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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

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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-tolipid ratio in salmon diets on mortality rates, biometric-, and qualityrelated 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.

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

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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 Analytig AS (Bergen, Norway) using their in-house real-time PCR assays (for detection of viral RNA) and Elongation factor 1A (Ef1 α) as guality 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 (±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, 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.

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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
 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) Diet code	3.5 mm		5 mm		7 mm		10 mm summer		10 mm autumn	
	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	22.2	20.5	20.0	18.7	20.3	18.0	21.6	15.0	16.2	14.0

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Notes. CP: crude protein; GE: gross energy; MJ: mega joule.

^aThe starch content was analysed chemically on 1–3 feed samples from representative batches of each particular pellet size. ^bThe astaxanthin values are based on the declared content due to lack of the chemical analysis of astaxanthin level for all diets. ^cThe gross energy content was calculated assuming 23.7, 39.5 and 17.2 MJ/kg of protein, lipids and starch, respectively.

sampled fish were selected so that the mean body weight (BW) of the 10 fish corresponded to the estimated mean weight of all the fish in the respective pen. All fish selected for samples were sedated (Benzoac[®] 2 ml \times 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).

Quality evaluations at harvest 2.6

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

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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 sum	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 <i>n</i> -6	0.2	0.2		
22:4 n-6	<0.1	<0.1		
18:3 n–3	6.2	6.7		
18:4 <i>n</i> –3	0.7	0.6		
20:3 n–3	<0.1	<0.1		
20:4 <i>n</i> –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.

of fat, pigment and texture of the fillet using the same analytical methods as described for onsite samplings. Harvest reports based on 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) × 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 = $100\% \times (1-\% \text{ mortality in test group/\% mortality in the control group).$

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 **TABLE 4**Overview over the inputvariables and the obtained data fromthe study used in the statistical model

	Statistical v	ariables use	d	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	

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

histopathological changes in line with PD and HSMI at 29 of June (see section Environmental conditions, diagnosis, and disease outbreak for more 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





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)

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)

Notes. The *p*-values are shown between brackets.

BW: body weight; ns: not significant; PD: pancreas disease.

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 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



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

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).

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 Journal of Fish Diseases

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

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FIGURE 5 Total sum of runts after an outbreak of pancreases disease in farmed SO 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 \pm SEM (n = 2)

conducted by our research group, significant reductions in HSMIrelated 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 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

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

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 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



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

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 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

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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

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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

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

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CONFLICT OF INTEREST

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

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FIGURE 7 Estimated growth (BW in

transferred to sea in mid-September 2014

grams) for farmed S0 Atlantic salmon

(High body weight group, HBW) and in

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