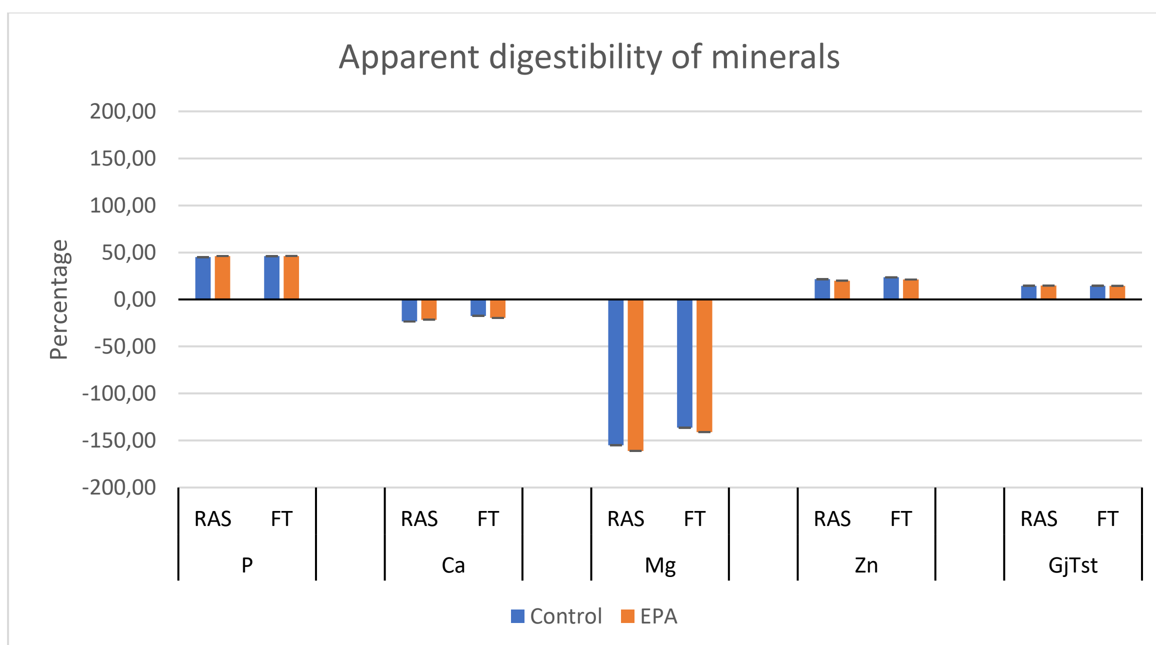
Note: - In the start of the experiment, Fishes in RAS and FT were fed same quantities of feed as per the feeding table. Later, feed input to RAS was reduced after observing more than 10% uneaten feed in the feeding tray.

5.3. Apparent digestibility of minerals

Table.7. *Digestibility coefficient of micronutrients such as phosphorus, calcium, magnesium and zinc were calculated for Atlantic salmon in the two production systems, RAS and FT, after feeding with control diet and EPA diet. A 2-way ANOVA is used to test for significant difference between the groups (n=6). The percentage values are represented in average. The standard error means (SEM) are represented as pooled S.E.M. P <,05 is considered as significant.*

	RAS		Flow-through				Two-way P-values			ANOVA
Variable	Control diet	EPA-diet	Control diet	EPA-diet	Pooled S.E.M	R^2	Model	System (FT×RAS)	Diet (Con× EPA)	Interaction
Phosphorous	44,94	46,17	46,02	46,25	2,56	0,06	0,91	0,70	0,63	0,74
Calcium	-23,47	-21,47	-17,40	-19,68	4,72	0,25	0,48	0,19	0,96	0,46
Magnesium	-155,00	-161,07	-136,42	-141,04	6,23	0,80	0,004	0,0007	0,18	0,85
Zinc	21,48	19,99	23,50	21,20	2,57	0,27	0,45	0,31	0,24	0,79
GjTst	14,57	14,71	14,57	14,45	0,27	0,15	0,71	0,42	0,96	0,44

Figure. 14 *shows the apparent digestibility coefficient of minerals Phosphorous (p), Calcium (Ca), Magnesium (Mg), Zinc (Zn) in the faecal sample from Atlantic salmon intestine, from two production systems, RAS and FT and further shows how the apparent digestibility of these minerals are affected by fatty acid composition control diet and EPA diet. The percentage values are represented as means ± S.E.M. (n =6).*



Digestibility is an indicator of absorption of nutrients into the body, however, this is not valid for minerals present in saltwater. We have calculated the apparent digestibility coefficient (ADC) of minerals such as calcium, magnesium which are found in saltwater, and for phosphorous and zinc in order to estimate the variability in utilization of these minerals in different systems with different diets.

Table.7 and figure.14 shows that ADC of calcium and magnesium are in negative values which indicates that the amount of these minerals in the faeces were higher than in the diet. This suggests that the fish were drinking water. In the RAS samples, the ADC value for the magnesium was increased by 14% compared to the FT samples, while remained unaffected by the diet groups. Simultaneously, the rest of the tested minerals showed no significant deference either between the production systems or between the diet groups.

5.4. **Total fat content**

Gill samples from the fish were analyzed for the total fat content (table.8). According to the statistical analysis, no significant difference was observed for fat content in gills either between production systems or between the diet groups.

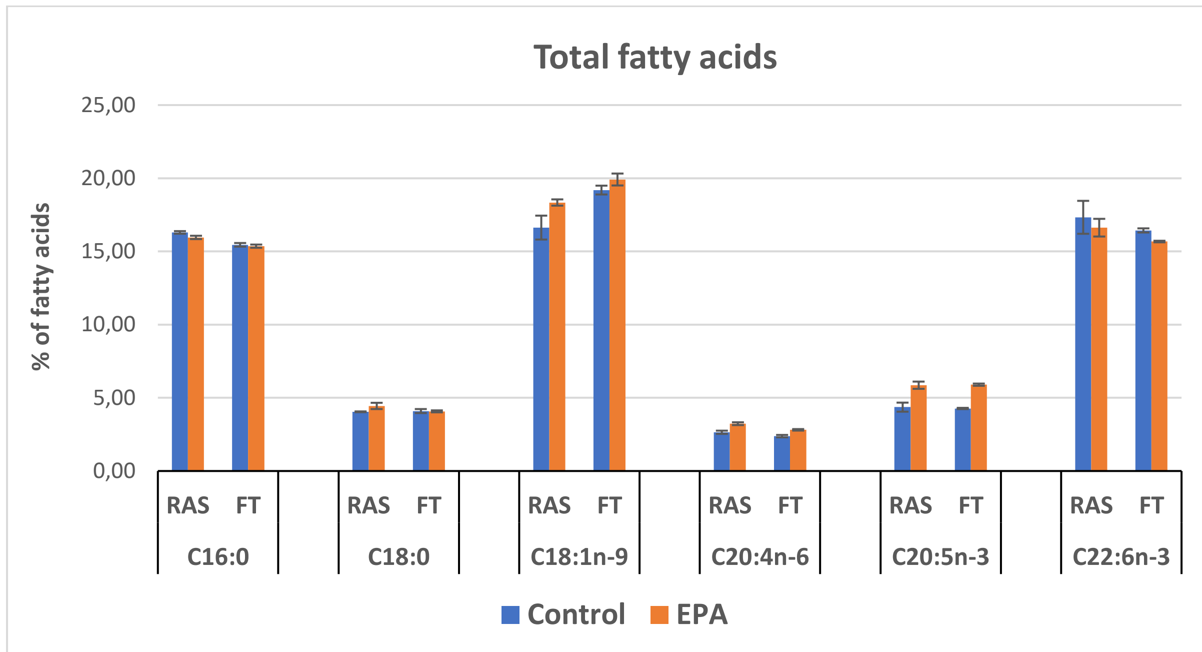
5.5. Total fatty acid composition and fat content in the gills

Table.8. *Percentage composition of total fatty acids and total fat content (Folch) in the gill tissue of Atlantic salmon in the two production systems, RAS and FT, after feeding with control diet and EPA diet. A 2-way ANOVA is used to test for significant difference between the groups (n=6). The percentage values are represented in average. The standard error means (SEM) are represented as pooled S.E.M. P <,05 is considered as significant.*

	RAS		Flow-through				Two-way ANOVA P-values			
Variable	**Control diet**	**EPA-diet**	**Control diet**	**EPA-diet**	**Pooled S.E.M**	**R^2**	**Model**	**System (FT×RAS)**	**Diet (Con× EPA)**	**Interaction**
Fat content (%)	2.26	2.30	2.27	2.22	0.06	0.11	0.80	0.55	0.90	0.45
14:0	1,72	1,42	1,84	1,51	0,05	0,83	0,002	0,09	0.0004	0,77
16:0	16,30	15,96	15,45	15,36	0,11	0,86	0,0009	0,0002	0,08	0,29
18:0	4,05	4,45	4,10	4,08	0,13	0,43	0,19	0,26	0,19	0,15
20:0	0,23	0,25	0,26	0,26	0,01	0,22	0,54	0,28	0,45	0,59
Σ SFA[1]	**23,29**	**22,92**	**22,62**	**21,84**	**0,32**	**0,59**	**0,06**	**0,02**	**0,11**	**0,52**
16:1n-7	2,34	1,93	2,13	1,69	0,12	0,66	0,03	0,10	0,009	0,90
18:1n-9	16,63	18,34	19,19	19,92	0,49	0,76	0,008	0,003	0,04	0,34
18:1n-7	1,66	2,01	1,78	1,75	0,15	0,25	0,48	0,67	0,34	0,26
20:1n-9	1,91	1,90	2,35	2,02	0,07	0,78	0,005	0,003	0,04	0,054
22:1n-11	0,98	0,86	1,55	1,09	0,06	0,90	0,0002	0,0002	0,001	0,03
Σ MUFA[2]	**29,40**	**30,15**	**32,27**	**31,47**	**0,41**	**0,79**	**0,005**	**0,0009**	**0,95**	**0,10**
18:2n-6	5,82	6,21	6,94	6,86	0,18	0,76	0,007	0,001	0,43	0,24
20:2n-6	0,88	0,97	0,94	0,96	0,03	0,40	0,23	0,52	0,09	0,29
20:3n-6	1,17	0,80	1,09	0,80	0,03	0,92	<,0001	0,21	<,0001	0,27
20:4n-6	2,65	3,24	2,38	2,81	0,09	0,86	0,0008	0,004	0,0004	0,40
Σ n-6[3]	**10,65**	**11,62**	**11,39**	**11,57**	**0,25**	**0,55**	**0,08**	**0,21**	**0,047**	**0,15**
18:3n-3	1,09	1,31	1,46	1,54	0,04	0,89	0,0003	0,0001	0,007	0,14
18:4n-3	1,27	1,00	1,00	1,03	0,13	0,27	0,45	0,39	0,39	0,29
20:5n-3	4,36	5,86	4,27	5,90	0,20	0,88	0,0005	0,90	<,0001	0,76
22:5n-3	0,97	1,35	10	1,34	0,04	0,90	0,0002	0,76	<,0001	0,68
22:6n-3	17,33	16,62	16,44	15,68	0,64	0,30	0,39	0,19	0,29	0,97
Σ n-3[4]	**25,20**	**26,46**	**24,49**	**25,87**	**0,78**	**0,31**	**0,38**	**0,43**	**0,13**	**0,94**
Σ PUFA[5]	**37,79**	**39,73**	**37,59**	**39,24**	**0,99**	**0,30**	**0,39**	**0,74**	**0,11**	**0,89**
EPA + DHA	21,70	22,49	20,71	21,58	0,84	0,22	0,55	0,29	0,35	0,96
n-3/n-6	2,37	2,28	2,15	2,24	0,05	0,54	0,09	0,04	0,97	0,12
Σ Others[6]	9,49	7,00	7,50	7,45	NA	NA	NA	NA	NA	NA

[1]Σ SFA includes 15:0, 17:0, 22:0.
[2]Σ MUFA includes 15:1, 17:1 Dimethyl, 17:1n-7, 16:1n-9, 18:1n-9T, 18:1n-11, 20:1n-7, 22:1n-7, 22:1n-9, 24:1n-9
[3]Σ n-6 includes 18:3n-6, 22:4n-6.
[4]Σ n-3 includes 20:4n-3, 20:3n-3.
[5]Σ PUFA includes 16:3 n-4.
[6]Σ Others correspond to the sum of the unidentified GC peaks in percentage values.
NA: Not applicable.

Figure. 15 *shows the percentage of fatty acids16:0, 18:0, 18:1n-9, 20:4n-6, 20:5n-3 and 22:6n-3 in total fatty acids composition of gill tissue of Atlantic salmon in the two production systems, RAS and FT and further shows how these fatty acids are affected by fatty acid composition of control diet and EPA diet. The percentage values are represented as means ± S.E.M (n =6).*



The total fatty acid composition in the gill is presented in figure.15 as percentage of total fatty acids. The major fatty acids were 16:0, 18:1n-9 and 22:6n-3. When comparing the total fatty acid composition of gills of Atlantic salmon between RAS and FT, significant differences in several fatty acids between production systems were observed.

The gills of experimental fishes in RAS had 4-5% higher 16:0 and 3-5% higher total SFA, 8-13% lower 18:1n-9 and 4-9% lower total MUFAs than fish in FT. Simultaneously, the PUFAs 18:2n-6 and 18:3n-3 was 9-16% and 5-10% lower in RAS than in FT, respectively. However, the percentage of longer chain n-3 fatty acids in the gills, eicosapentaenoic acid (EPA) 20:5n-3 and docosahexaenoic acid (DHA) 22:6n-3 were not significantly different between the production systems, whereas arachidonic acid (ARA) 20:4n-6 was significantly higher in RAS than in FT. The ratio between n-3 and n-6 fatty acids were also higher in RAS compared to that in FT (table.3).

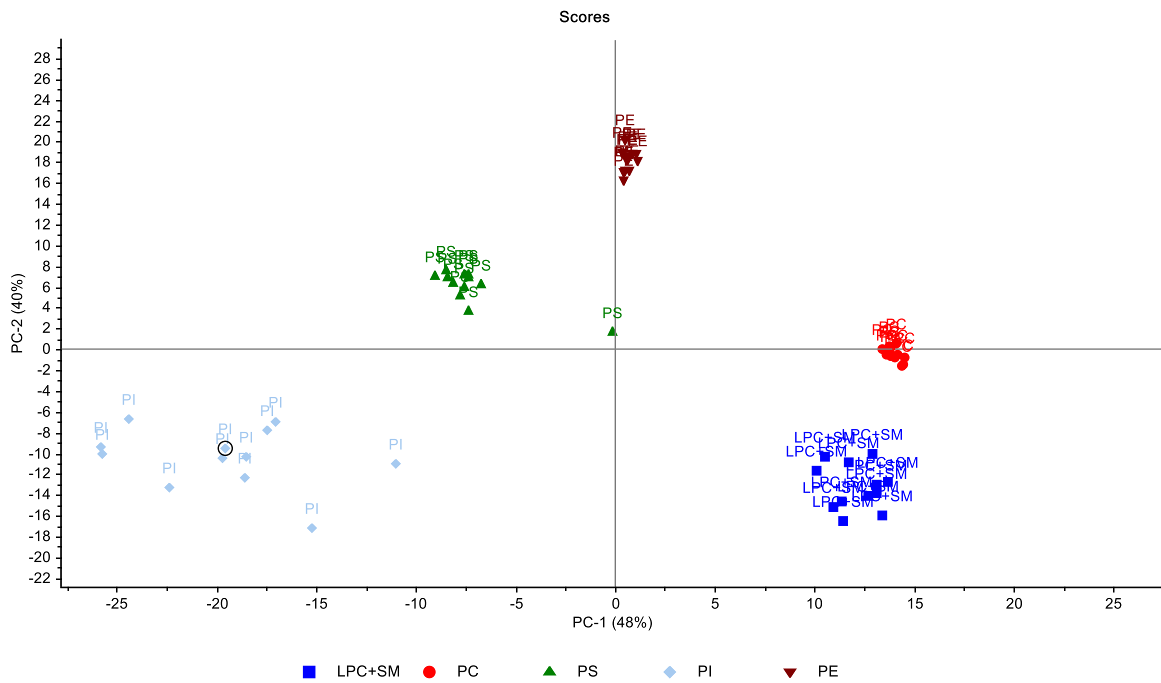
The total fatty acid composition of the gills reflected the fatty acid composition of the diets. Fish fed with EPA diet had 34-38% higher percentage of EPA and 18-22% higher ARA in the gills than the control group and a similar trend was found for the elongation product 22:5n-3 (34-39%). Increased dietary levels of MUFAs also resulted in higher levels of these in the EPA group than in the control group. Additionally, there was an interaction between system and diet for 22:1n-11 (table.3).

5.6. A multivariate analysis of fatty acids in different gill phospholipid classes

A principal component analysis (PCA) was done to get an overview of distribution of different fatty acids in phospholipid classes such as Lysophosphatidylcholine (LPC) + Sphingomyelin (SM), Phosphatidylcholine (PC), Phosphatidylserine (PS), Phosphatidylinositol (PI) and Phosphatidylethanolamine (PE). The entire data set from both production system groups and diet groups were used for this analysis. The analysis results are described in 2 different plots. Here, the score plot (figure.16) shows the grouping of different phospholipid classes, whereas the correlation loading plot shows the fatty acid composition and its correlation to each phospholipid class in the score plot. Those fatty acids seen in the outer circle of the score plot was significantly different between the phospholipid classes, while those inside the inner circle showed no significant difference.

It is clearly seen that this analysis has divided the samples into 5 distinct groups for LPC+SM, PC, PS, PI and PE which indicates that these phospholipids have a very distinctive composition of fatty acids. By plotting the different scores based on the principal components (PC), we can look for fatty acids in the data that are specifically related to the properties of the different phospholipid classes. Here, PC is characterized by higher levels of 16:0 and 18:1n-9, PE is characterized by higher DHA, PS is characterized by higher 18:0 and DHA, PI is characterized by higher 18:0 and ARA and LPC+SM is characterized by higher levels of 16:0 (figure.17 and 18) The figure.4 shows that the 16 fatty acids (18:1n-9, 18:2n-6, 18:4n-3, 16:1n-7, 16:1n-9, 14:0, 16:0, 17:1n-7, 15:0, 15:1, 18:3n-3, 18:0, 20:4n-6, 22:5n-3, 20:1n-9, 22:6n-3), those grouped towards the outer circle are significantly different between the phospholipid classes. Here, six fatty acids are chosen for the presentation of diet effect in two different production systems (RAS and FT), and distribution of those fatty acids were significantly different between the various phospholipid classes (figure 17 and 18).

Figure.16 *Multivariate analysis (PCA) of lipid composition of gills of Atlantic salmon grown in RAS and FT systems and fed two different diets. Here, the score plot shows the grouping of different phospholipid classes, whereas the correlation loading plot shows the fatty acid composition and its correlation to each phospholipid class in the score plot.*



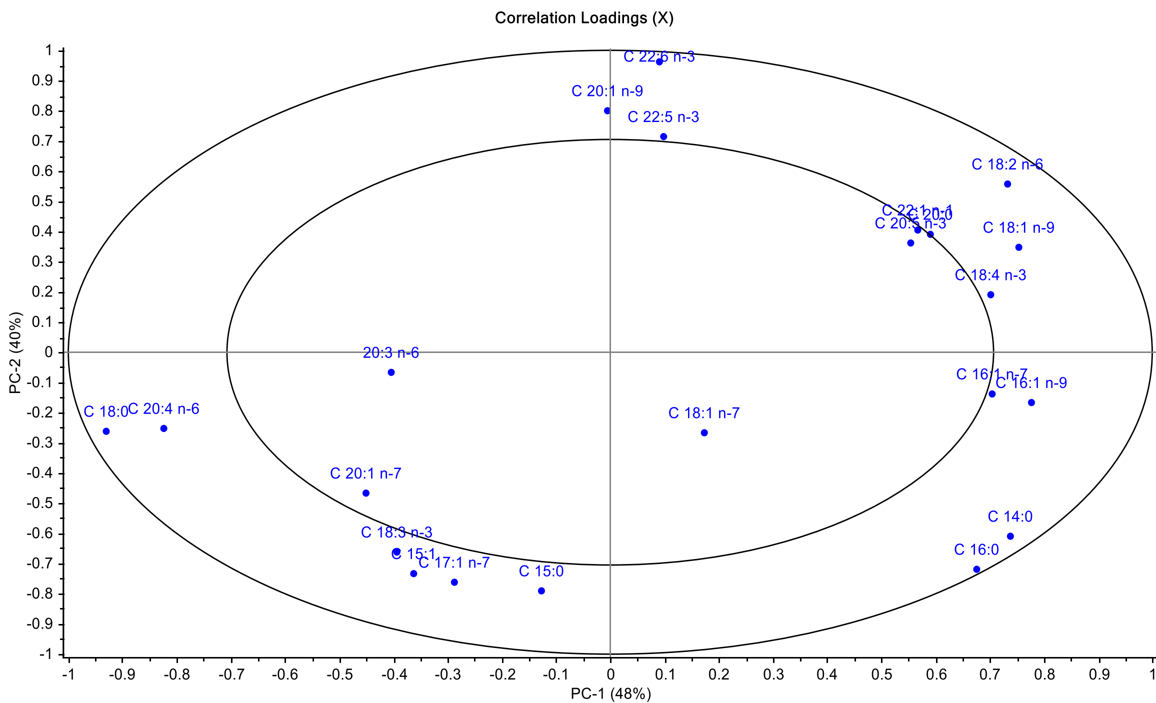
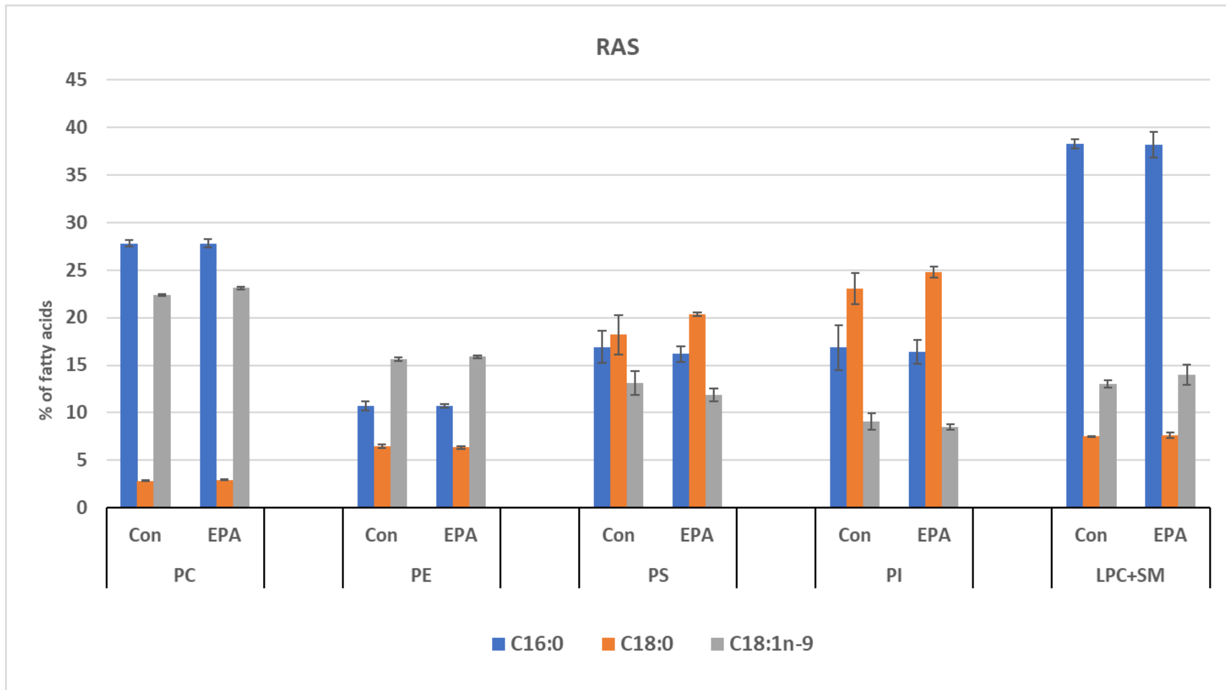


Figure.17 Figure 4a and 4b shows the distribution of fatty acids16:0, 18:0 and 18:1n-9 in phospholipid classes PC, PE, PS, PI and LPC+SM in the gill tissue of Atlantic salmon in the two production systems, RAS and FT respectively, further shows how these fatty acids are affected by fatty acid composition of control diet and EPA diet. The percentage values are represented as means ± S.E.M (n =6).

17a



17b

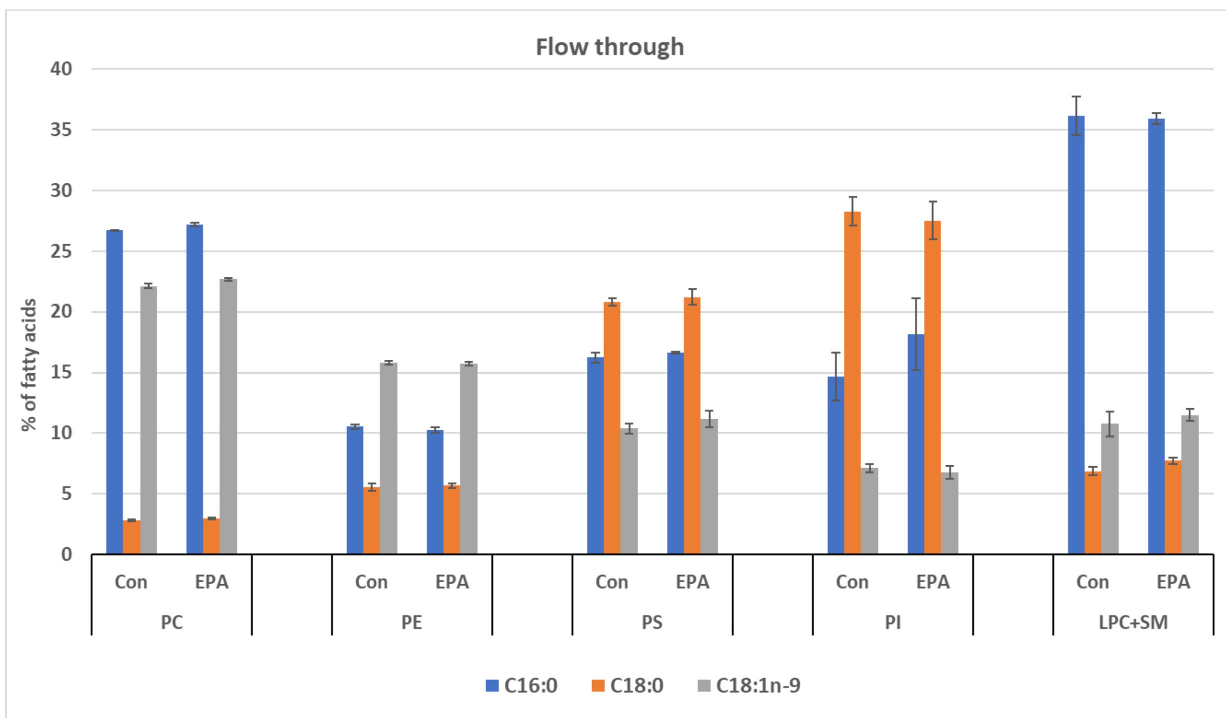
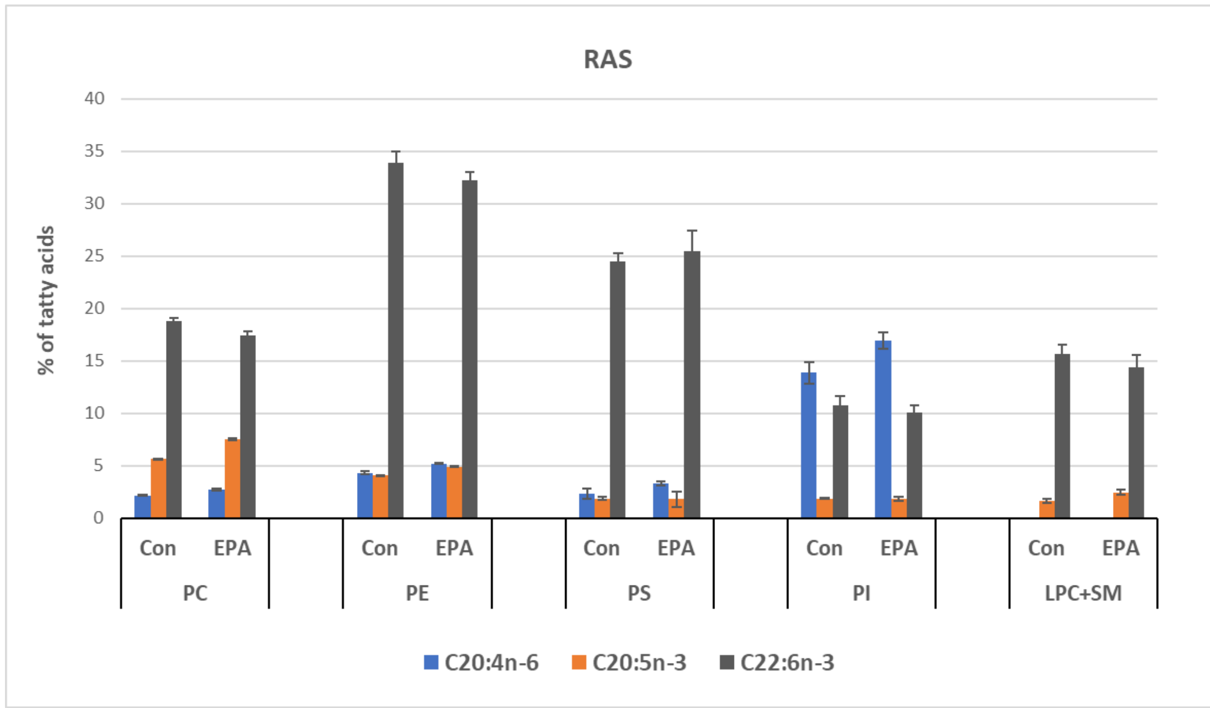
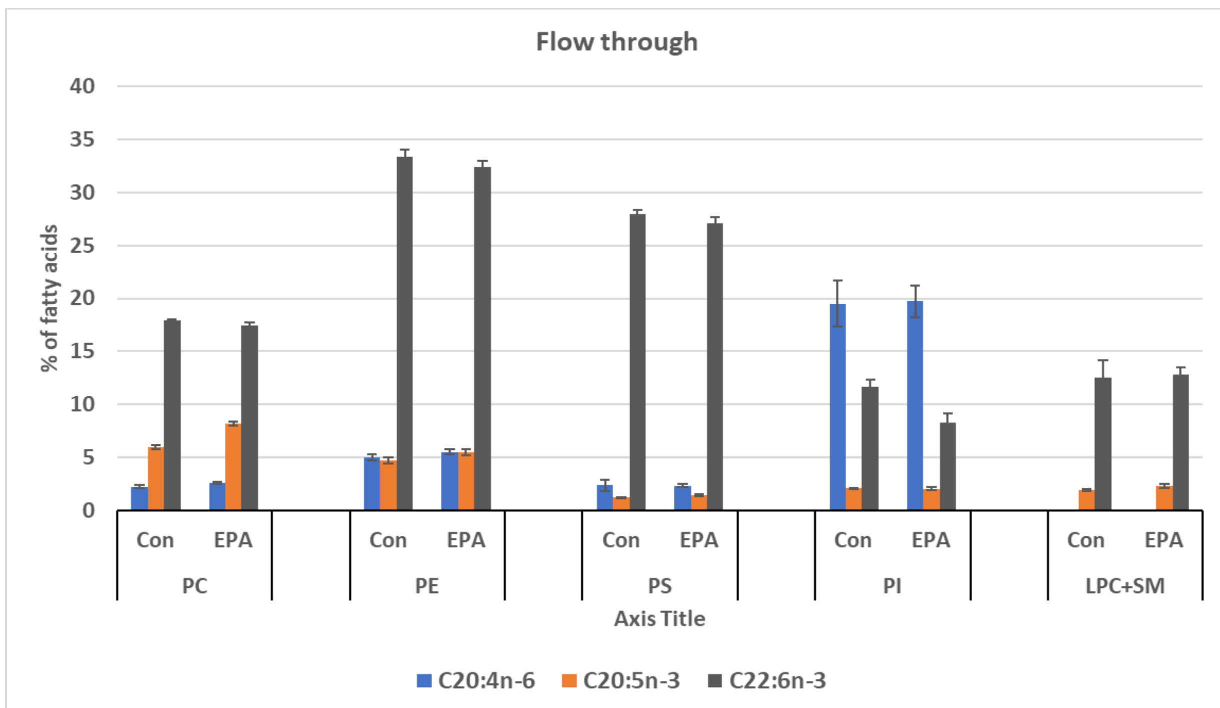


Figure.18 *Figure 5a and 5b shows the distribution of fatty acids 20:4n-6, 20:5n-3 and 22:6n-3 in phospholipid classes PC, PE, PS, PI and LPC+SM in the gill tissue of Atlantic salmon in the two production systems, RAS and FT respectively, further shows how these fatty acids are affected by fatty acid composition of control diet and EPA diet. The percentage values are represented as means ± S.E.M (n =6).*

18a



18b



5.7. Quantitative and Relative levels, and ratios of PL classes

Table.9 shows that the total amount of phospholipids (mg/g) in the gill tissue of the Atlantic salmon were not seen to be affected by either production systems or diet groups.

PC is the major PL class in the gills constituting more than 50% of total PL, which is more than double that of PE. PC and PE together constitute more than 80% of total PL. Similarly, the percentage of PS (9-10% of total PL) is more than 2-fold that of PI and LPC+SM each. The relative levels of each individual phospholipid class were not significantly affected by production systems or diet groups and showed no significant interaction between them. However, the concentration of PE showed a tendency (p=0,07) to be lower in the gill samples from RAS system than FT.

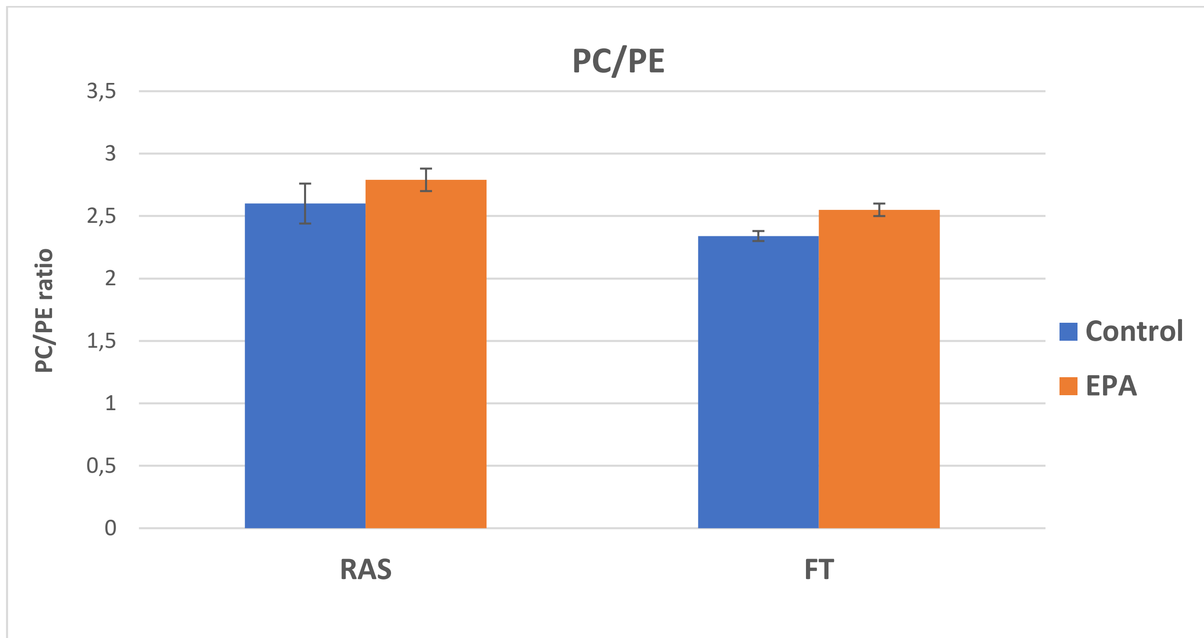
The ratios between major phospholipid classes were analyzed. The results (table.9) indicate that the ratio between PC to PE in fishes grown in RAS was 9-11% higher than FT (figure.19) and a similar tendency (p=0,08) for rise in this ratio was seen the fishes fed with EPA diet. At the same time, other ratios remain unaffected by either system or diet.

Table.9 *This table shows the sum of PL classes in mg/g in terms of their fatty acid content, the relative concentration of each phospholipid class, the ratio between important PL classes and their variability in the gill tissue of Atlantic salmon in two production systems, RAS and FT, after feeding with control diet and EPA diet. A 2-way ANOVA is used to test for significant difference between the groups (n=6). The values are represented in average. The standard error means (SEM) are represented as pooled S.E.M. P <,05 is considered as significant.*

	RAS		Flow-through				Two-way ANOVA P-values			
Variable	**Control diet**	**EPA-diet**	**Control diet**	**EPA-diet**	**Pooled S.E.M**	**R^2**	**Model**	**System (FT×RAS)**	**Diet (Con× EPA)**	**Interaction**
Σ PL[1] (mg/g)	3,94	3,72	4,07	3,64	0,31	0,13	0,76	0,93	0,33	0,75
						Percentage				
% PC	58,09	59,77	57,95	60,08	1,09	0,28	0,43	0,94	0,12	0,84
% PE	22,54	21,45	24,79	23,62	1,04	0,42	0,21	0,07	0,31	0,97
% PS	10,38	9,77	8,85	8,54	1,10	0,18	0,64	0,24	0,69	0,89
% PI	4,16	4,29	4,34	4,03	0,54	0,03	0,98	0,94	0,87	0,70
% LPC+SM	4,83	4,73	4,08	3,74	0,73	0,16	0,68	0,27	0,77	0,87
						Ratio				
PC/PE	2,60	2,79	2,34	2,55	0,10	0,58	0,07	0,03	0,08	0,95
PC/PS	5,97	6,24	6,64	7,10	0,76	0,14	0,74	0,34	0,64	0,90
PC/LPC+SM	12,20	13,09	16,74	17,29	3,19	0,19	0.60	0,21	0,83	0,96
PE/PS	2,35	2,24	2,84	2,79	0,33	0,24	0,51	0,16	0,81	0,94
PE/ LPC+SM	4,76	4,73	7,18	6,85	1,39	0,25	0,49	0,14	0,90	0,92

[1]**Σ PL** is the sum of FA in all PL classes such as PC, PE, PS, PI, LPC+SM, PA in mg/g.

Figure.19 *shows the ratio between phosphatidylcholine (PC) to phosphatidylethanolamine (PC/PE) in gill tissue of Atlantic salmon in the two production systems, RAS and FT and further shows how these fatty acids are affected by fatty acid composition of control diet and EPA diet. The percentage values are represented as means ± S.E.M. (n =6).*



5.8. Fatty acid composition of different Phospholipid classes

5.8.1. Phosphatidylcholine (PC)

Table.10. *Percentage composition of fatty acids in phospholipid class PC in the gill tissue of Atlantic salmon in the two production systems, RAS and FT, after feeding with control diet and EPA diet. A 2-way ANOVA is used to test for significant difference between the groups (n=6). The percentage values are represented in average. The standard error means (SEM) are represented as pooled S.E.M. P <,05 is considered as significant.*

	RAS		Flow-through				Two-way ANOVA P-values			
Variable	**Control diet**	**EPA-diet**	**Control diet**	**EPA-diet**	**Pooled S.E.M**	**R^2**	**Model**	**System (FT×RAS)**	**Diet (Con× EPA)**	**Interaction**
14:0	2,07	1,78	2,25	1,89	0,04	0,93	<0,0001	0,004	<0,0001	0,35
16:0	27,82	27,8	26,7	27,17	0,26	0,62	0,04	0,01	0,42	0,37
18:0	2,86	2,95	2,85	2,96	0,06	0,27	0,44	0,97	0,12	0,94
Σ SFA[1]	**34,2**	**34,01**	**33,48**	**33,55**	**0,29**	**0,37**	**0,30**	**0,08**	**0,83**	**0,67**
16:1n-7	1,65	1,43	1,53	1,28	0,04	0,87	0,0006	0,005	0,0002	0,66
18:1n-9	22,39	23,12	22,11	22,67	0,15	0,75	0,009	0,04	0,003	0,59
20:1n-9	1,16	1,19	1,57	1,37	0,15	0,37	0,27	0,09	0,62	0,46
22:1n-11	0,75	0,56	0,99	0,86	0,07	0,71	0,01	0,005	0,05	0,69
Σ MUFA[2]	**29,83**	**29,98**	**29,62**	**29,34**	**0,17**	**0,49**	**0,13**	**0,04**	**0,70**	**0,26**
18:2n-6	5,16	4,67	5,41	4,73	0,11	0,79	0,005	0,20	0,0009	0,42
20:3n-6	1,48	0,95	1,57	0,98	0,02	0,98	<0,0001	0,04	<0,0001	0,34
20:4n-6	2,18	2,73	2,23	2,60	0,10	0,76	0,008	0,65	0,001	0,37
Σ n-6	**8,82**	**8,35**	**9,21**	**8,31**	**0,18**	**0,67**	**0,03**	**0,37**	**0,006**	**0,29**
18:3n-3	BDL	BDL	BDL	BDL	NA	NA	NA	NA	NA	NA
18:4n-3	0,98	0,98	1,07	0,94	0,02	0,75	0,009	0,19	0,01	0,01
20:5n-3	5,63	7,50	6,00	8,20	0,15	0,96	<0,0001	0,007	<0,0001	0,30
22:5n-3	1,00	1,34	1,43	1,81	0,10	0,81	0,003	0,001	0,006	0,85
22:6n-3	18,77	17,39	17,91	17,42	0,30	0,64	0,04	0,20	0,01	0,17
Σ n-3	**26,38**	**27,21**	**26,42**	**28,38**	**0,33**	**0,76**	**0,008**	**0,10**	**0,002**	**0,12**
ΣPUFA[3]	**35,2**	**35,56**	**35,63**	**36,69**	**0,34**	**0,57**	**0,07**	**0,05**	**0,07**	**0,33**
EPA + DHA	24,40	24,89	23,91	25,62	0,29	0,70	0,02	0,68	0,006	0,07
n-3/n-6	2,99	3,26	2,87	3,42	0,09	0,76	0,008	0,83	0,002	0,15
Σ Others[4]	0,75	0,46	1,27	0,43	NA	NA	NA	NA	NA	NA

[1]Σ SFA includes 15:0, 17:0, 20:0.
[2]Σ MUFA includes 14:1n-5, 15:1, 16:1T, 17:1 Dimethyl, 17:1n-7, 16:1n-9, 20:1n-11, 24:1n-9.
[3]Σ PUFA is the sum of Σ n-6 and Σ n-3.
[4]Σ Others correspond to the sum of the unidentified GC peaks in percentage values.
BDL: Below detection level.
NA: Not applicable.

***Figure. 20** shows the percentage of fatty acids16:0, 18:0, 18:1n-9, 20:4n-6, 20:5n-3 and 22:6n-3 in phospholipid class phosphatidylcholine (PC) of gill tissue of Atlantic salmon in the two production systems, RAS and FT and further shows how these fatty acids are affected by fatty acid composition of control diet and EPA diet. The percentage values are represented as means ± S.E.M. (n =6).*

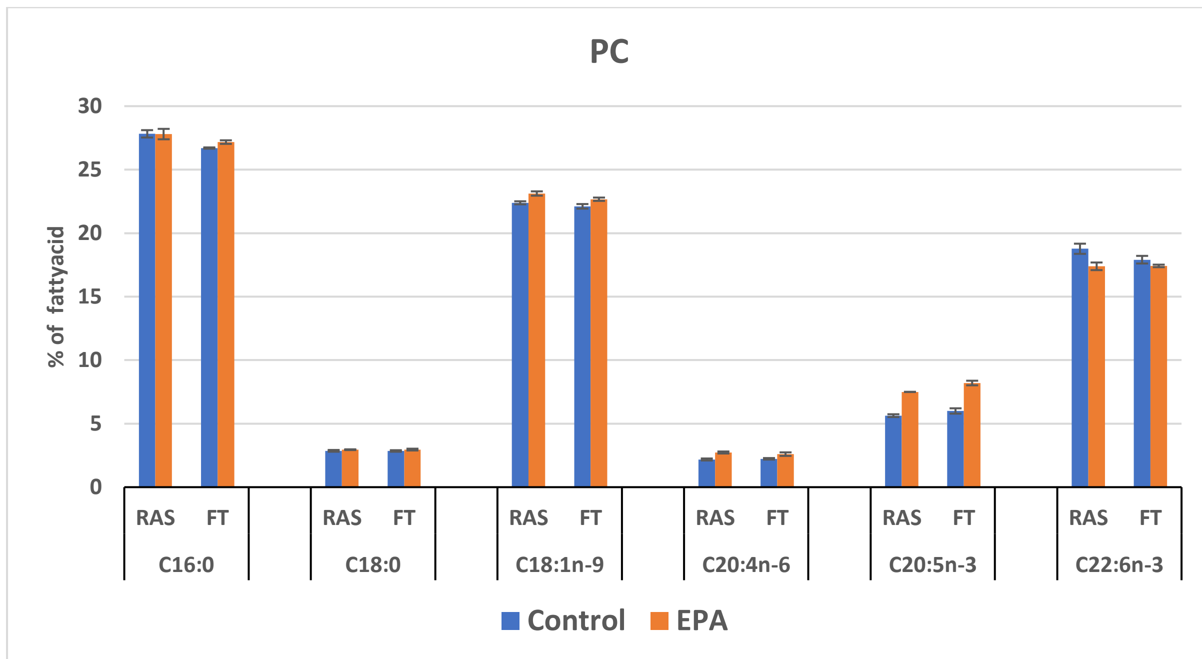


Figure.20 shows major fatty acids in the gill phospholipid class PC were 16:0, 18:1n-9, 20:5n-3 and 22:6n-3, with a higher percentage of 16:0.

The PC in gills of experimental fish in RAS had 2-4% higher 16:0, 6-8% lower 14:0, 1-2% higher 18:1n-9 and 0,7-2% higher total MUFAs than fish in FT. In addition to this, fish in RAS had a tendency for higher total SFA (0,08) than in FT. At the same time, EPA and total PUFA were 6-9% and 1-3% lower in RAS than in FT, respectively. However, percentages of DHA and ARA were not significantly different between the systems (table.10).

The fatty acid composition of phospholipid PC reflected the composition of experimental diets, where EPA and ARA were 33-37% and 17-25% higher in EPA diet group than control group, respectively and a similar trend was seen for 22:5n-3 (27-34%) (table.4). However, DHA was 3-7% lower in EPA diet group. A higher dietary level of MUFAs in EPA diet resulted in higher levels of these fatty acids in EPA group than control group. In addition to this, there was an interaction between system and diet for 18:4n-3 (table.10).

5.8.2. Phosphatidylethanolamine (PE)

Table.11. *Percentage composition of fatty acids in phospholipid class PE in the gill tissue of Atlantic salmon in the two production systems, RAS and FT, after feeding with control diet and EPA diet. A 2-way ANOVA is used to test for significant difference between the groups (n=6). The percentage values are represented in average. The standard error means (SEM) are represented as pooled S.E.M. P <,05 is considered as significant.*

	RAS		Flow-through				Two-way ANOVA P-values			
Variable	Control diet	EPA-diet	Control diet	EPA-diet	Pooled S.E.M	R^2	Model	System (FT×RAS)	Diet (Con× EPA)	Interaction
14:0	0,27	0,23	0,32	0,26	0,02	0,55	0,08	0,09	0,04	0,87
16:0	10,72	10,73	10,53	10,28	0,29	0,16	0,68	0,31	0,70	0,67
18:0	6,47	6,34	5,56	5,70	0,22	0,62	0,04	0,007	0,98	0,55
Σ SFA[1]	**18,55**	**18,34**	**17,31**	**17,40**	**0,46**	**0,41**	**0,21**	**0,04**	**0,91**	**0,76**
16:1n-7	0,21	0,17	0,22	0,17	0,008	0,82	0,002	0,49	0,0003	0,43
18:1n-9	15,63	15,88	15,81	15,73	0,15	0,17	0,67	0,92	0,60	0,29
20:1n-9	2,29	2,49	2,72	2,47	0,17	0,29	0,42	0,25	0,90	0,23
22:1n-11	0,52	0,49	0,67	0,56	0,05	0,50	0,12	0,055	0,17	0,43
Σ MUFA[2]	**22,42**	**22,26**	**22,75**	**22,23**	**0,27**	**0,23**	**0,52**	**0,59**	**0,24**	**0,51**
18:2n-6	4,85	4,78	5,49	5,22	0,13	0,72	0,01	0,003	0,22	0,46
20:3n-6	1,04	0,69	0,97	0,61	0,05	0,88	0,0004	0,18	<0,0001	0,90
20:4n-6	4,31	5,22	4,98	5,50	0,21	0,68	0,02	0,05	0,01	0,39
Σ n-6	**10,20**	**10,68**	**11,45**	**11,33**	**0,34**	**0,52**	**0,10**	**0,02**	**0,61**	**0,41**
18:3n-3	BDL*	BDL*	BDL*	BDL*	NA	NA	NA	NA	NA	NA
18:4n-3	0,89	0,97	0,92	0,93	0,03	0,29	0,41	0,98	0,17	0,35
20:5n-3	4,07	4,89	4,70	5,51	0,19	0,78	0,005	0,01	0,003	0,98
22:5n-3	0,98	1,38	1,18	1,56	0,08	0,77	0,006	0,06	0,002	0,89
22:6n-3	33,93	32,26	33,36	32,46	0,77	0,28	0,43	0,82	0,13	0,63
Σ n-3[3]	**40,26**	**39,89**	**40,41**	**4063**	**0,66**	**0,08**	**0,86**	**0,51**	**0,90**	**0,65**
ΣPUFA[4]	**50,46**	**50,54**	**51,85**	**51,96**	**0,61**	**0,40**	**0,23**	**0,05**	**0,88**	**0,98**
EPA + DHA	38	37,14	38,06	37,97	0,68	0,13	0,75	0,53	0,51	0,59
n-3/n-6	3,95	3,74	3,54	3,60	0,14	0,38	0,26	0,09	0,62	0,38
Σ Others[5]	8,57	8,84	8,07	8,39	NA	NA	NA	NA	NA	NA

[1]Σ SFA includes 15:0, 17:0, 20:0.
[2]Σ MUFA includes 15:1, 16:1T, 17:1 Dimethyl, 17:1n-7, 16:1n-9, 20:1n-7, 20:1n-11.
[3]Σ n-3 includes 18:3n-3.
[4]Σ PUFA is the sum of Σ n-6 and Σ n-3.
[5]Σ Others correspond to the sum of the unidentified GC peaks in percentage values.
BDL*: Some concentrations are below detection level.
NA: Not applicable.

Figure. 21 *shows the percentage of fatty acids16:0, 18:0, 18:1n-9, 20:4n-6, 20:5n-3 and 22:6n-3 in phospholipid class phosphatidylcholine (PE) of gill tissue of Atlantic salmon in the two production systems, RAS and FT and further shows how these fatty acids are affected by fatty acid composition of control diet and EPA diet. The percentage values are represented as means ± S.E.M. (n =6).*

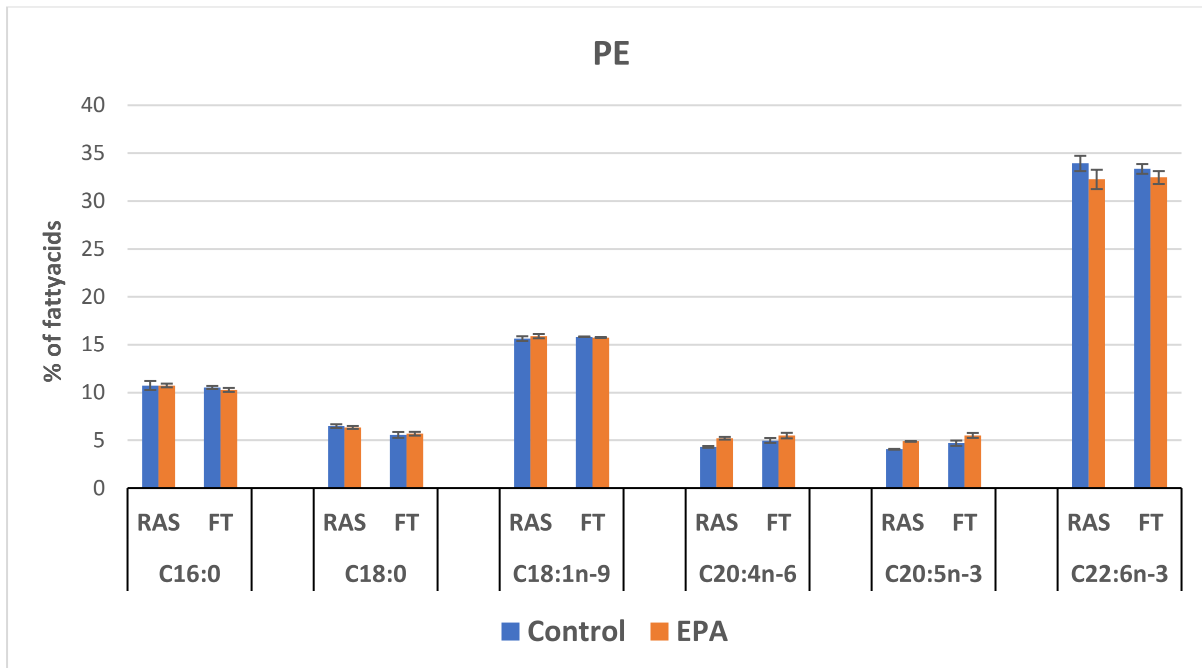


Figure.21 shows that the major fatty acid in the PE in the Atlantic salmon gill was 22:6n-3, with percentage composition more than 2 times that of 18:1n-9 and 3 times that of 16:0. Additionally, 50% of PE was comprised of PUFAs, which is more than 2 times the percentage of both total MUFA and total SFA. However, within the total PUFA, percentage of total n-3 fatty acids was observed to be 4 times greater than that of n-6 fatty acids.

Phospholipid class PE in the gills of experimental fish in RAS had 5-7% higher 18:0 and 11-16% higher total SFA than fish in FT. However, the MUFAs were not significantly different between production systems. The PUFA 18:2n-6 and total n-6 fatty acids were 8-12% and 6-11% lower in RAS fish than in FT fish, respectively. Similarly, EPA and ARA were 11-13% and 5-13% lower in RAS than in FT, respectively and a similar trend was found for total PUFA (3%). However, the percentage of DHA was not significantly different between the production systems. The ratio between n-3 and n-6 fatty acids showed a tendency (0,09) to be higher in samples from RAS system than in FT (table.11).

The experimental fish fed with EPA diet had 17-20% higher EPA and 10-21% higher ARA in gills than control feed and a similar trend was found for 22:5n-3 (32-40%). However, the SFAs and MUFAs were not significantly different between the different diet groups (table.11).

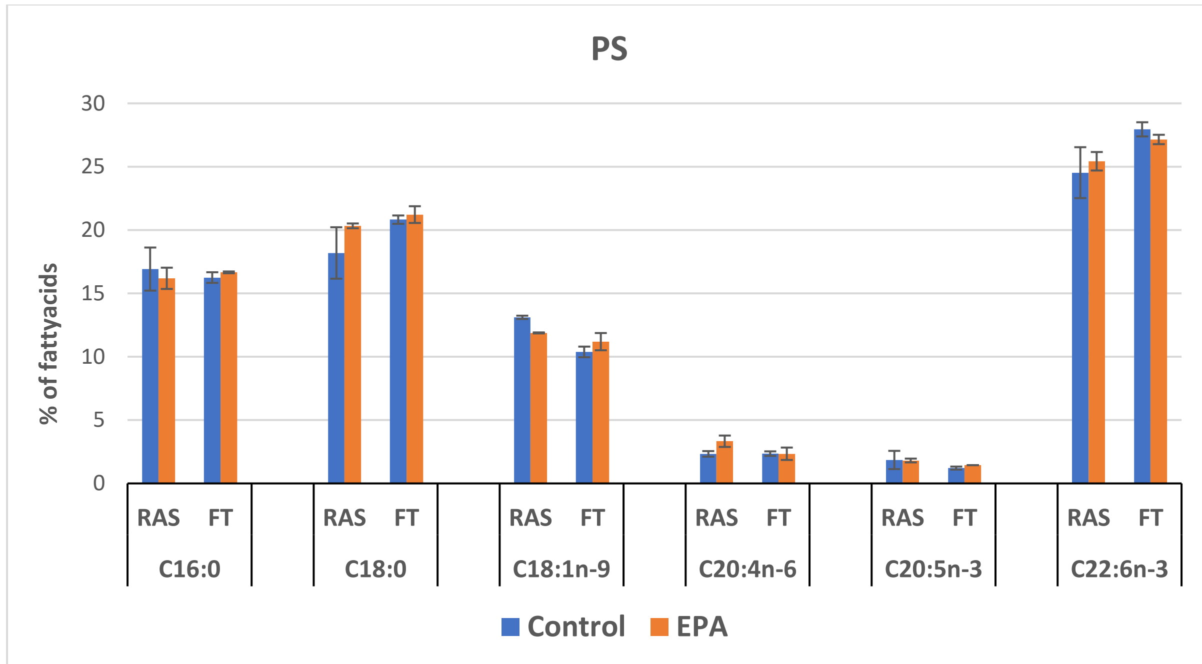
5.8.3. Phosphatidylserine (PS)

Table.12 *Percentage composition of fatty acids in phospholipid class PS in the gill tissue of Atlantic salmon in the two production systems, RAS and FT, after feeding with control diet and EPA diet. A 2-way ANOVA is used to test for significant difference between the groups (n=6). The percentage values are represented in average. The standard error means (SEM) are represented as pooled S.E.M. P <,05 is considered as significant.*

	RAS		Flow-through				Two-way ANOVA P-values			
Variable	**Control diet**	**EPA-diet**	**Control diet**	**EPA-diet**	**Pooled S.E.M**	**R^2**	**Model**	**System (FT×RAS)**	**Diet (Con× EPA)**	**Interaction**
14:0	0,55	0,41	0,36	0,37	0,08	0,29	0,42	0,20	0,47	0,42
16:0	16,92	16,19	16,25	16,66	0,97	0,10	0,84	0,55	0,52	0,94
18:0	18,19	20,33	20,82	21,22	1,09	0,37	0,27	0,14	0,28	0,44
Σ SFA[1]	**36,96**	**38,22**	**38,46**	**38,53**	**0,69**	**0,30**	**0,39**	**0,23**	**0,36**	**0,41**
16:1n-7	0,42	0,29	0,34	0,17	0,08	0,41	0,22	0,23	0,09	0,84
18:1n-9	13,11	11,88	10,38	11,19	0,82	0,43	0,19	0,07	0,81	0,25
20:1n-9	2,53	2,17	2,27	2,28	0,13	0,33	0,33	0,60	0,22	0,21
22:1n-11	BDL	BDL	BDL	BDL	NA	NA	NA	NA	NA	NA
Σ MUFA[2]	**23,76**	**21,21**	**19,97**	**20,20**	**0,59**	**0,76**	**0,007**	**0,003**	**0,09**	**0,047**
18:2n-6	2,95	2,69	2,90	2,67	0,15	0,26	0,46	0,80	0,13	0,91
20:3n-6	1,28	0,95	1,20	0,51	0,20	0,52	0,10	0,23	0,04	0,40
20:4n-6	2,33	3,33	2,35	2,34	0,36	0,41	0,21	0,22	0,21	0,20
Σ n-6	**6,56**	**6,97**	**6,45**	**5,51**	**0,55**	**0,32**	**0,35**	**0,19**	**0,64**	**0,25**
18:3n-3	1,32	1,01	0,89	1,10	0,14	0,39	0,24	0,26	0,73	0,10
18:4n-3	0,84	0,86	0,77	0,86	0,06	0,19	0,62	0,53	0,34	0,55
20:5n-3	1,85	1,81	1,21	1,44	0,38	0,20	0,60	0,22	0,81	0,72
22:5n-3	1,23	1,70	1,29	1,98	0,14	0,72	0,01	0,23	0,003	0,43
22:6n-3	24,53	25,43	27,95	27,15	1,12	0,42	0,20	0,05	0,97	0,47
Σ n-3	**29,76**	**30,80**	**32,11**	**32,53**	**0,95**	**0,40**	**0,23**	**0,06**	**0,47**	**0,75**
ΣPUFA[3]	**36,32**	**37,77**	**38,56**	**38,04**	**0,66**	**0,44**	**0,18**	**0,09**	**0,50**	**0,17**
EPA + DHA	26,38	27,23	29,16	28,59	0,80	0,48	0,13	0,03	0,87	0,40
n-3/n-6	4,62	4,46	5,05	6,16	0,65	0,35	0,31	0,14	0,48	0,36
Σ Others[4]	2,95	2,80	3,00	3,23	NA	NA	NA	NA	NA	NA

[1]Σ SFA includes 15:0, 17:0, 20:0.
[2]Σ MUFA includes 15:1, 16:1n-9, 16:1T, 17:1 Dimethyl, 17:1n-7, 18:1n-7, 20:1n-7, 20:1n-11.
[3]Σ PUFA is the sum of Σ n-6 and Σ n-3.
[4]Σ Others correspond to the sum of the unidentified GC peaks in percentage values.
The standard error means are represented as pooled standard error means (pooled SEM).
BDL: All below detection level.
NA: Not applicable.

Figure. 22 *shows the percentage of fatty acids16:0, 18:0, 18:1n-9, 20:4n-6, 20:5n-3 and 22:6n-3 in phospholipid class phosphatidylcholine (PS) of gill tissue of Atlantic salmon in the two production systems, RAS and FT and further shows how these fatty acids are affected by fatty acid composition of control diet and EPA diet. The percentage values are represented as means ± S.E.M. (n =6).*



PS of Atlantic salmon gills had a higher percentage composition of 18:0 (figure.22) and total PUFA. Besides that, total PUFA and total SFA had a similar composition in PS.

The fish in RAS had a 5-19% higher total MUFA in their gill PS than in FT. Besides that, 18:1n-9 showed a tendency (0,07) to be higher in RAS system than in FT. However, the SFAs were not significantly different between the systems. The DHA was 6-12% lower and, sum of EPA and DHA was 5-10% lower in RAS than in FT, whereas EPA and ARA were not significantly different between the system. Additionally, the total PUFA showed a tendency (0,09) to be lower in RAS than in FT (table.12).

The fatty acids in the gill phospholipid PS were not affected by dietary composition. For instance, the percentage composition of neither EPA nor ARA were significantly different between the systems. However, the elongation product 22:5n-3 was 38-53% higher in fish fed with EPA diet than control group. Additionally, there was an interaction between the system and diet for total MUFA (table.12).

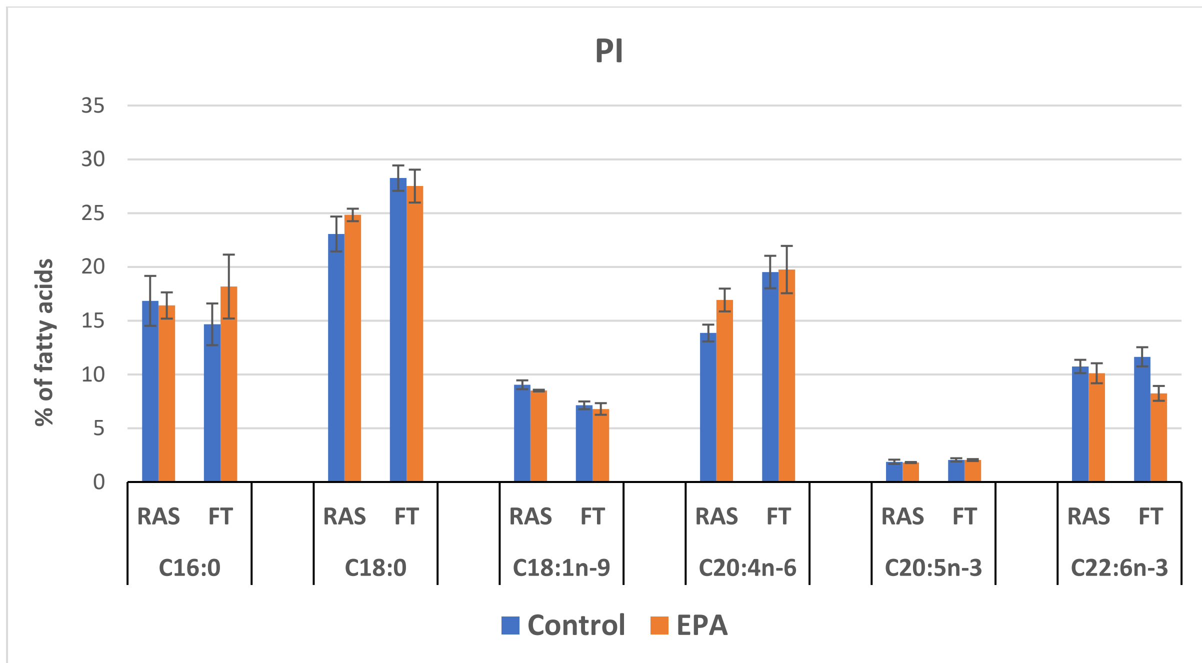
5.8.4. Phosphatidylinositol (PI)

Table.13 *The percentage composition of fatty acids in phospholipid class PI in the gill tissue of Atlantic salmon in the two production systems, RAS and FT, after feeding with control diet and EPA diet. A 2-way ANOVA is used to test for significant difference between the groups (n=6). The percentage values are represented in average. The standard error means (SEM) are represented as pooled S.E.M. P <,05 is considered as significant.*

	RAS		Flow-through				Two-way ANOVA P-values			
Variable	Control diet	EPA-diet	Control diet	EPA-diet	Pooled S.E.M	R^2	Model	System (FT×RAS)	Diet (Con× EPA)	Interaction
14:0	0,75	0,61	0,43	0,80	0,12	0,41	0,21	0,59	0,38	0,07
16:0	16,84	16,41	14,66	18,17	2,21	0,14	0,74	0,92	0,51	0,40
18:0	23.06	24,83	28,25	27,51	1,29	0,56	0,07	0,01	0,70	0,36
Σ SFA[1]	**41,95**	**43,04**	**44,30**	**47,42**	**1,36**	**0,53**	**0,09**	**0,04**	**0,16**	**0,48**
16:1n-7	BDL	BDL	BDL	BDL	NA	NA	NA	NA	NA	NA
18:1n-9	9,04	8,50	7,13	6,79	0,56	0,58	0,06	0,01	0,46	0,86
20:1n-9	1,28	0,89	1,05	0,79	0,23	0,24	0,50	0,49	0,20	0,78
22:1n-11	BDL*	BDL*	BDL*	BDL*	NA	NA	NA	NA	NA	NA
Σ MUFA[2]	**23,22**	**20,70**	**16,27**	**17,33**	**1,36**	**0,67**	**0,02**	**0,005**	**0,61**	**0,23**
18:2n-6	1,89	1,68	1,40	1,18	0,09	0,83	0,002	0,0004	0,04	0,98
20:3n-6	2,50	1,52	2,04	1,12	0,53	0,32	0,35	0,44	0,11	0,96
20:4n-6	13,85	16,92	19,52	19,75	1,49	0,56	0,07	0,02	0,30	0,37
Σ n-6	**18,25**	**20,12**	**22,96**	**22,05**	**1,50**	**0,42**	**0,20**	**0,06**	**0,76**	**0,38**
18:3n-3	3,15	2,06	1,99	2,38	0,42	0,38	0,26	0,34	0,42	0,11
18:4n-3	BDL*	BDL*	BDL*	BDL*	NA	NA	NA	NA	NA	NA
20:5n-3	1,88	1,81	2,06	2,04	0,13	0,24	0,50	0,16	0,77	0,85
22:5n-3	BDL*	BDL*	BDL*	BDL*	NA	NA	NA	NA	NA	NA
22:6n-3	10,74	10,11	11,64	8,24	0,79	0,55	0,08	0,56	0,03	0,12
Σ n-3[3]	**15,77**	**14,85**	**16,12**	**12,85**	**1,15**	**0,38**	**0,26**	**0,50**	**0,10**	**0,34**
ΣPUFA[4]	**34,02**	**34,96**	**39,08**	**34,90**	**1,69**	**0,40**	**0,22**	**0,18**	**0,36**	**0,17**
EPA + DHA	12,61	11,92	13,70	10,28	0,86	0,51	0,11	0,75	0,04	0,15
n-3/n-6	0,86	0,74	0,71	0,60	0,08	0,43	0,19	0,09	0,16	0,98
Σ Others[5]	1,20	1,93	0,54	0,55	NA	NA	NA	NA	NA	NA

[1]Σ SFA includes 15:0, 17:0.
[2]Σ MUFA includes 15:1, 16:1n-7, 1, 6:1n-9, 17:1n-7, 17:1 Dimethyl, 20:1n-7, 20:1n-11.
[3]Σ n-3 includes 18:4n-3, 22:5n-3.
[4]Σ PUFA is the sum of Σ n-6 and Σ n-3.
[5]Σ Others correspond to the sum of the unidentified GC peaks in percentage values.
BDL: All below detection level.
BDL*: Some concentrations are below detection level.
NA: Not applicable.

Figure. 23 *shows the percentage of fatty acids16:0, 18:0, 18:1n-9, 20:4n-6, 20:5n-3 and 22:6n-3 in phospholipid class phosphatidylcholine (PI) of gill tissue of Atlantic salmon in the two production systems, RAS and FT and further shows how these fatty acids are affected by fatty acid composition of control diet and EPA diet. The percentage values are represented as means ± S.E.M. (n =6).*



The Phospholipid class PI in the gill tissue had higher percentage composition of 18:0 and 20:4n-6 (ARA) and lower composition of total n-3 PUFA and lower n-3 to n-6 ratio in general (figure.23).

The PI in gills of experimental fish in RAS was 10-18% lower 18:0 and 5-9% lower total SFA, 25-27% higher 18:1n-9 and 19-42% higher total MUFA than fish in FT system. The PUFA 18:2n-6 was 35-42% lower and total n-6 fatty acids had a tendency (0,06) to be lower in RAS than in FT. Similarly, the percentage composition of ARA was 14-29% lower in RAS compared to FT. However, the percentage of EPA and DHA were not significantly different between the systems. Additionally, the ratio between n-3 and n-6 fatty acids had a tendency (0,09) to be higher in RAS than in FT (table.13).

The percentage composition of EPA and ARA in gill PI were not significantly different between the diet groups. Additionally, diet had no effect on either the composition of SFAs or MUFAs. However, the DHA was 6-29% lower and 18:2n-6 was 35-42% higher in the fish fed with EPA diet than control group and this corresponds to the composition diet (table.13).

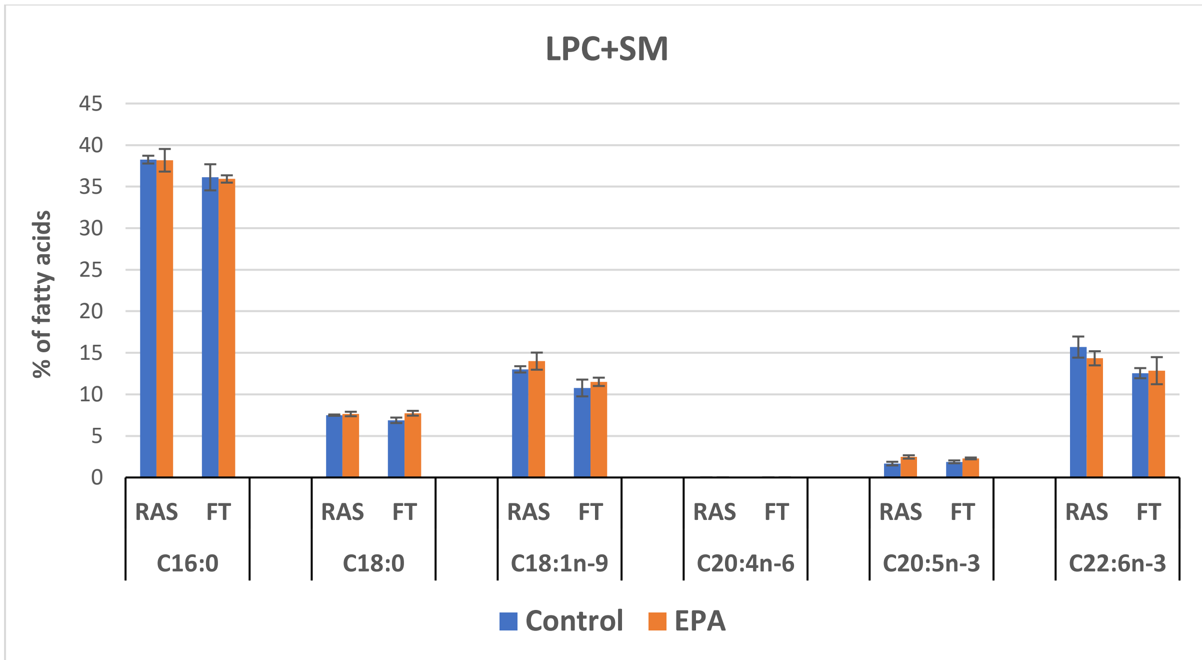
5.8.5. Lysophosphatidylcholine (LPC) + Sphingomyelin (SM)

Table.14 *Percentage composition of fatty acids in phospholipid class LPC+SM in the gill tissue of Atlantic salmon in the two production systems, RAS and FT, after feeding with control diet and EPA diet. A 2-way ANOVA is used to test for significant difference between the groups (n=6). The percentage values are represented in average. The standard error means (SEM) are represented as pooled S.E.M. P <,05 is considered as significant.*

	RAS		Flow-through				Two-way ANOVA P-values			
Variable	Control diet	EPA-diet	Control diet	EPA-diet	Pooled S.E.M	R^2	Model	System (FT×RAS)	Diet (Con× EPA)	Interaction
C14:0	2,00	1,78	2,25	1,95	0,19	0,28	0,43	0,30	0,21	0,83
C16:0	38,25	38,17	36,12	35,92	1,09	0,34	0,32	0,08	0,90	0,96
C18:0	7,50	7,64	6,88	7,73	0,26	0,45	0,17	0,35	0,09	0,21
Σ SFA[1]	**48,56**	**49,18**	**46,82**	**47,44**	**1,36**	**0,19**	**0,62**	**0,24**	**0,66**	**0,998**
C16:1n-7	BDL*	BDL*	BDL*	BDL*	NA	NA	NA	NA	NA	NA
C18:1n-9	13,01	14,00	10,77	11,51	0,79	0,56	0,07	0,02	0,31	0,89
C20:1n-9	BDL*	BDL*	BDL*	BDL*	NA	NA	NA	NA	NA	NA
C22:1n-11	BDL*	BDL*	BDL*	BDL*	NA	NA	NA	NA	NA	NA
Σ MUFA[2]	**26,61**	**25,82**	**28,35**	**26,75**	**1,16**	**0,24**	**0,51**	**0,28**	**0,33**	**0,74**
C18:2n-6	3,15	3,02	3,80	3,76	0,35	0,33	0,34	0,09	0,82	0,90
C20:3n-6	BDL*	BDL*	BDL*	BDL*	NA	NA	NA	NA	NA	NA
C20:4n-6	BDL*	BDL*	BDL*	BDL*	NA	NA	NA	NA	NA	NA
Σ n-6[3]	**4,55**	**4,34**	**4,97**	**5,25**	**0,44**	**0,25**	**0,49**	**0,17**	**0,93**	**0,59**
C18:3n-3	1,88	1,67	2,41	1,87	0,21	0,46	0,16	0,12	0,11	0,45
C18:4n-3	BDL*	BDL*	BDL*	BDL*	NA	NA	NA	NA	NA	NA
C20:5n-3	1,67	2,47	1,88	2,29	0,18	0,61	0,04	0,93	0,009	0,31
C22:5n-3	BDL*	BDL*	BDL*	BDL*	NA	NA	NA	NA	NA	NA
C22:6n-3	15,69	14,34	12,55	12,85	1,16	0,37	0,27	0,08	0,66	0,50
Σ n-3[4]	**20,27**	**20,12**	**17,76**	**18,47**	**1,50**	**0,20**	**0,59**	**0,20**	**0,86**	**0,78**
ΣPUFA[5]	**24,82**	**24,46**	**22,73**	**23,72**	**1,59**	**0,11**	**0,80**	**0,40**	**0,85**	**0,68**
EPA + DHA	17,36	16,81	14,42	15,14	1,28	0,30	0,38	0,11	0,95	0,63
n-3/n-6	4,74	4,64	3,57	3,59	0,56	0,33	0,33	0,08	0,94	0,91
Σ Others[6]	0	0,78	2,06	2,09	NA	NA	NA	NA	NA	NA

[1]Σ SFA includes 15:0, 17:0, 20:0, 22:0.
[2]Σ MUFA includes 15:1, C16:1n-9, 16:1n-7, 16:1T, 17:1n-7, 17:1 Dimethyl, 18:1n-9T, 18:1n-7, 20:1n-7, 20:1n-9, 20:1n-11, 22:1n-11, 24:1n-9.
[3]Σ n-6 includes 20:3n-6, 20:4n-6.
[4]Σ n-3 includes 18:4n-3, 22:5n-3.
[5]Σ PUFA is the sum of Σ n-6 and Σ n-3.
[6]Σ Others correspond to the sum of the unidentified GC peaks in percentage values.
The standard error means are represented as pooled standard error means (pooled SEM).
BDL*: Some concentrations are below detection level.
NA: Not applicable.

Figure. 24 *shows the percentage of fatty acids16:0, 18:0, 18:1n-9, 20:4n-6, 20:5n-3 and 22:6n-3 in phospholipid class phosphatidylcholine (LPC+SM) of gill tissue of Atlantic salmon in the two production systems, RAS and FT and further shows how these fatty acids are affected by fatty acid composition of control diet and EPA diet. The percentage values are represented as means ± S.E.M. (n =6). In LPC+SM the concentration of fatty acid 20:4n-6 was below detection level.*



The SM fraction was not able to separate from the LPC using TLC method. The presence of higher percentages of SFA in this mixture suggests a relatively higher composition of SM than LPC.

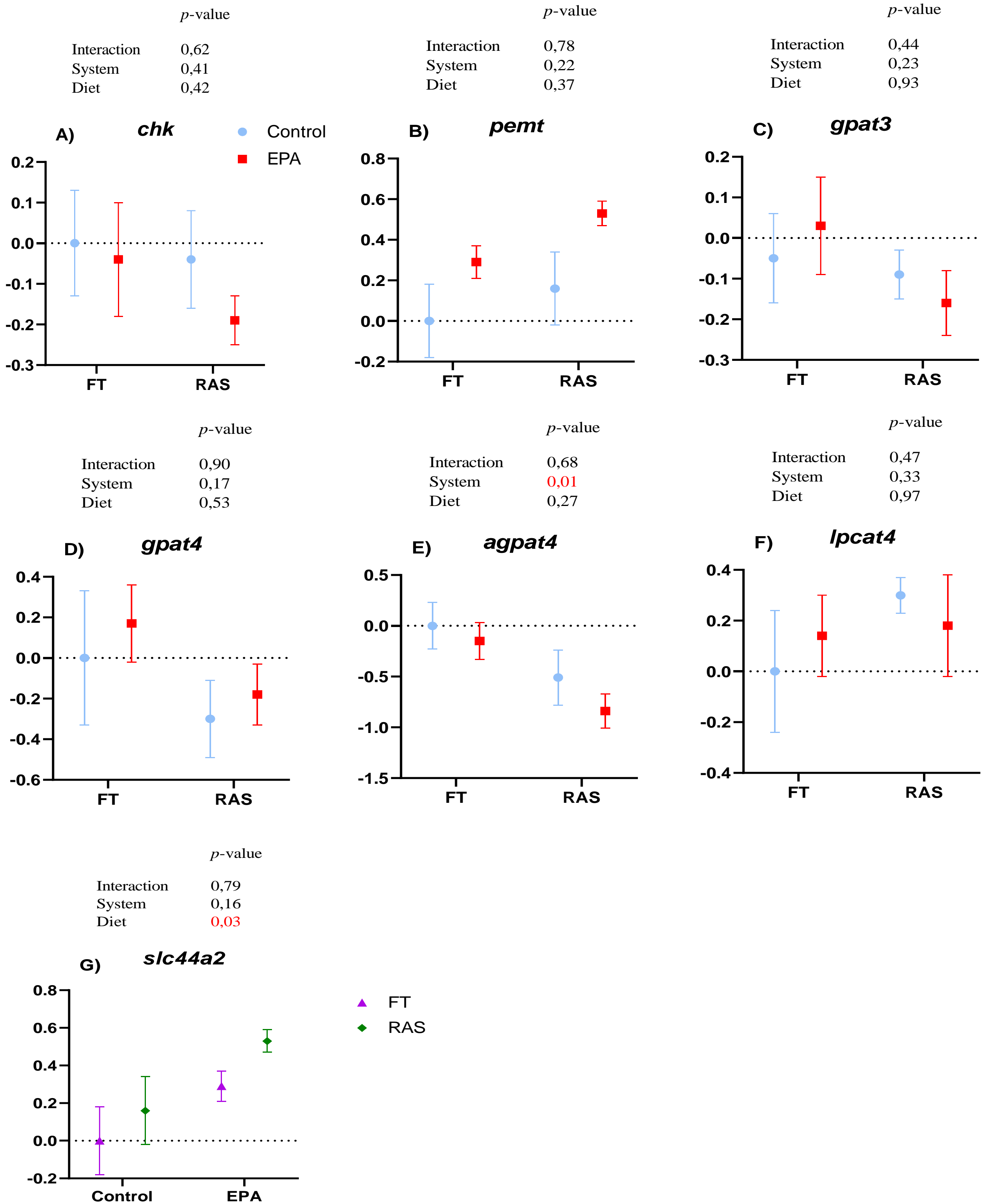
This mixture was characterized by a high percentage of SFA 16:0 as mentioned above. The percentage composition of total SFA was 2-fold higher than total PUFA (figure. 24)

In this mixture, the percentage composition of 18:1n-9 was 21% higher and 16:0 had a tendency to be higher in RAS compared to FT. The PUFA and EPA were not significantly different between the production systems. However, the DHA (0,08) and ratio between n-3 and n-6 (0,08) had a tendency for increase in RAS than in FT system (table.14).

The fish fed with EPA diet had 22-48% higher EPA in gill SM of fish fed with EPA diet than in control group. However, the remaining fatty acids were not significantly different between the diet groups (table.14).

5.9. Gene expression

Figure.25 *shows the relative expression of genes involved in phospholipid synthesis and remodeling in the gill tissue of Atlantic salmon in the two production systems, RAS and FT and further shows how these genes are affected by fatty acid composition of control diet and EPA diet. Relative expressions of A) chk, B) pemt, C) gpat3, D) gpat4, E) agpat4, F) lpcat4, G) slc44a2 are presented. A 2-way ANOVA is used to test for significant difference between the groups (n=6). The percentage values are represented in means ± S.E.M. P <,05 is considered as significant.*



Gene expressions of *slc44a2, chk, pemt, gpat3, gpat4, agpat4, lpcat4* were assessed in the gill tissue of all 4 treatment groups (figure.25). These genes were selected based on their relevance and implications in phospholipid *de novo* synthesis and remodeling pathway. The result showed that the relative expression of *agpat4* was lower in RAS compared to FT (figure.25E), while other genes showed no significant difference between the production systems. In addition to that, the fish fed with EPA diet had a higher relative expression of *slc44a2* in the gills (figure.25G), whereas no significant difference was observed for other genes between the diet groups.

6. Discussion

This study has, as the first of its kind, explored the effects of two production systems and two different diet compositions, RAS and FT, and different dietary EPA levels respectively, on fish growth and lipid composition of gill tissue. The study primarily aimed to provide a better understanding of how different production systems and diet composition impact the membrane dynamics of fish gills, which function as a barrier tissue. The results of this study can have significant implications for the aquaculture industry, as changes in the lipid composition of gill tissue can influence the overall health and productivity of fish.

6.1. Growth and survival

The study showed that only the production systems, but not dietary EPA levels, affected fish growth. Atlantic salmon post-smolts produced in the RAS system and FT system at the same water temperature, oxygenation level and water velocity showed a difference in growth, where fish in the RAS had lower growth rate than fish in FT. The stocking density at the end of the experiment was 40 kg/m^3 for RAS and 43 kg/m^3 for the FT system. However, previous studies of Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) in RAS and FT system showed no significant difference in growth performance at a stocking density of 60 kg/m^3 (d'Orbcastel, Blancheton, *et al*., 2009; Kolarevic *et al*., 2014). While the rainbow trout from the RAS system showed a higher growth compared to FT system at stocking density higher than 85 kg/m^3 (d'Orbcastel, Person-Le Ruyet, *et al*., 2009). The reduction in growth in RAS in our study can possibly be explained by the lower amount of feed eaten in the RAS than in the FT (figure.13). Feed intake measurements were not done in this trial; therefore, we don't know the exact feed intake in each tank and feed conversion ratio (FCR) could not be calculated in this study. However, appetite reduction in salmonids is considered as one of the prominent behavioral responses towards stress (Overli *et al*., 1998). The prevalence of environmental stress in RAS compared to FT is well documented (Martins *et al*., 2011; Robinson *et al*., 2021) in Atlantic salmon, which may indicate that the lower growth rate of fish in RAS than in FT in our trial may possibly be explained by higher stress level of fish in RAS.

There were very low mortalities in the trial, only two fish were died in FT Tanks fed with EPA diet at the same date. There is no clear reason for this mortality, it is most likely a random incident not linked to experimental treatments. However, earlier studies in larvae of tilapia and Atlantic cod have shown a higher mortality in FT than in RAS because of the more unstable conditions in FT (Attramadal *et al*., 2012; Deng *et al*., 2022)

6.2. High accumulation of magnesium (Mg^{2+}) in RAS

Digestibility analysis of feacal matter of the fish in RAS compared to FT showed a significantly higher amount of Mg than that supplemented through the feed. This is an indication of accumulation of these minerals in the RAS system and subsequent accumulation of these minerals in faeces through drinking of this water (figure.14). This result is in line with the finding of Palm *et al*., (2018), who reported that Ca and Mg enter the production systems not only through the feed, but mainly with the water exchange and these minerals are constantly precipitated and therefore are disproportionate to feed input in appearance.

6.3. Higher ARA in total lipid content of gill tissue in RAS

The omega-6 fatty acid, ARA is the precursor of proinflammatory eicosanoids and equally crucial for various physiological functions in fish, including growth, stress tolerance, reproduction, immunity, pigmentation and bone formation (Xu *et al*., 2022). In the present study, the relative values of ARA in TFA of gills were higher in RAS than in FT (table.8, figure.15). In addition, the lower levels of 18:2n-6 in RAS than in FT support a higher metabolic conversion of this fatty acid into ARA. Further, lower levels of ARA in PL classes, in particular PI and higher levels of ARA in total may indicate that this fatty acid is found in the free fatty acid fraction of total lipids and released for potential eicosanoid production. However, this assumption will be verified with analysis of eicosanoids production in the gills (not included in this thesis). Several studies have shown the relationship between stress and ARA. A similar elevated levels of ARA observed in body tissue of striped bass larvae was associated with elevated levels of whole-body cortisol (Harel *et al*., 2001). On the other hand, increased dietary inclusion of ARA from 1.5% to 6.3% TFA showed a significant down-regulation of genes related to cortisol synthesis in European sea bass (*Morone saxatilis*) larvae (Montero *et al*., 2015). Additionally, a significant reduction in stress response was achieved after increasing the diet concentration of ARA from 0.9% to 2.4% TFA in gilthead seabream (*Sparus aurata*) (Van Anholt *et al*., 2004). It may be possible that the elevated levels of ARA in the gill tissue was an adaptive mechanism to increased cortisol levels. The finding of moderately increased cortisol levels in faeces of fish in RAS compared to fish FT study (results not included in this thesis), support the assumption of a link between ARA and low-grade stress in this trial. Cortisol is a crucial hormone that indicates stress in fish, produced by the hypothalamic-pituitary-inter renal axis in response to external stimuli. Previous studies have shown that plasma cortisol and faeces cortisol and cortisol metabolites can serve as reliable indicators of stress levels in fish (Strange & Schreck, 1978).

6.4. **Higher PC/PE ratio and tendency to lower relative PE in RAS**

The ratio between PC and PE is considered as the key regulator of membrane integrity (Li *et al*., 2006). Several studies have documented variations in PC/PE ratio in response to the environmental stressors such as temperature and salinity (Cordier *et al*., 2002; Hazel, 1990; Tocher *et al*., 1995; Tocher & Sargent, 1990). In fish, during the acclimatization to low temperature, the proportion of PE increases and that of PC decreases in response to maintaining the fluidity (Hazel & Williams, 1990). However, no studies have investigated the variations in PC/PE ratio in fish between production systems like RAS and FT so far. In our study, the gill tissue of fishes in RAS were characterized with a higher PC/PE ratio (Table.19) and tendency for lower relative PE ($P = 0{,}07$) composition than gills of fish in FT.

The physiological impact of the change in membrane PC/PE on gill function is not possible to interpret based on our data. The PC and PE are the major phospholipids in the endoplasmic reticulum (ER) membrane. The ER is the initial part of secretory pathway and plays a crucial role in folding and modifying proteins for their secretion or integration into cellular membranes. The ER membranes are fluid-like, and the fluidity is necessary to enable the movement of protein molecules in and out of the ER compartment. A higher PC/PE ratio will result in an accumulation of the unfolded proteins in the lumen of ER, and this will trigger a stress response which is called unfolded protein response (UPR) (Patel & Witt, 2017; Ron & Walter, 2007). In mice liver, higher molar ratio of PC/PE leads to ER stress and obesity (Patel & Witt, 2017). However, lower ratio causes leakage of membrane of hepatocytes, and which again leads to steatohepatitis (Vance, 2013). Therefore, the PC/PE ratio is considered as an indicator of ER stress.

PE is a very important phospholipid in the gills of fish. The previous *in vivo* experiments with eels (*Anguilla anguilla*), rainbow trout (*Oncorhynchus mykiss*), crustaceans (*Eriocheir sinensis*) and toads (*Bufo bufo*) have shown (Chapelle & Zwingelstein, 1984; Hansen, 1991; Hansen, 1987) a clear connection between PE synthesis and osmoregulation in fish and crustacean gills, fish esophagus and amphibian skin. PE has been proposed to contribute to the stabilization of ion channel proteins as it has been shown to enhance the integration of membrane proteins and lipids in an *in vitro* model (Bazzi *et al*., 1992). This suggests that impaired PE synthesis may affect the osmoregulatory function of the fish. Even though the PC/PE ratio in RAS was moderately higher than in FT, no major negative effect on fish performance was observed during our study. The main project report will include all data on gill morphology.

6.5. Effect of AGPAT4 on phospholipid levels and PC/PE ratio in RAS

Acylglycerophosphate acyltransferase/lysophosphatidic acid acyltransferase (AGPAT/LPAAT) family is a group of proteins that have been identified according to their homologous sequence where AGPAT4 is known to localized in mitochondria and ER, and described to indirectly regulate brain PC, PE, and PI levels in mammals, however its function in fish gills is not known (Bradley *et al*., 2015).

In our study, the gene coding for the enzyme AGPAT4 had lower expression in gills of fish in RAS than in FT (figure. 25E). Previous study in mice brain tissue revealed that knock-down of AGPAT4 did not result in any change in brain PA but resulted in a decrease in the levels of PI, PC and PE (Bradley *et al*., 2015). In contrast to this, in the present study, no significant changes in the levels of PC and PI except PE were observed with lower expression of this specific gene. There are 11 homologs for the AGPAT that have been identified so far and many tissues express more than one AGPAT/LPAAT isoform (Takeuchi & Reue, 2009). Besides that, AGPAT isoforms from 6-11 have been reported to have additional acyltransferase enzyme activities (Prasad *et al*., 2011). Our results may indicate that the AGPAT homologs in salmon gill tissue may be involved in the regulation of PC/PE ratio.

The choline transporter like proteins 2 (SLC44A2) which belong to the solute carriers 44A (SLC44A) family are known to facilitate the transport of choline and ethanolamine across both the plasma membrane and mitochondria for the *de novo* synthesis of PC and PE (Taylor *et al*., 2021). Our results showed a higher SLC44A2 in the gills of fish fed with higher EPA diet (figure. 25G). Further, Richardson & Wurtman, (2007) previously reported that an increased synthesis and levels of PC in undifferentiated adrenal phaeochromocytoma (PC12) cell lines in response to increased availability of PUFAs such as ARA, EPA and DHA. However, in our study, the levels of PL classes remain unchanged in response to standard and higher dietary EPA.

6.6. Effect of genes on the altered composition of PUFAs in PL classes in RAS

In this study, major fatty acids in PC of Atlantic salmon gills were 16:0, 18:1n-9, EPA, and DHA. On the other hand, PE had lower levels of saturated fatty acids, particularly 16:0, and higher levels of DHA. PS exhibited a higher concentration of 18:0 and DHA, while PI was characterized by a dominance of 18:0 and ARA (figure 10,11,12&13). These results agree with Ghioni *et al*. (1997), who reported similar composition of fatty acid in skin and opercular membrane of rainbow trout.

In general, saturated fatty acids such as 16:0 and 18:0 are esterified at the sn-1 position, whereas PUFAs such as ARA, EPA, and DHA are commonly esterified at sn-2 position (MacDonald &

Sprecher, 1991). Although not studied in fish, the role of AGPAT4 (also called LPLAT4 or LPAAT4) has been studied in many tissues including brain, muscle, and white adipose tissue (WAT) and its role in incorporation of unsaturated fatty acids into sn-2 position of lysophosphatidicacid (LPA) to produce PA has been demonstrated (Eto *et al*., 2014; Takeuchi & Reue, 2009). However, an *in vitro* study in mice showed that lack of AGPAT4 enzyme barely affects the fatty acid profile of PC and PE (Bradley *et al*., 2015). This is, however, contradictory to our results where the fatty acid composition of phospholipid classes in the gill tissue showed some variation between the production systems. In general, all PL classes had a relatively lower percentage of unsaturated fatty acids (ARA, EPA or DHA) and higher saturated fatty acids (16:0 or 18:0) except PI, which had a lower 18:0. The gill phospholipids classes of salmon in RAS had lower EPA in PC and lower EPA and ARA in PE, lower DHA in PS and a lower ARA in PI.

The phospholipids containing PUFAs such as ARA, EPA and DHA are the main sources of fatty acid-derived lipid mediators. Lyso-PC acyltransferase 2 (LPCAT2), LPCAT3, lyso-PI acyltransferase 1 (LPIAT1), lyso-PA acyltransferase3 (LPAAT3) and LPAAT1 are known to incorporate PUFA into lysophospholipids during the phospholipid remodeling. The LPLAT family except AGPAT4 (only utilize lyso PA) utilizes all the major lyso phospholipids as their substrates and often shows overlapping substrate specificity (Hishikawa *et al*., 2014). For instance, the knockdown of LPCAT 3 (MBOAT5) resulted in a reduction in ARA incorporation into PC, PE and PS but not PI, in human HeLa cells (Matsuda *et al*., 2008), whereas LPAAT3 (AGPAT3) has preference for ARA and has a role in both *de novo* and remodeling pathways (Yuki *et al*., 2009). In humans, LPAAT3 shows acyltransferase activity towards LPC, LPS and LPI, only when ARA used as an acyl donor (Prasad *et al*., 2011). On the other hand, LPIAT1 (MBOAT7) catalyzes the incorporation of ARA and EPA into LPI in nematodes (*Caenorhabditis elegans)* (Lee *et al*., 2008). In addition to that, Mice deficient in LPIAT1 showed a decrease 18:0 and ARA (Anderson *et al*., 2013; Lee *et al*., 2012) and similar trend was observed in the present study also. *In vitro*, LPAAT1 has been shown to incorporate DHA in both LPC and LPE (Abe *et al*., 2014).

Although none of the above-mentioned genes are studied in fishes, it may be possible that the PUFA composition in gills may be harmoniously determined by the activity of these genes together with AGPAT4. At the same time, It is also worth mentioning that most of the studies related to the genes involved in phospholipid remodeling for incorporation of PUFA, were done either in mammals or in other species, in which the ARA is the major PUFA but not EPA (Lee *et al*., 2008). The fall in the PUFA level especially ARA, EPA and DHA in all PL classes in our study shows that RAS production system may be altered the expression of a large number of genes involved in phospholipid remodeling. The phospholipids are the major source for PUFA, which are the

substrates for the synthesis of eicosanoids, especially ARA. The levels of fatty acids particularly EPA, DHA and ARA are crucial for maintaining the health of the fish as they are involved in various process such as the synthesis of cells, ontogenesis, regulation of endocrine and immune systems, pigmentation, and development and function of neural tissue (Bou *et al.*, 2017; Glencross, 2009). Our study gives very limited understanding of the mechanism behind the changes in PUFA levels in phospholipid classes. Since the lower PUFA in RAS may affect the robustness of the fish, there is a need for further study.

On the other hand, the gills of fish fed with different diets reflected the fatty acid composition of the diet. The EPA diet contained higher levels of EPA and ARA, this was reflected in the PC and PE. However, EPA and ARA composition in PS and PI were not seen to be affected by the dietary composition of these fatty acids. This was in line with what was shown by Ruyter (2000), who reported earlier that PS and PI are more conserved and resistant to dietary fatty acid changes.

7. Conclusion

In the present study, a lower growth rate and a higher level of ARA in the total fatty acids of Atlantic salmon gill tissue were found, which supports the assumption that RAS provides a more stressful environment than FT. Moreover, our study shows that the RAS environment has altered the composition of gill membrane phospholipids by increasing the PC/PE ratio and changing the levels of fatty acids in phospholipids, especially PUFAs by altering the genes associated with phospholipid synthesis and remodeling. Based on our current results, it is not possible to conclude that all the observed changes were solely caused by stress in the RAS. Since the PUFAs especially ARA, EPA and DHA play a crucial role in maintaining the health and welfare of the Atlantic salmon, we recommend further investigation, specifically targeting the study of stress-induced alterations in genes that are associated with PUFA incorporation to phospholipid classes during synthesis and remodeling.

8. Reference

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