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# Growth, feed utilization, health and biometric parameters in Atlantic salmon (*Salmo salar* L.) - Influence of dietary protein-to-lipid ratio and body fat status

Vekst, fôrutnyttelse, helse og biometriske parameter hos Atlantisk laks (*Salmo salar* L.) – Effekt av protein-til-fett forholdet i fôret og fettinnholdet i laksen

Jens-Erik Dessen

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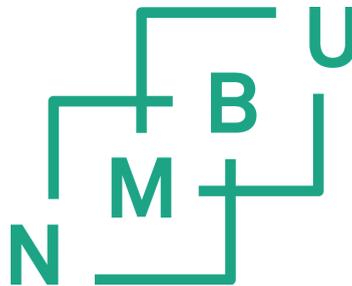
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Philosophiae Doctor (PhD) Thesis

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# 1. Abbreviations

List of the main abbreviations used throughout this work. The rest will be described in the text as they appear.

**ALT: alanine aminotransferase**

**AP: alkaline phosphatase**

**CMS: cardiomyopathy syndrome**

**DP/DE: digestible protein and digestible energy**

**FCR$_g$: Biological feed conversion ratio based on carcass weight**

**HSMI: heart- and skeletal muscle inflammation**

**PD: pancreas disease**

**PRV: piscine reovirus**

**P/L ratio: protein-to-lipid ratio**

**PUFA: polyunsaturated fatty acids**

**SAV3: Salmonid alphavirus subtype 3**

**S0: Under year-old smolt**

**S1: Year-old smolt**

**TGC: thermal growth coefficient**

**VSI: visceral somatic index**

## 2. List of publications

The thesis is based on the following publications, which will be referred to in the text by their roman numerals.

Paper **I**: Dessen, J. E., Weihe, R., Hatlen, B., Thomassen, M. S., & Rørvik, K. A. (2017). **Different growth performance, lipid deposition, and nutrient utilization in in-season (S1) Atlantic salmon post-smolt fed isoenergetic diets differing in protein-to-lipid ratio**. Aquaculture, 473, 345-354.

Paper **II**: Rørvik, K. A., Dessen, J. E., Åsli, M., Thomassen, M. S., Hoås, K. G., & Mørkøre, T. (2018). **Low body fat content prior to declining day length in the autumn significantly increased growth and reduced weight dispersion in farmed Atlantic Salmon *Salmo* salar L.** Aquaculture Research, 49(5), 1944-1956.

Paper **III**: Dessen, J. E., Mørkøre, T., Bildøy, J. I., Johnsen, S. N., Poppe, L. T., Hatlen, B., Thomassen, M. S., & Rørvik, K. A. (2018). **Increased dietary protein-to-lipid ratio improves survival during naturally occurring pancreas disease in Atlantic salmon, *Salmo salar* L.** Journal of fish diseases.

Paper **IV**: Dessen, J. E., Østbye, T. K., Ruyter, B., Bou, M., Thomassen, M. S., & Rørvik, K. A. **Sudden increased mortality in large seemingly healthy farmed Atlantic salmon (*Salmo Salar* L.) was associated with environmental and dietary changes**. *Manuscript*

# 3. Summary

Today, commercial diets for large farmed Atlantic salmon (*Salmo salar* L.) commonly contain 30-35 % protein and 35-39 % lipids, i.e. a ratio of protein-to-lipids below 1. Such energy dense diets have generally been shown to improve feed utilization and growth. However, reducing the dietary protein-to-lipid ratio may lead to increased deposition of fat in the muscle and visceral cavity. There is evidence that high levels of body lipids may reduce feed intake and growth in salmonids, and is often referred to as lipostatic regulation. Thus, there is a risk of lowered growth prior to and during periods with increased feed intake and high fat accumulation when feeding high-fat diets. The present thesis test the hypothesis that increased dietary protein-to-lipid ratio, and the possible involvement of lipostatic regulation on body fat levels can be utilized to significantly improve fitness-related traits including growth, survival and nutrient deposition of farmed Atlantic salmon.

Paper **I** describes the effects of isoenergetic diets with different protein-to-lipid (P/L) ratio on growth, feed intake, feed conversion, biometrics, nutrient retention and deposition in S1 Atlantic salmon post-smolt. The study was conducted during the early seawater phase from April to September. Significantly lower muscle fat, whole body lipid, and energy level was observed in the post-smolt fed high compared to low P/L ratio in July, approximately three months after the trial started. Reduced level of muscle fat/body fat in July significantly improved feed intake, growth and weight gain compared to fish fed low P/L ratio from July to September. The high P/L ratio group had also a significantly lower feed conversion ratio based on gutted weight (FCRg). In line with this, the visceral somatic index (VSI) of the group fed the high dietary P/L ratio was relatively stable during the experiment, whereas the VSI of the group fed the low dietary P/L ratio increased gradually, resulting in significantly higher VSI at the end of the trial. The increased protein content in the high P/L ratio diets was efficiently utilized for growth and weight gain, assessed by nutrient retention, particularly in the last period of the trial.

In Paper **II**, low and high P/L ratios and restricted ration (~ 50%) of the high P/L diet were used to alter the lipid deposition prior to the autumn in large salmon. In this study, a clear treatment effect on body fat was observed after three months (May - August). The salmon fed low P/L ratio had higher fat content than those fed high P/L ratio, both in muscle (16.4 vs. 13.2 %) and viscera (39 vs. 29 %). Restricting the ration of the high P/L diet to 50 % further reduced the fat content to 11.3 % in muscle and 27 % in the viscera. Tagged individuals from the groups with different lipid content were restocked and mixed in the same pens, and then fed the same diet for seven months (August – March). As in Paper **I,** reducing the level of muscle fat prior to autumn significantly increased growth and weight gain from August to October. In other words, the weight gain was the highest for the restricted group, intermediate for the high P/L ratio and the lowest for the low P/L ratio group. In October, after two months of feeding a common diet, the muscle fat content was similar in fish from all three groups, whereas the differences in visceral fat content disappeared after four months (December). Although the differences in body weight, length and lipid content between the groups had been offset, the high P/L ratio and restricted group showed a significantly increased growth compared to the low P/L-ratio group in the latter stages of the trial (December – March), resulting in an overall weight gain difference of up to 1 kg. The high P/L ratio group had significantly higher final body weight, whereas the restricted group ended up with a numerically higher final body weight than in the low P/L ratio group. In addition, the variation in body weight and shape was significantly higher in the low P/L ratio group.

The results from Paper **I** and **II** demonstrate that in early autumn, the salmon is able to replenish lipid stores rapidly after dietary lipid restriction and that energy intake and storage is of high priority. In Paper **I**, unlike Paper **II**, low and high P/L ratio were fed throughout the trial and it is therefore not possible to isolate the direct effect of diet on growth from the indirect effect caused by different body fat accumulation. Hence, paper **I** may also indicate that dietary P/L-ratio of 1.12 (DP/DE of 15.2 g MJ kg$^{-1}$) was below the P/L ratio for optimal growth during the early seawater phase for S1 salmon. This result is in line with previous studies, although now verified using isoenergetic diets under ambient environmental conditions.

Paper **III** describes the effects of increased dietary P/L ratio for S0 salmon on mortality rates, biometric and quality related parameters during the entire grow-out period in sea, within the SAV3 endemic zone. The low P/L group was fed a typical standard diet with 35% protein and 35% fat (P/L: 1), versus the high P/L group that was fed a diet with 47% protein and 24% fat content (P/L: 2). During the first summer at sea, a co-infection of SAV3 and PRV was detected and a natural PD outbreak was observed. The increased dietary P/L ratio improved survival during the natural outbreak of PD. In addition to diet, body weight and delousing mortality (induced stress) prior to the PD outbreak were also found to contribute significantly to explain the observed variation in PD associated mortality. The high P/L group had a mean mortality rate of 1.9 %, whereas the low P/L group had a mean mortality rate of 3.7 %. Subsequent to the PD outbreak, a large amount of fish failed to grow and caused an accumulation of runts (severely thin diseased fish). At the end of the trial, a significantly lower amount of runt fish was detected among fish fed high P/L ratio (12 vs. 21%) and among large compared to small body weight groups (11 vs. 20%) prior to PD.

In Paper **IV**, an event of sudden mortality of large (2.5 kg) seemingly healthy farmed salmon during the winter period in northern Norway is reported. The experimental fish were reared in four net-pens and two dietary treatments were established; a high or low P/L ratio diets. An increased mortality (of 6 and 10%) was only observed within the two net-pens receiving the high P/L ratio experimental diets, following an abrupt reduction in dietary P/L ratio (increase in dietary fat level) six weeks earlier. The moribund/dying fish had significantly higher lipid content in the liver, altered hepatic fatty acid composition, and increased levels of ALT and AP in the blood plasma compared to non-dying fish, indicating impaired hepatic function. A possible hypothesis involving reduced recruitment of fat cells in high P/L-salmon is presented.

Taken together, the results from this thesis show that alterations in dietary protein-to-lipid ratio have profound potential effects on growth, lipid deposition, nutrient retention and health of farmed Atlantic salmon. . The results obtained during this work related to fat deposition and subsequent growth may be crucial knowledge when developing new dietary concepts in semi-closed and closed RAS production units, where water temperature and photoperiod can be manipulated.

## Sammendrag

I dag inneholder kommersielle dietter for stor oppdrettet Atlantisk laks (*Salmo salar* L.) vanligvis 30-35% protein og 35-39% fett, dvs. et forhold mellom protein-til-lipider under 1. Slike energitette dietter har generelt vist seg å kunne forbedre fôrutnyttelse og vekst. Imidlertid kan en reduksjon av protein-til-lipid forholdet i fôret føre til økt deponering av fett i muskel og rundt innvollene. Det er vist at høye nivåer av kroppsfett kan redusere fôrinntak og vekst hos laksefisk og blir ofte referert til som lipostatisk regulering. Det er derfor en økt risiko for redusert vekst dersom man benytter et fôr med et høyt fettinnhold før og under perioder hvor fôrinntaket og fettakkumulering er høy. Denne avhandlingen tester hypotesen om at økt protein-til-lipid-forhold i fôret til laks, samt en mulig involvering av lipostatisk regulering ved å redusere kroppsfettet, kan utnyttes for å forbedre egenskaper som tilvekst, overlevelse og fôrutnyttelse hos oppdrettslaks.

Artikkel **I** beskriver effekten av isoenergetiske dietter med forskjellig protein-til-lipid (P/L) forhold på vekst, fôrinntak, fôrutnyttelse, biometri og retensjon av næringsstoffer hos S1 post-smolt. Denne studien ble gjennomført fra sjø-utsett i april til september. Det ble funnet et signifikant lavere nivå av fett og energi i muskel og helkropp for laks gitt et fôr med høyt sammenlignet med lavt P/L forhold i juli, omtrent tre måneder etter at forsøket startet. Det redusert nivå av kroppsfett i juli forbedret fôrinntaket, veksten og vektøkningen betydelig sammenlignet med fisken som ble gitt et lavere P/L forhold i perioden juli til september. Gruppen gitt et høyt P/L forhold hadde også bedre fôrutnyttelse basert på sløyd vekt (FCRg). I tråd med dette var den relative innvollsvekten (viscerale somatiske indeksen, VSI) for gruppen gitt et høye P/L forholdet relativt stabilt under forsøket, mens VSI for gruppen gitt et lavere P/L forhold økte gradvis, noe som resulterte i signifikant høyere VSI på slutten av forsøket. Det økte proteininnholdet i diettene med høyt P/L forhold ble effektivt utnyttet for vekst og vektøkning, vurdert ved retensjonsberegninger, særlig i den siste perioden av forsøket.

I artikkel **II**, ble lave og høye nivåer P/L forhold i fôret, samt begrenset rasjon (~ 50%) av det høye P/L fôret benyttet for å endre fettnivået/status før høsten i stor laks. I denne studien ble det påvist en klar effekt av de ulike fôrbehandlingen tre måneder etter forsøksstart (mai - august). Gruppen gitt et lavt P/L forhold hadde høyere fettinnhold enn gruppen gitt et høyt P/L forhold, både i muskel (16,4 vs. 13,2%) og innvollsmassen (39 vs. 29%). Ved å gi halv rasjon av fôret med et høyt P/L forhold ble fettet deponering reduserte ytterligere til 11,3% i muskel og 27% i innvollsmassen. Gruppene (markert med PIG-tagg) med forskjellig fett innhold ble deretter overført til samme enheter/merder oppsamlet og gitt lik diett (samme P/L forhold) i syv måneder (august - mars). I likhet med artikkel **1** ble det vist at gruppene med et redusert nivå av kroppsfett før høsten hadde økt tilvekst fra august til oktober. Vektøkningen var høyere for gruppene gitt halv rasjon og et høyt P/L forhold sammenliknet med gruppen gitt et fôr med lavt P/L forhold. I oktober, etter to måneder med fôring av lik diett, var det ingen forskjeller i muskelfett mellom de ulike gruppene og forskjellene innvollsfettet var borte etter fire måneder (desember). Selv om forskjellene i kroppsvekt, lengde og fettinnhold mellom gruppene var blitt kompensert, ble det observert en signifikant økning i tilvekst for gruppen gitt det høye P/L forholdet og halv rasjon av dette fôret (fra mai-august) sammenlignet med gruppen gitt et lave P/L forhold i den siste perioden av forsøket (desember - mars). Dette resulterte i en total relativt vektøkning for gruppene gitt halv rasjon og et høyt P/L forhold på opptil 1 kg. Gruppen gitt et høyt P/L forhold hadde derfor en signifikant høyere sluttvekt, mens gruppen gitt halvrasjon av dette fôret endte opp med en numerisk høyere sluttvekt sammenliknet med gruppen gitt et lave P/L forhold.  I tillegg var variasjonen i fiskevekt og kroppsform signifikant høyere i gruppen gitt lavt P/L forhold.

I artikkel **III** blir effekten av et økt P/L forholdet i fôret for S0 laks på overlevelse, biometriske registeringer og kvalitets parametere i løpet av hele sjøfasen, innenfor den SAV3-endemiske sonen testet. En gruppe ble fôret med et standard høyfett-fôr med 35% protein og 35% fett (P/L: 1), men den andre gruppen ble gitt et fôr med 47% protein og 24% fett (P/L: 2). I løpet av den første sommeren i sjø ble det oppdaget en samtidig infeksjon av SAV3 og PRV, og det ble observert et naturlig utbrudd av PD. Økt forhold mellom P/L i fôret forbedret overlevelsen under det naturlige utbruddet av PD. I tillegg

til diett ble kroppsvekt og dødelighet ved avlusing (indusert stress) før PD utbruddet også funnet å bidra betydelig til å forklare den observerte variasjonen i PD-relatert dødelighet. Gruppen gitt et fôr med høyt P/L forhold hadde en gjennomsnittlig dødelighet på 1,9%, mens gruppen gitt lavt P/L forhold hadde en gjennomsnittlig dødelighet på 3,7%. Etter PD-utbruddet mislyktes en stor mengde fisk med å gjenoppta inntak av mat og forårsaket en kraftig akkumulering av såkalte «runts/taperfisk», som er alvorlig avmagret klinisk syk fisk. På slutten av forsøket ble det registrert en signifikant lavere mengde med runts blant gruppen gitt et fôr med høyt P/L forhold (12 vs. 21%) og blant stor sammenliknet med liten kroppsvekt (11 vs. 20%) før PD utbruddet inntraff (1.9 vs. 1.3 kg).

Artikkel **IV** beskriver en hendelse med plutselig økt dødelighet av stor (2,5 kg) tilsynelatende frisk oppdrettslaks i løpet av vinteren i Nord-Norge. I dette forsøket ble fisken oppdrettet i fire merder, hvorav to ble gitt en fôrserie med høyt P/L forhold og de to andre et fôrserie med lavt P/L forhold. Den økte dødelighet (på 6 og 10%) ble kun observert i de to enhetene/merdene som ble gitt fôrserien med høyt P/L forhold først etter at fettinnholdet i denne fôrserien ble økt (reduserte P/L forholdet) seks uker tidligere. Den døende fisken hadde betydelig høyere fettinnhold i leveren, endret fettsyresammensetning, og økte nivåer av ALT og AP i blodplasma sammenlignet med ikke-døende frisk fisken. Dette kan indikere en nedsatt leverfunksjon hos den døende fisken, som trolig har negative konsekvenser for helse og robustheten til denne fisken. En mulig hypotese som involverer redusert rekruttering av fettceller i laks gitt et høy P/L forhold blir presentert.

Samlet sett viser resultatene fra denne avhandlingen at endringer i protein-til-lipid forhold i fôr til oppdrettslaks har store effekter på fôrinntak, tilvekst, fett deponering og helse.  potensielle effekter på vekst, lipidavsetning, næringsreservering og helse av oppdrettslaksatlantisk laks. Resultatene i denne avhandlingen knyttet til fettdeponering og påfølgende vekst, kan være viktig kunnskap ved utvikling av nye fôrkonsepter i semi-lukkede og lukkede RAS-produksjonsenheter hvor vanntemperatur og fotoperiode kan manipuleres.

# 4. General introduction

## 4.1 Atlantic salmon aquaculture

In the world aquaculture production of diadromous fish, Atlantic Salmon (*Salmo salar* L.) is the dominating fish species, with a total production of 2.38 million tons in 2015 (FAO). Norway is the world leading producer and exporter of Atlantic salmon, with a sale of over 1.23 million metric tons, representing a first-hand value of 61.6 billion NOK in 2017 (*Statistics Norway*, 2018; figure 1). Atlantic salmon was first cultivated in the beginning of the 1970s in Norway, and the industry began with small family-owned businesses. Today, the farming of Atlantic salmon is a modern, intensive and globalised industry, with large multinational companies involved in rearing, feed production and processing of farmed salmon. The exponential increase in production can be attributed several factors, such as genetic selection, research & development, technical innovations, improved inputs, and production practices. This tremendous productivity growth has allowed production cost to be reduced, making farmed salmon a high quality competitive product (Asche, 2008).
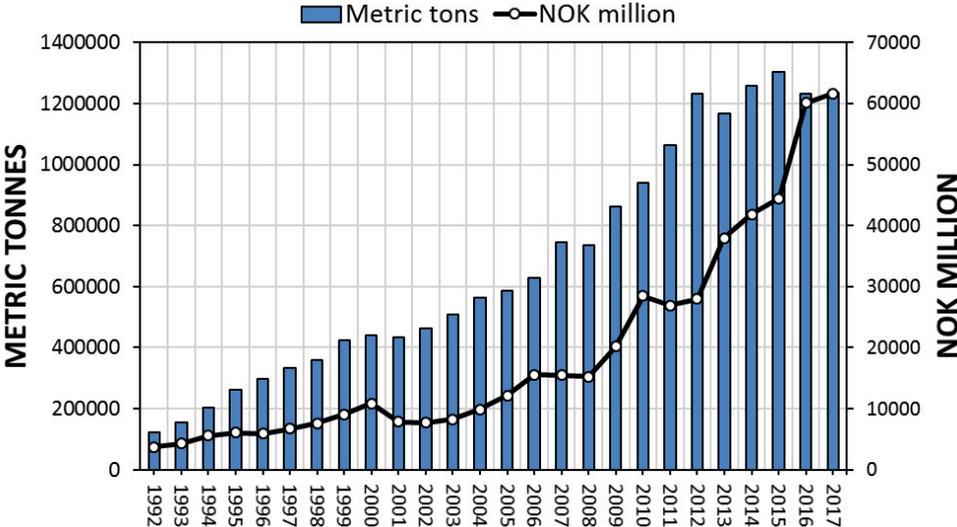


**Figure 1** Sales of Atlantic salmon (metric tons) and first hand value (NOK million) from 1992 to 2017. Source: Statistics Norway, aquaculture (2018) ("Statistics Norway," 2018)

## 4.2 Feed for farmed Atlantic salmon

The traditional salmon diets in 1990s contained up to 90 % of marine ingredients, mainly consisting of fish meal (FM) and fish oil (FO) (Ytrestøyl et al., 2015). Current modern diets contain less than 30% of marine ingredients, and this has been due to a static production of FM and FO during the last decades, combined with the fact that wild fisheries are being fully- or over-exploited (FAO, 2012; Ytrestøyl et al., 2015). In addition, the competitive pressure for FM and FO is high, due to that these sources are also used in feed production for other farmed livestock and in pharmaceutical industries. To reduce the dependency on wild fisheries and maintain an increase in sustainable aquaculture production, protein and oil sources of vegetable origin have been introduced and are used together with marine ingredients in modern diets for salmon (Turchini et al., 2009; Ytrestøyl et al., 2015).

In current intensive aquaculture production, high energy extruded diets are extensively used for Atlantic salmon. The introduction of extrusion technology together with vacuum coating has led to an increase of the dietary lipid level in salmonid diets during the last decade. Traditional pelleted diets used from the 1970s until the 1990s had a protein content of 45-55 % and lipid content of 10-20 %. Today, extruded vacuum coated diets containing 30-35 % protein and 35-39 % lipids are commonly used as grow-out diets (Torrissen et al., 2011). In Norway, the increase in dietary lipid inclusion has partly been driven by governmental restrictions (feed quotas), in order to control the biomass and restrict production growth from 1996 until 2005. The response from the aquaculture industry to this restriction was to increase the dietary lipid level (increasing the dietary energy) in order to improve feed utilization, and thereby gain higher fish biomass with less feed inputs (Torrissen et al., 2011). Protein sources are a major input cost in aquaculture feeds, and the global demand and prices for protein sources have been and are high. Lipids are generally a cheap source of energy compared to protein, and overall reductions in dietary protein together with an increase in lipid level are often utilized to reduce feed prices and increase sustainability. The proportion of lipid in salmon diets has approximately doubled during the last 30-40 years (Torrissen et al., 2011).

Today there is a variety in dietary protein-to-lipid ratio of commercial salmon feeds. However, the general trend is that the lipid content is gradually increased with higher fish weights, regardless of season. Feed costs account for more than 50 % of the total production costs in salmon farming, and fish farmers perception of low feed prices acts partially as a driver of the feed industries focus on producing the cheapest possible feed. Salmonids have high ability to utilize large amounts of lipids in high-energy diets efficiently for growth, resulting in good feed conversion and a favourable protein sparing effect (Azevedo et al., 2004; Karalazos et al., 2011, 2007). Based on these factors and the high demand and prices of preferred protein sources, it is likely to assume that the trend of high dietary lipid content can affect the protein-to-lipid ratio in diets during the whole seawater phase. However, small post-smolt that undergo rapid body growth require a high portion of digestible protein than larger salmon (Einen and Roem, 1997). It is therefore important that the protein content in current and future dietary regimes support optimal growth and health at different fish sizes and phases of the seawater production.

## 4.3 Diseases in salmon aquaculture

A major challenge during farming of Atlantic salmon is high levels of mortalities observed during the seawater phase. In Norwegian salmon aquaculture, the total mean production losses during the seawater phase was 13.1 % from 2010 to 2017 ("Statistics Norway," 2018). Large proportions of this loss are due to mortality caused by viral diseases (Hjeltnes et al., 2018). Currently, the most widespread, severe fatal viral diseases in Norwegian aquaculture are pancreas disease (PD), heart- and skeletal muscle inflammation (HSMI), and cardiomyopathy syndrome (CMS) (Hjeltnes et al., 2018, figure 2).
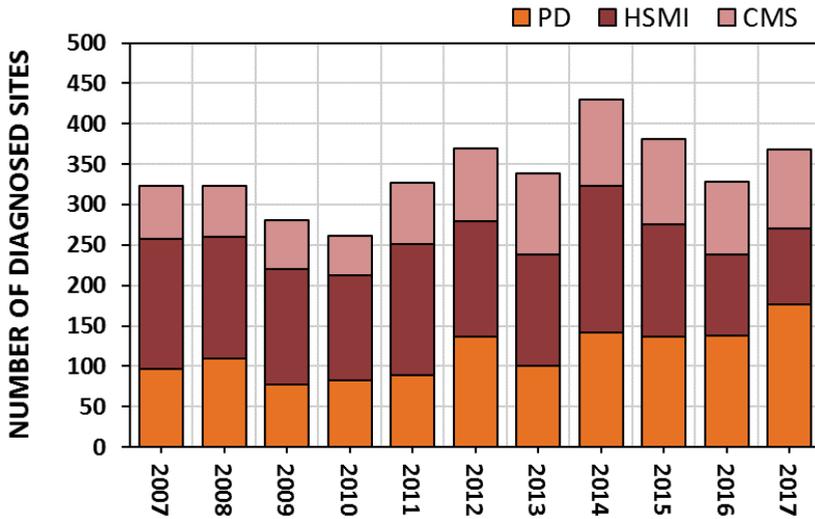
**Figure 2** Number of Norwegian production sites diagnosed with the viral diseases pancreas disease (**PD**), heart- and skeletal muscle inflammation (**HSMI**), and Cardiomyopathy syndrome (**CMS**) from 2007 – 2017. Data sources: Hjeltnes et al. (2018).

### 4.3.1 Pancreas disease

PD is a widespread contagious viral disease affecting Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) and is a significant problem in the European salmonid farming industry (Graham et al., 2011; Jansen et al., 2017). The first recognized and described cause of the disease was conducted in Scotland in 1976 (Munro et al., 1984), whereas the first detected cases in North America and Norway were in 1987 and 1989, respectively (Kent and Elston, 1987; Poppe et al., 1989). *Salmonid alphavirus* (SAV) is the causative agent of PD in farmed Atlantic salmon and rainbow trout, and is allocated to the genus alphavirus within the family *Togaviridae* (Weston et al., 1999). In Norway, the SAV subtype 3 and a marine subtype 2 have been detected (Hjortaas et al., 2013; Hodneland et al., 2005). Since 2003, PD caused by SAV3 has been endemic along the west coast of Norway up to Hustadvika in Møre and Romsdal (63° latitude – SAV3 endemic zone), particularly in counties of Hordaland and Rogaland (Jansen et al., 2015; Jensen and Gjevre, 2018). PD can occur throughout the yearly cycle, but the risk of outbreaks is highest during the spring and summer months when the seawater

temperature is increased (Hjeltnes et al., 2017; McLoughlin and Graham, 2007; Rodger and Mitchell, 2007; Stene et al., 2014). PD causes pathological changes that involve partly or severe loss of exocrine pancreatic tissue, cardiac and skeletal myopathies, epicarditis and white skeletal muscle degeneration and/or inflammation (Christie et al., 2007; McLoughlin and Graham, 2007; Taksdal et al., 2007). Mortality rates can reach up to 63% for sites that are severely affected by PD and among surviving fish, subsequent failure to grow may cause thin fish with poor condition (runts) and high number of discarded fish at slaughter (reviewed by Jansen et al., 2017). Several studies have found that PD may also impair the fillet quality of slaughter sized salmon (Larsson et al., 2012; Lerfall et al., 2012; Taksdal et al., 2012). A newly published economical simulation showed that PD caused a total direct cost for Norwegian fish farmers in 2015 of 2.4-2.8 billion NOK (Vedeler, 2017). This was equivalent to an increase in production cost of about 2.2 NOK/kg head on gutted salmon (HOG).

## 4.3.2 Heart and skeletal muscle inflammation

HSMI is another common fatal disease of farmed salmon and the disease have been link to the *piscine orthoreovirus* (PRV) (Palacios et al., 2010). However, PRV seem to be ubiquitous among farmed salmon in Norway (Løvoll et al., 2012), and can be present in high titers without causing mortality or marked lesion in the heart (Garseth et al., 2013). Severe HMSI outbreaks gives direct inflammatory lesions in cardiac and skeletal muscle and such damage may occur at an early stage in the disease progression, and may persist for many months after clinical disease (Kongtorp et al., 2006). The lesions observed during outbreaks of HSMI are similar to those described for PD and CMS (R. T. Kongtorp et al., 2004). The histopathological changes associated with HSMI in salmon has previously been thoroughly described, of which epi-, endo- and myocarditis, myocardial and skeletal muscle necrosis and signs of liver damage are central features (R T Kongtorp et al., 2004). Outbreaks of HSMI may lead to lowered appetite, reduced feed utilization and increased mortality, and although the mortality and duration of the outbreak can vary, mortality rates up to 20 % are observed (Alne et al., 2009; Kongtorp et al., 2006). Natural outbreaks of HSMI have normally been recorded 5 to 9 months after transfer to sea. However, observation shows that outbreaks may occur during the

whole seawater phase and as early as 14 days following seawater transfer (Bornø and Lie, 2015; R. T. Kongtorp et al., 2004).

### 4.3.3 Cardiomyopathy syndrome and non-infectious cardiovascular disease

CMS is a serious cardiac related disease that affect large Atlantic salmon in sea and the diseases is associated with the totivirus *Piscine myocarditis virus* (PMVC) (Hjeltnes et al., 2018). Salmon that are affected by CMS are presumably in good condition prior to the time of death, which occur suddenly (Brun et al., 2003). CMS is regarded as a chronic disease, which can cause prolonged periods of moderate to elevated mortality (Brun et al., 2003). Mortality of seemingly healthy salmon has also been linked to non-infectious cardiovascular diseases/failures and are often observed in salmonid aquaculture (Dalum et al., 2017; Hjeltnes et al., 2018; Poppe et al., 2007; Tørud and Hillestad, 2004). Stress is generally related to the outbreaks of diseases and increased mortality caused by HSMI, PD and CMS are often reported in association with handling and operation measures at site level, e.g. delousing and relocating fish (Bornø and Lie, 2015; Hjeltnes et al., 2018).

### 4.3.4 Disease prevention

In terms of viral disease prevention, a vaccine against PD is available in Norway since 2007 and recently a DNA-vaccine has been introduced. However, the immunity of the DNA-vaccine (Clynav, Elanco Europe Ltd) has only shown a duration of three months post vaccination (Felleskatalogen, 2018). In a cohort study conducted by Jensen *et al*. (2012), PD vaccinated fish had lower PD-associated mortality, number of discarded fish at slaughter and better growth than non-vaccinated fish. However, the efficacy of the monovalent vaccines under field conditions has been questioned and high PD associated mortality has been observed in PD vaccinated fish (Hjeltnes et al., 2018). No commercially available vaccines for HSMI and CMS seem to exist. However, several breeding companies are now marketing genetic lines with increased resistance towards PD, HSMB and CMS, using quantitative trait locus (QLT) analysis and marker-assisted selection.

Today, there are several commercially available feeds designed for better performance in connection with viral infections, for clinical use after infection or as feeds with prophylactic, immune stimulating or anti-inflammatory effects (often referred to as *functional feeds*). The effectivity of some of these functional feeds has been reported in scientific studies. Low-lipid diets containing high levels of specific polyunsaturated fatty acids (PUFAs) have been used as a potent tool to increase the tolerance/resistance towards HSMI and CMS in Atlantic salmon by modulating tissue fatty acid composition and eicosanoid production (Martinez-Rubio et al., 2014, 2012). Commercial available "PD feeds" are frequently used within the SAV3 endemic zone (Jansen et al., 2015), and these feeds are often formulated to contain lower amounts of lipids and increased levels of protein (pers. comm. 2016). However, no scientific studies are published on the potential effects of such feeds related to PD associated mortality of large salmon. High intake of dietary lipids is associated with metabolic risk factors (Johnson et al., 2008; Nicholls et al., 2006), and it can be suggested that several diseases in salmon aquaculture are worsened by the use of high-fat diets. This can be particularly prominent for diseases that also affect the heart, such as PD, HSMI and CMS.

## 4.4 Growth and feed utilization

Attaining optimal growth is one of the most important parameter in aquaculture, which defines the production efficiency and is a good indicator of a robust and healthy fish. The growth rates of salmonids depend on feed intake and utilization, which are highly dependent on water temperature, photoperiod, and affected a wide range of other internal and external factors such as genetics, health status, adiposity, physiological processes (smoltification, maturation etc.), water quality, fish size, dietary composition and feeding regime (Aksnes et al., 1986; Austreng et al., 1987; Bendiksen et al., 2003; Einen and Roem, 1997; Gjedrem, 2000; Sveier and Lied, 1998; Thorarensen and Farrell, 2011). The thermal growth coefficient (TGC) is a highly used growth model for fish, that express growth rate independent of temperature and fish size, which makes the model both useful and flexible (Cho, 1992; Thorarensen and Farrell, 2011). As other models, the TGC has some assumptions and limitations (Jobling, 2003), but is relatively robust within the normal temperature range (Thorarensen and Farrell, 2011). Feed conversion

ratio (FCR) is a useful indicator of feed utilization and efficiency, and describes how much feed is required to produce 1 kg of fish (direct inputs to outputs). Both the TGC and FCR vary to a large degree between different growth related studies of Atlantic salmon conducted both in net-pens and tanks, and an excellent overview of this is given in Thorarensen and Farrell (2011), showing TGC from 0.1 to 4.8 and FCR from 0.7 to 1.7. As discussed in this article, these differences show that salmon growth rates depend on experimental conditions, and they reflect the seasonal cycles in growth performance of Atlantic salmon. High growth rates and efficient production during the seawater phase are fundamental goals for all fish farmers. Thus, factors that can limit and increase salmon growth are of great importance.

## 4.5 Seasonal variations in growth and lipid deposition

The majority of farmed Atlantic salmon is reared in net-pens that are exposed to seasonal environmental changes. Several studies report seasonal variation in growth and lipid deposition of Atlantic salmon, mainly due to changes in seawater temperature and photoperiod (Alne et al., 2011; Mørkøre and Rørvik, 2001; Nordgarden et al., 2005, 2003c, 2003a; Weihe et al., 2018). However, some of these variations also seem to be influenced by smolt-type and timing of sea transfer. In the salmon industry, smolt are regularly transferred to the sea in the spring as "in season" year-old smolt (S1) or in the autumn as "out of season" under-year-old smolt (S0). Seawater adaption is an energy demanding process, and a reduction in appetite, growth and body lipid content is often observed after sea transfer, particularly for S1 post-smolt during the spring (Alne et al., 2011; Toften et al., 2003; Usher et al., 1991). S0 smolt on the other hand, are in some studies reported to not experience low-performing periods after sea transfer in autumn (Alne et al., 2011; Lysfjord et al., 2004). In line with this, the S1 salmon show increased feed intake, growth and lipid deposition during the late summer and autumn period (Alne et al., 2011; Mørkøre and Rørvik, 2001). However, it is shown that S0 smolt reared in mid-west part of Norway can experience reduced feed intake, growth and lipid deposition during the first spring in sea, 5-7 months after sea transfer (Alne et al., 2011). High feed intake and TGCs are generally observed summer and until late autumn for large salmon (Mørkøre and Rørvik, 2001; Nordgarden et al., 2003a), whereas reduced

feed intake, TGC and FCR are often observed during the winter period (Mørkøre and Rørvik, 2001). Photoperiodic manipulation using continuous light during the winter and early spring period is shown to induce increased feed intake and growth during the spring and early summer (Nordgarden et al., 2003a). Taken together, seasonal shift with its associated environmental conditions and internal factors seem to induce metabolic changes that significantly affect growth and feed utilization in salmon.

Important factors as body size, dietary lipid content and feed ration are shown to alter lipid deposition and fat content in salmonids (Hemre and Sandnes, 1999; Hillestad et al., 1998; Shearer, 1994; Torstensen et al., 2001). In relation to season, farmed salmon show high fat deposition and increased condition factor, with a concomitant increase in feed intake and weight gain during the summer and autumn period (Mørkøre and Rørvik, 2001; Nordgarden et al., 2003b). This pattern is particularly pronounced for S1 salmon at high latitudes that experience long winter and late spring (Mørkøre and Rørvik, 2001). Prolonged high seawater temperatures and declining day length characterizes the autumn period. During the late autumn and winter, a decline or stagnation in the level of muscle fat in salmon is often observed, which relates to reduced feed intake and increased FCR during low sea temperatures and short day length (Mørkøre and Rørvik, 2001). This pattern of fat deposition is commonly observed in commercial farming of salmon and during large-scale studies under ambient environmental conditions.

## 4.6 Fat storage as a reproductive adaption?

A model develop by Professor Kjell-Arne Rørvik links the pattern of fat storage to environmental adaption and the reproduction life-strategy of Atlantic salmon. Sexual mature Atlantic salmon spawn during the late autumn period. The sexual maturation process requires, in addition to photoperiodic stimuli, sufficient fat and energy reserves (Kadri et al., 1997, 1996; Rowe and Thorpe, 1990; Taranger et al., 2010). Development of gonads are energetically demanding process and require severe energy investment (Fleming, 1996; Jonsson et al., 1997). Appropriate and available fat reserves during the spring period seems to be a major factor controlling the initiation and progression of the maturation process in salmon (Thorpe, 1994; Thorpe et al., 1998; Wright, 2007). To low energy and fat levels may arrest the maturation process and postpone reproduction

(Duston and Saunders, 1999; Rowe et al., 1991; Rowe and Thorpe, 1990; Thorpe, 1994; Thorpe et al., 1990). To assure sufficient energy stores, salmon need to utilize the late summer and early autumn for accumulation of fat and build-up of energy stores prior to the initiation of the maturation process the following spring. This reproductive fat storage cycle hypothesis is illustrated in figure 3. The maturation process starts and continues if the salmon has sufficient energy stores in the spring before (black arrow). Thus, the overall reproduction process starts with the crucial accumulation of fat and build-up of energy stores during late summer and early autumn prior the initiation of the maturation process the following spring (grey arrow), one year ahead of spawning. The fat content in the beginning of the autumn may therefore be a dominant factor for feed intake, growth and the accumulation of fat.
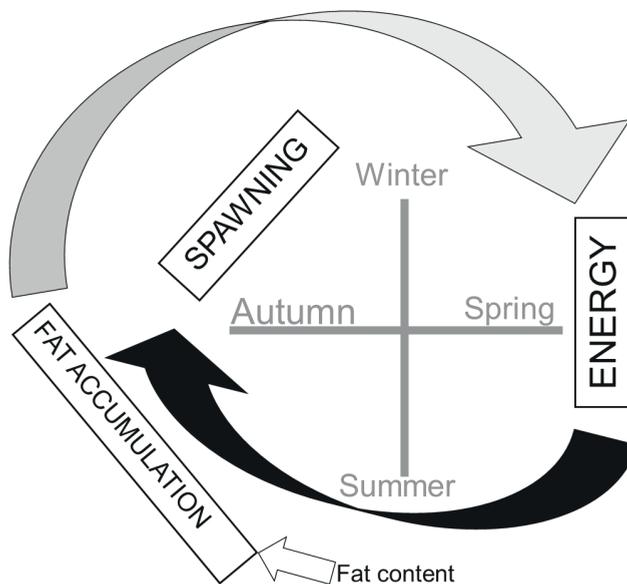


**Figure 3** The fat storage cycle hypothesis. Atlantic salmon spawn during the autumn and the maturation process starts (in addition to light stimuli) and continues if the salmon has sufficient energy stores in the spring prior to the autumn (**black arrow**). The overall reproduction process start with the accumulation and build-up of the energy stores during late summer and autumn period prior to the initiation of the maturation process the following spring (**grey arrow**), one year ahead of spawning. The fat content prior to the autumn may therefore be of importance.

## 4.7 Compensatory growth and lipostatic regulation

Healthy animals exposed to optimal environmental and nutritional conditions display good growth, whereas animals that encounter setbacks induced by nutritional deficits or sub-optimal conditions often display accelerated growth rate to recover lost body mass when circumstances are normalized (Ali et al., 2003; Arendt, 1997; Metcalfe and Monaghan, 2001). The majority of this evidence arise from studies in which animals exhibit accelerated growth after a period of growth depression, often referred to as compensatory growth (Ali et al., 2003; Dobson and Holmes, 1984; Hayward et al., 1997; Jobling, 2010). The compensatory growth response phenomena has been observed in several animals, including several fish species (Ali et al., 2003; Jobling et al., 1993; Sæther and Jobling, 1999; Wilson and Osbourn, 1959). The most common method to provoke compensatory growth responses in fish is by the means of complete or partial food deprivation prior to periods with food availability (Ali et al., 2003; Dobson and Holmes, 1984; Nikki et al., 2004). However, compensatory growth may also occur for fish periodically exposed to low temperatures, hypoxia and disease treatment (Foss and Imsland, 2002; Mortensen and Damsgård, 1993; Speare and Arsenault, 1997). The degree of compensatory growth in fish vary and is often  categorized based on the capacity of the fish to catch-up, and hence to achieve lower, similar or higher size/mass as their non-restricted counterparts, referred to as partial-, complete- and over compensation, respectively (Ali et al., 2003).

Feed restriction or deprivation induce changes in body energy by depleting lipid stores, and during the course of compensatory growth and hyperphagia, body weight and lipid reserves are gradually restored (Ali et al., 2003; Bull and Metcalfe, 1997; Jobling and Miglavs, 1993; Metcalfe and Thorpe, 1992). The lipostatic model is often discussed within the circumstances of compensatory growth responses in fish (Jobling and Johansen, 1999; Johansen et al., 2001), and this identifies adipose tissue and stored lipids as important factors governing appetite (Jobling and Johansen, 1999; Keesey and Corbett, 1984; Kennedy, 1953). The model implies that the amount of stored fat has a negative feedback control on feed intake and is important for the regulation of energy

homeostasis. Hence, compensatory growth is not only a response to recover body weight, but also a strong response to restore lipid levels and compensatory growth will therefore cease once this is achieved (Ali et al., 2003; Jobling and Johansen, 1999; Johansen et al., 2002). Johansen et al. (2002) showed that altering body lipids of juvenile salmon by feeding low-fat diets induced similar growth responses as deprivation and feed restriction.

Salmonids increase the deposition of fat in the muscle and visceral cavity as the fat content in the feed increases (Bendiksen et al., 2003; Hillestad et al., 1998). In view of the possible involvment of lipistatic regulation, it can be assumed that a diet with a lowered lipid level but with sufficient energy content (increasing the dietary protein-to-lipid ratio), can be an approach to reduce the deposition of lipids and enhance feed intake. It may also indicate that lowering the body fat levels may increase feed intake and growth, particlarly prior to or during periods with high fat acummulation.

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# 5. Objectives

The general objective of the present thesis was, in both small- and large-scale studies, to investigate the impact of dynamic changed dietary protein-to-lipid ratios throughout the production cycle of farmed Atlantic salmon with focus on:

- The effects of isoenergetic diets with different protein-to-lipid ratio on growth, feed intake, feed conversion, biometrics, nutrient retention and deposition in S1 Atlantic salmon post-smolt (Paper **I**).

- The influence of body fat levels prior to the autumn on subsequent growth, weight dispersion, biometrics and lipid deposition in Atlantic salmon. (Paper **II**).

- The effects of seasonal changes in dietary protein-to-lipid ratio on growth, survival and quality in Atlantic salmon during health challenging periods under commercial condition in the southern (Paper **III**) and northern (Paper **IV**) Norway.

Based on these aims, the initial main hypothesis was that increased dietary protein-to-lipid ratio and the possible involvement of lipostatic regulation on body fat levels can be utilized to significantly improve fitness-related traits including growth, survival and nutrient deposition of farmed Atlantic salmon.

# 6. Experimental overview
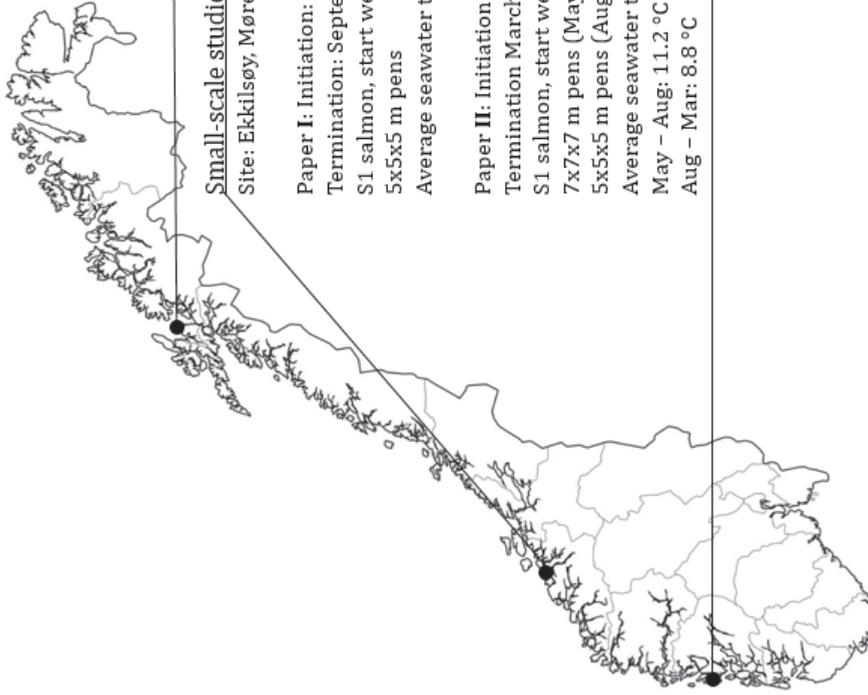


Commercial-scale trail (Paper **IV**).
Site: Dypingen, Bjarkøy - Harstad

Initiation: May 2015
Termination/Slaughter: July – August 2016
Post-smolt salmon, start weight 148 gram
130 m pens
Average seawater temp: 8.1°C

**Sudden increase in mortality**: January 2016

Commercial-scale trail (Paper **III**).
Site: Otterholmen, Radøy - Hordaland

Initiation: September 2014
Termination/Slaughter: Jan-May 2016
S0 salmon, start weight ~80 gram
120 m pens
Average seawater temp: 9.5°C

**PD outbreak**: July 2015

Small-scale studies (Paper **I** and **II**).
Site: Ekkilsøy, Møre and Romsdal

Paper **I**: Initiation: March 2012
Termination: September 2012
S1 salmon, start weight 95 gram
5x5x5 m pens
Average seawater temp: 9.8°C

Paper **II**: Initiation in May 2011.
Termination March 2012.
S1 salmon, start weight 1 kg
7x7x7 m pens (May – Aug)
5x5x5 m pens (Aug – Mar)
Average seawater temp:
May – Aug: 11.2 °C
Aug – Mar: 8.8 °C

# 7. Main results and general discussion

The following section summarizes and discusses the main results from Paper **I–IV**. In this section, protein-to-lipid ratio is referred to as P/L ratio. To simplify the discussion, the term high and low dietary P/L ratio is used instead of the specific P/L ratio for each experimental period within Paper **I-IV**. Table 1 summarizes the specific P/L ratios that were used in Paper **I-IV** and how they are referred to in the text (high or low). The seasonal timing of the dietary induced changes from low to high lipid level within the low P/L groups (test variable), is shown in figure 4.

**Table 1** Overview of the specific protein-to-lipid (P/L) ratios and the corresponding ratios between digestible protein and energy (DP/DE) used for each experimental period within Paper **I-IV**, and how they are referred to in the text (high or low).

| Paper | Time period | P/L ratio | | DP/DE ratio | |
|---|---|---|---|---|---|
| | | **High** | **Low** | **High** | **Low** |
| I | April - June | 1.86 | 1.55 | 20.5 | 18.5 |
| | June - July | 1.59 | 1.26 | 18.9 | 16.5 |
| | July - September | 1.40 | 1.12 | 17.4 | 15.2 |
| II | May - August | 2.85 | 0.98 | 23.7 | 13.5 |
| | August - December | 1.33 | | 16.6 | |
| | December - March | 0.95 | | 13.5 | |
| III | During PD outbreak | 2.00 | 1.01 | 21.6 | 15 |
| IV | May - August | 1.85 | 1.43 | - | |
| | August - October | 1.64 | 1.31 | - | |
| | October - December | 1.52 | 1.01 | - | |
| | December - February | 1.17 | 1.02 | - | |
| | March - July | 1.33 | 1.02 | - | |

PD; Pancreas disease
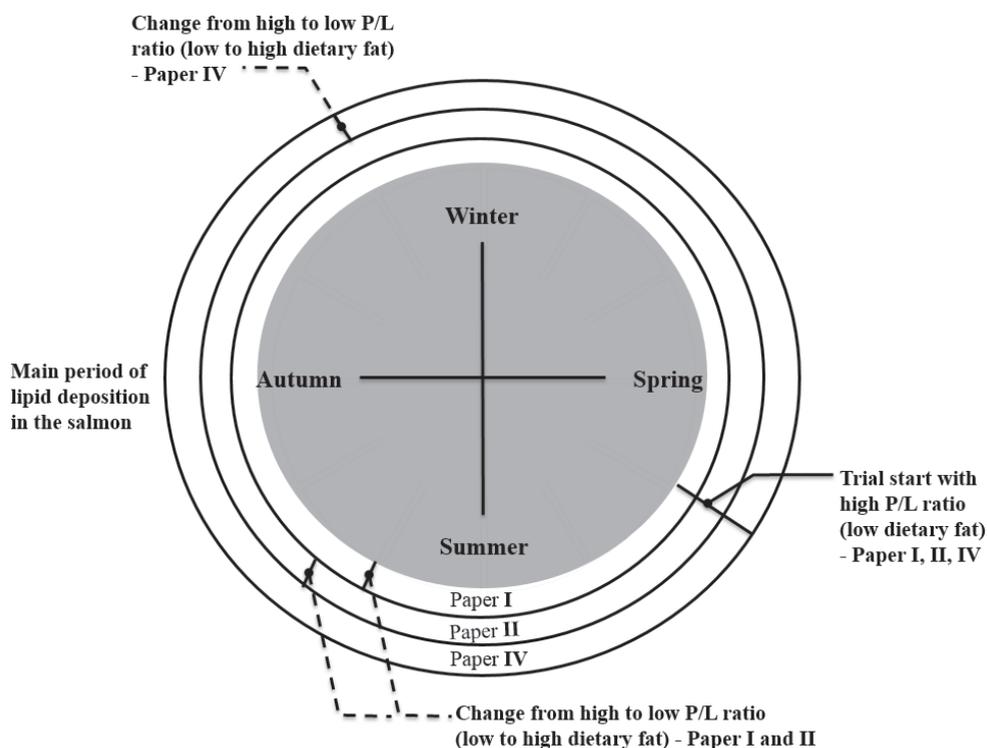
**Figure 4** The seasonal timing of the dietary induced changes from low to high lipid level within the high P/L groups (test groups).

## 7.1 Body fat levels and growth

For the control groups, the lipid deposition observed in Paper **I, II** and **IV** are generally in line with previous studies showing elevated lipid deposition with increased body weight, feed intake, dietary lipid content and growth (Aksnes, 1995; Einen et al., 1999; G I Hemre and Sandnes, 1999; Hillestad et al., 1998; Mørkøre and Rørvik, 2001; Shearer, 1994; Torstensen et al., 2001). The amount of stored fat seems to be an important regulator of appetite and feed consumption in fish and high fat levels correlates with subsequent reductions in feed intake in salmonids (Johansen et al., 2003; Silverstein et al., 1999). To evaluate the effect of altered lipid deposition on growth may be difficult in salmonids, since they tolerate long periods with food restriction or deprivation before

fat stores are changed. Factors like duration, fish size, dietary composition, environmental cues and life stage are important to consider in the planning of such trials and when interpreting the results. The different dietary P/L ratio used in Paper **I, II** and **IV** triggered significant effects on lipid deposition in the muscle and visceral cavity, which are the main lipid stores in salmon (Aursand et al., 1994; Sheridan, 1994). In Paper **I**, significantly lower muscle fat, whole body lipid, and energy level was observed in the post-smolt fed high compared to low P/L ratio in July, approximately three months after the trial started. A negative correlation between the level of muscle fat in July and the feed intake from July to September was observed. The fish fed high P/L ratio displayed a significantly higher feed intake, growth and weight gain compared to fish fed low P/L ratio from July to September. At the end of the trial, no differences in muscle fat was observed between the groups. High body lipid levels may reduce feed intake and growth in salmon (Johansen et al., 2003), and this observation may indicate a possible involvement of lipostatic regulation. However, in Paper **I**, low and high P/L ratio was fed throughout the trial and it is therefore not possible to isolate the effect of body fat accumulation. To be able to elucidate this, the groups should have been fed the same diet from July to September. The isolated effect of body fat levels was tested in Paper **II**. In Paper **II**, low, high P/L ratios, and restricted ration (~ 50%) of the diet with high P/L ratio, were used to alter the lipid deposition (primarily muscle fat content) prior to the autumn in large salmon. In this study, clear treatment effect on body fat was observed after three months (May - August). The salmon fed low P/L ratio had higher fat content than those fed high P/L ratio, both in muscle (16.4 vs. 13.2 %) and viscera (39 vs. 29 %). Restricting the ration of the high P/L diet to 50 % further reduced the fat content to 11.3 % in muscle and 27 % in the viscera. PIT-tagged individuals from the groups with different lipid content were restocked and mixed in the same pens, and then fed the same diet for seven months (August – March). The results showed that the the lipid content in August was negatively correlated to the subsequent TGC and weight gain from August to October. In other words, the weight gain was the highest for the restricted group, intermediate for the high P/L ratio and the lowest for the low P/L ratio group. In October, after two months of feeding a common diet, the muscle fat content was similar in fish from all three groups, whereas the differences in visceral fat content disappeared after four months (December). In the commercial scale trial (Paper **IV**), no differences in fat content or growth between the groups fed high and low P/L ratio were

detected prior to or during the autumn. This could be related to lower temperatures in spring and early summer prior to the autumn compared to Paper **I** and **II**. Differences in fish size and dietary composition can also be influencing factors. The main increase in fat content among the group fed high P/L ratio in Paper **IV** took place in December. This late dietary change seemed to have negative consequences for health-related parameters, which are discussed in section **7.5**. The results from Paper **I** and **II** show that alterations in body lipid levels prior to falling day length in the autumn, significantly affected feed intake and growth in salmon. The results also demonstrate that salmon is able to replenish lipid stores rapidly after dietary lipid restriction and that energy intake and storage is of high priority. The increased growth and rapid replenishment of lipid stores suggest the existence of a robust mechanism for the regulation of body fat in salmonids, and are in line with the previous observation from Silverstein *et al.* (1999).

## 7.2 Compensatory growth

The strength of compensatory growth responses seem to depend on the reduction in body condition, length and mass in the restricted or deprived fish groups compared to their fully fed conspecifics (Alvarez and Nicieza, 2005; Johansen et al., 2001; Johnsson and Bohlin, 2006, 2005). In Paper **II**, feeding a high P/L feed at full or restricted rations resulted in slightly and markedly lower body weight, respectively, compared to feeding the low P/L feed from May to August. Thus, the subsequent increased subsequent growth in fish from these groups from August to October, when all fish were fed a common diet, may partly be explained by the differences in mass and length compared to the low P/L group. However, the small difference in weight between the high and low P/L group in August and the strong correlation between body fat and growth indicate that fat content (i.e. energy status) seem to be a key growth regulator from August to October. This is in line with the observation in Paper **I**. In the the two last periods of the trial in Paper **II**, the high P/L ratio and restricted group showed a significantly increased growth compared to the low P/L-ratio group. The high P/L ratio group had significantly higher final body weight than the low P/L ratio group, whereas the restricted group ended up with a similar final body weight as the low P/L ratio group. There are evidences indicating that compensatory growth  responses will cease as lipid stores and body condition are restored to levels similar to the unrestricted conspecifics (Ali et al.,

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2003; Alvarez and Nicieza, 2005; Johansen et al., 2001; Johnsson and Bohlin, 2005). However, the increased growth in the two last periods of the trial in Paper **II**, from October to March, was evident although the differences in body weight, length and lipid content between the groups had been offset, except for a small difference in length between the restricted and the low P/L ratio group. This apparent overcompensation may indicate the existence of a more complex explanation than just lipostatic regulation.

The growth responses found in Paper **II,** are somewhat similar to compensatory growth responses and lipostatic regulation observed in previous studies (Ali et al., 2003; Jobling and Johansen, 1999; Johansen et al., 2002, 2001). However, some of the novelty of Paper **II** is that alterations in body fat content were dietary induced prior to the autumn. Thus, it differs from many previous experiments related to compensatory growth and lipostatic regulation in the exposure of the fish to ambient temperature and day length, which are rigid environmental cues in fish. The autumn is a period associated with optimal temperatures, declining day length, increased feed intake and high lipid deposition. In Paper **II**, it is discussed how the strong growth response shown by the high P/L ratio and the restricted group may have been triggered by the reproductive life strategy of the Atlantic salmon. The marked increase in lipid deposition and weight gain of the groups fed with a low fat diet may be a response to deposit and store energy for the upcoming spring period, which seems to be a critical period for the initiation and proceeding of the maturation process. Too low energy and fat levels may arrest the maturation process and postpone reproduction (Duston and Saunders, 1999; Rowe et al., 1991; Rowe and Thorpe, 1990; Thorpe, 1994; Thorpe et al., 1990). However, to verify this, the groups of salmon needs to be studied for a longer period and measurements of relevant plasma hormones, gonad development and transcript abundance of relevant genes should be conducted. Although the muscle and visceral cavity are the main sites for lipid deposition in salmon, lipids are also allocated to other parts of the body such as head and bones (Jobling et al., 2002). These structural compartments can also be affected by dietary lipid level and it has been suggested that the lean body mass relative to fat can be important for lipostatic regulation (Jobling and Johansen, 1999; Johansen et al., 2003). The indexes or the fat content of such structural components were not measured in Paper **II**. However, it should be noted that the visceral fat content was consistently lower in the high P/L ratio and in the restricted group compared to the low

P/L ratio group throughout the trial, but being significantly lower only in October. In addition, the high P/L and restricted groups had lower dispersion/variation in final body weights and shape compared to the low P/L group. This can indicate that several individuals in the low P/L group showed lowered or impaired growth in latter stages of the trial. These factors may also be a contributing to an increased growth and the obtained weight differences. Even though it could be difficult to pinpoint the causes of the observed growth response, the results in Paper **I** and **II** show that increasing the dietary P/L ratio prior to the autumn increase growth and weight gain in salmon.

## 7.3 Dietary protein-to-lipid ratio and growth

In Paper **I**, unlike Paper **II**, low and high P/L ratio were fed throughout the trial and it is therefore not possible to isolate the direct effect of diet on growth from the indirect effect caused by different body fat accumulation. Einen and Roem (1997) evaluated different dietary DP/DE-ratios for Atlantic salmon in seawater and concluded that salmon grown from 1 to 3 kg required a DP/DE above 16.4 g MJ kg$^{-1}$ (P/L-ratio of 1.23) for optimal growth performance. Although using somewhat smaller fish, the growth data in Paper **I** are in agreement with these results. Hence, the low P/L ratio group was fed a dietary DP/DE ratio of 15.2 (P/L ratio of 1.12), whereas the high P/L-ratio group was fed a DP/DE ratio of 17.4 (P/L ratio of 1.40) from July to September. However, in the study of Einen and Roem (1997) diets with different energy content were tested, whereas in Paper **I** isoenergetic diets were used. Salmonids seem to adjust their appetite according to the dietary energy level (Bendiksen et al., 2002), and it has been suggested that this could be the predominant factor for feed intake and utilization. Studies using isoenergetic diets, or adjusting the dietary ration level so that the diets tested were fed isoenergetically, found no negative effects of reduced dietary P/L ratio on growth performance in Atlantic salmon (Azevedo et al., 2004; Hillestad et al., 1998; Karalazos et al., 2011, 2007). These finding differ from the results from Paper **I**. In the experiments of Karalazos et al. (2011, 2007), isoenergetic diets with a P/L ratio in the range of 0.75 – 1.19 (DP/DE ratio: 12.5 - 15.5 g MJ kg$^{-1}$) and low fishmeal inclusion were used, whereas Azevedo et al. (2004) tested the diets using a wild salmon strain reared in freshwater with a constant temperature of 8 °C. Thus, the mentioned studies have clear dissimilarities in dietary inputs and experimental design compared to those from Paper

**I**. In addition, the salmon used were smaller (0.1 – 1 kg) than the salmon used in the studies of Karalazos et al. (2011, 2007). In Paper **I**, the seawater temperature from July to September had a mean of 13.6ºC, which is associated with optimal growth rates for salmon post-smolt (Handeland et al., 2008). Small salmonids require higher dietary proportions of digestible protein than larger salmonids (Cho and Kaushik, 1990; Einen and Roem, 1997), and this seems to be particularly important at high temperatures and during rapid somatic growth (Bendiksen et al., 2003). These mentioned factors, together with the potential *lipostatic* regulation, may explain why the results obtained in previous studies using isoenergetic diets differ from those presented in Paper **I**. The results also suggest that a DP/DE of 15.2 g MJ kg$^{-1}$ (P/L-ratio of 1.12) is below the required P/L ratio to attain optimal/maximum growth of S1 salmon in the weight segment from 0.1 – 1 kg.

## 7.4 Nutrient utilization

FCR and nutrient retention are commonly used to evaluate the efficiency and sustainability of aquaculture systems and diets. For an optimal and sustainable production, low FCR and high nutrient retention are favorable. High dietary energy content seems important for maintaining low FCR when fish size increases (Einen and Roem, 1997; Hillestad et al., 1998). In Paper **I**, no significant differences in FCR were detected between the low and high dietary P/L groups. However, when the FCR was calculated based on gutted weight (FCRg), the low P/L ratio group had a significantly higher FCRg from July to September compared to the high P/L ratio group. While visceral somatic index (VSI) of the group fed the high dietary P/L ratio was relatively stable during the experiment, the VSI of the group fed the low dietary P/L ratio increased gradually. Consequently, the low P/L group had significantly higher VSI and lower carcass yield compared to the high P/L group at the end of the trial. In Paper **II**, feeding the high P/L feed or a restricted ration of this diet from May to August also significantly reduced the VSI and reduced the visceral fat content compared to feeding a low P/L ratio. Refstie et al. (2001) found that feeding high-fat diets resulted in increased visceral fat content, lower carcass yield and larger visible fat deposits. In their study, the high-fat diets increased the weight gain by 122 grams compared to medium-fat diets; however, 91 grams of this weight gain were lipid. This and the results from Paper **I** and **II** shows that a major part of the fat accumulated in salmon when feeding high-fat diets

ends up around the intestines and in other fat deposits, which are often removed by gutting and trimming during slaughter. Hence, high dietary fat content may increase adiposity and do not primarily enlarge the carcass weight, that is the main edible product for sale and holds the most value. Although, it is possible to utilize the viscera for oil extraction and/or native protein and hydrolysates (Villamil et al., 2017; Wu and Bechtel, 2008), salmon feeding strategies should be optimized to maximize the carcass production.

The nutrient retention gives a measure of the proportion of the dietary nutrient that is retained in the fish. Simplified, there is generally an inverse relationship between the dietary inclusion rate of protein and lipid, and the retention efficiency of these nutrient in salmon (Bendiksen et al., 2003; Einen and Roem, 1997; Hillestad et al., 1998). Thus, reducing the P/L ratio can increase the retention efficiency of protein and facilitate a favorable "protein sparing" effect (Azevedo et al., 2004; Hillestad et al., 1998; Johansen et al., 2003; Karalazos et al., 2011, 2007), whereas the retention of lipid can potentially decrease to some extent (Weihe et al., 2018). It should be noted that lipids, unlike energy and protein (as N x 6.25) can be synthesized *de novo* by the fish. The term "lipid retention" should, therefore be used with care. However, it is a useful tool to express dietary or seasonal variations in energy deposition. In Paper **I,** the apparent nutrient retention efficiency of protein, lipid and energy was measured based on whole body composition (referred to as relative nutrient retention). The releative retention of lipid was low from April to June (25-28%), intermediate from June to July (45-50%) and high from July to September (67-75%). Generally, the lipid and energy retention increased with increasing feed intake, growth, weight gain and dietary energy level. These seasonal patterns are consistent with the observations from Alne et al. (2011). In their study, S1 post-smolt had lower lipid retention during the spring (∼20%) compared to the autumn (∼60%) and this coincided with low and high growth rates, respectively. In general, energy retention increases with increased feed intake and growth, because the relative proportion of the eaten energy used for maintenance is reduced, as shown for salmon by Helland et al. (2010). The relationship between growth and retention is even more marked for lipids, since fish eating at a high rate will deposit surplus energy as body fat stores. Accordingly in Paper **I**, the retention of protein was reasonably stable (at approximately 50%) and less dynamic than the retention of lipid, as previously reported

(Alne et al., 2011; Nordgarden et al., 2003b). This can be explained by the relatively stable body content of protein compared to that of lipid, which vary largely with energy supply in salmonids. This is also shown by assessing the whole body nutrient composition of the dietary groups during the experiment described in Paper **I**.

The nutrient retention efficiency (referred to as in Paper **I** as relative nutrient retention) expresses utilization efficiency and do not show how much of the nutrient that is deposited in the fish from the diet, when diets that differ in protein and lipid content are compared. To illustrate this in a simple manner, the retention of the nutrients from the feed were calculated in Paper **I** and expressed in grams (referred to as absolute nutrient retention). The increase in P/L ratio was not synonymous with reduced retention of protein in Paper **I**. No significant differences in relative protein retention were detected between the dietary treatments during the initial and latter stages of the trial. However, a significantly lower protein retention among the high P/L ratio group compared to the low P/L-ratio group was detected from June to July. In the periods when there were no differences in relative protein retention, the fish fed high the P/L ratio had significantly increased absolute protein retention. Thus, the fish fed the high P/L ratio retained more protein from the diet compared to the fish fed the low P/L ratio. This indicates that the increased protein content in the high P/L ratio diets was efficiently utilized for growth and weight gain. In the last period of the trial, the increased absolute protein retention in the high P/L group could be explained by a combination of increased whole body protein content, significantly improved feed intake, growth and weight gain compared to the low P/L ratio group. This was also accompanied by higher condition factor and carcass yield. The relative and absolute retention of lipid was lower in the high compared to the low P/L ratio group in the period from June to July. This was reflected in a significantly lower whole body lipid and energy content within the group fed the high compared to the low P/L ratio in the end of July. Hence, a large part of the dietary lipid content was used for energy production and less for storage in the high P/L ratio group. The period from June to July has previously been identified as a period when S1 salmon smolt have low feed intake, growth and lipid retention (Alne et al., 2011; Rørvik et al., 2007). From July to September, a significantly higher lipid retention was observed for the high compared with the low P/L ratio group. However, no significant differences were detected in absolute lipid retention during the period from July to September.

Thus, similar amount of lipid were retained in the two dietary groups, but the difference in dietary lipid content resulted in a significant different relative lipid retention. The increased dietary P/L-ratio did therefore not improve lipid retention during the initial stages of the trial, whereas in the last period the dietary lipid was more efficiently utilized in the high P/L ratio group.

Protein is usually the most expensive component of fish feed. It is therefore important that the requirement of this nutrient is accurately determined for each life stage and that the protein inputs are efficiently utilized. From a sustainability perspective, the similar relative and increased absolute retention of protein for the high P/L ratio group is positive. This shows that the dietary protein is efficiently utilized for somatic growth, and that salmon at this size and stage of production is in need of a high P/L ratio for optimal growth performance. In Paper **II**, the FCR and nutrient retention of the different groups were not obtained due to the mixing of the different groups in the net-pens. This was unfortunate as assessments of feed utilization and nutrient retention during compensatory or lipostatic growth responses in salmonids are scarce. However, some previous studies show that the increased growth and weight gain shown by salmonids during the compensatory phase is achieved by hyperphagia, rather than improved feed conversion (Johansen et al., 2003; Nikki et al., 2004).

## 7.5 Dietary protein-to-lipid ratio and health

The first spring in sea for S0 and S1 Atlantic salmon is an energy-demanding period associated with a high risk for outbreaks of viral diseases such as IPN, HSMI and PD (Alne et al., 2011, 2009; Hjeltnes et al., 2017; Rørvik et al., 2007). PD can occur throughout the annual cycle, but the risk of outbreaks is highest during the spring and summer months with increasing day length and sea temperature (Hjeltnes et al., 2017; McLoughlin and Graham, 2007; Rodger and Mitchell, 2007; Stene et al., 2014). Historically, June to July is the period with the highest reported number of PD positive diagnoses among commercial salmonid fish farms along the Norwegian coastline (Hjeltnes et al., 2017). In Paper **III**, the effects of increased dietary P/L ratio for S0 salmon on mortality rates, biometric and quality related parameters during the entire grow-out period in sea, within the SAV3 endemic zone was evaluated. The study was

designed to test a diet with a marked increase in P/L ratio during the spring and summer months when the risk of viral disease outbreaks is high. The contrast group (low P/L group) was fed a typical standard diet with 35% protein and 35% fat, versus the high P/L group that was fed a diet with 47% protein and 24% fat content. During the first summer at sea, a co-infection of SAV3 and PRV was detected and a natural PD outbreak was observed. The increased dietary P/L ratio improved survival during the natural outbreak of PD. In addition to diet, body weight and delousing mortality (indicating induced stress) prior to the PD outbreak was also found to contribute significantly to explain the observed variation in PD associated mortality. The high P/L group had a mean mortality rate of 1.9 %, whereas the low P/L group had a mean mortality rate of 3.7 %. Subsequent to the PD outbreak, a large amount of fish failed to grow and caused an accumulation of runts (severely thin diseased fish). At the end of the trial, a significantly lower amounts of runt fish was detected among fish fed the increased P/L ratio and among the fish with the largest body weight prior to PD.

The first winter in sea has also been identified as a challenging period for S1 salmon, associated with low temperature, little day light, reduced lipid deposition, lowered growth, feed intake and conversion (Mørkøre and Rørvik, 2001). In Paper **IV**, an event of sudden mortality of seemingly healthy farmed salmon during the winter period in northern Norway is reported. The experimental fish were reared in four net-pens and two dietary treatments were established; a high or low P/L ratio diets. An increased mortality of 6 and 10% was only observed within the two net-pens receiving the high P/L ratio experimental diets, following an abrupt reduction in dietary P/L ratio when the fish went from 7 to 10 mm diets a few weeks before. The moribund/dying fish had significantly higher lipid content in the liver, altered hepatic fatty acid composition, and increased levels of alanine aminotransferase (ALT) and alkaline phosphatase (AP) in the blood plasma compared to non-dying fish, indicating impaired hepatic function. A significant and instant reduction in mortality was observed when the high P/L ratio group were starved. After about 3 weeks of starvation, feeding was resumed (with a lower dietary fat content) and no further mortality was observed.

Diseases in fish are often identified as loss of immuno-competence/defense and the ability to resist this loss is often defined as disease resistance. Viral diseases such as HSMI, CMS and PD may all cause epicarditis and myocarditis, whereas HSMI and PD also lead to skeletal muscle inflammation and degeneration (R T Kongtorp et al., 2004). Thus, viral diseases cause severe inflammatory responses in salmon and have distinct similarities. The dietary supplementation with n-3 poly unsaturated fatty acids (PUFA) is generally beneficial for the health of marine fish and other species through the modulation of both inflammation and immune cell function (Calder, 2001; Montero et al., 2003). In Paper **III**, the two dietary treatments used during the natural outbreak of PD had identical premixes of vitamins and similar levels of PUFA (percentage of total FA). Thus, the high dietary P/L ratio and reduced energy content alone seemed to increase the tolerance and/or resistance towards PD in this study (PRV was also present). In previous studies, diets with reduced lipid content, and increased PUFA and phospholipids content, affected the innate immune response, reduced inflammatory responses, associated heart lesions, and hepatic steatosis in salmon experimentally challenged with ASRV and PMCV virus (Martinez-Rubio et al., 2012; 2013, 2014). In these studies, transcriptomic analysis using microarray showed that gene expression correlated with the innate immune response and reduced histopathological scores and lowered viral load, which again were associated with altered tissue fatty acids composition (in heart, kidney, and liver) and eicosanoid metabolism. The reduced lipid content combined with high PUFA and PL levels made it difficult to separate the dietary causative effects in these trials. However, in another study conducted by the same research group, reductions of dietary lipid and energy content alone showed to alter the expression of key genes involved in lipid metabolism in the liver, resulting in an up-regulation of biosynthetic pathways relevant for eicosanoid metabolism and immune responses (Martinez-Rubio et al., 2013b). Whether increased dietary P/L ratio and lowered energy content facilitates positive effects on lipid metabolism, immune and/or inflammatory responses in large salmon during natural PD outbreaks need to be elucidated. In Paper **IV**, the moribund/dying fish had indications of impaired hepatic function and PRV was detected in the heart tissue. Thus, the impaired hepatic function may have reduced the ability to resist the long-term inflammation associated with PRV and/or HSMI among the group given high P/L ratio. However, this theory is weakened

by the detection of the same PRV virus load and no differences in histopathological scores between normal living fish and moribund/dying fish.

Hjeltnes et al. (2017) refers to PD as a typical stress-related disease and states that sub-clinical SAV3 infections may develop into severe outbreaks after lice treatments with increased mortality rates. In addition to diet, the mortality after delousing and body weight prior to the PD outbreak were important variables for explaining the observed variation in PD associated mortality in Paper **III**. The fact that these variables contributed significantly in the statistical model imply that PD associated mortality is influenced by several different factors. The outbreaks of many disease conditions occur as the result of intricate interactions between environmental conditions, metabolic distress, nutritional imbalances and the presence of pathogens, combined with effects of site management and handling stress (Contessi et al., 2006; Wheatley et al., 1995). In Paper **III**, the induced handling stress due to delousing may have lowered the immune-competence of the salmon and made it more susceptible for PD. Regarding the effect of body weight; large salmon has usually more mass and stored energy than smaller salmon. If the amount of stored energy available is of importance for combating viral diseases, this could be a contributing factor for the increased survival among large compared to small salmon. No handling or delousing was conducted prior or during the mortality event observed in Paper **IV**. Hence, the observed mortality in this study can therefore be assigned to dietary and environmental changes.

The significant reduction in runts of the groups fed the high P/L ratio and with increased body is an important observation in Paper **III**. Runts represents a severe problem and animal welfare issue in salmon aquaculture and measures that can reduced the amount of runts are highly needed. PD causes loss of exocrine pancreatic tissue and pancreatic necrosis (McLoughlin et al., 2002), reducing the ability to a digest and absorb nutrients from the feed. High intake of dietary lipids and saturated FAs are associated with metabolic syndrome, inflammation, impaired anti-inflammatory properties of high-density lipoprotein and endothelial function in mammals (Johnson et al., 2008; Kien et al., 2005; Nicholls et al., 2006). In addition, lipid digestion for individuals with pancreatitis are reduced due to insufficient pancreatic lipase secretion, reduced concentrations of bile acids and bicarbonate section (Meier and Beglinger, 2006).

Protein digestion, on the other hand, is initiated by intragastric proteolytic enzymes and can be sustained by intestinal brush-boarder peptidases even in the lack of pancreatic proteolytic activity (DiMagno et al., 1975; Meier and Beglinger, 2006). Experimental induced pancreatitis in adult dogs has shown to be intensified by a high-fat diet (Haig, 1970), and feeding high-fat diets to rats with hyperlipidemia induce pancreatic injuries and oxidative stress (Yan et al., 2006). Increased dietary fat seems therefore to act as a stressor during periods with reduced pancreatic function, whereas dietary protein can be digested to some extent. Whether this is the case for salmon remains to be elucidated. However, if some of these factors apply for salmon, they can be potential reasons for the observed differences in mortality, number of runts and weight gain between the dietary groups in Paper **III**.

PD has shown to impair fillet quality by reducing the CF, fillet color, pigment and fat content in the muscle, in addition to induce hardening of the fillet texture (Larsson et al., 2012; Lerfall et al., 2012; Taksdal et al., 2012). In Paper **III**, the groups fed low P/L-ratio had lower CF, astaxanthin in muscle and harder texture after the PD outbreak, compared to fish of similar weight fed high P/L-ratio. This observation can be related to several different factors: 1) Reduced ability to absorb nutrients from the feed. 2) Increased oxidative stress due to severe impact of the disease, and/or higher intake of FAs that are more susceptible to oxidation, resulting in lowered pigment content. 3) Higher degree of collagenous scar tissue, fibrosis and/or skeletal muscle lesions/inflammation, resulting in increased fillet texture (Larsson et al., 2012; Lerfall et al., 2012). At slaughter, no significant differences in fillet pigment, texture or body shape were detected between the dietary groups. This is line with previous observations of Lerfall et al. (2012), showing that salmon with significantly altered fillet quality can to a large extent recover after a natural outbreak of PD. In Paper **III**, a significant negative linear relationship was observed between the amount of superior graded fish and the total mortality, whereas a positive linear relationship was detected between the percentage of fillets with melanin and the total mortality. The degree of quality deviation correlated to the severity of PD measured as cumulative mortality and amount of runts. Thus, increased dietary P/L ratio seemed to reduce the mortality and impaired slaughter quality associated with PD.

In Paper **IV**, the VSI of the fish fed the low P/L ratio was significantly higher compared to the high P/L ratio group prior to the acute mortality, indicating increased visceral fat deposition. Adipose tissue achieves the safe storage of lipids by increasing the recruitment of new adipocytes (hyperplasia) and/or the expansion of the existent ones (hypertrophy) (Otto and Lane, 2005). The expandability capacity of adipose tissue has a limit, and sustained energy overload may lead to the deposition of lipids in non-adipose tissues, leading to lipotoxicity and inflammation in mammals (Carobbio et al., 2017; Solinas et al., 2015; Unger et al., 2010). In Paper **IV**, it is discussed that the use of a low-fat diet during a period where salmon is known to increase the amount of visceral adipose tissue may have impaired the recruitment capacity of adipocytes. Thus, the capacity of this fish to accommodate the excess of energy when suddenly increasing the dietary lipid content could be challenged. As a result, the excess of energy would be stored in other organs, such as liver and heart, contributing to a low-grade inflammatory state and an increased risk of cardiometabolic diseases. However, the understanding of factors orchestrating salmon fat distribution and adipocyte recruitment is scarce and these factors need further research for deeper understanding of basic mechanisms and consequences of fat recruitment and accumulation in different tissues to ensure optimal health.

# 8. Concluding remarks

The results from this thesis show that alterations in dietary protein-to-lipid ratio have profound effects on growth, lipid deposition, nutrient retention and health of Atlantic salmon. Alterations in body lipid levels affect feed intake and growth in salmon, possibly through lipostatic regulation. The results show that salmon is able to replenish lipid stores rapidly after dietary lipid restriction, and that lipid deposition in the muscle is high during the autumn. Dietary P/L-ratio of 1.12 (DP/DE of 15.2 g MJ kg$^{-1}$) was below the P/L requirement for optimal growth during the early seawater phase for S1 salmon (from 0.1 to 1 kg), and the effects of altered dietary P/L ratio should be evaluated by taking into account the size of the fish and the environmental conditions. This result is in line with previous studies, although now verified using isoenergetic diets under ambient environmental conditions. The increased protein content in the high P/L ratio diet was efficiently utilized for growth and weight gain during the early seawater phase for S1 salmon. The reduced P/L ratio during this stage did therefore not result in protein sparing effect, but increased fat accumulation and adiposity. This reduced carcass yields and led to a significantly lower feed conversion based on gutted weight.

Feeding a high protein-to-lipid diet improved survival, reduced number of runts and impaired slaughter quality during a natural outbreak of PD, compared to a regular high-fat diet. In addition to diet, delousing mortality and body weight prior to the PD outbreak were important statistical variables for explaining the observed variation in mortality, number of runts and deviating slaughter quality. Reducing PD associated mortality, deviating slaughter quality, and runts leads to a higher tradeable biomass for sale, increased proportion of superior fish and improved animal welfare. Considering that PD is widespread disease with high associated mortality, the potential of using a dynamic dietary protein-to-lipid strategy for improving the production efficiency in PD affected areas is high.

Sudden increased mortality of large seemingly healthy farmed salmon during the winter period in the northern Norway was linked to abrupt dietary change from low to high lipid level. It is suggested that this change may have triggered a dysregulation in lipid metabolism, indicated by a higher lipid content in the liver, altered hepatic fatty acid

composition, and increased levels of ALT and AP in the blood plasma among moribund/dying fish.

As far as the results from the present thesis can be converted to practical advice for the salmon farmers, the following points can be made:

- S1 salmon require high dietary energy and protein content during the early seawater phase for optimal growth performance
- Increased dietary protein-to-lipid ratio reduce adiposity, increase growth and carcass yield
- Increased dietary protein-to-lipid ratio could be utilized during the first spring and summer in sea for S0 salmon for sites with high risk of PD to improve survival and reduce the accumulation of runts.

# 9. Future perspectives

The aquaculture industry is in rapid growth and is general investing and building large recirculation aquaculture system (RAS) both for production of larger smolt and rearing of slaughter-sized salmon on land. RAS enables the use high water temperature and the control over photoperiod compared to traditional flow through systems, and this can significantly increase feed intake and growth of salmonids. The use of high fat diets in these systems will most probably lead to increased fat accumulation and adiposity during the rearing and prior to sea transfer, which can reduce growth and increase the risk of early sexual maturation among males. Thus, the use of different dietary strategies together with photoperiodic manipulation in semi-closed and closed RAS production units is a field that needs to be explored and addressed in future research. Although the dietary protein-to-lipid ratio used to alter the body lipid content in Paper **II** was high, it illustrates an important principle related to fat deposition and subsequent growth that is crucial knowledge when developing new dietary concepts. Thus, detecting periods with high risk of excessive lipid accumulation using high-fat diets should be of future scientific interest in order to develop optimal feeding strategies in commercial fish farming.

The increased survival during natural occurring PD by using high a protein-to-lipid ratio is an important observation in the present thesis. HSMI and CMS has similarities to PD and the influence of dietary protein-to-lipid on these diseases should be investigated. Conducting research in commercial scale trials can detect factors that affect mortality during natural disease outbreaks, such as operating practices and environmental conditions. Future trials should focus on obtaining such relevant data to increase understanding of the interaction between biology and production.

The feed industry has been replacing fishmeal with other protein alternative of vegetable origin (i.e. soy protein concentrate, sunflower and pea/bean protein) gradually over the last decade. It is assumed that this trend will continue in the future due to limited and static supply of fishmeal. Protein of vegetable origin is often of lower quality and less dens compared to fishmeal. The high demand of quality protein for

direct human consumption and other livestock may also limit the future supply of protein sources to the industry. Thus, the use of high protein-to-lipid diets in the future may be challenging and difficult. This stresses the need for future research and development of obtaining new sustainable protein sources for aquaculture. In addition, future trials using a larger variety of diets with different protein-to-lipid ratios with more vegetable protein sources can give useful information, especially during the post-smolt stage.

Future research will benefit from additional molecular techniques evaluating the level of expression different genes, such as microarray. This would have provided useful information about different metabolic processes. This could have been used as a supplementary tool to increase the understanding about factors regulating feed intake, growth, nutrient utilization, lipid metabolism and fish health. In particular, new insights of mechanisms regulating the fat recruitment and deposition in different tissues are need to ensure optimal health. In-depth immune-histopathological investigations of relevant organs and with more individuals/units could also be used as key tool in future research. The mentioned analytical tools may give essential information when tissues that are involved in immune and inflammatory responses such as head kidney, spleen, heart, liver and adipose tissue are investigated. Further studies on metabolic distress and nutritional aspects of viral infection in farmed Atlantic salmon using these techniques is needed to understand the etiology of these diseases, so that relevant prophylactic measures can be introduced in large-scale production.

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# Paper I

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# Different growth performance, lipid deposition, and nutrient utilization in in-season (S1) Atlantic salmon post-smolt fed isoenergetic diets differing in protein-to-lipid ratio

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## ARTICLE INFO

## ABSTRACT

The aim of the present study was to evaluate how isoenergetic diets with different protein-to-lipid ratio affects feed intake, growth performance, lipid deposition, feed and nutrient utilization in Atlantic salmon post-smolt. A 6-month's feeding trial was conducted with in-season (S1) Atlantic salmon post-smolt reared in the sea under natural conditions (May–September). Quadruple groups of salmon (initial weight 95 g) were fed two isoenergetic diet series formulated to contain a high (HP) and low (LP) protein-to-lipid ratio designed to resemble upper and lower levels of ratios used in commercial feeds. The group fed the HP diet had a significantly ($P \leq 0.05$) lower muscle fat content (HP = 4.7%, LP = 5.7%), whole body lipid (HP = 9.0%, LP = 9.6%) and energy content (HP = 7.7 MJ kg$^{-1}$, LP = 8.0 MJ kg$^{-1}$) than the group fed the LP diet after the period June–July. These differences were mainly due to significantly lower absolute apparent lipid retention in the summer period for post-smolt fed HP diet. In the subsequent experimental period (July–September), a significantly higher specific feed intake (HP = 1.38%, LP = 1.33%), thermal growth coefficient (HP = 3.82, LP = 3.46) and weight gain (HP = 658 g, LP = 552 g) were observed for fish fed the HP diet. The period from July–September was associated with high water temperatures and declining day length. The reduced feed intake in the LP group coincided with increased visceral mass and lipid deposition, indicating a possible involvement of lipostatic regulation. The retention efficiency of nutrients increased with the up-regulation in feed consumption. The HP fed fish had a significantly higher whole body lipid retention (HP = 74.4%, LP = 67.2%), but significantly reduced visceral mass compared to LP fed fish during the autumn. The overall improved growth, good protein utilization and reduced visceral adiposity among the HP fed fish resulted in significantly improved final condition factor (HP = 1.46, LP = 1.40), carcass yield (HP = 86.0%, LP = 84.1%), feed conversion based on gutted weight (HP = 0.98, LP = 0.93) and whole body protein (HP = 17.6%, LP = 16.9%). The present study reveals that low dietary protein-to-lipid ratios for salmon post-smolt may negatively affect production parameters, although digestible energy contents in the diets are similar.

*Statement of relevance:* The present study confirms the importance of balanced dietary lipid-to-protein ratios for optimal production efficiency and nutrient utilization, and the significant effects of dietary and seasonal interaction on lipid deposition and production related parameters. To our knowledge, few have investigated the effect of isoenergetic diets differing in protein-to-lipid ratio on growth performance and nutrient utilization of juvenile Atlantic salmon reared in seawater under natural conditions. The experiment used feed formulations, fish breed and rearing conditions relevant for current commercial salmon farming practices.

Considering the current increase in the cost of lipid sources, it would be beneficial for the aquaculture industry if dietary lipid content could be reduced without compromising growth and feed utilization of the fish. We believe our findings will provide useful and relevant information regarding dietary formulations and nutritional knowledge for the global fish feed industry and salmon producers.

## 1. Introduction

The majority of Atlantic salmon (*Salmo salar* L.) is farmed in open sea pens that are exposed to seasonal variations in environmental

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conditions. Important production parameters such as appetite, feed utilization and growth rate are modulated by temperature and photoperiod, and by a wide range of other internal and external factors such as genetics, health status, adiposity, water quality, fish size, dietary composition and feeding regime (Austreng et al., 1987; Bendiksen et al., 2003a; Bendiksen et al., 2003b; Einen and Roem, 1997; Gjedrem, 2000; Gjøen and Bentsen, 1997; Hillestad et al., 1998; Jobling and Johansen, 1999; Johansen and Jobling, 1998; Sveier and Lied, 1998; Thodesen et al., 1999; Thorarensen and Farrell, 2011). Farmed salmon in the mid-west part of Norway encounter periods with low feed intake, decreased growth rate, low lipid retention and the depletion of energy stores during their first spring in the sea (Alne et al., 2011). In contrast, the salmon experience high feed intake, rapid growth, and altered deposition and retention of lipids during the late summer and early autumn (Alne et al., 2011; Hemre and Sandnes, 2008; Mørkøre and Rørvik, 2001; Måsøval et al., 1994; Oehme et al., 2010). This phenomena seems to occur both for smolt transferred to the sea during the autumn and for those transferred during the spring (Alne et al., 2011), which suggests that salmon have a seasonal growth pattern that is triggered by external photoperiodic information. Thus, season-specific signals and internal factors induce metabolic changes in salmon that significantly affect the production efficiency in natural environments.

The minimum requirements of salmonids for protein, amino acids and energy have been partly established (NRC, 2011; Wilson, 2002). Juvenile salmonids undergoing rapid body growth require a higher portion of digestible protein than larger salmonids (Cho and Kaushik, 1990; Einen and Roem, 1997; Grisdale-Helland et al., 2013b), which use large amounts of the dietary energy for maintenance (Azevedo et al., 2004a, 2004b; Jobling, 1994). However, sufficient dietary energy is important to ensure optimal feed utilization (Hillestad and Johnsen, 1994; Hillestad et al., 1998). Several studies do not detect significant differences in growth performance between groups of salmon fed diets varying in protein/lipid ratio (Azevedo et al., 2004b; Hillestad and Johnsen, 1994; Hillestad et al., 1998; Karalazos et al., 2007; Karalazos et al., 2011). In particular, studies using isoenergetic grower diets identified no negative influence of low protein/lipid ratio on growth performance or feed utilization, but a favorable protein sparing effect (Karalazos et al., 2007; Karalazos et al., 2011). These observations imply that salmon have high ability to utilize large amounts of lipids in high-energy diets efficiently for growth. The above mentioned factors together with the fact that lipid has historically been a cheap source of energy compared to protein, have lead the industry to reduce the amount of protein and increase the lipid content in the diets (Torrissen et al., 2011). Consequently, the dietary protein/lipid ratio in modern diets is thus lower compared with the traditional diets for salmonids. However, high demand of lipids and competitive pressure from competing industries, including direct human consumption, has increased the cost of lipids. Nutritional knowledge, raw material availability and world markets are under constant change and development, and thus, cost-effective and sustainable salmon production requires optimal utilization of both protein and lipids.

Most studies examining different dietary protein-to-lipid concentrations for salmon use non-isoenergetic diets (Einen and Roem, 1997; Grisdale-Helland and Helland, 1997), although several adjusted the dietary ration level so that the diets tested were fed isonitrogenously or isoenergetically (Hillestad and Johnsen, 1994; Hillestad et al., 1998). In addition, some studies indicate that salmonids are able to adjust their feed consumption according to the dietary energy level (Bendiksen et al., 2002; Boujard and Medale, 1994). As a result, this may complicate the direct comparisons among studies. To our knowledge, few have investigated the effect of isoenergetic diets differing in protein-to-lipid ratio fed ad-libitum on growth performance of juvenile salmon (0.1–1 kg) reared in seawater under natural conditions. In-house laboratory studies with constant light and temperature or short-term experiments may disregard the vital impact of seasonal environmental variations that influence the growth pattern.

Salmon increase the deposition of muscle fat and visceral adiposity as the fat content in the feed increases (Bendiksen et al., 2003a, 2003b; Einen and Roem, 1997; Hillestad et al., 1998; Jobling et al., 2002a). The carcass yield consequently decreases (Hillestad et al., 1998). Increased amount of lipid deposition correlates with decreased feed intake in salmonids (Jobling and Johansen, 1999; Jobling et al., 2002b; Johansen et al., 2002; Johansen et al., 2003; Shearer et al., 1997a; Shearer et al., 1997b; Silverstein et al., 1999). This finding is consistent with the lipostatic regulatory hypothesis (Jobling and Johansen, 1999; Keesey and Corbett, 1984; Kennedy, 1953; Schwartz and Seeley, 1997), which suggests that the amount of stored fat is an important regulator of energy intake and the homeostasis of adiposity. The hypothesis suggests that adipose tissue exerts a negative feedback control on appetite and feed consumption in fish. There is, thus, a risk of impaired growth as lipid deposition becomes excessive.

In view of the above-mentioned studies, it can be assumed that a diet with a low lipid level but with sufficient energy content, (i.e. increasing the dietary protein/lipid ratio), is an effective approach to reduce the deposition of lipids and enhance feed intake. This may be especially prominent for S1 juvenile salmon the first autumn in sea, since this period is associated with rapid growth and, elevated deposition and retention of lipids (Alne et al., 2011; Hemre and Sandnes, 2008; Mørkøre and Rørvik, 2001; Måsøval et al., 1994). However, excessive dietary protein or lipids, may lead to increased catabolism of the dietary nutrients and reduce the retention efficiency of protein and lipids, respectively (Kaczanowski and Beamish, 1996; Refstie et al., 2001; Walton et al., 1984).

During a five month period after sea transfer, the present study test the hypothesis that increased dietary protein-to-lipid ratio improves the feed intake and growth of S1 Atlantic salmon, compared to lower dietary protein-to-lipid ratio (using commercially formulated ratios). The dietary and seasonal effects on lipid deposition, feed conversion, whole body composition, nutrient retention, body shape and carcass yield were assessed.

## 2. Materials and methods

### 2.1. Experimental diets

The diets used in the study were manufactured by Havsbrún (Fuglafjørður, Faroe Islands) by extrusion and vacuum coating with oil. Two diet series that differed in protein/lipid ratio, but were isoenergetic with respect to digestible energy (DE), were formulated. Diets were produced as 3, 4 and 6 mm pellets according to fish size. The ingredients used and the compositions of macronutrients in diets for pellets of each given size are shown in Table 1. The approximate chemical compositions of the diets are shown in Table 2. The high-protein diet series (HP) had a higher content of protein and a lower content of lipid than the low-protein diet series (LP). The formulations were

**Table 1**
Formulation (g kg$^{-1}$) of the experimental diets.

| Pellet size | 3 mm | | 4 mm | | 6 mm | |
|---|---|---|---|---|---|---|
| Diet code | LP | HP | LP | HP | LP | HP |
| *Formulation, (g kg$^{-1}$)* | | | | | | |
| Micro ingredients[a] | 25 | 25 | 25 | 25 | 15 | 15 |
| Wheat | 119 | 105 | 138 | 100 | 140 | 125 |
| Wheat gluten | 20 | 58 | 20 | 63 | 28 | 69 |
| Soy protein concentrate | 38 | 26 | 15 | 61 | 56 | 45 |
| Fish meal | 492 | 531 | 520 | 511 | 387 | 425 |
| Krill meal | 55 | 55 | 15 | 15 | - | - |
| Porcine blood meal | - | - | - | - | 45 | 30 |
| Fish oil | 110 | 95 | 127 | 116 | 151 | 136 |
| Rapeseed oil | 110 | 95 | 127 | 116 | 151 | 136 |
| Pigment[b] (mg kg$^{-1}$) | 50 | 50 | 50 | 50 | 50 | 50 |

[a] Vitamin and mineral premixes.
[b] Astaxanthin.

**Table 2**
Approximate chemical compositions (g kg$^{-1}$) of the diets.

| Pellet size | 3 mm | | 4 mm | | 6 mm | |
|---|---|---|---|---|---|---|
| Diet code | LP | HP | LP | HP | LP | HP |
| *Chemical composition, (g kg$^{-1}$)* | | | | | | |
| Crude protein ($N \times 6.25$) | 444 | 483 | 413 | 452 | 390 | 441 |
| Crude lipid | 286 | 260 | 328 | 285 | 347 | 316 |
| Ash | 89 | 94 | 85 | 90 | 55 | 58 |
| Water | 71 | 73 | 64 | 79 | 62 | 62 |
| Crude fiber | 1.6 | 1.2 | 0.8 | 0.7 | 1.1 | 1.0 |
| Total starch | 73 | 73 | 77 | 69 | 101 | 88 |
| NFE[a] | 108.4 | 88.8 | 109 | 93 | 145 | 122 |
| Gross energy, (MJ kg$^{-1}$) | 23.8 | 23.3 | 24.4 | 23.4 | 25.2 | 24.9 |
| Crude protein/lipid ratio | 1.55 | 1.86 | 1.26 | 1.59 | 1.12 | 1.40 |
| *Digestibility calculations[b]* | | | | | | |
| Calculated DP, (g kg$^{-1}$) | 382 | 415 | 355 | 389 | 335 | 379 |
| Calculated DE, (MJ kg$^{-1}$) | 20.6 | 20.3 | 21.5 | 20.6 | 22.1 | 21.8 |
| Estimated DP/DE ratio (g MJ$^{-1}$) | 18.5 | 20.5 | 16.5 | 18.9 | 15.2 | 17.4 |

[a] NFE = Nitrogen-free extracts = 1000 − (protein + lipids + ash + fiber + water).
[b] The amounts of digestible protein (DP) and digestible energy (DE) were estimated assuming 23.7, 39.5 and 17.2 MJ kg$^{-1}$ as the gross energy content of protein, lipids and carbohydrates, respectively. The apparent digestibility coefficients (ADCs) for protein and lipids used were 0.86 and 0.94, respectively (Einen and Roem, 1997), whereas 0.50 was used for NFE (Arnesen and Krogdahl, 1993).

designed to resemble high and low protein-to-lipid ratios of commercial feeds used for salmon. The level of protein was decreased whereas the level of lipid was increased with the increase in pellet size, in accordance with commercial feed formulations. This upregulated the total energy level in order to account for the increase in fish weight. The difference in crude protein content (~40 g kg$^{-1}$) between the experimental diets was kept constant within all the pellet sizes, and the lipid level was adjusted to obtain equal levels of DE. The feed batches were stored in a refrigerated room (4 °C) and the amounts of feed corresponding to one-week consumption was taken out and kept in boxes at room temperature. Feed samples were taken on arrival from the manufacturer and stored frozen (−20 °C) until they were analyzed as described below. The diets were formulated to meet the NRC nutritional recommendations for salmonid fish (NRC, 2011).

### 2.2. Fish, rearing conditions and experimental design

On the 29 March 2012, 8000 S1 Atlantic salmon smolt from the Rauma strain (Rauma Broodstock AS, Sjøholt, Norway) were sorted out, weighed in bulk and distributed among eight tanks with 1000 fish in each, on a truck at the Straumsnes Settefisk AS hatchery at Tingvoll, Norway. The smolts were visually examined and individuals with similar size were selected and weighted in bulk. Fish with obvious signs of wounds, parr-marks or runts were removed. The fish were then transferred to Marine Harvest research station (former Nofima) at Ekkilsøy (63°03′N, 7°35′E) on the west coast of Norway during the same day. On arrival, fish from each tank on the truck were allocated to one of eight pens (5 × 5 × 5 m, 125 m³ volume). The smoltification status was checked by conducting a seawater challenge test, followed by determination of plasma osmolality, chloride content and gill Na$^+$,K$^+$-ATPase activity (Clarke et al., 1996), before the fish were exposed to seawater. The mean initial body weight of the smolt was 95.1 ± 0.2 g (mean ± SD). Each pen was assigned to one of two dietary groups in a randomized block design of quadruple net pens.

The eight pens were initially illuminated by four submerged 400 W light sources, 24 h day$^{-1}$. This was done in order to promote schooling behavior and avoid physical contact with the net wall in the pens. The submerged lights were removed on 29 May, and the salmon were subsequently exposed to the natural photoperiod until the feeding trial ended on 25 September 2012 (Fig. 1A). Daylight hours were defined as the period from twilight in the morning until the center of the sun was 6° below the horizon in the evening, referred to as civil twilight (data obtained from the website; www.timeanddate.no). The

experiment was divided into three periods: April–June (spring), June–July (summer) and July–September (autumn) (Table 2). The periods were adjusted to fit with the guidelines of the feed manufacturer with respect to pellet sizes, which have been determined according to the weight of fish (Table 3). The ambient seawater temperature and oxygen level were recorded daily at a depth of 3 m. The seawater temperature at transfer was 6 °C, and it increased to a maximum of 15 °C in late August. The average for the complete trial was 9.8 °C (Fig. 1A). The average temperatures for the three periods were: 7.5 °C in April–June, 11.5 °C in June–July and 13.6 °C in July–September. The oxygen level decreased with increasing water temperature, and ranged from 12.8 to 7.2 mg l$^{-1}$, with an average of 9.8 mg l$^{-1}$ (Fig. 1B).

### 2.3. Feed-monitoring system and feed administration

The feed-monitoring system used in the trial was established by combining the methods described by Einen et al. (1999) and Helland et al. (1996). Feed was administered by automatic feeders (Betten Maskinstasjon AS, Vågland, Norway) and uneaten pellets were collected in a plastic funnel at the bottom of each net pen. The uneaten feed was pumped up into wire mesh sieves through a plastic pipe using pressurized air. The uneaten feed was collected after each meal and quantified each day, in order to determine the daily feed intake and feed conversion ratio accurately. The daily feed intake was calculated as described by Helland et al. (1996). All feeds were tested for the recovery of dry matter in empty net pens after the trial. The fish were fed to satiation (four times a day), and the feed ration was set such that they received approximately 10–20% more than the estimated daily feed intake. Adjustments of the feed ration were done according to the amount of uneaten feed collected.

### 2.4. Weighing and sampling procedures

All fish were counted and weighed in bulk at the end of each feeding period. The fish were collected from each experimental pen using a fish-landing net and anesthetized in batches with MS-222 (Metacaine 0.1 g l$^{-1}$; Alpharma, Animal Health Ltd., Hampshire, UK) in a 1000-l tank of fresh seawater. All fish with obvious signs of wounds, runts or sexual maturation were removed and killed during the weighing procedure (the weights and numbers of such fish were recorded). An initial sample of 30 fish (three pooled samples with 10 fish in each) was taken before sea transfer, and 10 fish from each pen were sampled (sampled fish presented a mean body weight corresponding to the mean weight of all fish in the net pen) at the end of each feeding period. Sampled fish were anesthetized in MS-222 and then killed by a blow to the head. The gill arches were cut and the fish were bled out in ice water. The fish were subsequently transported to the processing hall nearby, where individual weights and fork lengths were measured. The fish were then cut open, sex was determined by inspection of the gonads, and visceral fat was assessed visually on a score from 1 to 5 according to the visibility of the pyloric caeca (1 = clearly visible, 2 = visible, 3 = visible through cracks 4 = visible through the fat, 5 = not visible). The viscera (including the spleen) and the liver were dissected and weighed, in order to calculate the viscerosomatic index (VSI) and the hepatosomatic index (HSI). The heart and kidney were then removed before the fish was rinsed with water and the gutted weight recorded. Finally, muscle samples (Norwegian Quality Cut, NQC, NS 9401, 1994) were taken for analysis of lipid content. In addition, 30 fish (3 × 10) were taken at the start of the experiment, and 10 fish per pen on each sampling point, for the analysis of the whole-body proximate composition. These selected fish presented a mean body weight corresponding to the mean weight of all fish in the pen, then exposed to a lethal concentration of MS-222, before being frozen at −20 °C. The fish were not starved before the sampling occasions in June and July, so feed matter was removed from the esophagus, stomach and intestines of all fish taken for analysis at these samplings. At the final sampling in
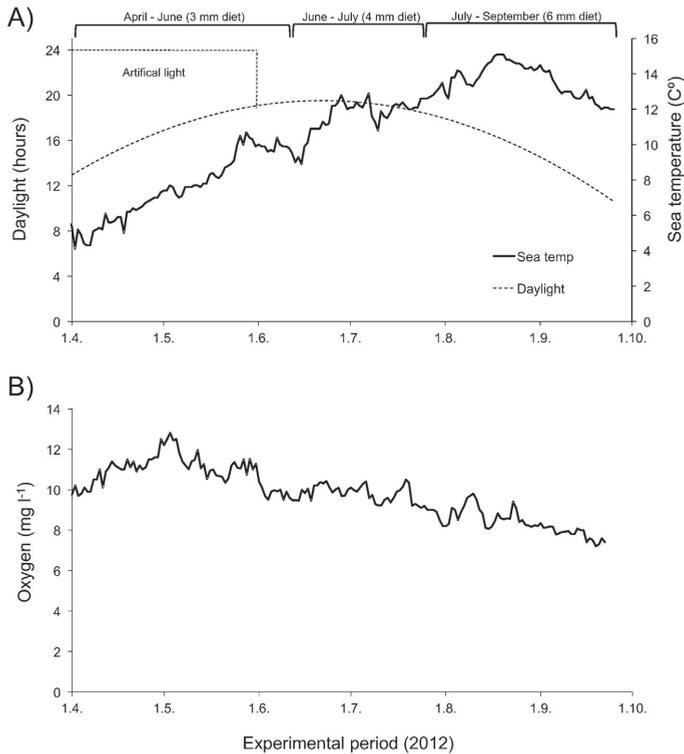
**Fig. 1.** A: Ambient sea temperature (°C) and hours of daylight during the trial. B: The measured oxygen level (mg l−1) during the trial. Diets used (3, 4 and 6 mm) and the duration of the feeding periods (months) are indicated in the top of the figure.

September, samples were taken 48 h after the last meal and no feed matter was observed in the gastrointestinal system.

The pens were checked for mortalities daily and all the dead fish, were collected and weighed. During period 1, 3 and 2 fish died in the HP and LP group, respectively. During period 2, the average morality rate was 1.0% for the HP group and 1.6% for the LP group. During period 3, the average morality rate was 1.4% for the HP group and 0.6% for the LP group. There were no significant differences in mortality.

*2.5. Analysis*

Feces and diets were analyzed gravimetrically for dry matter (DM) after drying at 105 °C until constant weight, and for ash by flame combustion and incineration at 550 °C. Nitrogen was analyzed using the semi-automated Kjeldahl method (Kjetec Auto System, Foss Tecator, Höganäs, Sweden) and crude protein calculated as N × 6.25. The amount of crude lipid after hydrolysis with hydrochloric acid (HCl) and petroleum ether extraction was determined using the Soxtec HT6 system and a Soxtec1047 hydrolyzing unit (Foss Tecator, Höganäs, Sweden). The gross energy content was determined by adiabatic bomb calorimetry (Parr 6400 oxygen bomb calorimeter, Parr Instrument Company, Moline, IL, USA). Starch was analyzed as glucose, after enzymatic hydrolysis using a Megazyme K-TSTA 05/06 total starch assay kit (Megazyme International Ltd., Wicklow, Ireland) according to the Association of Analytical Communities (AOAC) method, number 996.11. The amount of crude fiber was determined using a modified version of ISO 5498, by means of a Fibertec system (Foss Tecator, Höganäs, Sweden).

The amounts of crude protein and energy in homogenates of whole-fish body samples were determined as described for feeds. Whole-body fat was analyzed using a semi-automatic Soxhlet extractor (Soxtec Avanti 2055 apparatus, Foss Tecator, Höganäs, Sweden) with petroleum ether as the extracting solvent. The total fat content in muscle (NQC) was determined by extraction with ethyl acetate as described in NS 9402 (1994). The chemical analyses of muscle fat were conducted on pooled homogenized NQC samples from 10 fish per pen.

**Table 3**
The experimental periods with duration, dates, pellet size used and sampling date. The preferred fish weight intervals of the different pellet sizes are also given.

| Feeding period | Duration | Dates | Pellet size used | Preferred fish weight (g) | Samplings |
|---|---|---|---|---|---|
| Apr - Jun | 11 weeks | 29 Mar. - 11 Jun. | 3 mm | 100 ~ 150 | 1: 11 Jun. |
| Jun - Jul | 6 weeks | 11 Jun. - 23 Jul. | 4 mm | 150 ~ 300 | 2: 23 Jul. |
| Jul - Sept | 9 weeks | 23 Jul. - 24 Sep. | 6 mm | 300 ~ 800 | 3: 24 Sep. |

## 2.6. Calculations

The growth rates of the fish are presented as the thermal growth co-efficients (TGC), calculated as described by Cho (1992).

$$TGC = \left(W_1^{1/3} - W_0^{1/3}\right) \times (\Sigma T)^{-1} \times 1000$$

where $W_0$ and $W_1$ are the initial and final weights, respectively, and $\Sigma T$ is the sum of day degrees during the period

(feeding days × average temperature, °C).

The biological feed conversion ratio (FCRb) was calculated as:

feed intake (kg) × (biomass increase + biomass of dead fish (kg))$^{-1}$.

The feed conversion ratio on gutted weight basis (FCRg) was calculated as:

FCRg = FCRb × carcass yield$^{-1}$.

The specific feeding rate (SFR) was calculated as:

(feed intake during the time period (kg)

× average biomass weight during the time period (kg)) × 100$^{-1}$.

The retention of nutrients were estimated on pen basis, using the values of cumulative feed intake, the chemical composition of the diets, and changes in the biomass and whole-body content of the nutrient: Relative nutrient retention (% of ingested) was calculated as:

100 × (final mass of nutrient in fish−initial mass of nutrient in fish) (mass nutrient ingested)$^{-1}$.

Absolute amount of nutrient retained from the feed (g 100 g$^{-1}$ feed) was calculated as:

$$\left((\text{nutrient in the diet} \times \text{percentage of nutrient retention}), \times, 100^{-1}\right).$$

For absolute nutrient retention of energy, MJ kg$^{-1}$ feed was used.

The authors acknowledge that the relative and absolute lipid retention is apparent as the fish have the ability to synthesize this nutrient de novo. However, in the text the term apparent is not used.

The body weight (BW) of bled fish was estimated by adding 3% to the bled weight: (BW = bled weight × 1.03) (Einen et al., 1998).

Viscerosomatic index (VSI) and carcass yield were calculated as:

Y(g) × body weight(g)$^{-1}$ × 100,

where Y is the weight of the measured visceral or carcass mass.

The condition factor (CF) was defined as:

100 × total body weight with blood (g) × length$^{-3}$.

The CF and carcass yield on gutted weight basis were calculated by applying the same formulas, but with gutted weight instead of the body weight.

## 2.7. Statistical analysis

The trial was conducted using a randomized block design and all data were analyzed using the GLM procedure in the SAS 9.3 computer software (SAS Institute Inc., Cary, NC, USA). Diet and block were used as class variables. If differences based on the block variable were not significant, the data were analyzed using diet as the only experimental factor.

Net pen was used as the experimental unit. Percentage data were subjected to arcsine square root transformation before the statistical analysis. Homogeneity of variances was tested using Bartlett's test, and for data with heterogeneous variances, Welch's test for differences among groups was performed. Non-parametric data (visual score) were tested using the Kruskal-Wallis test. The Pearson product-moment correlation coefficient was used to describe the association between two variables. The level of significance was chosen at $P \leq 0.05$, and the results are presented as mean $\pm$ standard error of mean (SEM), unless stated otherwise.

## 3. Results

### 3.1. Feed intake, growth performance and feed utilization

The feed intake was low after sea transfer and throughout the first feeding period from April–June. It then increased gradually during the experiment. The feed intake did not differ between the dietary groups in April–June and June–July. The duration of daylight decreased in the period July–September and the water temperature was high (Fig. 1A and B). During this period, the fish fed the HP diet had significantly higher feed intake than those fed the LP diet (Table 4).

The growth rate reflected the feed intake, and TGC, FCRb and BW did not differ significantly between the dietary treatments in April–June or June–July (Fig. 3 and Table 4). The highest growth for both groups was observed during July–September (Fig. 3). In addition, during this period fish fed the HP diet presented a significantly higher TGC compared to fish fish fed the LP diet (HP = 3.82 $\pm$ 0.00, LP = 3.46 $\pm$ 0.03, $P < 0.001$). FCRb did not differ between the two groups (Table 4). Thus, the final body weight of fish in the HP group (945 $\pm$ 4 g) was significantly ($P < 0.0001$) higher than that of fish in the LP group (836 $\pm$ 11 g). Consequently, the weight gain (corrected for differences in start weight) for the HP group was 106 g higher (i.e. almost 20% higher weight gain) than the LP group. Fish given the HP diet had a significantly lower FCR on gutted weight basis (FCRg) than fish given the LP diet during the period July–September (Table 4).

### 3.2. Fat deposition, proportional visceral weight and body shape

The developments in muscle fat content and VSI for the two diets are shown in Fig. 2. The amount of muscle fat was the same in both dietary groups until the second sampling in July, when the group fed the HP diet had lower muscle fat content than the LP group (HP = 4.7 $\pm$ 0.3%, LP =

**Table 4**
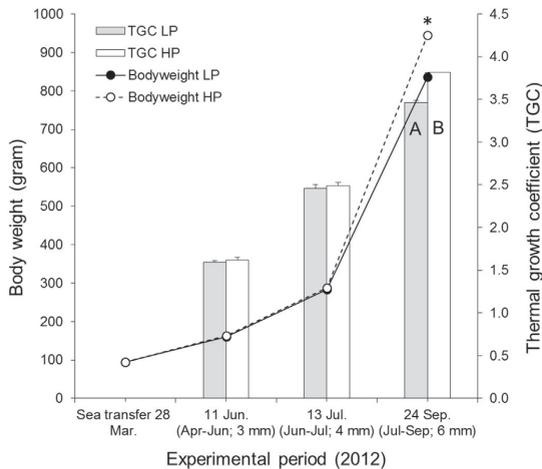Weight gain, feed intake and feed utilization (mean $\pm$ SEM, n = 4).

| Dietary group | LP | HP | Dietary effect (P-value) |
|---|---|---|---|
| *April – June, 3 mm diet* | | | |
| Weight gain, g | 66 $\pm$ 1 | 67 $\pm$ 2 | 0.533 |
| SFR | 0.55 $\pm$ 0.01 | 0.54 $\pm$ 0.00 | 0.205 |
| FI, g$^{-1}$ fish$^{-1}$ | 52 $\pm$ 1 | 51 $\pm$ 1 | 0.487 |
| FCRb | 0.79 $\pm$ 0.01 | 0.76 $\pm$ 0.02 | 0.277 |
| FCRg | 0.93 $\pm$ 0.02 | 0.88 $\pm$ 0.01 | 0.087 |
| *June – July, 4 mm diet* | | | |
| Weight gain, g | 123 $\pm$ 2 | 126 $\pm$ 2 | 0.383 |
| SFR | 1.03 $\pm$ 0.02 | 1.05 $\pm$ 0.02 | 0.536 |
| FI, g$^{-1}$ fish$^{-1}$ | 92 $\pm$ 1 | 95 $\pm$ 2 | 0.280 |
| FCRb | 0.74 $\pm$ 0.00 | 0.75 $\pm$ 0.01 | 0.210 |
| FCRg | 0.89 $\pm$ 0.01 | 0.88 $\pm$ 0.01 | 0.372 |
| *July – September, 6 mm diet* | | | |
| Weight gain, g | 552 $\pm$ 9.3 | 658 $\pm$ 2.3 | <0.001 |
| SFR | 1.33 $\pm$ 0.02 | 1.38 $\pm$ 0.01 | 0.054 |
| FI, g$^{-1}$ fish$^{-1}$ | 452 $\pm$ 9 | 527 $\pm$ 2 | <0.001 |
| FCRb | 0.81 $\pm$ 0.01 | 0.80 $\pm$ 0.00 | 0.126 |
| FCRg | 0.98 $\pm$ 0.01 | 0.93 $\pm$ 0.00 | 0.013 |

SFR, specific feed intake; FI, feed intake; FCRb, biological feed conversion ratio. FCRg, feed conversion based on gutted weight.
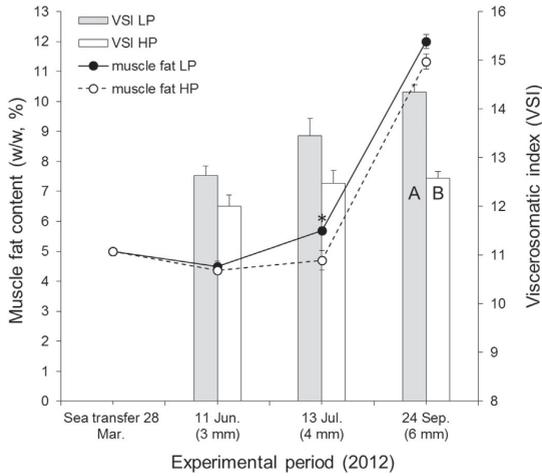
**Fig. 2.** Changes in muscle fat content, % w/w (lines) and viscero-somatic index, % (bars) for S1 Atlantic salmon fed isoenergetic diets with high (HP) or low (LP) protein/lipid ratio. Significant differences between dietary groups within each sampling (11 Jun, 13 Jul and 24 Sep) are indicated by * over the lines and different letters on bars. The diets used (3, 4 and 6 mm) before the samplings are shown in the parenthesis. Data are presented as means ± SEM, n = 4.

**Table 5**
Biometric parameters at each sampling point (mean ± SEM, n = 4).

| Dietary group | LP | HP | Dietary effect (P-value) |
|---|---|---|---|
| *11 June, Sampling 1, end of 3 mm diet* | | | |
| Body weight, g | 150 ± 3 | 151 ± 4 | 0.849 |
| Gutted body weight, g | 129 ± 3 | 131 ± 3 | 0.646 |
| Body length (fork), cm | 23.9 ± 0.1 | 24.0 ± 0.2 | 0.554 |
| Condition factor (CF) | 1.10 ± 0.01 | 1.09 ± 0.01 | 0.571 |
| Condition factor gutted (CFg) | 0.94 ± 0.01 | 0.94 ± 0.01 | 1.000 |
| Carcass yield, % | 86.1 ± 0.2 | 86.8 ± 0.3 | 0.102 |
| Visceral score, 1-5 | 1.3 ± 0.1 | 1.5 ± 0.1 | 0.139 |
| *23 July, Sampling 2, end of 4 mm diet* | | | |
| Body weight, g | 274 ± 2 | 277 ± 2 | 0.393 |
| Gutted body weight, g | 233 ± 2 | 238 ± 2 | 0.087 |
| Body length (fork), cm | 29.0 ± 0.0 | 29.2 ± 0.2 | 0.234 |
| Condition factor (CF) | 1.12 ± 0.01 | 1.11 ± 0.01 | 0.526 |
| Condition factor gutted (CFg) | 0.95 ± 0.01 | 0.95 ± 0.01 | 1.000 |
| Carcass yield, % | 85.0 ± 0.4 | 86.0 ± 0.3 | 0.072 |
| Visceral score, 1-5 | 1.4 ± 0.1 | 0.9 ± 0.1 | 0.017 |
| *24 September, Sampling 3, end of 6 mm diet* | | | |
| Body weight, g | 815 ± 20 | 926 ± 6 | 0.002 |
| Gutted body weight, g | 685 ± 16 | 796 ± 7 | 0.001 |
| Body length (fork), cm | 38.7 ± 0.3 | 39.9 ± 0.2 | 0.023 |
| Condition factor (CF) | 1.40 ± 0.01 | 1.46 ± 0.02 | 0.025 |
| Condition factor gutted (CFg) | 1.18 ± 0.01 | 1.25 ± 0.01 | 0.008 |
| Carcass yield, % | 84.1 ± 0.2 | 86.0 ± 0.2 | <0.001 |
| Visceral score, 1-5 | 2.7 ± 0.1 | 2.4 ± 0.1 | 0.106 |

Initial sampling before sea transfer, 29 March: body weight; 92.8 ± 0.3 g, length; 19.1 ± 0.0 cm, condition factor; 1.33 ± 0.01.

5.7 ± 0.1%, P = 0.03). Muscle fat content of both groups increased substantially (P < 0.001) from July to September (6.5%-units on average) and no significant differences in muscle fat content were detected between the two dietary groups in September (Fig. 3). The VSI of the group fed the LP diet increased steadily during the trial, whereas the VSI of the group fed the HP diet remained almost constant. At the final sampling in September (Fig. 3), the VSI of the HP group was lower than that of the LP group (HP = 12.6 ± 0.1, LP = 14.3 ± 0.2, P < 0.001), and thus the final carcass yield was significantly higher

(Table 5). The CF and CFg followed a similar pattern throughout the trail as that from the lipid level: they did not increase during the two first periods, but then increased sharply in the period July–September. At the final sampling in September, the length, CF, CFg, and gutted weight were all significantly higher for salmon fed the HP diet compared to those fed the LP diet (Table 5).

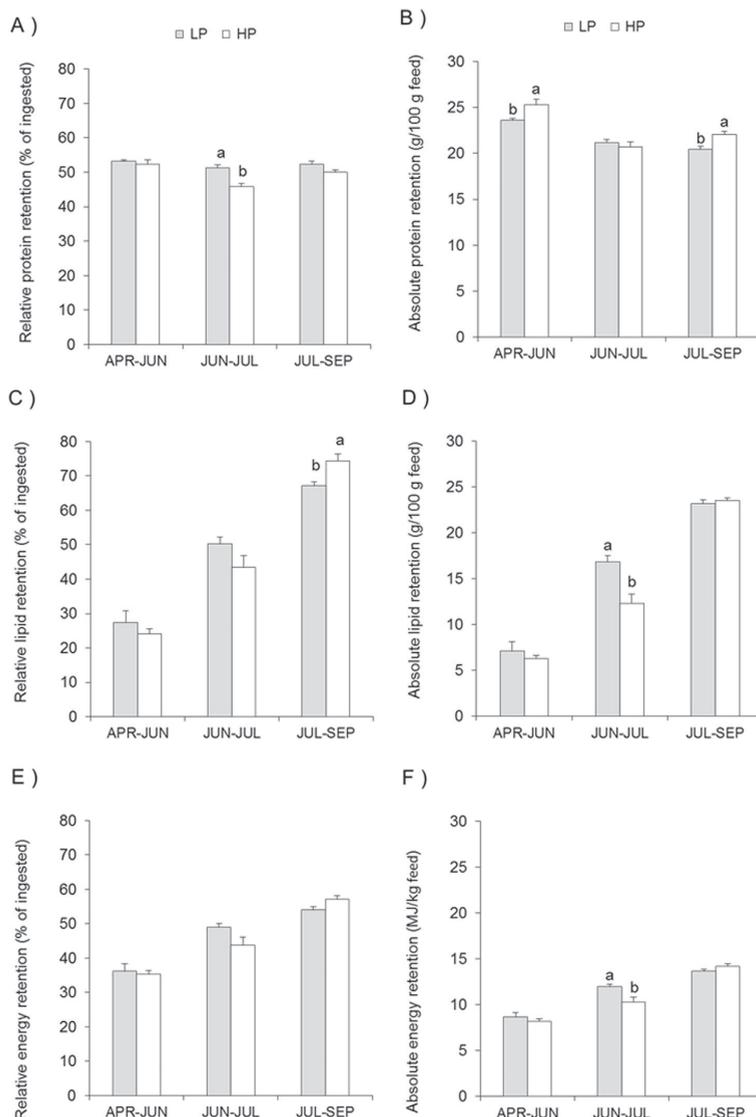### 3.3. Whole body analysis and nutrient retention

The fish fed the LP diet had significantly higher whole body lipid and energy content than the fish fed the HP diet at the sampling in July. The levels of whole body fat and energy were not different between the two groups at the final sampling in September. However, fish in the HP group had a significantly higher protein content than those in the LP group at the September sampling (Table 6). The relative retention of protein (% of ingested) did not differ between the dietary groups in

**Table 6**
Whole body composition of lipids, protein and energy at each sampling point (mean ± SEM, n = 4).

| Dietary group | LP | HP | Dietary effect (P-value) |
|---|---|---|---|
| *11 June, Sampling 1, end of 3 mm diet* | | | |
| Lipids (%) | 9.6 ± 0.3 | 9.0 ± 0.0 | 0.075 |
| Protein (%) | 17.9 ± 0.1 | 18.1 ± 0.2 | 0.287 |
| Energy (MJ kg$^{-1}$) | 8.0 ± 0.1 | 7.7 ± 0.0 | 0.098 |
| *23 July, Sampling 2, end of 4 mm diet* | | | |
| Lipids (%) | 10.9 ± 0.1 | 9.2 ± 0.3 | 0.003 |
| Protein (%) | 17.1 ± 0.1 | 17.2 ± 0.1 | 0.357 |
| Energy (MJ kg$^{-1}$) | 8.4 ± 0.1 | 7.8 ± 0.2 | 0.011 |
| *24 September, Sampling 3, end of 6 mm diet* | | | |
| Lipids (%) | 16.4 ± 0.1 | 16.0 ± 0.3 | 0.301 |
| Protein (%) | 16.9 ± 0.1 | 17.6 ± 0.1 | 0.004 |
| Energy (MJ kg$^{-1}$) | 10.3 ± 0.1 | 10.3 ± 0.1 | 0.867 |

Initial sampling before sea transfer, 29 March: Lipids; 12.0 ± 0.2 %, Protein; 17.3 ± 0.1 %, Energy; 8.8 ± 0.0 MJ kg$^{-1}$.



**Fig. 3.** Changes in body weight (lines) and thermal growth coefficient (bars) for S1 Atlantic salmon fed isoenergetic diets with high (HP) or low (LP) protein/lipid ratio. Significant differences between dietary groups within each sampling (11 Jun, 13, Jul and 24 Sep) or feeding period (Apr–Jun; 3 mm, Jun–Jul;4 mm and Jul–Sep; 6 mm) are indicated by * over the lines and different letters on bars. Data are presented as means ± SEM, n = 4.

**Fig. 4.** Relative nutrient retention (% of ingested) of protein (A), lipid (C) and energy (E), and the absolute nutrient retention of protein (g 100 g−1 feed; B), lipid (g 100 g−1 feed; D) and energy (MJ kg−1 feed; F) for S1 Atlantic salmon fed isoenergetic diets with either a high (HP; white bars) or a low (LP; gray bars) protein/lipid ratio. Significant differences between dietary groups within each feeding period (Apr–Jun, Jun–Jul and Jul–Sep) are indicated by different letters over the bars. Data are presented as means ± SEM, n = 4.

the periods April–June or July–September. However, the absolute protein retention (g 100 g$^{-1}$ feed) in the HP group was significantly higher than in the LP group during April–June (HP = 25.3 ± 0.6, LP = 23.6 ± 0.2, P = 0.05) and July–September (HP = 22.1 ± 0.3, LP = 20.4 ± 0.3, P = 0.01) (Fig. 4A and B). The relative protein retention differed significantly between the two diets only during June–July, when the retention of the protein was lower in the HP group than in the LP group HP = 45.8 ± 0.9%, LP = 51.2 ± 0.9%, P = 0.01 (Fig. 4A). No differences in absolute protein retention during this period were detected. In line with the whole body lipid in July, the LP group showed a trend towards higher relative lipid retention and significantly higher absolute lipid

retention (HP = 12.4 ± 1.0, LP = 16.9 ± 0.6, P = 0.01) compared to the group fed the HP diet during the period June–July (Fig. 4C and D). In the period July–September, the HP group had a significantly higher relative lipid retention than the group fed the LP diet (HP = 74.4 ± 2.0%, LP = 67.2 ± 1.1%, P = 0.02, Fig. 4C), but no differences in absolute retention were observed (Fig. 4D). The relative retention of energy was not significantly different between the two dietary groups during the experiment (Fig. 4E). However, the absolute energy retention (MJ kg$^{-1}$ feed) coincided with the absolute lipid retention with a significant difference between the groups in June–July (HP = 10.3 ± 0.5, LP = 11.9 ± 0.3, P = 0.03) (Fig. 4F).

*3.4. Relationships between overall feed intake and other parameters*

The overall daily feed intake was highly correlated with the temperature during the experiment ($r = 0.96$, $P < 0.001$). The relative lipid retention efficiency was positively correlated to the increase in feed intake ($r = 0.98$, $P < 0.001$). The SFR during the period July–September was negatively correlated with the level of muscle fat at the sampling in July ($r = -0.82$; $P = 0.01$).

## 4. Discussion

The feed intake and growth of salmon smolt are generally low during the first 4–8 weeks after seawater transfer (Alne et al., 2011; Jobling et al., 2002a; Oehme et al., 2010; Rørvik et al., 2007), and the manner by which feed intake and growth return to normal vary (Jobling et al., 2002a; Usher et al., 1991). After sea transfer, the fish need to adapt to new environmental conditions, a new feeding system and a new social hierarchy, and these are all factors that may influence feed intake and growth during the initial stages of a trial (Gilmour et al., 2005). In the present study, feed intake and growth improved as time progressed, and high SFRs (1.27–1.39) and TGCs (3.37–3.83) were observed during the latter stage of the trial in the period July–September. These corresponded to 120% of the growth predicted by Austreng et al. (1987) compared to only 40% during the April–June period. Condition factor, body lipids and energy all increased markedly during this period. These parameters often increase during the autumn (Alne et al., 2011; Mørkøre and Rørvik, 2001; Måsøval et al., 1994), which is a period when the duration of daylight declines rapidly and the water temperature is high. The changes by time in feed intake, growth, fat content and body shape are in line with those of previous studies of S1 smolt reared at the same site and under similar conditions (Alne et al., 2011; Mørkøre and Rørvik, 2001; Oehme et al., 2010). As in most poikilothermic species, feed intake was highest during the period July–September, when the average water temperature was 14 °C. This is in agreement with a study done by Handeland et al. (2008), showing that the feed intake of Atlantic salmon post-smolt is higher for those reared at 14 °C than for those reared at other temperatures (6, 10 and 18 °C).

Our results differ from those from Karalazos et al. (2007 and 2011), in which the dietary protein/lipid level did not affect growth when kept at a normal temperature (11 °C) or at low a temperature (4.2 °C). However, fish fed a diet with a low protein/lipid ratio tended to have lower final weights than fish fed other diets (Karalazos et al., 2011). Karalazos et al. (2007 and 2011) studied larger salmon (with initial weights of 1168 and 2053 g, respectively) and tested diets with a low inclusion of fishmeal and low protein/lipid ratios, ranging from 390/330 to 290/380 g kg$^{-1}$. Small salmonids require higher dietary proportions of digestible protein than larger salmonids (Cho and Kaushik, 1990; Einen and Roem, 1997), and this may explain why the results obtained in the previous studies differ from those presented here. Azevedo et al. (2004b) found no difference in weight gain or growth of rainbow trout or Atlantic salmon fed isoenergetic diets with different protein/lipid ratios. They used, however, a wild salmon strain, and both species were reared in freshwater with a constant temperature of 8 °C.

Salmonids seem to adjust their feed intake according to the dietary energy level (Bendiksen et al., 2002; Boujard and Medale, 1994), and this may be an influencing factor in trials in which feeds with different energy content are evaluated. Therefore, the use of isoenergetic diets eliminates this issue. Most studies that have investigated different protein/lipid levels for fish used diets with different total energy contents. Einen and Roem (1997) fed salmon reared from 1.0–2.9 kg in seawater diets that contained different protein/lipid levels and different energy levels. In this study, the TGC of a group fed a diet with a protein/lipid level of 480/308 g kg$^{-1}$ (corresponding to a DP/DE ratio of 18.8 g MJ$^{-1}$) was significantly higher than that of a group fed a diet with a protein/lipid level of 425/364 g kg$^{-1}$ (DP/DE of 16.4 g MJ$^{-1}$). The difference in growth observed in the latter study was only recorded during the last phase of the study, when the growth rates were high following a 60-day period with low appetite and growth. The results of Einen and Roem (1997) agree with those of the present study, and both indicate that a low ratio of dietary protein to lipids (below 16– 17 g MJ kg$^{-1}$ DP/DE) reduces feed consumption in salmon. This in turn affects the intake of protein and other nutrients and reduces the availability of essential nutrients for optimal growth (Bendiksen et al., 2003b; Johansen et al., 2002; Shearer et al., 1997a; Shearer et al., 1997b; Silverstein et al., 1999). Our findings confirm this line of results using feed formulations, fish breed and rearing conditions commonly used in commercial farming of salmon.

The observed negative relation between muscle fat in July and the subsequent feed intake in the period July–September suggest that the significantly higher lipid deposition in the LP group may have suppressed appetite and reduced feed consumption. This, together with a leaner HP diet, may have contributed to a higher feed intake among HP fed fish in latter stages of our trial. The lower feed intake in the LP group than in the HP group is consistent with the theory of lipostatic regulation (Jobling and Johansen, 1999; Keesey and Corbett, 1984; Kennedy, 1953; Schwartz and Seeley, 1997). In accordance with this, the VSI of the group fed the LP diet increased continuously, indicating increased adiposity. However, the pure effect of body fat content on feed intake cannot be separated in the present trail. To be able to elucidate this, the two groups should have received the same feed in the period after achieving differences in lipid content.

The VSI of fish in the HP group did not increase during the experiment, whereas that of fish in the LP group increased gradually. Normally, an increase in VSI reflects a higher deposition of visceral fat (Bendiksen et al., 2003b; Hillestad et al., 1998; Jobling et al., 1998; Jobling et al., 2002a). The VSI correlated with both the visual assessment of visceral fat and the level of whole body lipids. This indicates that the HP group stored dietary lipids preferentially in the muscle, whereas the LP group stored lipids in both muscle and viscera. The muscle is the major site of fat deposition and storage in salmonids, accounting for 60–65% of the body mass (Aursand et al., 1994; Jobling et al., 2002a; Polvi and Ackman, 1992). The increase in VSI and consequent decrease in carcass yield of the LP group may suggest that dietary lipids were in excess, and the protein/lipid ratio unbalanced.

The increase in feed intake throughout the experiment (Table 4) correlated with the increased relative and absolute retention of energy and lipids (Fig. 4). Increased energy and lipid retention with increased feed intake are in accordance with the results obtained by Grisdale-Helland et al. (2013b). Our results are also consistent with the observation from Alne et al. (2011), who showed that S1 smolt had low relative lipid retention (~20%) during the spring and high relative lipid retention (~60%) during the autumn. The absolute lipid retention was identical between the two dietary groups during the autumn period, due to a significant up-regulated relative lipid retention for the HP group. This shift in relative lipid retention indicates that fat deposition and storage during this period are a high priority. However, it is noteworthy that although the absolute lipid retention was equal between the groups during autumn, the VSI of HP group was significantly lower than that in the LP group in September. The relative retention of protein was reasonably stable (at approximately 50%) and far less dynamic than the retention of lipid, as previously reported (Alne et al., 2011). The significantly higher absolute protein retention of the HP group compared with LP group during April–June and July–September, suggests that dietary protein was efficiently incorporated to body protein in the fish fed the HP diet during these periods. For the period Jul-Sep, the increased absolute protein retention coincided with the high CF, carcass yield and body protein content among the HP fed fish. These factors are again interrelated with the improved feed intake and growth in the fish fed the HP diet. The lower protein retention in the fish fed the HP diet compared to that in the fish fed LP diet in June–July is in accordance with several trials showing a protein sparing effect of reduced protein-to-lipid ratio within certain ranges (Einen and Roem, 1997; Grisdale-Helland and Helland, 1997; Grisdale-Helland et al., 2013a).

FCRb did not change significantly during the experiment. However, FCRg was significantly higher in fish fed the HP diet than it was in fish fed the LP diet during the period July–September (Table 4). This indicates that less of the dietary nutrients were used to increase the visceral mass, and more nutrients were used for carcass growth. This is consistent with the observed nutrient retention and is an important observation, as the carcass is the primary edible product for sale and holds the most value (often referred to as head on gutted, HOG, in relation to sale and price estimations).

## 5. Conclusion

Muscle fat content in fish fed high dietary protein-to-lipid ratio (HP) was significantly reduced compared to that in fish fed low dietary protein-to-lipid ratio (LP) prior to first autumn in sea, without any negative effects on growth and feed conversion. In the subsequent autumn period, fish fed the HP diet showed a significantly higher feed intake, growth rate and weight gain (almost 20%). During this period, HP fed fish presented a significantly higher absolute protein retention and reduced the visceral mass compared to LP fed fish, resulting in significantly higher whole body protein, condition factor, improved carcass yield and feed conversion based on gutted weight. The present study shows that it is possible to modulate lipid deposition and growth by seasonal and dietary interaction.

## Acknowledgments

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# Paper II

## ORIGINAL ARTICLE

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# Low body fat content prior to declining day length in the autumn significantly increased growth and reduced weight dispersion in farmed Atlantic salmon *Salmo salar* L.

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## Abstract

Based on the regulatory effects of body fat on appetite and seasonal variations in fat deposition and growth of Atlantic salmon, the present study tested the hypothesis that body fat content prior to declining day length in the autumn can significantly modulate growth rate. The growth rate of salmon (mean initial body weight, BW = 2.3 kg) with different muscle fat content prior to autumn, subjected to natural photoperiod and temperature, during a 7-month period (mean final BW = 6.6 kg) was studied. In August, three fish groups (HF, LF and 0.5LF group) with significantly different muscle fat content (HF = 16.4%, LF = 13.2% and 0.5LF = 11.3%), individually marked with PIT-tag, were mixed into the four net-pens and fed a standard high-energy diet until March the following year. The muscle fat content prior to the autumn had a highly significant ($p < .0001$) effect on growth during the 7-month main-dietary period, even after identical fat stores among the groups were restored, indicating a more complex explanation than just a lipostatic regulation mechanism. Mean thermal growth coefficients were HF = 2.9, LF = 3.4 and 0.5 LF = 3.9, resulting in increased final weight gain for LF and 0.5LF of 590 g and 980 g, respectively, compared to the HF group. The LF groups obtained a significantly higher homogeneity in BW and shape than HF-fed fish in March, optimizing automatic gutting and filleting at slaughter. The improved growth response among the LF groups by reducing lipid levels can potentially be utilized in closed and semi-closed production units where photoperiod can be manipulated.

**KEYWORDS**
body lipids, growth response, salmon, seasonal cues

## 1 | INTRODUCTION

Fish that encounter setbacks induced by nutritional deficit, feed deprivation or sub-optimal conditions often display increased feed consumption (hyperphagia) and compensatory growth (CG) when circumstances are normalized (Ali, Nicieza & Wootton, 2003; Foss & Imsland, 2002; Metcalfe & Monaghan, 2001). The degree of CG in fish varies and is often categorized based on the growth catch-up ability (Ali et al., 2003). Feed restriction or deprivation induces changes in body energy by depleting lipid stores, and during the course of CG and hyperphagia, body weight and lipid reserves are gradually restored (Ali et al., 2003; Bull & Metcalfe, 1997; Jobling & Miglavs, 1993; Metcalfe & Thorpe, 1992). The lipostatic model is often discussed within the circumstances of CG responses in fish (Jobling & Johansen, 1999; Johansen, Ekli, Stangnes & Jobling, 2001). The lipostatic regulation hypothesis identifies adipose tissue

and stored lipids to have an important role in governing appetite (Jobling & Johansen, 1999; Keesey & Corbett, 1984; Kennedy, 1953). The model implies that the amounts of stored fat has a negative feedback control on feed intake and is important for the regulation of energy homeostasis. Hence, CG is not only a response to recover body weight, but also a strong response to restore lipid levels and thereof CG will cease once this is achieved (Ali et al., 2003; Jobling & Johansen, 1999; Johansen, Ekli, & Jobling, 2002). Johansen, Ekli and Jobling (2002) showed that altering body lipids of juvenile salmon by dietary administration of low-fat feeds yield similar growth responses as deprivation or feed restriction per se.

In modern high-fat diets for salmonids, lipids of marine and vegetable origin are the main sources of energy and support growth efficiently if essential fatty acids requirements are met (Bell et al., 2001; Thomassen & Røsjø, 1989; Torstensen, Lie & Frøyland, 2000). Because salmonids have a high ability to utilize large amount of lipids efficiently for growth, high-fat diets with up to 380 g/kg of fat are commonly used in intensive salmon farming (Torrissen et al., 2011). However, salmonids also have the capacity to store large amounts of excess fat as triacylglycerols mainly in the muscle and visceral cavity (Aursand, Bleivik, Rainuzzo, Leif & Mohr, 1994). Body lipid content of farmed salmonids correlates with fish size, dietary fat level and feed intake (Aksnes, 1995; Hemre & Sandnes, 1999; Torstensen, Lie & Hamre, 2001). Like other anadromous species, Atlantic salmon display seasonal changes in feed intake, growth and lipid deposition during the seawater phase (Mørkøre & Rørvik, 2001). Farmed Atlantic salmon display elevated deposition of lipids in muscle and increased retention of lipids in whole body during declining day length in autumn, with a concomitant increase in feed intake, somatic growth and condition factor (CF) (Alne, Oehme, Thomassen, Terjesen & Rørvik, 2011; Dessen, Weihe, Hatlen, Thomassen & Rørvik, 2017; Mørkøre & Rørvik, 2001; Rørvik et al., 2010). This is particularly pronounced for salmon reared at high latitudes that experience long winters and late spring, which results in reduced lipid levels and CF prior to summer and autumn.

The recent increase in automation of fish processing at slaughter requires uniform body weight (BW) and shape among the salmon for optimal efficiency and quality of products such as gutted fish and fillets. Increased uniformity of BW and CF reduces the need for manual gutting/filleting of very small or large individuals. Due to this, the homogeneity in body shape and mass of salmonids are important parameters in salmon farming industry and low dispersion in BW and CF are beneficial at the time of harvest. The homogeneity of BW may be strongly influenced by events occurring during the production cycle, that is, disease outbreaks, handling stress, reduced seawater tolerance or competition of feed (McLoughlin, Nelson, McCormick, Rowley & Bryson, 2002; Ryer & Olla, 1996; Taksdal et al., 2007; Usher, Talbot & Eddy, 1991). The dispersion in the distribution of BW, length and CF are often assessed by calculating the coefficient of variation (CV). The CV of BW for farmed salmon grown from 70 until 300 g and from 60 until 500 g fed either in excess or restrictively for period followed by unrestricted feeding, are reported to vary from 9% to 13% and 16% to 21%, respectively (Johansen et al., 2001). In the latter study, no significant differences were observed in the CV of BW between fish fed in excess and fish fed restrictively.

The majority of studies regarding growth responses related to lipid content are based on in-house laboratory experiments with small juvenile salmonids under constant conditions. To our knowledge, few have investigated grow out salmon with different lipid content subjected to seasonal environmental changes in photoperiod and temperature. Due to the regulatory effects of body fat on appetite and the observed fat storage in salmon linked to the seasonal cues, the present study tested the hypothesis that lipid status prior to declining day length in the autumn functions as a significant growth regulator. Accordingly, the growth rate for three groups of salmon with different muscle fat content prior to autumn, subjected to natural photoperiod and temperature, was studied throughout a 7-month period. About each second month, weight samplings and analysis of muscle fat content were conducted to investigate any relationship between fat accumulation and periodic growth rate, and to identify the duration of a potential lipostatic regulatory effect. Changes in visceral fat, CF, length and the dispersion in BW and CF were further assessed.

## 2 | MATERIALS AND METHODS

This experiment was conducted in accordance with laws and regulations that control experiments and procedures in live animals in Norway, as overseen by the Norwegian Animal Research Authority. Stunning and sampling of fish were performed in accordance with the Norwegian Animal Welfare act. Fish were treated as production fish up to the point of tissue sampling which was only conducted after the fish were put to death.

The experiment was conducted in seawater on the Norwegian west coast (Ekkilsøy, Norway 3°03′N, 7°35′E) at Nofima research centre from August 2011 to March 2012. In July 2010, the fish were transferred to seawater as S1 smolt, at which time the BW was 62 g. From the 10–12 of May 2011, the post-smolt were re-stocked into three net-pens (343 m³) with 650 fish per pen. Prior to this, all individual fish were measured for weight and length, and tagged using passive integrated transmitter tags (PIT-tags) placed in the body cavity just posterior to the gut. The average BW per pen was 1,085 g (SD = 79 g) and each pen received different dietary treatments: a high-fat diet (HF), a low-fat high-protein diet (LF) or half the ration of the low-fat high-protein diet (0.5LF). The 0.5LF group was given half the amount of the feed provided to fish administrated the LF diet the day before. Skretting (Averøy, Norway) produced the feeds and the composition of the HF diet was (wet weight, as is basis): dry matter 93.4%, crude protein 33.5%, crude lipid 34.1%, nitrogen-free extract (NFE) 21.2%, ash 4.6% and gross energy of 25.1 MJ/kg. The composition of the LF diet was (wet weight, as is basis) as follows: dry matter 91.7%, crude protein 49.9%, crude lipid 17.5%, NFE 17.1%, ash 7.2% and gross energy 21.7 MJ/kg. The

**TABLE 1** Biometrics and fat content of Atlantic salmon in August 2011 fed a diet high-fat diet (HF), low-fat high-protein diet (LF) or half ration of the low-fat high-protein diet (0.5LF) from May until August 2011, referred to as pre-dietary feeding phase

| Dietary treatment | HF | LF | 0.5LF |
|---|---|---|---|
| Biometric parameters, all fish | | | |
|   Number of fish, n | 584 | 584 | 602 |
|   Bodyweight, g | 2651 ± 335 | 2506 ± 287 | 1865 ± 253 |
|   Fork length, cm | 59.1 ± 2.3 | 59.1 ± 2.1 | 55.8 ± 2.3 |
|   CF | 1.28 ± 0.09 | 1.21 ± 0.07 | 1.07 ± 0.08 |
| Biometric parameters, sampled fish, n = 20 | | | |
|   Bodyweight, g | 2619 ± 70[a] | 2515 ± 63[a] | 1881 ± 47[b] |
|   Fork length, cm | 59.0 ± 0.5[a] | 59.0 ± 0.4[a] | 55.7 ± 0.5[b] |
|   CF | 1.22 ± 0.02[a] | 1.18 ± 0.02[a] | 1.03 ± 0.01[b] |
|   VSI | 11.3 ± 0.4[a] | 9.6 ± 0.2[b] | 8.5 ± 0.1[c] |
| Fat content, sampled fish, n = 20 | | | |
|   Muscle fat, % | 16.4 ± 0.3[a] | 13.1 ± 0.2[b] | 11.3 ± 0.3[c] |
|   Visceral fat[†], % | 39.0 | 29.0 | 26.6 |

CF, condition factor; VSI, Visceral-somatic index.

[†]The analysis of visceral fat content was conducted on pooled samples in August 2011 (n = 1).

Values in the same row with different letters are significantly different ($p \leq .05$) determined by one-way ANOVA followed by Duncan's multiple range test. Biometric parameters for all fish are presented as means ± SD, whereas biometric parameters and fat content for sampled fish are presented as means ± SEM together with indications of significant differences.

three dietary treatments were fed from 12 of May until 9 of August (pre-dietary phase). May 12th, the fish were sampled for analysis of initial muscle fat content and biometric data. The analysis showed the following (mean ± SE, n = 30): BW: 1087 ± 97 g, initial muscle fat: 12.2 ± 1.1% and initial CF: 1.10 ± 0.06. After ending the pre-dietary phase, the PIT-tag, BW and length of all individual fish in the

three pens were recorded. In addition, fish from each pen were sampled for analysis of muscle and visceral fat content. The pre-dietary feeding phase generated three fish groups with significantly different ($p < .05$) muscle fat content, visceral fat and visceral mass (Table 1). During the pre-dietary phase, 2.5%, 0.6% and 0.3% fish died in the HF, LF and 0.5LF group, respectively. The majority of mortality occurred from May until mid-June and was not related to any disease outbreak (non-specific morality).

At the 10–11 of August, the fish were restocked from the three original pens used in pre-dietary phase into four new pens (125 m³). Each of the four pens contained 50 fish from each of the three pre-dietary treatments (HF, LF and 0.5LF), 150 fish in total (Figure 1). During the period from 11 of August until termination at 20 of March 2012 (main-dietary phase), the pens were fed isonitrogenous and isoenergetic diets produced by Ewos (Bergneset, Norway) (Table 2). The current experiment was an integrated part of a large study were potential effects of dietary oil source were investigated. Therefore, two pens in the main-dietary phase were fed a diet with a marine oil profile (MO), whereas the two other pens were fed a diet with a rapeseed oil profile (RO). The MO diet had an inclusion of 70% South American fish oil and 30% of rapeseed oil. The RO diet had an inclusion of 70% rapeseed oil and 30% South American fish oil. During the main-dietary phase, the pellet size was changed from 7 to 9 mm in December due to the increase in BW of the fish.

In both periods, feed was administrated using automatic feeders (Betten Maskinstasjon AS, Vågland, Norway) and uneaten feed was collected as described in Einen, Mørkøre, Røra and Thomassen (1999) and corrected for the recovery of dry matter as described by Helland, Grisdale-Helland and Nerland (1996). The fish groups (except the 0.5LF group during the pre-dietary phase) were fed to satiation and the feed ration was set at 5%–10% in excess (ad libitum feeding). The fish were fed four times a day until October 2011; after this, the fish were fed three times a day until termination in
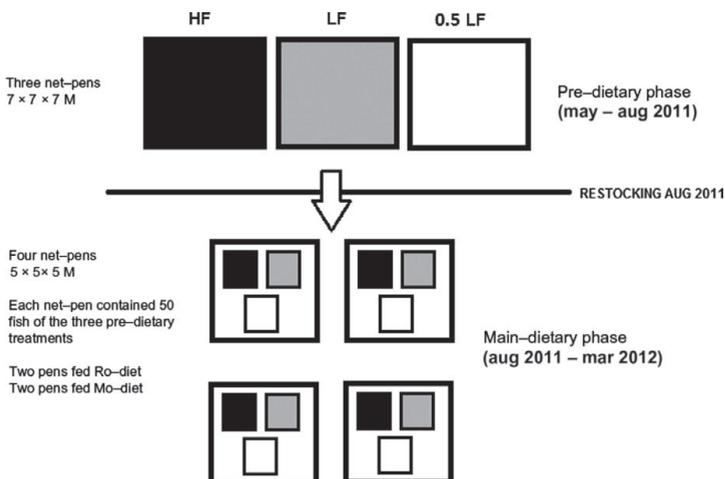


**FIGURE 1** Schematic overview of the experimental design during the pre- and the main-dietary phase. The squares during the pre-dietary phase represent net-pens fed different diets; HF, high-fat diet (black filled square); LF, low-fat diet (grey filled square); 0.5LF, half ration of the low-fat diet (white filled square). The large squares in the main-dietary phase represent the net-pens and the squares within the net-pens are the pre-dietary groups

**TABLE 2** Chemical compositions (wet weight, as is basis) and fatty acid composition (% of total fatty acids) of the diets used in the main-dietary phase

| Diet code | 7 mm pellet | | 9 mm pellet | |
|---|---|---|---|---|
| | MO | RO | MO | RO |
| Chemical composition (wet weight, as is basis) | | | | |
| Dry matter, % | 93.2 | 94.0 | 93.8 | 93.9 |
| Crude protein (N × 6.25), % | 41.2 | 41.7 | 34.4 | 34.6 |
| Crude Lipid, % | 31.2 | 31.4 | 37.0 | 35.7 |
| Starch, % | 6.2 | 6.1 | 6.7 | 6.8 |
| Ash, % | 4.8 | 4.8 | 5.1 | 5.1 |
| NFE[†], % | 16.0 | 16.1 | 17.3 | 18.5 |
| Crude protein/lipid ratio | 1.32 | 1.33 | 0.93 | 0.97 |
| Calculated values[‡] | | | | |
| Gross energy, MJ/kg | 24.8 | 25.1 | 25.7 | 25.5 |
| DP, g/kg | 354 | 359 | 296 | 298 |
| DE, MJ/kg | 21.4 | 21.5 | 22.2 | 21.9 |
| DP/DE ratio, g/MJ/kg | 16.6 | 16.6 | 13.3 | 13.6 |
| Fatty acid composition (% of total fatty acids) | | | | |
| C 16:0 | 12.7 | 8.5 | 14.3 | 9.3 |
| C 18:0 | 3.2 | 2.7 | 3.7 | 2.9 |
| ∑SFA[§] | 22.6 | 15.1 | 24.0 | 15.9 |
| C 18:1 n-9 | 26.8 | 42.1 | 23.3 | 42.5 |
| ∑MUFA[¶] | 38.1 | 49.8 | 36.2 | 52.8 |
| C 18:2 n-6 | 8.1 | 13.9 | 7.4 | 13.9 |
| C 18:3 n-3 | 3.4 | 6.5 | 2.9 | 6.0 |
| C 20:5 n-3 | 10.1 | 4.6 | 11.1 | 4.0 |
| C 22:5 n-3 | 1.3 | 0.6 | 1.4 | 0.5 |
| C 22:6 n-3 | 7.2 | 3.5 | 7.5 | 3.6 |
| ∑PUFA[¥] | 34.3 | 30.4 | 32.7 | 29.0 |
| SUM EPA + DHA | 17.4 | 8.1 | 18.6 | 7.5 |
| n-6/n-3 ratio | 0.4 | 0.9 | 0.4 | 1.0 |

MO, Marine oil profile; RO, Rapeseed oil profile; N, Nitrogen; NFE, Nitrogen-free extracts; DP, digestible protein; DE, digestible energy; MJ, Mega joule; SFA, Saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

[†]NFE was calculated as = 100 − (protein + lipids + ash + water).

[‡]Gross energy, DP and DE were estimated assuming 23.7, 39.5 and 17.2 MJ/kg as the gross energy content of protein, lipids and carbohydrates, respectively. The apparent digestibility coefficients (ADCs) for protein and lipids used were 0.86 and 0.94, respectively (Einen & Roem 1997), whereas 0.50 was used for NFE (Arnesen & Krogdahl 1993).

[§]SFA; C14:0. C15:0, C16:0, C18:0 and 22:0.

[¶]MUFA; C16:1n-9, C16:1n-7, C17:1n-7, C18:1n-7, C:18:1n-9, C20:1n-7, C20:1n-9,C20:1n-11, C22:1n-9,C22:1n-11,C24:1n-9.

[¥]PUFA; C16:2n-3, C16:3n-4, C18:2n-6, C18:3n-6, C18:3n-3, C18:4n-3, C20:4n-3, C20:2n-6, C20:3n-6, C20:4n-6,C20:5n-3, C22:5-n-3, C22:6n-3.

dietary phase. The pens were checked for mortalities daily and the dead fish were collected and weighed. The fish were exposed to natural variations in photoperiod and sea temperature during the experiment (Figure 2).

Three samplings were performed during the main-dietary phase; from 9 to 11 October 2011, from 6 to 9 December 2011 and the final sampling and termination of the experiment was conducted from 20 to 22 March 2012. At each sampling, all fish were anaesthetized (MS-222 metacaine 0.1 g/L, Alpharma, Animal Health, Hampshire, UK) and the PIT-tag, fork length and weight of each individual fish were recorded. All fish were starved 2 days prior to the samplings in August and October, and 3 days prior to the samplings in December and March to avoid feed matter in the gastrointestinal system. At each sampling, 10 fish from each pre-dietary group in all the pens were sampled. The sampled fish at each sampling point were selected so that the mean weight corresponded to the mean weight of all the fish in the respective fish group within each pen (as all possible fish were weighted and PIT-tag read). After anesthetization, a blow to the head was used to kill fish sampled for analysis. Then, the gill arches were cut and the fish were bled out in ice seawater. Length and weight of each individual fish sampled for analysis were recorded after bleeding and the fish visually tagged. The fish were then gutted and filleted by hand during the pre-rigor state. Norwegian Quality Cut, NQC (NS9401, 1994) from the left fillet was photographed and the fat content was predicted by digital image analyses (PhotoFish, AKVAgroup, Bryne, Norway), as described by Folkestad et al. (2008). The visceral mass of the sampled fish were pooled on group level, homogenized and frozen at −20°C for later analyses of total lipid content as described by Folch, Lees and Stanley (1957). The proximate composition of crude protein, lipid (acidic-hydrolysis method), starch and moisture of the diets were analysed according to the methods described by Oehme et al. (2010). To determine the fatty acid (FA) composition of the diets, lipids were first extracted according to Folch et al. (1957), and a sample of 2 ml from the chloroform–methanol phase was dried under $N_2$ gas, then the residual lipid extract was trans-methylated overnight with 2′,2′-dimethoxypropane, methanolic HCl and benzene at room temperature according to Mason and Waller (1964). Finally, the methyl esters were separated by gas chromatography and individual FA were identified as described in Røsjø et al. (1994).

The growth rates of the fish are presented as the thermal growth coefficient (TGC), and are calculated as described by Iwama and Tautz (1981): $TGC = [(M_1^{1/3} − M_0^{1/3}) × (\Sigma T)^{-1}] × 1000$, where $M_0$ and $M_1$ are the initial and final BW, respectively, and $\Sigma T$ is the sum of day degrees during the period (feeding days × average temperature, °C). The mean TGC for the total main-dietary phase was calculated as the weighted arithmetic mean of the periodical TGC to balance these values in relation to their relative contribution to the weight gain.

All fish sampled and killed for analysis were starved and bled. The calculation of visceral-somatic index is therefore based on BW with minimal blood content and no feed material in the
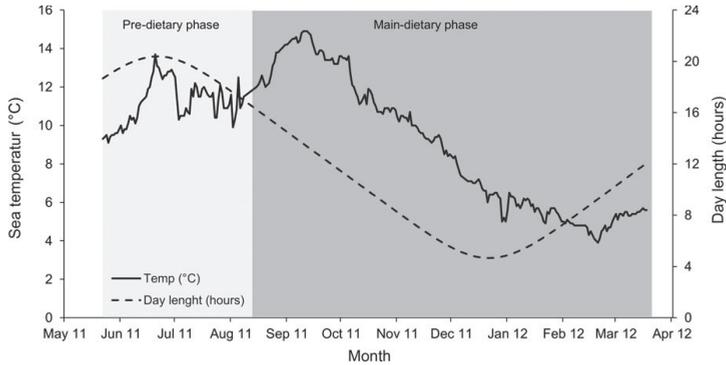
March 2012. Adjustments of the feed ration were done according to the daily amount of uneaten feed collected. Due to the stocking of 50 fish from each of the pre-dietary treatments into each net-pen, it was not possible to determine the feed intake or feed utilization of the different pre-dietary groups during the main-

**FIGURE 2** Ambient daily sea temperature (°C, y-axis) and hours of daylight (hours, z-axis) during the pre-dietary phase (May to August 2011) and the main-dietary phase (August 2011 to March 2012). The length of the different periods are indicated by the different grey colours (light grey, pre-dietary phase; dark grey, main-dietary phase)

gastrointestinal system. Visceral-somatic index (VSI) was calculated as follows: $Y$ (g) $\times$ $BW$ (g)$^{-1}$ $\times$ 100, where $Y$ is the measured visceral mass. The visceral mass was defined as all mass in the abdominal cavity except liver, heart, kidney and swim bladder. The CF was calculated as follows: 100 $\times$ $BW$ (g) $\times$ fork length (cm)$^{-3}$. The dispersions in the distribution of BW, length and CF were assessed by calculating the CV: (standard deviation $\times$ mean value$^{-1}$) $\times$ 100.

The results were analysed by the General Linear Model (GLM) procedure in the SAS 9.4 computer software (SAS Institute Inc., Cary, NC, USA). Mean results per fish group in each pen were initially subjected to a two-way analysis of variance (ANOVA) to evaluate the effects of muscle fat content due to the pre-dietary phase (0.5LF, LF and HF), main-dietary treatment (oil source; MO diet and RO diet) and their interaction (pre-diet $\times$ main diet). As the statistical analysis showed that neither oil source nor the interaction term has significant effects on the traits studied, the data were analysed using pre-dietary treatment as the only experimental factor (one-way ANOVA). Significant differences among experimental groups within treatments were indicated by Duncan's multiple range test. Least-square means (lsmeans) comparison was also used to identify differences among variables within treatments. The Pearson product-moment correlation coefficient was used to describe the association between two variables. Linear regression analysis was conducted using Microsoft excel. The proportion of total variance explained by the model was expressed by $R^2$ and the level of significance was chosen at $p \leq .05$. Tendencies were identified at $p = .05$–.1. The results are presented as means $\pm$ $SEM$, if not otherwise stated.

No significant effects of the main-dietary treatment (RO diet and MO diet) or interaction term (main $\times$ pre-diet) per se were detected on the traits examined during the main-dietary phase. Thus, only the effects of body fat content due to the pre-dietary treatment are presented in the section "Results." No significant differences in mortality among the pre-dietary groups were observed during the main-dietary phase (24 out of 650 fish, 3.6%).

# 3 | RESULTS

The muscle fat content increased by 8.1% for 0.5LF fish, 5.6% for the LF group and 3.6% for HF group from August to October (Figure 4A1). Thus, during an 8-week period of declining day length, the initial significant differences in muscle fat content were equilibrated. TGC was highest for the 0.5LF group, intermediate for the LF group and lowest for the HF group (Figure 5A). The growth rate and the increase in muscle fat content from August to October showed a significant positive linear relationship, and the increase in muscle fat explained 81% of the variation in growth (Figure 3). From August to October, the growth rates were therefore highly affected by the pre-dietary treatment (ANOVA: $R^2 = 0.97$, $p < .001$). The muscle fat



$y = 0.2674x + 2.063$
$R^2 = .8114$
$P = .0001$
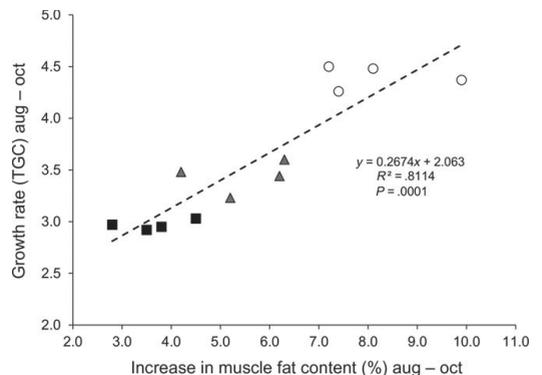
**FIGURE 3** Regression line between thermal growth coefficients (TGC) and the increase in muscle fat (%) from August to October in Atlantic salmon fed three different pre-dietary treatment from May to August 2011; HF, high-fat diet (black filled squares); LF, low-fat diet (grey filled triangles), 0.5LF, half ration of the low-fat diet (white filled circles). Each point represents average per fish group/experimental unit ($n = 12$)

did not differ significantly between the pre-dietary treatments in October or December (Figure 4A1), but pre-diet still significantly influenced the growth rates (ANOVA: $p < .05$, $R^2 = .51$) and the TGCs were similar, relatively, to the period from August to October (0.5LF > LF > HF) although no significant differences was found between LF and HF group. In the period December to March, the TGC for the 0.5LF and LF group were significantly higher ($p < .05$) than the HF group (Figure 5A). At the end of the main-dietary phase, the muscle fat content of the LF group was significantly lower ($p < .05$) than the 0.5LF group, and tended to be lower ($p < .1$) than the HF group (Figure 4A2).

The BW of the LF group reached a similar BW as the HF fish in October, whereas the 0.5LF group reached a similar BW as the HF group in December (Figure 4B1). At the end of the trial in March, the LF group ($6.87 \pm 0.07$ kg) had a significantly higher ($p < .05$) BW than the HF group ($6.40 \pm 0.16$ kg) (Figure 4B2). The 0.5LF group ($6.62 \pm 0.12$ kg) had numerical higher BW than the HF group; however, no statistically significant difference was detected. From

August 2011 to March 2012, the 0.5LF group gained 980 g and the LF group gained 590 g more relative to the BW of the HF group (Figure 5B). The overall weighted mean TGC during the main-dietary phase were 3.9 for the 0.5LF group, 3.4 for the LF group and 2.9 for the HF group. Hence, the pre-dietary treatment and consequently the fat status in August 2011 had a clear and significant effect on growth, weight gain and the changes in BW throughout the whole main-dietary phase, with a total duration of 7 months.

No significant differences in length between LF and HF group were detected during the trial (Figure 6B1). The strong growth spurt of the 0.5 LF group resulted in no significant differences in length between the 0.5 LF ($75.9 \pm 0.2$ cm) and HF group ($76.4 \pm 0.8$ cm) at the trial termination in March. However, the LF ($77.9 \pm 0.1$ cm) group was significantly longer ($p < .05$) than the 0.5LF group (Figure 6B2). The 0.5LF group that had the lowest CF in August, ended up having the significantly highest CF at termination (Figure 6A1 and A2). The overall development in CF correlated well with the changes in muscle fat during the study ($r = .98$, $p < .01$). Significant
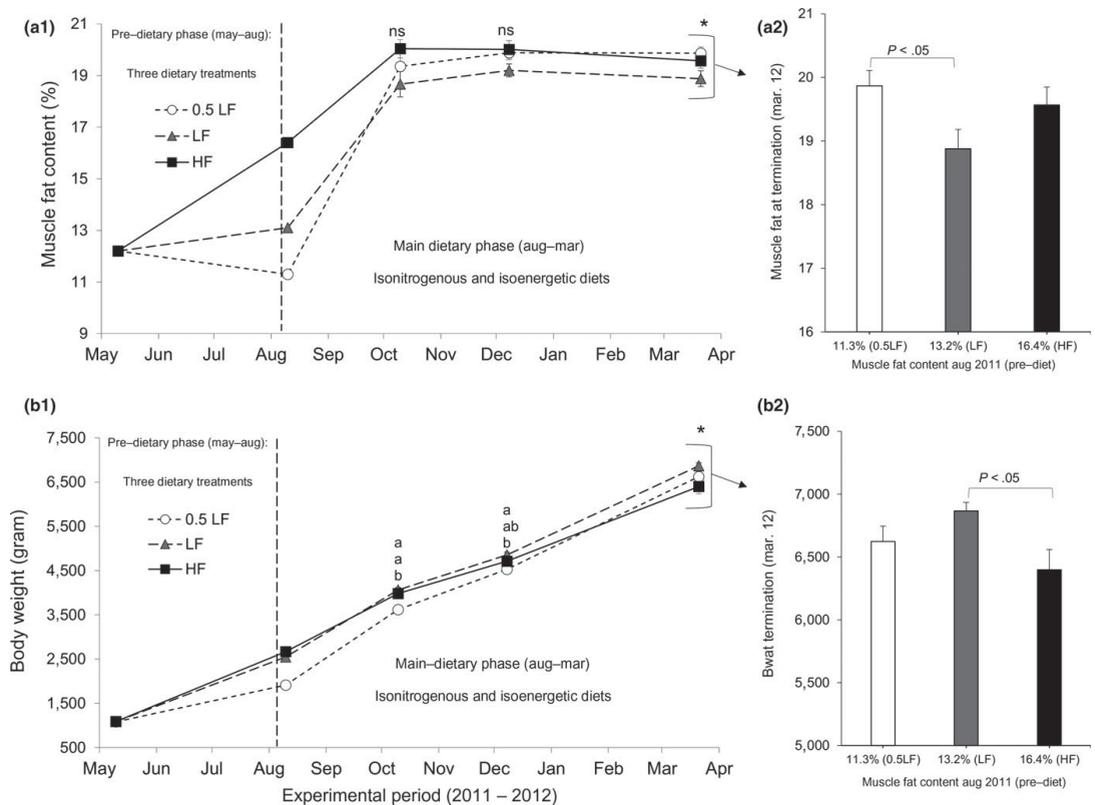


**FIGURE 4** Muscle fat content (a1) and body weight (b1) development of Atlantic salmon fed three different pre-dietary treatment from May to August 2011. Values are means $\pm$ SEM, $n = 4$ ($n = 1$ at termination of the pre-dietary phase). Values not sharing common superscript letters within each sampling period are significantly different ($p \leq .05$). a2 and b2 present the final muscle fat and BW of the groups, respectively. The values 11.3%, 13.2% and 16.4% represent the obtained fat content at the beginning of the main-dietary phase (August 2011) for the 0.5LF, LF and HF groups, respectively. ns, not significant. *trend ($p < .1$)
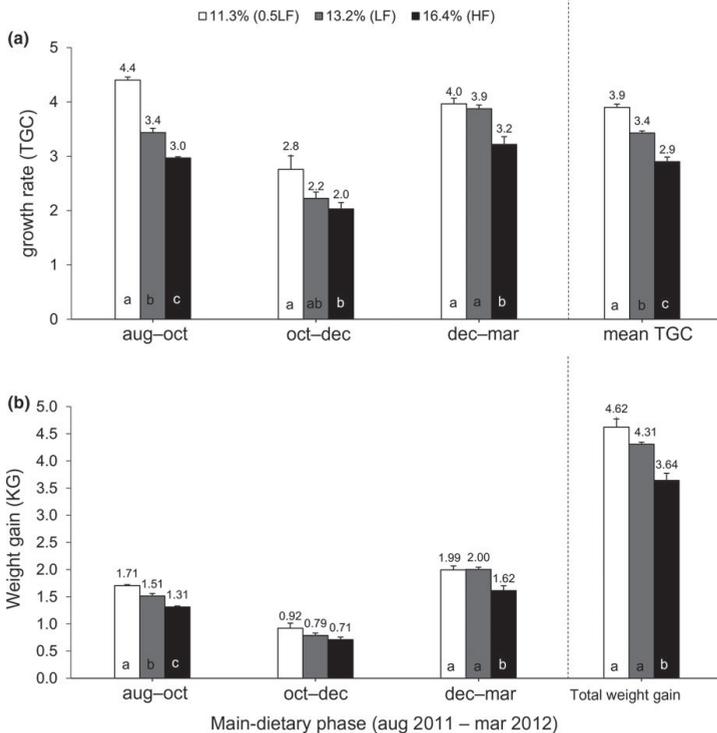
**FIGURE 5** Thermal growth coefficients (TGC) (a) and weight gain (kg) (b) of Atlantic salmon fed three different pre-dietary treatment from May to August 2011. Values are means ± *SEM*, *n* = 4. Values not sharing common superscript letters within each sampling period are significantly different (*p* ≤ .05). The values 11.3%, 13.2% and 16.4% represent the obtained fat content at the beginning of the main-dietary phase (August 2011) for the 0.5LF, LF and HF groups, respectively

positive overall correlations were also observed between the final CF and mean TGC (*r* = .88; *p* < .001), and between the final CF and total weight gain (*r* = 0.88; *p* < .001).

The visceral fat content of the HF group was consistently highest although only significant in October (Figure 7). The VSI of the LF group (8.5 ± 0.1) was significantly lower (ANOVA: *p* < .01) than the HF group (9.0 ± 0.1) in October, whereas the VSI of the 0.5LF group (8.7 ± 0.1) was not different from the LF or HF group. No significant differences in VSI were detected in December (overall mean; VSI: 8.8 ± 0.1) or March (overall mean; VSI: 9.8 ± 0.2).

The 0.5LF group had the highest CV$_{BW}$ at the end of the pre-dietary phase (Figure 8A). From August to October, the CV$_{BW}$ of the 0.5LF group decreased and no significant difference in CV$_{BW}$ was observed at the samplings in October and December. However, at termination in March, the HF group had a significantly (*p* < .05) higher CV$_{BW}$ compared to both LF and 0.5LF groups. The CV$_{CF}$ was lowest for the LF group and similar for the HF and 0.5LF groups at the end of the pre-dietary phase (Figure 8B). At the sampling in October, after the large increase in fat deposition, growth and CF, the 0.5LF group had the highest CV$_{CF}$. The variation within the CV of CF for the 0.5LF group was at this time very high and no significant differences between the groups were detected. The CV$_{CF}$ for the HF group increased gradually from October to March. In line with the CV$_{BW}$, the HF group had a significantly (*p* < .05) higher CV$_{CF}$ compared to the 0.5LF and LF group at termination. No

significant differences in the CV$_{LENGHT}$ were detected during the experiment (results not shown).

## 4 | DISCUSSION

The coinciding increase in fat and improved growth shown by the 0.5LF and LF groups, compared to the HF group in the beginning of main-dietary phase (August and September), seem to reflect a growth response similar to CG and lipostatic regulation observed in previous studies in the field and laboratory (Ali et al., 2003; Jobling & Johansen, 1999; Johansen et al., 2001, 2002). The obtained growth rates, fat increase and weight gain from August to October, together with the high feed intake (on pen basis), indicate that the 0.5LF and LF groups had increased feed consumption and hyperphagic behaviour. In addition to the high growth rate of the 0.5LF and LF groups, the increase in muscle and visceral fat content during August and September was substantial for these two groups. However, the muscle fat of the HF group also increased during this period (16.4%→20.0%). The TGC of the HF group had an average of 3.0, which is regarded as a normal and sufficient growth rate (Austreng, Storebakken & Åsgård, 1987; Thorarensen & Farrell, 2011). Thus, improved growth in the LF groups from August to October, compared to the HF group, is not a result of impaired growth due to adiposity in the latter group, but rather a stronger response among
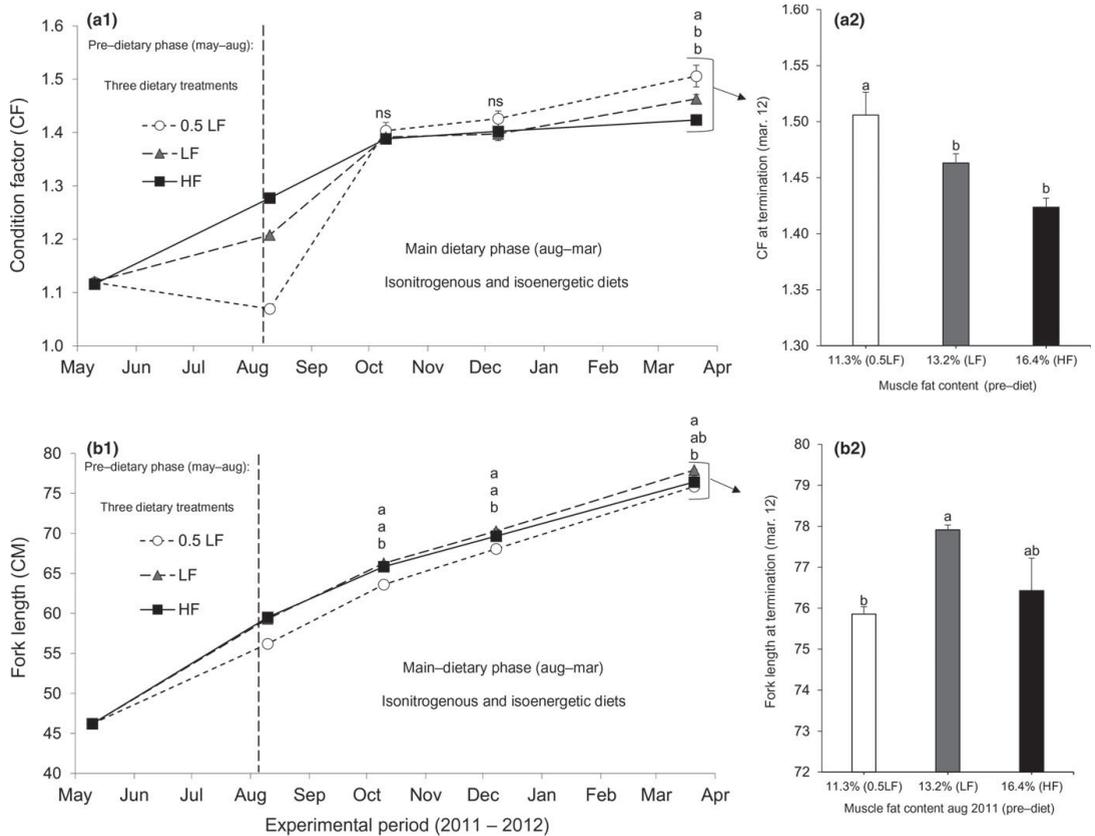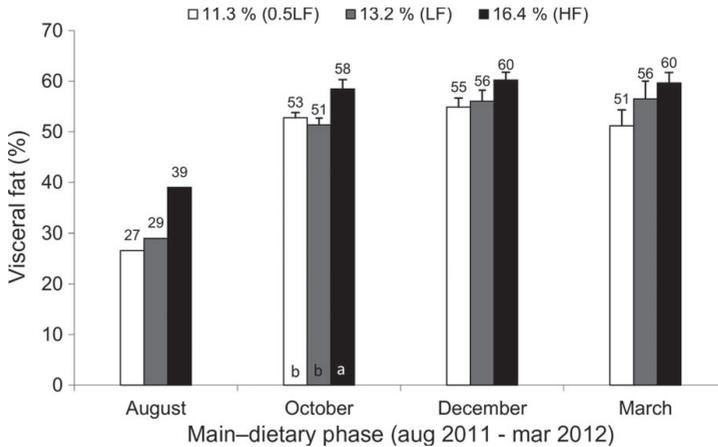
**FIGURE 6** Condition factor (CF) (a1) and fork length (cm) (b1) development of Atlantic salmon fed three different pre-dietary treatment from May to August 2011. Values are means ± SEM, n = 4 (n = 1 at termination of the pre-dietary phase). Values not sharing common superscript letters within each sampling period are significantly different (p ≤ .05). a2 and b2 present the final CF and fork length of the groups, respectively. The values 11.3%, 13.2% and 16.4% represent the obtained fat content at the beginning of the main-dietary phase (August 2011) for the 0.5LF, LF and HF groups, respectively

the fish in the LF and 0.5LF groups. The growth responses from August to October differ from the observations of Johansen, Sveier and Jobling (2003), where Atlantic salmon fed a high-fat diet during both the build-up and main phase, maintained their body fat levels after the build-up phase, at the same time as feed intake was down-regulated and growth impaired. In the present study, the salmon were exposed to natural photoperiod, as opposed to the study by Johansen et al. (2003), where the salmon were held under continuous light (24L:0D). It has been suggested that reduction in day length is an important environmental factor that triggers the salmon to assess its current mass during this time of the year (Maclean & Metcalfe, 2001). It may also apply for energy status and body condition (Kadri, Mitchell, Metcalfe, Huntingford & Thorpe, 1996). In addition, high retention of dietary lipid, elevated fat deposition, increased CF and rapid growth are observed during the autumn period (Alne et al., 2011; Dessen et al., 2017; Kadri et al., 1996; Mørkøre & Rørvik, 2001). Hence, the influence of natural seasonal cues might

be the main reason for the observed differences in growth between the present study and the one of Johansen et al. (2003).

In October, 2 months after the start of the main-dietary phase, muscle fat and CF were restored in both the LF and 0.5LF groups compared to the HF group. This observation shows that Atlantic salmon is able to rapidly replenish lipid stores and body condition during the autumn following a feeding period of a low-fat diet or restricted ration of this diet. In contrast, the visceral fat content among the groups maintained about the same pattern throughout the study. The level or severity of restricting lipid deposition during pre-dietary phase was highly negatively related with the magnitude of the subsequent growth response from August to October. This was particularly linked to the relative muscle fat content at termination of the pre-dietary phase prior to autumn. The degree of CG response seems also related to the level of deviance in body condition, length and mass in the restricted or deprived fish groups compared to their non-treated counter-specifics (Alvarez & Nicieza,
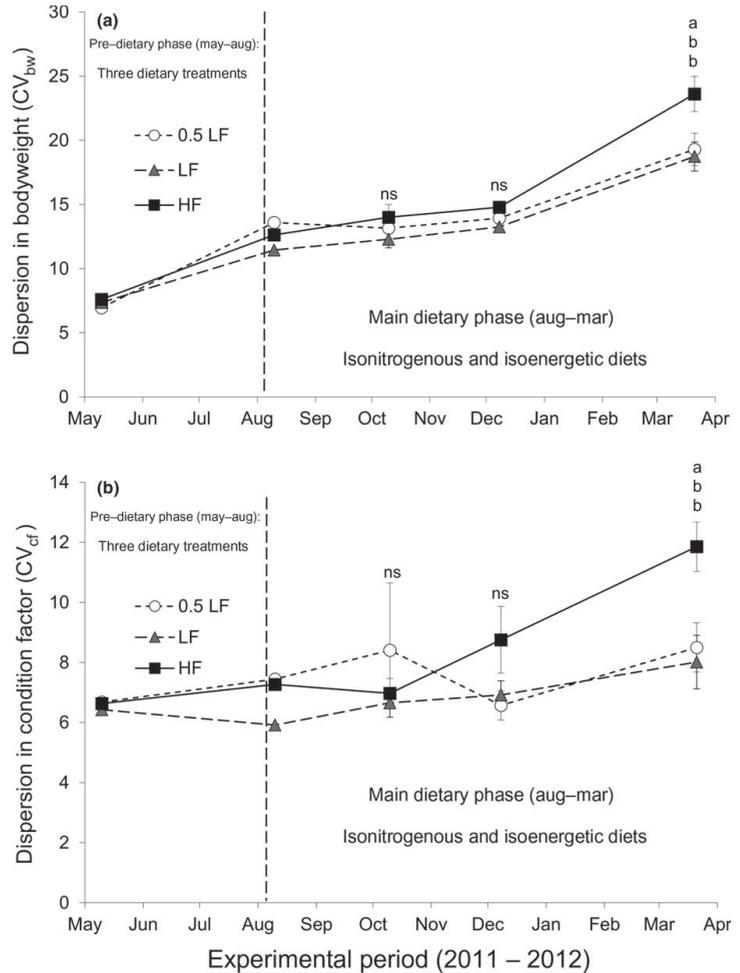
□ 11.3 % (0.5LF)  ■ 13.2 % (LF)  ■ 16.4 % (HF)

**FIGURE 7** Visceral fat development of Atlantic salmon fed three different pre-dietary treatment from May to August 2011. Values are means $\pm$ SEM, $n = 4$ ($n = 1$ at termination of the pre-dietary phase). Values not sharing common superscript letters within each sampling period are significantly different ($p \leq .05$). The values 11.3%, 13.2% and 16.4% represent the obtained fat content at the beginning of the main-dietary phase (August 2011) for the 0.5LF, LF and HF groups, respectively

2005; Johansen et al., 2001; Johnsson & Bohlin, 2005, 2006). Although the deviance in mass and length may have contributed to the growth response in the present study, the small difference between the LF and HF groups in August and the strong correlation between muscle fat and growth indicate that fat/energy status seems to be the most important growth regulator during August and September. The increased growth and rapidly replenishment of lipid stores suggest a robust mechanism for the regulation of body fat, and are in line with the observation of Silverstein, Shearer, Dickhoff and Plisetskaya (1999).

Several studies have indicated that animals displaying CG prioritize the restoration of body condition and fat stores before more resources are allocated to support structural and skeletal growth (Broekhuizen, Gurney, Jones & Bryant, 1994; Johnsson & Bohlin, 2006). In part, the results of the present study support these observations, as both the relative muscle fat content and CF were quickly restored in the 0.5LF group, but not that quickly restored for BW and length. Some studies have also suggested that structural restoration can be delayed due to the effects of food deprivation or restriction on the endocrine system, involved in the regulation of growth (Björnsson, 1997; Johnsson, Jönsson & Björnsson, 1996). There is evidence that skeletal and muscle growth are independent processes and that the relationship between length and weight is approximately cubic (Einen, Waagan & Thomassen, 1998; Jobling, 2002; Mørkøre & Rørvik, 2001). Thus, changes in weight are relatively greater than in length, and the rapid increase in BW and fat content observed among the 0.5LF group in the autumn, may be a factor explaining why length is restored later than body shape and fat content.

The stabilization of the muscle fat in October coincides with the study of Mørkøre and Rørvik (2001). This may suggest that the capacity of muscle fat deposition has reached an upper limit at this time point. There is documentation that CG responses will cease as lipid stores and body condition are restored to similar levels as the non-affected conspecifics (Ali et al., 2003; Alvarez &

Nicieza, 2005; Johansen et al., 2001; Johnsson & Bohlin, 2005). In the present study, the LF and 0.5LF groups continued to grow faster than the HF group both during the periods October to December and December to March. The improved growth of the LF groups from December to March was evident although the relative muscle fat content, CF and BW were restored prior to this period and not significantly different from the HF group. Hence, the observed growth response in this period is not directly related to restoration of fat or BW. The sexual maturation process in Atlantic salmon requires, in addition to photoperiod, sufficient fat and energy reserves (Kadri et al., 1996; Rowe & Thorpe, 1990; Taranger et al., 2010). The production of gonads is energetically expensive and acquire high-energy investment (Fleming, 1996; Jonsson, Jonsson & Hansen, 1997). Appropriate and available energy and fat reserves during the spring period seem to be a major factor controlling initiation and proceeding of the maturation process (Thorpe, 1994; Thorpe, Mangel, Metcalfe & Huntingford, 1998; Wright, 2007). Too low energy and fat levels may arrest the maturation process and postpone reproduction (Duston & Saunders, 1999; Rowe & Thorpe, 1990; Rowe, Thorpe & Shanks, 1991; Thorpe, 1994; Thorpe, Talbot, Miles & Keay, 1990). Hence, well-growing salmon with a high and stable fat content are more likely to adopt the development pathway of becoming sexual mature (Thorpe, 1994). Following this line of arguments, the stronger growth response observed in both LF groups compared to the HF group prior to the spring period in the present study may have been triggered by the salmon reproductive life strategy. However, to verify this, the groups of salmon must be studied for a longer period (during late spring, summer and autumn) and measurements of relevant plasma hormones, gonad-somatic index and gene expression of, for example, myosin should be conducted. Unfortunately, this was not possible in the present study. Anyhow, observation of a long-term improved growth response is important for a further development of a dynamic seasonal feeding concept in salmon farming. Not only for traditional sea cage farming, but

**FIGURE 8** Variation in body weight (gram) (a) and condition factor (CF) (b) assessed using coefficient of variation (CV; mean $\times SD^{-1}$) among Atlantic salmon fed three different pre-dietary treatment from May to August 2011. Values are means $\pm SEM$, $n = 4$ ($n = 1$ at termination of the pre-dietary phase). Values not sharing common superscript letters within each sampling period are significantly different ($p \leq .05$). ns, not significant

also in closed and semi-closed production units where photoperiod may be manipulated. Taken into consideration that the initial BW of the 0.5LF group was 738 g less than the HF group, a relative increase in weight gain of 950 g more than the HF group is impressive.

When feed availability is restricted, competition for the feed often increases and dominant individuals may try to monopolize the feeding area to obtain larger amounts of feed that is supplied (Maclean & Metcalfe, 2001; Ryer & Olla, 1996). High competition for feed may therefore lead to increased disparities in feed intake and growth that consequently will give higher variation in BW. To minimize such effects, the 0.5LF group was administrated all daily feed in only one ration during the pre-dietary phase. The high dispersion in BW and CF among the HF group at termination of the main-dietary phase indicates that the 0.5LF and LF groups had an increase in weight and CF that was more homogeneous than the HF group. This was probably due to the increased growth of LF groups

in latter stages of the trial. The possibility that fish among the LF groups displayed aggressive behaviour and tried to monopolize food in this period seem unlikely due to three main factors: (i) the HF group showed a normal and satisfying growth with mean TGC of 3.2, (ii) feed was administered in excess during the main-dietary phase to ensure ad libitum feeding and (iii) no or little fin damage were observed at termination.

In summary, salmon with low body fat levels (LF groups) prior to declining day lengths in the autumn displayed significantly higher growth rate and weight gain compared to the control fish (HF group). The initial differences in muscle fat and CF were restored after only 2 months, displaying rapid replenishment of lipid stores and body condition. Differences in body fat content prior to autumn had significant effect on growth throughout the whole 7-month main-dietary phase, even after similar body fat stores among the groups were obtained, indicating a more complex explanation than just a lipostatic regulation mechanism. The

LF-and 0.5LF-fed fish obtained a significantly lower variation in BW and CF than the HF-fed fish at trial termination. This increased uniformity of BW and CF may reduce the amount of manual gutting and filleting of large and small individuals, which optimizes the efficiency of automatic gutting and filleting of salmon at the time of slaughter.

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# Paper III

ORIGINAL ARTICLE

WILEY  *Journal of* **Fish Diseases**

# Increased dietary protein-to-lipid ratio improves survival during naturally occurring pancreas disease in Atlantic salmon, *Salmo salar* L.

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## Abstract

This study demonstrated that increased dietary protein-to-lipid ratio (P/L-ratio) improved survival of farmed Atlantic salmon naturally affected by pancreas disease (PD). In addition to diet, body weight (BW) and delousing mortality prior to the PD outbreak also contributed significantly ($p < 0.05$) to explain the observed variation in PD-associated mortality. Subsequent to the PD outbreak, large amount of fish failed to grow and caused thin fish with poor condition (runts). At the end of the trial, significantly ($p < 0.05$) lower amounts of runt fish and increased amount of superior graded fish where detected among fish fed increased P/L-ratio and within the fish with the largest BWs prior to PD. Diet, BW and delousing mortality contributed significantly ($p < 0.05$) to explain the variation in the amount of superior graded fish, whereas BW and diet explained the variation in the amount of runt fish. A significant ($p < 0.01$) negative linear relationship was observed between the amount of superior graded fish and the total mortality, whereas a positive linear relationship was detected between percentage of fillets with melanin and the total mortality. Thus, increased dietary P/L-ratio seem to reduce the mortality and impaired slaughter quality associated with PD.

**KEYWORDS**
Atlantic salmon, body weight, dietary protein-to-lipid ratio, mortality, pancreas disease, runts

## 1 | INTRODUCTION

Pancreas disease (PD) is a widespread contagious viral disease affecting Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*) representing a significant problem in the European salmonid farming industry (Graham et al., 2011; Jansen et al., 2017). *Salmonid alphavirus* (SAV) is the causative agent of PD in farmed Atlantic salmon and rainbow trout (Weston, Welsh, McLoughlin, & Todd, 1999). In Norway, the SAV subtype 3 and a marine subtype 2 have been detected (Hjortaas et al., 2013; Hodneland, Bratland, Christie, Endresen, & Nylund, 2005). Since 2003, PD caused by SAV3 has been endemic along the west coast of Norway up to

Hustadvika in Møre and Romsdal (63° latitude), referred to as the SAV3 endemic zone (Jansen, Jensen, & Brun, 2015; Jensen & Gjevre, 2018). PD causes pathological changes that involve partly or severe loss of exocrine pancreatic tissue, cardiac and skeletal myopathies, epicarditis, and white skeletal muscle degeneration and/or inflammation (Christie et al., 2007; McLoughlin & Graham, 2007; Taksdal et al., 2007). Mortality rates can reach up to 63% for sites that are severely affected by PD and among surviving fish, subsequent failure to grow may cause thin fish with poor condition (runts) and high number of discarded fish at slaughter (reviewed by Jansen et al., 2017). Several studies have found that PD may also impair the fillet quality of slaughter sized salmon (Larsson et al., 2012; Lerfall et al.,

2012; Taksdal, Wiik-Nielsen, Birkeland, Dalgaard, & Mørkøre, 2012). A newly published economic simulation showed that PD caused a total direct cost for Norwegian fish farmers in 2015 of 2,366–2,775 million NOK (Vedeler, 2017). This is equivalent to an increase in production cost of about 2.2 NOK/kg head on gutted salmon (HOG).

Heart and skeletal muscle inflammation (HSMI) is another common fatal disease in farmed salmon and the disease has been linked to the piscine orthoreovirus (PRV) (Palacios et al., 2010). HMSI gives direct inflammatory lesions in cardiac and skeletal muscle and such damage may occur at an early stage in the disease progression, and may persist for many months after clinical disease (Kongtorp, Halse, Taksdal, & Falk, 2006). The lesions observed during outbreaks of HSMI are similar to those described for PD and cardiomyopathy syndrome (CMS) (Kongtorp, Kjerstad, Taksdal, Guttvik, & Falk, 2004). Outbreaks of HSMI may lead to decreased appetite, reduced feed utilization and increased mortality, and although the mortality and duration of the outbreak can vary, mortality rates up to 20% are observed (Alne et al., 2009; Kongtorp et al., 2006). Stress is generally related to the outbreaks of diseases and increased mortality caused by HSMI and PD are often reported in association with handling and operation measures at site level, for example, delousing and relocating fish (Bornø & Lie, 2015). Sea lice infestation levels have increased dramatically in salmonid aquaculture over the last decades, which in turn has increased the resistance against pharmaceuticals commonly used for delousing (Aaen, Helgesen, Bakke, Kaur, & Horsberg, 2015). Due to this, the development and use of nonpharmaceutical treatments that remove sea lice by mechanical or thermal treatments has increased (Lekang, Salas-Bringas, & Bostock, 2016). Mechanical or thermal treatments units are usually placed on barges or ships, which makes it necessary to crowd and pump the fish into these systems. These handling procedures have shown to cause stress, mortality, and reduce fish health (Erikson, Gansel, Frank, Svendsen, & Digre, 2016).

Energy dense diets with high lipid content are extensively used in intensive salmonid fish farming (Torrissen et al., 2011), in which the lipid content is gradually adjusted with fish weight regardless of season. PD and HSMI can occur throughout the annual cycle, but the risk of outbreaks is high during the spring and summer months as sea temperature and day length increase (Hjeltnes, Bornø, Jansen, Haukaas, & Walde, 2017; McLoughlin & Graham, 2007; Rodger & Mitchell, 2007; Stene, Bang Jensen, Knutsen, Olsen, & Viljugrein, 2014). In particular, the majority of PD detections are recorded in June and July within the SAV3 endemic zone (Hjeltnes, Walde, Bang Jensen, & Haukaas, 2016), which often coincides with increased lice infestation and handling procedures. The pathology of PD in salmonids has some resemblance to exocrine pancreatic injuries in canine (Säteri, 1975). Feeding a high-fat low-protein diet has shown to induce pancreatitis and lead to extensive infiltration of fat in the liver of dogs (Lindsay, Entenman, & Chaikoff, 1948). Experimentally induced pancreatitis in adult dogs was also shown to be intensified by a high-fat diet (Haig, 1970). Additionally, long-term intake of high-fat diet in rats with hyperlipidaemia induced pancreatic injuries and oxidative stress

(Yan, Li, Meng, Ren, & Kou, 2006). Today, there are several commercially available feeds for salmonids that are marketed as functional feeds for viral diseases, either for clinical use after infection or as feeds with prophylactic, immune stimulating, or anti-inflammatory effects. The effectivity of some of these functional feeds has been reported in scientific studies. In line with this, low-lipid diets containing high levels of specific fatty acids have been used as a potent tool to control HSMI and CMS in Atlantic salmon by modulating tissue fatty acid composition and eicosanoid production (Martinez-Rubio et al., 2012, 2014). Commercial available "PD feeds" are frequently used within the SAV3 endemic zone (Jansen et al., 2015), and these feeds are often formulated to have lower lipid and higher protein content than standard diets. To our knowledge, little scientific literature is published on the potential effects of such feeds on mortality- and quality-related parameters in large farmed salmon.

This study evaluated the effects of increased dietary protein-to-lipid ratio in salmon diets on mortality rates, biometric-, and quality-related parameters during the entire grow-out period in sea, within the SAV3 endemic zone. The study was designed to test a diet with a significant reduction in fat content during the spring and summer months, when the risk of viral disease outbreaks is high. During the first summer at sea, a co-infection of SAV3 and PRV was detected and a natural PD outbreak was observed. Significant dietary and disease-related effects on mortality rates, biometrics, quality, and slaughter parameters are reported.

## 2 | MATERIAL AND METHODS

### 2.1 | Ethical statement

The research reported in this study was approved by The Norwegian Directorate of Fisheries, allowance H-R-19 and H-R-20, and was carried out in accordance with national guidelines, laws and the animal welfare act. Fish were treated as production fish up to the point of tissue sampling which was only conducted *postmortem* (according to regulation FOR-2015-06-18-761).

### 2.2 | Research facility and fish material

The present trial was carried out at Nofima large-scale research and development (R&D) facility in collocation with Blom Fiskeoppdrett AS, at the site Otterholmen (Radøy in Hjeltefjorden, Hordaland, west coast of Norway). The R&D facility consisted of six 120 m circumference net pens and a feed barge with an automated feeding system. Atlantic salmon under-yearling smolt (S0) were transferred to sea and stocked in the net pens at three time points, with two pens being stocked on 17 of September 2014 (average weight 88 g), three pens on 30 of September 2014 (average weight 75 g) and the final pen on 7 of October (average weight of 76 g). The smolts (SalmoBreed strain, SalmoBreed AS, Bergen, Norway) were reared at the same fresh water facility (Strømsnes Akvakultur AS, Hordaland, Norway) prior to sea transfer. The fish were vaccinated

with NORVAX® Compact PD (MSD Animal Health, Bergen, Norway) followed by ALPHA JECT® micro 6 (Pharmaq AS, Oslo, Norway). Before sea transfer, all fish were fed a commercial BioMar intro 40 diet with the following chemical composition (wet weight, as is basis): dry matter 92.8%, crude protein 48.3%, crude lipid 21.5%, starch 7.0% and ash 9.7%. Pen number, dietary treatment group, smolt group, specific time of sea transfer, average weight at sea transfer, and number of fish for all pens at the site are shown in Table 1.

## 2.3 | Environmental conditions, diagnosis, and disease outbreak

To reduce early sexual maturation, four underwater lamps (800 W) that provided artificial light (L:D 24:0) were placed in each pen from January 2015 until May 2015. Figure 1 gives an overview of the seawater temperature and the day length during the total production cycle. The average temperature during the trial was 9.5°C, with the highest recorded temperature of 16.2°C in late august 2015 and the lowest temperature of 5.2°C in the beginning of March 2016. The fish were reared under standard farming conditions representative for commercial production.

From the 2 to 5 of June 2015, all fish were deloused by thermal treatment using a Thermolicer® machine (Steinsvik AS, Tysvær, Norway) exposing the fish for short term to moderately heated water (30–34°C) to inactivate the sea lice, which subsequently detached from the fish (Grøntvedt et al., 2015). Subsequent to the thermal delousing treatment, an acute increase in mortality was observed (referred to as delousing mortality, see Table 4). In the end of June 2015, the appetite of the fish was reduced and a small increase in mortality was registered, indicating a possible disease outbreak. Thus, a routine inspection of the site was performed by the local veterinary service (Fishguard AS, Bergen, Norway) on 29 of June. Fish from three of the six pens, representing both dietary treatments, were sampled and different organs were for dissected and evaluated. Gill, heart ventricle and atrium, pancreas, and, red and

white skeletal muscle were fixed in 10% buffered phosphate formalin and sent to the National Veterinary Institute of Norway (NVI, Bergen, Norway) for histopathological examination. After fixation, the samples were processed and embedded in paraffin wax according to standard NVI procedures, and sections were stained with haematoxylin and eosin, before they were inspected by light microscopy. Small pieces of heart ventricle from the same fish were also sampled in RNA *later*® for real time RT-PCR analysis. The RT-PCR analysis were conducted by PHARMAQ Analytiq AS (Bergen, Norway) using their in-house real-time PCR assays (for detection of viral RNA) and Elongation factor 1A (Ef1α) as quality measure/reference gene. The histopathological examination revealed atrophy of exocrine pancreatic tissue and, necrosis and hyper cellularity of the connective tissue between the muscular threads in the red skeletal muscle. Necrosis and degeneration were also observed in the white skeletal muscle, but less pronounced than in the red muscle. In the heart samples, there were varying degrees of epicarditis, hyper cellularity, and necrosis in the compact layer (compacta) and spongiosa. The RT-PCR analysis showed that all samples were found positive for SAV3 and PRV with a mean ($\pm SEM$) obtained cycle-threshold (Ct) values of 18.1 ± 0.8 and 27.2 ± 1.8, for SAV and PRV, respectively. Based on the tissue changes detected by histopathology, which were similar to those characteristic for PD (Taksdal et al., 2007), combined with the detection of SAV3, the fish at the site were diagnosed with PD. The varying histopathological changes in the heart, skeletal muscle, and the detection of PRV may also resemble a possible HSMI.
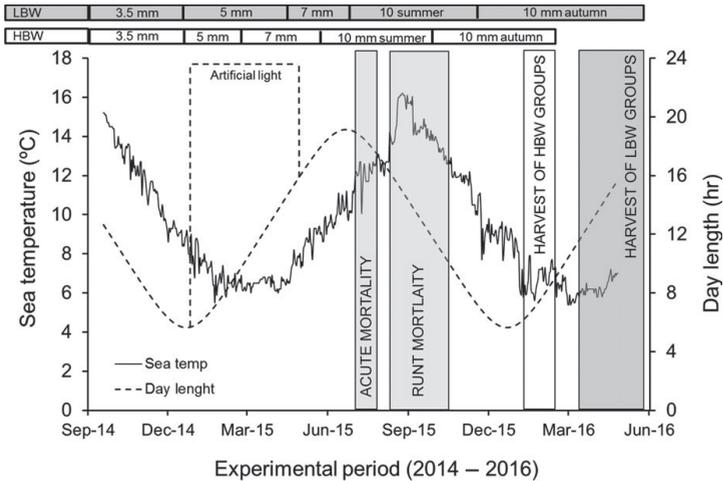
In the beginning of July 2015, a marked increase in mortality and severe reduced appetite was observed among all net pens. The increase in mortality lasted for 1 month and was defined as cumulative acute mortality (30 of June to 30 of July). From the 13 of August and until 23 of October, mortalities of runts were observed in all pens and the mortality in this period was defined as cumulative runt mortality. This was especially high in conjugation with the delousing treatments (Thermolicer treatment the 15 to 17 of September and hydrogen peroxide ($H_2O_2$) treatment with well boat the 9 to 12 of October). Runts were defined as marked skinny/thin salmon that had stopped eating and were smaller and/ or weaker than the other individuals from the same group.

## 2.4 | Dietary treatments, feeding, and daily routines

Two diet series (Test and Control) were defined and each diet was fed to one cage of salmon that was transferred to sea in mid-September and two replicate cages of fish that were transferred to sea in late September/early October 2014. BioMar AS (Karmøy, Norway) formulated and produced all feeds, according to target dietary protein and lipid contents defined by Nofima. Identical premixes of vitamins were used in the control and test diets. For each production batch of feed, both diets were as far as possible made from the same batches of raw materials. However, as the feed was produced during the entire experimental period (19 months), different batches of commercial raw materials were used during the production cycle. The experimental diets were produced as 3.5, 5, 7,

**TABLE 1** Pen number, dietary treatment group, time of sea transfer, average body weight (BW) at sea transfer and number of fish for all pens at NOFIMA large-scale research & development (R&D) facility in collocation with Blom Fiskeoppdrett AS, at the site Otterholmen

| Pen no. | Dietary treatment | Sea transfer, date | Average BW at sea transfer, gram | Number of fish |
|---|---|---|---|---|
| 1 | Control | 30.09.2014 | 75 | 125,304 |
| 2 | Control | 17.09.2014 | 88 | 99,097 |
| 3 | Control | 30.09.2014 | 75 | 100,884 |
| 4 | Test | 30.09.2014 | 75 | 104,627 |
| 5 | Test | 17.09.2014 | 88 | 99,758 |
| 6 | Test | 07.10.2014 | 76 | 126,353 |

**FIGURE 1** Ambient daily sea temperature (°C, y-axis) and day length (hours of daylight, z-axis) during the production cycle (September 2014 to June 2016). The feeding period of the different diets for the fish groups transferred to sea in mid-September 2014 (High bodyweight group, HBW) and in late-September/ October (Low bodyweight groups, LBW) are shown on the top of the figure. The timing of mortality events and harvest periods are indicated within the figure

and 10 mm pellets. The 10 mm pellets were produced in two different versions within both experiment diets, denoted 10 mm summer and 10 mm autumn. This was done in order to test the effect of a diet with a marked reduction in fat content within the test series during the summer period (Test – 10 mm summer), before the fat level was upregulated during the autumn and winter (Test – 10 mm autumn). The feeding period of the different diets are indicated in Figure 1.

Crude protein, lipid, and water content in all pellet sizes were assessed by on-line NIR analysis by BioMar. Dietary astaxanthin concentrations were not analysed and are given as declared content in each diet. Total starch content was analysed in 1–3 representative feed samples of each pellet size, using a modification of the glucoamylase method (developed by Wenger Manufacturing, Inc., USA) in accordance with Mason, Gleason, and Rokey (1982). Table 2 gives the proximate chemical composition of the different diets. The protein-to-lipid ratio of the control diets gradually decreased, and the total energy level increased, with the increase in pellets size. Hence, the composition of the control diets resembled typical commercial diets for the respective sizes of fish, whereas the test diets had higher protein and lower lipid content. The test and control diets had similar gross energy content, except for the 10 mm summer diets, for which the test diet contained less energy (21.8 MJ/kg) than the control (23.8 MJ/kg) and had twice as high protein-to-lipid ratio (test = 1.9 vs. control = 1.0). This was done in order to test a dietary composition with a substantially reduced fat content during the spring and summer months, when the risk of viral disease outbreaks is high. The 10 mm summer diets were used before and during the natural outbreak of PD, and the fatty acid (FA) composition of diets are shown in Table 3. The FAs were analysed according the AOCS Official Method (Ce 1b-98) using gas chromatography after lipid extraction (Bligh & Dyer, 1959).

The feeds were distributed to the net pens using an automated central pneumatic feeding system (CCS feed system unit, AkvaGroup AS, Bryne, Norway) similar to the system described in Aas et al. (2011). The size and frequency of meals were adjusted based on day length fish size, biomass, and appetite. The fish were fed to apparent satiation, assessed using an underwater camera system (AkvaGroup AS, Bryne, Norway). Feeding tables based on fish size and temperature was used as guidance, but the daily appetite was as far as possible interpreted by camera. Dead fish were pumped up into containers on each pen through an elastic plastic pipe using pressurized air (LiftUP system, Eiklandsosen, Norway) on daily basis. The feeding volume (kg/day), type of feed, number of dead fish, and seawater temperature were recorded daily and registered in the Mercatus Farmer software (Steinsvik AS, Tysvær, Norway). The biomass and weight increase were estimated weekly using the data from Mercatus Farmer software, as a function of feed intake, feed type, season, temperature and assumed feed conversion ratio depending on fish weight. If the number of dead fish exceeded ~1,000 individuals daily (massive runt mortality in conjunction with delousing during September and October), dead fish were pumped directly into 1,000 L tanks. The total number of runt fish that one 1,000 L tank is able to contain was recorded once, and this number was then used as a measure to calculate the total number of dead runt fish (depending on the number of 1,000 L tanks filled).

## 2.5 | On-site samplings

One cage of salmon transferred to sea in mid-September and one cage of fish transferred in late September 2014, within both dietary groups (pen 2, 3, 4, and 5), were sampled at several occasions in sea, including before and after the PD outbreak (June and August 2015). At each sampling point, 10 fish from each pen were sampled. The sampled fish were selected so that the mean body weight (BW) of the 10 fish corresponded to the estimated mean weight of all the

**TABLE 2** The proximate chemical composition of the different experimental diets. The values for protein, lipid and water content are based on the mean Near Infrared (NIR) analysis of each batch, weighted for the total amount of feed produced in each batch of the particular pellet size

| Pellet size (mm) | 3.5 mm | | 5 mm | | 7 mm | | 10 mm summer | | 10 mm autumn | |
| Diet code | Test | Control | Test | Control | Test | Control | Test | Control | Test | Control |
|---|---|---|---|---|---|---|---|---|---|---|
| Crude protein, % | 50.8 | 46.6 | 46.2 | 44.4 | 47.4 | 42.1 | 47.1 | 35.7 | 39.9 | 34.7 |
| Crude lipid, % | 24.0 | 26.4 | 27.0 | 29.3 | 27.2 | 30.7 | 23.6 | 35.5 | 34.7 | 38.2 |
| Water, % | 5.3 | 5.2 | 5.3 | 4.8 | 5.6 | 5.5 | 6.7 | 5.5 | 6.1 | 5.8 |
| Total starch[a], % | 7.8 | 7.4 | 8.4 | 9.3 | 7.7 | 7.5 | 7.7 | 7.5 | 8.4 | 8.1 |
| Astaxanthin[b], mg/kg | 20.0 | 20.0 | 20.0 | 20.0 | 37.5 | 37.6 | 40.0 | 40.0 | 56.5 | 57.9 |
| Estimated gross energy[c], MJ/kg | 22.9 | 22.7 | 23.1 | 23.7 | 23.3 | 23.4 | 21.8 | 23.8 | 24.6 | 24.7 |
| Estimated CP/GE ratio, g MJ kg$^{-1}$ | 22.2 | 20.5 | 20.0 | 18.7 | 20.3 | 18.0 | 21.6 | 15.0 | 16.2 | 14.0 |

*Notes.* CP: crude protein; GE: gross energy; MJ: mega joule.
[a]The starch content was analysed chemically on 1–3 feed samples from representative batches of each particular pellet size. [b]The astaxanthin values are based on the declared content due to lack of the chemical analysis of astaxanthin level for all diets. [c]The gross energy content was calculated assuming 23.7, 39.5 and 17.2 MJ/kg of protein, lipids and starch, respectively.

fish in the respective pen. All fish selected for samples were sedated (Benzoac® 2 ml × 10 L$^{-1}$) and killed by a blow to the head, gill arches were then cut and the fish were bled in sea water. Length and weight of each individual fish sampled were recorded, before the fish were gutted and the gutted weight (GW) registered. The Norwegian Quality Cut, NQC (NS 9401, 1994) were then cut, packed in plastic bags and stored in styrofoam salmon boxes filled with ice. The samples were then transported to Nofima laboratories in Ås and stored at 3–4°C. After 4 days on ice, by which time they had entered a postrigor state, the left NQC fillet was photographed using PhotoFish (AKVAgroup, Bryne, Norway). The fat (%) and astaxanthin content (mg/kg) were predicted by PhotoFish digital image analyses, as described by Folkestad et al. (2008) and Rørvik, Rørvik, Salberg, and Larsson (2014). Texture analyses were performed instrumentally (TA-XT2, Stable Mirco Systems Ltd, Surrey, England) by pressing a flat-ended cylinder (12.5 mm diameter, P/0.5) into the NQC-fillet perpendicular to the muscle fibres at 1 mm/s, according to the procedure described by Larsson et al. (2014). The force (Newton, N) required to puncture the fillet surface was registered and defined as the firmness of the fillet. The analysis of astaxanthin was conducted from the sampling in March 2015 and onwards (due to the low levels of astaxanthin before this time point), whereas the texture analysis was conducted from the sampling in April 2015 and onwards (due to the size of the NQC).

On the 23 of September, during the runt mortality, an additional sampling was conducted to register the biometrics and characteristics of the dying runt fish (BW, length, body shape, visual appearance). These fish were also filleted on site and the colour of the fillets (evaluated using DSM SalmoFan™ score, 20–34), prevalence, size, score, and location of melanin spots on the fillets (Mørkøre, 2012), were recorded for all sampled fish. During the delousing treatment using hydrogen peroxide with well boat on 9 to 11 of October, over 80,000 individuals from each pen were scanned using a pipe CSF-counter (AquaScan AS, Bryne, Norway)

on the well boat, in order to estimate the average weight and weight distribution in each pen. The data from the scans were continuously transferred to a control unit and the data were processed using AquaScan Win computer program (AquaScan AS, Bryne, Norway).

## 2.6 | Quality evaluations at harvest

All pens were harvested according to Blom Fiskeoppdrett AS standard procedures and slaughtered at Sotra Fiskeindustri AS (Glesvær, Norway) from 12 of January 2016 to 3 of June 2016 (Figure 1), trying to achieve a mean BW of approximately 4.8 kg for all pens, equal to ~4 kg GW. The harvest procedure included a starvation period of 5 days prior to transportation with well boat from the R&D site to the slaughter facility. At the slaughter facility, all fish were stunned with electricity, gill arches were cut and the fish were transported to a bleeding tank with a water temperature between 1°C and −1°C. After the fish had bleed out, the fish were gutted and rinsed, GW was individually recorded and the fish were then graded into the main categories "Superior," "Ordinary," "Production," or "Discard" according to Norwegian Industry Standards for Fish, regarding quality grading of farmed Atlantic salmon (Norsk Bransjestandard for Fisk, 1999). On the first day of slaughter for each pen, 200 salmon in the weight class segment of 4–5 kg were automatically filleted using BAADER 581 filleting machine (BAADER Group, Lübeck, Germany) and trimmed using BAADER 988 trimming machine (BAADER Group, Lübeck, Germany), removing the vertebra, ribs and belly membrane. A total of 400 fillets from these salmons (200 right and 200 left) were visually screened for prevalence, size/ score and location of melanin spots according to Mørkøre (2012). In addition, 10 fish with an average BW of 5 kg were taken from net pens 2, 3, 4, and 5 on the slaughter line (before gutting) for analysis of fat, pigment and texture of the fillet using the same analytical methods as described for onsite samplings. Harvest reports based on

**TABLE 3** The fatty acid (FA) composition of the test and control 10 summer diet used in the period before and during the natural outbreak of pancreas disease

| Pellet size (mm) | 10 mm summer | |
|---|---|---|
| Diet | Test | Control |
| FA composition (% in B&D extract) | | |
| 14:0 | 1.8 | 1.9 |
| 16:0 | 8.9 | 9.0 |
| 18:0 | 2.6 | 3.1 |
| 20:0 | 0.5 | 0.6 |
| 22:0 | 0.7 | 0.9 |
| 16:1 n−7 | 2.0 | 2.0 |
| 18:1 (n−9) + (n−7) + (n−3) | 38.2 | 40.9 |
| 20:1 (n−9) + (n−7) | 3.0 | 2.5 |
| 22:1 (n−11) + (n−9) + (n−7) | 2.9 | 2.2 |
| 24:1 n−9 | 0.3 | 0.3 |
| 16:2 n−4 | 0.2 | 0.2 |
| 16:3 n−4 | 0.2 | 0.2 |
| 18:2 n−6 | 14.4 | 15.0 |
| 18:3 n−6 | 0.1 | 0.1 |
| 20:2 n−6 | 0.1 | 0.1 |
| 20:3 n−6 | <0.1 | <0.1 |
| 20:4 n−6 | 0.2 | 0.2 |
| 22:4 n−6 | <0.1 | <0.1 |
| 18:3 n−3 | 6.2 | 6.7 |
| 18:4 n−3 | 0.7 | 0.6 |
| 20:3 n−3 | <0.1 | <0.1 |
| 20:4 n−3 | 0.2 | 0.2 |
| 20:5 n−3 (EPA) | 3.0 | 3.1 |
| 21:5 n−3 | 0.1 | 0.1 |
| 22:5 n−3 | 0.3 | 0.4 |
| 22:6 n−3 (DHA) | 3.3 | 2.8 |
| Sum saturated FAs | 14.5 | 15.5 |
| Sum monounsaturated FAs | 46.4 | 47.9 |
| Sum n−6 PUFA | 14.8 | 15.4 |
| Sum n−3 PUFA | 13.8 | 13.9 |
| Sum PUFA | 29.0 | 29.7 |
| Sum identified FAs | 89.9 | 93.1 |
| EPA (% of feed) | 0.7 | 1.0 |
| DHA (% of feed) | 0.8 | 0.9 |
| EPA+DHA (% of feed) | 1.5 | 1.9 |

*Note.* B&D: Bligh & Dyer; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; FA: fatty acid; PUFA: polyunsaturated fatty acid.

the recorded number of fish, GWs and quality grading were generated for all experimental pens. As almost all fish graded as discards were runts (by visual inspection and recorded GW), the number of discards (overall average of 6.6% discards) were registered and used together with cumulative runt mortality during the sea phase in order to calculate the total amount of accumulated runt fish in each pen.

## 2.7 | Calculations

The condition factor (CF) was calculated as: $100 \times BW$ (g) $\times$ fork length (cm)$^{-3}$. CF based on GW (CFg) was calculated using GW instead of BW. GW was defined as the weight of the fish when all organs and intestines in the abdominal cavity were removed (including kidney and heart). CFg was used to assess the body shape since the fish were not starved prior to the on-site samplings. The feed intake may normally vary from 0.2% to 2% of the BW on daily basis, depending on appetite, water temperature, and fish size. The CFg removes this issue and helps standardize this measurement for fed fish allowing comparison over time. To evaluate differences in mortality between groups, cumulative mortality, and the relative survival percentage (RPS) were used. RPS was calculated as described by Amend (1981): RPS = $100\% \times (1 - \%$ mortality in test group/% mortality in the control group).

## 2.8 | Statistical analyses

The results were analysed by multiple regression analysis using the General Linear Model (GLM) procedure in the SAS 9.4 computer software (SAS Institute Inc., Cary, NC, USA). Diet was used as class variable, whereas BW on the 29 of June 2015, prior to disease outbreak, and mortality after delousing (2 to 5 of June 2015) were used as covariates to control for differences in BW and handling stress prior to the disease outbreak. To test differences in BW, diet was used as class variable, whereas BW at sea transfer was used as covariate. The pens were used as experimental units and Table 4 shows the statistical variables used in the model and the obtained data registered in the study. The proportion of the total variation explained by the model is expressed by $R^2$ and calculated as the marginal contribution of the mean square of the parameter (type III sum of squares). The proportion of variance explained by each of the significant factors was also calculated. Simple linear regression analysis and figures were computed using Microsoft® Excel. Simple t test was used to test differences in fillet fat, pigment and firmness at slaughter. The level of significance was chosen at $p \leq 0.05$ and tendencies were identified at $p = 0.05$–0.1. To simplify the figures, pens are grouped based on dietary treatment and mean body weight prior to disease outbreak (low body weight, LBW = 1.3 kg; high body weight, HBW = 1.9 kg).

## 3 | RESULTS

In the beginning of July 2015, a simultaneous increase in mortality and reduced appetite were observed among all net pens. This occurred after positive detection of SAV3 and PRV with histopathological changes in line with PD and HSMI at 29 of June (see section Environmental conditions, diagnosis, and disease outbreak for more

**TABLE 4** Overview over the input variables and the obtained data from the study used in the statistical model

| | | Statistical variables used | | Obtained data from study | | | | |
|---|---|---|---|---|---|---|---|---|
| Pen no | Dietary treatment | BW prior to PD, 29.06.15 (g) | Delousing mortality prior to PD (%) | Total acute mortality (%) | Total amount of runt fish (%) | Superior graded fish (%) | Fillets with melanin (%) | BW after PD, 11.10.15 (g) |
| 1 | Control | 1,222 | 0.49 | 6.09 | 27.9 | 73.1 | 34.8 | 2,037 |
| 2 | Control | 1,768 | 0.68 | 2.30 | 14.8 | 83.3 | 19.8 | 2,888 |
| 3 | Control | 1,383 | 0.34 | 4.01 | 21.7 | 79.7 | 24.1 | 1,810 |
| 4 | Test | 1,355 | 0.60 | 4.75 | 14.4 | 85.9 | 16.8 | 2,160 |
| 5 | Test | 1,996 | 0.57 | 0.76 | 7.3 | 96.4 | 13.5 | 3,429 |
| 6 | Test | 1,236 | 0.33 | 1.24 | 15.2 | 89.8 | 16.5 | 2,532 |

*Note.* BW: body weight; PD: pancreas disease.

info). At end of July, the acute mortality levelled off and differences in mean cumulative mortality among the dietary and BW groups were observed (Figure 2 and Table 4). In the statistical model, diet, BW and delousing mortality prior to the disease outbreak were all found to significantly ($p < 0.05$) influence the acute mortality, explaining together 97.6% of the observed variation in mortality (Table 5). Diet, BW prior to PD, and delousing mortality explained 29%, 39%, and 29% of this observed variation in mortality, respectively (Table 5). No significant effect was observed when diet, BW or delousing mortality was used alone as single variables, or if diet and BW were used as variables. Reduced acute mortality was detected among fish fed the test diet and with the largest BWs prior to the disease outbreak (Figure 2). The overall average RPS in the end of July for fish in the test group was 48% compared with the fish in the control group.

Prior to the increase in mortality, only small differences and/or systematic changes in CFg, pigmentation in the flesh and firmness of the fillet were observed among the groups. However, after the natural disease outbreak, clear differences within these parameters were observed on the 12 of August (Figure 3). The test groups had higher

CFg and astaxanthin level compared to the control groups within both weight segments (Figure 3a,b), whereas the opposite was observed for firmness of the fillet (Figure 3c). In addition to the differences in biometric and quality parameters, large amounts of runts were visually observed in all pens. On the 13 of August, runt mortality was detected and high mortality rates of runts were observed during and after handling/delousing in mid-September and in start of October (Figure 4). During October, the runt mortality levelled off and no marked runt mortality was observed during the latter stages of the production. Low BW (752 ± 30 g), CF (0.66 ± 0.02), visual fillet colour (SalmoFan score of 22), and high prevalence of melanin spots in muscle (58 ± 10%) characterized all runt fish during the sampling on the 23 of September (overall mean on pen level, $n = 4$). In addition to be very skinny and have a high prevalence of melanin, postmortem inspection of the runts showed fluid in the abdomen, swollen discoloured liver with fibrin layer, no feed matter in the gastrointestinal system, low levels of visceral fat and petechial haemorrhages in the visceral fat tissue which surrounds the pyloric caeca.

At the end of the trial, a lower total amount of runts among HBW fish compared to that in the LBW fish was detected, and
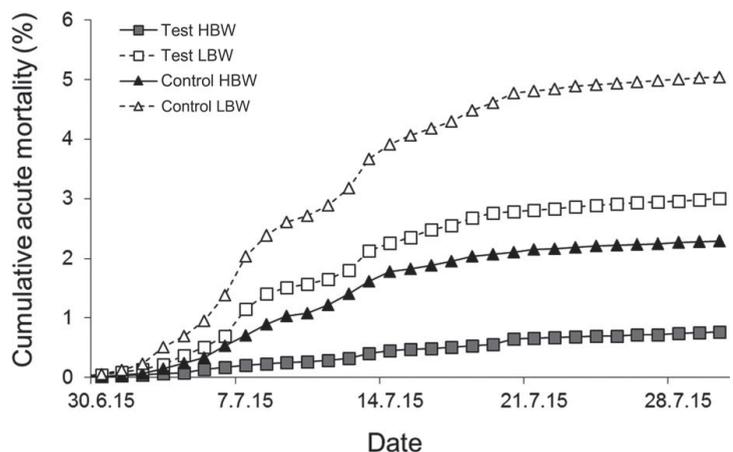


**FIGURE 2** Daily cumulative acute mortality during an outbreak of pancreases disease in farmed S0 Atlantic salmon (30.06.15–30.07.15), fed either a high protein-to-lipid ratio diet (test) or low protein-to-lipid ratio diet (control). The dietary groups are divided into the fish size (High bodyweight, HBW = 1.9 kg and Low bodyweight, LBW = 1.3 kg) at the time of disease outbreak

**TABLE 5** The proportion of the total variance explained by the total model expressed by $R^2$ and each of the significant factors calculated as the marginal contribution of the mean square of the parameter (Type III Sum of Squares)

| Statistical factors | Total acute mortality (%) | Total runt fish (%) | Superior graded fish (%) | Fillets with melanin (%) | BW after PD outbreak (g) |
|---|---|---|---|---|---|
| Total model | 0.98 (0.04) | 0.92 (0.02) | 0.99 (0.002) | 0.57 (0.08) | 0.96 (0.01) |
| Single variables in the model | | | | | |
| Diet | 0.29 (0.03) | 0.45 (0.03) | 0.61 (0.001) | 0.57 (0.08) | 0.16 (0.04) |
| BW prior to PD | 0.39 (0.02) | 0.48 (0.03) | 0.32 (0.002) | ns | – |
| Delousing mortality prior to PD | 0.29 (0.03) | ns | 0.07 (0.007) | ns | ns |
| BW at sea transfer | – | – | – | – | 0.80 (0.01) |

*Notes*. The *p*-values are shown between brackets.
BW: body weight; ns: not significant; PD: pancreas disease.

almost twice as many runts in the control compared to the test group within both weight segments (Table 4 and Figure 5). The statistical model revealed that both diet and BW prior to PD significantly ($p < 0.05$) influenced the total sum of runts, together explaining 92.1% of the observed variation. Diet explained 45% and BW 48% of the observed variation in this model (Table 5). The delousing mortality prior to PD was not found to significantly influence the total amount of runts. Percentages of superior graded fish were higher among the HBW than the LBW groups, and for the test compared to the control group (Table 4). The statistical analysis revealed that all; diet, BW and delousing mortality prior to PD, significantly ($p < 0.05$) influenced the percentage of superior graded fish, together explaining 99% of the observed variation in the model. Diet explained 61% of this variation, whereas BW and delousing mortality explained 32% and 7%, respectively (Table 5). The same trend was observed for percentage of fillets with melanin spots (Table 4), but here the statistical analysis revealed only a tendency ($p < 0.1$) towards a dietary effect, explaining 57% of the observed variation in melanin (Table 5). The percentage of superior graded fish showed a significant ($p < 0.01$) negative linear relationship with the total sum of mortality (acute mortality and accumulated runts) related to the disease outbreak (Figure 6a). Likewise, a significant positive ($p < 0.01$) linear relationship was observed between the total sum of mortality and the percentage of fillets with melanin spots (Figure 6b).

Based on the estimated growth within both weight classes, the test pens had a higher weight gain compared to the control pens, just before, during and after the natural disease outbreak (Figure 7). The obtained data from the scanning of fish during the delousing in October showed also that the test group had a higher BW than the control group within both weight segments (Table 4). The statistical run revealed a significant effect ($p < 0.05$) of diet corrected for the differences in BW at sea transfer, explaining 16% of the variation in BW at this time point (Table 5). In order to achieve approximately equal weight of slaughter, the fish were slaughtered at different times. Thus, the test pens were harvested somewhat before the control pens within each weight segment. At slaughter, no significant differences in muscle fat (16.0 ± 0.3%), fillet pigment (6.2 ± 0.1 astaxanthin mg/kg) or firmness (9.4 ± 0.3 N) were observed between the dietary treatments within the 5 kg BW groups (overall mean on

pen level, n = 4). However, a slightly but significant ($p < 0.05$) higher CFg (test: 1.09 vs. 1.07) was detected for the test group compared to the control group.

## 4 | DISCUSSION

Based on the histopathological observation of the pancreas and detection of SAV3, the salmon in this study were diagnosed with PD at the onset of increased mortality in the end of June 2015. It also resembled other natural outbreaks of PD by (a) the geographical location of the site within the PD endemic zone (b) the timing of the increased mortality and (c) the accumulation of runts after the outbreak. However, we cannot discard the potential influence of the presence of PRV. Bornø and Lie (2015) mentioned that field observations indicated that mortality rates caused by SAV2 were increased when HSMI was previously detected. In contrary, Lund et al. (2016) found that experimental PRV infection mediated protection against PD in small Atlantic salmon postsmolt. PRV seem to be ubiquitous among farmed salmon in Norway (Løvoll et al., 2012), and can be present in high titres without causing mortality or severe lesion in the heart (Garseth, Fritsvold, Opheim, Skjerve, & Biering, 2013).Thus, the following discussion will mainly focus on literature related to PD.

There are several recognized factors affecting the risk for PD outbreaks, such as: farming site (with a previous history of PD at the site or in the neighbourhood) type of smolt used (increased PD among S0 vs. S1 smolts) and sea lice burden (Kristoffersen, Viljugrein, Kongtorp, Brun, & Jansen, 2009; Rodger & Mitchell, 2007). All the mentioned risk factors were met in this study. The PD-associated mortality was also observed 3–4 weeks after the thermal delousing treatment in the beginning of June 2015. The mortality after this delousing treatment explained 29% of the observed variation in cumulative acute mortality. Hjeltnes et al. (2017) refers to PD as a typical stress-related disease where subclinical infections may develop into serious outbreaks after lice treatments. It is generally acknowledged that outbreaks of many disease conditions in aquaculture occur as a result of intricate interactions between the host, agent, and environmental conditions, combined with effects of site management and handling stress (Wheatley, McLoughlin, Menzies, & Goodall, 1995).
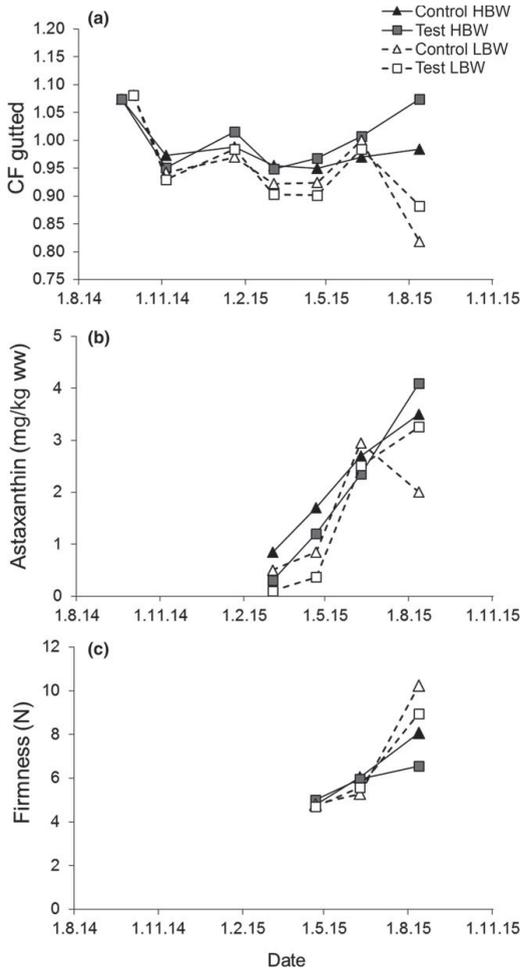
**FIGURE 3** Development in gutted condition factor (CF) (a), astaxanthin content in Norwegian Quality Cut (NQC) (b) and texture firmness of the fillet (c) until the sampling after the outbreak of pancreases disease (12. August 2015) in farmed S0 Atlantic salmon. Definition of HBW and LBW as in figure 2

In this study, the mean PD mortality was 2.8% with a duration of approximately 1 month. When the cumulative runt mortality was included, the total average mortality rate was about 15% with a duration of 4 months. This value is in the mid-range compared to the reported mortality rates (0.7%–26.9%) and duration (1–6 months) associated with natural outbreaks of PD in Norway (Jansen et al., 2010). SAV infections often lead to clinical diseases due to the severe loss of exocrine pancreatic tissue, pancreatic necrosis, and fibrosis that results in lethargy and anorexia combined with increased mortality (Ferguson, Rice, & Lynas, 1986; McLoughlin, Nelson, McCormick, Rowley, & Bryson, 2002). In Ireland and Scotland, it is reported that up to 15% of the fish that survive PD outbreaks may
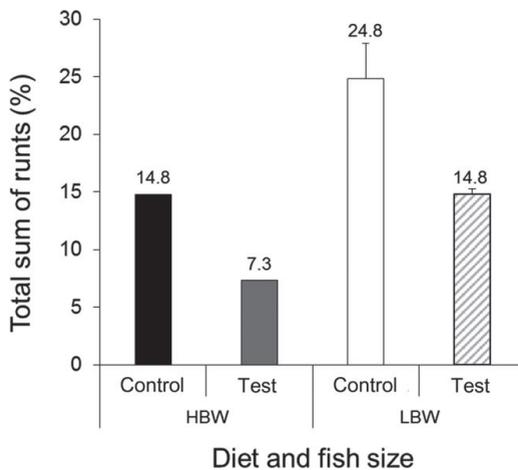
develop into runts (Munro, Ellis, McVicar, McLay, & Needham, 1984; Ruane, Graham, & Rodger, 2008). Most epidemiological studies related to PD in Norway do not categorize the dead fish during PD outbreaks or report statistics related to the amount of runts. However, Taksdal et al. (2007) discuss that the persistent pancreatic damage observed in Norwegian PD outbreaks may results in higher number of runts than in Irish and Scottish farms. Rodger and Mitchell (2007) observed that sites in Ireland with a low PD-associated mortality often have a higher percentage of runts compared to sites with high PD mortality. In this study, large number of runts died during handling in conjunction with delousing, which might not have died otherwise, and a relatively high number of runts were observed at slaughter. In addition, relatively low acute PD-associated mortality was observed. Thus, the results from this study seem to have good consistency with the observation of Rodger and Mitchell (2007).

The delousing mortality and body weights of the pens prior to the disease outbreak were necessary inputs in the statistical model to detect the dietary effects on acute mortality. The fact that these variables contributed significantly to explain the observed variation in acute mortality may indicate that PD-associated mortality is affected by several different factors. In addition to the dietary influence on the amount of runt fish, a highly significant effect of body weight was also observed. The high body weight groups had lower acute mortality and amount of runts compared to the low body weight groups. Normally, larger individuals have more mass and stored energy than smaller individuals. If the amount of stored energy available is of importance for combating viral diseases, this could be a factor contributing to the increased survival among large fish compared to the small ones.

The lower mortality and large reduction in runts among fish fed the test diet compared to those fed the control diet, is an important observation in this study. Previously, diets with reduced lipid content have been shown to reduce the inflammatory responses and associated heart lesions in salmon experimentally challenged with Atlantic salmon reovirus (ASRV) and piscine myocarditis virus (PMCV), that are associated with HSMI and CMS, respectively (Martinez-Rubio et al., 2012, 2014). In these studies, however, the reduced dietary lipid content was combined with increased levels of polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA) and phospholipids, and the dietary causative effects could not be separated. However, it seems that the reduced dietary lipid content was primarily responsible for the positive alterations of different genes involved in lipid metabolism (Martinez-Rubio, Wadsworth, González Vecino, Bell, & Tocher, 2013). Raynard et al. (1991) showed that the progression of PD in salmon was markedly increased by vitamin E deficiency, and that salmon fed reduced levels of PUFAs had increased development of PD compared to salmon fed higher levels of PUFAs. The latter study specifies the importance of antioxidants and how nutritional stress may influence the susceptibility to PD. In a previous study conducted by our research group, significant reductions in HSMI-related mortality among salmon fed tetradecylthioacetic acid (TTA) was observed (Alne et al., 2009). In this study and other similar experiments, it was suggested that the observed reduced HSMI
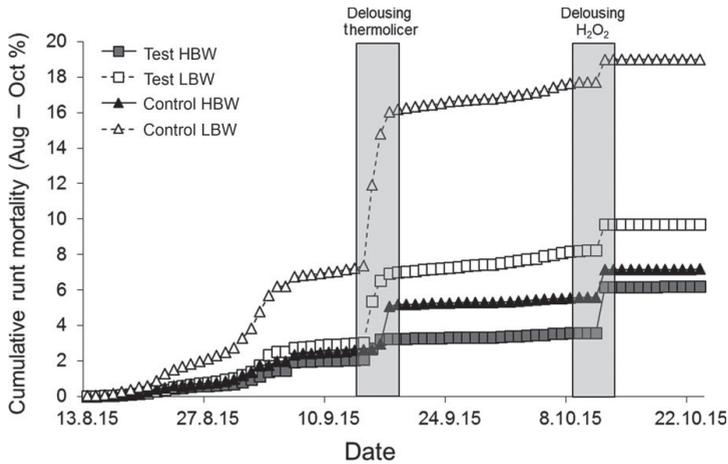
**FIGURE 4** Daily cumulative runt mortality (13.08.15–23.10.15) after an outbreak of pancreases disease in farmed S0 Atlantic salmon, fed either a high protein-to-lipid ratio diet (test) or low protein-to-lipid ratio diet (control). Definition of HBW and LBW as in Figure 2
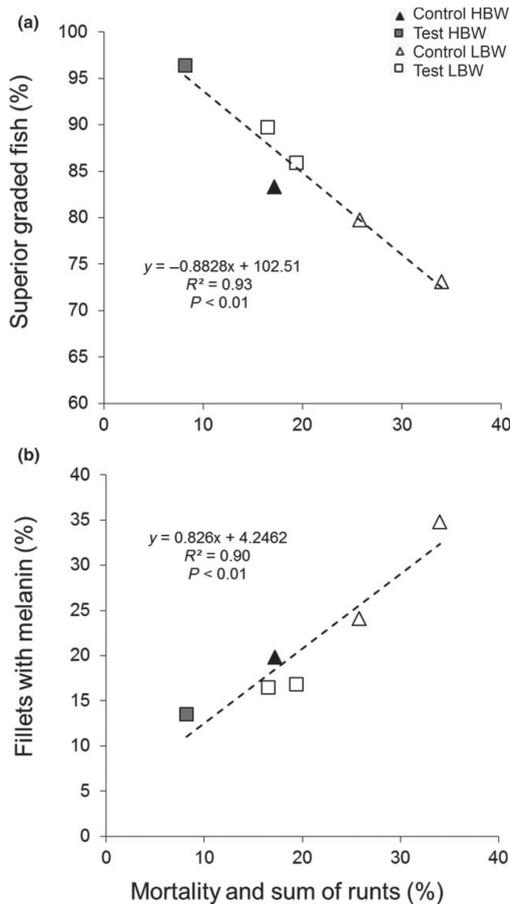


**FIGURE 5** Total sum of runts after an outbreak of pancreases disease in farmed S0 Atlantic salmon, fed either a high protein-to-lipid ratio diet (test) or low protein-to-lipid ratio diet (control). Definition of HBW and LBW as in Figure 2. The low BW groups are shown as mean ± *SEM* (n = 2)

amount of vitamin E between the experimental diets was similar, as identical premixes of vitamins were used in the control and the test diets. The level of PUFA and EPA as percentage of total FA was also similar in the 10 mm summer diets. Hence, reduced fat and increased protein, increasing the dietary protein-to-lipid ratio, seemed to play an important role in this study and significantly affected the tolerance and/or resistance towards PD.

In mammals, high intake of lipids is correlated with metabolic syndrome risk factors and inflammation, and increased consumption of saturated FAs may impair the anti-inflammatory properties of high-density lipoprotein and endothelial function (Johnson, Mander, Jones, Emmett, & Jebb, 2008; Kien, Bunn, & Ugrasbul, 2005; Nicholls et al., 2006). Exocrine pancreatic insufficiency due to chronic pancreatitis in humans may often lead to major weight loss that is strongly associated with maldigestion of fat (Meier & Beglinger, 2006). Meier and Beglinger (2006) stated that the luminal lipid digestion within the small intestine in humans seem to depend on the synergetic effects of pancreatic lipase and cofactors such as bile acids and colipase. The digestion of fat among individuals with pancreatitis is therefore often reduced due to insufficient pancreatic lipase secretion, reduced concentration of bile acids and bicarbonate secretion. There are also no enzymes for triglyceride degradation within the bush-boarder membrane (Meier & Beglinger, 2006). Protein digestion, on the other hand, is initiated by intragastric proteolytic enzymes and can be sustained by intestinal brush-boarder peptidases that are maintained even in the lack of pancreatic proteolytic activity (DiMagno, Malagelada, & Go, 1975; Meier & Beglinger, 2006). If the same factors apply for salmon, this could potentially be a reason for the reduction in runts and higher weight gain among salmon fed the test diet in this study. Regarding growth, the observation of somewhat improved growth within the test group prior to the mortality, may also indicate a dietary effect alone without influence of the disease. Accordingly, diets with increased protein-to-lipid ratio compared to current commercial practices, have previously been shown to promote good growth and improve feed utilization in

mortality due to the increase in available energy was related with an enhanced FA-oxidation capacity, an increased cardio somatic index and the anti-inflammatory effects of TTA (Arge et al., 2018; Grammes, Rørvik, & Takle, 2012; Grammes & Takle, 2011). HSMI, CMS and PD may all cause epicarditis and myocarditis, whereas HSMI and PD also lead to skeletal muscle inflammation and degeneration (Kongtorp, Taksdal, & Lyngøy, 2004). Thus, several of the viral diseases in salmon have similarities and may all cause severe inflammatory responses. The results from the mentioned studies suggest that diets with anti-inflammatory properties and/or low fat, fed before and during inflammatory viral infections, may have a partly preventative effect on the development of disease. In this study, the

**FIGURE 6** Linear regression of the mortality and sum of runts (%) with the amount superior graded fish (a) and fillets with melanin (b) observed at slaughter

large and small scale experiments carried out in the Faroes Island and Mid-Norway, respectively (Weihe et al., 2018).

Pancreas disease has shown to significantly impact fillet quality by reducing the body shape, fillet colour, astaxanthin, and fat content in the muscle, in addition to induce hardening of the fillet texture among affected salmon (Larsson et al., 2012; Lerfall et al., 2012; Taksdal et al., 2012). The results of the measured quality traits in this study are in agreement with the results of these studies, showing that groups with the highest PD-associated mortality had reduced condition factor and astaxanthin content in the muscle, and the hardest texture/firmness of the fillets after the acute mortality. Reductions in condition factor are probably related to the decrease in feed intake and reduced ability to absorb essential nutrients from the feed due to exocrine pancreatic insufficiency associated with PD. Thus, internally stored pigment, fat, and protein are utilized to maintain metabolic functions. Astaxanthin is as effective as vitamin E inhibiting radical-initiated lipid peroxidation

and functions as a potent antioxidant in membrane models in interaction with this vitamin (Mortensen & Skibsted, 1997; Palozza & Krinsky, 1992a,b). Hence, oxidative stress related to PD may be a factor for depigmentation of the muscle. This was particularly observed in the fish fed the high-fat diet, which may also be related to a higher dietary intake of lipids that are more susceptible to oxidation, and hence an increase in the degradation of carotenoids (Boon, McClements, Weiss, & Decker, 2010). Increased fillet firmness among PD affected salmon seem to be correlated with increased levels of collagenous scar tissue, pH, fibrosis and hydroxyproline (Larsson et al., 2012). Hence, the observed increase in firmness can be related to a higher amount of connective tissue and skeletal muscle lesions. Taken together, the results from this study indicate that increased dietary protein-to-lipid ratio and body weight reduced the severity of negative and/or degenerative muscle changes that is normally associated with impaired PD fillet quality.

No large differences in fillet coloration, texture, and shape were detected between the 5 kg groups at slaughter, indicating that a large proportion of the salmon with deviating fillet quality after the PD outbreak had recovered by the time of harvest. This is line with previous observations from Lerfall et al. (2012), showing that salmon with significantly altered fillet quality can to a large extent recover after a natural outbreak of PD. The overall obtained proportion of fish graded as superior at slaughter were within previously reported ranges for sites infected by SAV3 (Jansen et al., 2015). Significantly higher levels of superior graded fish and lower levels of melanin spots in fillet observed in the test and high body weight groups coincide with their positive effects on PD mortality. Increased levels of melanin have previously been found in salmon affected by PD (Lerfall et al., 2012), and our findings are in line with this observation. All quality traits measured in this study showed a highly significant relationship with the observed total mortality. The degree of quality deviation seemed therefore to be related to the severity of PD, measured as total mortality including amount of runts.

It should be noted that this paper used data extracted from a large-scale field trial with the limitations that accompany this kind of experiments. Natural viral infections may not always results in massive mortalities, and it should be noted that this study describes mortality rates and not the development in the number of fish infected by SAV3 or PRV. Thus, important information on viral load and infection pressure over time is not described. During massive runt mortality, exact number of dead runts are difficult to obtain due to the large scale of the experiment. In addition, it is not possible to separate the effects of the long-term feeding with increased protein-to-lipid ratio and the short-term feeding of the test 10 mm summer diet.

The main findings in this study were that increased dietary protein-to-lipid ratio improved survival of farmed Atlantic salmon naturally affected by PD. Significant relations between different quality attributes and the total mortality were observed, where groups with the lowest mortality had increased proportion of superior graded fish and lower amount of fillets with melanin at slaughter. Considering the reduction in mortality and runts in this study, increased dietary
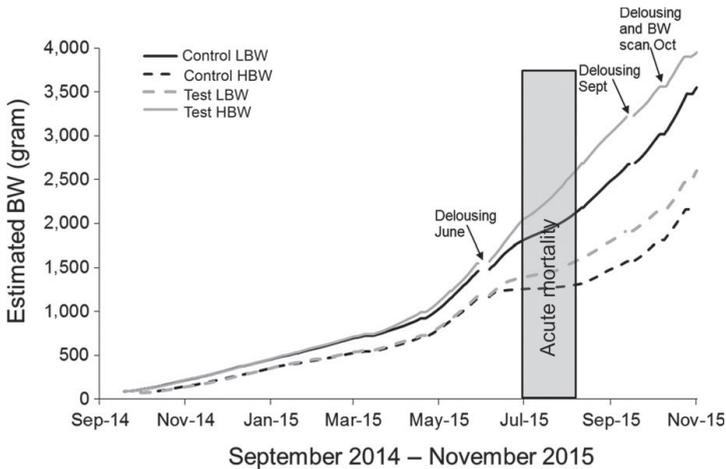
**FIGURE 7** Estimated growth (BW in grams) for farmed S0 Atlantic salmon transferred to sea in mid-September 2014 (High body weight group, HBW) and in late-September/October (Low body weight group, LBW) until November 2015, fed either a high protein-to-lipid ratio diet (test) or low protein-to-lipid ratio diet (control). The acute mortality associated with an outbreak of pancreases disease and time points for delousing treatments are indicated in the figure

protein-to-lipid ratio can improve fish welfare during and after naturally occurring PD.

## CONFLICT OF INTEREST

The authors declare that there are no potential sources of conflict of interest with this work.

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# Paper IV

1 **Sudden increased mortality in large seemingly healthy farmed Atlantic**
2 **salmon (*Salmo Salar* L.) was associated with environmental and dietary**
3 **changes**

4
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15 **fat cells.**

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## Abstract

Mortality of seemingly healthy farmed Atlantic salmon (*Salmo salar* L.) is a large problem in Norwegian aquaculture, and has been linked both to infectious and non-infectious cardiovascular diseases. In this study, an event of sudden mortality of seemingly healthy farmed salmon during the winter period in northern Norway is reported. The experimental fish reared in net-pens were fed two dietary treatments; control and test experimental diets in duplicates. An increased mortality of 6 and 10% was only observed within the two net-pens receiving the experimental test diets. The moribund/dying fish had significantly higher lipid content in the liver, altered haptic fatty acid composition, and increased levels of alanine aminotransferase and alkaline phosphatase in the blood plasma compared to non-dying fish, indicating impaired hepatic function. A significant and instant reduction in mortality was observed when the fish fed the test were starved. The observed mortality was associated with dietary induced changes, shifting sea temperature and day lengths. The possible underlying mechanism for the increased mortality is discussed.

## Introduction

Mortality of seemingly healthy farmed Atlantic salmon (*Salmo salar* L.) is frequently observed in Norwegian salmonid aquaculture, and has been primarily linked both to infectious and non-infectious cardiovascular diseases (Dalum et al., 2017; Hjeltnes et al., 2018; Poppe et al., 2007). Cardiomyopathy syndrome (CMS) and heart and skeletal muscle inflammation (HSMI) are widespread, contagious and fatal cardiovascular diseases in Norwegian salmon farming (Hjeltnes et al., 2018), and are associated with piscine myocarditis virus (PMCV) and piscine reovirus (PRV), respectively (Haugland et al., 2011; Løvoll et al., 2010; Palacios et al., 2010). PRV seem to be ubiquitous among farmed salmon in Norway (Løvoll et al., 2012), whereas PMCV seem to be more associated with clinical CMS outbreaks (Wiik-Nielsen et al., 2012). CMS and HSMI may affect and cause mortality among large and seemingly well fit salmon, and is a great economic burden for the farming industry. In many cases, however, cardiovascular and circulatory failure are also observed without viruses present and may represent a significant proportion of the non-specific mortality in fish farms (Dalum et al., 2017; Tørud, 2004). The agents and causes to increased mortality in aquaculture are complex and outbreaks of many disease conditions occur as results of intricate interactions between environmental conditions, metabolic distress, nutritional imbalances and the presence of pathogens, combined with effects of site management and handling stress (Contessi et al., 2006; Crane and Hyatt, 2011; Wheatley et al., 1995).

Photoperiod and temperature are important environmental cues in fish, changing dramatically with the season, particularly at high latitudes. Several studies show that seasonal changes may alter the utilization of lipids for energy production or storage (Aksnes et al., 1986; Mørkøre and Rørvik, 2001; U Nordgarden et al., 2003; U. Nordgarden et al., 2003). The late summer

83    and autumn period is characterized by optimal sea temperatures for growth and the duration

84    of daylight becomes noticeably shorter. For salmon, this period is associated with increased

85    somatic growth and high lipid deposition (Dessen et al., 2017; Mørkøre and Rørvik, 2001;

86    Rørvik et al., 2018). The winter period is generally associated with reduced feed intake,

87    growth and feed utilization (Mørkøre and Rørvik, 2001), which correlates with low

88    temperatures and little day light. Studies also reported a decline or stagnation in the level of

89    muscle fat in salmon during this period (Mørkøre and Rørvik, 2001; U. Nordgarden et al.,

90    2003). The dietary lipid level and inclusion of vegetable oils (VO) in salmonid diets has

91    increased gradually over the recent years (Torrissen et al., 2011; Turchini et al., 2009).

92    Previous studies has shown that increased dietary energy level, VO inclusion and temperature

93    can alter lipid metabolism, hepatic fat content and fatty acid (FA) composition in fish (Leaver

94    et al., 2008; Martinez-Rubio et al., 2013b; Ruyter et al., 2006). In many cases of infectious

95    and non-infectious heart diseases in salmon farming, few specific etiologies related to

96    seasonal changes and nutritional aspects seems to have been identified or described.

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98    The present study was a part of a large scale project evaluating the effects of seasonal dietary

99    changes in protein-to-lipid ratio on growth, feed utilization and quality related parameters

100   during the growth out phase in sea. During this trial, an unexpected event of sudden mortality

101   of seemingly healthy farmed salmon during the winter period was observed. During the

102   period of increased mortality, samples were secured to investigate possible causes for the

103   mortality. Chemical analyses of liver lipid content, FA composition and blood parameters of

104   moribund and live salmon were conducted. In addition, all fish that was sampled were

105   screened for detection of a range of RNA viruses that are known to infect salmonids in this

106   area. The results of these analyses are linked to seasonal and dietary induced changes.

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## Material and methods

The research reported in this study was approved by The Norwegian Directorate of Fisheries, allowance T-H-8 and T-H-19, and was carried out in accordance with national guidelines, laws and the animal welfare act. Fish were treated as production fish up to the point of tissue sampling which was only conducted *post-mortem (*according to regulation FOR-2015-06-18-761).

The present trial was carried out at NOFIMA large-scale research & development (R&D) facility in collocation with Nordlaks Oppdrett AS, at the sea site Dypingen (Kvernsundet, Bjarkøy, Troms, Northern Norway). The R&D facility consisted of four net-pens (130 m circumference cages) and a feed barge with an automated feeding system. Atlantic salmon yearling smolt (S1) (AquaGen strain, AquaGen AS, Hemne, Norway) vaccinated with Pentium Forte Plus (Novartis Animal Vaccines Ltd, Essex, UK) and transferred to sea on the 3 of May 2015 were used in the study. The mean body weight at sea transfer was 148 gram with a mean number of 99 263 salmon per net-pen (SEM = 355). Two diet series (test and control) were formulated and produced by BioMar AS (Myre, Norway) according to target dietary protein and lipid contents defined by Nofima. Net-pen 2 and 3 were fed the test series, whereas pen 1 and 4 were fed the control series. The pens receiving the same dietary treatment were placed diagonally in relation to each other and on the opposite sides of the site (to randomize the localisation). The experimental diets were produced as 3.5, 5, 7 and 10 mm pellets. Crude protein and lipid content in all pellet sizes were assessed by on-line near-infrared (NIR) analyses by BioMar and dietary levels of fat and protein used are illustrated in figure 1A and B, respectively. The test diet series was designed to have a low fat and a high protein content, whereas the control series was designed to have generally higher lipid and a

133  lower protein content. The salmon were exposed to ambient environmental conditions (Fig

134  2A).

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136  On the 22nd of January 2016, a sudden and marked increase in mortality among large

137  seemingly healthy fish in the two test pens (pen 2 and 3) was observed (Fig 2B). The local

138  veterinary service (Vesterålen Fiskehelsetjeneste AS, Sortland, Norway) performed an

139  inspection of the site on 25th of January 2016, whereas Nofima carried out samplings at the

140  site on the 3rd and 17th of February 2016. On the 3rd of February, five fish with normal

141  appearance and swimming behaviour (defined as normal not dying individuals) were sampled

142  from each of the four experimental pens. In addition, five moribund (dying) fish were

143  sampled from the test pens 2 and 3 with the increased mortality. Moribund fish were alive and

144  had a seemingly normal outer appearance, but were collected from the dead fish collection net

145  (bottom of the pen) and had lethargic behaviour. During this sampling, the colour appearance

146  of the liver (scoring scale from 0- 5; pale/yellow - normal/brown) was evaluated and pieces of

147  liver were stored at -20 °C prior to analysis of fat content and FA composition as described by

148  Rørvik et al. (2018). On the 17th of February, ten normal and ten moribund fish were sampled

149  from both test pens, and blood was drawn from the caudal vein using vacuum tubes with

150  ethylene diamine-tetra-acetic acid (EDTA). All sampled fish were sedated (Benzoac® 2 ml x

151  10 $L^{-1}$, ACD Pharmaceuticals AS, Leknes, Norway) and killed by a blow to the head, before

152  blood was taken and different organs were dissected. Plasma was separated by centrifugation,

153  stored on dry ice and then sent to the Central Laboratory at the Norwegian University of Life

154  Sciences (Oslo, Norway) for analysis of alanine aminotransferase (ALT), aspartate

155  aminotransferase (AST) and alkaline phosphatase (AP) based on the methods described by

156  Tietz (1995). In addition to the mentioned samplings, fish were routinely sampled from all

157  experimental pens at several occasions for analysis of biometric parameters (visceral somatic

158  index and visceral fat score) and muscle fat content as described by Dessen at al. (2018). The

159  specific feeding rate (SFR), condition factor and organ indices were also calculated as

160  described in study.

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162  Different tissues (liver, kidney and hears) were collected for histopathological examination

163  (fixed in formalin) and RT-PCR analysis (fixed in RNA *later*® Thermo Fisher Scientific,

164  Massachusetts, USA) during the veterinary inspection, and these analyses were conducted by

165  the National Veterinary Institute of Norway, NVI (Harstad, Norway). In addition, heart tissue

166  from all fish sampled on the 3 of February were also evaluated by histopathological analysis

167  (blinded scoring of lesions in the heart, epicardium and ventricle) and RT-PCR analysis by

168  Fish Vet Group (Oslo, Norway) and PatoGen (Ålesund, Norway), respectively. The samples

169  were screened by RT-PCR analysis for the detection of a range of RNA viruses that are

170  known to infect salmonids in this area; PRV, infectious salmon anemia virus (ISAV),

171  salmonid alphavirus (SAV), PMCV and infectious pancreatic necrosis virus (IPNV). Due to

172  the fact that the increased mortality was only observed within the dietary test group, the test

173  diet used in this period was also screened for toxins and abnormalities in the content of

174  nutrients and FA composition (standard procedures and analysis conducted by BioMar).

175  However, no toxins or abnormalities in the content of nutrient were detected in the test diet.

176  The FA composition of the test and control 10 mm winter diets used is shown in table 1.

177

178  The moribund fish from the test pens were defined as the Moribund-T group, whereas the live

179  fish with normal behavior from the test and control pens were defined as the Normal-T and

180  Normal-C group, respectively (n = 10). Statistical analysis were preformed using the General

181  Linear Model (GLM) procedure in the SAS 9.4 computer software (SAS Institute Inc., Cary,

182  NC, USA). The effect of diet was tested using the normal fish from the test and control group.

183    If no significant differences were detected, the normal fish were pooled and tested against the

184    moribund fish to test the effect of health status (dying vs. not dying). In cases were significant

185    differences in diet was detected, the normal and moribund fish from the test group was tested

186    against each other. Non-parametric data (scores) were tested using the non-parametric

187    Kruskal-Wallis test and Pearson product-moment correlation coefficient was used to describe

188    the association between two variables. The level of significance was chosen at $P \leq 0.05$. The

189    results are presented as mean ± standard error of mean (SEM), unless stated otherwise.

190

191    **Results**

192

193    The increase in acute mortality on the 22nd of January was only observed in the two pens fed

194    the test diet series. The mortality increased with about 4 and 6 % within test-pens 2 and 3,

195    respectively versus 0.1% for the controls during January, February and March (Fig 2B). Prior

196    to the increased mortality, the lipid content in the test diet series was elevated from 28.7 % to

197    34.2 % in early December 2015, whereas the lipid content in the control series was gradually

198    increased to such high dietary fat during the autumn (Fig 1A). The dietary shift for the test

199    group was done after a period with substantial somatic growth and deposition of lipids in the

200    muscle for both dietary groups. On the 7th of January, both groups had accumulated about the

201    same level of fat in the muscle (Fig 3). However, the VSI was significantly ($P < 0.05$) lower

202    in the test than in the control group on this date (Fig 4).

203

204    ISAV, SAV, PMCV or IPNV were not detected by the RT-PCRs analyses. However, PRV

205    (average Ct-value of 22.3) was found in all fish sampled on the 3rd of February. Hence, no

206    significant differences in Ct-PRV values between the dietary groups nor any differences

207    between normal and moribund fish in the test pens were detected. The gross evaluation of all

208    moribund fish often revealed pale hearts with blood filled atrium. Liver was regularly

209    yellowish and pale, and swollen spleen was often observed. Feed matter was found in the

210    gastrointestinal tract among most of the moribund fish. The histopathological findings from

211    moribund fish sampled during the veterinarian inspection showed mild to moderate

212    myocarditis and epicarditis in the heart, in addition to moderate degrees of hemorrhagic

213    necrosis in the liver (results not shown). The majority of the hearts sampled from both normal

214    and moribund fish on the 3rd of February had normal histopathological appearance. However,

215    some hearts within all groups displayed mild changes that can be associated with CMS or

216    mild HSMI. This was mainly indicated by diffuse infiltration of inflammatory cells in the

217    epicardium and minor inflammatory changes in the compact layer of the ventricle. The

218    histopathological score was overall numerically higher for the fish sampled in the test pens

219    (both moribund and normal live fish), but no significant differences were detected between

220    moribund and normal fish groups, or between the dietary groups (results not shown).

221

222    There were no significant differences in biometrics between the groups of fish sampled on the

223    3rd of February (Table 2). However, the Moribund-Test group had a significantly ($P < 0.05$)

224    higher liver lipid content compared to the other groups (Fig 5). In addition, the Moribund-Test

225    group had numerically higher hepatosomatic index (HSI) and a significantly lower liver color

226    score (more pale/yellow) than the Normal-T group (Table 2). A significant ($P < 0.001$) overall

227    negative correlation was found between liver fat content and liver color score (r = -0.66, n =

228    30). Regarding the blood samples, significantly ($P < 0.05$) higher levels of ALT and AP in

229    plasma were observed among the moribund compared to the normal fish within the pens with

230    high mortality (Fig 6A and B). A similar pattern was observed for AST, although no

231    significant differences was detected (results not shown). The analysis of the FA profile in the

232    liver showed that moribund fish had significantly ($P < 0.05$) higher levels of palmitoleic acid

233 (16:1 $n$-7), vaccenic acid (18:1 $n$-7) and oleic acid (18:1 $n$-9), irrespective of dietary treatment

234 (Table 3). In addition, the $\Delta^9$ desaturate index ($\Delta^9$ DI) was 1.3 folds higher ($P < 0.05$) in

235 moribund versus the normal fish groups (Table 3).

236

237 Due to an exponential increase in mortality, the feeding of the two test pens was stopped on

238 the 15th of February to test the potential effect of starvation on mortality rates. After five

239 days, a marked reduction in daily mortality was observed and on the 25th of February, the

240 mortality levelled off to normal levels (Fig 7). The 12th of March, the feeding was resumed

241 using a diet with lower lipid content (30 % fat, Fig 1A). No increase in mortality was

242 observed after re-feeding and during the latter stages of the production (Fig 2B). Figure 2C

243 shows that daily specific feed intake did not differ between the test and control groups during

244 the first six weeks after the dietary change. However, the daily specific feed intake in the test

245 group dropped during the period of acute mortality and recovered after the starvation period.

246 All net-pens were slaughtered during August 2016.

247

248 **Discussion**

249

250 The described results were obtained during a large-scale study that was initially designed to

251 evaluate the effects of diets diverging in protein-to-lipid ratio on growth, feed utilization and

252 quality related parameters. Previous studies show that lipid dens diets may improve feed

253 utilization, especially at low temperatures (Bendiksen et al., 2003; Hillestad et al., 1998;

254 Karalazos et al., 2007). Thus, the lipid level in the 10 mm winter test diet was increased so

255 that it contained both a high protein and lipid level. The test group switched to another diet

256 during early December, when there was nearly no daylight (polar night) and the water

257 temperature was 8°C. Both experimental groups showed substantial increase in weight and fat

258 deposition prior to winter, which is consistent with previous observations of growth and lipid

259 storage in farmed salmon during late summer and autumn (Alne et al., 2011; Dessen et al.,

260 2017; Mørkøre and Rørvik, 2001). The muscle fat content did not increase after January,

261 which may indicate that the possibility and/or ability to store fat has reached an upper limit

262 relative to this point of time.

263

264 The liver of the moribund fish had a higher lipid content and percentages of the FA 16:1 *n*-7,

265 18:1 *n*-7 and 18:1 *n*-9 compared to the other groups. The increase in hepatic lipid content and

266 the aforementioned FAs, together with slightly lower percentages of saturated FAs

267 (particularly C18:0) may indicate an up-regulated $\Delta^9$-desaturase hepatic activity. This

268 assumption is strengthened by the significant increase in $\Delta^9$ desaturase index among the

269 moribund fish. In salmon, lipid dens diets with increased inclusion of vegetable oils at low

270 water temperatures have shown to increase liver lipid content (Karalazos et al., 2007). Ruyter

271 et al. (2006) found higher lipid content and percentages of monounsaturated FAs, especially

272 18:1 *n*-9, in the liver and intestine of salmon kept at 5°C compared with salmon reared at 12

273 °C, suggesting a higher $\Delta^9$ desaturase activity. In the present study, salmon was switched to a

274 diet with higher lipid content (similar vegetable oil inclusion as the control) when the water

275 temperature was 8 °C, and when the water temperature was reduced to 5 °C, the mortality

276 started. Thus, the dietary alterations together with the environmental conditions in the present

277 study may have increased the $\Delta^9$ desaturase activity. However, it may also indicate a reduced

278 capacity of the liver to secret fat as very low density lipoproteins. These factors may

279 consequently explain the increased levels of lipid and monounsaturated FAs in the liver. The

280 moribund fish did also have significantly higher levels of ALT and AP in plasma than

281 previously reported in healthy salmon (Sandnes et al., 1988). AST, AP and ALT have been

282 used as useful diagnostic tool to detect liver and kidney disturbances in salmonids (Racicot et

283    al., 1975; Sandnes et al., 1988). In mammals, increased levels of AST, AP and ALT is

284    associated with impaired liver function, liver tissue damage and necrosis (Giannini et al.,

285    1999; Oguz et al., 2013).

286

287    The presence of PRV was detected in all groups, in addition to some sings of

288    histopathological changes that can resemble HSMI. However, it has been observed that PRV

289    can be present in high titers without causing mortality or marked lesion in the heart (Garseth

290    et al., 2013). Diets with increased lipid content have previously been shown to increase

291    inflammatory responses, heart lesions, hepatic fat content, and signs of hepatic steatosis in

292    salmon experimentally challenged with Atlantic salmon reovirus (ASRV) (Martinez-Rubio et

293    al., 2013a). The presence of PRV and the clinical signs of the moribund fish may have

294    reduced the ability to resist the long-term inflammation associated with PRV and/or HSMI

295    among the test group. However, this theory is weakened by the detection of the same PRV

296    virus load and similar histopathological heart scores in both normal and moribund fish. A

297    significant and instant reduction in mortality was observed when the fish within the test group

298    were starved. Starvation is known to increase β-oxidation in rodents (Osmundsen et al.,

299    1991), and may have altered the metabolic state of the fish. Reduced dietary energy is known

300    increase the resistance towards HSMI and CMS in Atlantic salmon (Martinez-Rubio et al.,

301    2014, 2012). Observation from the salmon industry indicate that starvation may reduce the

302    mortality of large salmon associated with CMS (pers. comm. 2014).

303

304    During the present trail, it was observed that the VSI was significantly lower among the test

305    group compared to the control group prior to the acute mortality. This seems to indicate a

306    lower visceral fat deposition in test group. In mammals, when the dietary energy intake

307    exceeds the amount of energy being expended, the excess of energy is normally stored in the

form of triglycerides in white adipose tissue. Adipose tissue achieves the safe storage of lipids by increasing the recruitment of new adipocytes (hyperplasia) and/or the expansion of the existent ones (hypertrophy) (Otto and Lane, 2005). However, the expandability capacity of adipose tissue has a limit, and sustained energy overload may lead to the deposition of lipids in non-adipose tissues, leading to lipotoxicity and inflammation (Carobbio et al., 2017; Solinas et al., 2015; Unger et al., 2010). Salmonids normally store lipids in visceral adipose tissue and intramuscularly, whereas very little is stored in the liver and heart (Sheridan, 1994; Weil et al., 2013). The understanding of factors orchestrating salmon fat distribution and adipocyte recruitment is scarce. However, the development of  mesenchymal stem cells isolated from Atlantic salmon white adipose tissue is characterized (Todorčević et al., 2010; Vegusdal et al., 2003). The capacity to recruit adipocytes in humans is increased during childhood and remains constant during adulthood regardless of total body fat content (Knittle et al., 1979; Spalding et al., 2008). Whether this is the case for salmon remains to be elucidated. Nonetheless, the results from the present study suggest that the use of a low-fat diet during a period where salmon is known to increase the amount of visceral adipose tissue may have impaired the recruitment capacity of adipocytes. Thus, the capacity of this fish to accommodate the excess of energy when suddenly increasing the dietary lipid content would be challenged. As a result, the excess of energy would be stored in other organs, such as liver and heart, contributing to a low-grade inflammatory state and an increased risk of cardiometabolic diseases. Considering that the salmon liver contributes to about only 1-1.5 % of the body mass, this organ might be affected quickly by overload of fat. The fact that feed matter was found in the gastrointestinal tract among most of the moribund fish indicates that the fish dies suddenly.

332   To summarize, the increase in mortality in the present study might be due to an interaction

333   between the nutritionally induced metabolic changes and challenging environmental

334   conditions. Risk factors such as increased liver fat, and increased blood plasma of levels ALT

335   and AP were identified among the moribund dying fish. It is suggested that the increase in

336   mortality may be related to reductions in the fat storage capacity among the test group. The

337   mortality can also be associated with the presence of PRV and reduced ability to resist long-

338   term inflammation associated with PRV and/or HSMI due to the impaired haptic function.

339   These suggested hypotheses/causes needs to be further investigated, tested, and verified.

340

352

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## Tables

**Table 1** Fatty acid composition (% of total) of the 10 mm winter test and control diets used prior to the increased mortality among the test group.

| 10 mm winter diet | Control | Test |
|---|---|---|
| 14:0 | 1.9 | 2.4 |
| 16:0 | 7.9 | 8.0 |
| 18:0 | 2.6 | 2.2 |
| **Σ SFA[1]** | 14.0 | 14.0 |
| 16:1n-7 | 1.6 | 1.9 |
| 18:1n-9 | 40.0 | 38.0 |
| 18:1n-7 | 5.4 | 4.6 |
| 20:1n-9 | 2.9 | 3.8 |
| **Σ MUFA[2]** | 52.8 | 52.2 |
| 18:2n-6 | 16.3 | 14.0 |
| 20:4n-6 | 0.2 | 0.2 |
| **Σ n-6[3]** | 16.8 | 14.7 |
| 18:3n-3 | 5.8 | 5.9 |
| 20:5n-3 | 4.4 | 5.5 |
| 22:5n-3 | 0.4 | 0.6 |
| 22:6n-3 | 2.6 | 3.5 |
| **Σ n-3[4]** | 13.5 | 15.7 |
| **n-3/n-6** | 0.8 | 1.1 |

[1]Includes 15:0, 17:0, 20:0, 22:0, and 24:0.
[2]Includes 14:1n-5, 16:1n-5, 17:1n-7, 22:1n-7, 22:1n-11, 22:1n-9, and 24:1 n-9.
[3]Includes 16:2n-6, 18:3n-6 and 20:2n-6
[4]Includes 16:2n-3 and 20:4n-3.

528　**Table 2.** Biometric data of Atlantic salmon fed the two experimental diets and presenting

529　differences in health status. (Mean values with their standard errors; n = 10)

| Dietary treatment | Control | Test | Test | P-value | |
|---|---|---|---|---|---|
| Health status | Normal | Normal | Moribund | Diet | Health status |
| Body weight, g | 2533 ± 227 | 2501 ± 152 | 2511 ±151 | ns | ns |
| Gutted weight, g | 2201 ± 192 | 2211 ± 129 | 2145 ±104 | ns | ns |
| Length, cm | 57.6 ± 1.6 | 58.3 ± 1.0 | 57.6 ± 1.0 | ns | ns |
| CF | 1.30 ± 0.04 | 1.25 ± 0.03 | 1.31 ± 0.04 | ns | ns |
| CFg | 1.13 ± 0.03 | 1.11 ± 0.02 | 1.12 ±0.02 | ns | ns |
| HSI | 1.49 ± 0.08 | 1.48 ± 0.05 | 1.56 ± 0.09 | ns | ns |
| Liver score, 0 - 5 | 1.7 ± 0.1 | 2.3 ± 0.3 | 1.3 ± 0.2 | ns | 0.001 |

530　CF; condition factor, CFg; condition factor gutted, HSI: hepatic-somatic index

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**Table 3.** Fatty acid composition (% of total) in the liver of Atlantic salmon fed the two experimental diets and presenting differences in health status. (Mean values with their standard errors; n = 10)

| Dietary treatment | Control | Test | Test | P-value | |
|---|---|---|---|---|---|
| Health status | Normal | Normal | Moribund | Diet | Health status |
| 14:0 | $1.0 \pm 0.12$ | $1.2 \pm 0.14$ | $1.4 \pm 0.03$ | ns | ns |
| 16:0 | $7.7 \pm 0.30$ | $7.3 \pm 0.19$ | $6.6 \pm 0.29$ | ns | ns |
| 18:0 | $3.9 \pm 0.14$ | $3.7 \pm 0.09$ | $3.5 \pm 0.15$ | ns | ns |
| **Σ SFA[1]** | $13.4 \pm 0.31$ | $13.0 \pm 0.25$ | $12.4 \pm 0.39$ | ns | ns |
| 16:1n-9 | $0.0 \pm 0.00$ | $0.1 \pm 0.04$ | $0.1 \pm 0.05$ | 0.025 | ns |
| **16:1n-7** | **$1.7 \pm 0.07$** | **$1.9 \pm 0.13$** | **$2.4 \pm 0.13$** | **ns** | **0.001** |
| **18:1n-9** | **$36.0 \pm 0.97$** | **$35.8 \pm 1.65$** | **$40.6 \pm 0.92$** | **ns** | **0.001** |
| **18:1n-7** | **$2.8 \pm 0.04$** | **$2.9 \pm 0.09$** | **$3.2 \pm 0.06$** | **ns** | **0.009** |
| 20:1n-9 | $4.7 \pm 0.13$ | $4.7 \pm 0.13$ | $5.0 \pm 0.20$ | ns | ns |
| **Σ MUFA[2]** | $48.5 \pm 1.23$ | $49.1 \pm 1.66$ | $54.9 \pm 1.24$ | ns | 0.006 |
| 18:2n-6 | $10.5 \pm 0.21$ | $9.5 \pm 0.33$ | $9.8 \pm 0.23$ | 0.023 | ns |
| 20:4n-6 | $0.8 \pm 0.08$ | $0.9 \pm 0.09$ | $0.6 \pm 0.04$ | ns | 0.027 |
| **Σ n-6[3]** | $11.4 \pm 0.18$ | $11.3 \pm 0.25$ | $10.6 \pm 0.21$ | ns | 0.051 |
| 18:3n-3 | $3.2 \pm 0.12$ | $3.3 \pm 0.10$ | $3.4 \pm 0.08$ | ns | ns |
| 18:4n-3 | $2.0 \pm 0.05$ | $1.6 \pm 0.23$ | $1.9 \pm 0.05$ | ns | ns |
| 20:5n-3 | $4.9 \pm 0.32$ | $4.1 \pm 0.30$ | $3.0 \pm 0.17$ | 0.031 | 0.010 |
| 22:5n-3 | $1.4 \pm 0.08$ | $1.5 \pm 0.16$ | $1.1 \pm 0.09$ | ns | 0.023 |
| 22:6n-3 | $11.1 \pm 0.71$ | $12.2 \pm 1.46$ | $8.2 \pm 0.71$ | ns | 0.011 |
| **Σ n-3[4]** | $23.6 \pm 1.02$ | $23.6 \pm 1.74$ | $18.9 \pm 0.93$ | ns | 0.02 |
| **Δ⁹ DI[5]** | **$3.3 \pm 0.18$** | **$3.5 \pm 0.21$** | **$4.3 \pm 0.24$** | **ns** | **0.008** |

[1]Includes 15:0, 17:0, 20:0, 22:0, and 24:0.
[2]Includes 14:1n-5, 16:1n-5, 17:1n-7, 20:1n-11, 22:1n-7, 22:1n-11, 22:1n-9, and 24:1 n-9.
[3]Includes 16:2n-6, 18:3n-6, 20:2n-6 and 20:3n-6.
[4]Includes 16:2n-3, 20:4n-3 and 20:3n-3.
[5]$\Delta^9$ DI = (16:1n-7 + 18:1n-9) / (16:0 + 18:0)

**Figure legends**

558

559 **Figure 1**. The weighted mean dietary lipid **(A)** and protein level **(B)** of the control and test

560 experimental diet series used during the production cycle (May 2015 to July 2016). The

561 values for protein and lipid levels are based on the mean Near Infrared (NIR) analysis of each

562 batch, weighted for the total amount of feed produced in each batch of the particular pellet

563 size.

564

565 **Figure 2.** Ambient daily sea temperature (°C, y-axis), day length (hours of daylight, z-axis)

566 **(A)** and weekly cumulative mortality during the production cycle for the control and test pens

567 **(B)** during the production cycle (May 2015 to July 2016). The specific feeding rate (%) for

568 the control and test pens from December 2015 to May 2016 **(C)**.

569

570 **Figure 3.** The development in and muscle fat (%) content (May 2015 – June 2016) of Atlantic

571 salmon fed control and test dietary series. Samplings were conducted in May, June and

572 October 2015, in addition to January and June 2016. Values are shown as pooled means of the

573 pens.

574

575 **Figure 4.** Visceral somatic index **(A)** and visceral fat score **(B)** of Atlantic salmon sampled on

576 7 of January 2016 fed control and test dietary series. Values are shown as pen means ± SEM,

577 n = 10.

578

579 **Figure 5.** Liver fat content of Atlantic salmon sampled on the 3rd of February 2016. Normal-

580 C; normal fish from control pens, Normal-T; normal fish from the test pens, Moribund-T;

581   diseased/moribund fish from the test pens. Values are shown as means ± SEM, n = 10.

582   Significant differences (P < 0.05) are indicated by different subscript letter on the bars.

583

584   **Figure 5.** Alanine aminotransferase **(A)** and alkaline phosphatase **(B)** and aspartate

585   aminotransferase (C) in plasma of Atlantic salmon sampled on the 17th of February 2016.

586   Normal-C; normal fish from control pens, Normal-T; normal fish from the test pens,

587   Moribund-T; diseased/moribund fish from the test pens. Values are shown as means ± SEM, n

588   = 10. Significant differences are indicated over the bars.

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590   **Figure 6.** Daily mortality in test-pens 2 and 3 from starvation on the 15th of February and

591   until the end of March 2016. Feeding was resumed on the 12th of March 2016.

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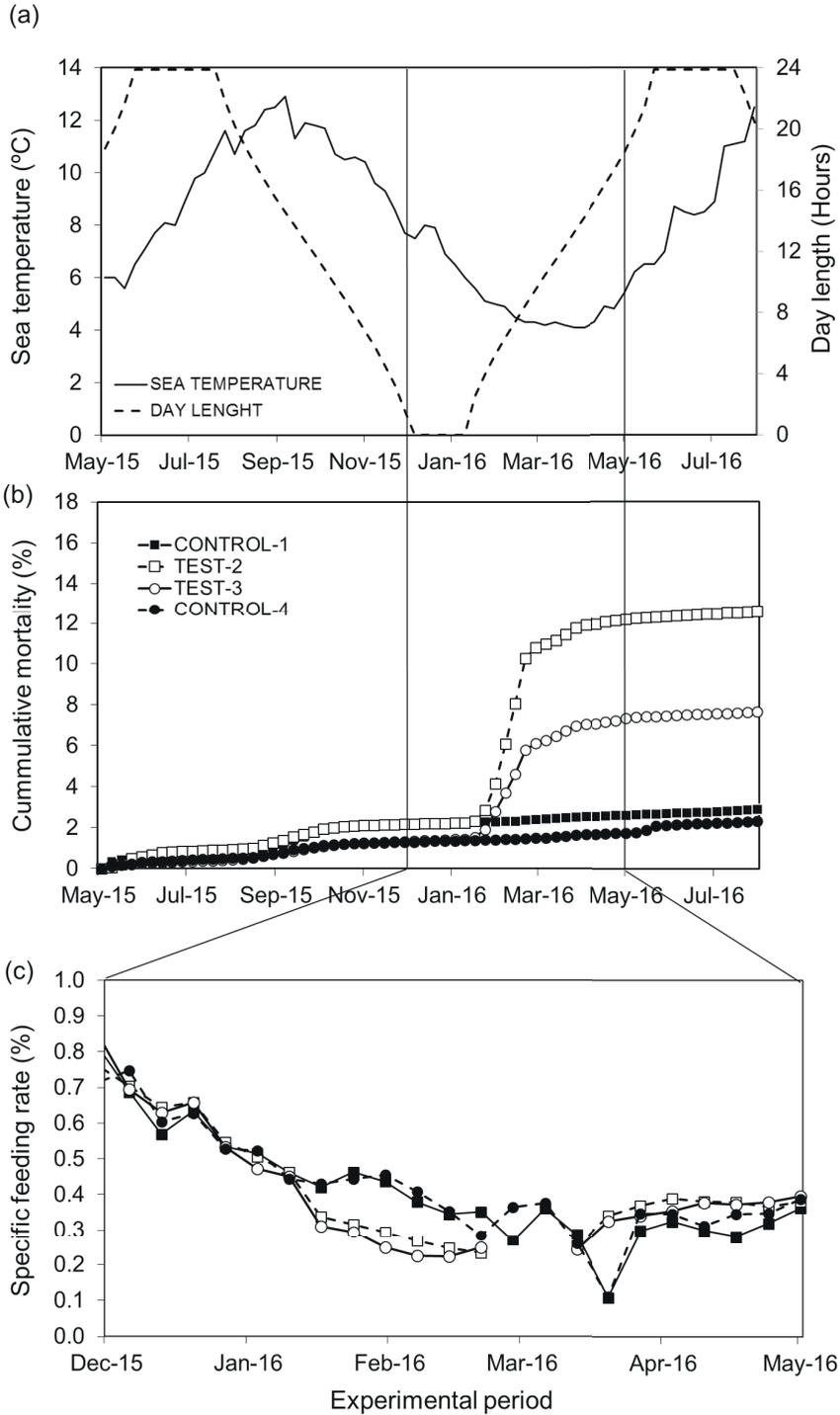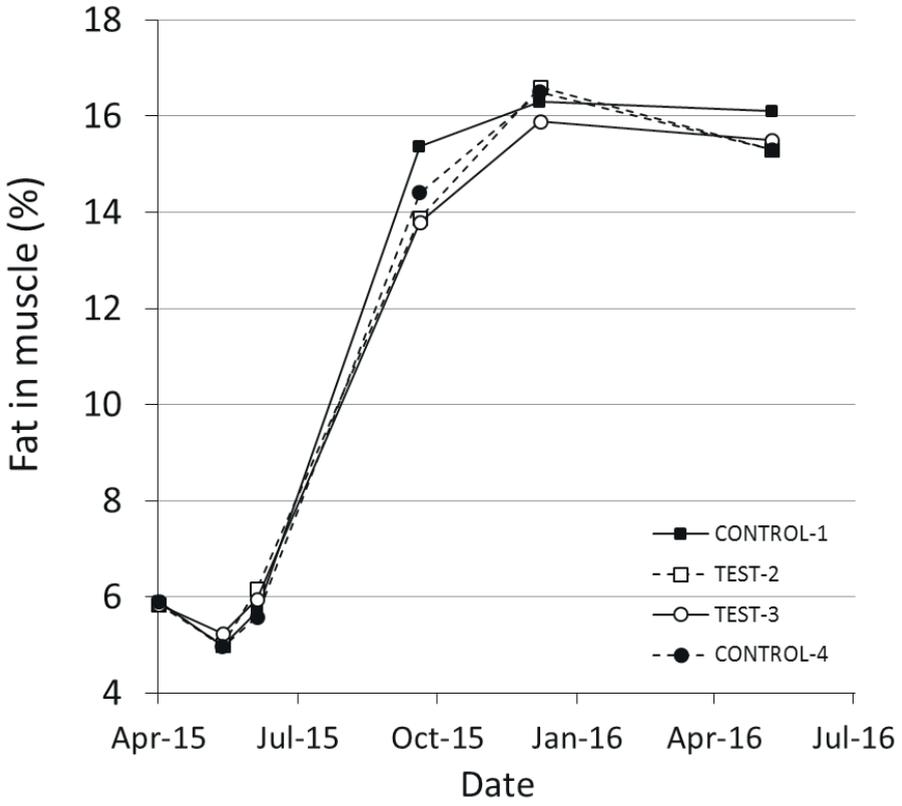
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607 **Figures**

608

609 **FIGURE 1**

(a)

| DIET | 3.5 mm | 5 mm | 7 mm | 10 mm winter | 10 mm spring |



(b)



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611 **FIGURE 2**

(a)



(b)

(c)
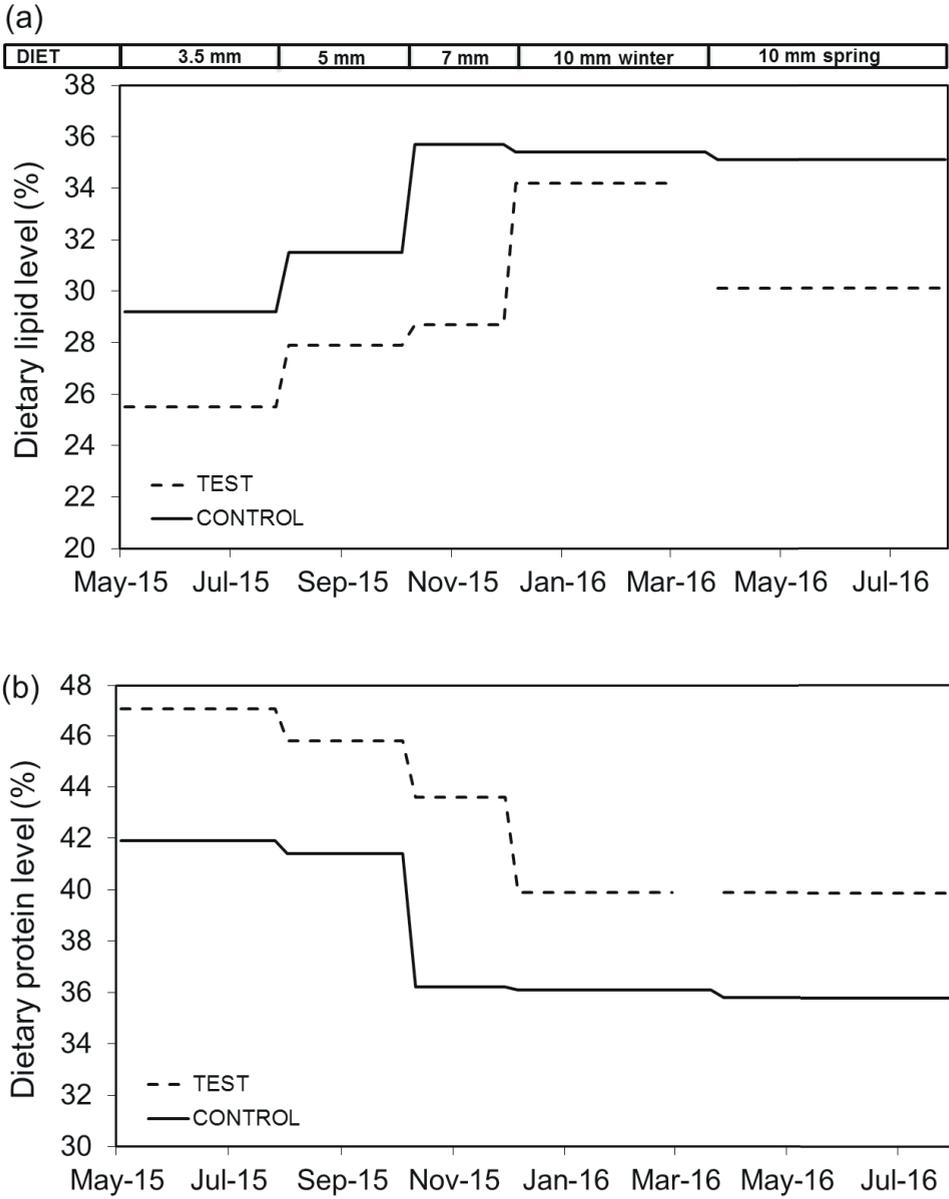
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613    **FIGURE 3**

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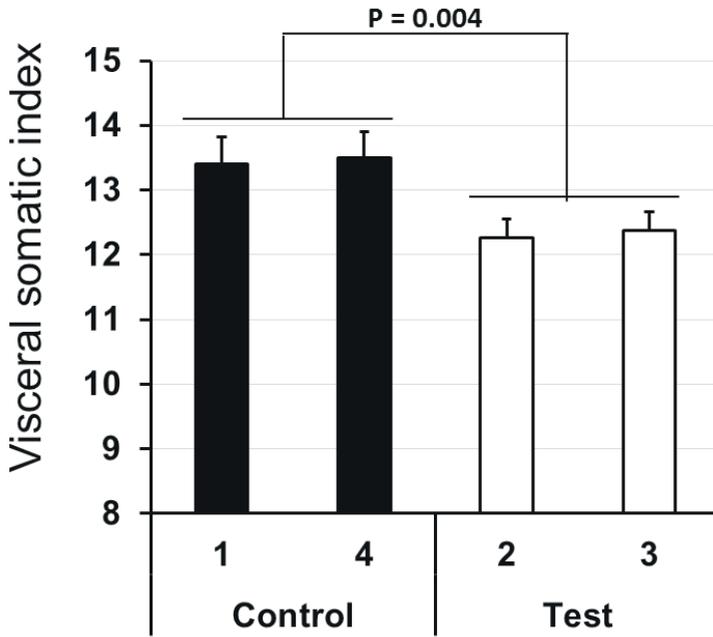
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626    **FIGURE 4**
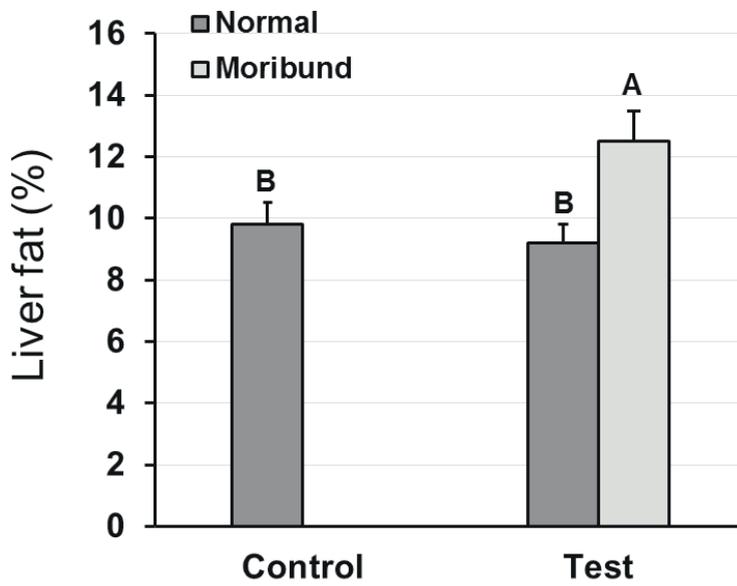
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641    **FIGURE 5**
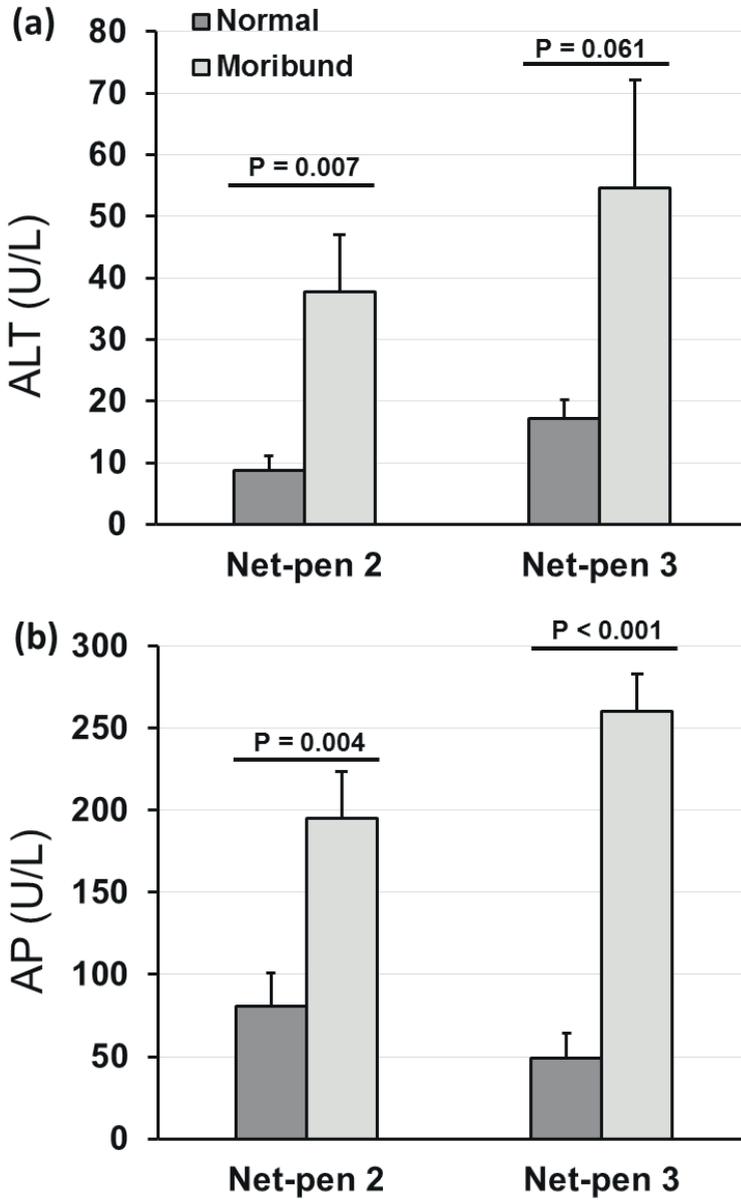
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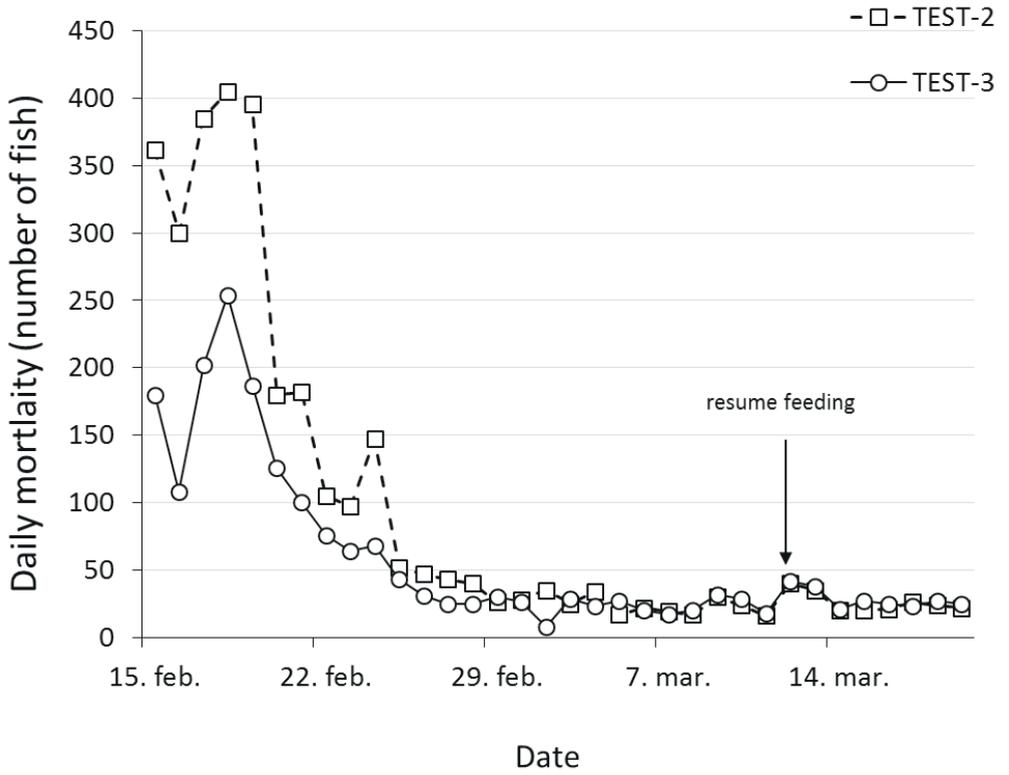
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657 **FIGURE 6**

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662 **FIGURE 7**

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