Dietary fatty acids and their ratio: impact on Atlantic salmon health, mineral status and intestinal lipid transport

Chandrasekar Selvam

Thesis for the degree of Philosophiae Doctor (PhD)
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Scientific environment

This Ph.D. project started in November 2018 as a collaboration between the Department of Biology at the University of Bergen (UiB) and the research group Requirement and Welfare at the Institute of Marine Research in Bergen (IMR). The work for this doctoral thesis was performed under the supervision of Dr. Nini H. Sissener (IMR), Dr. Øystein Sæle (IMR), Dr. Antony J. Prabhu Philip (IMR), and Prof. Rune Waagbø (IMR, UiB) at the Institute of Marine Research (IMR), Bergen, Norway.

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Chandru

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Abstract

The rapid growth of the aquaculture industry and the sharp decline of capture fisheries necessitates finding alternative sources for fish oil and fish meal in aquafeeds. However, the inclusion of VOs in aquafeed alters the dietary fatty acid composition, significantly reducing the amount of essential n-3 LC-PUFA, inducing the n-3/n-6 ratio, and increasing the MUFA contents. In the last few decades, numerous studies have been conducted to demonstrate the possibility of partial or complete replacement of FO with vegetable oils (VOs) without any adverse effects on the growth and welfare of the fish, provided sufficient n-3 LC-PUFA from other dietary sources. However, most feeding trials are run in controlled, stable environmental conditions, where there is minimal stress on fish. In contrast, under demanding environmental conditions in sea cages, fish are exposed to various stressors, including fluctuating water temperatures, handling, parasitic pressure, delousing, etc. Therefore, we need increased knowledge on how optimal FA nutrition can be used to maintain a healthy and robust fish that can cope with stressful situations, such as fluctuating environmental conditions and disease pressure. Besides the change in dietary FA profile, increased inclusion of plant ingredients also reduces the supply and availability of dietary minerals to fish. Further, little is known about how this change in the FA profile affects the intracellular fate of these fatty acids in intestinal cells. Therefore, this Ph.D. project investigates how stressful conditions combined with the change in dietary FA level affect the absorption and intracellular fate of dietary fatty acids, stress and immune responses, and the utilization of minerals in the fish.

In the present thesis, the three trials were conducted i) short-term challenge experiment, ii) long-term seawater trial, and iii) in vitro trial in RTgutGC cells. In the short-term challenge trial (**Paper I**), Atlantic salmon were fed diets containing different ratios of n-6/n-3 FA (at 1.3, 2.4, and 6.0 and one diet with a ratio of 1.3 combined with a higher level of n-3 FA and n-6 FA) and challenged with amoebic gill disease (AGD). In the long-term seawater trial (**Paper II**), Atlantic salmon were fed diets containing graded levels of EPA+DHA (10, 13, 16, and 35 g/kg of feed, and one diet with 13g/ kg of EPA+DHA with reduced total fat content) and fish were subjected to physical stress

during delousing. In both trials (**Paper I** and **Paper II**), diet did not affect growth under optimal conditions, while a high n-6/n-3 ratio (6:1) (Paper I) and low EPA+DHA (10-16 g/kg of feed) (**Paper II**) diet had negative impact on growth under AGD challenge and delousing stress, respectively. Furthermore, despite AGD challenge and delousing stress altering the mRNA expression inflammatory and immune marker and oxidative stress markers, both dietary n-6/n-3 ratios and EPA+DHA levels did not alter the expression of any of these genes. In the challenge trial, disease progression, gross gill score, and associated gill pathology were much lower compared to previously reported studies, suggesting the possible genetic resistance of the experimental fish against AGD. In the long-term trial (**Paper II**), despite increased plasma cortisol level after delousing, dietary EPA+DHA levels had no effect on plasma cortisol, vertebrae deformities, or any other external welfare indicators. Interaction of dietary EPA+DHA on tissue trace mineral status was assessed at delousing (~2.5 kg) and at harvest stage (~ 5 kg). The liver Se, Zn, Fe, Cu, and Mn and plasma Se levels were increased in fish fed a diet high in EPA+DHA (35 g/kg of feed) upon delousing stress. Further, a high dietary EPA+DHA also significantly increased the whole-body Zn, Se, and Mn levels at harvest size fish.

This thesis is one of first attempt to use fish intestinal cells (RTgutGC cells) as an in vitro model to study the intracellular fate of FA upon uptake and transport (**Paper III**), following the 3R principles. The RTgutGC cells offered oleic acid accumulated higher amounts of TAG in the cells, and lead to higher (x6 times) cytosolic lipid droplets (CLDs) accumulation. The accumulation of TAG in CLDs were lower for arachidonic acid (ARA) and palmitic acid (PA) compared to oleic acid (OA). The lower uptake of OA from apical compartment than other FAs (PA and ARA) might indicate the negative consequence of excessive CLDs accumulation and physical barrier to uptake of this FA. A significant amount of ARA was transported as TAG to basolateral compartment and suggesting the better regulated transport for LC-PUFA. Based on the results of this Ph.D. work, it could be concluded that low dietary EPA+DHA (10-16 g/kg of feed) and a high n-6 /n-3 FA ratio (6:1) can have negative impact on growth under challenging environmental conditions. Thus, the optimal dietary n-3 FA needs to be revisited under different challenging situations, and the balance of n-6/n-3 FA in the diet is an important

factor that needs to be considered while formulating the diet for Atlantic salmon. Higher inclusion of EPA+DHA (35 g/ kg) in the diet increased the trace mineral levels in plasma, liver, and whole body. Knowledge on the interaction between dietary fatty acids on body mineral stores is crucial for the aquaculture industry to improve the bioavailability and status of these minerals in fish and limit their discharge into the environment. Further, results from this thesis increased the Knowledge on how uptake and transport of FA are affected by their chain length and saturation level. The lipid fraction in current commercial diets for Atlantic salmon contains higher inclusion of rapeseed oil, resulting in a major reduction in SFA and LC-PUFA and increased MUFA levels in diets. This thesis demonstrated that higher inclusion of VOs rich in MUFA results in excessive accumulation of large lipid droplets in the enterocytes, reducing fatty acid absorption efficiency.

List of publications

Paper I

Selvam C, Powell MD, Liland NS, Rosenlund G, and Sissener NH. 2021. Impact of dietary level and ratio of n-6 and n-3 fatty acids on disease progression and mRNA expression of immune and inflammatory markers in Atlantic salmon (*Salmo salar*) challenged with *Paramoeba perurans*. PeerJ 9:e12028. doi.org/10.7717/peerj.12028

Paper II

Selvam, C., Antony J Prabhu, P., Lutfi, E., Sigholt, T., Norberg, B., Bæverfjord, G., Rosenlund, G., Ruyter, B., Sissener, N.H., 2022. Long-term feeding of Atlantic salmon with varying levels of dietary EPA+DHA alters the mineral status but does not affect the stress responses after mechanical delousing stress. British Journal of Nutrition, 1-42. doi.org/10.1017/S0007114522000514 doi.org/10.1017/S0007114522000514

Paper III

Selvam, C., Takaya Saito, Nini H Sissener, Antony J. Prabhu Philip, Øystein Sæle. Intracellular trafficking of fatty acids in the fish intestinal epithelial cell line RTgutGC. **Manuscript.**

Abbreviations

AGD	Amoebic gill disease
ARA	Arachidonic acid
BSA	Bovine serum albumin
BSDL	Bile salt dependent lipase
Caco-2	human colon adenocarcinoma cell line
CLD	Cytosolic lipid droplets
COX	Cyclooxygenase
DHA	Docosahexaenoic acid
DPC	Days post-challenge
EPA	Eicosapentaenoic acid
FA	Fatty acids
FABP	Fatty acid binding proteins
FATP	Fatty acid transport protein
FFA	Free fatty acids
FM	Fish meal
FO	Fish oil
HPI	Hypothalamic-pituitary-interrenal axis
LC-PUFA	Long chain polyunsaturated fatty acids
LOX	Lipoxygenase
LTB	Leukotriene B
MAG	Monoacylglycerol
MUFA	Monounsaturated fatty acids
n-3	Omega 3
n-6	Omega 6
OA	Oleic acid
PA	Palmitic acid
PGE_2	Prostaglandin E_2
PL	Phospholipid
PLINs	Periplins
RTgutGC	Rainbow trout intestinal epithelial cell line
SFA	Saturated fatty acids
TAG	Triacylglyceride
VLC-PUFA	Very long chained polyunsaturated fatty acids
VO	Vegetable oil

1. Introduction

1.1. General introduction

Aquaculture is one of the rapidly growing food sectors in the world, contributing 46% of global fish production in 2018 (FAO, 2020) (Figure 1). Salmonids are one of the most valued farmed species contributing 4.6% of global seafood supply (Salmon farming industry Handbook, 2021). Among the salmonids, Atlantic salmon is most widely farmed. Global production of Atlantic salmon reached almost 2.6 million tons in 2019. Norway is the leading producer of Atlantic salmon, reaching about 1.3 million tons (Norwegian Directorate of Fisheries, 2019). The Norwegian salmon industry uses nearly 1.6 million tons of feed every year (Aas et al., 2019). However, salmon nutrition faces current issues in developing high energy-low pollution feeds, efficient feed conversion, and sustainable ingredients replacing fish meal (FM) and fish oil (FO) (Aas et al., 2019). Therefore, for an efficient aquaculture production, high quality feed that meets the nutritional demand of salmon is essential.

1.2. Feeds for Atlantic salmon

Rapid growth of the aquaculture industry has raised concerns about its sustainability and environmental impacts. One concern is the high reliance on marine ingredients such as FM and FO in aquafeeds, increasing the pressure on the already vulnerable global fisheries, which is being fully or over exploited. As a result, there has been a significant shift in dietary composition in the last two decades, replacing most marine ingredients with other available ingredients, such as vegetable oils (VOs) and plant proteins. For example, feeds for farmed Atlantic salmon have changed from essentially a marine-based diet in the early 90s to a diet with more than 80% plant ingredients (Figure 2). Concurrently, the protein/fat ratio in the diet also gradually changed from 3:2 to approximately 1:1 today (Aas et al., 2019). However, most alternatives to FO in salmon diets are VOs, completely lacking the very long-chained polyunsaturated fatty acids (>20C fatty acids, VLC-PUFA, e.g., eicosapentaenoic acid, 20:5n-3, EPA; docosahexaenoic acid, 22:6n-3, DHA; arachidonic acid, 20:4n-6, ARA), but contain

high levels of n-6 fatty acids (18:2n-6) and monoene fatty acids. For example, the lipid content in the current commercial diets for Atlantic salmon is based on 70 % of rapeseed oil, which contains a higher amount of monounsaturated FA (MUFA) (oleic acid, 18:1n-9). Likewise, the other VOs used in Atlantic salmon diets, such as palm oil is high in saturated FA (SFA) (palmitic acid, 16:0), soybean and sunflower oils are high in the n-6 FA (linoleic acid, 18:2n-6). Thus, the shift in the ingredient composition, mainly increased VOs in salmon feed at the expense of FO dramatically affects the FA profile in the feeds, with decreasing LC-PUFA (EPA and DHA) levels and increasing both absolute content of MUFA and n-6 FAs as well as the dietary n-6/n-3 ratio.

Several studies in Atlantic salmon have demonstrated the possibility of partial or complete replacement of FO with VOs without any negative effects on fish performance and welfare (Hixson et al. 2017; Katan et al. 2019; Liland et al. 2013; Sissener et al. 2016; Torstensen et al. 2005; Turchini et al. 2009). However, in a long-term trial in sea cages, Atlantic salmon subjected to delousing stress had significantly increased mortality in fish fed low EPA+DHA (≤10 g/kg) in the diet than fish fed a higher EPA+DHA diet (16 g/kg of feed) (Bou et al., 2017b). In comparison, 11 g/kg feed EPA+DHA seemed to be sufficient for salmon during seawater phase in land-based tanks, and despite some negative health effects even salmon fed only 5 g/kg feed had survival rates ≥99% (Rosenlund et al., 2016). This clearly shows that the robustness of salmon fed low dietary EPA and DHA needs to be tested in the challenging conditions experienced by the fish in sea cages rather than the controlled and stable conditions of land-based tanks.

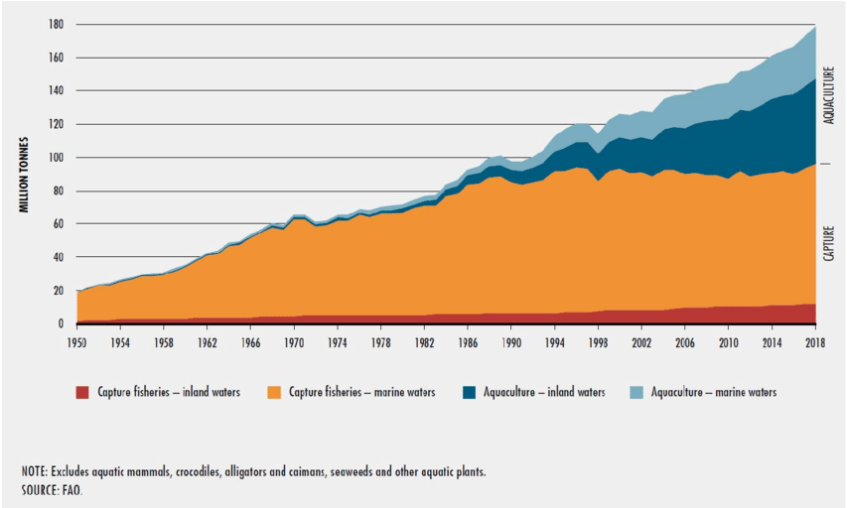

Figure 1. Development of world capture fisheries and aquaculture production. Adapted from FAO (2020).

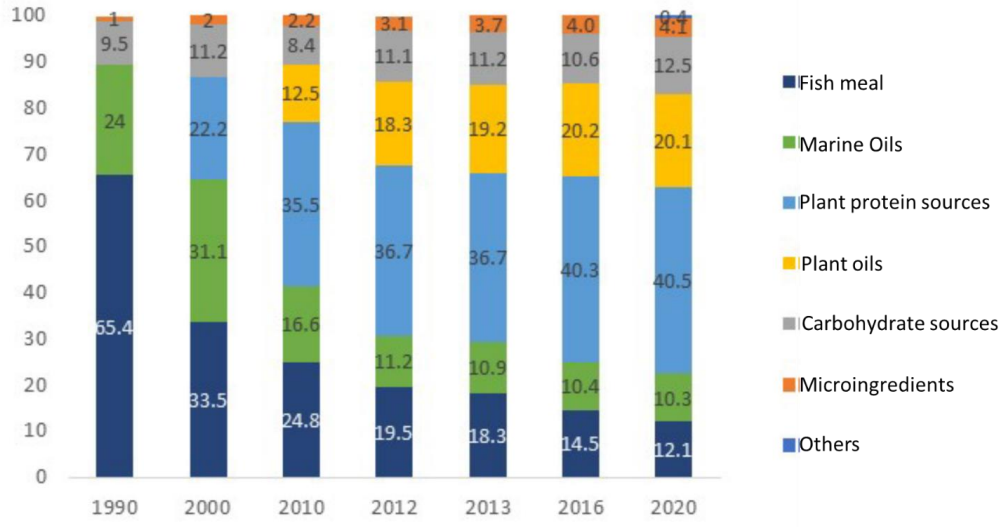

Figure 2. Ingredients used in Norwegian salmon feed from 1990 to 2020, given as% of feed. Adapted from Nofima (2022).

1.3. Challenges in salmon farming

Even though the salmon industry is closely monitored and regulated, there are challenges faced by the industry that can lead to significant production losses (Sommerset et al., 2021). In recent years, one of the major challenges that has been a point of focus on the growing salmon industry is the control of diseases and parasites. Among the parasitic infection, sea lice and amebic gill disease have caused significant problems in recent years in the salmon industry. Furthermore, both parasitic infection and subsequent management control or treatment can significantly affect the welfare of the fish.

1.3.1. Gill health and amoebic gill disease (AGD) in farmed Atlantic salmon

Gill health attributing to fish well-being represents a significant challenge in marine and freshwater finfish aquaculture globally. The fish gill is a multipurpose organ that is responsible for many critical physiological functions, including gas exchange, osmoregulation, acid-base regulation, excretion of nitrogenous wastes, and plays a vital role in immune function and hormone production (Evans et al., 2005; Rombough, 2007). However, fish gills are constantly exposed to external environment and often encounter various infectious and noninfectious agents which can cause gill diseases. Poor gill health can lead to increased mortality during treatment, disease susceptibility, and reduced performance and welfare of the fish. Amoebic gill disease, caused by the protozoan *Paramoeba perurans* (syn. *Neoparamoeba perurans*; Feehan et al., 2013), is established in many salmon producing areas and poses a serious challenge to salmonids farming (Marcos-López and Rodger, 2020).

AGD and other infections represent a source of progressive stress for salmon (Nowak and Archibald, 2018; Robledo et al., 2020), and contribute around 20% as an additional cost for disease control in Atlantic salmon farming (Nowak, 2018). The parasite colonizes the gills and induces the proliferation of lamellar epithelium and increases mucus production making the fish difficult to breathe (Nowak and Archibald, 2018; Powell et al., 2008). The pathological symptoms include edema, decreased chloride

cells, and infiltration of neutrophils (Nowak and Archibald, 2018; Taylor et al., 2009). Gill scoring is often employed in salmon farms for monitoring the severity and stage of disease (Taylor et al., 2009). Regular monitoring can be a useful aid in early diagnosis and offering appropriate treatment. The disease can be diagnosed by lethargy, elevated body position in the water column, high rate of respiration and enhanced opercular movement with white or grey mucoid patches on the gill surface. It has been observed that high temperature, and salinity facilitate disease development. Fresh water and hydrogen peroxide exposure are the currently available treatments for AGD (Rodger, 2014), among which freshwater treatment proved to be an effective option. Multidisciplinary approach in health management and advances in nutrition is a contributing factor in controlling AGD (Marcos-López and Rodger, 2020).

1. 3. 2. Sea lice and delousing stress in farmed Atlantic salmon

The salmon lice (*Lepeophtheirus salmonis*) are naturally occurring injurious ectoparasites that attach to the skin of salmon, feeding on mucus, blood, and skin. High infestations can lead to direct injury, secondary infestations, and immune suppression and negatively affect salmon health and welfare (Overton et al., 2019). Permissible lice number to be maintained is below 0.2 mature females per salmon during spring and 0.5 for the rest of the year. According to the Norwegian lice surveillance programme, all the farms need to update their lice management plans to the Norwegian Food Safety Authority (Sommerset et al., 2021). Delousing often involves removing the lice using chemotherapeutics, hydrogen peroxide, or a thermal and mechanical process. Among different available methods, thermal and mechanical delousing (physical removal) are the most frequently applied methods for the immediate removal of salmon lice and have been discussed as one of the reasons for the increased mortality (Bang Jensen et al., 2020). The procedure of de-lousing includes crowding, handling, transportation, or confinement, thereby creating a series of stressful conditions resulting in direct physical/mechanical injury to gills, fins, eyes, skin, etc., which might cause a considerable challenge to fish welfare (Overton et al., 2019). Fish weight and

temperature are also important factors influencing the fish mortality after delousing. Medicated feeds and anti-lice lasers are also used to prevent the sea-lice infestation (Hjeltnes et al. 2018). It is important to know about the effect of different treatment methods and their side effects on deciding the type of treatment for delousing. Detailed scoring for the fish health (Stien et al. 2013; Folkedal et al. 2016), disease status of fish, and consistent reporting of the treatment method are some of the future recommendations for improving the delousing efficiency thereby fish welfare.

1.4. Effects of dietary fatty acids on stress response in fish

The primary response to any stressor involves the induction of a neuroendocrine cascade system, which involves the synthesis and secretion of stress hormones, importantly corticosteroids (cortisol) and catecholamines (adrenaline and noradrenaline). When a stressor is perceived, the hypothalamic-pituitary-interrenal (HPI)-axis becomes active, which in turn releases the Corticotropin Releasing Factor (CRF) from the hypothalamus. In the pituitary, CRF through its receptor CFR-R1 induces the release of adrenocorticotropic hormone (ACTH) into the blood stream. The released ACTH induces the synthesis and release of cortisol from the interrenal tissue as a final product of primary stress response (Schreck and Tort, 2016). Thus, the increase of plasma cortisol is recognized as a primary stress indicator and is strongly involved in regulating energy balance and homeostasis (secondary response) (Marcel Martínez-Porchas, 2009; Sadoul & Geffroy, 2019).

In mammals, it has been demonstrated that the prostaglandin E_2 PGE_2 derived from ARA modulates the hypothalamus-pituitary-adrenal axis to release ACTH, which is responsible for the release of cortisol in response to stress in mammals. Thus, the role of fatty acids on stress response seems to be mediated through eicosanoids production. Similarly, in fish, it has been shown that eicosanoids derived from LC-PUFA such as EPA and ARA can modulate the ACTH release from HPI-axis and thereby influence the cortisol production from inter-renal tissue (Ganga et al., 2011a; Ganga et al., 2006; Montero et al., 2015b). Several studies have reported increased plasma cortisol in

marine fish fed low n-3 LC PUFA (Conde-Sieira et al., 2018; Ganga et al., 2011b; Montero et al., 2003). Further, an increased plasma cortisol in response to low n-3 LC-PUFA in marine fish exposed to acute stress was also reported earlier (Conde-Sieira et al., 2018; Ganga et al., 2011b; Jutfelt et al., 2007; Montero et al., 2003). A modulation of stress-related gene expression by vegetable oils has also been reported in different fish species (Montero et al., 2003; Montero et al., 2015b; Pérez-Sánchez et al., 2013). Nevertheless, no effects on plasma cortisol and stress resilience were reported in fish (Conde-Sieira et al., 2018; Gesto et al., 2021). Further, it has then been suggested that the effects of VO inclusion on fish stress physiology depend on the combination of VOs used in the diet (Carvalho et al., 2019; Conde-Sieira et al., 2018; Gesto et al., 2021).

1.5. Effect of dietary fatty acids on inflammatory and immune responses in fish

The modulatory effects of dietary lipids on immune system functions have been well documented in many vertebrates. Dietary FA can modulate the immune response by altering the membrane lipid composition of immune cells or altering the signal transduction, thereby altering the cytokine production or influencing via production of their immunologically active eicosanoids (Arts and Kohler, 2009; Ashton et al., 1994; Balfry and Higgs, 2001; Calder, 2008a) (Figure 3). Eicosanoids are key mediators of inflammation and immune regulation (Bruce German et al., 1986; Rowley et al., 1995). The general tendency is that eicosanoids derived from the n-6 family, such as leukotriene B4 (LTB_4) and (PGE_2), are more abundant and have greater biopotency and promote proinflammatory responses, while eicosanoids (LTB_5 and PGE_3) from the n-3 family are considered as anti-inflammatory regulators (Lands, 1992). However, over synthesis of n-6 PUFA derived eicosanoids, especially PGE_2, may also lead to immunosuppression. Studies in human whole blood cells reported the inhibitory effect of PGE_2 on classical proinflammatory cytokines such as TNF-α and IL-1β (Miles, Allen & Calder, 2002). Similar inhibitory effects of PGE_2 on proinflammatory cytokines were also reported in fish *in vitro* (Fast, Ross & Johnson, 2005; Furne et al., 2013). Further, it has been described that PGE_2 has both pro-inflammatory and anti-inflammatory roles

and that some lipoxins derived from ARA, especially lipoxin A4, may be important for switching off inflammation (Calder, 2009; Liu et al., 2017).

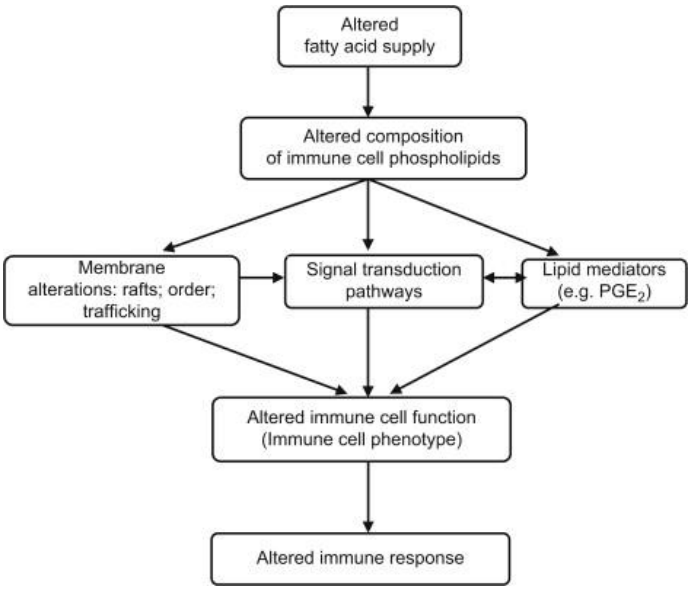

Figure 3. Schematic representation of the mechanisms by which dietary fatty acids altered the immune responses. Adapted from Calder (2008b).

Further, while ARA is the preferred substrate for the main enzymes cyclooxygenase (COX) and lipoxygenase (LOX) involved in eicosanoid production pathways, increased EPA levels in the immune cell membrane can competitively inhibit the production of pro-inflammatory eicosanoids. As dietary FA directly influences the membrane lipid composition, manipulating dietary n-6 and n-3 FA will alter the ratio of ARA and EPA in the cell membrane, which in turn alters the production of the eicosanoids and modulates the inflammatory and immune responses in fish (Bell et al., 1991; Bell et al., 1994). Thus, the balance between n-6 and n-3 FA in the diet is critical for maintaining optimal immune function and health (Calder, 2008a; Simopoulos, 2002). The increased VOs in fish diets resulted in increased n-6 content, and a high n-6/n-3 ratio can be directly reflected in cell membrane FA composition. Thus, the potential modulation of immune and inflammatory responses by dietary n-6/n-3 needs further investigation. Despite the foregoing, in fish, the information about the relationship between dietary FAs, immune responses and specific disease resistance is contradictory. Variable results

on the health impact of increased n-6/n-3 ratios are reported, ranging from no apparent negative effects (Andresen et al., 2019; Bransden et al., 2003; Gjøen et al., 2005; Grisdale-Helland et al., 2002; Montero et al., 2008; Montero et al., 2003), beneficial effects (Lopez-Jimena et al., 2015) to significantly increased mortality following transportation induced stress (Bell et al., 1991), reduced resistance to infection (Martinez-Rubio et al., 2012; Thompson et al., 1996), and effects on the humoral immunity and expression of immune related genes (Farndale et al., 1999; Montero et al., 2015a; Montero et al., 2010; Yan et al., 2020).

1.6. Physiological role of minerals in stress

Minerals are essential inorganic elements that are important for several physiological and biochemical processes, including ionic homeostasis, metabolic processes, antioxidant systems, hormone synthesis, immunity, etc. Particularly, the macro-minerals like phosphorus, calcium, and magnesium are major structural components of bones and hard tissues, while potassium and sodium are involved in regulating osmoregulation, acid-base balance, and cellular nutrient transport (Lall and Kaushik, 2021; Lall and Lewis-McCrea, 2007). The trace minerals such as selenium (Se), zinc (Zn), copper (Cu), manganese (Mn), and iron (Fe) are involved as co-factor or co-enzymes for several enzymatic reactions, playing an important role to regulate the metabolism, antioxidant and redox regulation, immunity and growth (Lall and Kaushik, 2021).

The role of trace minerals in stress responses is well recognized in both mammals and fish. Stress-induced changes in tissue mineral status are among the physiological and metabolic responses attributed to increased energy demands during stress. For instance, stress situation in animals causes oxidative stress, which in turn reduces antioxidant status and increases oxidative damage, with a concurrent reduction in the concentration of plasma antioxidant minerals such as Zn and Se (Kucukbay et al., 2009). Selenium is an integral part of several selenoproteins, and among them, selenoprotein P (Sepp) plays a significant role in antioxidant protection function (Burk and Hill, 2009). Furthermore,

the most studied selenoprotein family is the antioxidant glutathione peroxidase (Gpx), which is crucial against oxidative cellular injury (Watanabe et al., 1997). Several studies in fish have demonstrated the increased Se requirement during stress situations (Antony J Prabhu et al. 2020; Betancor et al., 2012; Kucukbay et al., 2009; Rider et al., 2009). Furthermore, the activity of metalloenzymes such as Gpx, Cu-Zn-SOD (superoxide dismutase), Mn-SOD, and Fe-CAT (catalase) are involved in scavenging the oxygen free radicals, thereby protecting the cells from oxidative damage (Espinosa-Diez et al., 2015).

1.6.1. Interaction of dietary fatty acids and mineral status

As discussed in the previous chapter (section 1.2), considerable efforts have been made to substitute the marine ingredients with plant-based ingredients in fish feed, and it is proven to be suitable for Atlantic salmon growth. However, the effect of this substitution in fish feed affects the level and bioavailability of other beneficial micronutrients such as minerals (Antony Jesu Prabhu et al., 2019; Antony Jesu Prabhu et al., 2018a; Aas et al., 2019). Although substitution of FO with VOs in diets and their subsequent change in n-3 LC-PUFA contents in fish are well reported, limited attention has been focused on the effect of this substitution on trace minerals (Waagbø & Magge, 1992; Waagbø et al., 1993). A recent long-term seawater trial in Atlantic salmon fed different inclusion levels of VOs diets with no additional Se supplements showed a significant reduction of Se content in fish tissue that received a higher inclusion of VOs diet than low VOs diet (Betancor et al., 2016). However, in that trial high VO diet had low Se compared to FO diet.

Additionally, an interaction of dietary FA and regulation of tissue minerals status has been documented in mammals. A study in rats fed diet rich in DHA demonstrated the downregulation of Zn-transporter and decreased the Zn level in different tissues (Jayasooriya et al., 2005; Mollard and Weiler, 2006). Likewise, the interaction of dietary fat levels and saturation level of FA on tissue iron status are reported in rats and other higher animals (Ahmed and Oates, 2013; Diaz-Castro et al., 2015; Miret et al.,

2003). Furthermore, similar *in vitro* results were also demonstrated in a human cell line (De Mel and Suphioglu, 2014). In fish, only a handful of studies have reported the effect of dietary FA on mineral metabolism with focus on bone mineral metabolism and vertebral deformities (Berge et al., 2009; Bou et al., 2017b; Gil Martens et al., 2010). However, to the best knowledge, the interaction of dietary FA on tissue mineral status and mineral metabolism in fish is not studied yet and warrants further research to study the underlying mechanism behind the regulation. Furthermore, besides the source of n-3 LC-PUFA, fish also serve as a major source of highly bioavailable trace elements such as Se and Zn that are essential for human health. Especially, ready to slaughter Atlantic salmon contain an average of about 0.27 mg/kg of Se and 35 mg/kg of Zn (Bell & Waagbø 2008; Daniel Holliman, 2006; Aas et al., 2019). Thus, knowledge on dietary FA and tissue minerals status would be beneficial for aquaculture industry.

1.7. Digestion, uptake, and transport of dietary lipids.

The major lipid constituents of salmonid diets are generally TAG with smaller amounts of phospholipids, cholesterol esters, lipid-soluble vitamins, and other lipids such as wax-esters. The pyloric caeca and midgut are the primary sites for lipid absorption and transport in Atlantic salmon (Olsen et al., 1999; Olsen et al., 2000). Mammals possess two different pancreatic lipases; pancreatic lipase in combination with colipase (substrate-specific) and bile salt-dependent lipase (non-specific). Digestion of TAG is primarily hydrolyzed by pancreatic lipase, which results in the release of two free FAs (FFAs) and one 2-monoacylglycerol (2-MAG) (Lowe, 1997). The substrate specificity of colipase helps the hydrolysis process that binds at the lipid-aqueous interface and provides a high-affinity anchor site for pancreatic lipase activity. The pancreatic lipase-colipase system is found to be predominating in vertebrates. However, colipase has been lost from the teleost genome multiple times (Sæle et al., 2018), consequently leaving bile salt-dependent lipase (BSDL) as the dominating lipase (Olsen and Ringø, 1997; Tocher, 2003). Thus, due to the potential loss of colipase, the dietary TAG is cleaved to three single FFA and one glycerol backbone by non-specific BSDL (Sæle et al., 2018). The complete digestion of TAG into FFA and glycerol have been demonstrated in *in*

vivo and *in vitro* studies in Atlantic salmon (Bogevik et al., 2008; Sigurgisladottir et al., 1992).

Like mammals, in fish, the absorption of digested lipids by enterocytes may both be passive by diffusion or also actively taken up via energy-dependent carrier-mediated transfer protein. Fatty acid transfer proteins (FATPs) are a family of integral membrane proteins that facilitate the transport of fatty acids into the cells, including the transport of dietary fatty acids into enterocytes (Ho and Storch, 2001; Matarese et al., 1989; Stahl et al., 1999). Furthermore, membrane-associated proteins such as fatty acid-binding protein (FABP) and fatty acid translocase (FAT/CD36) are known to play a role in the uptake of FA (particularly of long-chain FA). The differential regulation of FABP2 and FATP expression when fed different levels of n-3 LC-PUFA were reported in different fish species, including Atlantic salmon (Betancor et al., 2017; Darias et al., 2012; Jin et al., 2018; Venold et al., 2013; Yan et al., 2015).

In mammals, the absorbed 2- monoacylglycerol (MAG) and FA by enterocytes enter the MAG-pathway, in which 2-MAG are re-acylated into TAG through the sequential action of monoacylglycerol acyltransferase (MGAT) and diacylglycerol acyltransferase (DGAT) (Coleman and Lee, 2004; Coleman et al., 2000; Lehner and Kuksis, 1996). A second pathway is the α-glycerophosphate (G-3-P) pathway. In this pathway, glycerol-3-phosphate is acylated to form phosphatidic acid that is further hydrolyzed to form DAG and then converted to TAG. In addition, the phospholipids and cholesterol esters may also be synthesized through this pathway (Coleman and Lee, 2004; Coleman et al., 2000). The relative importance of the MAG pathway and the G-3-P pathway depends on the supply of 2-MAG and FA. In fish, due to the non-specificity of BSDL, the hydrolyzed TAG is mostly of FFA and glycerol. Thus, the G-3-P pathway appears to be the predominant pathway for biosynthesis of both TAG and PL (Oxley et al., 2005). The resynthesized TAGs can be either stored as lipid droplets in cytosol (CLDs) or packed with PL, dietary cholesterol and apolipoproteins into large lipoprotein complexes, mainly chylomicron particles and very low-density lipoproteins (VLDL). These are then exported into blood stream and are transported to the liver and other target tissues, for storage or energy production (Sheridan, 1988; Sire et al., 1981). The

formation of LP is facilitated by the microsomal TAG transfer protein (MTP), which shuttles resynthesized TAG onto a newly formed apolipoprotein molecule.

Cytosolic lipid droplets (CLDs) are cellular organelles composed of a core of neutral lipids surrounded by a monolayer of phospholipids coated with one or more proteins called perilipins (PLINs). While initially believed to be a temporary site for lipid storage, it is now appreciated that lipid droplets are dynamic organelles with roles in cellular lipid homeostasis, protection from lipotoxicity and ER stress, viral and parasitic infection, and host defense (Guo et al., 2009; Olzmann and Carvalho, 2019). However, excessive accumulation of CLDs may also cause damage to the cells and create pathogenicity (Schaffer, 2003).

Induction of CLDs by dietary FA is well documented in mammals. Especially, the nature of dietary FA (i.e., carbon-chain length and saturation) can alter the CLDs accumulation. For instance, several studies in Caco-2 cells derived from human colon adenocarcinoma cells demonstrated that oleic acid (18:1n-9) increased the TAG synthesis and CLDs accumulation, while the palmitic acid (16:0) led to higher phospholipid (PL) and lowered the TAG synthesis (Bateman et al., 2007; Dashti et al., 1990; van Greevenbroek and de Bruin, 1998). Similarly, a number of studies have demonstrated the excessive accumulation of large lipid droplets (mainly consisting of TAG) in the intestine of fish that received higher inclusion VOs in the diet (Caballero et al., 2003; Caballero et al., 2002; Deplano et al., 1989; Liland et al., 2018; Olsen et al., 1999; Olsen et al., 2000). A delayed export of TAG due to an insufficient production rate of lipoprotein is thought to be the main cause of more severe lipid accumulations, which have been shown to be reduced by the addition of dietary PL (Fontagné et al., 1998; Hansen et al., 2020; Morais et al., 2007; Morais et al., 2005; Olsen et al., 1999). Further, Olsen et al. (2003) reported that replacing a part of dietary linseed oil by 16:0 (important component of PL) ensures a high rate of PL synthesis and subsequently reduced the CLDs accumulation in the enterocytes. Although CLDs are temporary lipids storage organelles, studies have also shown mild to extensive damage to enterocytes due to mass accumulation of CLDs in fish fed VOs rich diets high in oleic acid have (Olsen et al., 1999; Olsen et al., 2000; Olsen et al., 2003). Furthermore, the

accumulation of lipid droplets within the gastrointestinal mucosa may present a physical barrier to efficient lipid absorption, which in turn may negatively affect the fish performance (Morais et al., 2007).

1.8. RTgutGC as an invitro intestinal model for fish nutrition research

The intestinal epithelium is an important biological barrier that regulates the uptake of nutrients. RTgutGC cell line is derived from the gastrointestinal tract of rainbow trout (*Oncorhynchus mykiss*), which is used as a model system for studying fish intestinal cells (Geppert et al., 2016) similar to the Caco-2 cell line (Kawano et al., 2011). The RTgutGC cells develop a functionally polarized epithelium if cultured on permeable supports, leading to a two-compartment intestinal barrier model. The apical and a basolateral compartment of RTgutGC can be mimicked to the intestinal lumen and the portal blood, respectively, which enable transport-dependent studies at this interface (Minghetti et al., 2017; Geppert et al., 2016; Kawano et al., 2011). The RTgutGC acts as enterocytes (Wang et al., 2019), that assists in FA assimilation and support the *de novo* synthesis of FAs, cholesterol and serve as a site for production of apolipoproteins (Minghetti et al., 2017). RTgutGC has been suggested as a physiologically suitable fish intestinal epithelial model and is currently used in fish nutrition studies to study the immune response of fish gut to different feed ingredients, nutrient uptake (Antony Jesu Prabhu et al., 2018b; Kim et al., 2018; Pumputis et al., 2018; Wang et al., 2019), test functional ingredients (Holen et al., 2021; Wang et al., 2019) and toxicity (Langan et al., 2017; Schug et al., 2020).

2. Research aims

The aim of this PhD thesis was to gain the knowledge on the effects of dietary FAs on salmon health and interaction with minerals under challenging environmental conditions and understand the mechanism of intracellular trafficking of dietary FAs.

The overall aim can be subdivided into the following specific objectives:

- To describe the impact of dietary level and ratio of n-6 and n-3 fatty acids on disease progression and mRNA expression of immune and inflammatory markers in Atlantic salmon challenged with amoebic gill disease (AGD) (**Paper I**)
- To elucidate the long-term feeding of different levels of EPA+DHA on stress responses and welfare indicators in Atlantic salmon after mechanical delousing stress (**Paper II**)
- To study the interaction/ influence of varying dietary EPA+DHA levels on body and tissue mineral status (**Paper II**)
- To increase the knowledge on uptake and transport of different chain length and saturation level of fatty acid *in vitro* using intestinal epithelial cell line (RTgutGC) (**Paper III**)

3. Methodological considerations

3.1. Experimental design and diets

With the intensification of aquaculture farming, fish are naturally exposed to different stressors such as transport and handling of the fish, delousing, treatment for parasites like AGD etc. Meanwhile, most feeding trials are run in controlled conditions in land-based tanks, with stable environmental conditions and minimal handling of the fish. However, practical nutrient requirements or tolerance levels need to be defined for producing a robust and healthy fish that can handle the challenges commonly experienced in commercial aquaculture. Two feeding trials included in this thesis had experimental designs focusing on specific nutrients to detect the differences due to changes in lipid composition, such as n-6/n-3 ratio (**Paper I**) and EPA+DHA (**Paper II**). The experimental designs (Figure 4A) and dietary composition (Figure 4B) are summarized here, and the detailed descriptions are given in their respective papers (**Paper I** and **Paper II**). The pre-feeding of fish with experimental diet in **Paper I** was performed at Skretting ARC Research station at Lerang, Norway, and the challenge trial was carried out in the indoor challenge facility at The Industrial and Aquatic Laboratory (ILAB) in Bergen (Norway). The long-term fish trial in **Paper II** was run at Gildeskål Research station (GIFAS) Norway. The ILAB, Skretting ARC and GIFAS all have long experience in running feeding trials with salmon, and the trials in **Paper I** and **Paper II** were executed without any complications.

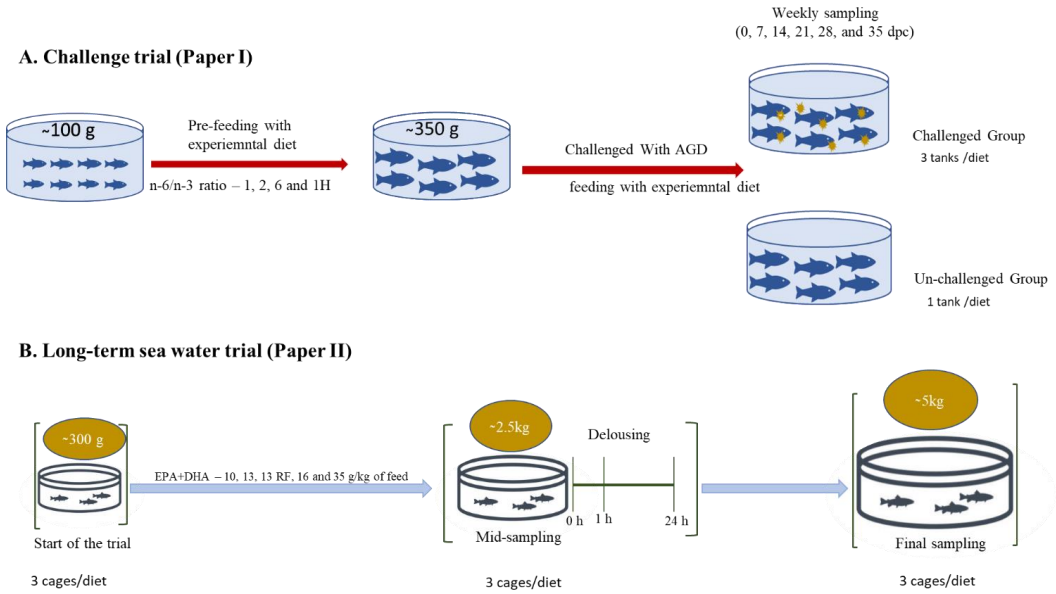

*Figure 4. An overview of the experimental design followed in **Paper I** (A) and **Paper II** (B)*

The dietary trial presented in **Paper I** included four different diets different in absolute contents of n-6 and n-3 FA and n-6/n-3 ratios from 1.0 – 6.0. A higher n-6/n-3 ratio was included to provoke potential effects of n-6 FA (Figure 5A). A Pre-feeding was performed to enable the fish to stabilize the tissues FA profile according to their dietary FA composition. In the long-term trial in **Paper II**, a total of five diets with different EPA+DHA levels were included, four different levels of EPA+DHA (10, 13, 16, 35 g /kg of feed), and one diet had an EPA+DHA of 13 g/kg of feed with reduced fat compared to the other four diets (Figure 5B). Atlantic salmon (~ 115 g) were procured from a commercial fish hatchery and acclimatized to the research facility at GIFAS with a commercial diet (BioMar). After the initial acclimatization, fish (~275 g) were randomly transferred into 15 outdoor cages and fed with experimental diets to apparent satiation. This trial was conducted in open sea cages relevant to commercial farming conditions, and there was no waste feed collection. Thus, due to lack of reliable data on

actual feed intake, this study does not provide the possibility to calculate nutrients retention.

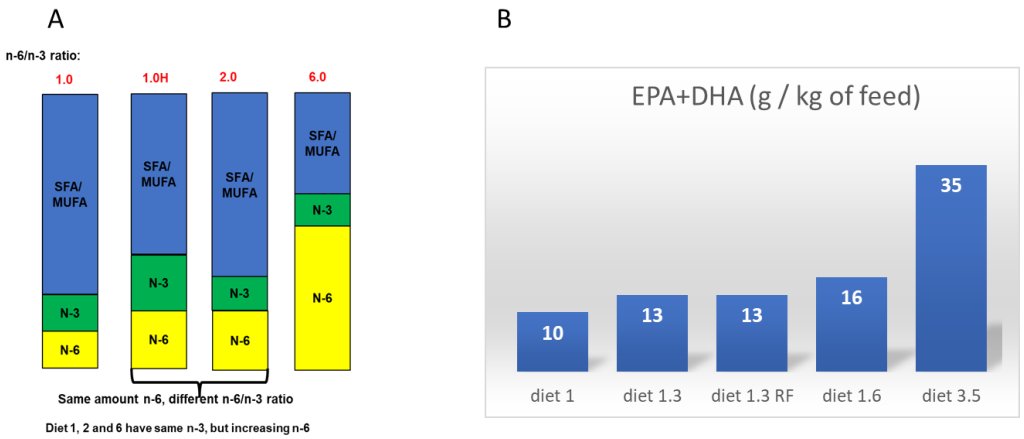

***Figure 5**. Overview of the experimental diets in **Paper I** (A) and **Paper II** (B). In figure 5A, diet 1/diet 2/diet 6/diet 1H, diet codes are set according to dietary n-6/n-3 ratio. The final diet is labelled 1H due to its higher absolute contents of n-3 and n-6 compared to the first diet. In figure 5B, diet 1/diet 1.3/diet 1.6/ diet 3.5, diet codes are set according to their g/kg of EPA+DHA in the feed. One diet labelled as diet 1.3 RF due to its reduced-fat level.*

The inclusion of different oils resources in feed can greatly alter the FA composition of the dietary lipids. In any feeding trial, when we want to study the effect of particular FAs, we add them in a higher amount or reduce them at the expense of other FA. This means that there will be an effect of added components to the diets, as well as the effect of reducing other factors in the diet. For instance, in **Paper I**, the VOs such as linseed, sunflower, olive, and coconut oils were used in different combinations to achieve the desired ratio and absolute content of n-6 and n-3 FA in the diets. However, changes in the combination of dietary oils will also affect the content of other FA such as SFA and MUFA. For example, olive oil contains a high MUFA (55-83%), whereas sunflower oil is high in n-6 linoleic FA (~ 55%) (Orsavova et al., 2015). Thus, the higher inclusion of olive oil in diets 1 and 2 resulted in higher content of MUFA, while other diets (diet 6 and diet 1H) had no or less olive oil, which resulted in the low amount of MUFA. Each

FA has its own metabolic and biological functions (Tvrzicka et al., 2011). Similar case for the diets in **Paper II**, where we used rapeseed oil as VO source along with FO to adjust the EPA+DHA levels. But rapeseed oil contains a higher amount of oleic acid (high in MUFA), which resulted in increased MUFA content with increased level of rapeseed oil in the diet (Tvrzicka et al., 2011). Therefore, it is difficult to discern whether these observed effects are caused by the modification of a single FA or by the combined effect of several altered FAs. However, the diets used in this study (**Paper I**) were designed to assess the dietary ratio of n-6/n-3 FA on immune and stress response where n-6 and n-3 are potential substrates for the biologically active eicosanoids which in turn activate the inflammatory and immune response. Further, the role of SFA and MUFA on eicosanoids productions and subsequent immune and inflammatory responses are insignificant. Like mammals, Atlantic salmon does not possess the enzymes capacity to convert SFA and MUFA to n-3 and n-6 FA. Thus, any observed changes in this study (**Paper I**) could be an effect of n-6 and n-3 FA in the diet. However, SFA and MUFA may influence the immune responses in different other pathways, which we were not able to address *in vivo* due to study design. Further, the *in vivo* trial was not aimed to assess the fate of dietary SFA and MUFA; particularly, very little is known about these FA on intestinal uptake and transport. Therefore, we designed an *in vitro* experiment (**Paper III**) to elucidate the fate of these FA in fish intestinal cells (RTgutGC). The other concern in the feeding trial, especially in the long-term trial is the production of different feed batches. In **Paper II**, since the trial was aimed to elucidate the long-term effect of different levels of EPA+DHA, three different pellet sizes (4, 6, and 9 mm) with specific formulations (five separate feed batches) were used to meet the dietary requirements at the different life stages of the fish. Therefore, the types of FM and FO (South American/North Atlantic) used in the diets were adjusted according to their availability at the time of feed production, which resulted in slightly different EPA+DHA levels within each feed production.

3.2. Challenge experiment

High quality challenge facilities and validated methods to maintain the pathogen and challenge protocol are crucial for conducting any challenge trials with pathogens. The ILAB has long experience in running challenge trials with AGD (Rosenlund, 2017). The amoebae (*P. perurans*) used in the **Paper I** were originally isolated from an AGD outbreak at Sotra in 2013 by ILAB, according to methods described by (Morrison et al., 2004) and were identified as *P. perurans* by the 18S rRNA PCR method (Young et al., 2008). The virulence of the amoeba was assessed regularly *in vitro* as in the method described by Collins et al. (2017). The concentration of amoeba chosen (1000 cells/ L) in this trial was in accordance with several other published AGD challenge trials and followed a well established and standard procedure to challenge the salmon with AGD (Benedicenti et al., 2019; Haugland et al., 2017; Rosenlund, 2017; Taylor et al., 2009).

In all samplings, to minimize the variance, samples were collected from the same location of the organs by the same person. In **Paper I**, sampling was carried out weekly, i.e., 0-, 7-, 14-, 21-, 28-, and 35-days post-challenge (dpc). When sampling the fish, they were anesthetized with a Finquel vet. (Tricainmesilat, 100 mg L−1). AGD progression was assessed by gross gill examination, and a well-experienced person was involved in assessing and scoring the gill in all sampling points. When scoring, all gill arches of the fish were visually assessed for the presence and severity of lesions and scored according to the method described by Taylor et al. (2009) with a scale from 0 to 5, with 0 representing no signs of infection and 5 representing a severe AGD infection. Although gill score was increased over the challenge period until 21 days, the recorded gill score was much lower (1.21 ± 0.11) than other challenge trials (~ 2) (Benedicenti et al., 2019; Haugland et al., 2017; Rosenlund, 2017; Taylor et al., 2009). Further, the gill score declined thereafter meaning the fish had recovered from AGD infection. In contrast, the same amoeba strain used in other studies showed higher gill scores and gill pathology. The possible reasons for the low gill score could be that the previous experience of AGD exposure induced low-grade infections from which fish recovered and were resistant to subsequent challenge (Findlay and Munday, 1998; Findlay et al.,

1995; Vincent et al., 2006) or genetic resistance to AGD infection (Robledo et al., 2020; Taylor et al., 2009; Taylor et al., 2007). However, in the present trial (**Paper I**), the fish population (salmon breed) obtained from the hatchery was not previously exposed to AGD infection. Further, the fish was screened for AGD before the start of the challenge experiment. Thus, it could be possibly due to genetic resistance to AGD infection, or it might also be immunological resistance. Therefore, these factors must be considered while selecting salmon for the AGD challenge trial. This thesis (**Paper I**) would have seen more diet effects if there was a more normal and stronger AGD infection. However, this trial provided the chance to study the recovery phase of AGD infection.

The second gill arch on the left side of the fish were dissected and snap frozen in liquid N2 and stored at -80 ^{0}C for gene expression studies. The remaining portion of the same gill arch was fixed in 10% neutral buffered formalin for histology analysis. The third gill arch on the left side was dissected out and snap frozen in liquid N_2 and stored in -80 ^{0}C for FA analysis. For screening amoeba by qPCR, only a small and consistent upper portion of the second gill arch on the right-hand side was collected to maintain the consistency in sampling, which might be the possible reason for very few positive in qPCR screening. Thus. if we would have taken visible gill infection spots more specifically, we probably would have obtained a more accurate picture of the infection. This challenge trial was planned for 28 days, and we had to give away our control tanks after 28 days. However, we extended the experiment with the challenge group to 35 days to ensure there was no artifact in results at 28 days. Since the gill score for the control group were very low, it did not affect the experiment. So, the sample for gene expression analysis were chosen from 0, 21 and 28 dpc.

3.3. Delousing sampling

In **Paper II**, the average sea lice count was monitored weekly on random cages. The average number of gravid lice per salmon, before the month of delousing was 0.13, which is lower than the allowed limit (0.5 matured female lice/ salmon) according to Norway regulations on farmed salmonids. The delousing of the fish was performed from

12 -14th of August 2018, when the average fish size reached around 2.5 kg. A mechanical delousing was performed according to GIFAS established delousing method. In this method, all fish in a cage were transferred to a small well boat and anesthetized, and the lice were removed using wet vacuuming with an adapted mouthpiece. After delousing, fish were dropped directly back in the cage. In this trial (**Paper II**), we chose to sample the fish before delousing (0 h) and 1 h and 24 h post-delousing stress. A sudden spike in the cortisol level after 1 h post-stress compared to pre-stress was noticed and then restored to basal level within 24 h post-stress. However, this trial failed to assess if longer time is needed before pre-stress status is restored. Thus, it would be interesting to sample the fish every 1 h after the stress, which would enable us to know how long time is required to restore from the stress. During sampling, the net pen was raised (decreased in size) to gather the fish before fish were caught by a hand net. The staff in GIFAS have a lot of experience in this method to minimize stress to the fish. After netting, fish were immediately anesthetized in a tub of water to prevent further stress and transported to the sampling area. Thus, the data in different time points in our data clearly shows that the stress induced by a general sampling of the fish is very low compared to that of the delousing procedure, which allowed us to study the stress response after delousing.

3.4. *In vitro* trial

To increase the knowledge on intracellular lipid trafficking and CLDs formation were studied *in vitro* using RTgutGC cell model (**Paper III**). Three different approaches have been used in this trial, (i) live-cell imaging for lipid droplets formation, (ii) radiolabeled FAs for lipid class analysis, and (iii) transcriptomic analysis. An overview of the experimental design is given in the Figure 6, and detailed descriptions are provided in **Paper III**.

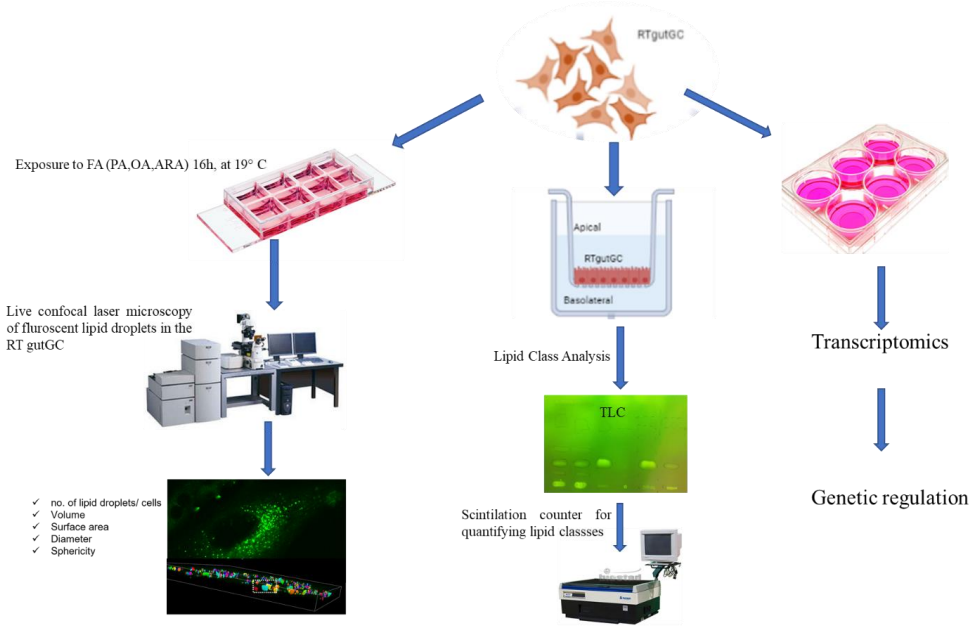

Figure 6. *An overview of the in vitro experiments conducted in* ***Paper III***. *(A) live-cell imaging for lipid droplets formation, (B) radiolabeled FAs for lipid class analysis, and (C) whole transcriptomic analysis. In all the experiment RTgutGC cells treated with 200 µM of FAs (PA, OA, or ARA) control for16 h at 19 ^{0}C.*

The *in vitro* cell line models may accurately provide firsthand information on the mechanism of the cellular process. In addition, it has many other advantages, which include the 3Rs, availability, reliability, reproducibility, less time consuming, etc. In this study, RTgutGC cells (**Paper III**) showed results comparable to what has been observed in the most studied Caco-2 cell model and to several *in vivo* studies in fish. Thus, it could be used as a potential *in vitro* intestinal model for lipid research in fish. However, there are some limitations using *in vitro* models. Particularly, *in vivo*, intestinal epithelium contains different types of cells that interact with each other, but this is not the case for RTgutGC composed only of enterocytes. Further, *in vivo* intestinal epithelium contains bile salts, mucus, and an unstirred water layer, which strongly influence the uptake and transport of intestinal FA *in vivo*. In contrast, no mucus and unstirred water layers are present in RTgutGC.

Long-chain FFAs are less soluble in aqueous solution, and FFAs are toxic to the cells; thus, they should be conjugated to a carrier molecule to deliver to the cells. Bovine serum albumin (BSA) is similar to the physiological system of nonesterified FA transport, and it is proved to be a suitable method to deliver FA to cells (Alsabeeh et al., 2018). In this study, FFAs were conjugated to FA-free BSA with molar concentration of 2.5:1, according to the method described by Nøstbakken et al. (2012). A cytotoxicity test was performed using xCELLigence Real Time Cell Analysis (RTCA) instruments use biosensors to continuously monitor cell behavior in a label-free manner and the result showed no toxic effect of FA+BSA conjugation even at very high concentration of FAs (1000µM). In addition to FA+BSA conjugation, sodium taurocholate micelles were also widely used for FA delivery. Studies in the Caco-2 cell line suggested that taurocholate micelles mimic the postprandial intestinal situation at the apical surface of the cells. As this study was the first attempt to use RTgutGC cells as an intestinal model, further experiments with FA+ taurocholate micelles complex for apical and FA+BSA complex for basolateral compartment should be explored.

In the radioactive FA exposure experiment in this thesis, the recovery percentage of radiolabeled FA was for PA and ARA compared to OA. Therefore, we suspect that there might be preferential increased β-oxidation in the cells. Furthermore, as these FA are ^{3}H radiolabeled, the oxidized products must be released into the water phase in lipid extraction. Unfortunately, we did not measure the radioactivity from the water phase of the lipid extract, which should be taken care of in further experiments. Alternatively, carbon capture methods using ^{14}C labeled FA could also be employed to measure the β-oxidation. To ensure uniformity, RTgutGC cells were treated with the same concentration of FA (200 µM) and hours of incubation (16 h) for all three experiments. Results obtained from live cell image analysis for CLDs were well correlated with quantitative analysis (radiolabeled FAs for lipid class analysis). However, data from transcriptomics were not correlated to the latter two experiments.

Transcriptomics is the study of complete set of RNA transcripts that are produced by the genome, under specific circumstances or in a specific cell which are used to learn more about how genes are differentially expressed in different cells in distinct cell

populations, or in response to different treatments at a given time point. With the introduction of next generation sequencing technology, RNA-seq based transcriptomics has become an important tool to explore the global transcriptome changes under different experimental conditions. The data generated from the RNA-seq based transcriptomics can provide deeper insights than the conventional procedures. Moreover, the great advantage of RNA-seq based transcriptomics is that it can be used to explore even the non-model organism including Atlantic salmon (Chalifa-Caspi, 2021; Sundaram et al., 2017). However, transcriptome analysis has some potential pitfalls when interpreting the data. The obtained results on transcriptome are like snapshots and only give information on the status of the cells or tissue transcriptome at one specific time point. Furthermore, over 95% of the RNAs in a cell are not translated into a protein at a given time point. So, transcriptomics also includes the study of these non-coding RNAs, which might have different other functions (Diamantopoulos et al., 2018). Thus, the description of transcriptome can be valuable, but it does not necessarily provide all information about mRNA transcription rate, turnover, or protein synthesis. In addition, changes to the transcriptome induced by experimental treatment are often time-dependent, and different genes may be regulated in opposite directions. Therefore, a time-course study may well be necessary to reveal the actual changes and find the peaks in the expression level of a regulated gene. However, with the limited budget in the present thesis, a time-course analysis of transcriptome was beyond the scope. Nevertheless, the transcription data is useful for identifying potential candidates for follow-up work at the protein level (proteomics).

4. Discussion

4.1. Dietary FA on growth performance and survival

In the present thesis, the effects of feeding Atlantic salmon with different dietary levels and ratios of n-6 and n-3 FAs (Figure 1A; **Paper I**), and different dietary EPA+DHA levels (Figure 1B; **Paper II**) on growth and survival of the fish were assessed under different challenge conditions. Previous studies in Atlantic salmon have concluded the dietary requirement of EPA+DHA between 10-15 g/kg of feed for optimal growth and performance (Bou et al., 2017a; Glencross et al., 2014; Rosenlund et al., 2016). In the present thesis, fish fed with diets containing constant EPA+DHA (11 g/kg of feed and increasing n-6/n-3 FA ratios had no effect on final weight of the fish during the pre-feeding phase in trial 1 (Figure 7A). This result is consistent with another trial in Atlantic salmon using the same diets as in **Paper I,** reporting no difference in growth after a three-month growth trial in seawater (Hundal et al., 2020). Nevertheless, during the challenge period in trial 1 in the present thesis (Paper I), fish that challenged with AGD showed a significant reduction in growth in fish fed high n-6/n-3 ratio diet (6:1) compared to other dietary groups. The observed reduction of growth in fish fed high n-6/n-3 FA ratio could be due to combined effects of diet, AGD, and also the repeated handling stress introduced by weekly sampling. Likewise, in feeding trial 2 with different EPA+DHA levels, no significant difference in growth was observed up until the delousing (~ 2.5 kg) (**Paper II**). However, toward the end of the experiment, fish that received lower EPA+DHA diets (10, 13, 13 (reduced fat), and 16 g/ kg of feed) had negative impact on the growth compared to fish received high EPA+DHA diet (35g / kg of feed) (~ 5 kg) (Figure 7B; Lutfi et al., 2022). Further, during that period (from delousing sampling (~ 2.5 kg) to towards end of the trial (~ 5 kg)), the onset of CMS outbreak and increased sea lice level were reported (Lutfi et al., 2022). Thus, the combined effect of the CMC outbreak and increased sea lice level in the post-delousing period might be the possible reason for the observed difference in the growth. Alternatively, it could be related to long term effects of low EPA+DHA. Especially DHA is selectively preserved in the fish when dietary levels are low, hence it takes a

long time to deplete body stores if fish are fed a borderline suboptimal diet. Further, although a notable difference in growth was observed, diet had no effect on mortality of the fish in both the feeding trials (**Paper I** and **Paper II**).

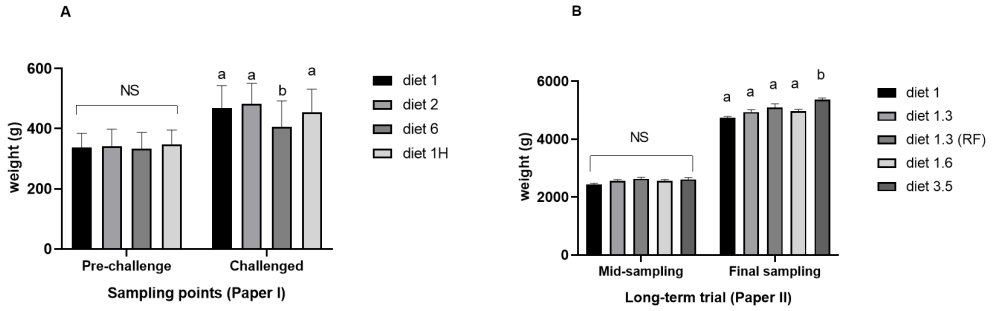

***Figure** 7. (A)Weight of Atlantic salmon in pre-challenge and at the end of the challenge trial (**Paper I**). (B) Weight of Atlantic salmon in mid-sampling (delousing) and at the end of the long-term trial (**Paper II**).*

In general, the AGD infection in salmonids is assessed based on gross gill scores, and a gill score of 2 and above is the point where treatment for AGD would be started (Taylor et al., 2009). However, in the present thesis (Paper I), although gill scores increased significantly over the challenge period, the maximum gill score was relatively low (only minor gill pathology was observed), and fish spontaneously recovered after the peak infection. Thus, the possible reason for the lack of mortality during the AGD challenge period (**Paper I**) could be due to a low-grade AGD infection or genetic resistance. The lack of mortality after delousing in feeding trial 2 (**Paper II**) might be due to the nutritional history of the fish prior to the start of the experiment. Thus, the fish used in the current trial (**Paper II**) received a commercial diet until it reached 270g, which is typically rich in n-3 PUFA and that might have provided enough n-3 PUFA until the delousing period. However, a long-term seawater trial with Atlantic salmon, showed a significant increase in mortality after delousing during high water temperature (17 ºC) in fish that received 10 g/kg EPA+DHA compared to 17 g/kg DHA+EPA diet (Bou et al., 2017c). The possible reason for the discrepancy of our results with those obtained by Bou et al. (2017c) could be due to differences in delousing procedures or water temperatures. In the later study (Bou et al., 2017c) the delousing was carried out

during a high-water temperature period 17 ºC compared to 13 ºC in the current study, which might have caused additional stress for the fish. Furthermore, others have also noted the stress-induced mortality in Atlantic salmon fed high amount of n-6 FAs (Bell et al., 1991; Thompson et al., 1996).

Concluding on growth performance and survival, results from both feeding trials in this thesis as well as earlier reports (Lutfi et al., 2022, Bou et al., 2017; Bell et al., 1991; Thompson et al., 1996) clearly emphasize that absolute content and ratio of n-6 and n-3 are important factors to be considered; thus, the use of diets with a high level of n-6 FA relative to n-3 FA should not be recommended.

4.2. Inflammatory and immune response to dietary FA

The influence of dietary FA on inflammatory and immune responses are well documented in many vertebrates (discussed in detail in sec. 1.5). In the present thesis, one would expect that high dietary n-6/n-3 ratios (**Paper I**) and sub-optimal levels of dietary EPA+DHA (**Paper II**) could enhance the incidence and severity of inflammatory responses to stressors under challenging environmental conditions, such as diseases, handling, delousing etc. Accordingly, several studies in fish, including Atlantic salmon, have demonstrated the possible regulation of inflammatory and disease resistance in response to changes in dietary LC-PUFA levels. In the present thesis, the mRNA expression of key inflammatory cytokines (TNF-α, iNOS, IL4-13b, GATA-3, IL-1β, p53, COX2, and PGE2-EP4) were significantly affected by treatment/stress of the fish both in the challenge experiment with AGD (**Paper I**) and as well as delousing trial (**Paper II**). However, despite the relatively high absolute content of n-6 FAs and a higher ratio of n-6/n-3 (6:1) in trial 1, we did not find dietary effects on the expression of these genes (**Paper I**). Similarly, feeding trial 2 with different levels of dietary EPA+DHA (1, 1.3, 1.3 RF (reduced fat), 1.6, and 3.5 % of total FA) did not affect the expression of inflammatory markers analyzed. The evidence of eicosanoids production in salmon gills is well documented (Beckman and Mustafa, 1992; Bell et al., 1992). Unfortunately, in the present thesis we were not able to analyze the eicosanoids levels

in gills. A feeding trial in Atlantic salmon using the same diets as in **Paper I** reported that the fish fed the high n-6/n-3 ratio diet (diet 6, 6:1) had significantly elevated liver PGE_2 levels both before and after stressing conditions (Hundal et al., 2021). Furthermore, despite no dietary effect, mRNA expression of COX-2 (an enzyme that catalyzes the conversion of ARA and EPA to prostaglandins) and PGE_2-EP4 receptor (preferentially activated by PGE_2) were significantly upregulated at peak infection time. While amoebic gill disease is the result of an ecto-parasite infection, there is evidence of inflammatory and immune responses in the infected gills (Marcos-Lopez et al., 2018; Marcos-Lopez et al., 2017; Powell et al., 2008; Talbot et al., 2021). Therefore, could be possible interactions of eicosanoids productions and inflammatory responses, but it was not reflected on the expression of analyzed pro-inflammatory markers (**Paper I**). Contradictory to our results, studies in Atlantic salmon fed high dietary n-6/n-3 ratio have reported the significantly increased mortality following transportation induced stress (Bell et al., 1991), reduced resistance to infection (Martinez-Rubio et al., 2012; Thompson et al., 1996), and effects on the humoral immunity and expression of immune related genes (Caballero-Solares et al., 2017; Seierstad et al., 2009). However, there are also studies that have reported no negative effects on immune and inflammatory response upon high dietary n-6/n-3 ratio. Thus, the possible reasons for no dietary effects on inflammatory markers in this current thesis remain unclear. As suggested by many others, dietary influence on gene expression is not always straight forward, there could be an interaction of several other factors like other dietary nutrients, availability of micronutrients, species differences, and experimental conditions may all impact the immune response (Kiron, 2012; Oliva-Teles, 2012).

The CLDs are generally considered as a site for temporary fat storage and beneficially reduce lipotoxicity and maintain intracellular homeostasis. However, the excessive accumulation of CLDs may negatively affect the epithelial permeability and barrier system of the cells (Schaffer, 2003). Further, several *in vivo* studies in fish have also demonstrated extensive damage to enterocytes (inflammation) due to excessive accumulation of CLDs caused by VO diets high in MUFA (Olsen et al., 1999; Olsen et al., 2000; Olsen et al., 2003) and loss of intestinal integrity due to low EPA+DHA diet (< 1% of total FA) (Bou et al., 2017a). Like other mammals, the intestinal tract serves

as a barrier between the environment and internal organs in fish. The consequence of loss of intestinal integrity and enhanced epithelial permeability may lead to enhanced uptake of intestinal pathogenic bacteria and toxins, which might cause pathogenesis. In the *in vitro* trial (**Paper III**) in the current thesis, RTgutGC cells treated with OA had accumulated a higher amount of CLDs (cytosolic lipid droplets) than PA and ARA. In addition, the size and volume of the CLDs was higher for OA than PA and ARA. This result is consistent with what has been reported in Caco-2 cells, a primarily used cell line for the intestinal epithelial barrier model (Bateman et al., 2007; Dashti et al., 1990; Field et al., 1988; Levin et al., 1992). Similarly, several *in vivo* studies in fish have shown excessive CLD accumulation in intestinal tissue in response to high MUFA diets (Caballero et al., 2003; Caballero et al., 2002; Fontagné et al., 1998; Olsen et al., 1999; Olsen et al., 2000; Olsen et al., 2003). Therefore, targeted studies will be required to investigate what has been observed on CLDs accumulation by MUFA in RTgutGC and how it will affect the intestinal health in Atlantic salmon *in vivo*.

4.3. Stress responses

Cortisol is a primary stress hormone released by the hypothalamus-pituitary-interrenal (HPI) axis in response to stress. Measuring plasma cortisol has long been a well-accepted approach to assess stress levels in fish and other vertebrates (discussed in section. 1.4.). Furthermore, in fish, it has been reported that LC-PUFA can stimulate the cortisol release by interrenal tissue and suggested that the dietary n-6 and n-3 FA can modify the stress response and cortisol production through their altered eicosanoid production (Ganga et al., 2011a; Ganga et al., 2011b; Ganga et al., 2006). In the present thesis, the delousing, where fish were subjected to handling and crowding stress, caused a significant increase in plasma cortisol level 1 h post-stress (**Paper II**). Similarly, a trial by Hundal et al. (2021), using the same diets as in **Paper I**, reported significantly increased plasma cortisol 1 h after the acute stress. Additionally, an increased mRNA expression of stress protein (hsp) was seen in both the trial in the current thesis (**Paper I** and **Paper II**) and as well as in Hundal et al. (2021). Thus, it should have been able to detect the differences between the diet groups if dietary n-6/n-3 ratios (**Paper I**) or EPA+DHA levels (**Paper II**) had any impact on stress responses and recovery.

However, neither dietary n-6/n-3 ratios (**Paper I** and Hundal et al. (2021)) nor EPA+DHA levels (**Paper II**) affected the cortisol levels in fish. In contrast, several other studies in marine fish have demonstrated the increased plasma cortisol production in response to low n-3 LC-PUFA, either in the presence or absence of stress conditions (Conde-Sieira et al., 2018; Ganga et al., 2011b; Jutfelt et al., 2007). The differences in the studies might be due to the fact that Salmonids are capable of bioconversion of dietary 18:3 n-3 PUFA to EPA and DHA and selectively retain them in the membrane. Thus, it can be concluded that even the lowest level of EPA+DHA used in the current study was sufficient to mount a cortisol response to acute stress, indicating that this may be a prioritized physiological function in the fish.

Activation and regulation of oxidative stress marker genes upon stress exposure are well recognized in fish as like in other animals (Ibrahim et al., 1997; Madeira et al., 2013; Olsvik et al., 2005; Olsvik et al., 2011). In the present thesis (**Paper II**) dietary EPA+DHA levels had no effects on the expression of these oxidative stress marker genes (SOD, CAT, Gpx1, Gpx4b, Gpx7, GST1, GR, SePP, and SePK). Our result agrees with the previous report by Betancor et al. (2016), where they found no effect on basal expression of oxidative genes/Se-related genes in Atlantic salmon fed different inclusion levels of vegetable oils in the diet. It is worth noting that delousing stress has caused significant downregulation of oxidative stress markers, and most of the assessed genes (except CAT and SePP) were downregulated after 1 h delousing stress compared to pre-delousing. Some of them (SOD, MnSOD, Gpx7, GR) remained downregulated even after 24 h post-stress, and the GPx1 and GST1 were further downregulated at 24 h post-stress. Only SePK and GPx4b were recovered at 24 h post-stress. Similar decreased activity of these oxidative markers in response to different stressors/chemicals were reported elsewhere (Ghelichpour et al., 2019; Penglase et al., 2014; Wischhusen et al., 2020). The probable reason for the downregulation could be, as described before (Huang et al., 2020; Puerto et al., 2010), when stress is within the critical limit, the antioxidant defense system of organisms can remove ROS and protect complex biological macromolecules from ROS attack. However, when ROS levels induced by stress surpass the scavenging capability may result in a weakening of the activity of the antioxidant defense system, which is seen in the significant

downregulation of oxidative markers in this study (**Paper II**). Thus, a significant downregulation of key oxidative genes might be due to the accumulation of more reactive oxygen species or free radical formation and directly indicating the severity of stressful environment during delousing procedure, which is further supported by increased mRNA expression of stress protein HSP70 at 1 h post-stress.

To conclude, although delousing stress significantly affected the expression of oxidative markers, dietary n-3 LC-EPA+DHA levels did not alter the expression of oxidative stress markers. Therefore, results from our study might indicate the adaptive nature of Atlantic salmon to a high pro-oxidative environment or the other sense, lower dietary EPA+DHA 10 g/kg of feed seems sufficient to maintain the oxidative defense system under stressful environment.

4.4. Dietary EPA+DHA and tissue mineral status

This discussion is based on the long-term trial in salmon fed with EPA+DHA diets (**Paper II**), as mineral status was not analyzed in the AGD challenge trial in fish fed n-6/n-3 ratio diets (**Paper I**) and also from the *in vitro* trial (**Paper III**). The plasma macromineral (Ca, Na, Mg, and P) and trace minerals (Cr, Mn, Fe, Co, Cu, Zn, and Mo) were significantly affected in response to delousing stress. Stress- induced hydromineral disturbance, demineralization and mobilization of trace minerals from other tissue to compensate immune and metabolic needs were reported in fish (Kucukbay et al., 2009; Kacem et al., 2004; Sahin & Kucuk 2003). As these minerals are involved in several physiological and biochemical processes, changes in mineral levels are attributed to increased metabolic and enzymatic demands during the course of stress.

In mammals, the negative effects of low n-3 LC-PUFA and high n-6/n-3 ratio on bone deformities are well documented (Watkins et al., 2000) and suggested to be due to their eicosanoids productions (Thanabalan and Kiarie, 2021). Similarly, the effect of dietary FA composition on bone mineral contents and demineralization were also reported in Atlantic salmon ((Berge et al., 2009; Bou et al., 2017; Gil Martens et al., 2010). However, despite observed changes in macromineral and trace minerals in the present

thesis (**Paper II**), dietary EPA+DHA levels did not cause vertebral deformities in fish. Similarly, a previous study in Atlantic salmon fed with an increase in n-6/n-3 LC-PUFA ratio from 0·2 to 6 (ratios 6:1, as in the **Paper I**) found no inflammation-related spinal deformities (Gil Martens et al., 2010). However, in the latter trial, the high n-6/n-3 diet fulfilled the EPA+DHA requirements (22 g/kg feed)).

Interestingly, in the present thesis, we observed the significant changes in trace minerals status (Zn, Se, Fe) in fish that received high EPA+DHA (diet 3.5) levels and low-fat diet (diet 1.3 RF) compared to other dietary groups. The higher Zn in the whole body of fish fed either high EPA+DHA (Diet 3.5) or low dietary fat (Diet 1.3L) indicate an important interaction between the levels of EPA + DHA and Zn in the diet. Thus, low EPA + DHA levels probably affect the uptake of Zn across the gut and result in reduced levels of Zn in the body, as previously observed in rats (Lobo et al., 2009). However, the exact mechanism of dietary fat level and zinc utilization remains unclear in fish. Studies in fish suggest the increased dietary requirement for Zn when increasing plant-based ingredients in the diet (Antony Jesu Prabhu et al., 2019; Antony Jesu Prabhu et al., 2018a), due to the presence of anti-nutritional factors like phytic acid, which can reduce the Zn availability. Additionally, a negative correlation of high fat or low EPA+DHA on Zn retention was also reported in mammals (Knudsen et al., 1990; Lobo et al., 2009; Tallman and Taylor, 2003; Weigand and Boesch-Saadatmandi, 2012). In the last few decades, in parallel with the increased use of plant-based ingredients and high-fat level in the feed for farmed salmon, a simultaneous decrease in body Zn status in ready-to-slaughter salmon was also noticed (4-5 kg; 55 to 35 mg/kg) (Shearer et al., 1994; Aas et al., 2019). Considering the significant importance of Zn in fish (discussed in sec. 3) and for the consumer, further research is necessary to understand the underlying mechanism. High EPA+DHA in the diet markedly increased the plasma and whole-body Se levels. Increased oxidative stress causes the increased demand for oxidative enzymes, consequently increasing the Se utilization, which in turn lowers the Se reserve in the body. Previous studies in fish have demonstrated the increased Se requirement during stress (Kucukbay et al., 2009; Rider et al., 2009). Thus, increased Se reserve in fish fed high dietary EPA+DHA could be a beneficial effect on stress mitigation and might, in turn, protect n-3 LC-PUFA from oxidation.

Hepcidin is a protein encoded by the HAMP gene, a key regulator of iron transport and utilization, and is considered an important marker of iron bioavailability (Ganz, 2003). HAMP transcript levels increase with increased iron load, which in turn diminish the iron uptake by acting reciprocally with iron import proteins such as DMT or TFR (Antony Jesu Prabhu et al., 2016). In this present study, plasma iron level was not affected by dietary EPA+DHA. However, fish fed high EPA+DHA had significantly increased the mRNA expression of HAMP, which is concurrent with downregulation of TFR and high iron status in the liver during delousing stress signifies a pro-oxidant environment. Similar increased expression of HAMP in response to DHA supplement was reported in mammals (Diaz-Castro et al., 2015). Thus, our results suggest the possible interaction of dietary EPA+DHA on the iron store in fish. Further, results from oxidative stress markers reiterate the robustness of Atlantic salmon to a pro-oxidative environment (Hamre et al., 2010).

To conclude, increasing EPA+DHA levels in the diet increased the trace mineral levels in plasma, liver, and whole-body (Zn, Se, Fe). Both Zn and Se both have upper limits regarding what can be legally added to the aquafeeds, and play important roles in maintaining fish health, knowledge on how the dietary fatty acid composition and lipid level affect body stores of these minerals is crucial for the aquaculture industry.

4.5. Intracellular trafficking of FA affected by chain length and saturation level of FA

This discussion is based on the RTgutGC *in vitro* trial exposed to SFA, MUFA or PUFA (**Paper III**). The results on increased CLDs accumulation by OA in RTgutGC with concurrent increase in TAG synthesis (Figure 8) from quantitative analysis was supported by several other studies in Caco-2 cell line (Bateman et al., 2007; Dashti et al., 1990; Field et al., 1988; Levin et al., 1992) and other cell types. This is consistent with a previous *in vitro* study done in Atlantic salmon adipocytes, where OA led to a higher accumulation of lipid droplets than PA and EPA (Bou et al., 2020b; Todorcević et al., 2008). Similarly, previous studies in fish fed diets rich in oleic acid have shown excessive CLDs accumulation in intestinal tissue (Olsen et al., 1999; Olsen et al., 2000; Olsen et al., 2003). However, it should be mentioned the diets used in those studies were

purified diets without the inclusion of phospholipids and might suggest that this accumulation could be due to insufficient synthesis of phospholipids required for lipoprotein synthesis (Sæle et al., 2018). The different n-6/n-3 ratios with varying amount of SFA and MUFA among diets in the present thesis (**Paper I**) could have also influenced the CLDs accumulation in the enterocyte and may warrant further research.

The low recovery of [^{3}H] labeled FAs for PA (58.7±7.0 %) and ARA (56±7.6 %) compared to OA (91.3±2.1 %) might be increased β-oxidation in the cells for PA and ARA. Unfortunately, the current study was not designed to measure the β-oxidation products. However, a study in isolated hepatocytes from Atlantic salmon reported that ARA, followed by PA, were the preferential substrates for β-oxidation compared to OA (Stubhaug et al., 2005). Similarly, a study in HepG2 (human hepatocyte carcinoma) cell line reported the induction of mitochondrial β-oxidation by ARA (Eynaudi et al., 2021). This increased β-oxidation could be suggested to be the lipid-lowering effects of LC-PUFA and/or an adaptive mechanism to relieve cytotoxicity effects of free FA. Accordingly, another report from feeding trial 2 (as in **Paper II**) demonstrated the lipid-lowering effect in the liver of Atlantic salmon fed a high EPA+DHA diet (35 g/ kg of feed) compared to a low EPA+DHA diet (10 and 13 g/ kg of feed) (Hundal et al., 2022). The lower uptake of OA from apical compartment than other FA (PA and ARA) might indicate the negative consequence of excessive CLDs accumulation and physical barrier to uptake of this FA (Morais et al., 2007). Further, the increased amount of TAG in the basolateral compartment for ARA might indicate a better regulated intracellular transport of LC-PUFA. Taken together, results from the current *in vitro* study, as well as earlier studies, suggest that OA has the capacity to promote more CLDs to a higher degree than ARA and PA, and suggesting that dietary inclusion of OA rich oils for fish feed should be limited.

It is worth to be mentioned that, in the current trial, a significant difference in DEGs were found in transcriptomics analysis between FA treatment (PA, OA, and ARA). However, transcriptomics data does not confirm what has been observed quantitatively (using confocal laser microscopy and radio labeled FA), that could be due to long hour of incubation with FA or negative feedback mechanism.

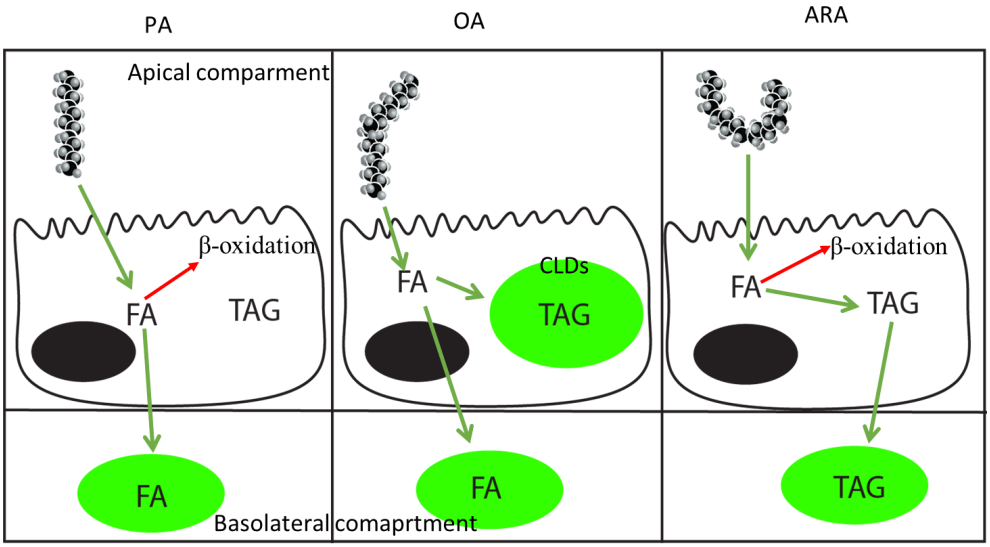

Figure 8. Summary of uptake, accumulation, and transport of FAs in RTgutGC cells. FA, fatty acid; PA, Palmitic acid; OA, Oleic acid; ARA, Arachidonic acid; TAG, Triacylglycerol; CLDs, cytosolic lipid droplets. RTgutGC cells treated with OA increased the TAG synthesis and stored in CLDs. The cells treated with ARA transport the FA as TAG to the basolateral side. A significant amount of PA and OA are transported as free fatty acids to the basolateral compartment. PA and ARA are assumed to be preferred substrate for β-oxidation over OA.

4.6. RTgutGC as a model in lipid nutrition research in fish

The RTgutGC cell derived from the rainbow trout intestine (Kawano et al., 2011) exhibits apical and basolateral characteristics of intestinal epithelial cells and is proposed to be equivalent to Caco-2 cell line (human colon adenocarcinoma cell line). In addition, RTgutGC contains functionally active polarized absorptive cells (enterocytes) that play a central role in lipid metabolism. The present thesis (**Paper III**) is the first of a kind using a fish enterocyte cell line (RTgutGC) as a model to investigate the intracellular fates of different FAs. The results obtained show promise and are comparable to the most studied Caco-2, as well as similar to several *in vitro* (Bou et al., 2020a; Todorcević et al., 2008) and *in vivo* studies in fish (Olsen et al., 1999; Olsen et al., 2000; Olsen et al., 2003). Additionally, RTgutGC cell lines showed encouraging results in several key aspects of *in vitro* research in fish nutrition, including nutrient

uptake (Antony Jesu Prabhu et al., 2018b; Kim et al., 2018; Pumputis et al., 2018), test functional ingredients and gut immune function (Holen et al., 2021; Wang et al., 2019a) and toxicity (Langan et al., 2017; Schug et al., 2020). Therefore, RTgutGC could serve as a useful model for studying the intracellular uptake, metabolism and transport of fatty acid at molecular, proteomics, and metabolomics levels. In general, *in vitro* cell line models are considered as the starting point in biological research and provide an insight into cells. Cell line offer several advantages such as cost effective, material availability, ease of use, controlled environment, ethical concerns associated with the use of experimental animals (Kaur and Dufour, 2012). Nevertheless, since cell lines are isolated from their natural environment, these models may not completely predict the effects on the entire organism. However, the scientific knowledge gained through the *in vitro* cell model can be utilized for further in vivo experiments.

5. Conclusions

- The results showed that the dietary n-6/n-3 ratios (1.3, 2.4, and 6.0) and EPA+DHA levels (10, 13, 16, and 35 g / kg of feed) tested did not affect the growth of Atlantic salmon under optimal conditions. Nevertheless, diets with higher n-6/n-3 ratios (6:1) and low EPA+DHA levels 10-16 g/ kg of feed reduced the growth of the fish under challenging conditions like AGD challenge and delousing / handling stress, respectively. Previous studies in Atlantic salmon reported the optimal dietary level of EPA+DHA between 10-15 g / kg of feed. However, results from present thesis indicate that increasing dietary EPA+DHA level to 35 g/kg of feed and maintain the optimal n-6/n-3 ratio (1-2:1) is beneficial for Atlantic salmon under different challenging environmental conditions.
- Neither dietary n-6/n-3 ratios nor EPA+DHA levels influenced inflammatory and immune marker genes during the challenge. Further, dietary EPA+DHA levels did not alter the plasma cortisol or genes related to oxidative stress, nor did it cause any vertebral deformities or any other negative external welfare indicators. Our results indicate that Atlantic salmon is capable to mount an adequate immune and stress response during experimental conditions, even on diets with high n-6/n-3 ratio (6:1) and sub-optimal level of EPA+DHA (10-16g/kg of feed).
- High dietary EPA+DHA (35 g/kg of feed) positively influenced the trace mineral (Zn, Se, Fe) levels in plasma, liver, and the whole body of fish. Increased knowledge in this area, especially dietary FA on body minerals retention, would be beneficial for better feed formulation considering the upper legal limits (eg. Zn, 180 mg/kg diet) to improve the body stores of these minerals, better fish health, and limit the discharge into the environment.
- In vitro trial in RTgutGC cells demonstrated that OA increased the TAG synthesis and was accumulated in CLDs to a higher degree (x6) than PA and ARA, whereas a significant amount of ARA was secreted as TAG to the basolateral side. Low recovery of radioactive [^{3}H] material for ARA and PA

might indicate that ARA and PA are the preferred substrate for β-oxidation, whereas OA is primarily synthesized as TAG and stored in the CLDs lipid droplets. Our data and other in vivo studies have demonstrated that higher inclusion of VOs rich in OA result in higher accumulation of large lipid droplets in the enterocytes, which may in turn reduce fatty acid absorption efficiency and may negatively affect the growth.

6. Future directions

- In this AGD challenge trial, even low grade AGD infection had negative impact on the growth of fish that were fed high n-6/n-3 ratio (6:1), but not for other dietary n-6/n-3 ratio (1:1 and 2:1) that might signify the beneficial effect of n-3 LC PUFA in the diet. However, the question is, would this effect persist if the severity of the infection was to be higher for this tested diet groups. Better understand of the interaction between dietary n-6/n-3 ratio and challenge pressure necessitate to design an experiment in order to test different challenge pressure like severe, mild or low with different n-6/n-3 ratio in the diets. This knowledge would certainly benefit the farming industry as the severity of the AGD infection fluctuates farm to fam, season to season, vary with temperature fluctuations.
- It is a known fact that dietary n-6 and n-3 can be converted into their respective eicosanoids. Considering the role of eicosanoids in inflammatory and immune response, one would expect that changes in the dietary n-6/n-3 ratio would directly be reflected on the animal's inflammatory and immune responses. However, the low-grade AGD infection during the challenge period did not provoke the effect of a high dietary n-6/n-3 ratio on inflammatory markers at mRNA expression level. Further studies aiming at direct evidence for pro- and anti-inflammatory eicosanoid metabolites and even a metabolomic approach would enable further understanding.
- This thesis and in Lutfi et al. (2022) showed the beneficial effect of higher dietary EPA+DHA (35 g/kg of feed) on growth performance under challenging environmental conditions. However, there is a gap between higher (35 g/kg of feed) and sub-optimal level (16 g/kg of feed) of EPA+DHA tested in this trial. Thus, further experiments in sea cages needed to be designed in a way to evaluate the dietary EPA+DHA with different levels (minimum of 5 levels) between 16 to 35 g/kg of feed as a dose response experiment to determine the optimum requirement and ensure the robust salmon.

- The mechanisms regarding dietary fatty acids on body mineral status remained unanswered in the current study needed further investigation. Especially the effect of dietary FA on uptake and transport of trace minerals needs to be studied in vitro and in vivo experiments for different FA, for instance, saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acid.
- Our results demonstrate the promising potential of RTgutGC as in vitro fish intestinal cell model for lipid transport study. Further, the non-correlation of quantitative data with transcriptomics in this present thesis might warrant the further experiments with time-series sampling to better understand the gene regulation at transcriptional levels. Further, the proteomics approach could be used to investigate at the wide-scale proteins involved in lipid transport. This thesis largely demonstrates that resynthesis of TAG in CLDs is based on carbon chain length and saturation levels of dietary FA. However, the different biological process involved that mediate the transport the dietary FA according to their chain length and saturation level need to be studied in detail at protein level.

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Paper I

Chandrasekar Selvam, Mark D. Powell, Nina S. Liland,
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Impact of dietary level and ratio of n-6 and n-3 fatty acids on disease progression and mRNA expression of immune and inflammatory markers in Atlantic salmon (Salmo salar) challenged with *Paramoeba perurans*

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Impact of dietary level and ratio of n-6 and n-3 fatty acids on disease progression and mRNA expression of immune and inflammatory markers in Atlantic salmon (*Salmo salar*) challenged with *Paramoeba perurans*

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ABSTRACT

The aim of the study was to investigate the influence of dietary level and ratio of n-6/n-3 fatty acids (FA) on growth, disease progression and expression of immune and inflammatory markers in Atlantic salmon (*Salmo salar*) following challenge with *Paramoeba perurans*. Fish (80 g) were fed four different diets with different ratios of n-6/n-3 FA; at 1.3, 2.4 and 6.0 and one diet with ratio of 1.3 combined with a higher level of n-3 FA and n-6 FA. The diet with the n-6/n-3 FA ratio of 6.0 was included to ensure potential n-6 FA effects were revealed, while the three other diets were more commercially relevant n-6/n-3 FA ratios and levels. After a pre-feeding period of 3 months, fish from each diet regime were challenged with a standardized laboratory challenge using a clonal culture of *P. perurans* at the concentration of 1,000 cells L^{-1}. The subsequent development of the disease was monitored (by gross gill score), and sampling conducted before challenge and at weekly sampling points for 5 weeks post-challenge. Challenge with *P. perurans* did not have a significant impact on the growth of the fish during the challenge period, but fish given the feed with the highest n-6/n-3 FA ratio had reduced growth compared to the other groups. Total gill score for all surfaces showed a significant increase with time, reaching a maximum at 21 days post-challenge and declined thereafter, irrespective of diet groups. Challenge with *P. perurans* influenced the mRNA expression of examined genes involved in immune and inflammatory response (TNF-α, iNOS, IL4-13b, GATA-3, IL-1β, p53, COX2 and PGE_2-EP4), but diet did not influence the gene expression. In conclusion, an increase in dietary n-6/n-3 FA ratio influenced the growth of Atlantic salmon challenged with *P. perurans*; however, it did not alter the mRNA expression of immune genes or progression of the disease.

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INTRODUCTION

The rapid growth of the aquaculture industry has raised concern about its sustainability and environmental impacts. One concern is the high dependency on marine ingredients such as fish meal and fish oil for aquafeeds, which could increase the pressure on the already vulnerable global fisheries. In many aquaculture-grown fish species, there has been a large shift in dietary composition in the last two decades, with a replacement of the majority of marine ingredients with other available ingredients, such as plant oils and proteins. Several studies in Atlantic salmon have demonstrated the possibility of partial or complete replacement of fish oil with plant oils without any adverse effects on growth, feed utilization, and survival of the fish (*Hixson et al., 2017*; *Katan et al., 2019*; *Liland et al., 2013*; *Sissener et al., 2016b*; *Torstensen et al., 2005*; *Turchini, Torstensen & Ng, 2009*). However, there are some limitations in using plant oils. For example, they completely lack the very long-chained polyunsaturated fatty acids (>20C fatty acids, LC-PUFA, *e.g.*, eicosapentaenoic acid, 20:5n-3, EPA; docosahexaenoic acid, 22:6n-3, DHA; arachidonic acid, 20:4n-6, ARA). These fatty acids (FA) are considered crucial for many physiological processes, and are thus important for optimal growth and health of the fish. Additionally, most plant oils contain high linoleic acid (18:2n-6) than α-linolenic acid (18:3n-3). Thus, increased plant oil in fish feed at the expense of fish oil greatly affects the FA profile in the feeds, with decreasing LC-PUFA levels and increases in both absolute content of n-6 FA as well as dietary n-6/n-3 ratio.

As dietary FA directly influences membrane lipid composition, manipulating dietary n-6 and n-3 FA will alter the ratio of ARA and EPA in cell membranes. This can, in turn, alter the production of eicosanoids and modulate the inflammatory and immune responses in fish (*Bell et al., 1991*; *Bell, Tocher & Sargent, 1994*). As in mammals, eicosanoids are key mediators of inflammation in fish and play a central role in immune regulation (*Bruce German, Bruckner & Kinsella, 1986*; *Rowley et al., 1995*; *Rowley et al., 2012*). Although this is a complex system (*Araujo et al., 2019*), eicosanoids derived from the n-6 family, such as leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2) are generally considered to have a greater ability to promote pro-inflammatory responses than eicosanoids from the n-3 family, which are considered to have more anti-inflammatory properties (*Lands, 1992*). However, there are studies that have reported the inhibitory effects of PGE_2 on classical proinflammatory cytokines such as TNF-α and IL-1β in human whole blood culture (*Miles, Allen & Calder, 2002*) and fish cell line models (*Fast, Ross & Johnson, 2005*; *Furne et al., 2013*). Further, it has been described that PGE_2 has both pro-inflammatory and anti-inflammatory roles and that some lipoxins derived from ARA, especially lipoxin A4, may be important for switching off inflammation (*Calder, 2009*; *Liu et al., 2017*). Although ARA is the preferred substrate for the main enzymes

cyclooxygenase (COX) and lipoxygenase (LOX) in eicosanoid production pathways, an increased EPA levels in cell membrane can competitively inhibit the production of n-6 derived eicosanoids (*Bell et al., 1996a*; *Bell et al., 1996b*). Similarly, a study in human cell models (HUVEC) reported the high production of COX metabolites when exposing the cells to ARA resulted in increased production. However, the high levels of COX metabolites were suppressed when ARA was combined with DHA, ALA (*Araujo et al., 2019*). Thus, the ratio between these FA is important for subsequent eicosanoids production. The balance between n-6 and n-3 FA in the diet is therefore important for a healthy inflammatory-and immune response (*Calder, 2008*; *Simopoulos, 2002*), and the potential modulation of immune and inflammatory responses by dietary n-6/n-3 ratio remains in the interest of research. In fish, information about the relationship between dietary n-6/n-3 ratio, immune responses and specific disease resistance is limited and contradictory. In Atlantic salmon, variable results on health impact of increased n-6/n-3 ratios have been reported, ranging from no apparent negative effects (*Andresen et al., 2019*; *Bransden, Carter & Nichols, 2003*; *Gjøen et al., 2005*; *Grisdale-Helland et al., 2002*; *Hundal et al., 2021*), to significantly increased mortality following transportation induced stress (*Bell et al., 1991*), reduced resistance to infection (*Martinez-Rubio et al., 2012*; *Thompson, Tatner & Henderson, 1996*), effects on the humoral immunity and expression of immune related genes (*Caballero-Solares et al., 2017*; *Seierstad et al., 2009*), and increased production of n-6 FA derived prostaglandins (*Hundal et al., 2021*).
The contradicting reports regarding the link between dietary lipids and immune regulation in fish remain unclear and it may be due to interactions between environment, pathogens and fish.

Amoebic gill disease (AGD) caused by the protozoan *Paramoeba perurans* (syn. *Neoparamoeba perurans*; *Feehan et al., 2013*), is established in many salmon producing areas and poses a major challenge for salmon aquaculture industry. AGD and other infections represent a source of progressive stress for salmon (*Nowak & Archibald, 2018*; *Robledo et al., 2020*). A few studies on functional diets and AGD infection are reported (*Bridle et al., 2005*; *Mullins et al., 2020*; *Powell et al., 2007*). However, to our knowledge, there are no controlled studies on the relation between dietary fatty acids and gill infections. Nevertheless, there are dietary studies on fatty acids where the fish happened to be affected with AGD during the trial, indicating a relation between the severity of the disease with the dietary n-6/n-3 ratio (*Glencross et al., 2014*; *Sissener et al., 2016b*). While AGD is the result of an ecto-parasitic infection, there is evidence of immune and inflammatory responses in the infected gills (*Marcos-Lopez et al., 2018*; *Marcos-Lopez et al., 2017*; *Pennacchi et al., 2014*), as well as a systemic physiological compromise (*Powell et al., 2008*), which means an effect of dietary FAs is plausible. Therefore, the present study was designed to determine the effects of both absolute levels of dietary n-3 and n-6 FA, and the n-6/n-3 ratios on disease progression, growth, and mRNA expression of selected immune and inflammatory markers during amoebic gill infection in Atlantic salmon.

Table 1 **Feed formulation in g/100 g of the four diets used in the challenge trial (4 mm pellet size).** Previously published in *Hundal et al. (2020)*.

	Diet 1	Diet 2	Diet 6	Diet 1H
Wheat	7.2	7.2	7.2	7.2
Soya protein concentrate	28.2	28.2	28.2	28.2
Sunflower meal	6.0	6.0	6.0	6.0
Wheat gluten	18.0	18.0	18.0	18.0
Faba beans dehulled	2.0	2.0	2.0	2.0
Fish meal North Atlantic	10.0	10.0	10.0	10.0
Linseed oil	1.2	1.2	1.3	2.7
Sunflower oil	0.6	4.8	19.3	5.1
Olive oil	12.2	7.7	0.0	1.6
Coconut oil	0.7	1.0	0.3	0.0
Fish oil North Atlantic	0.0	0.0	3.9	4.9
Fish oil Capelin	10.0	10.0	0.0	10.5
Premixes	3.7	3.7	3.7	3.7
Yttrium	0.1	0.1	0.1	0.1

Note:
Diet 1/Diet 2/Diet 6/Diet 1H, diet codes are set according to dietary n-6/n-3 ratio. The final diet is labelled 1H due to its higher absolute contents of n-3 and n-6 compared to the first diet.

MATERIALS AND METHODS

Experimental diets

The experimental diets were produced by Skretting ARC (Stavanger, Norway).
The formulation for the experimental diets is shown in Table 1 (previously published in *Hundal et al., 2020*). A total of four different diets were formulated to contain different absolute contents of n-6 and n-3 FA and n-6/n-3 ratios from 1.0–6.0. The n-3 FAs were kept constant for the first three diets and constituted approximately 8% of total FA. Roughly half of the n-3 FAs were provided as EPA + DHA (10 g/kg of EPA + DHA in the final feed) to meet the minimum requirement (*Rosenlund et al., 2016*). In the same three diets, the n-6 FA levels were adjusted to create n-6/n-3 ratios of 1, 2 and 6. The diet with the n-6/n-3 ratio of 6.0 was included to make sure potential n-6 effects were revealed, while the three other diets had more commercially relevant n- 6/n-3 ratios of averaging 0.9 (*Sele et al., 2018*). The fourth diet was formulated to contain double the amount of n-3 FA as compared to first three diets (20 g/kg of EPA + DHA in the final feed), but with an n-6/n-3 ratio of 1, like the first diet. The experimental diets are referred in the text according to their dietary n-6/n-3 ratios and named diet 1, diet 2, diet 6 and diet 1H (diet 1H, due to its higher absolute contents of n-3 and n-6 FA compared to diet 1) (previously published in *Hundal et al., 2020*). All the experimental diets were produced from a common dry meal mixture and differed only in the combination of oils used to adjust the n-3 and n-6 FA content of the extruded pellets. Proximate and FA composition of the experimental diets (3 and 4 mm pellet) have been described previously in detail by *Hundal et al. (2020)* and are also presented in Table 2 (4 mm pellet) for a better understanding of the current study.

Table 2 Analysed dietary proximate (g/100 g) and fatty acid composition (% of total FA), total FA (mg/g) of the four diets used in the challenge trial (4 mm pellet). Previously published in *Hundal et al. (2020)*.

	Diet 1	Diet 2	Diet 6	Diet 1H
Proximate composition g/100 g				
Lipid	29.8	30.0	28.6	28.8
Protein	44.5	44.1	44.5	44.8
Ash	5.1	5.2	5.5	5.4
Fatty acids (% of total FA)				
ΣSFA	19.7	19.7	16.6	19.8
12:0	1.3	1.7	0.4	0.1
14:0	3.3	3.5	1.5	4.4
16:0	11.2	10.4	9.4	11.5
18:0	2.5	2.6	3.7	2.7
ΣMUFA	59.4	52.2	27.3	43.5
16:1n-7	3.8	3.7	1.5	5.1
18:1n-7	2.2	2.0	1.1	2.0
18:1n-9	37.1	30.1	23.1	18.2
20:1n-9	6.4	6.4	0.7	7.0
22:1n-11	7.7	7.7	0.7	8.5
Σn-6	11.1	18.2	46.9	18.4
18:2n-6	10.8	17.9	46.7	17.6
20:4n-6 (ARA)	0.1	0.1	0.1	0.2
Σn-3	7.7	7.7	7.8	15.1
18:3n-3	2.9	2.9	3.1	5.7
18:4n-3	0.5	0.5	0.4	0.9
20:5n-3 (EPA)	2.4	2.4	2.0	4.4
22:6n-3 (DHA)	1.5	1.5	1.8	3.1
EPA + DHA	3.9	3.9	3.8	7.5
ΣPUFA	20.9	28.1	56.1	36.8
n-6/n-3	1.4	2.4	6.1	1.2
Sum FA (mg/g)	270.7	272.7	296.5	255.3

Note:
Diet 1/Diet 2/Diet 6/Diet 1H, diet codes are set according to dietary n-6/n-3 ratio. The final diet is labelled 1H due to its higher absolute contents of n-3 and n-6 compared to the first diet; FA, fatty acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acids.

Pre-feeding

The feeding trial was started with a pre-feeding period and performed at Skretting ARC Research station at Lerang, Norway (December 2017). Atlantic salmon (SalmoBreed, Erfjord, Stamfisk AS) with a mean weight of 80 g were randomly distributed to four large circular tanks (3 m inner diameter and water holding capacity of around 7,000 L), 735 individuals in each tank. The tanks were supplied with flow through sea water (25–27ppt) at 8 °C and 24 h light photoperiod was maintained throughout the pre-feeding period. The fish were fed to satiation with experimental diets (one tank per diet; 3 mm pellet size,

proximate and FA composition published in *Hundal et al., 2020*) and the pre-feeding period lasted for about 4 months (till March 2018) to stabilize FA composition of tissues. During the pre-feeding period, fish were examined routinely and no signs of AGD related infection or any other disease conditions were found.

Amoeba

The amoebae (*P. perurans*) for the challenge experiment were obtained and used under the license from ILAB (Industrial and aquatic laboratory, Bergen Norway). The original amoeba clone (ES301013 C2) was isolated from an AGD outbreak in Sotra in 2013 by ILAB, according to methods described by *Morrison, Crosbie & Nowak, 2004* and 18S rRNA gene PCR method (*Young et al., 2008*) were used to detect and identify the species and confirmed as *P. perurans*. From this, an *in vitro* monoclonal culture was established as described by *Crosbie et al. (2012)*. The amoebae were sub-cultured regularly and occasional PCR tests were carried out to confirm the presence of *P. perurans* (*Young et al., 2008*). The virulence of the amoebae was also assessed regularly, as described by *Collins et al. (2017)* and the same amoeba clone were previously used for other challenge experiments and a reliable and reproducible infection developing AGD to mean gill score of 2 within 21 days (*Rosenlund, 2017*). For this study, the amoebae were cultured in liquid media (0.1% Malt Yeast Seawater Broth) in plastic culture flasks maintained at 15 °C and isolated by scraping before counting using a hemocytometer.

Challenge experiment and sampling

A challenge experiment was performed at the ILAB challenge facility, Bergen, Norway. After the pre-feeding period, fish-(250 fish from each diet group) were transported to ILAB by truck designed for transportation of live fish (Jarle Tveiten Transport, Tørrvikbygd, Norway), and maintained full strength salinity (34 ppt) and water temperature 8 °C. After arrival, fish were distributed into the experimental tanks (500 L); three technical replicates for each diet group (three tanks containing 60 fish/diet) were used as challenge groups and one tank for each diet group was assigned as a negative control group. The average weight and length of the fish assigned for each experiment tank were 335.4 ± 20.3 g (mean ± SD) and 28.6 ± 0.6 cm (mean), respectively. Prior to the challenge, the fish were acclimated to the tank conditions for 2 weeks. During this period, the water temperature was gradually increased from 8 to 13 °C and 34 ppt salinity was maintained. The experimental tanks were supplied with flow-through filtered seawater (34 ppt) at 13 °C and 12:12 light:dark photoperiod was maintained. The fish were fed to satiation with the experimental diets (4 mm pellet) throughout the challenge study. Water quality parameters were regularly monitored, and standard husbandry procedures were followed as per the guidelines from ILAB.

The fish in the challenge groups were exposed to *P. perurans* trophozoites at a concentration of approximately 1,000 cells L^{-1}. This challenge concentration has been established and is recognized to induce disease at a moderate rate at 13 °C (*Downes et al., 2017*; *Pennacchi et al., 2016*; *Rosenlund, 2017*). Prior to inoculation with amoebae, the water flow to the tanks (including negative control tanks) was stopped. The appropriate

amount of amoebae isolate was added to each tank and the fish were maintained under static water conditions for 60 min with constant aeration before the water flow was reinstated. The behavior of the fish was carefully observed throughout the entire exposure, and in the immediate hours thereafter.

A pre-challenge sample point (week 0, prior to the addition of amoebae) was performed, followed by weekly sampling point throughout the 5-week trial. At the first four samplings (0 dpc; days of post challenge, 7 dpc, 14 dpc and 21 dpc), 10 fish per tank were sampled and at the last two samplings, 7 fish per tank were sampled. At each sampling event, fish from the tank were randomly removed by dip net and euthanized by an overdose of Finquel vet. (Tricainmesilat, 100 mg L^{-1}). Weight and length of the fish were recorded. All gill arches of the fish were visually assessed for the presence and severity of lesions and scored on a scale from 0 to 5, with 0 representing no signs of infection and 5 representing a severe AGD infection, according to (*Taylor et al., 2009b*). To reduce blood contamination of gill samples, fish were first bled by caudal venous puncture. Gills from the left second gill arch was carefully dissected out and filaments from the apex region of the gill arch were flash frozen in liquid N_2 and stored at −80 °C for gene expression analysis. The remaining part of the gill arch was fixed in a 10% neutral buffered formalin solution (VWR®) and processed for histology (3–5 µm sections stained with H&E). The third left gill arch was dissected, and flash frozen in frozen in liquid N_2 and stored in −80 °C for FA profile analysis.

mRNA expression in gill tissue

To study the effect of diets on gill mRNA expression, samples from 0 dpc (pre-challenge), 21 dpc (maximum gill score) and 28 dpc (recovery phase) samplings were used (n = 6 individual fish per tank). The selected target genes for mRNA expression analysis include pro-inflammatory cytokines (TNF 1α, IFN γ, IL1β, IL4-13b), genes involved in eicosanoids production pathway (PGE2-EP4, COX-2), cellular stress markers (HSP70, HSP90), tumor suppressor protein p53 (p53), the inducible isoform of nitric oxide synthase (iNOS) and T-Cell-specific transcription factor GATA-3 (GATA-3).
The procedures for RNA extraction, reverse transcription and qPCR followed are described in detail in *Hundal et al. (2021)*. In brief, total mRNA was extracted from gill tissue using EZ1 RNA Universal Tissue Kit (Qiagen, Crawley, UK) and the Bio Robot EZ1 according to the manufacturer's instructions. Quality and integrity of RNA were assessed with the NanoDrop ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). A two-step, real-time RT-PCR protocol was followed to assess the mRNA transcriptional levels of the selected target genes. The stability of the reference genes (β-actin and EF1ab) and mean normalized expression of the target genes were calculated using CFX Maestro software (Bio-Rad CFX maestro version 1.1, Bio-Rad laboratories, Hercules, CA). The primer sequences of the selected target genes as well as the reference genes are given in Table 3 (Invitrogen, Life Technologies, Waltham, MA, USA).

Table 3 Fatty acid composition (percentage of total fatty acids) of gills from Atlantic salmon fed different diets at 21 days of post-challenge with *P. perurans*.

Target genes	Forward primers	Reverse primers	Accession number
PGE_2-EP4	CTGATTATGATGCACAAGCGGTTCA	GTTTACAAAAATCCGCAGCACCAAAG	KM519440
COX2	GATCGCTGGAAGGGTGGCTG	GCCAGCTCTGTCTCTCCTGTGAGGT	AGKD04000045
TNF-α	GTGTATGTGGGAGCAGTGTT	GAAGCCTGTTCTCTGTGACT	NM_001123617
IL-1 β	GCTGGAGAGTGCTGTGGAAGAAC	CGTAGACAGGTTCAAATGCACTTTGTG	AY617117
iNOS	GCTACACGACATGAAACACCCAGAGTT	GGACATCCTGGACATAGACCTTTGG	*Benedicenti et al. (2019)*
IL4-13b	CTCAATGGAGGTTTGGAGTTTCAGG	TGCAGTTGGTTGGATGAAACTTATTGT	HG794525
GATA3	ACCCAAGCGACGACTGTCTG	GGTGAGAGGTCGGTTGATATTGTG	XM_014153208
p53	CTTGGGAGGGATATGATAATTTC	AGGGTAGAGATGGAGGGCTG	XM_014195886
Hsp70	CATCGACTTCTACACCTCCATCAC	CTGAAGAGGTCGGAACACATCTC	AJ632154
Hsp90	GTGTGAACAATGGGAAATGGAACA	CAGCGTGCATGTTATGTTGCA	NM_001173702.1
β-actin	CCAAAGCCAACAGGGAGAA	AGGGACAACACTGCCTGGAT	BG933897
EF1αb	TGCCCCTCCAGGATGTCTAC	CACGGCCCACAGGTACTG	AF321836

Note:
Results are means ± SD (for challenged group $n = 3$, for non-challenged group. 3 fish per diet). P-values of two-way ANOVA are presented for factors 'diet', 'AGD challenge' and interaction between diet and AGD challenge. ns, not statistically significant ($p > 0.05$). Different superscript letters within an individual row denote statistically significant differences in fatty acid content according to Tukey's multiple comparison test. FA, fatty acid; ΣSFA, sum of saturated fatty acids; ΣMUFA, sum of monounsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acids. Diet 1/Diet 2/Diet 6/Diet 1H, diet codes are set according to dietary n-6/n-3 ratio. The final diet is labelled 1H due to its higher absolute contents of n-3 and n-6 compared to the first diet. PGE_2-EP4, prostaglandin E2-EP4 receptor; COX2, cyclooxygenase2; TNF-α, tumour necrosis factor- α; IL-1β, induction of interleukin-1β; iNOS, inducible nitric oxide synthase; IL413-b, interleukin 4/13b; GATA3, transcription factor GATA binding protein3; p53, tumor suppressor protein p53; Hsp70, Heat shock protein 70; Hsp90, Heat shock protein 90; β-actin, Beta-actin; EF1 αb, Elongation factor 1 alpha B.

Fatty acid composition

The FA composition of gill tissue was determined according to the method by (*Lie & Lambertsen, 1991*) as previously described in detail (*Jordal et al., 2005*; *Sissener et al., 2016a*), using gas liquid chromatography (Scion 436-GC, Scion Instruments, UK). The FA were identified by their retention times using a standard mixture of methyl esters (Nu-Chek-Prep, Elysian, MN, USA) and the FA composition (area %) was determined. Quantification of FA was done using 19:0 as an internal standard and integration of peak areas was done using software Chromeleon® version 7.2 (Thermo Scientific, Waltham, MA, USA). The FA composition of the feed was analysed by Skretting ARC, according to the method described by *Rosenlund et al. (2016)*.

Ethics statement

The feeding trial and subsequent disease challenge was conducted according to the guidelines of the Norwegian State Commission for Laboratory Animals and the protocol for the challenge experiment was approved by the National food safety authority (Mattilsynet, Norway) under the permit number 14333.

Statistics

Statistical analysis was performed using the software Statistica 13.4 (Statsoft Inc., Tulsa, OK, USA) and GraphPad Prism version 8.0 (Graphpad Software Inc., San Diago, CA, USA). Data were tested for normality and homogeneity of variance using a Kolomogorov–Smirnov test and Shapiro-wilk test. Data from gene expression analysis were log

transformed for statistical analysis. All data were tested for tank effect using nested ANOVA (tanks nested under groups), and no significant difference was observed between the replicates. The data were subjected to a two-way analysis of variance (ANOVA), with diet and days post-challenge as the two factors. Only in the cases where a significant effect was observed within a factor, one-way ANOVA followed by Tukey's multiple comparison were performed for each factor separately. For all statistical tests, p-values < 0.05 were considered significant. All results are expressed as mean ± standard deviation (SD).

RESULTS

Growth

The body weight and length were registered for all fish sampled at each sampling point (Fig. 1; Table S1). No differences were found in the mean individual start body weight (335.4 ± 20.3 g; mean ± SD) or length (28.6 ± 0.6 cm; mean ± SD) of the fish assigned to each experimental tank for the challenge trial. No dietary effects on weight or length were seen in the non-challenged fish. In the challenged fish, diet had a significant effect on the weight and length of the fish. The fish fed with the highest n-6/n-3 ratio (6:1, diet 6) had a significantly reduced weight compared to the other dietary groups (diet 1, diet 2 & diet 1H) at 21 dpc and 35 dpc (p = 0.0004 and 0.038, respectively; Fig. 1). Similarly, length of the fish were also significantly affected at 35 dpc (p = 0.017, Fig. 1) in the group fed with the highest n-6/n-3 ratio (6:1, diet 6) compared to other dietary groups. There was a borderline negative interaction on weight of the fish between time after AGD exposure and dietary treatments (p = 0.07, Fig. 1). No mortality was observed during the experiment.

Gill pathology

Gill scores and severity of gill lesions were recorded at all the sampling points (n = 10 per tank, three tanks per diet group challenged fish, one tank per diet in non-challenged control-groups) to assess how different dietary n-6/n-3 ratios influence the gill response and health of the fish upon infection with *P. perurans*. No signs of AGD were visible upon gross examination of the gills of the uninfected control fish at any of the sampling points. In the challenged fish, the gill score for all surfaces and severity of gill lesions showed a significant increase with time, reaching a maximum at 21 dpc, declining after that (Fig. 2; Table S2). At the pre-challenge sampling point (0 dpc), fish showed no signs of AGD. At 7 dpc, the gill scores had increased to 0.52 ± 0.03 (mean ± SD) in the challenged fish, displaying only a few focal, raised white patches on the gills. Thereafter, gill scores further increased at 14 dpc (0.84 ± 0.13), reaching a maximum at 21 dpc (1.21 ± 0.11; mean ± SD; Fig. 2). There was a clear temporal change in the severity of gross pathology, with distinguishable AGD-like lesions across the gills at 21 dpc. At sampling points 28 dpc and 35 dpc, mean gill scores were decreased to 0.53 ± 0.28 and 0.53 ± 0.08 (mean ± SD), respectively (Fig. 2). There were significant differences in gill score between the different sampling points after infection (p < 0.0001; Fig. 2), but no dietary effects were

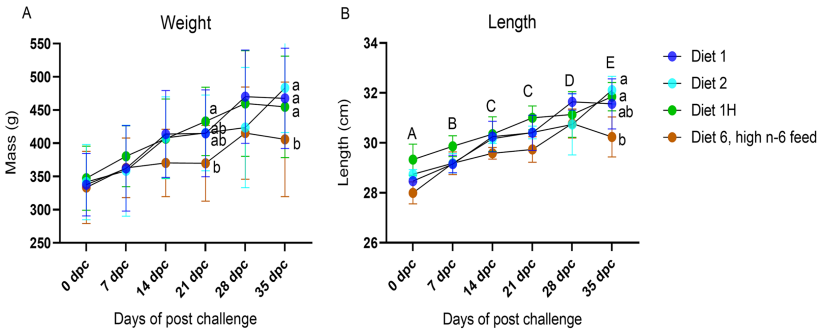

Figure 1 Weight (A) and Length (B) of Atlantic salmon fed different level and ratio of n-6 and n-3 FA and challenged with *P. perurans*. Data presented as mean with standard deviation (n = 3). Two-way ANOVA followed by Tukey's Multiple comparison was performed for factors diet and AGD challenge. Capital letters (A, B) indicate significant differences between the time-points ($p < 0.05$) and small letters (a, b) indicate significant differences between the dietary group ($p < 0$ .05) detected in nested one-way ANOVA. Diet 1/Diet 2/Diet 6/Diet 1H, diet codes are set according to dietary n-6/n-3 ratio. The final diet is labelled 1H due to its higher absolute contents of n-3 and n-6 compared to the first diet.
Full-size DOI: 10.7717/peerj.12028/fig-1

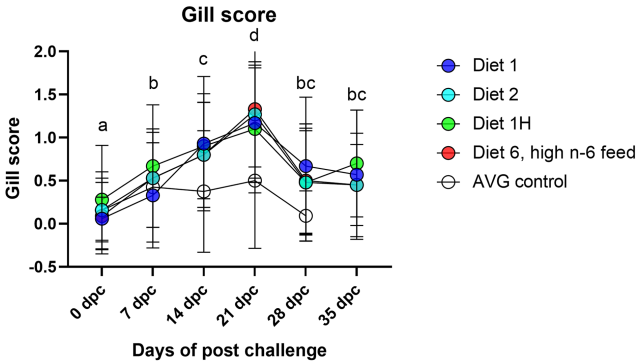

Figure 2 Gill score of Atlantic salmon fed different level and ratio of n-6 and n-3 FA and challenged with *P. perurans*. Data presented as mean with standard deviation (n = 3). Two-way ANOVA followed by Tukey's Multiple comparison was performed for factors diet and AGD challenge. Different letters indicate the significant differences between the time-points ($p < 0.05$). No significant effects between diet groups were detected at any time points. Diet 1/Diet 2/Diet 6/Diet 1H, diet codes are set according to dietary n-6/n-3 ratio. The final diet is labelled 1H due to its higher absolute contents of n-3 and n-6 compared to the first diet. AVG control, unchallenged fish. Full-size DOI: 10.7717/peerj.12028/fig-2

found during challenge period. Histologically, no classical characteristic signs of AGD lesions were observed and no amoebae were associated with the lesions. However, a few fish at 21 dpc, showed a mild hyperplastic lesion (ht) with fused adjacent lamellae and stratified layer of epithelial tissue at the lesions surface (Fig. 3). At 21 dpc, three fish were positive for *P. perurans* by qPCR (Pharmaq analytiq, Bergen, Norway).

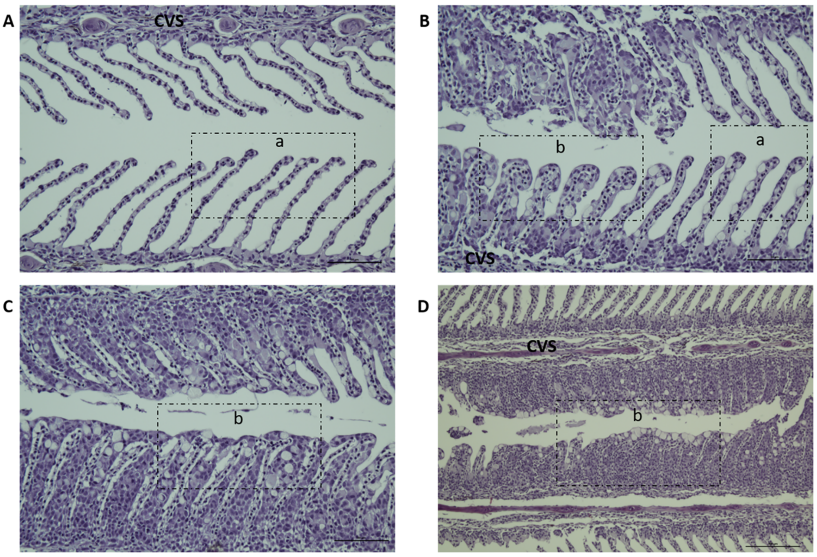

Figure 3 Histopathology representative examples of *P. perurans* challenged Atlantic salmon gills at 21 dpc. Lowercase letter (a) indicates the healthy gill and (b) indicates the hyperplastic lesions with fused lamellae and the stratified layer of epithelial tissue at the lesions surface. The central venous sinus is denoted as CVS; bar = 50 µm (A, B, C); bar = 100 µm (D). Full-size DOI: 10.7717/peerj.12028/fig-3

Fatty acid composition of the gills

The FA composition of the gills was analyzed for both challenged and non-challenged fish at 21 dpc, where we observed the maximum gill score (Table 4). The FA composition of the gills largely reflected the dietary FA composition. In both challenged and non-challenged fish, the percentage of ΣSFA (~25% of total FA) in the gills showed no significant difference between the dietary groups despite diet 6 having lower absolute dietary SFA contents than the other dietary groups. The percentage of ΣMUFA, however, was significantly different between dietary groups and reflected the dietary MUFA content. In both challenged and non-challenged fish, total n-6 FA content in the gills increased with increasing dietary n-6/n-3 ratio. The fish fed diet 2 had slightly elevated level of some of the n-6 FA (20:3n-6, 20:4n-6) compared to diet 1H, despite both diets containing equal amounts of n-6 FA. In the challenged fish, the ARA content was significantly increased with increasing dietary n-6/n-3 ratio (diets 1, 2 and 6), and for the dietary group 1H, ARA content was intermediate between the dietary groups 1 and 2. In the non-challenged fish, only the fish fed diet 6 showed a higher ARA in gill tissue compared to the other dietary groups. The EPA levels were significantly reduced with increasing dietary n-6/n-3 ratio in the diets (diets 1, 2 and 6). However, levels of DHA were similar between all dietary groups, despite differences in dietary DHA content. Further, AGD challenge did not significantly influence the levels of these FA (EPA and DHA). There was a significant interaction effect between challenge and diet on total n-6 FA in gill, challenged fish fed diet

Table 4 Nucleotide sequence of primers used in qPCR for mRNA expression analysis of target and house-keeping genes.

	Challenged group				Non-challenged group				Two-way ANOVA *P* value		
	Diet 1	Diet 2	Diet 6	Diet 1H	Diet 1	Diet 2	Diet 6	Diet 1H	Diet	Challenge	Diet* Challenge
ΣSFA	24.5 ± 0.9	25.2 ± 0.5	24.8 ± 0.4	25.4 ± 0.9	24.5 ± 0.8	23.8 ± 1.0	23.6 ± 0.8	25.5 ± 1.2	ns	ns	ns
ΣMUFA	34.3 ± 3.0^{a}	30.6 ± 1.5^{ac}	20.2 ± 1.2^{b}	26.9 ± 2.1^{c}	33.4 ± 2.8^{a}	32.4 ± 2.8^{a}	21.8 ± 0.3^{b}	25.6 ± 1.4^{bc}	<0.0001	ns	ns
18:2n-6	6.1 ± 0.5^{a}	8.7 ± 1.5^{b}	17.9 ± 0.8^{c}	8.4 ± 1.1^{d}	5.9 ± 0.6^{a}	9.7 ± 0.9^{b}	21.0 ± 0.7^{e}	7.6 ± 0.9^{d}	<0.0001	0.0103	0.0005
20:2n-6	0.7 ± 0.1^{a}	0.9 ± 0.1^{b}	1.9 ± 0.2^{c}	0.9 ± 0.1^{b}	0.7 ± 0.1^{a}	1.0 ± 0.0^{b}	1.8 ± 0.1^{c}	0.8 ± 0.2^{ab}	<0.0001	ns	ns
20:3n-6	1.4 ± 0.2^{a}	1.9 ± 0.2^{b}	2.2 ± 0.1^{c}	0.7 ± 0.1^{d}	1.4 ± 0.2^{a}	1.6 ± 0.1^{ab}	2.0 ± 0.2^{c}	0.8 ± 0.1^{d}	<0.0001	ns	ns
20:4n-6 (ARA)	3.4 ± 0.5^{a}	4.3 ± 0.4^{ab}	5.4 ± 0.6^{c}	3.7 ± 0.5^{ab}	3.8 ± 0.6^{ab}	3.7 ± 0.5^{ab}	4.7 ± 0.1^{b}	4.1 ± 0.3^{ab}	0.0002	ns	0.0137
Σn-6	11.9 ± 0.4^{a}	16.3 ± 0.4^{b}	27.2 ± 0.7^{c}	14.2 ± 0.5^{d}	12.2 ± 0.2^{a}	16.4 ± 0.3^{b}	30.6 ± 0.7^{e}	13.7 ± 0.7^{d}	<0.0001	0.0946	0.0006
18:3n-3	1.0 ± 0.2^{a}	0.8 ± 0.4^{a}	0.8 ± 0.1^{a}	1.9 ± 0.4^{b}	0.90 ± 0.2^{a}	1.0 ± 0.2^{a}	1.1 ± 0.1^{a}	1.6 ± 0.4^{b}	<0.0001	ns	ns
20:4n-3	0.4 ± 0.1^{a}	0.3 ± 0.1^{a}	0.3 ± 0.0^{a}	0.5 ± 0.1^{b}	0.4 ± 0.1^{a}	0.4 ± 0.1^{a}	0.2 ± 0.1^{a}	0.5 ± 0.1^{b}	<0.0001	ns	ns
20:5n-3 (EPA)	4.4 ± 0.5^{a}	3.9 ± 0.3^{a}	3.0 ± 0.3^{b}	5.6 ± 0.6^{c}	4.8 ± 0.4^{a}	4.2 ± 0.2^{a}	3.0 ± 0.2^{b}	6.0 ± 0.5^{c}	<0.0001	ns	ns
22:5n-3	1.1 ± 0.2^{a}	1.1 ± 0.1^{a}	1.0 ± 0.1^{a}	1.5 ± 0.2^{b}	1.2 ± 0.1^{a}	1.2 ± 0.0^{a}	0.9 ± 0.1^{a}	1.5 ± 0.1^{b}	0.0006	ns	ns
22:6n-3 (DHA)	16.9 ± 2.7^{ab}	17.0 ± 1.2^{ab}	16.5 ± 1.0^{ab}	18.7 ± 1.7^{b}	17.8 ± 1.7^{a}	16.0 ± 2.0^{a}	16.6 ± 0.3^{a}	20.9 ± 1.7^{b}	0.0005	ns	ns
Σn-3	24.4 ± 2.6^{a}	23.7 ± 0.9^{a}	21.9 ± 1.1^{a}	29.0 ± 1.7^{b}	25.9 ± 1.8^{a}	23.3 ± 1.9^{a}	22.2 ± 0.3^{a}	30.9 ± 0.8^{b}	<0.0001	ns	ns
n-6/n-3	0.50 ± 0.1^{a}	0.7 ± 0.0^{b}	1.3 ± 0.1^{c}	0.5 ± 0.1^{a}	0.5 ± 0.0^{a}	0.7 ± 0.1b	1.4 ± 0.0^{c}	0.4 ± 0.1^{a}	<0.0001	ns	0.0116

Notes:

PGE_2-EP4, prostaglandin E2-EP4 receptor; COX2, cyclooxygenase2; TNF-α, tumour necrosis factor- α; IL-1β, induction of interleukin-1β; iNOS, inducible nitric oxide synthase; IL413-b, interleukin 4/13b; GATA3, transcription factor GATA binding protein3; p53, tumor suppressor protein p53; Hsp70, Heat shock protein 70; Hsp90, Heat shock protein 90 ; β-actin, Beta actin; EF1 αb, Elongation factor 1 alpha B.

Results are means ± SD (for challenged group $n = 3$, for non-challenged group. three fish per diet). *p*-values of two-way ANOVA are presented for factors 'diet', 'AGD challenge' and interaction between diet and AGD challenge. ns, not statistically significant ($p > 0.05$). Different superscript letters within an individual row denote statistically significant differences in fatty acid content according to Tukey's multiple comparison test.

FA, fatty acid; ΣSFA, sum of saturated fatty acids; ΣMUFA, sum of monounsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acids. Diet 1/Diet 2/Diet 6/Diet 1H, diet codes are set according to dietary n-6/n-3 ratio. The final diet is labelled 1H due to its higher absolute contents of n-3 and n-6 compared to the first diet.

6 had significantly reduced total n-6 FA ($p = 0.0356$) compared to the other dietary groups. This was due to a reduction in 18:2n-6 ($p = 0.019$) in the challenged fish, while other n-6 FA showed no significant differences between challenged and non-challenged fish. There also was a significant interaction between diet and challenge for ARA, appearing to have a response in the opposite direction than 18:2n-6 in diet group 6 when challenged.

mRNA expression in gills

The mRNA expression of the gills of the control and challenged fish were analyzed, and samples from 0 dpc (pre-challenge), 21 dpc (maximum gill score) and 28 dpc (recovery phase) samplings were used (Fig. 4). In general, AGD influenced the mRNA expression of examined genes involved in immune and inflammatory response (TNF-α, iNOS, IL4-13b, GATA-3, IL-1β, p53, COX2 and PGE_2-EP4), and most of these genes were either upregulated or downregulated at 21 dpc compared to 0 dpc and 28 dpc. The mRNA expression of all these genes remained equivocal at the pre-challenge and recovery phase than compared to the maximum gill score phase. However, diets did not affect all the examined gene expression at 21 dpc (Fig. S1) so the dietary treatments are presented grouped in the Fig. 4. At 21 dpc (maximum gill score), the mRNA expression of TNF-α and iNOS were significantly upregulated (TNF-α, $p = 0.0002$; iNOS, $p < 0.0001$) when

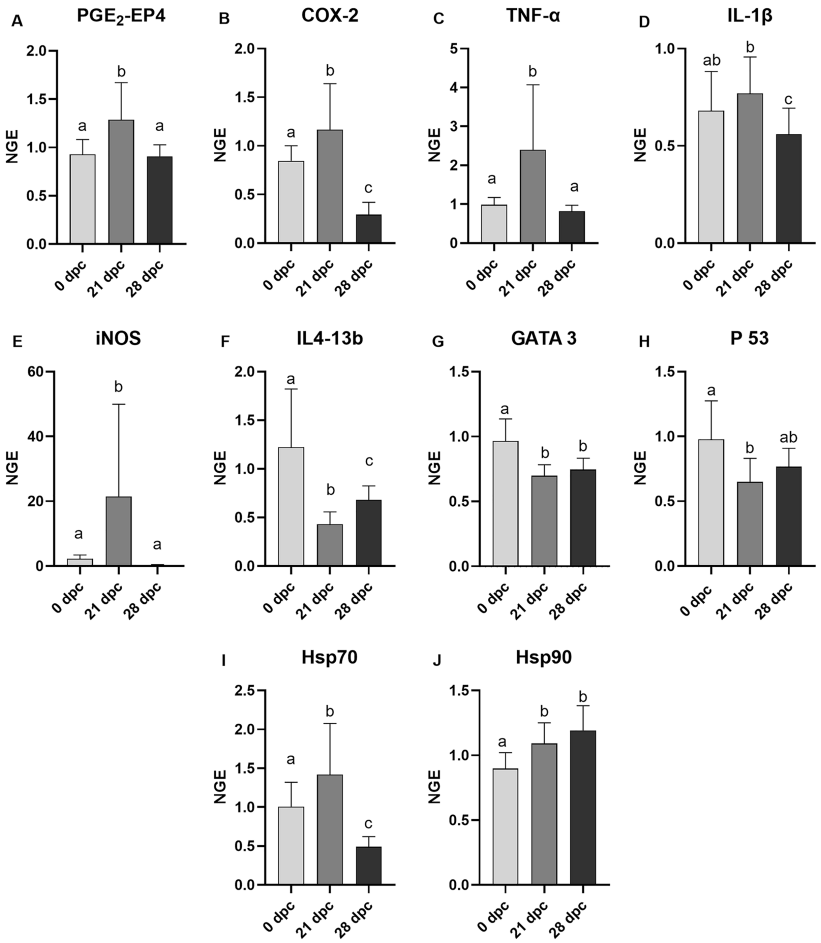

Figure 4 Mean normalized expression of gill mRNA from Atlantic salmon fed different level and ratio of n-6 and n-3 FA and challenged with *P. perurans*. Due to the lack of significant differences between dietary groups at any time point (Fig. S1), these were pooled in the figure to show the effects of the challenge. (A) PGE2-EP4, prostaglandin E2-EP4 receptor; (B) COX-2, cyclooxygenase2; (C) TNF-α, tumour necrosis factor- α; (D) IL-1β, induction of interleukin-1β; (E) iNOS, inducible nitric oxide synthase; (F) IL413-b, interleukin 4/13b; (G) GATA 3, transcription factor GATA binding protein; 3 (H) p53, tumor suppressor protein p53; (I) Hsp70, Heat shock protein 70; (J) Hsp90, Heat shock protein 90. Challenge effects were considered significant when $p < 0.05$ upon nested ANOVA followed by Tukey's multiple comparison analysis between time-points. Different letters (a,b,c) represent significant difference between time-points. Data are presented as mean with standard deviation (n = 6/tank). Days of post-challenge, dpc.
Full-size DOI: 10.7717/peerj.12028/fig-4

compared to both 0 dpc (pre-challenge) and 28 dpc (recovery phase). Similarly, expression of stress proteins Hsp70 and Hsp90 mRNA expression were also significantly upregulated at 21 dpc (Hsp70, $p = 0.0005$; Hsp90, $p < 0.0001$) when compared to 0 dpc (pre-challenge).

However, at 28 dpc, Hsp90 mRNA expression remained elevated, whereas Hsp70 mRNA expression was significantly downregulated when compared to 0 dpc and 21 dpc. Expression of IL4-13b, GATA-3 (the transcriptional factors for IL4-13 cytokines), and p53 mRNA were significantly downregulated at 21 dpc when compared to 0 dpc and 28 dpc (IL4-13b, $p < 0.0001$; GATA-3, $p = 0.0011$; p53, $p = 0.0088$; Fig. 4). The IL-1β mRNA expression was significantly high at 21 dpc compared to 28 dpc; however, no difference was found between 0 dpc and 21 dpc. The mRNA expression of COX2 and EP4 (Prostaglandin E2 receptor 4) genes were also significantly upregulated ($p < 0.0001$) at 21 dpc compared to 0 dpc and 28 dpc.

DISCUSSION

A series of investigations have reported that substitution of fish oil with different vegetable oils or different dietary ratios n-6/n-3 FA does not affect the growth performance or robustness of Atlantic salmon (*Bransden, Carter & Nichols, 2003*; *Gjøen et al., 2005*; *Glencross et al., 2014*; *Katan et al., 2019*; *Sissener et al., 2016b*; *Torstensen et al., 2005*). Accordingly, two trials using the same diets as in the current work, reported no dietary effects on final weight of Atlantic salmon after a three-month growth trial in seawater (*Hundal et al., 2020*) or after a 4 weeks stress trial following the growth trial, involving both repeated and acute stress (*Hundal et al., 2021*). However, in the present study, the diet with highest n-6/n-3 ratio (diet 6, 6:1) negatively affected the growth of the fish, but only in the fish challenged with *P. perurans*. This reduction of weight gain in fish fed high n-6/n-3 FA ratio could be due to combined effects of diet, AGD and the added handling stress introduced by weekly sampling. Further, the hypothesis of increased stress in all *P. perurans* challenged fish is supported by increased mRNA expression of stress markers Hsp70 and Hsp90 genes in gills at 21 dpc (Fig. 4), concurrent with the peak of gross pathology scores in gills (Fig. 2). Thereby, the added factor of a sub-optimal dietary n-6/n-3 ratio could have added additional stress to the fish and thus causing the observed changes in growth. Although the present study was of relatively short duration and more focused to test the effects of FA on AGD development, the results could suggest that the dietary n-6/n-3 FA ratio may affect growth during an AGD challenge and is therefore an important factor to be considered. As Amoebic gill disease (AGD) is currently one of the major challenges for the Atlantic salmon farming industry (*English et al., 2019*; *Oldham, Rodger & Nowak, 2016*; *Shinn et al., 2015*), thus the use of diets with a high level of n-6 FA relative to n-3 FA should not be recommended, based on our results.

In general, dietary FA can either be incorporated into cell membranes (polar lipids, PLs) as phospholipids or be stored as reserve lipids (neutral lipids, NLs), mainly as triglycerides. In fish, as in mammals, the dietary FA composition greatly influences the membrane FA composition (*Tocher, 2003*). The three FAs ARA, EPA and DHA are considered crucial for cell membrane functions which includes membrane permeability, fluidity, membrane fusion and ion transportation. In addition, these FA also serve as precursors for production of immunologically active eicosanoids and resolvins, hence being important for proper health status (*Arts & Kohler, 2009*; *Hagve, 1988*; *Valentine & Valentine, 2004*). Any diet-induced changes in membrane FA compositions could directly influence the

fish's immune system and health (*Bell et al., 1996b*; *Bell, Sargent & Raynard, 1992*; *Tocher et al., 2003*). Interestingly, we found that fish fed a high dietary n-6/n-3 ratio (diet 6) had significantly reduced 18:2n-6 content in gills when challenged compared to the fish in non-challenged conditions. As there was also a significant interaction between diet and challenge for ARA, where ARA seemed to be affected in the opposite direction to 18:2n-6 in diet group 6 when the fish were challenged, this could suggest an increased conversion from 18:2n-6 to the eicosanoid precursor ARA in challenged fish. Eicosanoids levels were not analyzed in the current study, but a feeding trial in Atlantic salmon using the same diets, reported that the fish fed the high n-6/n-3 ratio diet (diet 6, 6:1) had significantly elevated liver PGE_2 levels both before and after stressing conditions (*Hundal et al., 2021*). Changes in n-6 FA have been seen in earlier studies with a typical inflammatory response, such as in *Sanden et al. (2018)* where they reported that dietary pesticide chlorpyrifos-methyl affected the arachidonic acid metabolism in Atlantic salmon. They found a significantly reduced ARA content in response to increasing dietary pesticide exposure, indicating a possible change in eicosanoid synthesis from n-6 precursors.

Despite low gill scores and slow disease progression, the mRNA expression of selected immune and inflammatory genes were significantly influenced by the *P. perurans* infection. There was a demonstrable difference in gills mRNA expression of TNF-α, iNOS, PGE_2-EP4 receptor, COX-2, IL4-13b, p53, GATA3, Hsp70 and Hsp90 during the challenge period. Notably, most of these genes were either up-regulated or down-regulated at 21dpc compared to the two other time points pre-infection and at a resolution stage. The expression of COX2 (enzyme that catalyzes the conversion of ARA to prostaglandins), inducible nitric oxide synthase (iNOS, key enzyme in NO synthesis) and PGE_2-EP4 receptor were all significantly increased at 21 dpc compared to the two other time points sampled. Earlier studies with Atlantic salmon fed soybean oil diets, rich in n-6 FA, have shown induction of COX-2 expression in gill and intestinal tissue (*Olsen et al., 2012*; *Oxley et al., 2010*), as well as in head kidney leucocytes (*Holen et al., 2018*). There were, however, no significant dietary effects on the expression of these genes in the current trial. The EP4 receptor, one of four subtypes of the EP prostanoid receptor, is preferentially activated by prostaglandin E_2 (PGE_2) (*Jones, 2007*; *Konya et al., 2013*), thus indicating an increased presence of the ARA-derived PGE_2 in the gills at this time point. In fish, the importance of induced iNOS expression and nitric oxide production as a host protective immune response against pathogen infection has been demonstrated (*Acosta et al., 2004*; *Bridle, Morrison & Nowak, 2006*). Further, the Co-expression of COX-2 and iNOS is also well documented (*Chiarugi, Magnelli & Gallo, 1998*; *Rahman et al., 2001*). Additionally, *Timoshenko, Lala & Chakraborty (2004)* demonstrated a PGE_2-mediated upregulation of iNOS in murine breast cancer cells through the activation of EP4 receptor. Therefore, the upregulation of the mRNA expression of COX2, iNOS, and PGE_2-EP4 at the same time point (21 dpc) where fish had the highest gross pathology score supports the hypothesis that there may have been an activation of the eicosanoid synthesis at this time point.

In the current study, an increased dietary n-6/n-3 ratio significantly reduced EPA levels in the gills but did not affect DHA levels. Neither FA were affected in fish exposed to

P. perurans. The reduction of the EPA with increasing n-6/n-3 ratio occurred even when dietary EPA and DHA were unchanged. This is in line with previous results using the same diets (*Hundal et al., 2020*), where they reported reductions of EPA in liver polar lipids with increased dietary n-6/n-3 ratio. In general, the FA composition of fish gills is mostly composed of polar lipids (fat content typically ~2.2% wet weight; *Fountoulaki et al., 2003*) and the fat is therefore assumed to be mostly polar and reductions in EPA according to what *Hundal et al. (2020)* explained. In brief, it is generally accepted that DHA is preferentially incorporated over EPA into membrane phospholipids as a structural component (*Brodtkorb, Rosenlund & Lie, 1997*; *Stillwell & Wassall, 2003*). A decrease in EPA as reported might also be utilized to produce anti-inflammatory and inflammation resolving resolvins (*Calder, 2010*; *Wall et al., 2010*). A decreased EPA with increasing n-6/n-3 ratios in the diets may also be due to increased competition for enzymes shared by n-6 and n-3 FA. (*Buzzi, Henderson & Sargent, 1996*; *Henderson & Tocher, 1987*; *Tocher, 2003*). The result of this decreased membrane EPA in fish fed high n-6/n-3 ratio, could be a change in the overall inflammation status of the fish.

A clinical sign of AGD is the colonization of the gills by *P. perurans* and, consequently, the formation of white mucoid spots and plaques on the gill surface. These white spots are grossly assessed and scored on a scale of clear to heavy (*Adams, Ellard & Nowak, 2004*; *Taylor et al., 2009b*). In the present study, although no AGD lesions were observed histologically, gross gill scores were increased over time in the challenged fish and reached a maximum at 21 dpc, declining thereafter, and leading to the fish spontaneously recovering. Fish at 21 dpc showed a mild hyperplastic lesion (ht) with fused adjacent lamellae and a stratified layer of epithelial tissue at the lesions surface. Diets did not appear to have any effect on gill score or disease progression. This is in agreement with previous studies where increased dietary n-6/n-3 FA had no influence one disease resistance of Atlantic salmon against bacterial pathogens (*Bransden, Carter & Nichols, 2003*; *Gjøen et al., 2005*). However, this result also contradicts other studies, where it has been reported that high dietary n-6 FA negatively affects disease resistance against bacterial infection in Atlantic salmon (*Thompson, Tatner & Henderson, 1996*).
In commercial aquaculture practice, a gill score of 2 and above is the point where intervention treatment would be started (*Taylor et al., 2009b*). However, in the present study, although there was a significant difference in gill scores over the challenge period, the maximum gill score was relatively low (only minor gill pathology was observed; Fig. 2) compared to many other studies (*Marcos-Lopez et al., 2018*; *Marcos-Lopez et al., 2017*; *Rosenlund, 2017*; *Taylor et al., 2009b*; *Wiik-Nielsen et al., 2016*). Similar reduced gill scores have been reported previously by *Bridle et al. (2005)*; however, unlike the present study, amoebae were found on the gills of the challenged fish. Moreover, they observed an enhanced survival of a sub-population of Atlantic salmon exposed to *P. perurans* with relatively minor gill pathology. The reason for this may be complex and a combination of acquired resistance, be it immunological or genetic in origin (*Robledo et al., 2020*; *Robledo et al., 2018*; *Taylor et al., 2009a*; *Taylor et al., 2007*; *Findlay & Munday, 1998*; *Findlay et al., 1995*; *Vincent, Morrison & Nowak, 2006*).

Macrophages are essential for host defense (*Mosser & Edwards, 2008*; *Rolot & Dewals, 2018*; *Van Dyken & Locksley, 2013*). In general, macrophages are activated in number of ways, including interaction with immune cells such as CD8+, NK cells, and other monocytes (eosinophils, and basophils), macrophages and pre-exposed to a diversity of PAMPs (pathogen associated molecular patterns) (*Jang & Nair, 2013*; *Martinez & Gordon, 2014*; *Mills et al., 2000*). However, recent evidence from AGD experiments (*Benedicenti et al., 2015*; *Marcos-Lopez et al., 2018*) suggests that the classical Th1-type pathway or the alternate Th2-type pathway are most likely involved during the development of *P. perurans* infection. In any case, pro-inflammatory cytokines, including those from Th1-type cells such as IFN-γ and TNF-α induce iNOs that stimulate the NO production by granulocytes are released. Alternatively, cytokines such as IL4/13, IL-1β are released and needed the Th2-type pathway. Previous studies in salmon have shown that there is a switching from Th1 to Th2-type pathway in gills as AGD progresses (*Benedicenti et al., 2015*; *Marcos-Lopez et al., 2018*). A similar a switching from Th1 to Th2 pathway in response to sea lice infection has also been reported in Coho salmon (*Oncorhynchus kisutch*) (*Braden, Koop & Jones, 2015*) and rainbow trout (*O. mykiss*) (*Chettri et al., 2014*). They found that an early pro-inflammatory Th1-type pathway as an initial host response during infection with Pacific Sea lice and demonstrates subsequent regulatory Th2-type processes as candidate defense mechanisms as disease progresses. In the present study, the mRNA expression of Th1 pro-inflammatory cytokines such as TNF-α and the enzymes iNOS were markedly increased at the time point of maximum gill scores (21 dpc), while the Th2 pro-inflammatory cytokine IL4-13b was downregulated at the same time. GATA-3 (the transcriptional factor for IL4-13 cytokines) was also significantly downregulated at 21 dpc compared to the pre-challenge and recovery phase. These results suggest that an activation of the Th1 pathway is an initial host response and also more prominent during a low-grade/early stage AGD. This contradicts the work of *Rosenlund (2017)*, where the opposite was found where AGD was more severe. IL-1β has been identified as one of the hallmarks of Atlantic salmon response to AGD (*Nowak et al., 2014*). In the present study, IL-1β expression was significantly upregulated at the maximum gill score compared to in the recovery phase. Further, there was a clear downregulation of tumor suppressor protein (p53), a well-studied protein involved in the cellular stress response pathway (*Guo et al., 2016*) at the maximum gill score phase (21 dpc) compared to pre-challenge (0 dpc) and recovery phase (28 dpc). This is in agreement with previous reports, where a significant downregulation of p53 expression is described as one of the underlying mechanisms for cell proliferation in AGD (*Morrison et al., 2006*). The elevated mRNA expression of stress-related genes Hsp90 and Hsp70 in the present study is also in agreement with previous results (*Marcos-López et al., 2017*), where they are suggested to be involved indirectly in cell proliferation. Taken together, this demonstrated gene expression in gills has provided novel evidence of a Th1 type immune response to a low-grade/early stage AGD compared to other studies where AGD is more severe.

The reports on the effect of dietary n-6 FA levels on the fish immune system are contradictory, ranging from no apparent dietary effects (*Andresen et al., 2019*; *Bransden,*

Carter & Nichols, 2003; *Gjøen et al., 2005*), as seen in the present study, to reduced resistance against or increased mortality from pathogens (*Thompson, Tatner & Henderson, 1996*; *Martinez-Rubio et al., 2012*; *Estensoro et al., 2012*; *Montero et al., 2008*; *Montero et al., 2010*; *Montero et al., 2019*). Furthermore, it has been suggested that other dietary nutrients such as the sources of protein, availability of micronutrients, species differences, and experimental conditions may all impact the immune response.

CONCLUSION

The result of the present study showed that dietary n-6 and n-3 FA or their ratios were ineffective in altering the mRNA expression of immune genes or the progression of the disease in Atlantic salmon challenged with *P. perurans*. However, a high n-6/n-3 ratio of 6 caused a significant reduction in growth during a *P. perurans* challenge and subsequent development of low-grade hyperplastic lesions, compared to fish fed diets with low n-6/n-3 ratios at 1 and 2. Therefore, this is an important factor to be considered when formulating Atlantic salmon diets. Additionally, our study indicates the activation of Th1 type immune response to low-grade hyperplastic lesions and reports the spontaneous recovery of fish.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

Mark D. Powell is employed by Marineholmen RAS Lab AS & University of Bergen, Norway. Grethe Rosenlund is employed by Skretting ARC.Stavanger, Norway. Otherwise there are no competing interests.

Author Contributions

- Chandrasekar Selvam performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Mark D. Powell conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Nina S. Liland performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

- Grethe Rosenlund conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Nini H. Sissener conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (*i.e.*, approving body and any reference numbers):

The feeding trial and subsequent disease challenge was conducted according to the guidelines of the Norwegian State Commission for Laboratory Animals and the protocol for the challenge experiment was approved by the National food safety authority (Mattilsynet, Norway) under the permit number 14333.

Data Availability

The following information was supplied regarding data availability:

Mean normalized expression of gill mRNA from Atlantic salmon fed different diets challenged with N. perurans and raw data are available in the Supplemental Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.12028#supplemental-information.

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Paper II

Chandrasekar Selvam, Chandrasekar Selvam, Antony J. Prabhu Philip, Esmail Lutfi, Trygve Sigholt, Birgitta Norberg, Grete Bæverfjord, Grethe Rosenlund, Bente Ruyter and Nini H. Sissener

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Long-term feeding of Atlantic salmon with varying levels of dietary EPA + DHA alters the mineral status but does not affect the stress responses after mechanical delousing stress

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Abstract

Atlantic salmon were fed diets containing graded levels of EPA + DHA (1·0, 1·3, 1·6 and 3·5 % in the diet) and one diet with 1·3 % of EPA + DHA with reduced total fat content. Fish were reared in sea cages from about 275 g until harvest size (about 5 kg) and were subjected to delousing procedure (about 2·5 kg), with sampling pre-, 1 h and 24 h post-stress. Delousing stress affected plasma cortisol and hepatic mRNA expression of genes involved in oxidative stress and immune response, but with no dietary effects. Increasing EPA + DHA levels in the diet increased the trace mineral levels in plasma and liver during mechanical delousing stress period and whole body at harvest size. The liver Se, Zn, Fe, Cu, and Mn and plasma Se levels were increased in fish fed a diet high in EPA + DHA (3·5 %) upon delousing stress. Furthermore, increased dietary EPA + DHA caused a significant increase in mRNA expression of hepcidin antimicrobial peptide (HAMP), which is concurrent with downregulated transferrin receptor (TFR) expression levels. High dietary EPA + DHA also significantly increased the whole-body Zn, Se, and Mn levels at harvest size fish. Additionally, the plasma and whole-body Zn status increased, respectively, during stress and at harvest size in fish fed reduced-fat diet with less EPA + DHA. As the dietary upper limits of Zn and Se are legally added to the feeds and play important roles in maintaining fish health, knowledge on how the dietary fatty acid composition and lipid level affect body stores of these minerals is crucial for the aquaculture industry.

Key words: **Atlantic salmon: EPA: DHA: Fatty acids: Cortisol: Stress**

Feeds for aquacultured Atlantic salmon have changed from essentially a marine-based diet with a protein/fat ratio of 3:2 in the early 90s to a diet with 70 % plant ingredients and protein/fat ratio of approximately 1:1 today[1]. The shift in the ingredient composition of the salmon diet has resulted in a reduced level of long-chain *n*-3 fatty acids (FA), EPA (20:5*n*-3) and DHA (22:6*n*-3), and increased *n*-6 FA content. The beneficial effects of EPA and DHA are well documented[2–4], and the dietary contents of these FA are conditionally essential for Atlantic Salmon[5–8]. The pooled dietary requirement of the *n*-3 FA *α*-linolenic acid, EPA and DHA for salmonids has been reported to range from 10 to 25 g/kg feed depending on the species and experimental conditions[9]. However, recent results have shown that salmon fed a diet containing 10 g/kg EPA + DHA in the feed throughout the whole production cycle had significantly higher mortality than salmon fed 16 g/kg EPA + DHA when the fish were subjected to repeated handling stress such as delousing at high water temperatures in sea cages[6]. In comparison, 11 g of EPA + DHA /kg feed seemed to be sufficient for salmon during long-term feeding during the seawater phase in land-based tanks, and despite some negative health effects, even salmon fed only 5 g of EPA + DHA/kg feed had survival rates ≥ 99 %[10].

Abbreviations: FA, fatty acid; FAS, fatty acid synthase; Gpx, glutathione peroxidase; GST, glutathione S-transferase; G6PD, glucose-6-phosphate dehydrogenase; HAMP, hepcidin antimicrobial peptide; IFN-γ, interferon gamma; SOD, superoxide dismutase; TFR, transferrin receptor; TGF-β 1, transforming growth factor β 1.

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This clearly shows that the robustness of salmon fed low dietary EPA and DHA needs to be tested under challenging conditions experienced by the fish in sea cages rather than the controlled and stable conditions of land-based tanks. The salmon lice (*Lepeophtheirus salmonis*) are naturally occurring injurious ectoparasites that cause direct injury to sea-farmed salmon and pose a detrimental effect on salmon health and welfare[11]. Among the various methods for salmon delousing, thermal and mechanical delousing are the most frequently applied methods for the immediate removal of the salmon lice[11]. These delousing procedures involve crowding, handling, transportation or confinement, thereby creating a series of stressful conditions resulting in direct physical/mechanical injury to gills, fins, eyes, skin, etc., which might cause a considerable challenge to fish welfare[11,12].

Besides the change in dietary FA profile, increased inclusion of plant ingredients also reduces the supply and availability of dietary minerals to salmonids[13]. Both animal model studies and *in vitro* cell model studies have identified a possible relationship between EPA + DHA supplements and mineral homoeostasis. A significant interaction of EPA + DHA supplements on the expression of selenoproteins[14], Zn transporters[15] and HAMP (hepcidin antimicrobial peptide)[16] were previously reported *in vitro*. Conversely, these relationships are poorly studied in fish. The negative effects of low dietary levels of EPA and DHA included reduced tissue integrity of the intestine[17] and increased plasma cortisol levels (both basal and after a stress challenge)[18]. In the latter trial, reductions in liver Fe, Zn and Mn were also observed 3 h after stress, with further reductions 24 h after stress (pers. Comm. NH Sissener, IMR). Recent reports point towards a relation between tissue mineral status and endoplasmic reticulum (ER) stress, lipid and antioxidant metabolism in fish[19,20]. Furthermore, environmental stressors cause significant oxidative stress, which in turn affects the antioxidant defence system of animals *in vivo* and subsequently increases oxidative damage[21,22]. Additionally, increased oxidative stress leads to increased demand for antioxidant minerals such as Se, Zn, Cu and Mn, thereby reducing the concentration of these minerals in circulation[23–25]. Although considerable efforts have been made to understand the effects of dietary FA on oxidative stress[26–28], very few studies have evaluated these effects under stressful conditions[29,30]. Therefore, the aim of the present study was to investigate the effect of different levels of dietary EPA and DHA on stress responses, trace mineral concentration in plasma and liver, and expression of antioxidant markers in Atlantic salmon during delousing and also long-term effect of these FA on the whole-body mineral status.

Materials and methods

Diets and experimental design

This study was part of a long-term feeding trial with five different diets produced by BioMar AS. The experimental design, diets and fish performance have been described in detail in Lutfi *et al.*[31] and are also presented in Table 1 (9-mm pellet) for a better understanding of the current study. In brief, four different diets were formulated to contain graded levels of EPA + DHA (10, 13, 16 and 35 g/kg in the diet), and the only differences between the diets were the oil blends used to achieve the desired FA composition. Additionally, the fifth diet used in the current study was formulated to contain 13 g/kg EPA + DHA with a reduced total fat content compared with other four diets. The difference in EPA and DHA levels was achieved with different combinations of rapeseed oil and fish oil. Increasing dietary EPA and DHA levels resulted in decreasing levels of 18:1, 18:2*n*-6 and 18:3*n*-3 (Table 2). The average EPA:DHA ratio in the feeds was about 1·1 from start until the fish were about 500 g and then changed to about 1·6 until the stress trial. The experimental diets are referred to in the text according to their percentage of EPA + DHA in the feed (diet 1·0, diet 1·3, diet 1·6, diet 3·5 and diet 1·3 RF (reduced fat)). The experimental diets were produced from a similar dry feed mixture except for the 1·3 RF diet that had higher inclusion of wheat gluten and less oil added. Three different pellet sizes (4, 6 and 9 mm) and specific formulations of experimental feeds (five batches) were used to meet dietary requirements for the different life stages. The diet formulation of the 9-mm pellet given from approximately 1·0 – 2·5 kg is shown in Table 1 and for the other pellet sizes and feed batches are given in Lutfi *et al.*[31]. The analysed proximate and FA composition of all diets are provided in Table 2 for the 9-mm pellets, while the results from the same analyses for the 4- and 6-mm pellets are given in Lutfi *et al.*[31]. The mineral composition of the 9-mm pellet (given from approximately 1·0–2·5 kg) was analysed, and no differences were found between diets (Table 2).

The feeding trial was carried out at Gildeskål Research station (GIFAS) from October 2017 to January 2019. Atlantic salmon post-smolts (about 115 g) were acquired from a commercial hatchery (Marine Harvest Glomfjord) and transferred to GIFAS research station. After an initial acclimatisation with a standard commercial diet (BioMar), fish with a mean initial weight of about 275 g were randomly distributed into 15 outdoor sea cages (125 m^3, 5 × 5 × 5 m) with 190 individuals per cage (in triplicates). Fish were fed the experimental diets to apparent satiation once (autumn and winter periods) or twice (spring and summer) a day. The fish were reared under standard farming conditions. The water temperature, salinity and O_2 (measured at 3 m depth) were recorded daily, and the average water temperature ranged from 3°C in winter to 16°C in summer. Mortality data were recorded throughout the experiment. The experiment was conducted according to the National Guidelines for Animal Care and Welfare published by the Norwegian Ministry of Education and Research (Norwegian Food Safety Authority (FOTS); approval 16 059).

Sampling

At the start of the experiment, individual fish length and weight were recorded. Sea lice counts were recorded on weekly basis by randomly selecting one cage per week as per Norwegian regulations and standard procedures at GIFAS. The delousing was performed from 12 to 14 August 2018, when the average fish size reached about 2·5 kg. Before delousing, the fish were starved overnight, and mechanical delousing was performed according to GIFAS standard protocol with manual delousing of each fish.

Table 1. Formulation and chemical composition of the experimental diets (9-mm pellet size)

Raw material	Dietary EPA + DHA levels				
	Diet 1	Diet 1·3	Diet 1·3 RF	Diet 1·6	Diet 3·5
Rapeseed oil*	25·89	24·34	19·42	22·84	14·89
Guar meal†	20·00	20·00	17·44	20·00	20·00
Soya SPC	11·35	11·35	8·00	11·35	9·80
Wheat‡	10·81	10·81	13·40	10·81	11·51
Pea protein§	10·74	10·74	10·00	10·74	11·25
Fish oil‖	4·29	5·83	5·83	7·32	15·00
Fishmeal¶	5·00	5·00	5·00	5·00	5·00
Maize gluten**	5·00	5·00	5·00	5·00	5·00
Wheat gluten††	2·18	2·18	11·50	2·18	2·78
Mono-calcium phosphate (MCP) 1·32		1·32	1·32	1·31	1·33
Vitamin and mineral premix‡‡	1·53	1·53	1·53	1·53	1·53
Amino acids§§	1·33	1·33	1·28	1·33	1·36
Lucatin Pink 10 %	0·055	0·055	0·055	0·055	0·055
Water change	0·51	0·52	0·24	0·53	0·52
Chemical composition					
Moisture	6·00	6·00	6·00	6·00	6·00
Gross energy (MJ/kg)	25·30	25·30	24·55	25·30	25·27
Crude protein	36·20	36·20	39·83	36·20	36·18
Crude fat	34·71	34·72	30·10	34·72	34·56
Ash	5·51	5·51	5·21	5·51	5·48

* Denmark.
† India.
‡ Denmark.
§ China.
‖ Peru/Denmark.
¶ Peru/Denmark.
** Ukraine.
†† EU.
‡‡ Sweden.
§§ Germany/Korea/China.
Diet 1/diet 1·3/diet 1·6/ diet 3·5, diet codes are set according to their percentage of EPA + DHA in the feed.
One diet labelled as diet 1·3 RF due to its reduced-fat level.

In brief, all fish in a cage were transferred to a small well boat and anaesthetised before lice were removed using wet vacuuming with an adapted mouthpiece. After delousing, fish were dropped directly back in the cage. At each sampling, the net of the pen was raised to gather the fish, before fish are caught by a hand net. After netting, fish were immediately anaesthetised in a tub of water to prevent further stress response before the tub was transported from the net pen to the sampling area. During the delousing period, fish were sampled at three different time points relative to acute stress: before delousing (pre-stress), 1 h after and 24 h after delousing stress. At each sampling point, seven fish per cage were killed using overdose of anesthesia (Tricaine Pharmaq, 0·3 g/l). Weight and length were measured on all fish before blood was taken from the caudal vein with vacutainers coated with EDTA. Blood was centrifuged for 7 min at 4000 g to separate plasma and erythrocytes. Erythrocytes were washed thrice in physiological saline. At each sampling, plasma samples were collected from six individual fish per cage and erythrocytes were collected from three individual fish per cage only at pre-sampling. External welfare indicators were recorded for all sampled fish. For gene expression analysis, individual liver samples from six fish per cage were collected and flash-frozen in liquid N_2 before they were stored in −80°C until analysis. Final sampling was performed at the end of the experiment on January 2019. Pooled whole-body homogenates (fifteen fish per cage) were collected for mineral analysis for this current study (n 3).

Plasma cortisol

Cortisol was extracted from blood plasma by a method modified from Pankhurst & Carragher[32]. Briefly, plasma samples (100 µl) were mixed with 1 ml of ethyl acetate, vortexed for 20 s and centrifuged for 3 min at 1870 rpm and 4°C. The organic phase was collected with a Pasteur pipette, before a second extraction with 1 ml of ethyl acetate. The extracts were evaporated in a Speed Vac centrifuge (Savant 1000) for 30 min and dissolved in 1 ml of buffer (phosphate 0·1 M (pH 7·4), 0·4 M NaCl, 1 mM EDTA) by heating (60°C for 10 min) and stored at −20°C until further analysis. The extracted cortisol was measured by ELISA[33]. Cortisol EIA Monoclonal antibody (cat; 400 362), Cortisol AchE (acetylcholinesterase) tracer (cat; 10005272) and 96-well microtitre plates-coated Goat Anti-Mouse IG (cat; 400 008) were purchased from Cayman Chemicals. Standard cortisol was purchased from Sigma Aldrich (Sigma reference standards). According to the manufacturer, the primary antibody shows a 100 % cross-reactivity with cortisol, 0·23 % with 11-deoxycorticosterone and 17-hydroxyprogesterone, 0·15 % with cortisol glucuronide, and 0·14 % corticosterone. The lower detection limit was 0·031 ng/ml. The accepted inter-assay CV was 10 %. The percentage recovery of cortisol was 80 % and final cortisol concentration was corrected according to percentage recovery.

Gene expression analysis by quantitative real-time PCR

The gene expression analysis was studied in three selected dietary groups, including low (diet 1), mid (diet 1·6) and high

Table 2. Fatty acid composition (% of total fatty acids) and mineral composition (mg/kg) of the experimental diets (9-mm pellet size)

	Dietary EPA + DHA levels				
	Diet 1	Diet 1·3	Diet 1·3 RF	Diet 1·6	Diet 3·5
C 14:0	0·96	1·14	1·45	1·56	3·02
C 16:0	7·55	8·01	8·79	8·96	12·41
C 18:0	2·68	2·92	2·86	3·02	3·45
Σ SFA*	12·32	14·60	15·29	15·95	20·92
C 16:1 *n*-7	1·14	1·34	1·72	1·82	3·52
C 18:1 *n*-7	2·97	2·94	2·95	2·90	2·85
C 18:1 *n*-9	47·84	46·08	44·46	43·71	34·61
C 20:1 *n*-9	1·25	1·24	1·24	1·24	1·22
C 22:1 *n*-11	0·13	0·16	0·20	0·24	0·47
Σ MUFA†	55·00	53·53	52·64	52·17	46·48
C 16:2 *n*-6	0·12	0·14	0·19	0·21	0·41
C 18:2 *n*-6	19·13	18·69	17·99	17·50	13·42
Σ *n*-6‡	19·63	19·01	18·42	17·94	14·25
C 18:3 *n*-3	8·17	7·89	7·46	7·44	5·36
C 20:4 *n*-3	0·07	0·07	0·09	0·11	0·18
C 20:3 *n*-3	1·02	0·26	0·34	0·36	0·73
C 20:5 *n*-3	1·95	2·35	3·00	3·22	6·40
C 22:5 *n*-3	0·29	0·35	0·45	0·48	0·96
C 22:6 *n*-3	1·27	1·52	1·91	2·04	3·95
Σ *n*-3§	12·85	12·51	13·48	13·81	17·66
Σ EPA + DHA	3·22	3·86	4·91	5·26	10·35
Σ PUFA	31·91	32·48	31·90	31·75	31·51
EPA/DHA	1·54	1·55	1·57	1·57	1·62
n-3/*n*-6	0·65	0·66	0·73	0·77	1·24
Mineral composition mg/kg					
Ca	5844·5	5927·8	5627·3	5960·4	5400·5
Na	1061·4	1080·4	1022·1	1097·9	1052·2
K	6458·1	6361·4	5509·1	6239·9	5131·6
Mg	1502·2	1507·7	1332·1	1470·4	1285·3
P	7446·1	7624·1	7030·6	7469·7	6984·5
Mn	28·2	27·2	27·8	30·0	26·7
Fe	159·5	164·8	146·9	169·2	150·7
Cu	6·8	6·9	6·4	7·1	6·7
Zn	109·0	107·0	104·6	107·3	103·9
Se	0·8	0·8	0·7	0·8	0·8

* Includes 15:0. 17:0. 22:0. 24:0.
† Includes 16:1 *n*-5. 16:1*n*-9. 17:1*n*-7. 18:1*n*-11. 20:1*n*–7. 22:1 *n*–7. 22:1 *n*-9. 22:1 *n*-11. 24:1 *n*-9.
‡ Includes 18:3 *n*-6.
§ Includes 20:3 *n*-3.
Diet 1/diet 1·3/diet 1·6/ diet 3·5, diet codes are set according to their percentage of EPA + DHA in the feed.
One diet labelled as diet 1·3 RF due to its reduced-fat level.

(diet 3·5) EPA + DHA levels. Candidate genes involved in trace mineral metabolism (HAMP; TFR, transferrin receptor; Met-B, metallothionein-B), stress response (CAT, catalase; SOD, superoxide dismutase; Gpx1, glutathione peroxidase 1; Gpx4b, glutathione peroxidase 4b; Gpx7, glutathione peroxidase 7; GR, glutathione reductase; GST1, glutathione S-transferase 1; FAS, fatty acid synthase; G6PD, glucose-6-phosphate dehydrogenase; SePP, selenoprotein P and HSP70, heat shock protein 70) and inflammatory markers (IFN-γ, interferon gamma; TNF1α, tumour necrosis factor 1 alpha; TGF-β 1, transforming growth factor β 1 and IL4/13a, interleukin 4/13a) were analysed in the liver. The procedure for RNA extraction, reverse transcription and quantitative PCR (qPCR) followed were as described in Hundal *et al.*[34]. In brief, the total RNA was extracted from liver tissue using EZ1 RNA Universal Tissue Kit (Qiagen) and the BioRobot EZ1 according to the manufacturer's descriptions. Quality and integrity of RNA were assessed with the NanoDrop ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies) and the Agilent 2100 Bioanalyzer (Agilent Technologies). A two-step real-time PCR protocol was followed to assess the mRNA transcriptional levels of the selected target genes. The stability of the reference genes (geometric mean of both βact and elf1α) and mean normalised expression of the target genes were calculated using CFX Maestro software (Bio-Rad CFX maestro version 1.1, Bio-Rad laboratories). The details of the qPCR primers used for amplification of the reference and target genes are provided in Table 3.

Mineral analysis

The concentration of minerals in diets, liver, plasma (from delousing stress sampling) and whole fish (from final sampling) were determined by inductively coupled plasma MS (ICP-MS) as described elsewhere[35]. Briefly, finely ground samples of the feeds and freeze-dried homogenates of whole fish (approximately 0·5 g) or plasma (0·5 ml) were digested using 10 ml of HNO3 (69 % w/w) and 10 ml of H2O2 (30 % w/w) in an UltraClave (Milestone Inc.). The digested samples were subsequently diluted to 50 ml with Milli-Q® water. The samples

Table 3. Primers used for quantitative PCR

Primers	Forward	Reverse	Accession number
CAT	CCAGATGTGGGCCGCTAACAA	TCTGGCGCTCCTCCTCATTC	NM_001140302·1
SOD	GTTTCTCTCCAGCCTGCTCTAAG	CCGCTCTCCTTGTCGAAGC	XM_014145196·1
Gpx1	TCTCCTGCCATAACGCTTGA	GTGATGAGCCCATGGCCTTA	EH033571
Gpx4b	GGGCAGGTGGTGAAGAGATA	CACGCTAGGTTTCATCAGGC	BT044014·1
Gpx7	TGGGAAAGTCATGGATGCCT	GCTCAGGGTGTTTTGTTGCA	NM_001140889·2
GR	CCAGTGATGGCTTTTTTGAACTT	CCGGCCCCCACTATGAC	XM_014199133·1
GST 1	ATTTTGGGACGGGCTGACA	CCTGGTGCTCTGCTCCAGTT	BT049972·2
SePP	CACCTTCACACCTTGCTGAG	CAGTCCCCACAGATGCTTTG	BT072527·1
Met-B	TGAATAAAGAAGCGCGATCAAA	CTGGTGCATGCGCAGTTG	BT047801·1
HAMP	CATTGAAAATCGTGCATTGG	AAGGCCTTCATTCTCGGTTT	NM_001140849·1
TFR	TTGTCGCAACCCCTATAACC	AAGACAGCCCACATCAGGTC	XM_014188394·1
FAS	CATTGCCATGACGATGTCATAAT	TAAACGCTGACGCCTCATTG	XM_014179800·1
G6PD	GAGCTGCATGATGACAAGGA	TGTTCAGGGAGGAGACCATC	NM_001141724·1
HSP 70	CATCGACTTCTACACCTCCATCAC	CTGAAGAGGTCGGAACACATCTC	BG933934
IFNγ	GATGGGCTGGATGACTTTAGGATG	CCTCCGCTCACTGTCCTCAAA	AY795563·1
TNFα	GTGTATGTGGGAGCAGTGTT	GAAGCCTGTTCTCTGTGACT	NM_001123617·1
TGF-β 1	CTCACATTTTACTGATGTC	GGACAACTGCTCCACC TTGTG	XM_014129261·1
IL4/13a	CCACCACAAAATGCAAGGAGTTCT	CCTGGTTGTCTTGGCTCTTCAC	NM_001204895·1
EF1αb	TGCCCCTCCAGGATGTCTAC	CACGGCCCACAGGTACTG	AF321836
β-actin	CCAAAGCCAACAGGGAGAA	AGGGACAACACTGCCTGGAT	BG933897

CAT, catalase; SOD, superoxide dismutase; Gpx1, glutathione peroxidase 1; Gpx4b, glutathione peroxidase 4b; Gpx7, glutathione peroxidase 7; GR, glutathione reductase; GST, glutathione S-transferase; SePP, selenoprotein P; Met-B, metallothionein-B; HAMP, hepcidin antimicrobial peptide; TFR, transferrin receptor; FAS, fatty acid synthase; G6PD, glucose 6 phosphate-1 dehydrogenase; HSP 70, heat shock protein 70; IFN-γ, interferon gamma; TGF-β 1, transforming growth factor beta 1; IL4/13a, interleukin4/13a; EF1αb, elongation factor alpha b.

were subsequently introduced into the nebuliser tube of the ICP-MS (iCapQ ICP-MS, Thermo Scientific) equipped with an auto sampler (FAST SC-4Q DX, Elemental Scientific), and the elements were detected at corresponding mass-to-charge ratios.

Fatty acids analysis

FA composition of erythrocytes was analysed using ultra-fast gas chromatography, as described by Sissener *et al.*[7]. In this method, MUFA is not separated according to the position of their double bond and these FA are stated as 16:1, 18;1, 20:1 and 22:1. In brief, samples were thawed and weighed. Nonadecanoic acid (19:0) was added as an internal standard to the samples, and then the samples were saponified and methylated by adding 1 ml NaOH (0·5M) and 2 ml BF_3 in methanol. The samples were evaporated and then purified with hexane. The final concentration of the samples was adjusted to 0·2–0·3 mg/ml and injected into FA detection system. The system used for FA detection was a Trace GC Ultra (Thermo Corporation) with SSL injector, flame Ionization Detector, and the column was a Wax column (P/N UFMC00001010501, 5-m long, 0·1-mm. Id., 0·1-µm film thickness). Chromeleon® version 7.2 was the integrator used (Thermo Scientific).

Evaluation of welfare indicators and X-ray analysis of vertebrae

The external welfare indicators (eye cataract, skin lesions, snout damage, and fin damage, including dorsal, pectoral and caudal fins) of fish exposed to delousing stress were evaluated by using a scoring system developed by Nofima and BioMar[36] and also described in details in Lutfi *et al.*[31]. In short, each of the welfare indicators was scored between 0 and 3 where the lowest value represents a good and the highest a poor condition of the fish.

The X-ray radiographic analysis of fish vertebrae was performed at the Nofima X-ray radiography laboratory in Sunndalsøra. The X-ray set-up was semi-digital, with a standard X-ray source (Shimadzu mobile art) and with the exposure (40 kV and 40 mAs) of reusable image plates. The X-ray radiographs were transferred to the computer as digital images and were analysed visually, and variations in bone structures were recorded and classified in a blind evaluation. The number of samples examined was 22–25 per diet. A detailed description of the X-ray analysis was given in Bou *et al.*[6].

Data analysis and statistics

Data were tested for homogeneity of variance and normality using a Kolomogorov–Smirnov test and Shapiro–wilk test, respectively. Data from gene expression analysis were log-transformed before statistical analysis. Data from plasma cortisol, plasma and liver trace minerals and gene expression analysis were subjected to a two-way ANOVA, with diet and delousing stress as the two factors. Only in those cases where a significant effect was observed within a factor, nested one-way ANOVA followed by Tukey's multiple comparisons were performed for each factor separately ($n = 6$/cage). One-way ANOVA followed by Tukey's multiple comparison were performed for whole-body mineral status ($n = 3$). For all statistical tests, P-values $< 0·05$ were considered significant. All results are expressed as mean ± standard error. Statistica 13.4 (Statsoft Inc.) and GraphPad Prism version 8.0 (Graphpad Software Inc.) were used in the statistical analyses.

Table 4. Growth performance of Atlantic salmon fed experimental diets (until mid-sampling)

	Diet 1		Diet 1·3		Diet 1·3 RF		Diet 1·6		Diet 3·5		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	ANOVA *P*
Initial weight (g)	275·4	1·40	275·2	2·88	274·3	1·3	276·4	0·6	277·1	0·7	NS
Mid sampling weight (g)†	2434·9	43·20	2552·8	54·8	2630·6	52·2	2554·9	50·3	2613·8	59·4	NS
Mid sampling length (cm)†	55·67	0·30	56·39	0·5	56·9	0·4	56·6	0·4	57·3	0·4	NS
Final weight (g)¤	4748^{a}	33·4	4938^{a}	85·5	5100^{a}	124·0	4964^{a}	68·4	5365^{b}	56·9	<0·001
Condition factor K†	1·4	0·01	1·41	0·014	1·42	0·013	1·4	0·01	1·37	0·008	NS
SGR†	0·72	0·01	0·73	0·003	0·75	0·005	0·73	0·004	0·74	0·008	NS
Survival %†,*	100	0·0	99·7	0·0	99·2	0·0	100	0·0	100	0·0	NS

NS, Non-significant; SGR, Specific growth rate.
Data are shown as mean values with their standard errors ($n = 3$).
Statistical significance analysed through one-way ANOVA followed by Tukey's multiple comparisons.
Significantly different means are denoted by different superscript letters.
Diet 1/diet 1·3/diet 1·6/ diet 3·5, diet codes are set according to their percentage of EPA + DHA in the feed.
One diet labelled as diet 1·3 RF due to its reduced-fat level.
† Data from mid sampling = Delousing sampling.
¤ Final weight of the fish at harvest size.
* Survival percentage presented for the the period of two weeks after delousing.

Table 5. Fatty acid composition (percentage of total fatty acids) of erythrocytes of Atlantic salmon fed experimental diets (at pre-delousing stress)

	Diet 1		Diet 1·3		Diet 1·3 RF		Diet 1·6		Diet 3·5	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
14:0	0·5	0·1	0·9	0·3	1·3	0·5	0·6	0·1	1·0	0·2
16:0	21·6	1·1	20·0	1·1	19·1	0·6	20·0	0·3	21·1	0·5
18:0	11·0	1·0	10·2	0·8	9·5	0·5	11·0	0·3	11·2	0·3
ΣSFA	33·1	2·0	31·1	1·8	30·0	1·3	31·7	0·5	33·3	0·9
16:1*n*-9	0·3^{a}	0·05	0·3^{a}	0·04	0·5^{a,b}	0·04	0·3^{a}	0·04	0·5^{b}	0·1
18:1*n*-9	11·2^{a}	0·6	11·1^{a}	0·6	10·2^{a}	0·3	10·0^{a}	0·3	7·6^{b}	0·2
20:1*n*-9	0·8^{a}	0·1	0·6^{b}	0·1	0·6^{a}	0·1	0·5^{a,b}	0·04	0·4^{b}	0·1
ΣMUFA	12·3^{a}	0·5	12·0^{a}	0·5	11·3^{a}	0·3	10·8^{a}	0·4	8·5^{b}	0·3
18:2*n*-6 (LA)	5·3^{a}	0·2	4·9^{a,b,c}	0·2	4·6^{b,c}	0·1	4·2^{c}	0·2	2·5^{d}	0·03
20:2*n*-6	0·8^{a}	0·04	0·9^{a}	0·1	0·8^{a,b}	0·1	0·6^{b}	0·03	0·4^{c}	0·04
20:3*n*-6	1·3^{a}	0·1	1·2^{a}	0·1	1·1^{a}	0·1	0·7^{b}	0·02	0·4^{c}	0·04
20:4*n*-6 (ARA)	4·2^{a}	0·1	4·4^{a}	0·1	4·5^{a,b}	0·1	4·5^{a,b}	0·1	5·0^{b}	0·1
Σ*n*-6	11·6^{a}	0·4	11·3^{a}	0·3	11·0^{a,b}	0·2	10·1^{b}	0·2	8·3^{b}	0·2
18:3*n*-3 (ALA)	<0·1		<0·1		<0·1		<0·1		<0·1	
20:4*n*-3	1·1^{a}	0·1	1·0^{a}	0·1	1·0^{a}	0·1	0·9^{a}	0·04	0·6^{b}	0·1
20:5*n*-3 (EPA)	8·8^{a}	0·4	9·4^{a}	0·5	9·8^{a}	0·3	10·2^{a}	0·2	10·0^{a}	0·2
22:5*n*-3	3·4^{a}	0·2	3·8^{a,b}	0·1	4·2^{b,c}	0·1	3·9^{a,b}	0·1	4·6^{c}	0·2
22:6*n*-3 (DHA)	29·3^{a}	0·9	31·0^{a}	1·0	32·2^{ab}	0·7	32·2^{a,b}	0·5	34·6^{b}	0·8
Σ*n*-3	42·9^{a}	1·3	45·5^{a,b}	1·5	47·6^{b,c}	0·9	47·5^{bc}	0·5	49·9^{c}	0·8
ΣEPA + DHA	38·0^{a}	1·1	40·4^{a,b}	1·4	42·0^{b,c}	0·9	42·3^{b,c}	0·5	44·7^{c}	0·9
ΣPUFA	54·6^{a}	1·6	56·8^{a}	1·6	58·6^{a}	1·1	57·5^{a}	0·4	58·2^{a}	0·9
n-3/*n*-6	3·7^{a}	0·1	4·0^{a,b}	0·1	4·3b^{c}	0·1	4·7^{c}	0·1	6·1^{d}	0·1

LA, linoleic acid; ARA, arachidonic acid; ALA, α-linoleic acid; FA, fatty acid.
Data are shown as mean values with their standard errors ($n = 3$).
Different superscript (small letters) indicates statistical significance as obtained through one-way ANOVA followed by Tukey's multiple comparisons.
Significantly different means are denoted by different superscript letters.
Diet 1/diet 1·3/diet 1·6/ diet 3·5, diet codes are set according to their percentage of EPA + DHA in the feed.
One diet labelled as diet 1·3 RF due to its reduced-fat level.

Results

Performance

The results of growth performance including fish weight, length, specific growth rate and condition factor K were measured from the start of the experiment and until the mid-sampling/delousing sampling and are presented in Table 4. The average body weight increased from about 0·27 to about 2·5 kg during this period with no significant differences in weight, length, specific growth rate and condition factor K between different dietary groups at this time point. As previously stated, the growth performance, FA and other lipid data from the final sampling are reported elsewhere[31]. The average size of the fish at the final sampling was about 5 kg. Fish that received a diet containing 3·5 % of EPA + DHA had significantly ($P < 0·001$) higher final weight compared with other dietary groups.

The fatty acid composition of the erythrocytes

The FA composition of the erythrocytes as affected by dietary treatments is presented in Table 5. Dietary effects were seen for total *n*-3 FA, which increased with increasing dietary

Table 6. Visual evaluation of external welfare indicators during delousing period (irrespective of stress conditions)

	Diet 1		Diet 1·3		Diet 1·3 RF		Diet 1·6		Diet 3·5		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	ANOVA *P*
Skin	0·9	0·1	1·1	0·1	1·0	0·0	1·1	0·1	1·0	0·0	NS
Eye damage	0·1	0·0	0·1	0·0	0·1	0·0	0·1	0·1	0·1	0·0	NS
Snout	0·1	0·5	0·2	0·4	0·3	0·5	0·3	0·6	0·4	0·5	NS
Dorsal fin	1·1	0·1	3·1	2·1	0·9	0·1	1·1	0·1	1·0	0·0	NS
Caudal fin	1·0	0·0	1·0	0·0	1·0	0·0	1·1	0·0	1·0	0·0	NS
Pectoral fin	1·0	0·0	0·9	0·1	1·0	0·0	0·9	0·0	0·8	0·1	NS
Pelvic fin	0·5	0·1	0·6	0·1	0·5	0·1	0·6	0·1	0·5	0·1	NS

Data are shown as mean values with their standard errors ($n = 3$).
Statistical significance analysed through one-way ANOVA followed by Tukey's multiple comparisons.
Significantly different means are denoted by different superscript letters.
Diet 1/diet 1·3/diet 1·6/ diet 3·5, diet codes are set according to their percentage of EPA + DHA in the feed.
One diet labelled as diet 1·3 RF due to its reduced-fat level.

EPA + DHA. Particularly, DHA levels were significantly increased with increasing dietary EPA + DHA, while no difference was seen for EPA. The contrary was seen for MUFA and total *n*-6 FA, and these FA were decreased with increasing dietary EPA + DHA. Particularly, fish fed higher dietary EPA + DHA (diet 3·5) had a significantly lower percentage of MUFA compared with all other dietary groups. The ratio of *n*-3/*n*-6 FA was significantly increased in response to increasing dietary EPA + DHA levels, and high *n*-3/*n*-6 ratio (6·1:1) was seen in the fish fed diet 3·5. The SFA levels in the erythrocytes were not significantly different among different dietary groups.

Welfare indicators

The mean sea lice count was 0·13 ± 0·027 (mean ± SEM) gravid female lice / salmon recorded from the start of the experiment until delousing procedure. The external welfare scores (eye cataract, skin lesions, snout damage, and damages in fins, including dorsal, caudal pectoral, and pelvic fins) were recorded during the delousing stress sampling (Table 6). Neither delousing stress nor diet caused any significant difference among external welfare scores. X-ray radiography analysis of vertebra showed few fish having specific pathological lesions in the spine, that is, vertebral fusions were recorded for 14 %, and cross-stitch vertebra was recorded for 7 % of total analysed fish. Despite some differences in the observed values, dietary effects were not statistically significant, neither in the percentage of affected fish nor in the extent of lesions, including fusion vertebra and across-stitch vertebra (Table 7).

Plasma cortisol

Plasma cortisol, a primary stress response marker, was measured before, 1 h after and 24 h after delousing stress (Fig. 1). The cortisol responses were not significantly affected by diet at any sampling point, and no interaction effect between diet and stress on cortisol levels was found. As expected, plasma cortisol levels were dramatically increased 1 h post-delousing stress ($P < 0·0001$) and then decreased and returned to pre-stress levels within 24 h, with no statistical differences observed between pre- and 24 h post-stress.

Plasma trace mineral levels

Dietary EPA + DHA levels had a significant impact on plasma Co, Cu, Zn and Se levels (Fig. 2). Significant increase in plasma Zn level was observed in fish fed with diet 1·3 RF compared with the other dietary groups (diet 1·0, diet 1·3, diet 1·6 and diet 3·5) ($P < 0·00001$), irrespective of stress factors. Similarly, Cu level was also increased in fish fed with diet 1·3 RF compared with other dietary groups ($P = 0·0006$); however, this difference was observed only 1 h post-stress. Fish fed with high dietary EPA + DHA level (diet 3·5) had significantly increased Co level compared with other dietary groups, irrespective of stress factors ($P = 0·0001$). As there was no stress effect for Se, all the values were nested under each diet group irrespective of stress condition for one-way analysis. Significant dietary effects were found for plasma Se levels. Fish fed high EPA + DHA (diet 3·5) had significantly increased plasma Se level compared with diet 1·0 and diet 1·3 ($P < 0·0001$). No significant difference in plasma Se was found between low-fat (diet 1·3 RF) and high-fat fed group (diet 1·3). A significant change in most analysed trace mineral levels (Cr, Mn, Fe, Co, Cu and Zn) were observed after delousing stress, except for Se (Fig. 2). Mn and Fe levels were significantly decreased at 1 h post-stress compared with pre-stress, and these levels were further decreased at 24 h post-stress (Mn, $P < 0·0001$; Fe, $P < 0·00001$). On the other hand, Co and Zn levels were increased after stress compared with pre-stress conditions. Zn and Co levels were increased at 1 h post-stress, and 24 h post-stress, Co levels remained elevated, whereas Zn level decreased back to pre-stress level (Co, $P < 0·0001$; Zn, $P = 0·03$). Similarly, plasma Cu levels were also increased at 24 h post-stress compared with pre- and 1 h post-stress conditions ($P = 0·0001$).

Liver trace mineral levels

Liver trace mineral (Se, Zn, Fe, Cu and Mn) levels during delousing stress were analysed in three selected dietary groups, which include low (diet 1·0), mid (diet 1·6) and high (diet 3·5) EPA + DHA. There was a significant effect of diet on liver trace minerals (Se, $P = 0·001$; Zn, $P = 0·015$; Fe, $P < 0·0001$; Cu, $P < 0·0001$; and Mn, $P = 0·009$; Fig. 3). Irrespective of stress conditions, liver Se, Zn, Fe, Cu and Mn were significantly increased in fish that received high EPA + DHA diet (diet 3·5) compared

Table 7. X-ray radiography analysis of vertebra showed from pre-delousing time point

	Diet 1	Diet 1·3	Diet 1·3 RF	Diet 1·6	Diet 3·5	Total sum	ANOVA *P*
Number of fish analysed	23	26	23	21	21	114	NS
Fish with fusion (*n*)	3	5	2	5	1	16	NS
Fish with fusion	13	19	9	24	5	14	NS
Fish with cross-stitch lesions (*n*)	1	3	1	1	1	7	NS
Fish with cross-stitch lesions	4	12	4	5	5	6	NS

Data are shown as mean values with their standard errors ($n = 3$).
Statistical significance analysed through one-way ANOVA followed by Tukey's multiple comparisons.
Significantly different means are denoted by different superscript letters.
Diet 1/diet 1·3/diet 1·6/ diet 3·5, diet codes are set according to their percentage of EPA + DHA in the feed.
One diet labelled as diet 1·3 RF due to its reduced-fat level.

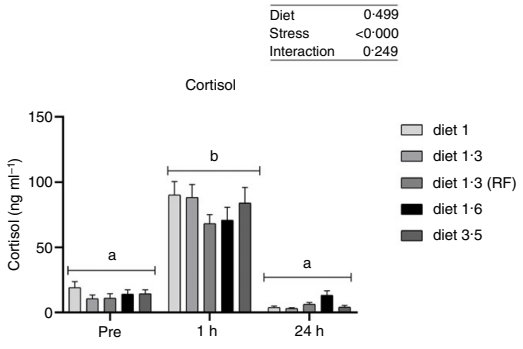

Fig. 1. Plasma cortisol (ng/ml) in Atlantic salmon subjected to delousing stress. Fish were sampled before delousing stress (0 h) and 1 h and 24 h post-delousing stress. Small letters (a, b and c) indicate the statistical difference in cortisol levels between time points detected with two-way ANOVA followed by Tukey's multiple comparisons. Nested one-way ANOVA was performed and no significant difference between dietary groups were detected at any of the sampling points. All data are shown as mean ± SEM, $n = 3$. RF, reduced fat.

with the two other dietary groups, while no significant difference was observed between diet 1·0 and diet 1·6. Similarly, liver trace mineral levels were significantly affected by delousing stress. Se, Fe and Cu levels were increased at 24 h post-stress compared with pre-stress, and for Cu this difference was observed already 1 h post-stress. However, Mn level was decreased at 1 h and 24 h post-stress compared with pre-stress. The Zn level remained the same in all stress conditions. There were no 'diet*stress' interactions observed for any mineral levels in the liver.

Liver mRNA expression analysis

The mRNA expression of genes involved in stress response and trace mineral metabolism was analysed (Fig. 4 and 5). The mRNA expression of the HAMP and TFR was significantly influenced either in response to dietary EPA + DHA or delousing stress (Fig. 4). Fish fed high EPA + DHA diet (diet 3·5) had significantly increased HAMP mRNA expression compared with other dietary groups (diet 1·0 and diet 1·6) at pre-stress ($P = 0·03$) and 1 h post-stress ($P = 0·02$). There was no statistical difference observed for diet at 24 h post-stress ($P = 0·25$). Additionally, there was a significant stress effect; HAMP mRNA expression was significantly downregulated at 1 h post-stress compared with pre-stress and 24 h post-stress, and no significant difference was detected between pre-stress and 24 h post-stress. The mRNA expression of TFR was significantly downregulated 1 h post-stress compared with pre- and 24 h post-stress, while at 24 h post-stress TFR expression was back to pre-stress level. Significant diet effects were observed for TFR; fish fed low EPA + DHA (diet 1) had increased TFR expression compared with fish fed diet 1·6 and diet 3·5 pre-stress ($P = 0·025$) and only to diet 1·6 at 1 h post-stress ($P = 0·03$), with a similar (non-significant) trend at 24 h post-stress.

No effects of dietary EPA + DHA on mRNA expression of oxidative stress markers (CAT, SOD, Met-B, Gpx1, Gpx4b, Gpx7, SePP, GR, GST 1 and HSP70) were detected at any sampling point (Fig. 5). However, expression of all the analysed oxidative stress marker genes, except CAT, were significantly affected by delousing stress (Fig. 5). The SOD expression was significantly downregulated at 1 h post-stress compared with pre-stress and remained downregulated 24 h post-stress ($P = 0·0001$). Similarly, the mRNA expression of Gpx1, Gpx4b and Gpx7 were also significantly downregulated at 1 h post-stress. However, at 24 h post-stress, Gpx4b was back to pre-stress level, whereas Gpx1 and Gpx7 levels remained downregulated (Gpx1, $P = 0·001$; Gpx4b, $P = 0·0001$ and Gpx7, $P = 0·0001$). The mRNA expression of SePP was upregulated 1 h post-stress and was even further upregulated 24 h post-stress ($P = 0·0001$). The mRNA expression of Met-B was significantly downregulated 1 h post-stress compared with pre-stress and further downregulated 24 h post-stress ($P = 0·0001$). The expression of GR and GST1 were also significantly downregulated 1 h post-stress. However, GR expression remained the same, whereas GST1 was further downregulated 24 h post-stress ($P = 0·000$, GR; $P = 0·0001$, GST1). The mRNA expression of HSP70 was upregulated 1 h post-stress and then back to pre-stress levels 24 h post-stress ($P = 0·000$). Significant interaction between diet and stress was observed for Gpx7 and GR (Gpx7, $P < 0·006$; GR, $P < 0·04$). The mRNA expression of FAS was significantly downregulated at 1 h and 24 h post-stress compared with pre-stress (Fig. 5). A dietary effect was observed pre-stress, where fish fed diet 1·0 had significantly increased FAS mRNA expression compared with the other dietary groups (diet 1·6 and diet 3·5). No dietary effects were seen in FAS mRNA expression 1 h and 24 h post-stress, and no 'diet*stress' interaction was detected. The mRNA expression of G6PD was upregulated 1 h post-stress and was back to pre-stress levels 24 h post-stress ($P = 0·0001$). No effects

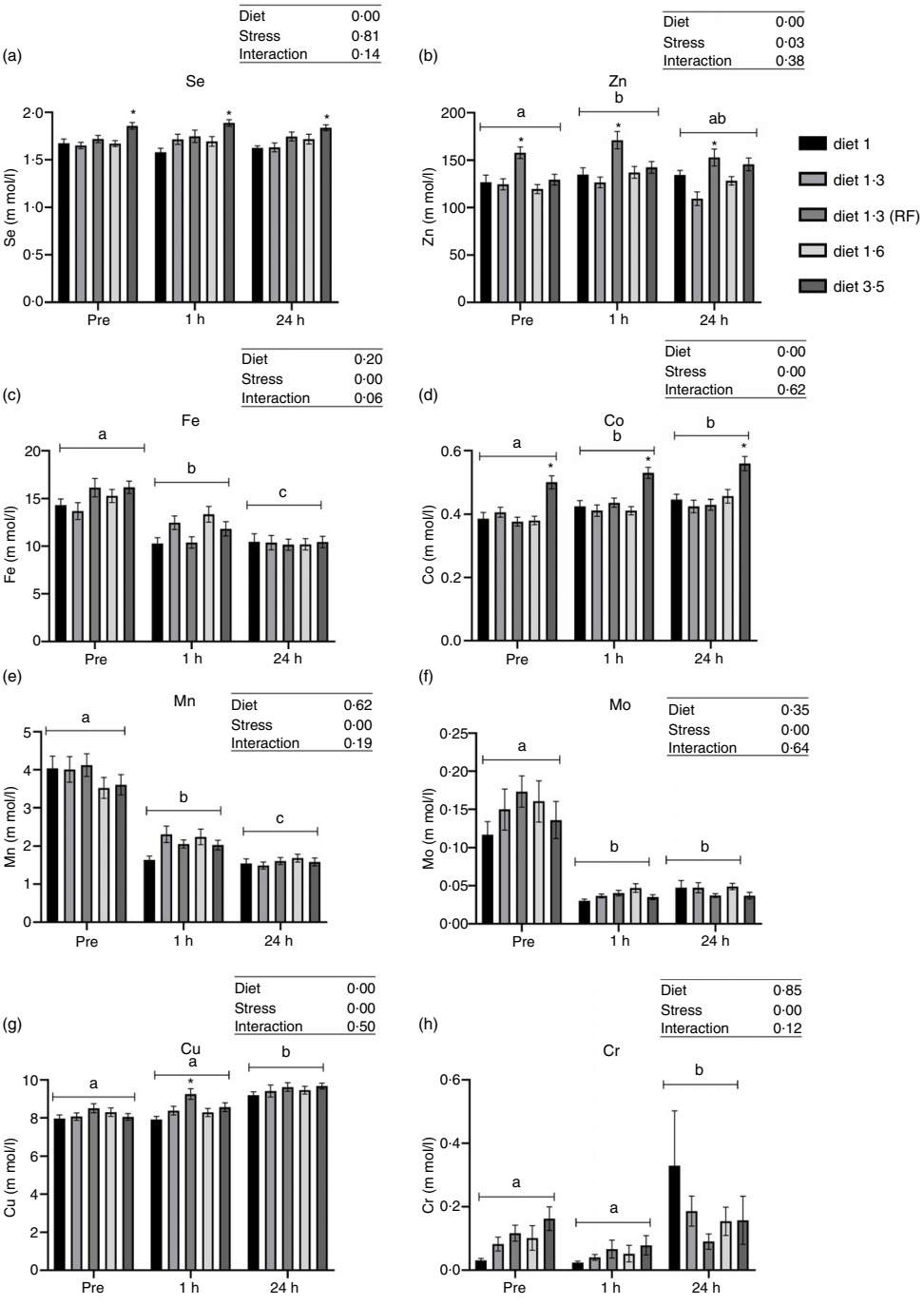

Fig. 2. Plasma trace minerals in Atlantic salmon subjected to delousing stress. Fish were sampled before delousing stress (pre-stress) and 1 h and 24 h post-stress. Small letters (a, b and c) indicate the statistical difference between stress conditions detected with two-way ANOVA followed by Tukey's multiple comparisons. Asterisks (*) indicate the statistical difference between dietary groups detected in nested one-way ANOVA followed by Tukey's multiple comparisons. All data are shown as mean ± SEM, $n = 3$. RF, reduced fat.

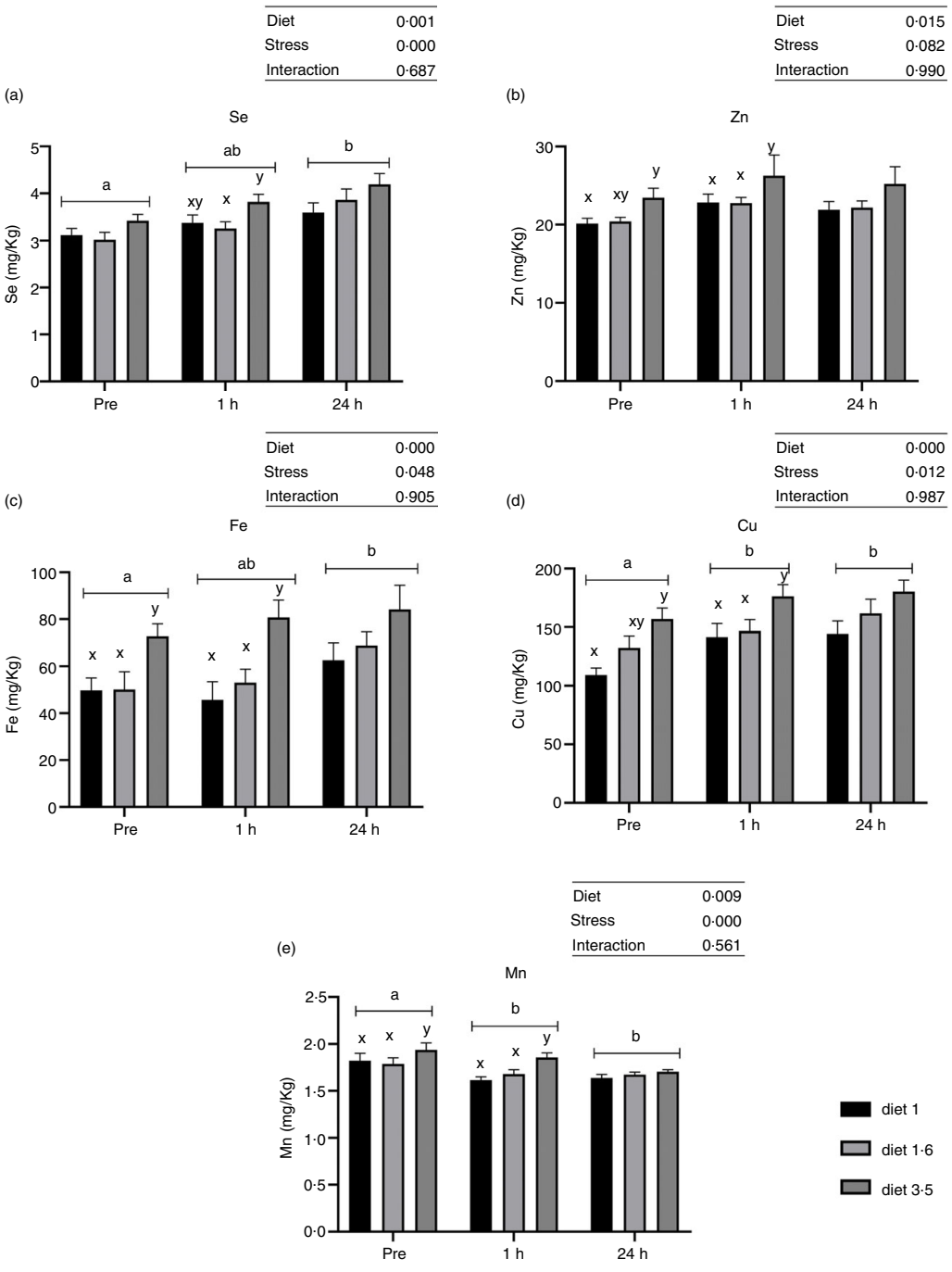

Fig. 3. Liver trace minerals in Atlantic salmon subjected to delousing stress. Fish were sampled before delousing stress (pre-stress) and 1 h and 24 h post-stress. Small letters (a, b and c) indicate the statistical difference between stress conditions detected with two-way ANOVA followed by Tukey's multiple comparisons. All data are shown as mean ± SEM, $n = 3$.

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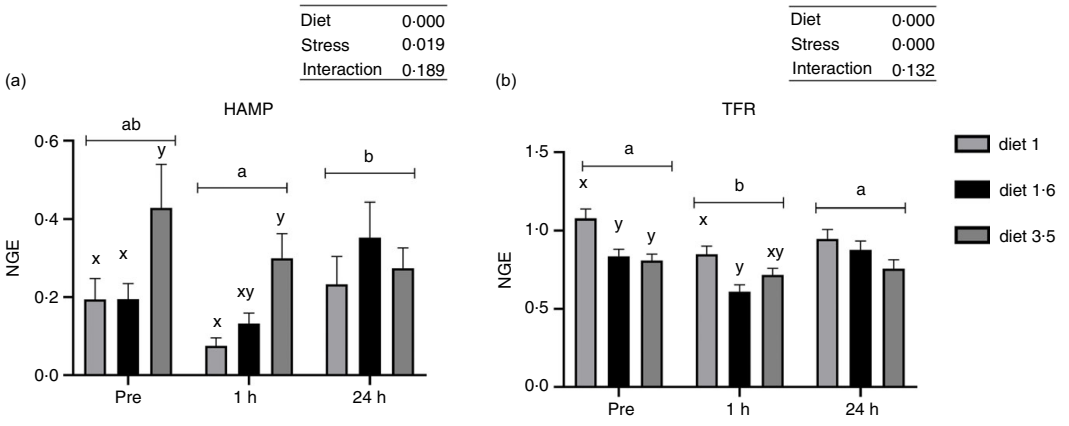

Fig. 4. The liver mRNA expression of hepcidin antimicrobial peptide (HAMP, a); transferrin receptor (TFR, b). Fish were sampled before delousing stress (pre-stress) and 1 h and 24 h post-stress. Small letters (a, b and c) indicate the statistical difference between stress conditions detected with two-way ANOVA followed by Tukey's multiple comparisons. Small letters x, y and z indicate the significant difference between dietary groups detected in nested one-way ANOVA. All data are shown as mean ± SEM, $n = 3$. Log-transformed values were used for statistical purposes. NGE, normalised gene expression.

of dietary EPA + DHA levels on mRNA expression of G6PD were detected at any sampling point; however, significant 'diet*stress' was observed for G6PD mRNA expression ($P = 0{\cdot}043$). Fish that received low EPA + DHA had significantly low G6PD mRNA expression compared with other dietary groups at 1 h post-stress, and this difference was not observed in other sampling points.

The mRNA expression of the analysed inflammatory genes (IFN-γ, TNF1α, TGF-β 1 and IL4/13a; Fig. 6) was also significantly downregulated at 1 h post-stress (IFN-γ, $P < 0{\cdot}00001$; TNF1α, $P < 0{\cdot}00001$; TGF-β 1, $P < 0{\cdot}00001$; IL4/13a, $P < 0{\cdot}00002$). IFN-γ and TNF1α expression were back to pre-stress levels 24 h post-stress. However, TGF-β 1 and IL4/13a remained downregulated even 24 h post-stress. No significant dietary effects or interaction between diet and stress were observed for these genes.

Long-term effect – whole-body mineral status

Whole-body trace mineral (Zn, Cu, Mn, Se and Fe) levels were analysed for the fish at the final sampling when the fish reached harvest size (about 5 kg) (Table 8). Higher inclusion of dietary EPA + DHA levels had a significant impact on increasing the whole-body Zn, Mn and Se levels (Zn, $P = 0{\cdot}0001$; Mn, $P = 0{\cdot}01$; Se, $P < 0{\cdot}0001$). The whole-body Zn level was significantly higher for fish that had received high dietary EPA + DHA (diet 3·5) compared with the ones fed diet 1·0, diet 1·3 and diet 1·6, while there was no difference between diet 3·5 and diet 1·3 RF. Further, in fish fed diet 1·3 RF significantly higher body Zn levels were found compared with diet 1·3. The whole-body Se and Mn levels were also significantly higher for fish fed the diet containing 3·5 % of EPA and DHA compared with the ones fed the other diets. The level of Fe and Cu were not significantly different among dietary treatments.

Discussion

The goal of the present study was to investigate the effects of different levels of EPA + DHA (1·0, 1·3, 1·3 (RF), 1·6 and 3·5 % of the diet) on the stress response in Atlantic salmon undergoing a delousing procedure. As mentioned earlier, the growth performance of salmon fed four of the experimental diets (diet 1, diet 1·3, diet 1·6 and diet 3·5) from the larger feeding trial has been reported elsewhere[31]. The minimum requirement for EPA + DHA in Atlantic salmon has been reported to range from 5 to 10 g/kg feed. However, the challenging environmental conditions and other stressors in sea cages, besides other biological factors such as the growth rate of the species, strain disease resistance, etc., are likely to influence this requirement. For example, in a recent long-term seawater trial in Atlantic salmon, Bou *et al.*[6] reported a significant increase in mortality after delousing at high water temperature in fish fed low 10 g/kg EPA + DHA compared with fish fed 17 g/kg DHA + EPA diet. Another study in Atlantic salmon reared in sea cages under commercial conditions and fed two different levels of EPA + DHA (16 and 26 g/kg feed) showed no difference in mortalities after sea lice treatments and also did not affect the fish performance and robustness[37]. However, in the later study, the lowest EPA + DHA (16 g/kg) was close to current commercial level. Nevertheless, in the current study, despite low dietary EPA + DHA in diet 1 (10 g/kg), we did not find any significant mortality after delousing stress. The probable reason for the lack of mortality might be the lower lice infection rate (0·13 gravid female lice / salmon), which was lower than allowed limits (0·5 gravid female lice / salmon) in Norway or the efficient delousing procedure followed during delousing compared with the previously mentioned experiment. In addition, in the current study, mean temperature during delousing was 13°C. In contrast, the delousing in Bou *et al.*[6] was carried out during a high water temperature period (17·5°C), which might have caused additional stress for the fish. The other possible reason for the lack of mortality could be the

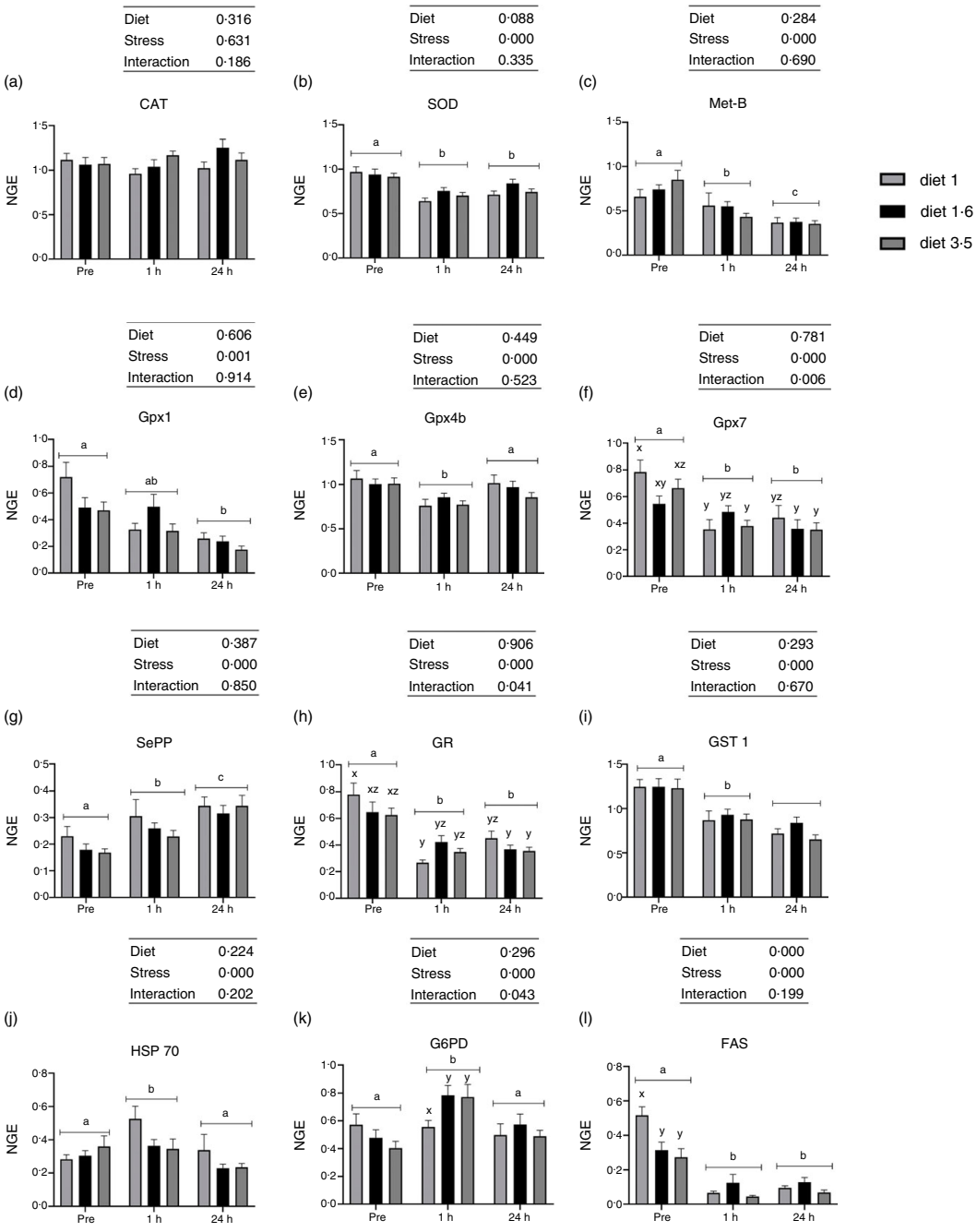

Fig. 5. The liver mRNA expression of oxidative stress marker genes in Atlantic salmon subjected to delousing stresses. Catalase (CAT, a); superoxide dismutase (SOD, b); metallothionein B (Met-B, c); glutathione peroxidase (Gpx1, d; Gpx4b, e; Gpx7, f); selenoprotein P (SePP, g); glucocorticoid receptor (GR, h); glutathione S-transferase (GST, i); heat shock protein 70 (HSP 70, j); glucose-6-phosphate 1-dehydrogenase (G6PD, k); fatty acid synthase (FAS, l). Fish were sampled before delousing stress (pre-stress) and 1 h and 24 h post-stress. Small letters (a, b and c) indicate the statistical difference between stress conditions detected with two-way ANOVA followed by Tukey's multiple comparisons. Small letters x, y and z indicate the statistical difference between dietary groups detected in nested one-way ANOVA. All data are shown as mean ± SEM, $n = 3$. Log-transformed values were used for statistical purposes. NGE, normalised gene expression.

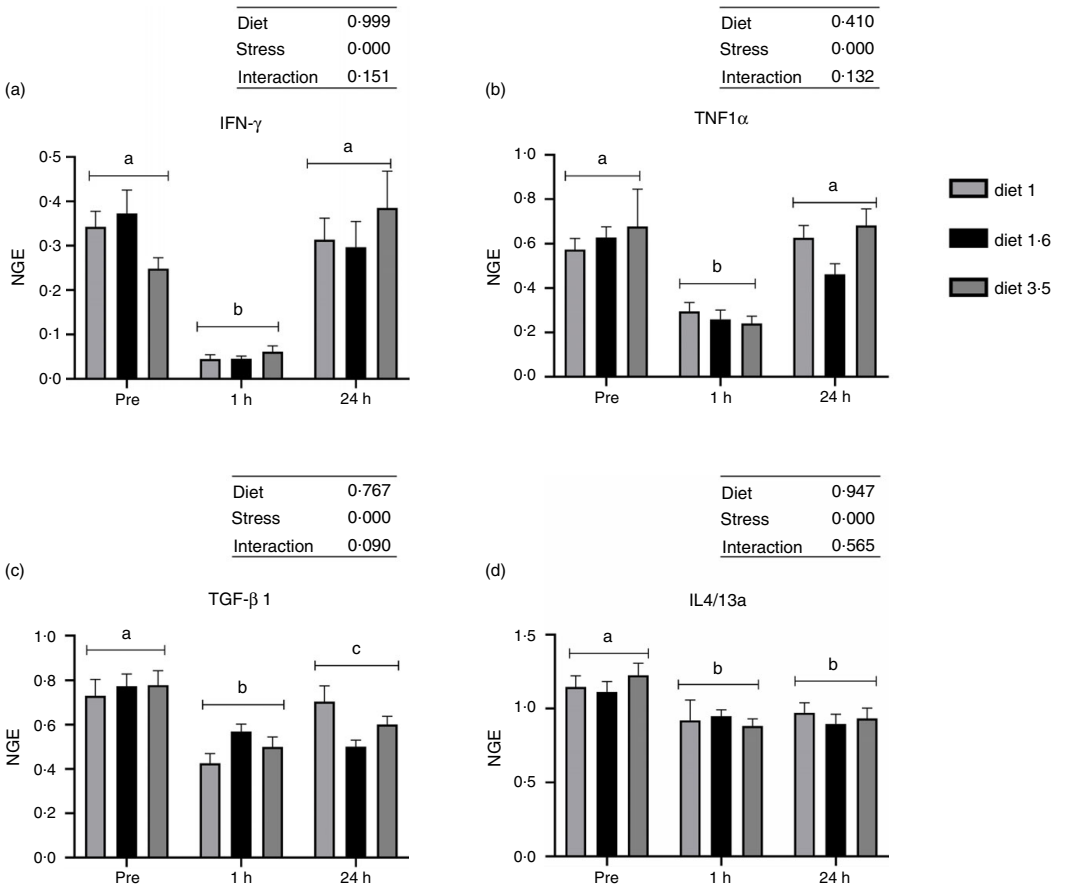

Fig. 6. The liver mRNA expression of selected immune genes in Atlantic salmon subjected to delousing stresses. Interferon-gamma (IFN-γ, a); TNF1α, b; transforming growth factor beta 1 (TGF-β 1, c); IL4/13a, d). Fish were sampled before delousing stress (pre-stress) and 1 h and 24 h post-stress. Small letters (a, b and c) indicate the statistical difference between stress conditions detected with two-way ANOVA followed by Tukey's multiple comparisons. Small letters x, y and z indicate the statistical difference between dietary groups detected in nested one-way ANOVA. All data are shown as mean ± SEM, $n = 3$. Log-transformed values were used for statistical purposes. NGE, normalised gene expression.

Table 8. Analysed whole-body trace mineral concentrations of Atlantic salmon from long-term seawater trial (mg/kg ww)

	Diet 1		Diet 1·3		Diet 1·3 RF		Diet 1·6		Diet 3·5	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Zn	22·7^{a}	1·2	23·0^{a}	1·0	31·7^{b}	3·2	24·0^{a}	1·2	33·0^{b}	1·5
Cu	1·6^{a}	0·0	2·0^{a}	0·2	1·6^{a}	0·1	1·7^{a}	0·1	1·7^{a}	0·1
Mn	1·3^{a}	0·1	1·4^{a}	0·1	1·4^{a}	0·0	1·4^{a}	0·2	2·3^{b}	0·3
Se	0·26^{a}	0·001	0·28^{a}	0·005	0·25^{a}	0·001	0·28^{a}	0·003	0·34^{b}	0·003
Fe	9·7^{a}	0·1	10·2^{a}	0·9	9·8^{a}	0·1	10·3^{a}	0·7	10·0^{a}	0·6

Data are shown as mean values with their standard errors ($n = 3$).
Different superscript (small letters) indicates statistical significance as obtained through one-way ANOVA followed by Tukey's multiple comparisons.
Significantly different means are denoted by different superscript letters.
Diet 1/diet 1·3/diet 1·6/ diet 3·5.
Diet codes are set according to their percentage of EPA + DHA in the feed. One diet labelled as diet 1 3 RF due to its reduced-fat level.

previous nutritional history of the animal, as fish in the current trial received commercial diets (17 g/kg EPA + DHA) before the start of the experiment (about 270 g), which was assumed to be sufficient. In addition, neither delousing stress nor diet caused any significant difference in external welfare scores of the fish. Further, although a prominent specific pathological lesion in the spine, that is, fusion vertebrae was recorded in 14 % of analysed vertebrae, but no significant relation to dietary EPA + DHA was found. The type of specific pathological lesion (fused vertebrae) and percentage (14 %) and fused vertebrae observed in this study resembles very much to Bou *et al.*[6], who reported 18 % fused vertebrae with no effect of low dietary EPA + DHA on vertebral deformities in Atlantic salmon in a long-term trial in sea cages. Furthermore, the fused vertebrae are typical observations in Atlantic salmon produced commercially and it might be due to early smoltification and seawater transfer[38] and may not be coupled with dietary contents. Taken together, data from mortality, external welfare scores and X-ray radiographic analysis of vertebrae indicate that low dietary levels of EPA + DHA (10 g/kg) do not negatively influence the robustness of the fish upon delousing stress.

The increase of plasma cortisol is recognised as a stress indicator[39,40] and was observed 1 h after the delousing procedure, returning to basal level 24 h post-stress. However, the response in cortisol was independent of the dietary EPA + DHA levels. An increased plasma cortisol in response to low *n*-3 LC-PUFA was observed in marine fish in presence or absence of stress[41–43]. Furthermore, in fish, it has been shown that eicosanoids derived from LC-PUFA such as EPA and arachidonic acid can modulate the ACTH release from the hypothalamic-pituitary-adrenal (HPI) axis and thereby influence the cortisol production from interrenal tissue[44,45]. However, in Atlantic salmon, different dietary *n*-6/*n*-3 ratio of LC-PUFA (from 1:1 to 6:1) and their absolute contents did not affect plasma cortisol level during acute stress[34]. Our results indicate that even the lowest level of EPA + DHA used in the current study was sufficient to mount a cortisol response to acute stress, indicating that this is a highly prioritised physiological function in the fish. The difference to studies in marine fish could be explained by the fact that Atlantic salmon can desaturate and elongate *n*-3 linoleic FA to EPA and DHA[46,47], unlike other marine fish, and further selectively retain them in the membrane when fed with low *n*-3 LC-PUFA[48,49].

FAS catalyses the *de novo* FA synthesis in the presence of NADPH[50]. The G6PD is a key regulatory enzyme in the pentose phosphate pathway involved in NADPH production, essential for FA biosynthesis and the maintenance of the redox state in fish and other vertebrates[51]. Thus, the increased mRNA expression of G6PD and subsequent high FAS expression during pre-stress in salmon fed low dietary EPA + DHA (diet 1) may indicate the possible up-regulation of FA biosynthesis in response to low *n*-3 LCPUFA diet, as previously described in Atlantic salmon[52,53]. Further, after delousing stress, mRNA expression of FAS was significantly downregulated, on the other hand G6PD expression increased dramatically compared with pre-stress levels. This result suggests the possible inhibition of FA biosynthesis under stress conditions or might also indirectly indicate the activation of lipolysis upon energy demand. Further, stress caused increased expression of G6PD, demonstrating the increased NADPH production to maintain the redox status and also to meet the enhanced energy demand during the stressful environment[54–56].

The enzymes SOD, CAT, Gpx and GST play a crucial role in glutathione metabolism and antioxidant defence system by scavenging the reactive oxygen species. The mRNA expression of these genes are recognised as important indicators of oxidative stress[57]. Further, metallothionine and selenoproteins are also linked to the glutathione metabolism in the redox cycle[58]. Accordingly, the down-regulation of genes in dismutation of the superoxide radical (SOD), glutathione metabolism (Gpx1, Gpx7, GR and GST1) and Met-B 1 h and 24 h post-stress with concurrent up-regulation of SePP was indicative of oxidative stress during delousing[59–61]. In Atlantic salmon fed graded levels of *n*-3 LC-PUFA (2·6 to 4·2 % of feed), mRNA expression of antioxidant genes in the liver was not affected[62]. In our study, even though the delousing procedure significantly affected the mRNA expression of oxidative stress markers, dietary EPA + DHA levels did not alter their expression either in the presence or absence of stress. However, an increase in HAMP expression, with concurrent down-regulation of TFR and high Fe status in the liver of fish fed the high EPA + DHA diet signifies a pro-oxidant environment. HAMP transcript levels increase with increased Fe load to degrade the Fe exporter ferroportin, which in turn diminish the Fe uptake by acting reciprocally with Fe import proteins such as DMT or TFR[63]. Similar increased expression of HAMP in response to DHA supplement was reported in mammals[64]. Thus, our results suggest the possible effect of EPA + DHA on the Fe store in fish as reported in mammals[64]. However, despite accumulation of a pro-oxidant (Fe) in the liver during delousing, a high dietary EPA + DHA (diet 3·5) does not induce oxidative stress, reiterating that Atlantic salmon is robust to a pro-oxidative environment[65].

Zn and Se are the most limiting trace elements in present-day plant-based salmonid feeds[13,66]. Increased use of plant-based ingredients containing anti-nutritional factors like phytic acid reduce Zn availability, thereby reducing their body status. Nevertheless, decreasing dietary EPA + DHA or increasing the fat level can also have consequence on body Zn retention, as shown in mammals. Zn retention in mammals is reduced by high-fat or low EPA + DHA diets thereby increasing the need for oral Zn supplements[67–69]. The higher Zn in the whole body of salmon fed either high EPA + DHA (diet 3·5) or low dietary fat (diet 1·3 RF) indicate an interaction between dietary fat or LC-PUFA and Zn in the diet. In this regard, the increase of dietary fat up to 38 % in salmon feeds over the years, and simultaneous decrease in body Zn status in ready-to-slaughter salmon (4–5 kg size fish; 55 to 35 mg/kg)[1,70], warrant further attention. Similarly, increased inclusion of EPA + DHA in the diets markedly increased the plasma and whole-body Se levels in salmon fed diet 3·5. Even though the dietary Se levels satisfied the known minimum requirements of salmon, stressors can affect Se utilisation[24,25,35]. In the present study, the increased Se status with 3·5 diet might indicate improved stress mitigation and could in turn protect *n*-3 LC-PUFA from oxidation.

The beneficial effects of dietary *n*-3 LC-PUFA on immune functions are well documented in many vertebrates[2–4], including Atlantic salmon[5,6,8]. However, many studies in Atlantic salmon have found no detrimental effects on health and immune

functions when fed low dietary *n*-3 LC-PUFA[71–73]. Similarly, in this current study, dietary EPA + DHA level did not alter the mRNA expression of analysed immune genes (IFN-γ, TNF-α, TGF-β and IL4/13a). As pointed out by others[71,73,74], the dietary manipulation of gene expression is not always straightforward. It may also be influenced by various other factors, which include ingredient composition, duration of feeding, species, studied tissue, rearing conditions and other environmental factors. Further, the delousing stress significantly downregulated the mRNA expression of all analysed immune genes (IFN-γ, TNF-α, TGF-β and IL4/13a) at 1 h post-stress, which is concurrent with increased cortisol production. The similar down-regulation of immune response upon stress exposure has been reported previously in mammals[75,76] and in fish[77,78].

In conclusion, although delousing causes considerable stress, dietary EPA + DHA levels and fat levels did not affect stress responses in Atlantic salmon. Further, it was noteworthy that increasing EPA + DHA levels in the diet increased the Se and Zn status in plasma and the whole body, while dietary fat level affected Zn status. As these minerals are crucial to fish health, and upper limits exist for their addition in feeds, knowledge on how other dietary factors affect their uptake and retention are of high relevance to the aquaculture industry. However, further studies are necessary in order to elucidate the underlying mechanism for how dietary FA and dietary lipid level affects body mineral status in fish.

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The authors declare that there are no conflicts of interest.

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