UiT
THE ARCTIC UNIVERSITY OF NORWAY

Norwegian College of Fishery Science

Functional feed for Atlantic salmon (*Salmo salar* L.)

Effect on gastrointestinal tract during parr-smolt transformation and susceptibility to infectious pancreatic necrosis virus (IPNV)

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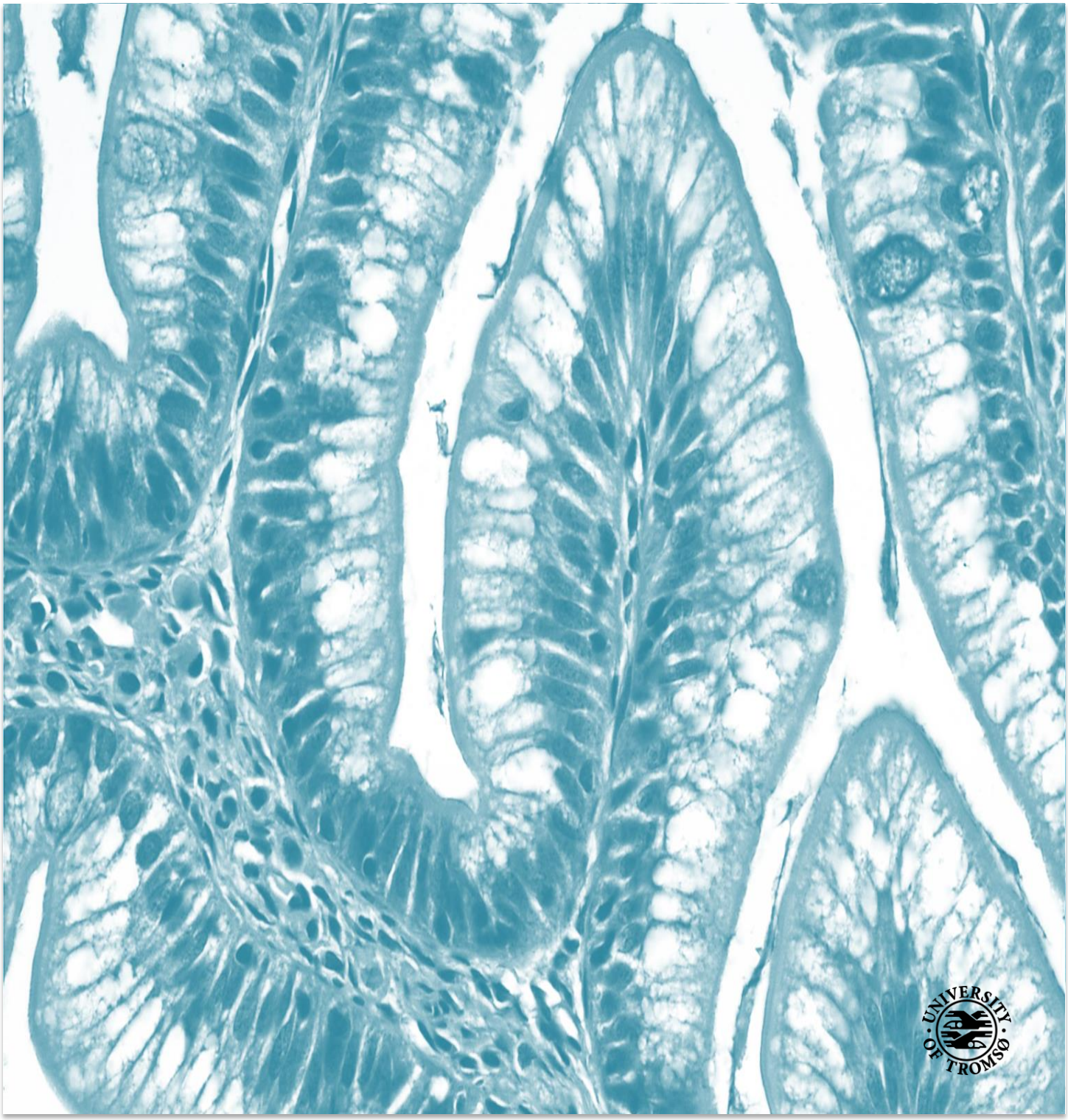

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Gunhild Seljehaug Johansson

Sammendrag (Norwegian)

Produksjon av atlantisk laks (*Salmo salar* L.) er en relativt ny næring i Norge og kan dateres tilbake til 1960-tallet. Siden da har den utviklet seg til en lønnsom næring. Kontinuerlig forbedring av hygienekontroll og kunnskap om fiskeernæring og helse har bidratt til denne utviklingen. Fiskeernæring har blitt i stadig større fokus, blant annet på grunn av økt bruk av planteråvarer som erstatning for de mindre bærekraftige marine råvarene. Jakten på nye fôrkomponenter som skal dekke de ernæringsmessige behov, bedre fiskehelsen, økt vekst og sykdomsresistens er kontinuerlig. Fôrindustrien produserer funksjonelt fôr ved å implementere funksjonelle ingredienser som har vist seg å ha positiv effekt på fisken.

I denne oppgaven ble atlantisk laks fôret med en kontroll diett og to ulike funksjonelle fôr under smoltifisering i ferskvann og senere i sjøvann. Fôrets virkning på laksens tarm ble undersøkt ved hjelp av lysmikroskopi, kombinert med et kvalitativt histologisk poengsystem og real time polymerase chain reaction (PCR). Resultatene fra de histologiske analysene viste at laks fôret med funksjonelt fôr får færre morfologiske endringer i tarmen sammenlignet med kontrollfôret. Det relative uttrykket av pro- og anti-inflammatoriske gener var lavt i både ferskvann- og sjøvannsfasen. Fôrets effekt på mottakeligheten for smitte ved sjøsetting ble testet i et kohabitantforsøk med infeksiøs pankreas nekrose virus (IPNV). Testen viste en signifikant lavere dødelighet hos fisk fôret med funksjonelt fôr. Morfologiske observasjoner ble gjort underveis i de histologiske undersøkelsene fra fôr- og smitteforsøk. Videre ble IPNV detektert med immunmerking og transmisjonselektronmikroskopi i enterocytter fra blindsekkene og tykktarm.

Resultatene av denne studien viste at laks fôret med en diett tilsatt vitaminer, prebiotika, nukleotider og betaglukaner i løpet av forsøksperioden hadde signifikant bedre tarmhelse enn laks fôret med kontrolldietten to uker etter overføringen til sjøvann. Det funksjonelle fôret tilført i ferskvannsfasen ga signifikant lavere mottakelighet til IPNV etter sjøsetting.

Abstract

Atlantic salmon (*Salmo salar* L.) aquaculture is a relatively new industry in Norway, dating back to the 1960s. It has since then developed in to a profitable industry. Continuous improvement of hygiene control, knowledge of fish nutrition and health has been important for this development. Fish nutrition is a research field that has been increasingly focused on partly due to more use of plant raw materials as substitute for the less sustainable marine sources. The search for feed components meeting the nutritional requirements, improving fish health, growth and disease resistance is continuous. Feed producers produce functional feed by implementing functional ingredients that have positive effect on the fish.

In this study, Atlantic salmon were fed a control diet and two different functional feeds during the parr-smolt transformation in freshwater and subsequently in seawater. The feed effects on the salmon gastrointestinal (GI) tract were investigated using light microscopy combined with a qualitative histological scoring system and real time polymerase chain reaction (PCR). Results from the histological analyses showed that feeding salmon with functional feeds produces less morphological changes to the GI tract compared to the control diet. The relative expression of pro- and anti-inflammatory genes was low during both periods. A mortality test using an Infectious pancreatic necrosis virus (IPNV) cohabitant challenge model revealed a significantly lower susceptibility and mortality in fish fed the functional diet. Numerous morphological observations were done during the histological examination from the feed- and challenge trial. Furthermore IPNV were detected by immunolabeling and transmission electron microscopy in pyloric caeca and distal intestine enterocytes.

The results of this study showed that salmon fed a diet containing added vitamins, prebiotics, nucleotides and beta-glucans during the parr-smolt transformation period had significantly better gut status than the control diet two weeks post seawater transfer. The functional feed fed during the freshwater period contributed to significantly lower susceptibility to IPNV post seawater transfer.

Contents

Abbreviations

A-100	Agar 100 Resin
AB	Applied Biosystems
ARA	Arachidonic acid
DHA	Docosahexaenoic acid
DI	Distal intestine
EGCs	Eosinophilic granular cells
EMS	Electron Microscopy Sciences
EtOH	Ethanol
EPA	Eicosapentaenoic acid
FOS	Fructooligosaccharide
FW	Freshwater
FW1	Freshwater feed 1
FW2	Freshwater feed 2
FW3	Freshwater feed 3
GCs	Goblet cells
GI	Gastrointestinal
HE	Harris hematoxylin and eosin
IELs	Intraepithelial lymphocytes
IL	Interleukin
IPN	Infectious pancreatic necrosis
IPNV	Infectious pancreatic necrosis virus
MI	Mid intestine
MKCs	McKnight cells
MOS	Mannanoligosaccharide
NTC	Non-template control
PBS	Phosphate buffered saline
PC	Pyloric caeca
PCN	Pycnotic cell nuclei
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PO	Propylene oxide
RCs	Rodlet cells
SBM	Soybean meal
scFOS	Short-chain fructooligosaccharide
SW	Seawater
SW1-1	Seawater feed 1, origin from FW1
SW2-2	Seawater feed 2, origin from FW2
SW2-3	Seawater feed 2, origin from FW3
SW3-2	Seawater feed 3, origin from FW2
SW3-3	Seawater feed 3, origin from FW3
TEM	Transmission electron microscopy
TNF	Tumor necrosis factor
TOS	Trans-galactooligosaccharide
VP	Virus protein

1 Introduction

1.1 Background

There are numerous different species of aquatic animals for that are produced in aquaculture worldwide for human consumption. These include fishes, crustaceans, mollusks, amphibians, reptiles, sea cucumber, sea urchin etc. (FAO 2011). Countries involved in aquaculture, ranked according to production in 2011 include; China, India, Vietnam, Indonesia, Bangladesh, Norway, Thailand, Egypt, Chile and Myanmar (FAO 2011). Species that are produced in Norwegian aquaculture include; Atlantic salmon (*Salmo salar* L.), rainbow trout (*Oncorhynchus mykiss* Walbaum), Arctic charr (*Salvelinus alpinus* L.), Atlantic cod (*Gadus morhua* L.) and Atlantic Halibut (*Hippoglossus hippoglossus* L.) (SSB 2013). Norwegian salmonid fish farming reached new production heights in 2012 of 1.3 million metric tons, and Atlantic salmon accounted for over 1.2 million tons of this total production (SSB 2013). The largest current producers of farmed Atlantic salmon in Norway are Marine Harvest, Leroy Seafood and SalMar (MarineHarvest 2013).

1.2 Aquaculture

According to Stickney and Treece (2012) fish farming can be dated to the period 2000 – 1000 B.C in China, where mainly common carp (*Cyprinus carpio*) were utilized and has subsequently spread to the entire world. Fish farming in Norway started in the 1960s, and by the 1970s extensive culture of Atlantic salmon and rainbow trout were established (Ford 1984). Salmon farming was shown to be very profitable, but its development was hampered in the 1980's by diseases (Tilseth *et al.* 1991). The basic knowledge regarding disease-control was limited, and treatments were restricted to antibiotics. By 1987 vaccine against *Vibrio salmonicida* the causative agents of cold water vibriosis was commercially available (Lillehaug 2014). Development of efficient vaccines against bacterial diseases, and increased knowledge on disease prevention has led to a reduction in the use of antibiotics with 99% since 1987 (Andersen *et al.* 2013).

Today there is a strong focus on fish health and prevention of diseases both within the industry and government. Governmental regulations are applied to prevent disease outbreaks, some examples of these include; clear distinctions between generations, distance between farms, disinfection of equipment and other infectious components and processing dead fish (Matloven 2003). Additionally, aquaculture employees are obligated to possess knowledge and competence concerning fish health, hygiene, diseases and disease prevention. Vaccination has become one of the most important preventative health work applied in modern aquaculture. Salmon is today vaccinated against a number of bacteria; *Aeromonas salmonicida* subsp. *Salmonicida*, *Listonella anguillarum*, *Vibrio salmonicida* and *Moritella viscosa*. Additionally three virus vaccines are available against the causative agents of Infectious pancreas necrosis (IPN), Infectious salmon anemia (ISA) and pancreas disease (PD). However, they have shown to be less effective.

1.3 Atlantic salmon

Atlantic salmon exists in nature in both anadromous and freshwater resident form, and are located at the east and west coast of the Atlantic Ocean (Klemetsen *et al.* 2003). Anadromous salmon lives in freshwater from the fertilized eggs to juvenile stage. Juveniles undergo a major transformation to be able to survive in seawater, the parr-smolt transformation (smoltification). In this process the parr-marks vanish due to increased silvering of the scales. Additionally, the dorsal, caudal and pectoral fins becomes lighter in color, and develops a black margin (Stefansson *et al.* 2008). Several internal changes occur during smoltification. The reversal in the ionic regulatory mechanism from losing ions by diffusion in freshwater to an ion influx in seawater (Jobling *et al.* 2010). Additionally an increase of intestinal Na^+, K^+-ATPase activity which in turn increase epithelial paracellular permeability (Sundell *et al.* 2003) and an increase in fluid transport in salmon distal intestine (DI) (Veillette *et al.* 1993). The gastrointestinal (GI) tract functions as a physical barrier between the organism and the external environment. During smoltification in freshwater and shortly after sea transfer the changes in fluid transport may disturb this barrier and thereby render salmon more susceptible to disease (Jutfelt *et al.* 2007). To ensure the best possible health status for the fish during their life cycle the quality of the feed is an important factor.

1.4 Fish feed

Fish feed consists of proteins, fat, carbohydrates, vitamins, minerals and non-essential nutrients. Traditionally, marine proteins and oils have been the main ingredients in Norwegian aquaculture diets. Over the last decade the industry has increased the use of plant proteins and plant oils due to costs and sustainability of the marine sources. The main sources of plant proteins in Norwegian aquaculture today are soybean meal (SBM), sunflower meal, pea protein concentrate, beans, wheat gluten and corn gluten (Sørensen *et al.* 2011). The increased use of plant proteins entails higher amount of fibers in fish diets. Feeding Arctic charr diets of 15% inclusion of inulin (fiber) has shown to be potentially harmful due to accumulation of inulin in the enterocytes which in turn impairs cell function (Olsen *et al.* 2001). The main plant oil sources are rapeseed oil, but also palm oil and soybean oil are being used in small amounts depending on costs (Sørensen *et al.* 2011). Using vegetable oils as the sole oil source for Atlantic salmon does not meet the nutritional requirements of n-3 polyunsaturated fatty acid (PUFA) and n-6 PUFA (Miller *et al.* 2008). To reduce feed production costs vegetable oils have been mixed in with marine oils. The use of marine raw materials has decreased from 90% in 1990 to 32% in 2012 (Ytrestøyl *et al.* 2014).

Several plant proteins have been tested as replacement for marine proteins. The alcohol soluble fraction of SBM meal has shown to induce enteritis in the distal part of the intestine of salmonids (van den Ingh *et al.* 1991, Bæverfjord and Krogdahl 1996, Burrells *et al.* 1999). Additionally, Knudsen *et al.* (2007) suggested that soybean-induced enteritis in Atlantic salmon is caused by soyasaponins, in combination with antigenic soybean proteins or intestinal gut microbiota. The soy source used today are extracted soy protein concentrate, inclusion of 17-22 % in 2010, where saponins and alcohols are low or absent (Sørensen *et al.* 2011). Romarheim *et al.* (2013) suggests that SBM induced enteritis could be prevented by adding bacterial meal containing bacteria grown on natural gas to a fish meal free diet. Jutfelt *et al.* (2007) discovered that bacterial translocation of *A. salmonicida* in Atlantic salmon fed sunflower oil was lower than if fed fish oils and concluded that sunflower oil could protect against enteric infections. Arctic charr fed diets with linseed oil containing 64% PUFA showed significant enterocyte damage in PC and MI due to extensive lipid accumulation (Olsen *et al.* 1999). Lipid accumulation increases with increasing levels of PUFA

and low amounts of saturated acids (Olsen *et al.* 2000) to the extent of intracellular damage and loss of cellular integrity. The latter study suggests that "the necrotic form of cell death may predominate in epithelial damage due to high lipid loading". Strains of rainbow trout (and probably other salmonids) selected for growth while fed plant diet with soybean are less sensitive to the factors producing inflammation in the DI (Venold *et al.* 2012).

Currently there are four fish feed producers operating in the Norwegian market: BioMar, Skretting, EWOS and Polarfeed (FHL 2013). BioMar were the contracting authority, providing diets for this study. Over the last three decades it has become well established that there is a link between preventative health and nutrition (Kiron 2012). The feed industry continuously develops new feeds to increase growth performance in the different life stages, increase fish health in terms of modulating the immune system, and reduce stress and mortality. Introducing different additives such as prebiotics and immunostimulants, and macronutrients such as vitamins and carotenoids, have shown positive effects on fish immune system and fish health and the industry has developed what are known as functional feeds. Functional fish feeds are described as "*fish food that has added benefits above and beyond the basic nutritional requirement and it is hoped these diets will improve health status and growth of the fish"* (Tacchi *et al.* 2011). Two functional feeds, one for pre- and one for post smolt, provided by BioMar were used in the present study.

1.4.1 Functional feed ingredients

The term "functional food" was created by the Japanese in the 1980s (personal communication). Functional feeds are an active part of preventive health care in the aquaculture industry in Norway. The main categories of components in functional feeds are; prebiotics, probiotics, immunostimulants, vitamins, nucleotides, minerals and plant or algal extracts (Tacchi *et al.* 2011). Functional ingredients applied in this study were: vitamin C and E, prebiotics, nucleotides and β-glucans. Additionally, adjustments were done to the profile of the fatty acids n-3 (eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA)) and n-6 (arachidonic acid (ARA)).

Vitamin C is an antioxidant that reduces molecular oxygen and incorporates it to other substrates (Englard and Seifter 1986). Deficiency of vitamin C increases susceptibility of infectious diseases, as shown by Hardie *et al.* (1991) on Atlantic salmon fed vitamin C depleted diet and challenged with *A. salmonicida*. Vitamin E (tocopherols) is anti-oxidants which protect membrane phospholipids (Halver 1982). Deficiency of vitamin E in diets for salmonids lead to erythrocyte anisocytosis, erythrocyte fragility and fragmentation, nutritional muscular dystrophy, exudative diatheses and lipid peroxidation (Halver 1982), including reduced survival and growth.

Prebiotics were defined by Gibson and Roberfroid (1995) as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves health". Some of the most common prebiotics in aquaculture are inulin, fructooligosaccharides (FOS), short-chain fructooligosaccharides (scFOS), oligofructose, mannanoligosaccharides (MOS) and *trans*-galactooligosaccharides (TOS) (Ringø *et al.* In press). A study conducted on Atlantic salmon fed a MOS rich diet containing 14% soybean meal, as substitute for fish meal, promotes growth, feed efficiency ratio and protein retention (Refstie *et al.* 2010), in addition elimination of enteritis induced by the soybean meal. MOS supplemented in diets for European sea bass (*Dicentrarchus labrax* L.) showed effect on disease resistance against *Vibrio anguillarum* and reduced stress of the microbiota diversity (Torrecillas *et al.* 2012).

An immunostimulant was described by Bricknell and Dalmo (2005) as "a naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens". β-glucans, nucleotides, lipopolysaccharides and some lipoproteins are immunostimulants, which comprises of repeating units such as glucose, riboses and fatty acids (Ringø *et al.* 2012). Burrells *et al.* (2001) suggested that an inclusion of exogenous nucleotides in commercial diets for Atlantic salmon, coho salmon (*Oncorhynchus kisutch* W.) and rainbow trout may have positive effect on disease resistance and resistance against ectoparasitic infection. β-glucan inclusion,

together with sunflower meal, in Atlantic salmon diet has shown to prevent salmon lice (*Lepeophteirus salmonis*) infection (Refstie *et al.* 2010).

1.5 Morphology and function of the gastrointestinal (GI) tract

The GI tract of salmonids can be divided into; esophagus, stomach, pyloric caeca (PC), mid intestine (MI), DI and the anal opening. Parts of the intestine included in this study were PC, MI and DI (Figure 1). In general, intestinal comprises of multiple layers; mucosa, submucosa, muscularis and serosa. Mucosa, with its folds and brush border membrane increases the overall absorptive surface in the gut (Harder 1975). Mucosa consists of enterocytes and underlying lamina propria (Jutfelt 2006). Figure 2 illustrates the mucosal fold.

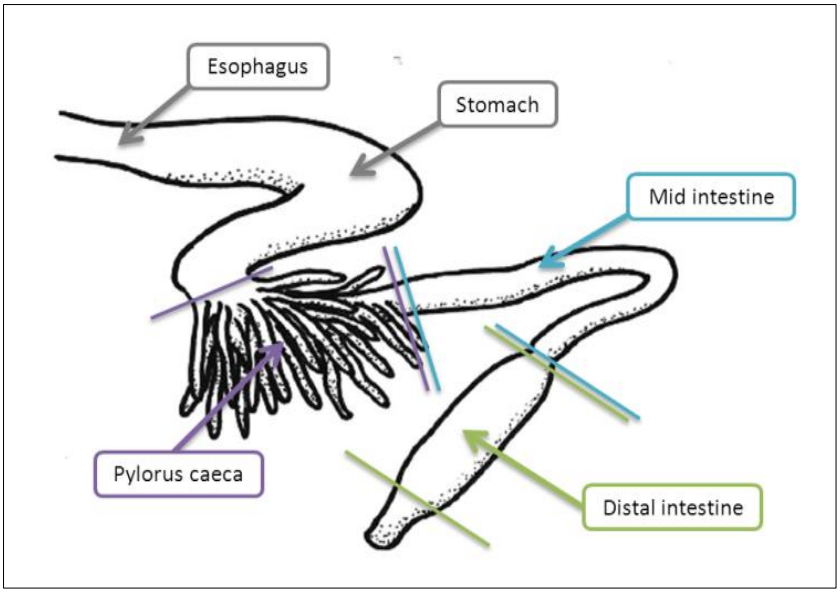

Figure 1. Gastrointestinal tract of Atlantic salmon can be divided in five segments; esophagus, stomach, pyloric caeca (PC), mid intestine (MI) and distal intestine (DI). Included in this study are the PC, MI and DI. PC protrudes from the area posterior to the pyloric muscle that separates stomach from the gut. MI starts posterior to the last caeca and ends at distal intestine. DI is thicker than mid intestine, with visible mucosal folds, and ends at the transition to the anal opening. Illustration: G. S. Johansson.

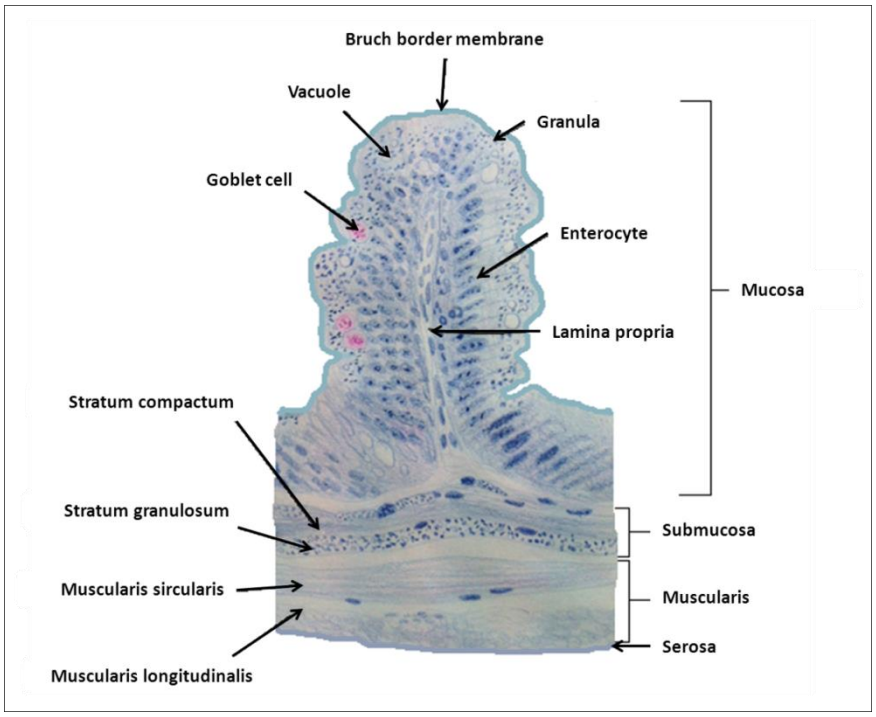

Figure 2. Schematic overview of intestine mucosal fold structure. Illustration: G. S. Johansson.

1.5.1 Pyloric caeca (PC), mid intestine (MI) and distal intestine (DI)

The stomach ends in the pylorus. The part of the stomach with reinforced circular musculature which regulate the passage of food to the MI (Harder 1975). PC are blind-sacs protruding from the pyloric region of MI. Their role is to ensures food retention and provide an increased area for food absorption, especially with high lipid diets, reviewed by Olsen and Ringø (1997). PC comprises of an outer smooth muscle layer (tunica muscularis), a layer of connective tissue and an inner mucosal layer (mucosa). This layer consist of enterocytes, goblet cells (GCs) and lamina propria with surrounding smooth muscle (muscularis mucosae) (Olsen *et al.* 2000). Surrounding the PC is adipose and pancreatic tissue. Pancreas comprises of two parts; exocrine (acinar cells) that secrets digestive enzymes through small tubules to PC and endocrine (islets of Langerhans) which are hormone producing. MI is the main site of nutrient absorption in the gut (Harder 1975). MI is categorized as the thin smooth part of the intestine, from the end of PC to the beginning of DI. MI comprises of an outer double muscle layer (muscularis longitudinalis and sircularis), layer of connective tissue comprising stratum compactum and stratum granulosum, and the mucosal layer. DI has less nutrient absorption capacity and more phagocytic activity then the other segments (Buddington and Diamond

1987). DI has the same layers as mid intestine, but with additional complex mucosal folds. DI is visibly more rough, thicker and darker in color compared to MI.

1.5.2 Enterocytes, goblet cells (GCs) and rodlet cells (RCs)

Epithelium in the GI tract of fish consists of GCs and enterocytes (Olsen *et al.* 2000) (Figure 3). Enterocytes are cylindrical shaped epithelial cells of the GI tract. Their cell nuclei are located in the center or toward the base of the cells (Harder 1975). Enterocytes are "bound" together by desmosomes, which strengthens the intercellular contact (Harder 1975). The cells surface facing the gut lumen, are the microvilli which are "plasma-containing tube-shaped extension of the cell membrane" (Harder 1975) which increases the cell surface significantly. The apical enterocyte surface, with its microvilli, is referred to as the brush border membrane. Enterocytes absorb nutrients through membrane transporters, pinocytosis and diffusion (Jutfelt 2006). At the base of the microvilli, pinocytosis and phagocytosis takes place moving particles and macromolecules into the cell (Jutfelt 2006), where they are transported to digestive lysosomes, or exported by exocytosis in the basal part of the cell.

Supranuclear vacuoles in DI enterocyte and mucosal branching distinguish this segment histologically from MI and PC. Supranuclear vacuoles occupy the apical part of DI enterocytes. Their function is uptake and transport from the gut lumen to the portal vein. In starved fish these vacuoles are small or absent. GI tract enterocytes function as a quick protein reserve in periods of starvation. Studies have shown that the weight of PC can decrease 25% during 2-4 days of starvation (Krogdahl 2001).

Goblet cells (GCs) is the second type of epithelial cell located in the cell lining of the GI tract, where they secret mucus onto the mucosa surface. Maxson *et al.* (1994) showed that the mucus layer protects against transmucosal passage of bacteria. Other functions of mucus are to protect the epithelium from chemical, enzymatic and physical aggressors that may be present in the gut lumen (Montagne *et al.* 2004). Mucus consists of water and glycoproteins,

which are synthetized in cytoplasm and secreted from vacuoles of goblet cells. GCs derived mucin (glycoproteins) have shown, *in vitro*, to inhibit binding of bacteria to the microvilli membrane (Drumm *et al.* 1988).

Rodlet cell (RCs) (Figure 3) was first described in 1892 by P. Thélohan as a protozoan parasite and were later named *Rhabdospora thélohani* by Laguesse in 1895 (Reite and Evensen 2006). However, some scientists suggest that rodlet cells are a type of host cell, while other keeps to the theory of the cell being a parasite (Reite 2005). Marianne Plehn described RCs as secretory cells in 1906 (Reite and Evensen 2006). The rodlet cell may represent a type of eosinophilic granulocyte that populates the tissue at its immature stage and mature in response to the appropriate stimuli, reviewed by Reite and Evensen (2006).

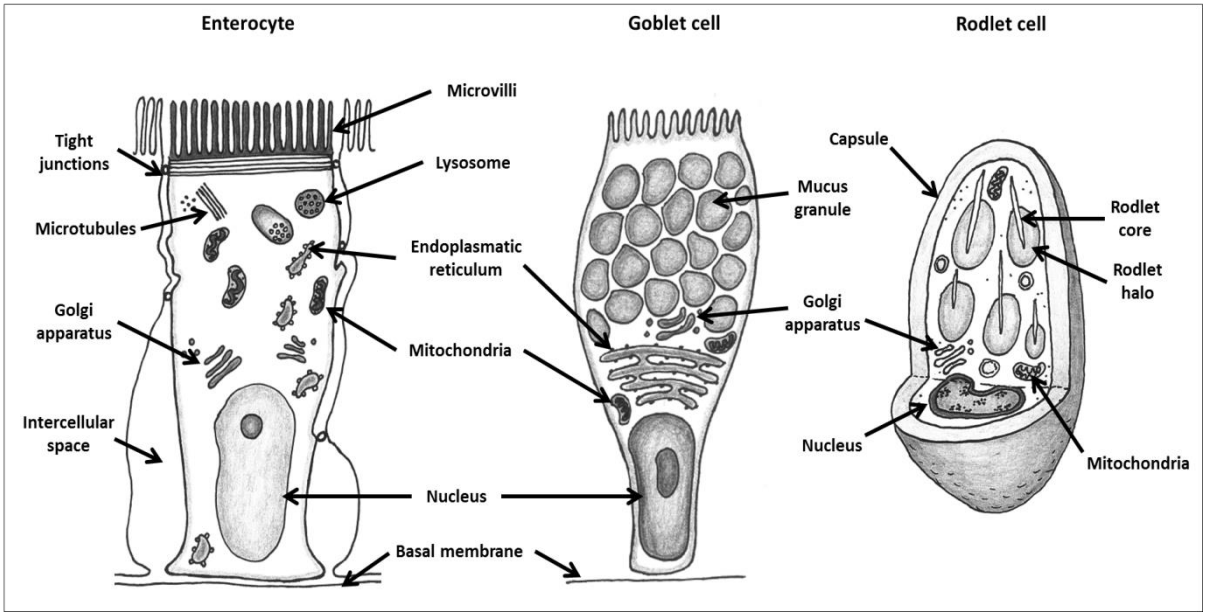

Figure 3. Schematic overview of enterocyte, goblet cell and rodlet cell. Enterocyte inspired by (Krogdahl 2001) and rodlet cell inspired by (Vetmed). Illustration: G. S. Johansson.

1.6 Gastrointestinal (GI) tract immunology

Fish immunological organs constitute of thymus, head kidney, spleen and mucosa-associated lymphoid tissue (Press and Evensen 1999). The mucosal immune system consists of a intraepithelial lymphocytes (IELs) (Figure 4A) macrophages, eosinophilic granular cells (EGCs) and neutrophilic granulocytes, which are not reported organized in Peyer's patches (bundles of lymphatic cells) as in mammals (Rombout *et al.* 2011). Gut-associated lymphocytes in fish

comprise of scattered IELs in mucosa of the GI tract, and are mainly T cells (Bernard *et al.* 2006). The function of these cells in the gut immune system is largely unknown. However, Bernard *et al.* (2006) suggest they have the same function as the systemic T cells some of which are to bind antigens and secrete cytokines. Histologically IELs are observed as small round cells with a thin line of cytoplasm surrounding the nucleus (Amin *et al.* 1991).

EGCs (Figure 4B) are normally observed in submucosa. EGCs are one of two granulocytes, and are part of the innate cells (Rombout *et al.* 2011). EGCs are abundant in mucosa and SM. Reite (1998) described these cells first as mast cell. However, Rombout *et al.* (2011) stated that there EGCs do not have IgE or histamine as mammalian mast cells, thus making the term mast cells inadequate. In cases of inflammation the EGCs migrate to lamina propria and submucosa, and release their granules (Urán *et al.* 2009). The complete functions of these cells are not established.

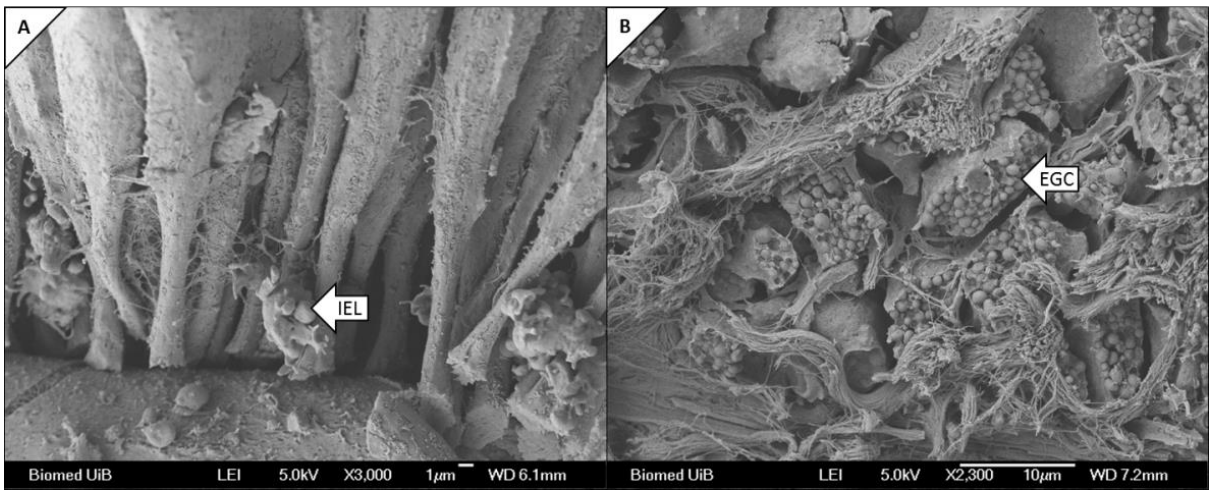

Figure 4. Eosinophilic granule cells (EGCs) and intraepithelial lymphocyte (IELs) in Atlantic salmon mid intestine (MI). (**A**) IEL located in the basal parts of mucosal epithelium. (**B**) EGCs located in stratum granulosum with surrounding connective tissue (collagen fibers). Unpublished photos kindly provided by Reidar Myklebust.

Inflammation is described as a "fundamental pathologic process consisting of a dynamic complex of histologically apparent cytological changes, cellular infiltration, and mediator release that occurs in the affected blood vessels and adjacent tissue" (MediLexicon 2006). These changes are a "response to an injury or abnormal stimulation caused by a physical,

chemical, or biologic agent, including the local reactions and resulting morphologic changes" (MediLexicon 2006). Inflammation of the intestine is referred to as enteritis. In DI morphological changes associated with enteritis includes loss of the supranuclear vacuoles, widening of lamina propria, increased amount of connective tissue between base of folds and stratum compactum, shortening of the mucosal folds height and infiltration of inflammatory cells in lamina propria (Knudsen *et al.* 2007). Cytokines involved in enteritis are: Interleukin 1 beta (*IL-1β*), interleukin 10 (*IL-10*) and tumor necrosis factor alpha (*TNF-α*). Both IL and TNF are reported in fish (Secombes *et al.* 1996). Cytokines are proteins secreted from macrophages and natural killer cells in the innate immune response, and mainly by T cells in the adaptive immune response. Synthesis of cytokines is triggered by gene transcripts as a result of cell stimulation (Secombes *et al.* 1996). *IL-1β* and *TNF-α* is pro-inflammatory while *IL-10* is anti-inflammatory. The involvement of cytokines in fish enteritis was first reported by Urán *et al.* (2008) in common carp. The study showed up-regulation of the pro-inflammatory genes and initial up-regulation followed by down regulation of the anti-inflammatory gene.

1.7 Evaluating pathological changes in the GI tract

To evaluate large amounts of gut samples it is important to base the evaluation on specific criteria. A system with scores indicating different categories of morphological changes makes it possible to run statistical analyses on histological examinations. Evaluating the effects of different feed ingredients on the GI tract has been undertaken by several scientists using morphological scoring systems (van den Ingh *et al.* 1991, Bæverfjord and Krogdahl 1996, Bakke-McKellep *et al.* 2007b, Knudsen *et al.* 2007, Urán 2008, Penn *et al.* 2011). Enteritis caused by SBM has been characterized as flattening of the intestinal mucosa, increased cell proliferation and apoptosis, lack of absorptive vacuoles, widening of lamina propria, leucocyte infiltration, reduced brush border enzyme activity and activation of the immune system (van den Ingh *et al.* 1991, Bæverfjord and Krogdahl 1996, Bakke-McKellep *et al.* 2000, Krogdahl *et al.* 2003, Bakke-McKellep *et al.* 2005, Bakke-McKellep and Krogdahl 2005, Bakke-McKellep *et al.* 2007a). van den Ingh *et al.* (1991) based the evaluation of SBM diets effect on the MI and DI on the ratio between surface areas and length of the different morphologies of the gut, in addition to GCs and microvilli length. Knudsen *et al.* (2007)

adapted a semi quantitative scoring system from Urán *et al.* (2004) for classification of enteritis in DI of Atlantic salmon. Knudsen *et al.* (2007) categorizes score 1-2 as normal morphology, and score 5 as severe enteritis.

1.8 Infectious pancreatic necrosis (IPN)

IPN was first described by Wood *et al.* (1955) in brook trout (*Salvelinus fontinalis*) in North America. Indications of IPN was reported in 1940 in Canada (Smail *et al.* 1995). In 1975, ten years after Wood first described the disease, the first case of IPN in Norway was reported in rainbow trout (Håstein and Krogsrud 1976) and the first clinical outbreak on Atlantic salmon fingerlings occurred in 1985 (Krogsrud *et al.* 1989). The virus is known to infect several fish species in both freshwater (FW) and seawater (SW), in addition to other aqueous organisms (Biering 2002). IPN can cause great loss in the FW phase of salmon rearing, and in the first weeks post SW transfer (Roberts and Pearson 2005). Increasing investments in the salmon production cycle makes the economic loss greater when the disease occurs in SW (Kirkemo 2013). The disease has been widely distributed in all major salmonid farming countries and was considered the most serious viral disease in salmon production (Ariel and Olesen 2002). Since 2009, the number of IPN outbreaks in Norway have declined from 223 to 56 in 2013 (Kristoffersen *et al.* 2014), Table 1. This decline is predominantly a result of the use of quantitative trait loci (QTL)-eggs, which were launched by Aqua Gen in 2009 (AquaGen 2010) from brood fish selected based on a genetic marker for IPN resistance, and the sanitation of broodstock for IPNV (Kristoffersen *et al.* 2014)

Table 1. Number of IPN outbreaks from 2009 to 2013, the Fish Health Report 2013 (Kristoffersen *et al.* 2014)

	2009	**2010**	**2011**	**2012**	**2013**
Infectious pancreatic necrosis - IPN	223	198	154	119	56

IPN is caused by an archetypal birnavirus – a distinctive, small, non-membrane double-stranded RNA virus (Roberts and Pearson 2005), called Infectious pancreatic necrosis virus (IPNV) (Figure 5). The virus belongs to the family *Birnaviridae* in the genus *Aquabirnaviridae* (Kirkemo 2013). IPNV has two genomic segments with bound viral RNA-dependent RNA

polymerase, virus protein (VP) 1 and encapsulated by the capsid protein VP2 and VP3 (Pedersen *et al.* 2007). VP1 exists in free form and bound to the genome (VPg), where VPg is a primer in the RNA synthesis (Calvert *et al.* 1991). VP2 is the outer capsid of the IPNV and is the antigenic region of the virus (Heppell *et al.* 1995) which induces antibody production in the host. VP3 is important in the organization of IPNV replication cycle (Pedersen *et al.* 2007).

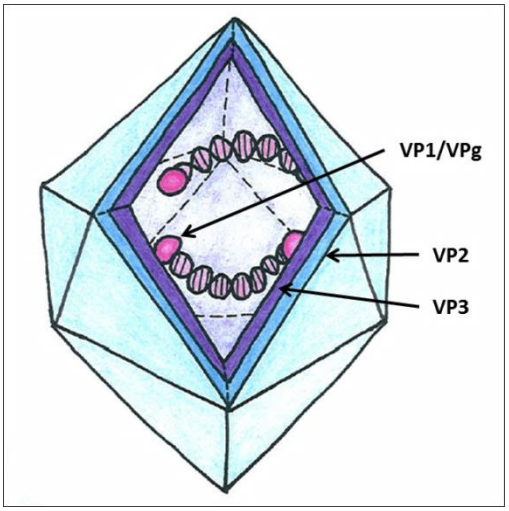

Figure 5. Structure of Infectious pancreatic necrosis virus (IPNV) from the family Birnaviridae. IPNV are built up of VP1, VPg, VP2 and VP3, and double stranded RNA. Illustration: G. S. Johansson.

Histologically the disease manifests in the liver and the exocrine cells of pancreas (cells that produces digestive enzymes) (Kirkemo 2013). In addition typical findings are eosinophilic casts in the lumen of PC and nests with necrotic cells in gut epithelium (Lumsden 2006). The nests consist of degenerated mucosal enterocytes called McKnight cells (MKCs) which are characteristic for IPNV infections (McKnight and Roberts 1976). Loss of epithelium occurs, but most frequently MKCs are formed and expelled through the epithelium (Roberts and Pearson 2005). MKCs are shed into the lumen (McKnight and Roberts 1976), together with the epithelium, and are seen as eosinophilic cast. IPNV has shown to translocate through the intestinal epithelium (Sundh *et al.* 2011). Pathological damage caused by IPNV to the pancreatic tissue has been suggested to be less lethal compared to the damage the virus inflict on the intestinal tissue (McKnight and Roberts 1976, Wolf 1988, Smail *et al.* 1995).

1.9 Aim

This master project was part of a project conducted by Nofima Tromsø in collaboration with BioMar Norway. The main objective of this study was to test functional feeds, produced by BioMar, in the parr-smolt transformation of Atlantic salmon followed by seawater transfer and an IPNV challenge. Two control diets and four test diets were included in this study and where tested for growth performance, pathological changes in the GI tract, and effect on the innate immune system. Three methods were applied;

1) Histological examination of feeds effect on the GI tract
2) Gene expression of genes encoding for cytokines associated with enteritis
3) Survival post seawater transfer in an IPNV cohabitation challenge

Additionally, general observations of cells in the GI tract with light microscopy and detection of IPNV with transmission electron microscopy (TEM).

2 Method

2.1 Fish

The fish used in the present study were IPNV sensitive Atlantic salmon obtained from AquaGen (Kyrksæterøra, Norway). Salmon eye stage eggs were delivered to the Aquaculture Research Station AS in Tromsø and had undergone traditional treatment that applies to intensive fish farming. One week before the experiment started; salmon (35 gram) in freshwater (FW) were acclimated from winter stimuli (6 °C and 6 hours of light) to summer stimuli (12 °C and 24 hour light regime). During the 6 day acclimation period all fish were fed control diet FW feed 1 (FW1). The feeding regime consisted of four hour continuous feeding in the FW period, and 4x30 minutes in the SW period. Smoltification status was checked (n=10 per tank) at experimental start and after 6 weeks using a 24h seawater (SW) challenge test as described by (Blackburn and Clarke 1987). Feeding, weighing and routine maintenance of the fish during the trial were conducted by the personnel at the research station.

2.2 Feed

The feed tested in this study are defined as functional feed, with ingredients which have shown to have a positive effect on salmon in terms of both health (Burrells *et al.* 2001, Torrecillas *et al.* 2012, Meena *et al.* 2013) and growth (Tacchi *et al.* 2011, Gultepe *et al.* 2012). The feeds are specifically developed to improve the performance and growth during the smoltification stage and in the first period post SW transfer (personal communication). BioMar provided six diets for this study, two control diets and four test diets all designed to be as equal as possible. The FW control diet (FW1) containing 49.1% protein, 22.0% fat, 9.1% ashes and 5.4% water and the SW control diet SW feed 1 (SW1) containing 45.9% protein, 22.2% fat, 9.2% ashes and 6.1% water, were formulated to give normal growth but to not include any extra health benefits. The four test diets were divided accordingly: two diets for the FW period and two for the SW period. Test diet two constituting FW feed 2 (FW2) and SW feed 2 (SW2) had additions to the proteins, fat, ash and water added ingredients: 1000 ppm C-vitamin, 400 ppm E-vitamin, prebiotics, nucleotides and β-glucans (package 1). Test

diet three constituting FW feed 3 (FW3) and SW feed 3 (SW3) had adjustment in the fatty acid profile of EPA, DHA and ARA (package 2). Presented in Table 2 is an overview of the feed ingredients.

Table 2. Feed ingredients of the six experimental diets.

	FRESHWATER (3 mm)			SEAWATER (4 mm)		
	FW1 (Control)	*FW2 (Package 1)*	*FW3 (Package 2)*	*SW1 (Control)*	*SW2 (Package 1)*	*SW3 (Package 2)*
Protein %	49.1	47.6	49	45.9	46.2	44.6
Fat %	22.0	22.3	22.2	22.2	21.8	21.4
Ashes %	9.1	9.8	10	9.2	9.6	8.6
Water %	5.4	4.6	5.1	6.1	5.5	6.3
Gross energy (MJ/kg)	22.3	22.5	22.3	21.9	22.3	21.9
Sum marine %	*51.7*	*52.7*	*52.6*	*51.8*	*51.9*	*40.7*
Sum fish meal %	*46.7*	*47.7*	*47.7*	*40.0*	*40.0*	*30.0*

***Package 1:** 1000 ppm C-vitamin, 400 ppm E-vitamin, prebiotics, nucleotides and β-glucans**. **Package 2:** Adjustment in the fatty acid profile of eicossapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (ARA)

2.3 Feed trial

2.3.1 The freshwater period

At the start of the acclimation period a total of 2952 fish were divided in to 12 groups, tagged with Alcian Blue (Panjet) subcutaneous at the abdomen and distributed in 12 tanks (500L) (246 fish per tank) located in two rooms (Figure 6). The different fish groups were randomly distributed in triplicate tanks to reduce possible tank effects. Post acclimation samples (n=10, day 0) were taken and four experimental groups were established with three tanks per group. One dietary group continued on FW1, the second group were given FW2 and the third group FW3 and the fourth given freshwater feed four (FW4, not included in this study). Forty two days later FW samples (n=6 per tank) were collected and the fish were either distributed in two challenge tanks (n=30 from each tank) or in new triplicate tanks with SW for subsequent feed trials (Figure 7).

2.3.2 The seawater period

Fish were distributed in new tanks (500L) with 85 fish per tank. Fish fed FW1 continued on SW1 (SW1-1). Fish fed FW2 and FW3 was split in two tanks where one was given SW2 and the other SW3. This established four feed groups: Fish fed SW2 originating from the FW2 group (SW2-2) and the FW3 group (SW2-3), and fish fed SW3 originating from the FW2 group (SW3-2) and the FW3 group (SW3-3) (Figure 7). After 14 days in SW samples (n=6 per tank) were collected. The feed trial was completed after 40 days in seawater and in total the trial lasted 82 days.

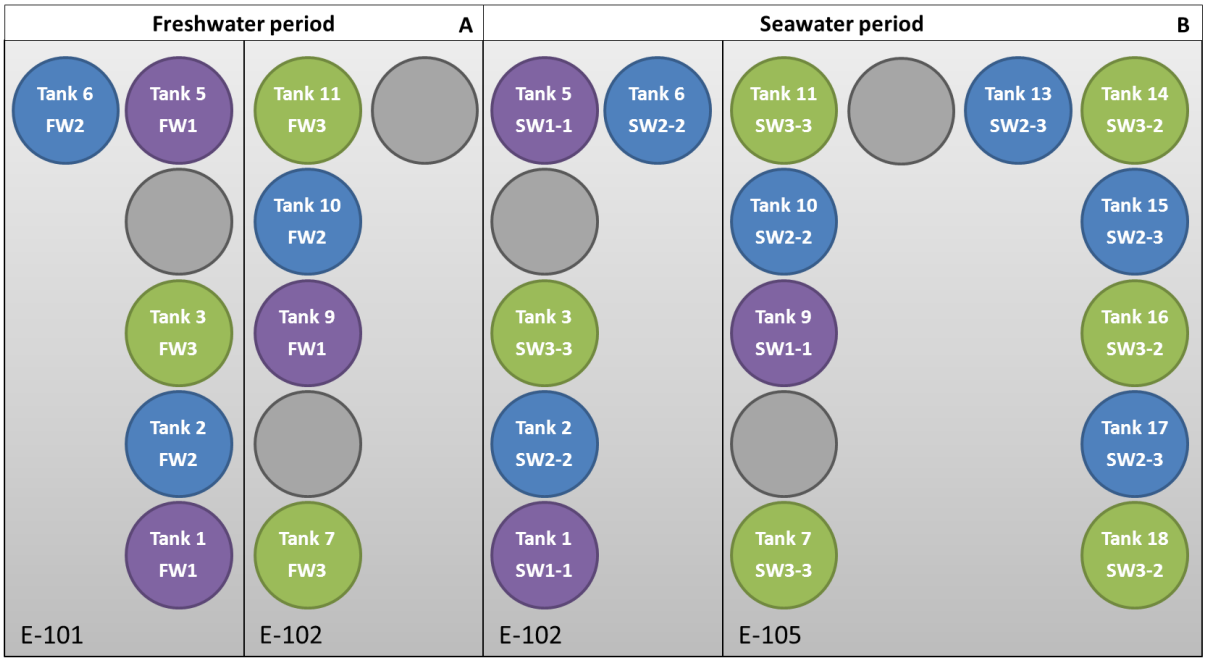

Figure 6. Tank design, 500 liter tanks. Tanks marked in grey held fish fed with feed 4 (FW4 and SW4). These were not included in this study. (**A**) Fish were held in nine tanks allocated to two rooms at the Aquaculture Research Station AS in Tromsø. The three feed groups were represented in triplicate tanks and spread randomly to give similar conditions for each tank. After sampling, at day 42, fish were transferred to SW (**B**) in new tanks in room E102 and E105. Fish on control diet FW1 continued on SW1-1, while the two remaining feed groups were divided in two and fed SW2 and SW3.

2.3.3 Infectious Pancreatic Necrosis Virus (IPNV) challenge test

The susceptibility to IPNV was tested by an IPNV cohabitation challenge model performed according to a confidential internal protocol developed by Nofima. The isolate used were the Nofima isolate FS12 (serotype Sp) collected from an outbreak on Atlantic salmon in Norway in 2001. No further details regarding challenge dose, percentage cohabitants and so on will

not be included in this report. Planning, execution and analyses of the challenge test were conducted by senior advisor Heidi E. Mikalsen at Nofima. At the end of the FW period at the research stations land facility, 30 fish from each tank (270 fish) were transferred to SW in duplicate tanks (900L) at the Fish Health Laboratory. During the IPNV challenge, fish were fed SW1 (Figure 7). Nineteen days post challenge the fish that were moribund and were likely to be dead within few hours were sampled (n=6) for histological analyses. By collecting dying fish the ongoing challenge test were not influenced or disturbed.

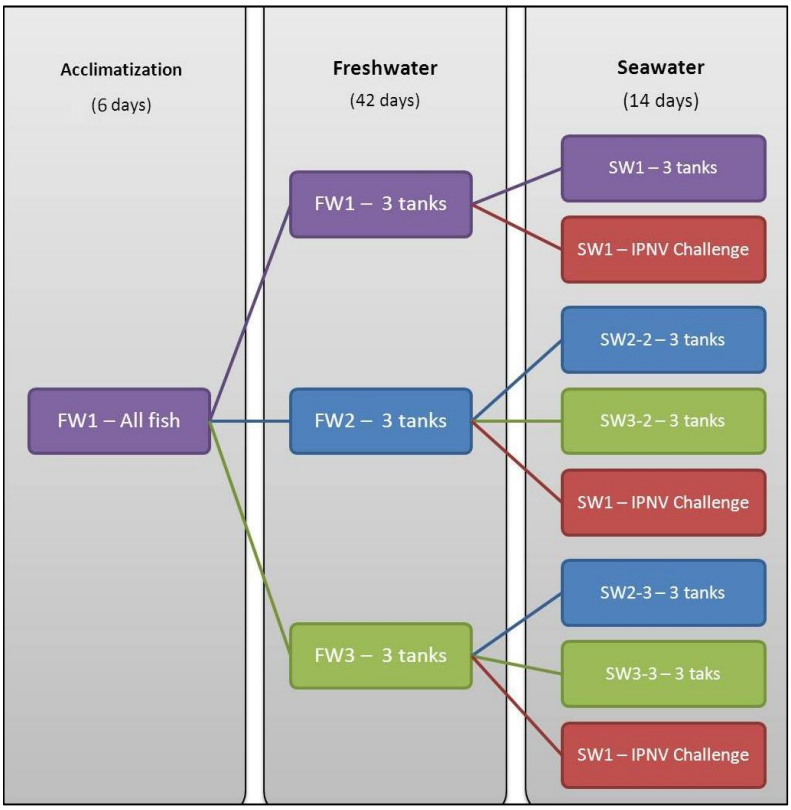

Figure 7. Feed groups and trial layout. Sample collections were conducted post acclimation (6 days) at day 0, post feeding in freshwater (day 42) and seawater (14 days, day 56). Smoltification status was monitored using seawater challenge test during freshwater phase. At day 42, after positive smoltification test, samples were taken and fish transferred to seawater in two batches; one continued in the feed trial, while the 2nd one was used in the IPNV challenge test. The last samples from the feeding trial were taken 14 days post seawater transfer, at day 56.

2.4 Tissue sampling

Six randomly selected fish were netted from the tanks into buckets containing 30 liters of water from the same tanks as the fish were collected from. Oxygen was added to the fish bucket to minimize the window of time between death and sampling. Intestinal tissue is especially susceptible to enzymatic degradation, it is therefore critical that the sampling time is as short as possible. During sampling notes were taken of the fish origin (tank number), fish number (from 1 to 6), fork length, weight, gender and the general exterior- and interior appearance of the fish. Because this master project was part of a larger BioMar/ Mabit project, sampling was also conducted on organs not included in this study; feces, blood, spleen, liver, gill and heart. The samplings were done in collaboration with staff from the fish health group at Nofima. An overview of samples included in this study is displayed in Figure 8.

Fish were sacrificed by cranial concussion. This method was chosen in preference to the use of the anesthetic agent because gut samples had to be taken successively to minimize degeneration of tissue due to *post mortem* enzyme activity. Length and weight was recorded *post mortem*. The abdomen was opened using scalpel and the GI tract was cut free. Fat tissue associated with the intestine was carefully removed lengthwise from the anus to the PC. Scissors were used to open the intestine lengthwise. Feces were gently squeezed out by hand and intestine was rinsed once with ice cold phosphate buffered saline (PBS) (Gibco, Life Technologies). The samples were taken as aseptically as possible, equipment rinsed in sterile water and 70% ethanol (EtOH) (Kemetyl, Vestby, Norway). Complete chemical list is given in Appendix I, and equipment list in Appendix II.

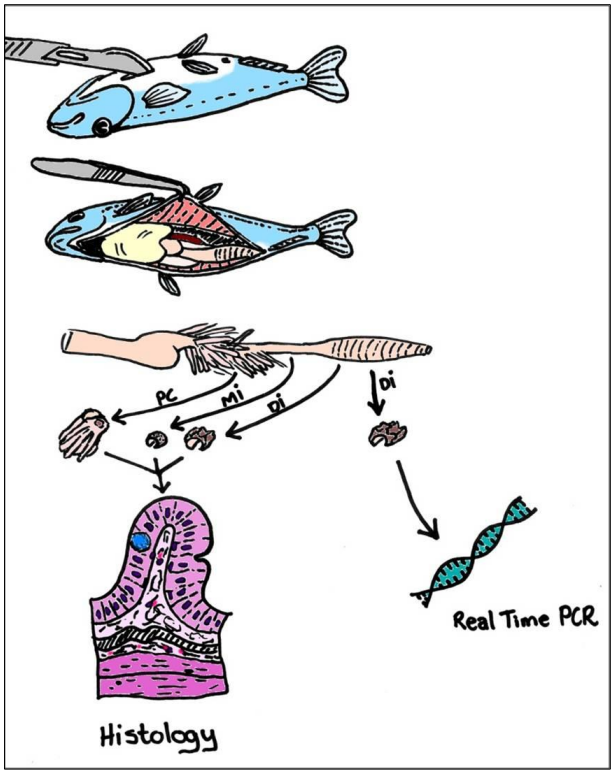

Figure 8. Tissue sampling from Atlantic salmon (*Salmo salar* L.). Samples for histology were taken from pyloric caeca, mid intestine and distal intestine. Samples were stored on 4% paraformaldehyde in phosphate buffered saline (PBS) (Gibco, Life Technologies). Real time PCR samples were taken from distal intestine and stored on RNAlater Solution (Ambion, Austin, Texas, USA). Illustration: G. S. Johansson.

Samples for histological analyses were collected from PC, MI and DI. MI and DI samples were approximately 0.5 cm wide. For histological analyses the orientation of the gut samples were important. When lifting the sample it was essential that the gut curled in the correct direction (Figure 9). Orientation of MI and DI was important since a longitudinal section reveals the simple folds and complex folds. Samples from PC were taken in the middle of the pyloric caeca bundle, a collection of three to four caeca. Gut samples were gently placed in marked histology cassettes (M490-Simport, Histonette I) and immediately transferred to precooled 4% Paraformaldehyde (PFA) (Aldrich) in PBS (Gibco, Life Technologies). Intestinal samples were stored in 4% PFA at 4°C in the dark for approximately 24 hours to ensure proper fixation of the tissue. After fixation, PFA was removed and samples were briefly washed in precooled PBS (Gibco, Life Technologies) before further storage in 70% EtOH (Kemetyl) at 4°C.

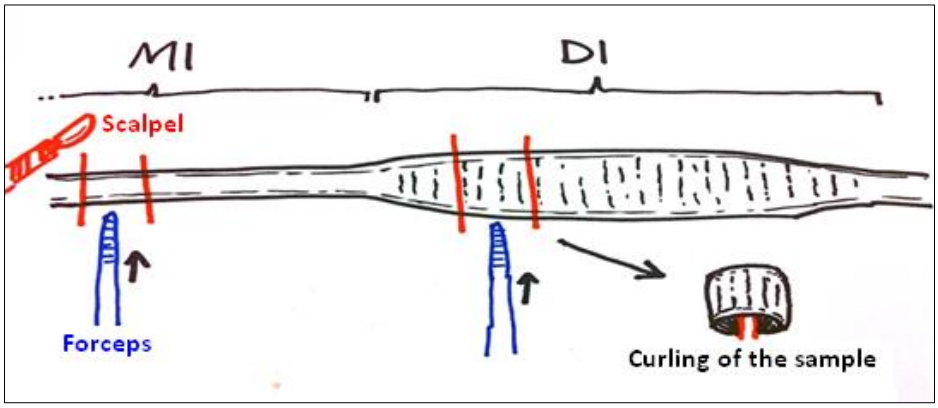

Figure 9. Sampling of MI and DI. The intestine were cut open lengthwise and rinsed one time in ice cold PBS. Tissue pieces were cut about 0.5 cm wide. Samples were lifted with forceps over in histology cassettes. Here it was important that the tissue sample was curled properly in the correct direction to ensure visualizing of all morphologic structures. Illustration: G. S. Johansson.

Real time PCR samples were taken from the DI and spleen of six fish from each feed group and transferred to 2.0 ml eppendorf tubes (Eppendorf AG, Hamburg, Germany) with RNAlater Solution (Ambion, Austin, Texas, USA), ca 1 ml. Sample size was approximately 0.5 cm, and was taken proximal to the DI section destined for histological analyses. Samples in RNAlater Solution (Ambion) were kept overnight at 4°C, before storing at -80°C until further use.

2.5 Histology – light microscopy

Histology was used to study tissue changes in sections from the GI tract of Atlantic salmon. Figure 10 gives an overview of the histological processing of tissue samples. All histology procedures for Harris hematoxylin and eosin (HE) stained tissue are presented in Appendix III.

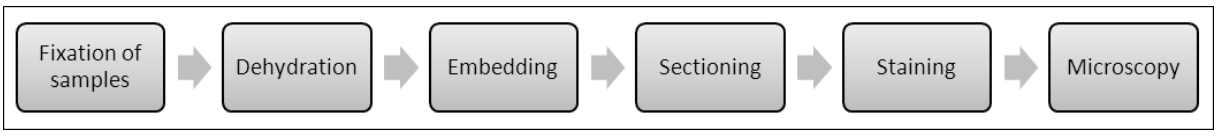

Figure 10. Schematic overview of the histological processing of tissue samples. Inspired by histology protocol by Mumford *et al.* (2007).

2.5.1 Dehydration and infiltration of tissue

Fixation of tissue samples for light microscopy histology were done using 4% PFA in PBS (Gibco, Life Technologies). The ratio of PFA volume to tissue volume should ideally be 1:20, and never less than 1:10. Tissue samples for light microscopy were processed and coated with Paraffin wax (Sigma) using a tissue processor (Shandon Citadel 1000). The following program was used: 2 x 2 hours in 96% EtOH (Kemetyl), 2 x 2 hours in 100% EtOH (Kemetyl), 2 x 2 hours in a 1:1 mix of 100% EtOH (Kemetyl) and Histo-Clear (National diagnostics, ND, Atlanta, Georgia, USA), 3 x 1 hours in Histo-Clear (ND), 1 hour in a 1:1 mix of Histo-Clear (ND) and melted Paraffin wax (Sigma).

2.5.2 Embedding samples in paraffin wax

Following dehydration the tissue samples were brought through the embedding process (Figure 11) using a heated paraffin embedding module (Leica EG1150 H). Molding trays were filled with hot paraffin wax (Sigma), placed on a cold plate and tissue samples were mounted in the middle of the tray. The histology cassettes were placed on top, extra paraffin wax (Sigma) was added and the tray was transferred to the cooling plate (Axel Johnson CP-4). After approximately ten minutes, wax had hardened and the tray could be removed. When embedding tissue samples in paraffin wax it was important to orientate the tissue in the right position (Figure 11C and 11D). All the samples were embedded in a position that gave a longitudinal section.

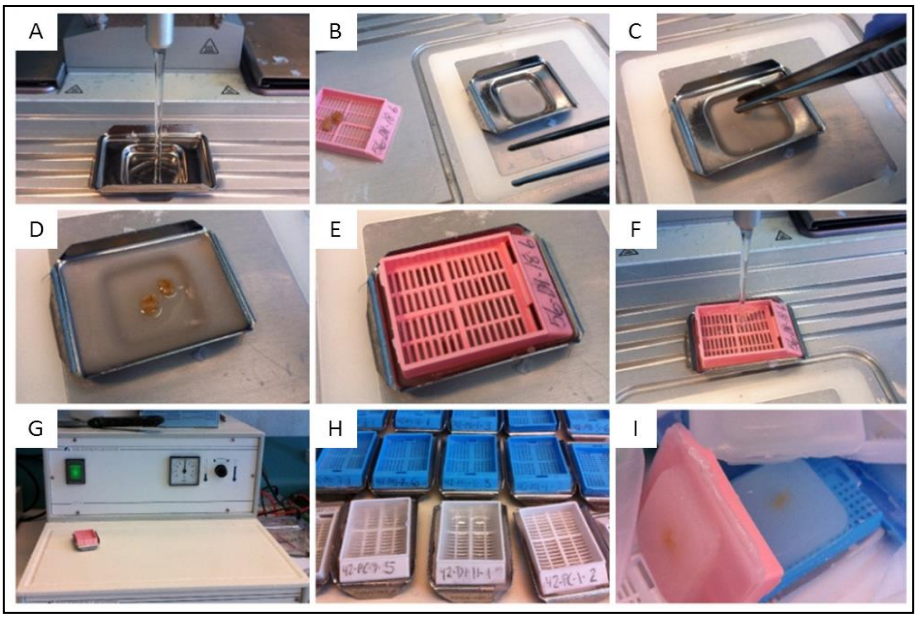

Figure 11. Embedding tissue samples using heated paraffin embedding module (Leica EG1150 H) and cooling plated (Axel Johnson CP-4). Tissue samples were oriented to ensure longitudinal sections. (**A**) Filling molding tray with paraffin wax (Sigma). (**B**) Placing filled molding tray on cold plate and (**C**) orienting tissue samples. (**D**) Tissue sample fixes to the molding tray stiffening of the wax. (**E**) Histology cassette (M490-Simport, Histonette I) were placed on top of mold and (F) mold replenished with wax. (**G**) Samples were set on cooling plate (Axel Johnson CP-4) for the paraffin wax to solidify (**H**). (**I**) Hardened molds were removed from molding tray and were ready for sectioning.

2.5.3 Sectioning of samples

Sections (5 µm) of paraffin wax embedded samples were cut using a microtome (Leica MR2235) and subsequently transferred to a water bath at 42°C (Leica HI 1210) and further to microscope slides (Superfrost Plus, Menzel Gläser) (Figure 12). The first cut was a bit thicker than the following cuts; this was done to reach the tissue inside the paraffin wax. It is of importance to mention that the first cut made wounds in the embedded tissue, and this cut was discharged by cutting about 30 slices prior to sample collection. The samples were stored at room temperature (23^{o}C) and dried over night at 37°C to ensure proper fixation to the glass slide. Figure 13 illustrates the sectioning prosess.

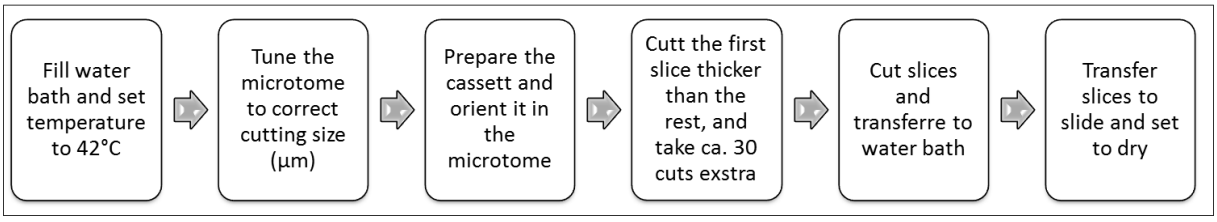

Figure 12. Schematic overview of the steps of sectioning the tissue samples using Leica MR2235 microtome and Leica HI 1210 water bath.

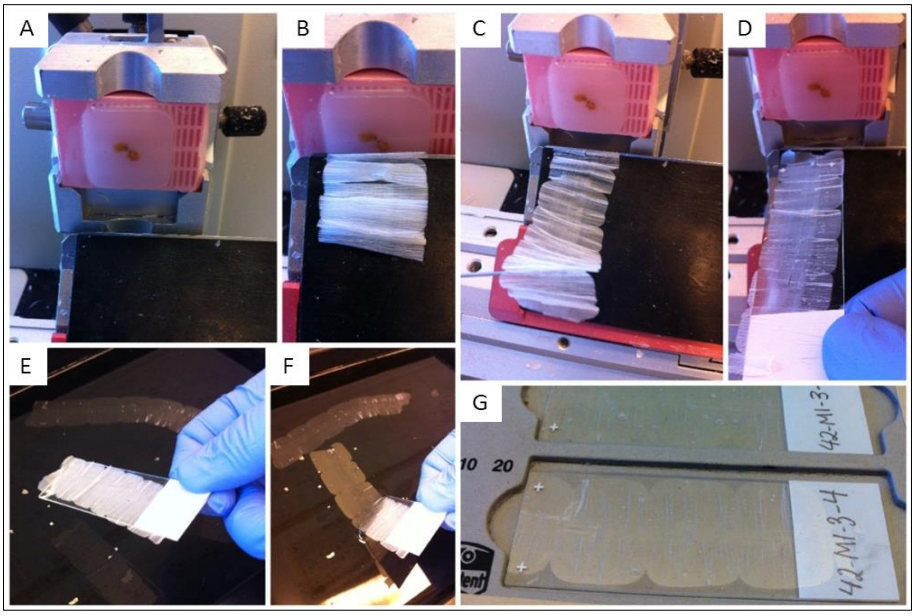

Figure 13. Sectioning of paraffin wax embedded tissue samples. (**A**) Positioning of knife for the initiating rough section (several µm). (**B**) Several discarded sections for smoothening of cut surface from the rough cut. (**C**) Cutting of a few sections and (**D**) placing sections on microscope slide dipped in ethanol (Kemetyl). (**E**) Transfer of sections to water bath (Leica HI 1210) and (**F**) carefully sliding them onto the water surface. Contact with water at 42 °C smooth the sections out. (**G**) Sections are fished out from the water with microscope slide dipped in 50% EtOH and set to dry.

2.5.4 Staining of sample slides

This process consists of several steps which include deparaffinization, rehydration, HE-staining and coverslipping (Figure 14 and 15). HE-staining is a natural dye which is commonly used in histology evaluations (Mumford *et al.* 2007). Briefly, the first step before staining was to remove paraffin from the tissue and reintroduce water. First the slides were dipped for 3 x 5 minutes in Histo-Clear, then 1 x 5 minutes in 100% EtOH, 1 x 5 minutes in 96% EtOH, 1 x 5 minutes in 70% EtOH and 1 x 5 minutes in 50% EtOH and finally a in MilliQ (distilled) water for 10 minutes. The slides were then ready for staining. The staining process was initiated with 2.5 minutes in Harris hematoxylin (Sigma-Aldrich Inc., St. Louis, MO, USA) following ten minutes in pouring water and two minutes in Eosin Y solution (alcoholic) (Sigma-Aldrich). The following steps were 3 x 2 minutes in 96% EtOH and 1x2 minutes in 100% EtOH. Next the slides were incubated for 3 x 2 minutes in Histo-Clear. While the Histo-Clear were still wet, the microscope cover glass (24x60 mm) was mounted using Histomount (National Diagnostics).

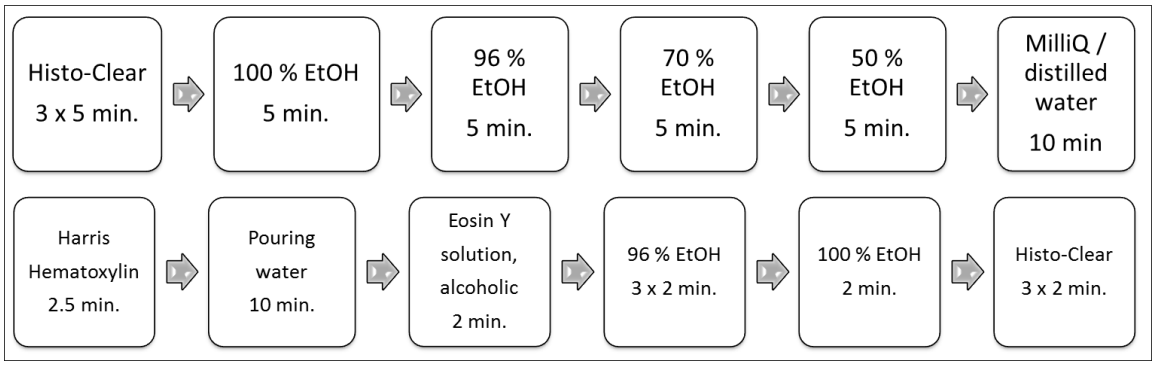

Figure 14. Schematic overview of the staining process of light microscopy histology. The first six steps remove paraffin wax and rehydrate the tissue sections, while the remaining six steps stain the tissue. Modified from a protocol by Fride Tonning (Engineer) University of Tromsø.

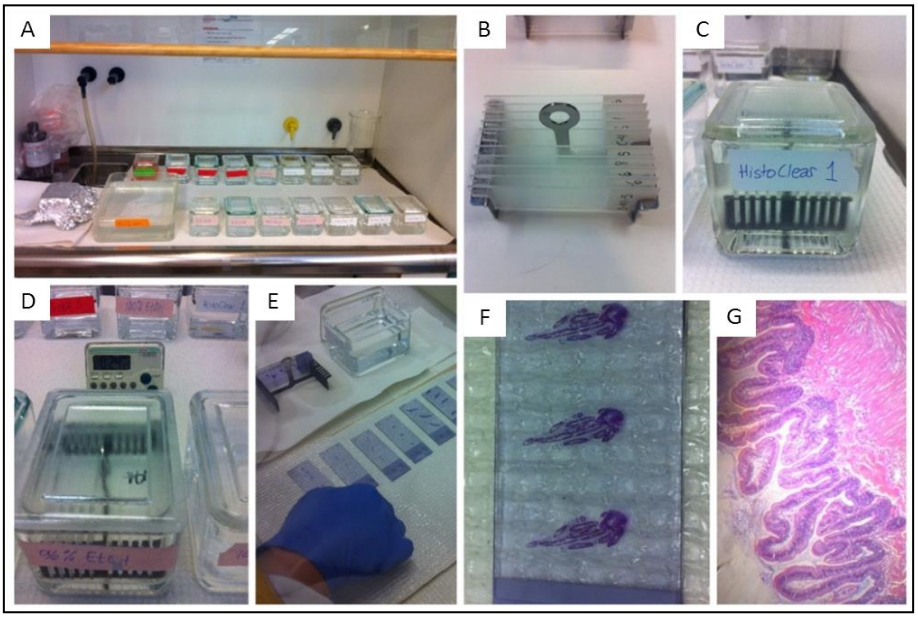

Figure 15. Overview of the staining process. (**A**) Setup of all baths included in staining of tissue sections. (**B**) Microscope slides with tissue sections placed in rack. (**C**) Racks were incubated in several baths of Histo-Clear (National diagnostics) and ethanol (Kemetyl) as part of the deparaffinization and re-hydration of the tissue. (**D**) After staining in Harris hematoxylin and eosin stain were rinsed of in Histo-Clear and water baths. (**E**) Coverslipping with microscope cover glass and Histomount (National diagnostics). (**F**) Coverslipped slides were set to dry in ventilation cabinet before (**G**) analyzing sections.

2.5.5 Microscopy - normal histology

Light microscope was used to evaluate HE-stained tissue slides, analyses were done using Nikon's inverted microscope (Eclipse TE2000-S). Micrographs were captured using Leica DMLB with digital camera (Leica DC 300) and imaging software (analysis Image Processing), and Leica DM 6000B with Leica application suite imaging software. Before analyzing tissue the microscope was tuned according to the microscope manual.

2.6 Scoring of pyloric caeca (PC), mid intestine (MI) and distal intestine (DI) – light microscopy

A qualitative scoring system modified from Knudsen *et al.* (2007) by Hanne Johnsen at Nofima was used for histology evaluation and was used to evaluate inflammatory reactions in the GI tract. This unpublished scoring system has previously been used in similar BioMar studies conducted by Nofima. It was developed for mass screening based on morphologic appearance, not measurements or counts, of GI tract samples (PC, MI and DI) with graded criteria assessing different stages of change in the morphology. The grading of morphological changes enables statistical analyses. A brief description of the criteria is presented here: Criteria 1 is based on the level of vacuolization, criteria 2 the amount of connective tissue and enlargement of lamina propria, criteria 3 the connective tissue between the base of folds and stratum compactum (MI and DI) and the amount of pycnotic cell nuclei (PCN), and criteria 4 the appearance of the mucosal fold length and thickness. Score 1 and 2 (no- and mild changes) are characterized as "normal" morphology, scores from 3 to 5 (moderate-, distinct- and severe changes) are sign of increasingly more damaged morphology. Hereafter, the term "normal" will be used as a collective term when referencing to score 1 and 2. All samples were analyzed by light microscopy. Two rounds of scoring were conducted and scores compared. In cases were slides had score discrepancies between the two rounds a new scoring was conducted. Samples were blindly analyzed meaning that information on neither diet nor expectations were given in advance. This information was shared when the results were completed.

2.6.1 Criteria 1 – level of vacuolization and presence of supranuclear vacuoles

Criteria 1 are divided in two sub-divisions, one for PC and MI and DI, Table 3. PC and MI are scored according to the degree of vacuolization, from low to high. The lowest score indicates none to very little vacuolization of the enterocytes. Increased level of vacuolization is indicated by higher scores. DI is scored in the opposite manner, with low score meaning high presence of supranuclear vacuoles and low scores a loss of vacuoles. Examples of vacuolization in DI versus PC and MI are presented in Figure 16.

Table 3. Scoring system for criteria 1.

Criteria 1 - Vacuolization				
PC and MI	Level of vacuolisation	Score	Changes	Appearance
		1	No	No to very little vacuolization of the enterocytes
		2	Mild	A slight increase in vacuolization of the enterocytes
		3	Moderate	A clear increase in vacuolization of the enterocytes
		4	Distinct	A clear increase in vacuolization that affects most of the enterocytes
		5	Severe	A clear increase in vacuolization that affects all of the enterocytes
DI	Large (Supranuclear) vacuoles	Score	Changes	Appearance
		1	No	Large vacuoles occupy almost the entire apical part of the enterocytes
		2	Mild	Medium-sized vacuoles, wich occupy less than half of the enterocytes, are persent
		3	Moderate	Small-sized vacuoles are near the apical membrane in most enterocytes
		4	Distinct	Scattered small vacuoles are still present in some enterocytes
		5	Severe	No supranuclear vacuoles are present

*PC = Pyloric caeca. MI = mid intestine. DI = distal intestine.

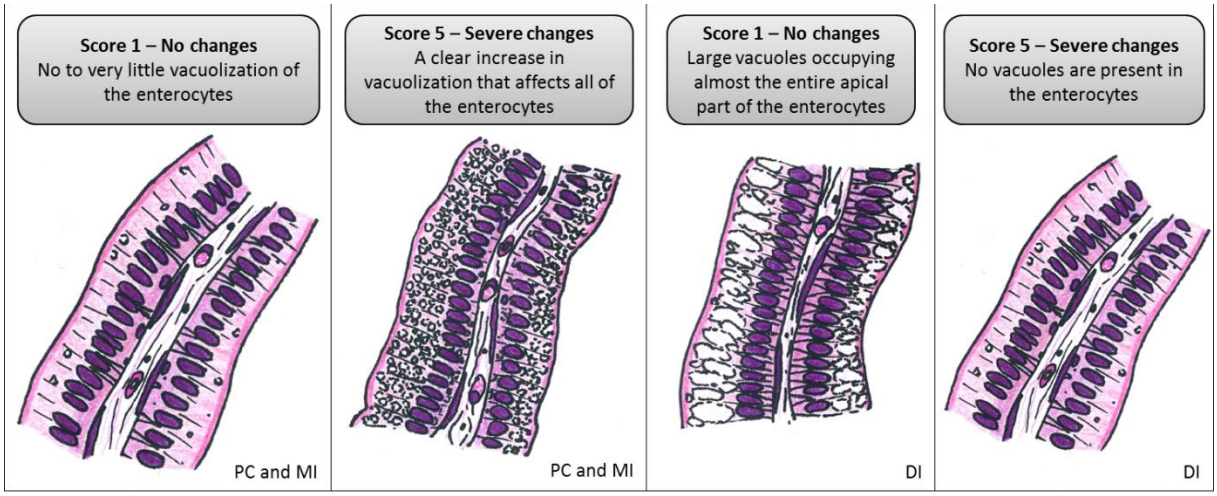

Figure 16. Illustration of criteria 1 (vacuolization) of Atlantic salmon pyloric caeca (PC), mid intestine (MI) and distal intestine (DI). PC and MI have no to very little vacuolization in normal tissue, and in severe pathological changes vacuolization affects all enterocytes. DI has large supranuclear vacuoles filling all enterocytes in normal tissue, and in severe changes these vacuoles are no longer present. Illustration: G. S. Johansson.

2.6.2 Criteria 2 – lamina propria of mucosal folds

Lamina propria is defined as the layer of connective tissue beneath the GI tract epithelium, surrounded by blood vessels and the basolateral membrane (Kryvi and Totland 1997). In case of inflammation lamina propria widens with a more distinct appearance. These observations are given scores from 3 to 5, Table 4. Score 1 and 2 indicates a very thin and delicate core of connective tissue (score 1), or lamina propria appears slightly more distinct in some of the folds (score 2). Examples of lamina propria changes are presented in Figure 17.

Table 4. Scoring system for Criteria 2.

Criteria 2 - Lamina propria				
PC, MI and DI	Lamina propria of mucosal folds	Score	Changes	Appearance
		1	No	There is a very thin and delicate core of connective tissue in all mucosal folds
		2	Mild	The lamina propria appears slightly more distinct and robust in some of the folds
		3	Moderate	There is a clear increase of lamina propria in most of the mucosal folds
		4	Distinct	There is a thick lamina propria in many folds
		5	Severe	There is a very thick lamina propria in many folds

*PC = pyloric caeca. MI = mid intestine. DI = distal intestine.

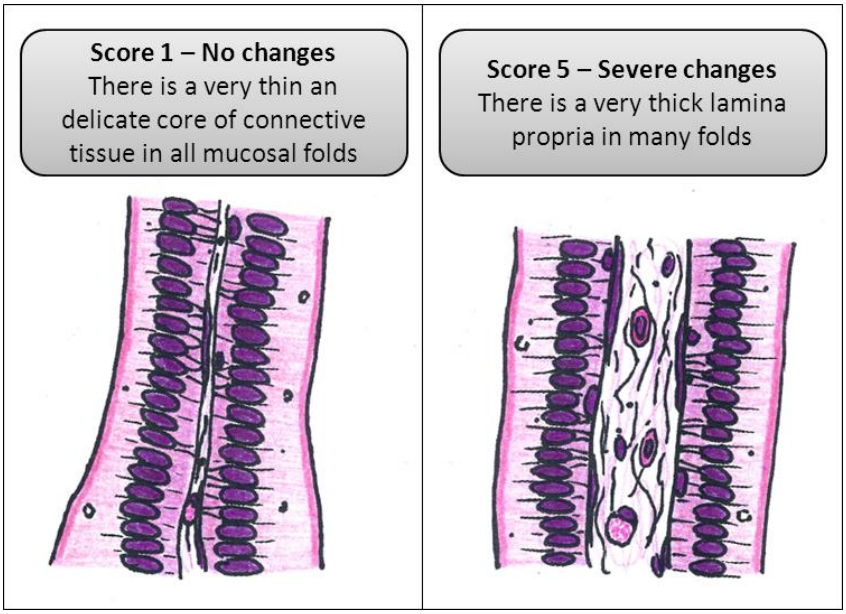

Figure 17. Illustration of criteria 2 (lamina propria) of Atlantic salmon pyloric caeca, mid intestine and distal intestine . In normal tissue there is a thin and delicate core of connective tissue in lamina propria, which in severe pathological changes gets very thick in many mucosal folds. Illustration: G. S. Johansson.

2.6.3 Criteria 3 – connective tissue between base of folds and *stratum compactum*, and presence of pycnotic cell nuclei (PCN)

Criteria 3 are divided in two sub-divisions, one for PC and one for MI and DI, Table 5. MI and DI are scored increasingly by the amount of connective tissue beneath base of the mucosal fold and stratum compactum. In normal gut morphology the layer of connective tissue is very thin (score 1) or there is a slight increase of connective tissue beneath some mucosal folds (score 2). Increasing amounts of connective tissue are signs of inflammation and scored from thickness and prevalence, up to the highest score (score 5) which represents an extremely thick layer of connective tissue beneath most of the mucosal folds (Figure 18).

PC is scored according to the magnitude of PCN present and they are observed during cell death and manifests as the nucleus becomes smaller and denser due to clogging of the

chromatin (Leuchtenberger 1949). Score 1 and 2 indicates that there are no to a slight increase of PCN present. As the amount of enterocytes with PCN increases, the score increases. Examples of PC's PCN are presented in Figure 19.

Table 5. Scoring system for Criteria 3.

Criteria 3 - Connective tissue and pycnotic cell nuceli				
MI and DI	Connective tissue (between base of folds and stratum compactum)	Score	Changes	Appearance
		1	No	There is a very thin layer of connective tissue between the base of folds and the stratum compactum
		2	Mild	There is a slightly increased amount of connective tissue beneath some of the mucosal folds
		3	Moderate	There is a clear increase of connective tissue beneath most of the mucosal folds
		4	Distinct	A tick layer of connective tissue is beneath many folds
		5	Severe	An extremely thick layer of connective tissue beneath most folds
PC	Presence of Pycnotic cell nuclei	Score	Changes	Appearance
		1	No	There are no to very few pycnotic cell nuclei
		2	Mild	There is a slight increase in pycnotic cell nuclei
		3	Moderate	There is a clear increase in pycnotic cell nuclei
		4	Distinct	There is a clear increase in pycnotic cell nuclei that affect most enterocytes
		5	Severe	There is a clear increase in pycnotic cell nuclei that affect all enterocytes

*PC = pyloric caeca. MI = mid intestine. DI = distal intestine.

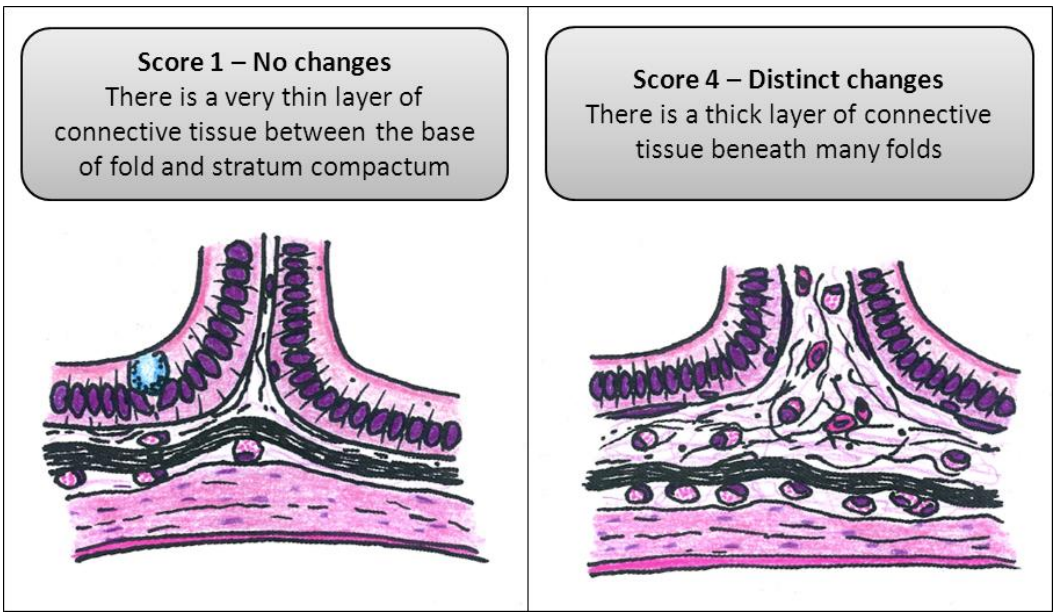

Figure 18. Illustration of criteria 3 (connective tissue) of Atlantic salmon mid intestine (MI) and distal intestine (DI). Criteria 3, MI and DI, characterize the amount of connective tissue beneath the base of fold and stratum compactum. Illustration: G. S. Johansson.

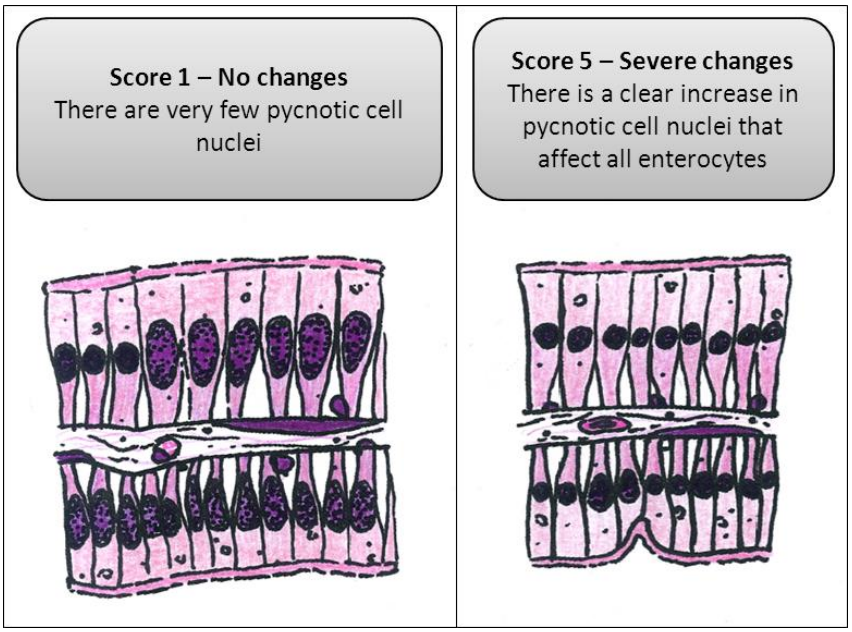

Figure 19. Illustration of criteria 3 (pycnotic cell nuclei) of Atlantic salmon pyloric caeca (PC). Criteria 3 characterize presence of pycnotic cell nuclei (PCN) in PC enterocytes from very few (score 1) to a clear increase (score 5). Illustration: G. S. Johansson.

2.6.4 Criteria 4 – mucosal folds

This criteria is based on the length and width of the mucosal folds, and are divided in two sub-divisions; one for DI and one for PC and MI, Table 6. DI mucosal folds are characterized from long and thin with thin side branches (low score) to loss of side branches and a stubby appearance (high score). PC and MI mucosal folds are simple compared to DI and have characteristics as long and thin (low score) to short and stubby (high score). Examples of criteria 4 are presented in Figure 20.

Table 6. Scoring system for criteria 4.

Criteria 4 - Mucosal folds				
PC and MI	Mucosal folds	Score	Changes	Appearance
		1	No	Mucosal folds appear long and thin
		2	Mild	Mucosal folds apear long to medium and are slightly thicker
		3	Moderate	Mucosal folds have short to medium length
		4	Distinct	Mucosal folds are short
		5	Severe	Mucosal folds appear very stubby
DI	Mucosal folds	Score	Changes	Appearance
		1	No	Simple and complex folds (CFs) appear long and thin. Thin sidebranches on the CF
		2	Mild	Simple mucosal folds have medium length. CFs are still long but appear thicker
		3	Moderate	Simple folds have short to medium length. Side branches on CF are stubby
		4	Distinct	Thick CFs are prevalent. Simple forlds are short. Almost no side branches are on the CF
		5	Severe	Both complex and simple folds appear very stubby

*PC = pyloric caeca. MI = mid intestine. DI = distal intestine. CFs = complex folds

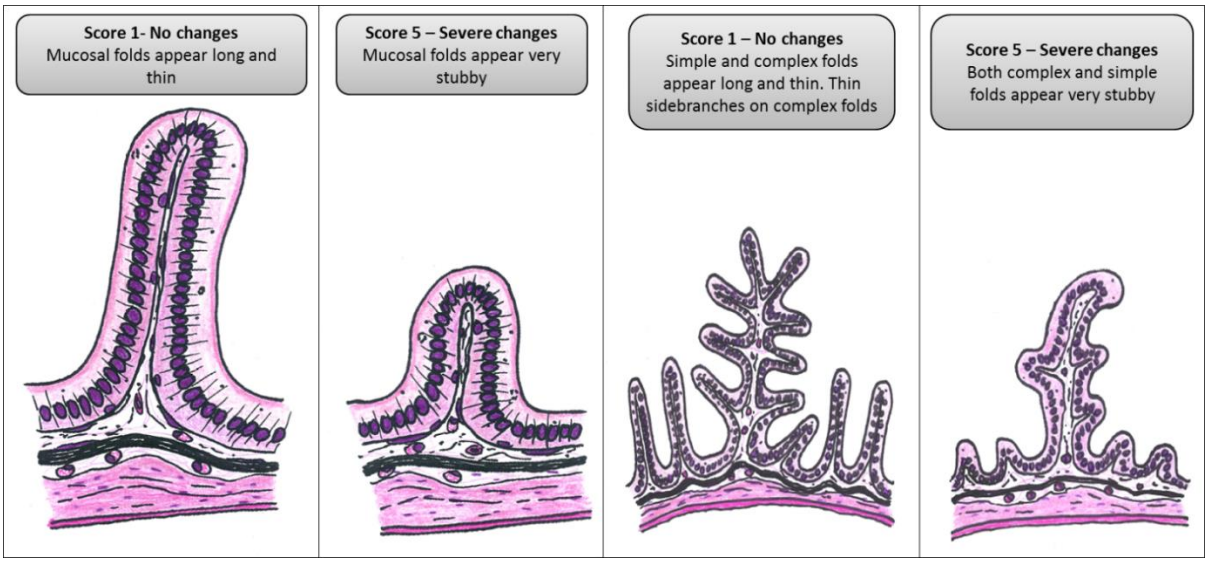

Figure 20. Illustration of criteria 4 (mucosal folds) of Atlantic salmon pyloric caeca (PC), mid intestine (MI) and distal intestine (DI). Criteria 4 characterize the height and width of the mucosal folds of PC and MI, height and width of DI simple folds and degree of branching of the complex folds. Illustration: G. S. Johansson.

2.7 Histology - transmission electron microscopy (TEM)

TEM was used to determine the nature of specific cells observed in the light microscopy evaluations. Transmission electron microscopes shoots electrons through an ultra-thin tissue section stained with heavy metals. The electrons passing through the sample produces an image in grey tones. TEM morphology analyses were conducted at the Molecular Imaging Center, University of Bergen, and their established protocol was followed. Normally tissue samples for TEM (with Agar 100 Resin embedding) are fixed in 2% glutaraldehyde in 0.1M Sodium Cacodylate buffer. Because samples for TEM were not taken during fish trial another approach was used. Paraffin wax embedded tissue samples from the IPNV-trial were chosen for further analyses; two PC and two DI samples from dietary group FW1 and one PC and one DI sample from group FW3.

2.7.1 Rehydration of paraffin embedded tissue

Paraffin wax embedded tissue samples were melted from wax at 70 °C for 4.5 hours. Millimeter sized cubes (approximately 2-3 mm) were cut from the tissue and transferred to 2.0 ml eppendorf tubes with Xylene (Sigma-Aldrich Inc., St. Louis, USA) for deparaffinization. The following day Xylene were removed and the tissue rehydrated through the following

steps in ethanol (Sigma); 100% EtOH for 1 hour, 96% EtOH for 1 hour, 70% EtOH for 1 hour, 50% EtOH for 30 minutes and 30% EtOH for 30 minutes.

2.7.2 Post fixation of tissue samples

After rehydration tissue samples were washed (3 x 30 minutes) in 0.1 M dimethylarsenic acid sodium salt trihydrate (Na-cacodylate) (Merck KGaA, Darmstadt, Germany). Post fixation in 1% osmium tetraoxside (Electron Microscopy Sciences (EMS) Hatfield, PA, USA) in 0.1M Na-cacodylate (Merck) for 1 hour. Osmium was used doe to its high electron density and ability to enhance image contrast in TEM. Osmium was neutralized by 5% ascorbic acid (VWR BDH Prolab, Oslo, Norway), and was removed by rinsing the tissue with sodium cacodylate buffer (Merck) before incubation in sodium cacodylate buffer (Merck) for 20 minutes.

Tissue samples were then ready for dehydration though five steps in increasing ethanol (Sigma) in the following steps; 30% EtOH for 30 minutes, 50% EtOH for 2x15 minutes, 70% EtOH for 30 minutes, 96% EtOH for 3x15 minutes and 100% EtOH for 3x15 minutes.

2.7.3 Embedding samples in epoxy (Agar 100 Resin)

Dehydrated tissue samples were coated and embedded in Agar 100 Resin (A-100) (Agar Scientific Ltd., Boras AS, Rasta, Norway) dissolved in 1,2 propylene oxide (PO) (EMS) for 30 minutes at each step (Figure 21).

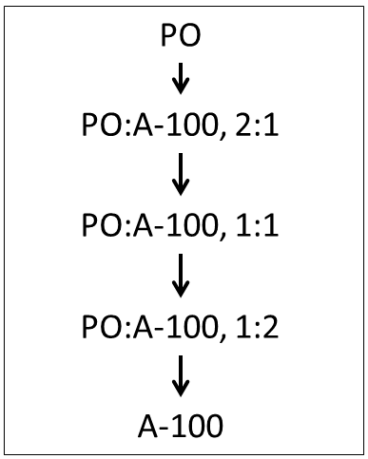

Figure 21. Embedding of tissue samples in Agar 100 Resin (A-100) (Agar Scientific Ltd.) through five steps (30 minutes in each step). Tissue samples were first coated with A-100 dissolved in 1,2 propylene oxide (EMS) and then embedded.

A-100 was slightly viscous which made it important to manually mix the tissue samples into the solution. A-100 coated tissue were transferred to silicon molding trays. Small paper strips with sample information was embedded together with each sample (Figure 22). The tray was stored at room temperature overnight and then oriented using magnifier and tweezers. Tissue was oriented to give longitudinal sections. Embedded tissue samples were stored at 60°C in 48 hours for the A-100 to harden.

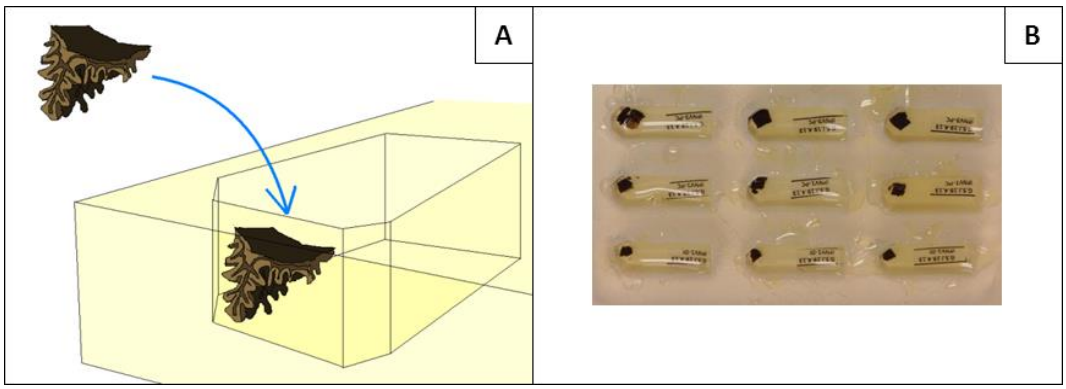

Figure 22. Embedding of tissue sample in Agar 100 Resin (**A**) Orientation of DI tissue sample in molding trays and (**B**) embedded tissue samples with label. Illustration: G. S. Johansson.

2.7.4 Sectioning and staining of TEM samples

Samples were broken out from the molding tray and cut into a diamond shape with four sides (Figure 23). The samples were mounted to the ultramicrotome (Reichert ultracut S, Leica). A diamond-knife on a small water bath (Diatome) was used to cut 45 nm sections of the samples. Sections were observed on the water bath surface during sectioning. The color of the section slides changed with adjusting section thickness, and was cross checked with a color scale to ensure the right thickness. The first sections were semi thick (70-80 nm), thereafter the section thickness were decreased to 45 nm. Sections were transferred onto circular copper mesh (grids) and left to dry at room temperature for a few minutes. Grids with sections were stained in droplets through the following steps; 1% uranyl acetate (Merck) in ddH_2O for 18 min, washed in ddH_2O, put in lead nitrate (Merck) (Reynolds 1963) for 8 min and washed in ddH_2O. Sectioning and dyeing was done by Engineer Anne Nyhaug. TEM micrographs from the feeding trial were captured by Professor Reidar Myklebust at the Molecular Imaging Center, University of Bergen.

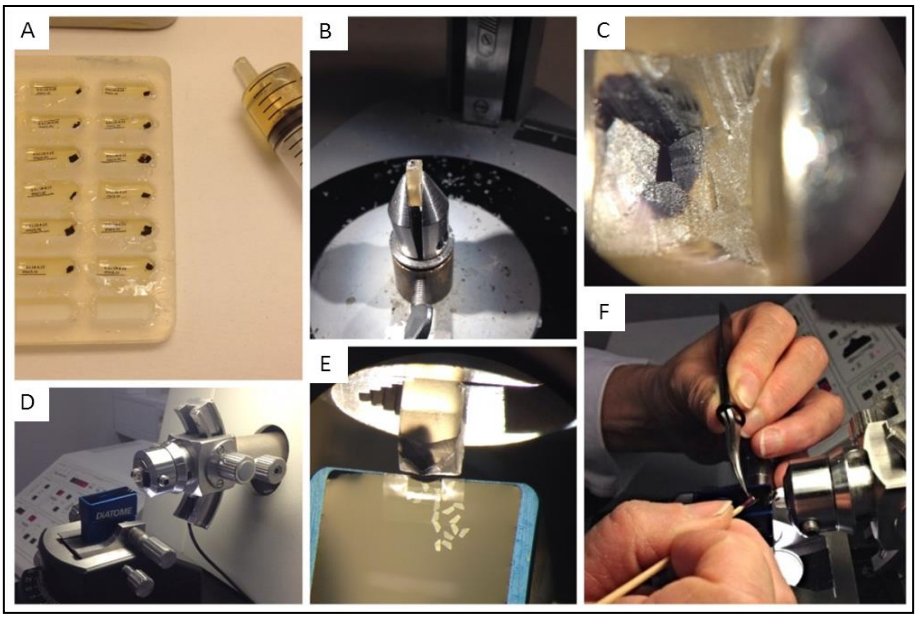

Figure 23. Sectioning of tissue samples for transmission electron microscopy (TEM). (**A**) Embedding of tissue samples with Agar 100 Resin in molding tray. (**B**) Trimming of the mold to design the cut area (**C**) for sectioning. (**D**) Sectioning of ultrathin slides. (**E**) Tissue sections were cut directly onto a water bath to visualize the sections. (**F**) Transfer of sections with forceps, grid and soft stick with dog hair.

2.8 Immunocytochemistry – transmission electron microscopy (TEM)

Two paraffin wax embedded tissue samples, PC and DI, from the dying fish collected from the IPNV challenge were chosen for immunocytochemistry and TEM. DI was chosen based on indications that IPNV uses proximal intestine and DI as route of infection (Sundh *et al.* 2011). Of the proximal segments (PC and MI), PC were chosen due to the greater pathological changes seen in this segment compared to MI. A detailed description of the method is described by Webster and Webster (2007).

2.8.1 Rehydration of paraffin embedded tissue

Tissue samples were cut out from the paraffin wax block and deparaffinated in Xylene (Sigma-Aldrich) for 2x30 minutes. Rehydration of tissue samples was done using the following steps in ethanol (Sigma-Aldrich) and PBS; 100% EtOH for 3x5 minutes, 96% EtOH for 15 minutes, 70% EtOH for 15 minutes and PBS for 2x10 minutes.

2.8.2 Fixation, cryprotection and freezing

Specimens were cut in smaller pieces (a few mm) and fixed with gelatin and sucrose through the following steps; 8% Gelatin from cold water fish skin (Sigma) for 1 hour at 37°C, 12% Gelatin from cold water fish skin (Sigma) for 1 hour at 37°C and 2.3 M sucrose in PBS (made by the laboratory engineers) overnight at 4°C. The following day tissue samples were oriented on specimen pins, to give longitudinal sections, and frozen in liquid nitrogen (Figure 24). Sucrose surrounding the tissue acted as glue.

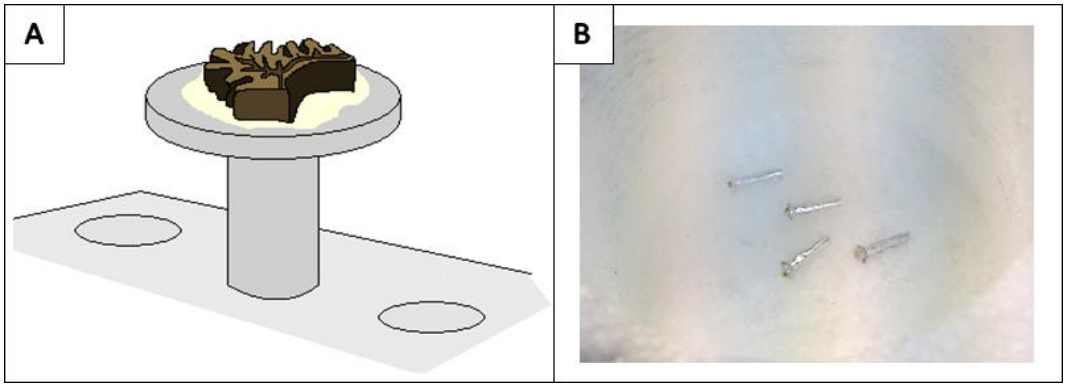

Figure 24. Fixed, cut and sucrose-infiltrated tissue samples were placed on a specimen pin (**A**) and frozen in liquid nitrogen (**B**). Illustration: G. S. Johansson.

2.8.3 Cryosectioning of frozen tissue

Specimen pin were mounted in the cryo ultra microtome (Reichert FCS Ultracuts, Leica), trimmed and sectioned at - 80°C (Figure 25). Sectioning was done by Staff engineer Helga-Marie Bye and Chief engineer Randi Olsen at Department of Electron Microscopy, Faculty of Medical Biology, The Arctic University of Norway, Tromsø, according to the method described by Webster and Webster (2007).

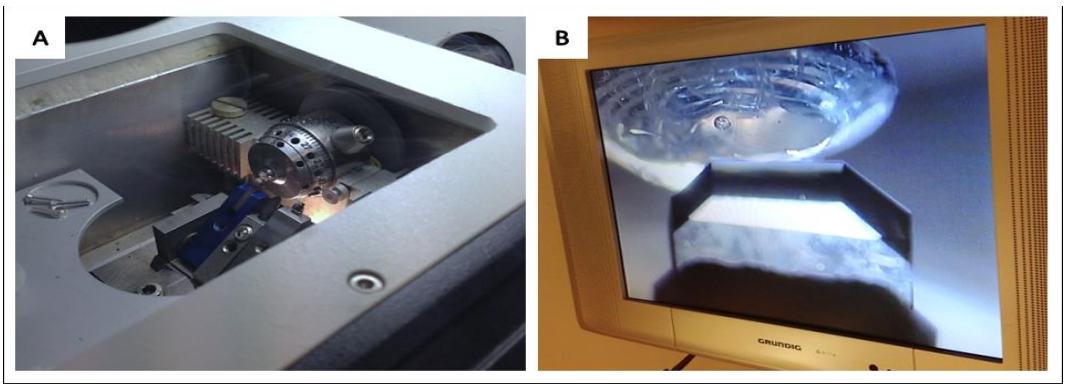

Figure 25. Cryo sectioning of TEM samples. (**A**) Sectioning of cryprotected tissue sample was done using Cryo Ultra Microtome (Reichert FCS Ultracuts, Leica). (**B**) Monitor connected to the microtome shows the trimming blade when trimming the tissue sample.

2.8.4 Immunolabeling of IPNV and contrasting and dying of tissue

Immunolabeling, contrasting and dying of tissue sections were conducted on laboratory film (Parafilm, Bemis Flexible packaging). Grids with tissue sections were incubated on droplets of the different solutions by the following steps; double distilled water (ddH_2O) for 20 minutes, blocking solution (0.8% Bovine serum Albumin (BSA) (Calbiochem, EMD Millipore, Merck KGaA, Darmstadt, Germany) dissolved PBS and 0.1% gelatin from cold water fish skin (FSG)(Sigma) in PBS (Sigma-Aldrich) for 15 minutes, monoclonal mouse anti IPNV antibody specific for virus protein 3 (VP3) (produced at Intervet Norbio, Bergen, Norway) for 20 minutes, PBS for 10 minutes, 1:150 bridging antibody (Rat anti mouse, RαM) (ICN Pharmaceuticals, Inc., Cappel, Aurora, Ohio, USA) for 15 minutes, PBS for 10 minutes, 1:50 Protein A-gold 10nm (PAG_{10}) (University of Utrecht, The Netherlands) diluted in blocking solution for 15 minutes, PBS for 10 minutes, 1% Glutaraldehyde (Merck) for 5 minutes, ddH_2O for 10 minutes and 9:1 with Methylcellulose (Sigma) and uranyl acetate (Fluka, Sigma-Aldrich) (MC/UA) for 6 minutes (Figure 26). This method is described in detail by Webster and Webster (2007). VP3-antibodies were kindly provided by Lill-Heidi Johansen, Nofima, Tromsø. Two samples were tested as negative control, and were not added the VP3 antibody, but only blocking solution to exclude unspecific binding of PAG_{10}. The bridging antibody binds to VP3 and will bind Protein A-gold (Webster and Webster 2007).

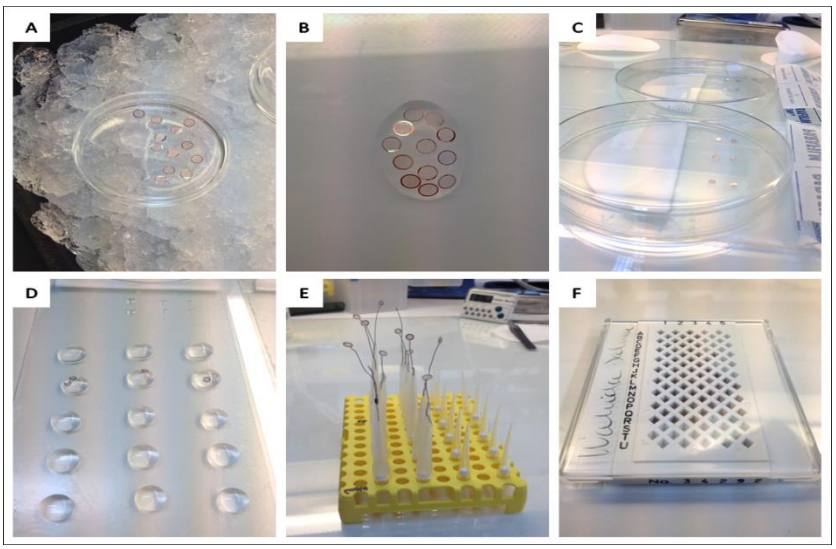

Figure 26. Immunolabeling of ultrathin tissue sections. (**A**) Grids were washed in ddH_2O to remove the methylcellulose. (**B**) Blocking the tissue in 0.8% BSA (Sigma) and 0.1% FSG (Sigma) to prevent unspecific binding of IPNV antibody (VP3). (**C**) Immunolabeling with VP3 specific antibody, linking of RαM-bridge to VP3, linking of gold to bridge, fixation in glutaraldehyde and incubation in MC/UA was all done with on grid per droplet. (**D**) Wash in PBS and ddH_2O was done with both grids in on larger droplet. (**E**) After Immunolabeling and contrasting grids were set to dry at room temperature. (**F**) Completed grids were stored in grid box.

2.8.5 Microscopy of cryosectioning fixed samples

For TEM analyses a Jeol JEM-1010 Electron microscope with digital camera (Morada – Soft Imaging System) and Image analysis software (iTEM, Olympus) were used. In searching for IPNV the magnification was set to 15.000, and micrographs were captured on 30.000 magnification.

2.9 Immunology – real time polymerase chain reaction (PCR)

Real time PCR is the leading tool in detection and quantification of DNA or RNA, and is based on using specific oligonucleotides, heat stable DNA polymerase and thermal cycling (LifeTechnologies 2012). Real time PCR was used for detecting expression of inflammatory genes from the distal intestine of Atlantic salmon. The main focus in this study was the pro-inflammatory *IL-1β* and *TNF-α* and the anti-inflammatory *IL-10*. The reference gene 18S-rRNA (*18S*) served as endogenous control. Presented in Figure 27 is a schematic overview of the real time PCR method. The material used for real time PCR analyses was DI tissue stored in RNAlater Solution (Ambion) according to the manufacturer's protocol.

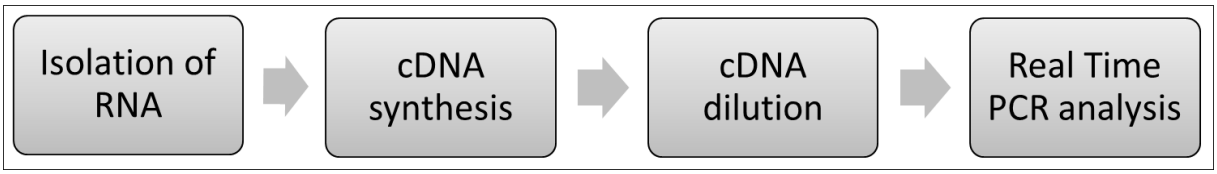

Figure 27. Schematic view on RNA isolation, cDNA synthesis and Real time PCR analyses.

2.9.1 Homogenization

Tissue samples on RNAlater Solution (Ambion) were thawed on ice. Twenty mg pieces were cut out, transferred to MagNaLyser Greenbeads tubes (Roche) added 1000 µl 2 x Nucleic Acid Purification Lysis solution (Applied Biosystems (AB), Foster City, CA, USA) diluted 1:1 in PBS (Gibco, Life Technologies). The tissue samples were homogenized using the MagNALyser instrument (Roche) for 4 x (7000 rpm x 10 seconds). Between each round, samples were cooled for 2 minutes. The homogenized tissue samples were frozen (-80°C) before RNA isolation.

2.9.2 Total RNA isolation

Total RNA was isolated using the ABI Prism 6100 Nucleic Acid Prep Station (AB). The semi-automatic vacuum filtration machine has two working positions, waste and collection. DNase treatment was applied to remove genomic DNA. To ensure no clogging of the RNA purification tray (AB) the tissue amount limits were set at 8-10 mg. The method was conducted according to the manufacturer's protocol and includes DNase treatment using 50 µl Absolute RNA Wash Solution (AB) as described in Figure 28 step 5. Elutes (100 µl) of total RNA were collected in 96-well half skirt tray (Bioplastics) and stored at -80 °C.

Total RNA were mixed using a vortex VF2 (JK Janke & Kunkel IKA-Labortechnik), and centrifuged for a few seconds at 2500 rpm (Centrifuge 5804 from Eppendorf). Subsequently, RNA quantity (ng/µl) and quality were measured using NanoDrop 8000 Spectrophotometer (Thermo Scientific). The ratio between RNA and protein contaminants was measured. RNA was considered pure at A_{260}/A_{280} ratio between 1.8 and 2.1.

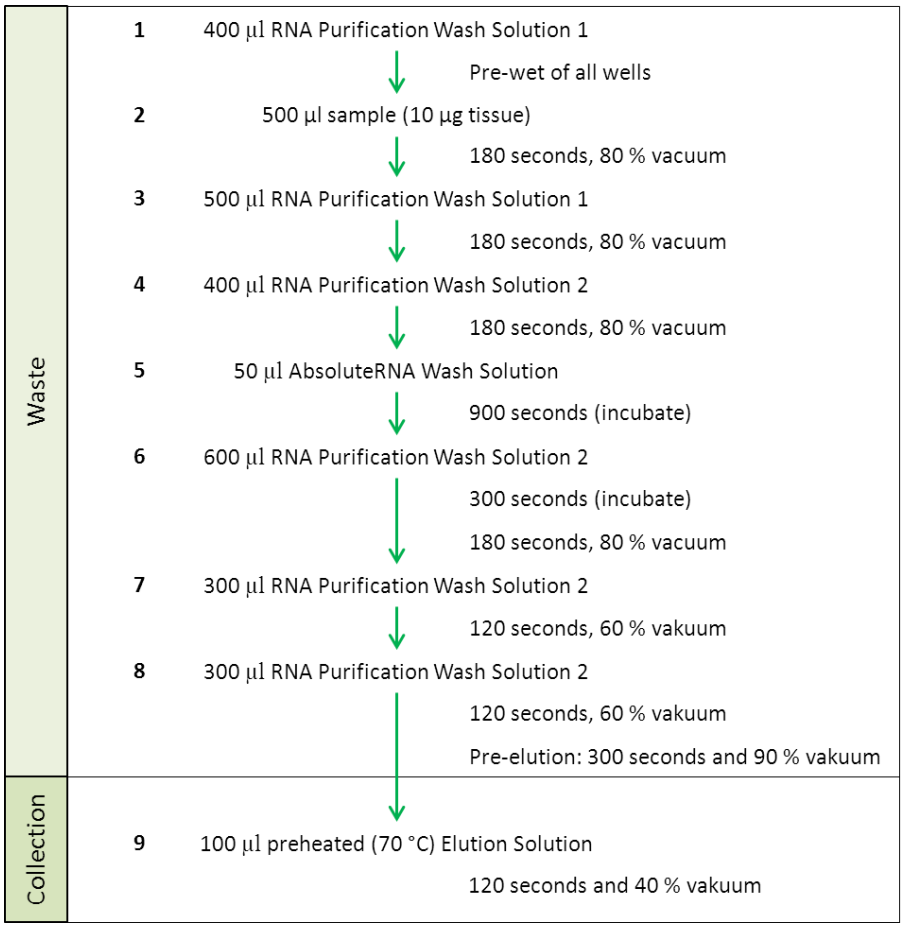

Figure 28. Main steps in isolation of total RNA using the ABI Prism 6100 Nucleic Acid Prep Station (AB).

2.9.3 cDNA synthesis

RNA is not suitable as target molecule for DNA polymerase and must therefore be transformed to complementary DNA (cDNA). Total RNA was reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (AB) according to the manufacturer's protocol. In brief, reaction volumes of 25 µl contained 300 ng total RNA, 2.5 µl 10x Reverse Transcription Buffer, 1.0 µl 25x dNTPs, 2.5 10x random primer, 1.0 Oligo d(T), 1.25 µl MultiScribe™ Reverse Transcriptase and 1.75 µl nuclease free water (Ambion). The reaction was set up using 96-well trays (Bioplastics) using the following cycling parameters: 25 °C for 10 minutes, 37 °C for 120 minutes, 85 °C for 5 minutes (2720 Thermal cycler, AB) and stored in -20 °C. The cDNA was diluted 1:6 in nuclease free water (Ambion) to a stock solution and additional 1:30 for further use in real time PCR analyses.

2.9.4 Real time PCR analyses

Real time PCR was used to quantify the expression of specific genes from DI. Power SYBR Green Master PCR mix (AB) was used as detection agent in the amplification steps of real time PCR analyses. It binds to double stranded DNA (dsDNA) and releases fluorescent light. In the amplification of PCR product the Power SYBR Green Master PCR mix (AB) provides a fluorescent signal that reflects the amount of PCR product (LifeTechnologies 2011) as shown in Figure 29. Real time PCR was conducted in duplicates in 384 well plates using the 7900 HT Fast Real-Time PCR System (AB). Each reaction contained 10 µl Power SYBR Green PCR Master Mix (AB), 0.6 µl (300 nM) of each primer, 8 µl diluted cDNA (1:30) and nuclease free water to a final concentration of 20 µl. Specific primers for target genes (*IL-1β*, *TNF-α* and *IL-10*) and endogenous controls (18S) (Listed in Table 7) were previously designed by Dr. Marit Seppola using Primer Express 3 and synthesized by Eurogentec. The sequences of the indicated primers can be viewed in Appendix IV. Three controls (negative, positive and non-template control (NTC)) were included in the real time PCR setup. To check for possible genomic contamination of the total RNA, ten random total RNA samples were included as template. The following cycling parameters were used: 90°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 1 minute at 60°C. To view the melting peak of each amplicon a third dissociation step of 95°C for 15 seconds, 60 °C for 15 seconds and 95°C for 15 seconds was

added. All primer pairs gave distinct melting peaks demonstrating the absence of unwanted amplification products, as primer-dimers or genomic DNA.

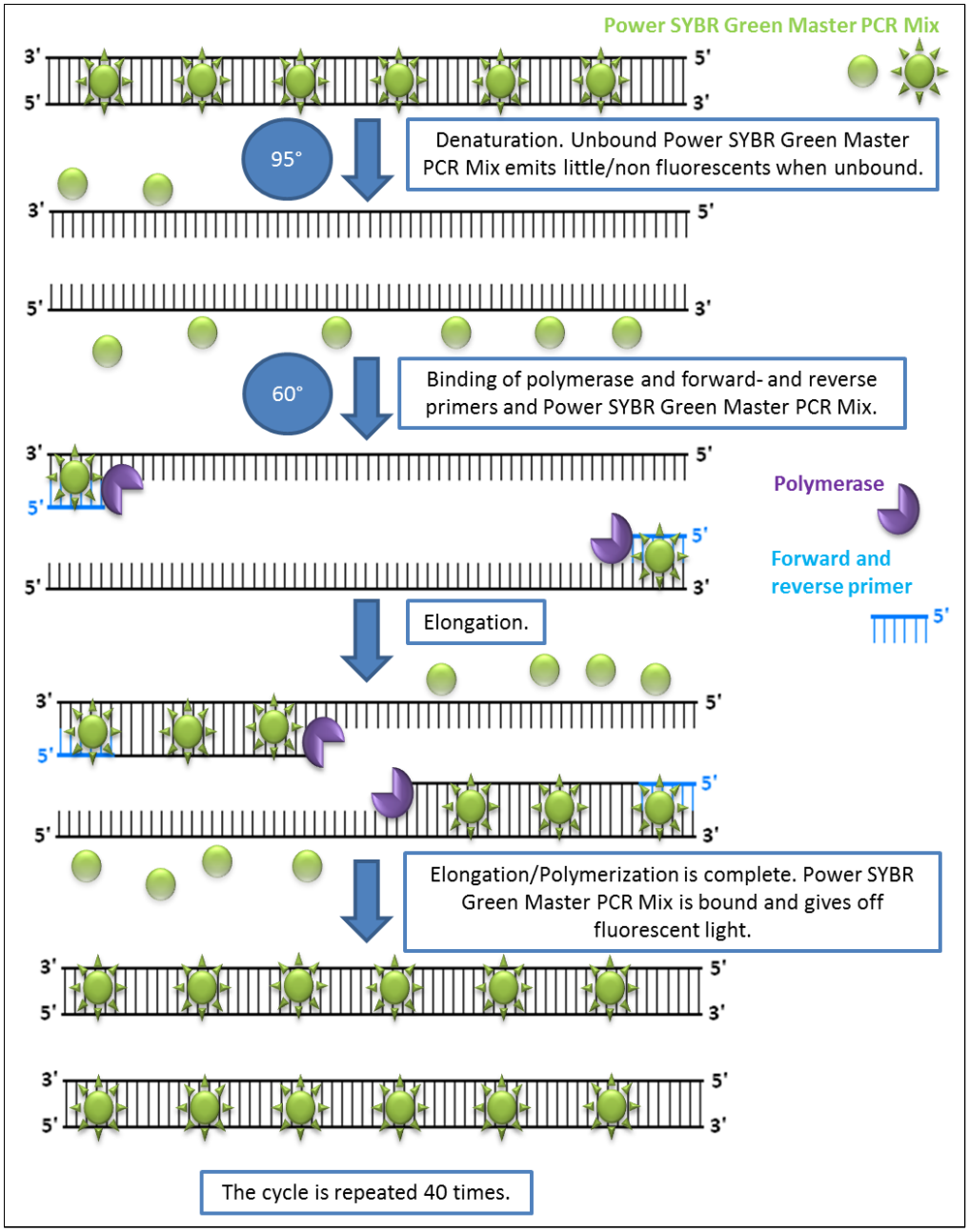

Figure 29. Binding of Power SYBR Green Master PCR mix (AB) to cDNA in Real Time PCR. Modified from AppliedBiosystems (2003).

All real time PCR assays had previously been established by Dr. Marit Seppola (unpublished). The amplification efficiency of each primer pairs had formerly been calculated by using a 2-fold dilution series using cDNA (diluted 1:10) from head kidney and spleen as starting material. Series of eleven dilutions were done. Primer efficiency (E) was calculated following

the equation E= $10^{(-1/slope)}$ (Pfaffl 2001). Amplification efficiency is often given in % (% E= (E-1) x100). All primers, sequences, GeneBank accession numbers and efficiency are shown in Table 7.

Table 7. Real time PCR primers for *IL-1β*, *IL-10* and *TNF-α* and *18S*.

Primer name	Sequence (5'- 3')	GeneBank acc. no.	Efficiency	E =
***18S* F**	TGTGCCGCTAGAGGTGAAATT	AJ427629	91.7%	1,84
***18S* R**	GCAAATGCTTTCGCTTTCG			
***IL1β*-539F**	CCCGTCCCCATTGAGACTAAA	NM_001123582.1	95.7%	1,91
***IL1β*-611R**	AGCAGGACAGGTAGAGGTTGGATC			
***IL10*-194F**	GAACGCAGAACAACCACTTTAAATCT	EF165028.1	95.1%	1,902
***IL10*-267R**	GATCTCTTTCTTCAGCTCGTGGAA			
***TNFα*-482F**	CGTGCAGTGGAGAAAGGATGA	NM_001123590.1	90.8%	1,816
***TNFα*-577R**	AAGAAGAGCCCAGTGTGTGGG			

*GenBank accession number. IL = interleukin. TNF = tumor necrosis factor. R = reverse. F = forward. E = efficiency.

2.10 Data analyses and statistics

Specific growth rate (SGR) was used to evaluate growth during the freshwater and seawater period, and statistical analyses were done using student t-test with significance level at <0.05. One-Way ANOVA tests were conducted using SPSS (*Statistical Package for the Social Sciences, IBM*) to detect possible weight differences of the sampled fish. Values of p<0.05 were considered significant.

Specific growth rate (SGR): $\left(\frac{(\ln(final\ weight)-\ln(\ initial\ weight))}{trail\ days}\right) * 100$

The histological scoring system consists of four criteria with five scores, each with a specific characteristic and purpose. Results were treated as categorical data and chi square test of independence ($p < 0.05$) was conducted between each feed group combining scores and each criteria. Contingency table for the results for each feed group criterions was set up, and tests conducted.

All the results from the real time PCR analyses were collected by the SDS 2.3 software (AB). The threshold was adjusted manually to 0.1, and the melting peak of each amplicon was checked. Melting peaks with one single peak (Figure 30 A) were approved. All Ct values were exported to Microsoft excel for further analyses. Mean Ct values from the two parallels were used for calculations and the mean Ct-value of samples from day 0 were used as calibrator for the gene expression in the six dietary groups. Relative expression was calculated using the Pfaffl method (Pfaffl 2001) (Figure 31). The Pfaffl method takes in to consideration that the primer efficiency is not 100%. One-Way ANOVA was chosen as statistical method and conducted between the groups relative expression of *IL-1β*, *IL-10* and *TNF-α* (p-value set to <0.05) using SPSS (*Statistical Package for the Social Sciences, IBM*).

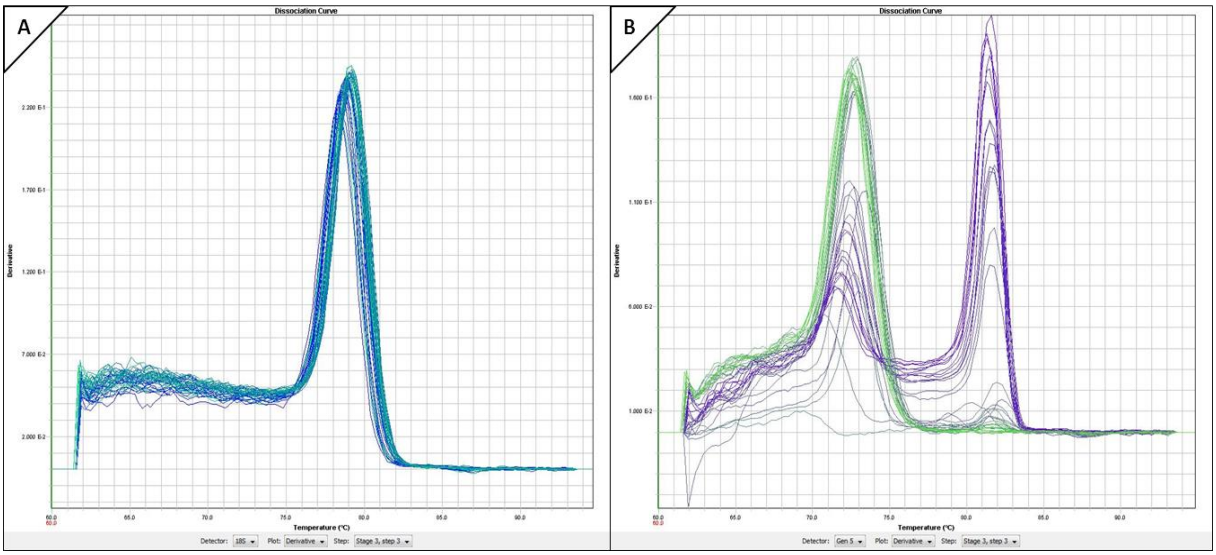

Figure 30. Dissociation curves showing one single melting peak (**A**) and one that shows unwanted amplification products (**B**). Photos kindly provided by Hanne Johnsen.

$$\text{Ratio} = \frac{(E_{target})^{\Delta CP\ target\ (control-sample)}}{(E_{ref})^{\Delta CP\ ref\ (control-sample)}}$$

Figure 31. Pfaffl model for calculation of relative gene expression (Pfaffl 2001).

3 Results

3.1 Growth

Statistical analyses were conducted on weight of the sampled fish (n=18 per dietary group) and specific growth rate (SGR). No significant differences in weight, between the different dietary groups were observed neither in the FW phase (p=0.841), nor in the SW phase (p=0.672) (Figure 31A). The same observation was done in SGR (Figure 31B). There was however a significant difference in weight (p=0.00001) and SGR (p=0.004) between the FW and SW period. In the FW phase (42 days), a mean SGR of 1.69 and mean weight of 65.3g was observed; in contrast the mean SGR was 1.17 and mean weight of 77.6g in the SW period.

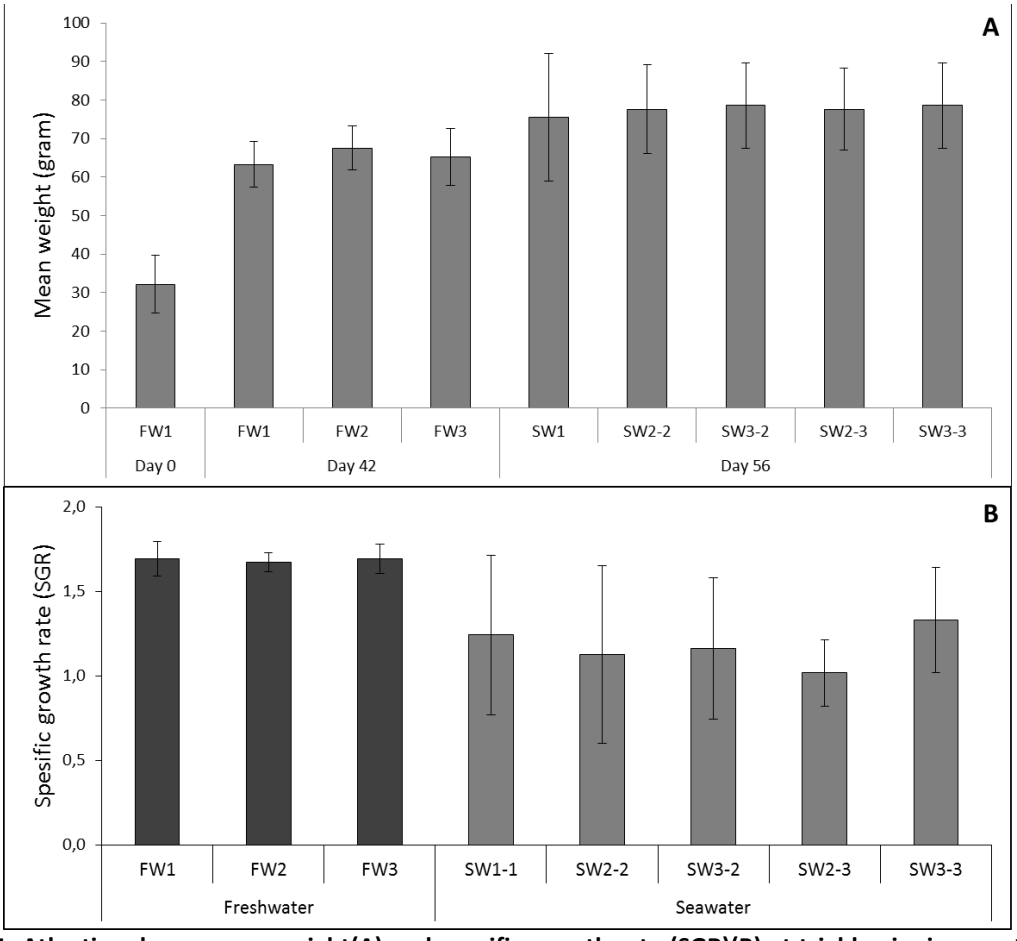

Figure 31. Atlantic salmon mean weight(A) and specific growth rate (SGR)(B) at trial beginning, post feeding on FW feed in 42 days and SW feed in 14 days (day 56). Data based on weight at day 0 taken post acclimation and feeding on FW1 (n=10), day 42 post feeding with FW1, FW2 and FW3 (n=18) and day 56 (14 days) post feeding on SW (SW) feeds (SW1, SW2 and SW3) (n=18).

3.2 Morphology

Morphological changes in Harris hematoxylin and eosin (HE) stained PC, MI and DI were observed with light microscope. Vacuolization of the enterocytes in PC and MI showed no- to severe vacuolization in both FW and SW. Presented in Figure 32 are examples of vacuolization in PC. DI showed no- to moderate changes in the presence of supranuclear vacuoles, with a few examples of severe changes and loss of vacuoles (Figure 33).

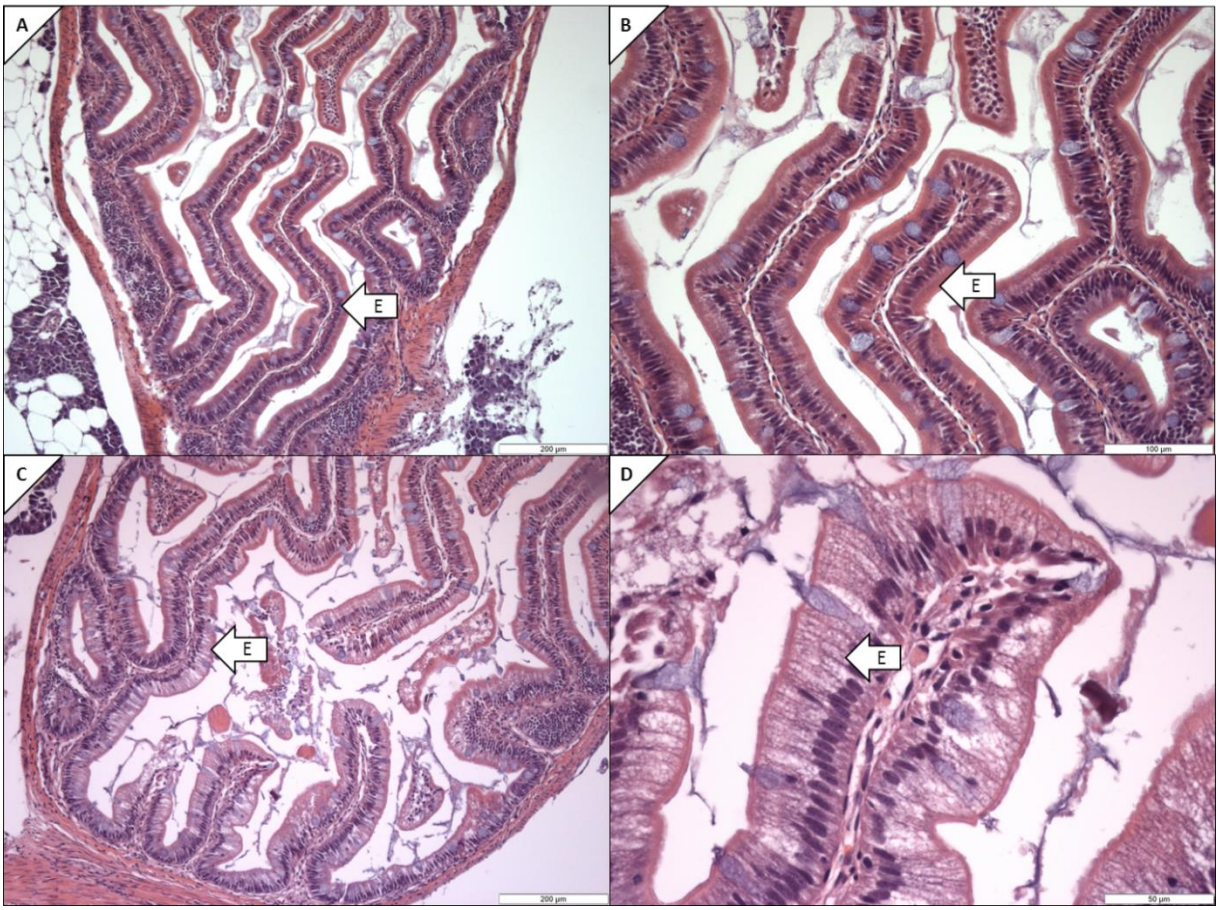

Figure 32. Atlantic salmon pyloric caeca Harris hematoxylin and eosin stained tissue illustrating vacuolization of the enterocytes. (**A**) Example of PC with enterocytes (E) showing no to very little vacuolization from fish fed FW2 (obj. x10). (**B**) Enterocytes (E) with no to very little vacuolization (obj. x20) from fish fed FW2. (**C**) Overview of enterocytes (E) with distinct vacuolization (obj. x10) from fish fed FW1. (**D**) Mucosal fold with distinct vacuolization of the enterocytes (E) from fish fed FW1 (obj. x40).

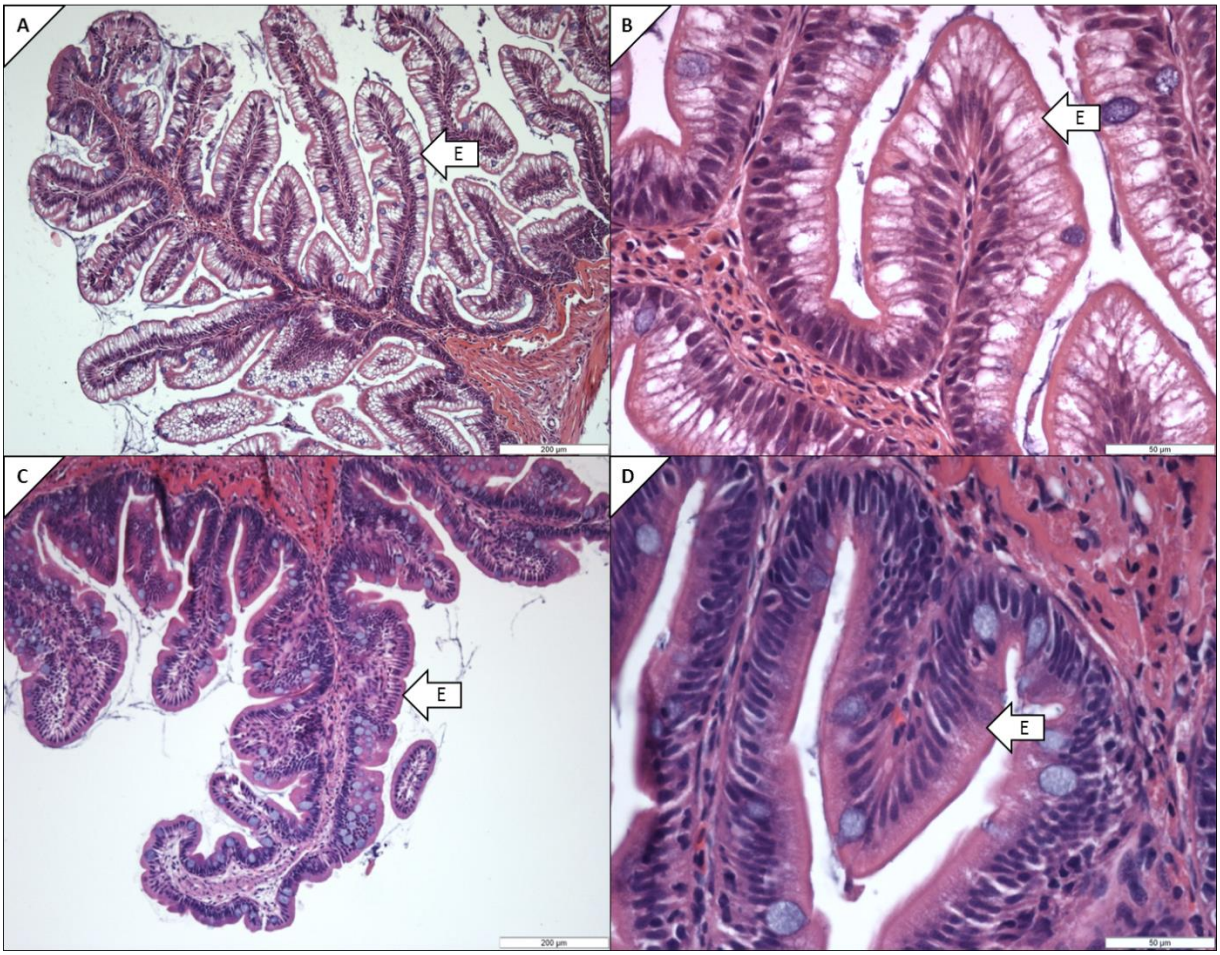

Figure 33. Atlantic salmon distal intestine Harris hematoxylin and eosin stained tissue illustrating differences in morphology due to presence of supranuclear vacuoles in the enterocytes. (**A**) Complex folds and side branches showing normal morphology, with high presence of supranuclear vacuoles in the enterocytes (E) from fish fed FW1 (obj. x10). (**B**) Simple mucosal folds having supranuclear vacuoles occupying almost the entire apical part of the enterocytes (E) from fish fed FW1 (obj. x40). (**C**) Complex and simple folds showing total loss of vacuoles in the enterocytes (E) from fish fed SW1-1 (obj. x10). (**D**) Simple mucosal fold having no vacuoles present in the enterocytes (E) from fish fed SW1-1(obj. x40).

Lamina propria in PC and DI showed normal morphology in both FW and SW, with a few exceptions of moderate changes observed in the SW control group. MI showed mainly mild and moderate changes to the lamina propria, with more moderate changes in SW than FW. Figure 34 illustrates two examples of lamina propria from the FW period.

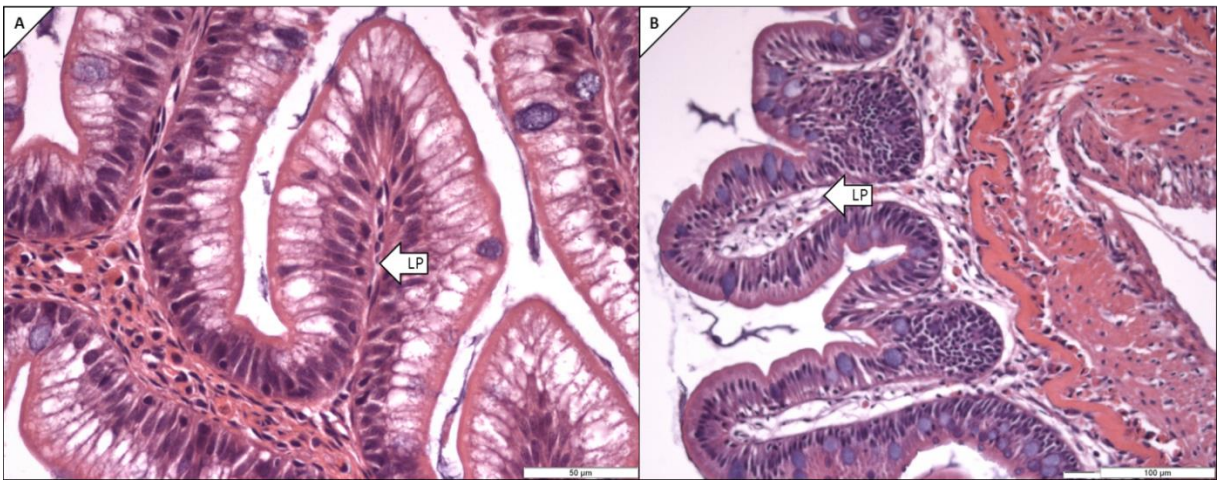

Figure 34. Lamina propria of Atlantic salmon Harris hematoxylin and eosin stained mid intestine (MI) and distal intestine (DI). (**A**) Lamina propria (LP) in side branches on complex mucosal folds of DI showing a thin delicate core of connective tissue from fish fed FW1 (obj. x40). (**B**) Lamina propria (LP) in MI of dietary group FW2 revealed clear increase in influx of connective tissue (obj. x10).

The layer of connective tissue in submucosa, beneath the base of folds and stratum compactum of MI and DI from the FW period showed no- to moderate influx of connective tissue. In the SW period MI displayed mild and moderate changes, while DI remained mainly unchanged. Figure 35 illustrates examples of connective tissue in submucosa. PC showed a decrease in PCN from the FW to the SW period. Figure 36 illustrates examples of normal nuclei and increased amount of PCN.

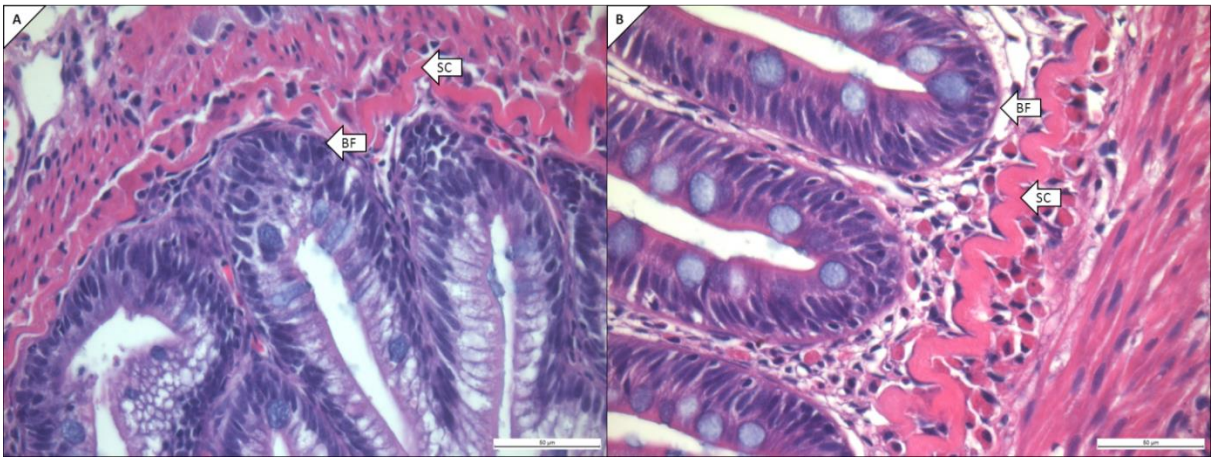

Figure 35. Atlantic salmon mid and distal intestine Harris hematoxylin and eosin stained tissue illustrating increase of connective tissue between base of folds and stratum compactum. (**A**) Distal intestine showing a thin layer of connective tissue between base of folds (BF) and stratum compactum (SC) from fish fed FW1 (obj. x40). (**B**) Mid intestine showing an increase in the amount of connective tissue between base of folds (BF) and stratum compactum (SC) from fish fed SW2-2 (obj. x40).

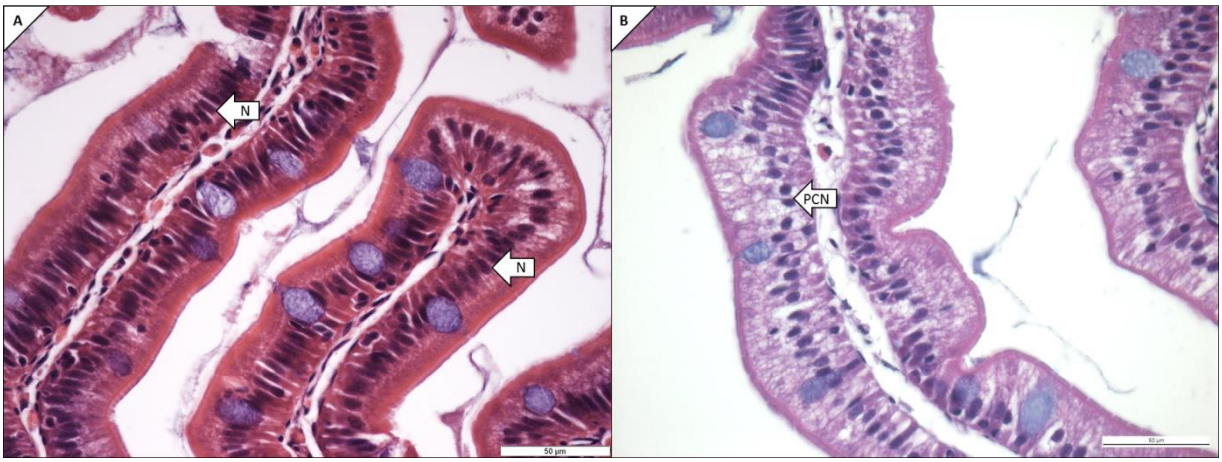

Figure 36. Atlantic salmon pyloric caeca Harris hematoxylin and eosin stained tissue illustrating presences of pycnotic cell nuclei (PCN). (**A**) Mucosal folds with enterocytes having undamaged nuclei (N) apically located, from fish fed FW2 (obj. x40). (**B**) Mucosal folds with increased vacuolization and presence of PCN from fish fed FW2 (obj. x40).

PC and MI mucosal folds showed mostly normal morphology in both the FW and SW period, with only a few moderate changes. Figure 37 illustrates examples of shortening of the mucosal folds in MI. DI mucosal folds showed more moderate to distinct changes compared to PC and MI, and the changes seemed to increase post SW transfer where the complexity of the mucosal folds decreased (Figure 38).

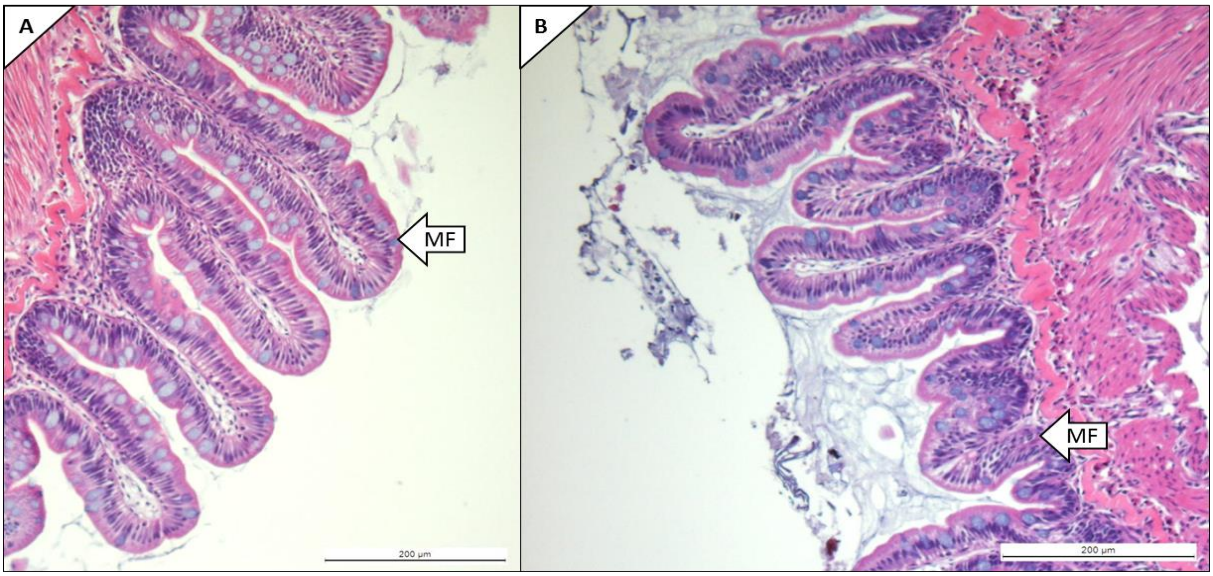

Figure 37. Atlantic salmon mid intestine Harris hematoxylin and eosin stained tissue exemplifying height of the mucosal folds. (**A**) Mucosal folds (MF) appear long and thin (obj. x10). Example from day 0. (**B**) Mucosal folds (MF) appears shorter and some even stubby (obj. x10), example from fish fed FW1.

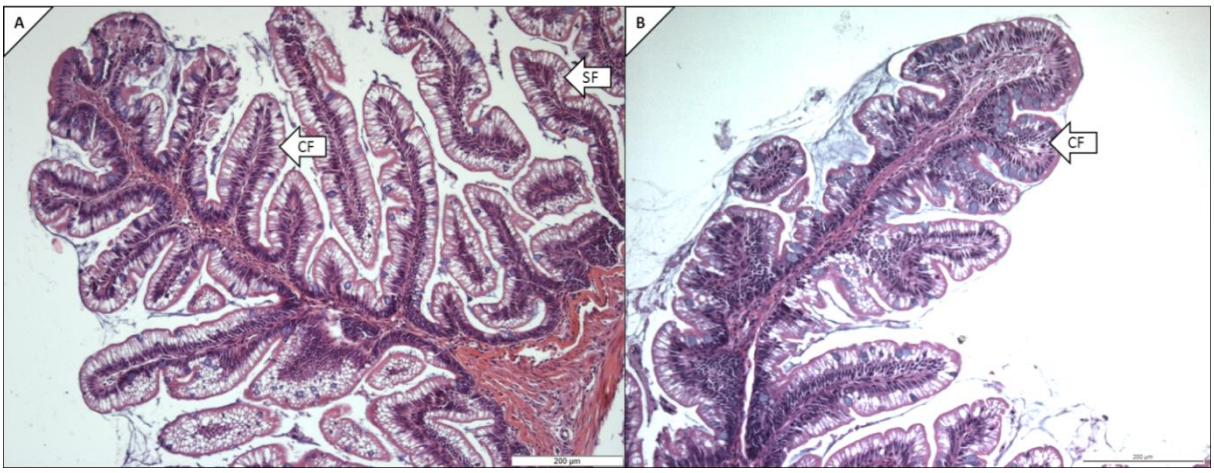

Figure 38. Atlantic salmon distal intestine Harris hematoxylin and eosin stained tissue illustrating mucosal folds height and complexity. (**A**) Complex mucosal (CF) fold with long and thin side branches, and long and thin simple folds (SF) (obj. x10). (**B**) Complex folds (CF) showing loss of complexity with fewer side branches, and shortening of the remaining branches (obj. x10).

3.3 Dietary effects on GI tract morphology

PC, MI and DI sampled from two of the three triplicate tanks (Figure 6) were selected for histological analyses, total amount of samples were 336. Sections were analyzed according to the scoring system presented in Table 3 to 6. Results of the scored samples were calculated as percentage of each score within group and criteria (Appendix VI). Results were treated as categorical data and statistical analyses were therefor conducted using frequency analyses (Chi-square test, p-value set to <0.05). Appendix V shows results from the statistical analyses.

No statistical differences were detected between the three feed groups combined scores post 6 and 42 days of feeding in FW (Figure 39A). Between 80.0% and 86.0% of all samples taken post 42 days in FW displayed normal morphology. Fish from day 0, fed a commercial diet prior to six days of acclimation feeding on FW1, showed the highest degree of changes (16.7% moderate-, 5.0% distinct- and 1.7% severe changes). Of the three dietary groups from day 42, fish fed FW2 showed most changes (14.3% moderate- and 5.0% distinct changes) compared to FW1 (10.7% moderate- and 3.6% distinct changes) and FW3 (11.8% moderate- and 2.8% distinct changes).

Statistical differences were detected between fish fed SW1-1 and SW2-2 ($p<0.05$), indicating that the functional feed has significantly better effect on the GI tract post SW transfer (Figure 39B). Between 74.0% and 86.0% of all samples taken 14 days post feeding in SW

showed normal morphology. Fish fed the SW1-1 had the highest amount of changes (20.8% moderate-, 2.8% distinct- and 2.1% severe changes), closely followed by SW2-3 (17.9% moderate-, 3.6% distinct- and 1.4% severe changes) and SW3-3 (20.3% moderate- and 0.8% severe changes). Lowest combined scores were observed in fish fed SW3-2 (18.5% moderate- and 2.3% distinct changes) and SW2-2 (10.4% moderate- and 2.1% distinct changes).

All scores and criteria combined showed an overview of the general health of the three gut segments. Further, health status will be presented by criteria of each gut segment for the FW and SW period.

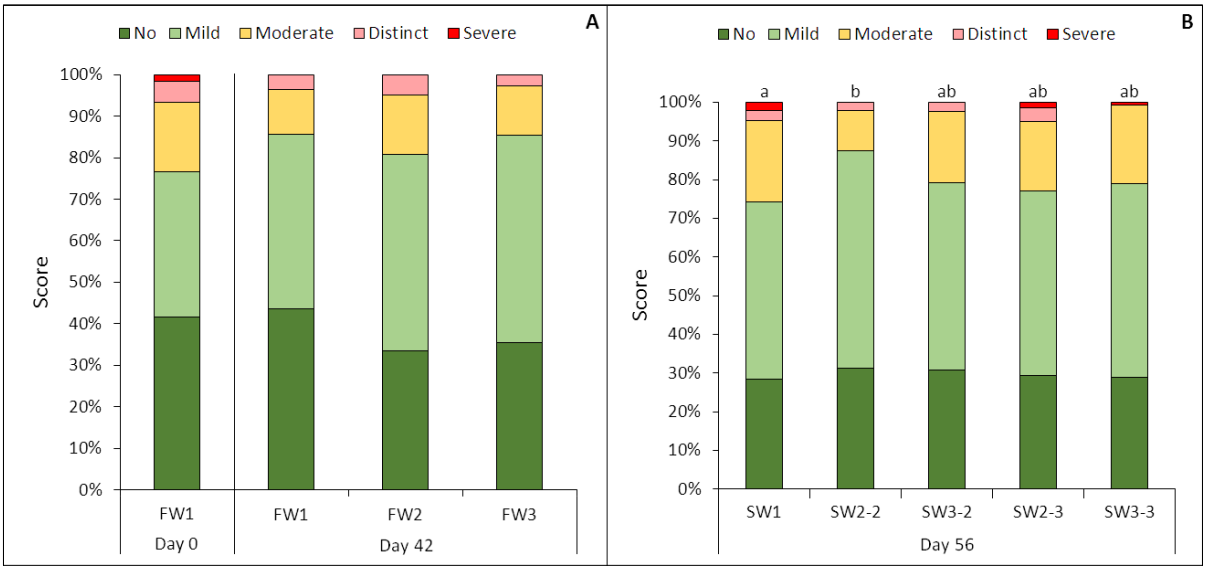

Figure 39. Histological analyses of gastrointestinal tract from Atlantic salmon fed three different test diets pre- and post SW transfer. Columns show the percentage of the different scores. Different letters indicates significant difference, $p<0.05$. (**A**) Score frequency from day 0 (fish fed FW1 in six days of acclimation, n=10) and day 42 (FW1, FW2 and FW3) (n=12) in the FW period, pre SW transfer. (**B**) Score frequency from day 56 (14 days post SW transfer) of fish fed SW1, SW2 and SW3 (n=12) with their different FW period origin indicated with a dash and feed number.

3.4 Dietary effects on GI tract morphology in the freshwater (FW) period

3.4.1 Pyloric caeca (PC)

The PC sampled in the FW period displayed changes in form of vacuolization and increased amount of PCN (Figure 40). PC sampled from day 0 scored highest in vacuolization (30% moderate-, 50% distinct- and 10% severe changes) in this period, closely followed by the three dietary groups from day 42; FW2 (16.7% mild-, 33.3% moderate- and 50.0% distinct changes), FW1 (16.7% mild-, 41.7% moderate- and 41.7% distinct changes) and FW3 (8.3% no-, 33.3% mild-, 33.3% moderate- and 25% distinct changes). No significant differences were detected.

Lamina propria showed normal morphology; day 0 (90.0% no, 10% mild), FW2 (75.0% no- and 25.0% mild changes), FW1 (58.3% no- and 41.7% mild changes) and FW3 (50.0% no- and 50.0% mild changes). Significant difference was detected between PC lamina propria from day 0 and FW3 ($p<0.05$).

PCN were most abundant in fish fed FW2 (50.0% moderate- and 8.3% distinct changes), followed by FW1 (50.0% moderate), FW3 (25.0% moderate- and 8.3% mild changes) and day 0 (30% moderate changes). No statistical differences were detected.

The mucosal folds of the PC displayed mostly normal morphology and a few moderate changes; FW3 (50.0% no- and mild changes), FW1 (16.7% no-, 75.0% mild- and 8.3% moderate changes), day 0 (70.0% no-, 20 mild- and 10% moderate changes) and FW2 (25.0% no-, 50.0% mild- and 25.0% moderate changes). Significant differences were detected between mucosal folds of the dietary groups FW1 and FW3, and between day 0 and FW1 ($p<0.05$).

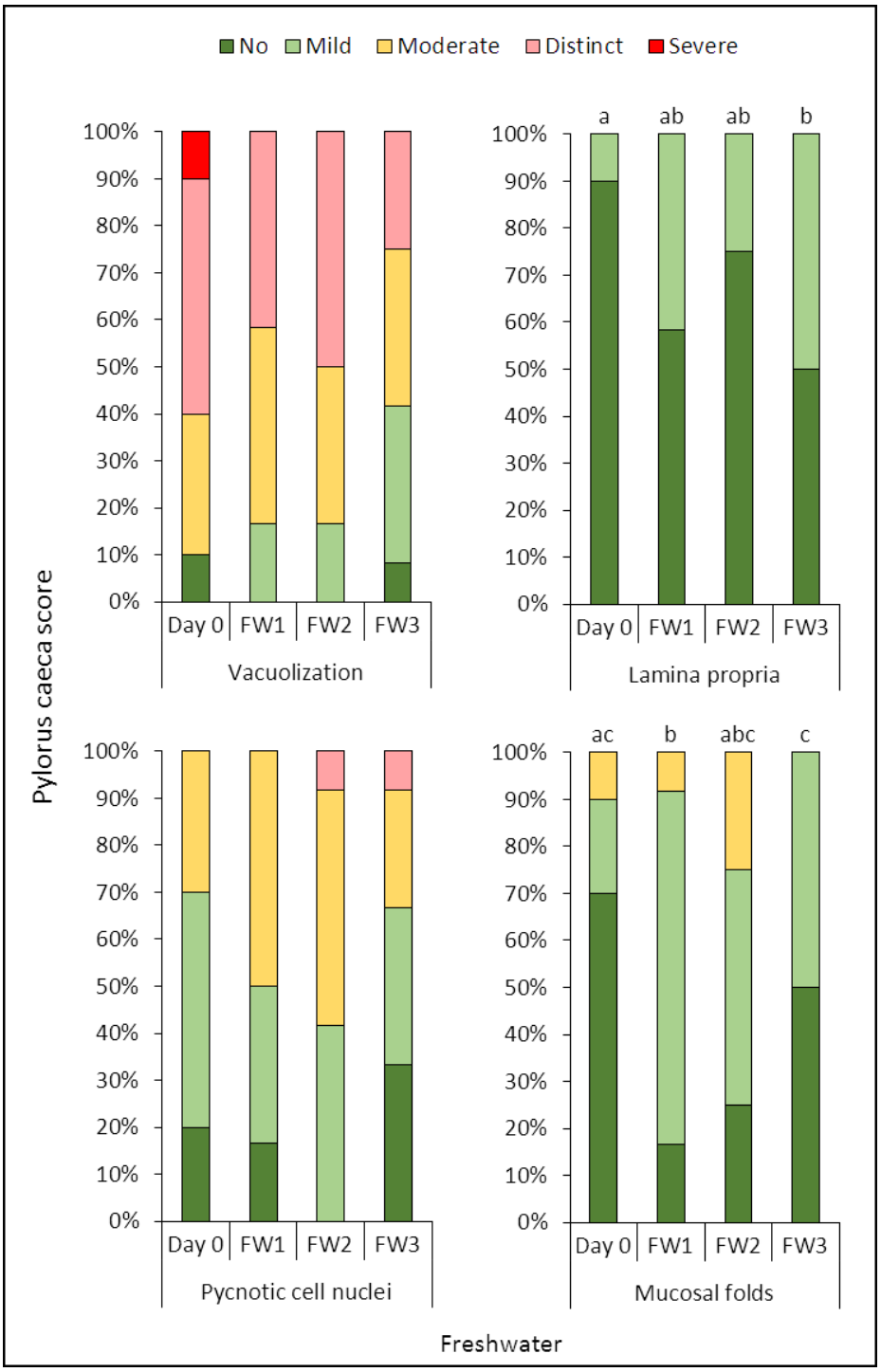

Figure 40. Histological analyses of pyloric caeca from Atlantic salmon fed three different test diets in FW. Columns show the score frequency from day 0 (fish fed FW1 in six days of acclimation, n=10) and day 42 (FW1, FW2 and FW3) (n=12) in the FW period, pre SW transfer. Different letters indicates significant difference, $p<0.05$.

3.4.2 Mid intestine (MI)

The MI from the FW period showed mostly normal morphology and a few moderate changes in all criteria, with one exception of the 10% of MI from day 0 having severe vacuolization (Figure 41). Apart from these ten percent, all feed groups displayed no- to mild vacuolization. Fish fed FW1 showed no changes, while FW2 and FW3 a low percentage of mild changes (25.0% and 16.7%). Significant difference was detected in vacuolization of MI from the FW1 and FW2 dietary group.

Lamina propria displayed normal morphology in all feed groups 42 days post FW feeding. Fish from day 0 showed the highest amount of change (30.0% moderate) in lamina propria of the FW samples. There were detected significant difference in score between fish fed FW1 for 42 days and fish from day 0 ($p<0.05$).

The amount of connective tissue in MI showed normal tissue to moderate morphological changes; FW1 (41.7% no-, 50% mild- and 8.3% moderate changes), FW3 (91.7% mild- and 8.3% moderate changes) and FW2 (25.0% no-, 58.3% mild- and 16.7% moderate changes). MI from day 0 displayed normal amounts of connective tissue (80.0% no- and 20.0% mild changes). MI from fish fed FW2 and FW3 both showed significant difference in the amount of connective tissue from day 0 ($p<0.05$). Additionally, fish fed FW3 were significantly different from fish fed FW1 ($p<0.05$).

Mucosal folds height and width showed normal folds in fish fed FW1 (58.5% no- and 41.7% mild changes) and FW2 (41.7% no- and 58.3% mild changes). Moderate changes to the mucosal folds were observed in MI from fish sampled at day 0 (10.0%) and fish fed FW3 for 42 days (25.0%). Statistical differences were detected between fish fed FW3 and FW1 ($p<0.05$).

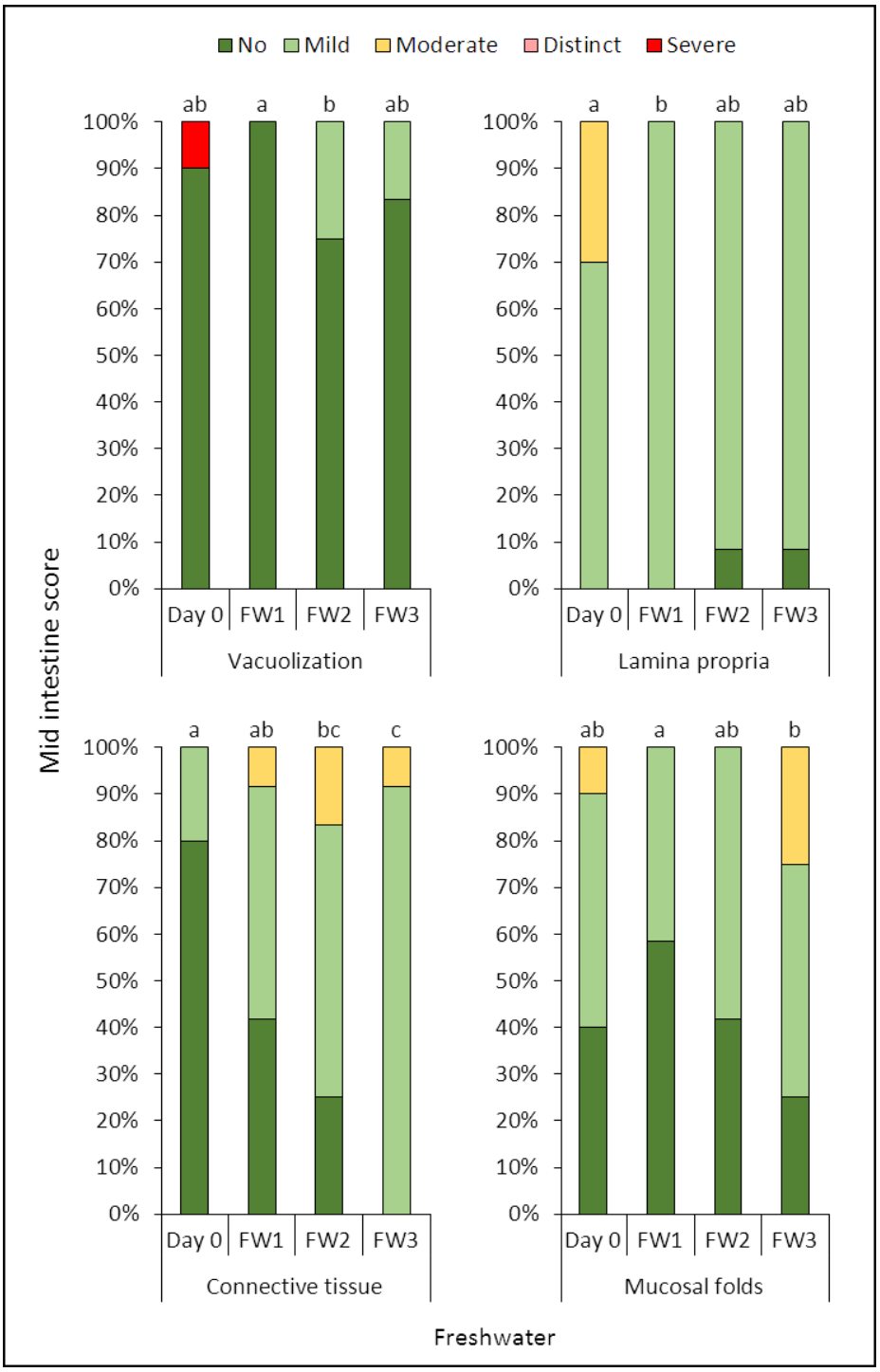

Figure 41. Histological analyses of mid intestine from Atlantic salmon fed three different test diets in FW. Columns show the score frequency from day 0 (fish fed FW1 in six days of acclimation, n=10) and day 42 (FW1, FW2 and FW3) (n=12) in the FW period, pre SW transfer. Different letters indicates significant difference, $p<0.05$.

3.4.3 Distal intestine (DI)

The DI from the FW period did not display many changes (Figure 42). The changes were mainly observed in the mucosal folds. Fish from day 0 showed the highest (70.0% moderate, 10% distinct) level of changes in the mucosal folds. A significant difference was detected between DI mucosal folds from day 0 and FW1. Fish fed FW1 had the lowest amount of moderate changes and the highest amount of no changes (36.4% no-, 45.5% mild- and 18.2% moderate changes), compared to day 0's 10.0% no-, 10% mild-, 70.0% moderate- and 10.0% distinct changes. Moderate morphological changes observed in the two remaining groups are 33.3% in FW2 and 41.7% in FW3, respectively.

The amount of connective tissue between the base of folds and stratum compactum in DI mainly showed no changes; FW1 having 100% no changes, FW2 75.0% no- and 25.0% mild changes, FW3 66.7% no- and 33.3% mild changes. Day 0 displayed 70.0% no- and 30% mild changes, and were significantly different from FW1 ($p<0.05$). Significant differences ($p<0.05$) were also detected in DIs lamina propria, where samples from day 0 (80.0% mild- and 20.0% moderate changes) were different from lamina propria of all three dietary groups 42 days post FW feeding (which all scored low, no- and mild changes).

The level of supranuclear vacuoles present was high for day 0 (20.0% no- and 80.0% mild changes) and fish fed FW1 (36.4% no- and 63.6% mild changes). Fish fed FW2 and FW3 showed a small (8.3%) percentage of samples with moderate loss of vacuoles, but mainly normal morphology.

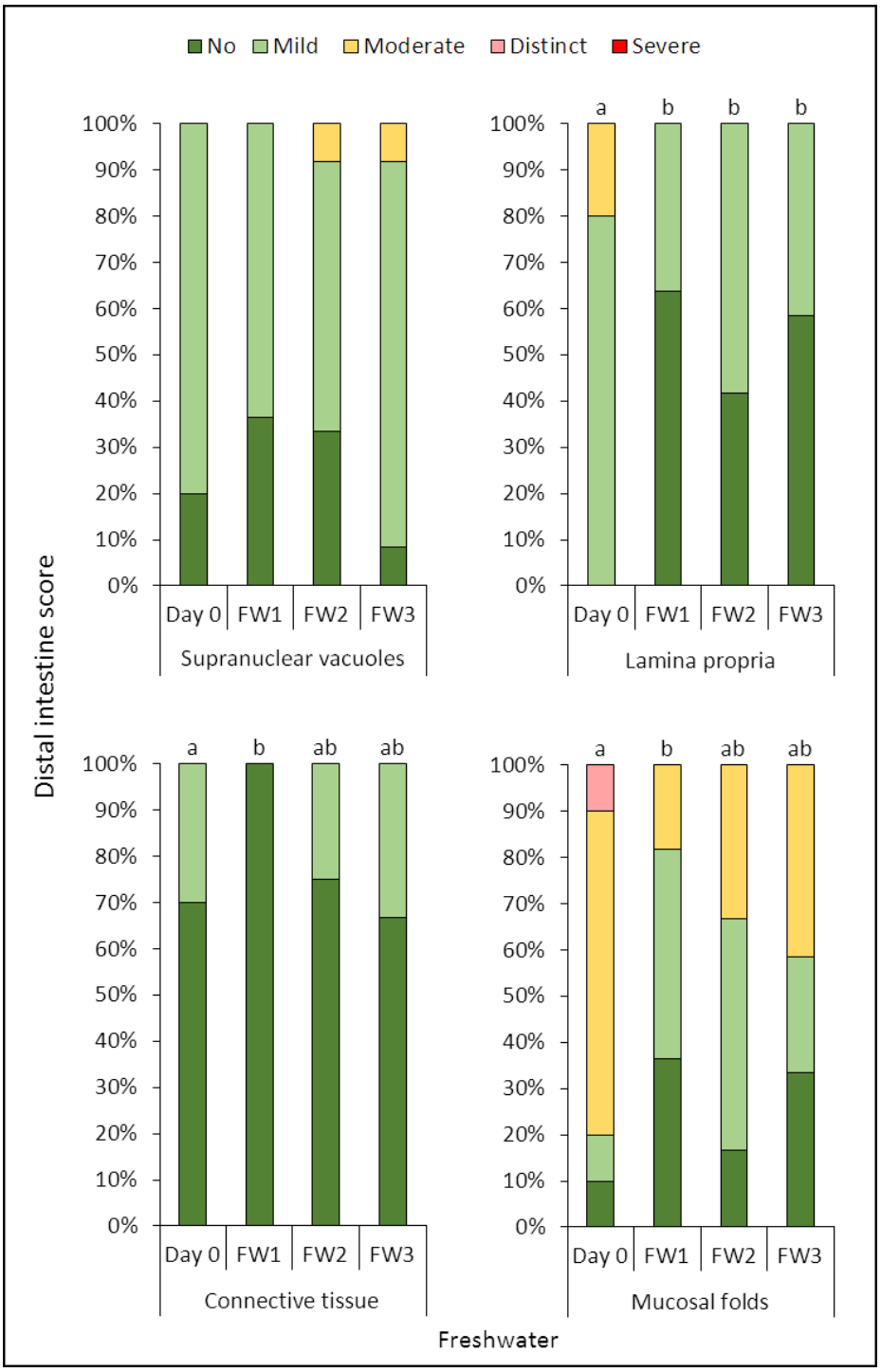

Figure 42. Histological analyses of distal intestine from Atlantic salmon fed three different test diets in FW. Columns show the score frequency from day 0 (fish fed FW1 in six days of acclimation, n=10) and day 42 (FW1, FW2 and FW3) (n=12) in the FW period, pre SW transfer. Different letters indicates significant difference, $p<0.05$.

3.5 Dietary effects on GI tract morphology in the seawater (SW) period

3.5.1 Pyloric caeca (PC)

The PC 14 days post SW transfer showed high levels of vacuolization (Figure 43). Fish fed SW1-1 showed the most changes with 7.0% severe-, 12.0% distinct- and 40.0% moderate vacuolization, and no statistical differences were detected. Significant differences in vacuolization were detected between fish fed SW2-2 and SW3-2 ($p<0.05$). Fish fed SW2-2 had the lowest amount of tissue samples with vacuolization (16.7% moderate- and 16.7% distinct changes) and fish from the same origin fed SW3-2 the highest of the two (63.6% moderate changes). Fish originating from FW3 did not display significant difference in vacuolization. SW2-3 showed 25.0% distinct and 25% moderate vacuolization, while SW3-3 only showed moderate vacuolization (58.3%).

PC lamina propria and mucosal folds showed normal morphology in all dietary groups. Statistical differences were detected between the dietary groups in terms of mucosal folds, where fish fed SW3-2 showed higher (90.9%) levels of mild changes than SW1-1 (58.3%), SW2-3 (33.3%) and SW3-3 (25%) respectively ($p<0.05$). No differences were observed in lamina propria between the dietary groups.

Low levels of PCN were observed in PC from this period. Fish fed SW2-3 had the highest amount of moderate changes in cell nuclei (33.3%), followed by SW3-2 (18.2%). Significant differences were detected between PCN from fish fed SW1-1 and SW2-2 ($p<0.05$), whereas SW1-1 showed 41.7% no-, 41.7 mild- and 16.7% moderate changes, and SW2-2 had 75.0% no-, 8.3% mild- and 16.7% moderate changes. SW2-2 displayed the highest amount of undamaged cell nuclei of the five dietary groups (Figure 41).

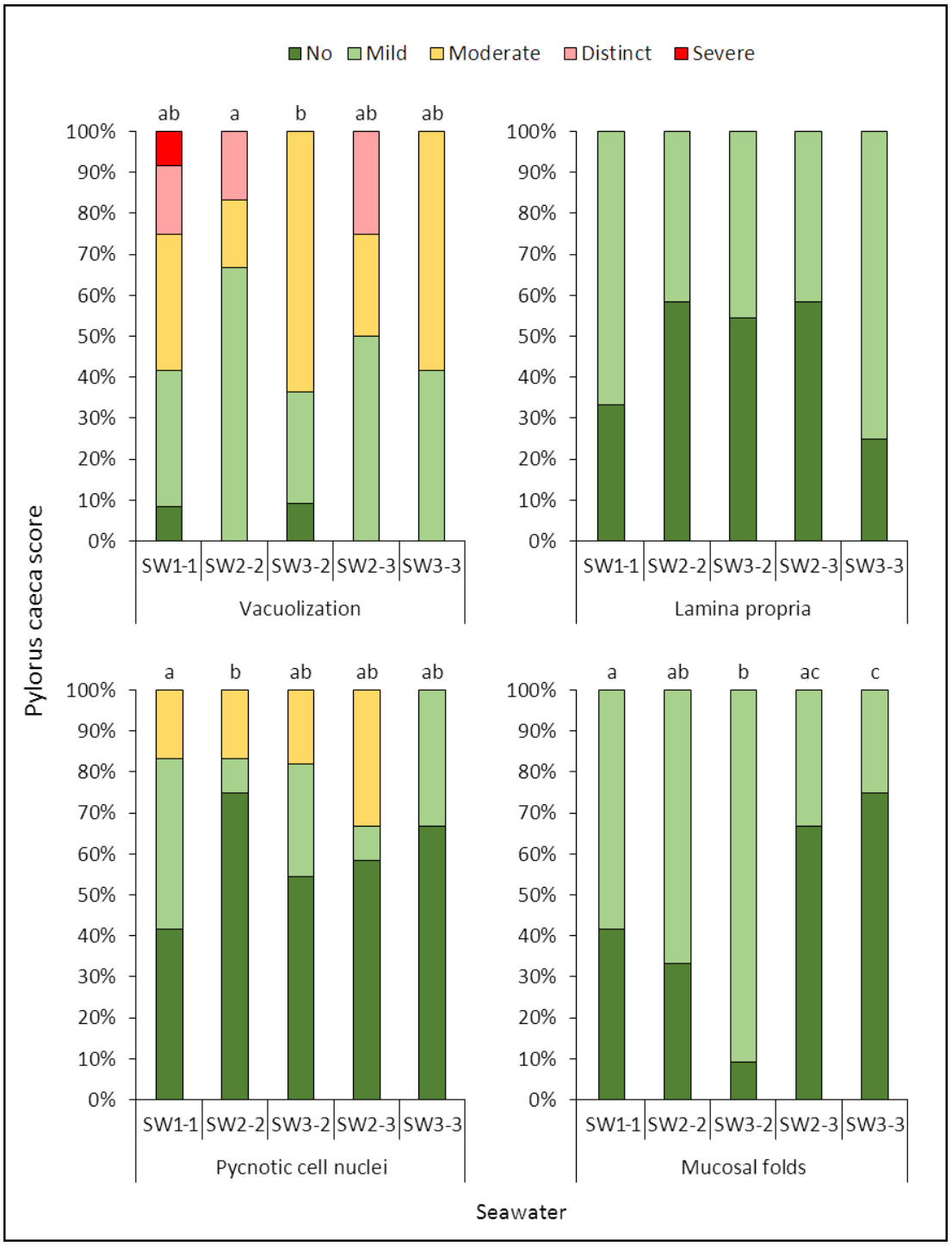

Figure 43. Histological analyses of pyloric caeca from Atlantic salmon fed three different test diets for two weeks post SW transfer. Columns show the score frequency from day 56 (14 days post SW transfer) of fish fed SW1, SW2 and SW3 (n=12) with their different FW period origin indicated with a dash and feed number. Different letters indicates significant difference, $p<0.05$.

3.5.2 Mid intestine (MI)

Vacuolization, lamina propria and the amount of connective tissue were the three criteria showing the highest level of changes in MI 14 days post feeding in SW (Figure 44). Statistical differences were detected between the lamina propria of fish fed SW1-1 (50.0% mild- and 50.0% moderate changes) and SW2-3 (90.9% mild- and 9.1% moderate changes) ($p<0.05$). Fish fed SW3-3 and SW2-2 showed similar changes as SW2-3, whereas lamina propria from the SW3-2 group was most comparable to SW1-1. No other statistical differences were detected in MI from this period.

The level of vacuolization varied between the MI samples. The highest level of vacuolization was observed in fish fed SW2-3 (27.3% moderate- and 18.2% severe changes), followed by SW3-2 (20.0% moderate- and 10.0% distinct changes) and SW2-2 (16.7% moderate changes). MI from the SW3-3 groups showed only low levels of vacuolization (50.0% no- and 50.0% mild changes).

All SW feed groups showed high levels of mild- and moderate changes in connective tissue between base of folds and stratum compactum. Moderate changes were highest (60.0%) in fish from SW3-3, followed by SW2-3 (54.5%), SW3-2 (50.0%), SW1-1 (33.3%) and SW2-2 (16.7%). Additionally, fish from dietary group SW2-2 displayed the highest level of mild- (75%) and distinct (8.3%) changes in the amount of connective tissue.

The mucosal folds of MI displayed normal morphology in fish fed SW2-2 (33.3% no- and 66.7% mild changes) and SW3-3 (40.0% no- and 60.0% mild changes). Moderate morphological changes in the mucosal folds were observed fish fed SW1-1 (33.3%), SW2-3 (18.2%) and SW3-2 (11.1%).

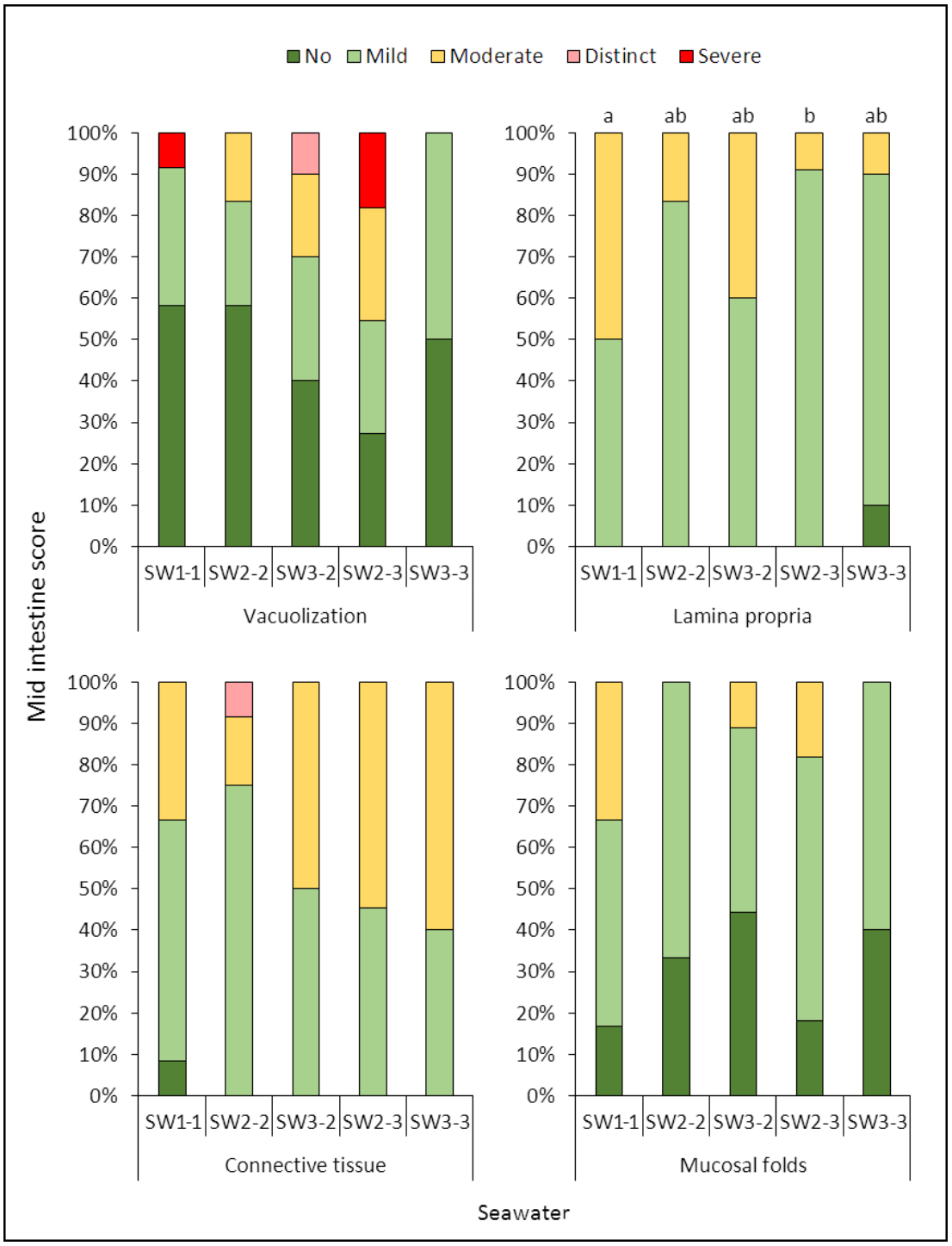

Figure 44. Histological analyses of mid intestine from Atlantic salmon fed three different test diets for two weeks post SW transfer. Columns show the score frequency from day 56 (14 days post SW transfer) of fish fed SW1, SW2 and SW3 (n=12) with their different FW period origin indicated with a dash and feed number. Different letters indicates significant difference, $p<0.05$.

3.5.3 Distal intestine (DI)

Low levels of changes were observed in DI after 14 days of feeding in SW, with lamina propria and connective tissue being the two criteria showing only normal morphology, with the exception of 8.3% of moderate changes in amount of connective tissue observed in fish fed SW1-1. Significant differences were detected between lamina propria of fish from the SW2-2 dietary group and SW2-3. SW2-3 exhibited 75% no- and 25% mild changes whereas SW2-2 showed 33.3% no- and 66.7% mild changes ($p<0.05$). In addition, the changes observed in the amount of connective tissue in the SW2-3 dietary group (75% mild) were significantly higher compared to SW1-1, SW3-2 and SW3-3 ($p<0.05$) (Figure 45).

Morphological changes were in the form of loss of supranuclear vacuoles and mucosal folds complexity. DI mucosal folds of fish fed SW1-1 showed the highest level of moderate and distinct changes (50% and 16.7%), closely followed by fish fed SW2-3 (33.3% and 16.7%) and SW3-2 (27.3% and 18.2%), while SW2-2 and SW3-3 displayed 25% and 58.3% moderate changes. Changes observed in the mucosal folds of fish fed SW1-1 were significantly different from SW3-2, SW2-3 and SW3-3, additionally SW2-2 and SW3-2 mucosal folds were significantly different ($p<0.05$).

The loss of supranuclear vacuoles was low in most feed groups, whereas DI from fish fed SW3-2 showed the lowest loss (8.3% no-, and 91.7% mild). Both groups fed SW2 displayed 16.7% of moderate changes in the amount of supranuclear vacuoles, whereas SW2-3 was the only one of the two with samples displaying no changes (16.7%). Severe and moderate loss of supranuclear vacuoles was observed in fish fed SW1-1 (8.3% and 16.7%) and SW3-3 (8.3% and 41.7%). Statistical difference in the amount of vacuoles were detected between SW3-2 and SW3-3 ($p<0.05$).

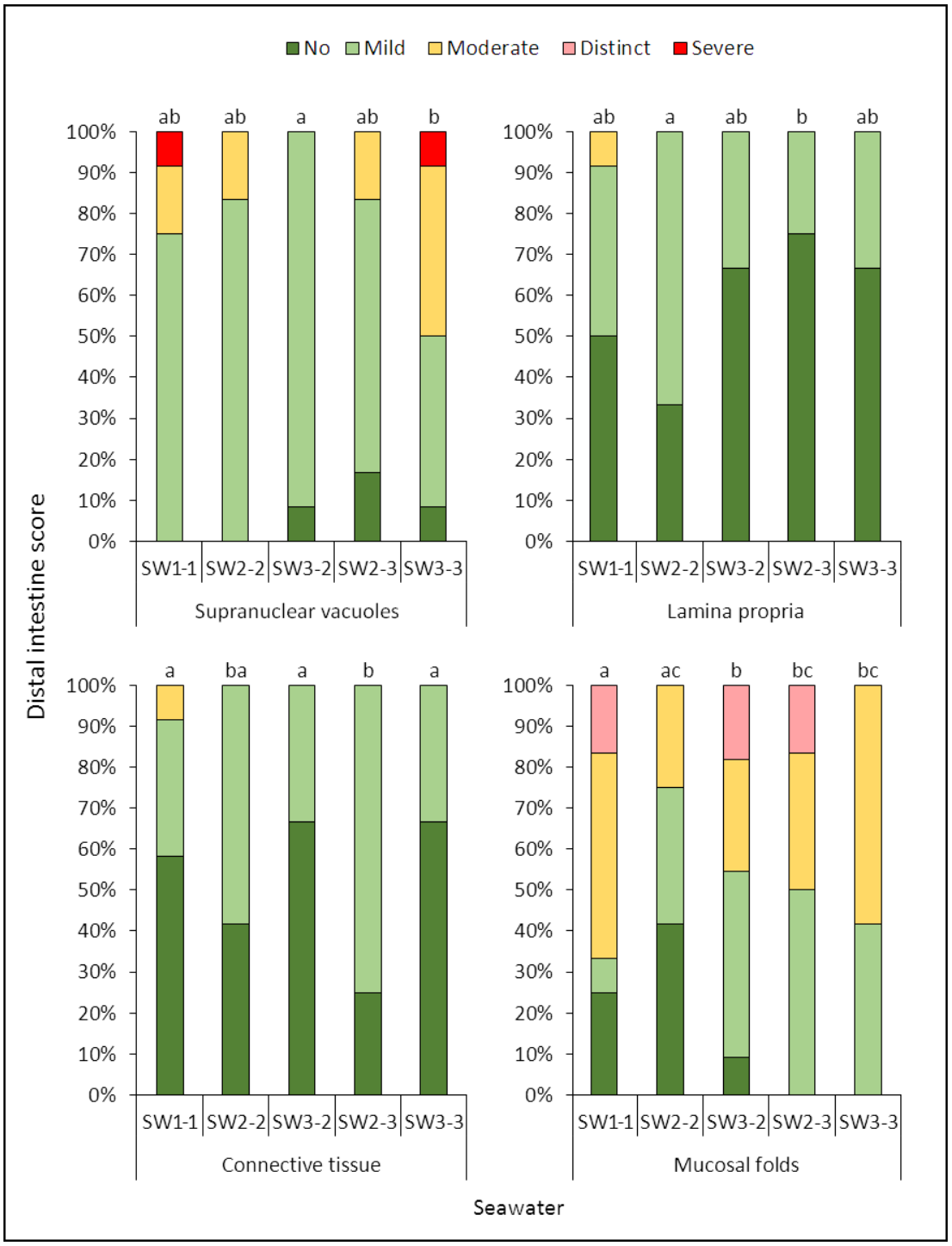

Figure 45. Histological analyses of distal intestine from Atlantic salmon fed three different test diets in two weeks post SW transfer. Columns show the score frequency from day 56 (14 days post SW transfer) of fish fed SW1, SW2 and SW3 (n=12) with their different FW period origin indicated with a dash and feed number. Different letters indicates significant difference, $p<0.05$.

3.6 Sub epithelial and intramuscular edema

The present study revealed large vacuoles in submucosa, muscularis and mucosal folds in samples from four fish from the SW1-1 group (four PC and three MI and DI samples) and three fish from the SW2-2 group (two PC and MI and one DI sample). The possibility of this observation being artifacts was considered. However, these vacuoles were observed in several slides, thus suggesting it may be pathological. Poppe, T. (personal communication, February 2014) suggested that these vacuoles were caused by fluid, for example water, and characterized it as sub epithelial and intramuscular edema (Figure 46). The vacuoles showed no correlation to fish weight and weight ranged from 53.3 g to 101.2 g.

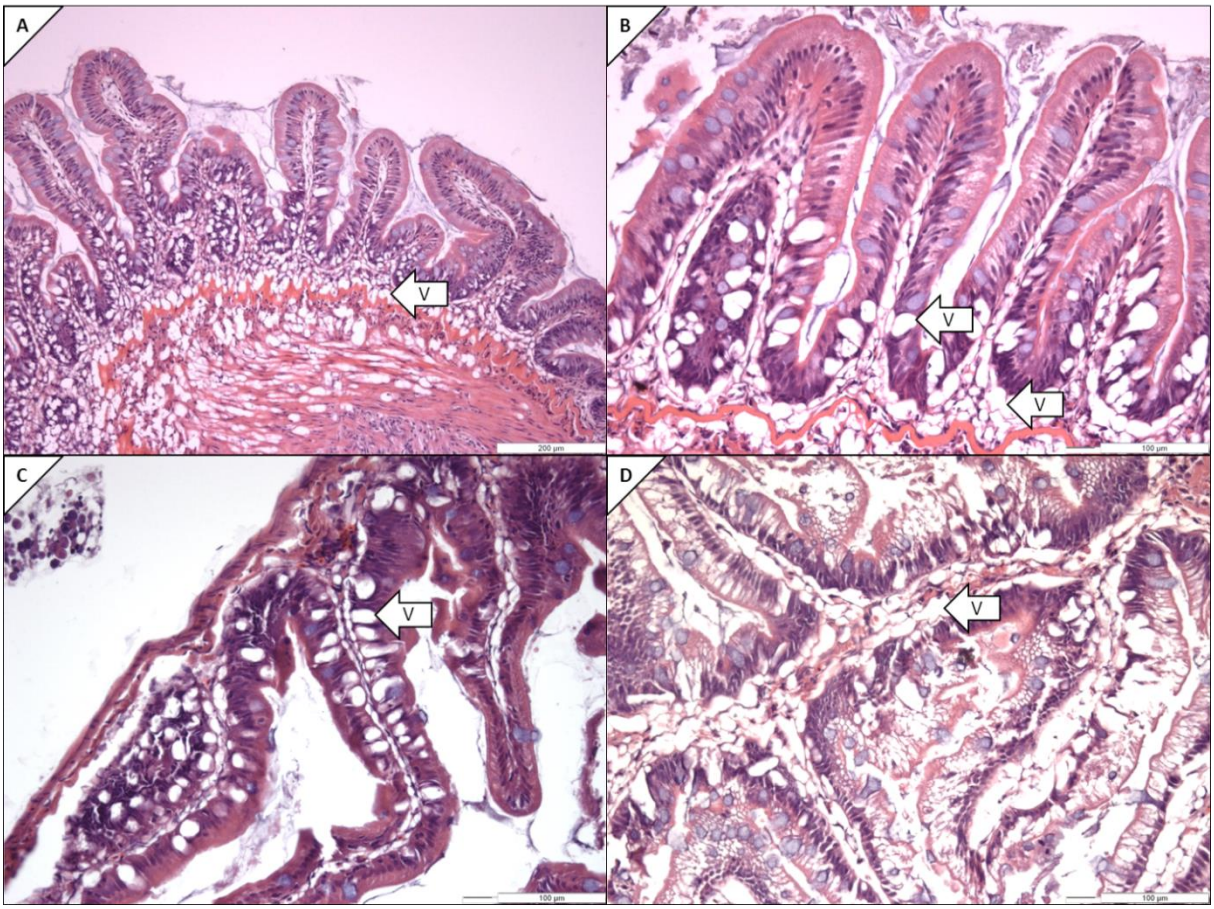

Figure 46. Atlantic salmon intestine Harris hematoxylin and eosin stained tissue from fish fed SW1-1 showing sub epithelial edema. Unidentified vacuoles (V) observed in all three gut segments of four fish from the group fed SW1-1 and three from SW2-2. (**A**) Vacuoles located in mid intestine (MI) muscularis sircularis, submucosa and the basal parts of mucosal folds (obj. x10). (**B**) MI vacuoles (V) in the areas surrounding stratum compactum (obj. x 20). (**C**) Vacuoles (V) in pyloric caeca mucosal folds (obj. x 20). (**D**) Vacuoles (V) in lamina propria and mucosa in distal intestine (obj. x 20).

3.7 Dietary effect on expression of *IL-1β*, *IL-10* and *TNF-α*

Real time PCR was conducted on DI samples collected at day 42 (before SW transfer) and 56 (two weeks post SW transfer). Expression of *IL-1β*, *IL-10* and *TNF-α* was low in both the FW and SW period (Figure 47) and no statistical differences were detected (Appendix V). Samples from naive fish (day 0) served as calibrator with the transcriptional level equal to 1. The pro-inflammatory gene *IL-1β* was slightly down-regulated in almost all dietary groups, except for SW3-2 which was very slightly up-regulated. *TNF-α* was slightly up-regulated in fish from the FW1, FW2 and SW3-2 dietary group, and slightly down-regulated in the remaining groups. The anti-inflammatory gene *IL-10* was slightly up-regulated in the FW1, FW2 and SW3-2 dietary groups, and slightly down-regulated in the other groups.

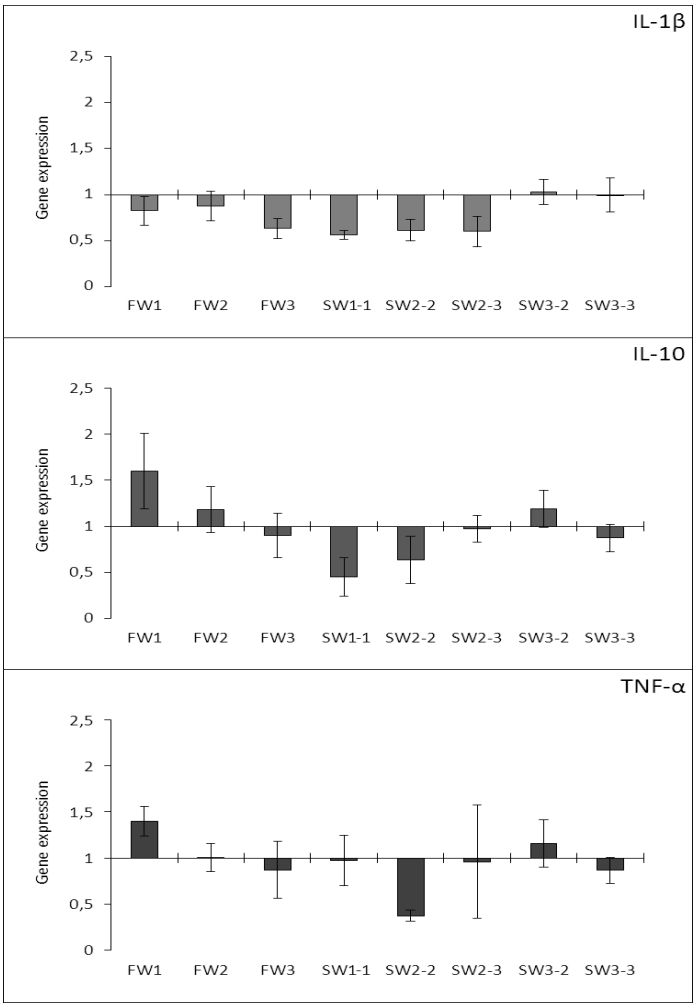

Figure 47. Gene expression of the gene *IL-1β*, *IL-10* and *TNF-α* in distal intestine of Atlantic salmon fed three test diets. The Y-axis shows fold increase in relative expression of the three genes. Samples from day 0 were used as control. Gene expression post feeding on FW feed in 42 days (FW1, FW2 and FW3) and SW feed in 14 days (SW1-1, SW2-2, SW2-3, SW3-2, SW3-3).

3.8 Cells of the gastrointestinal (GI) tract

Enterocytes are the main cells of intestine epithelium and, to the author's knowledge, have a life span of two to three weeks before being replaced by new enterocytes. Enterocyte replacement was observed in all samples (Figure 48).

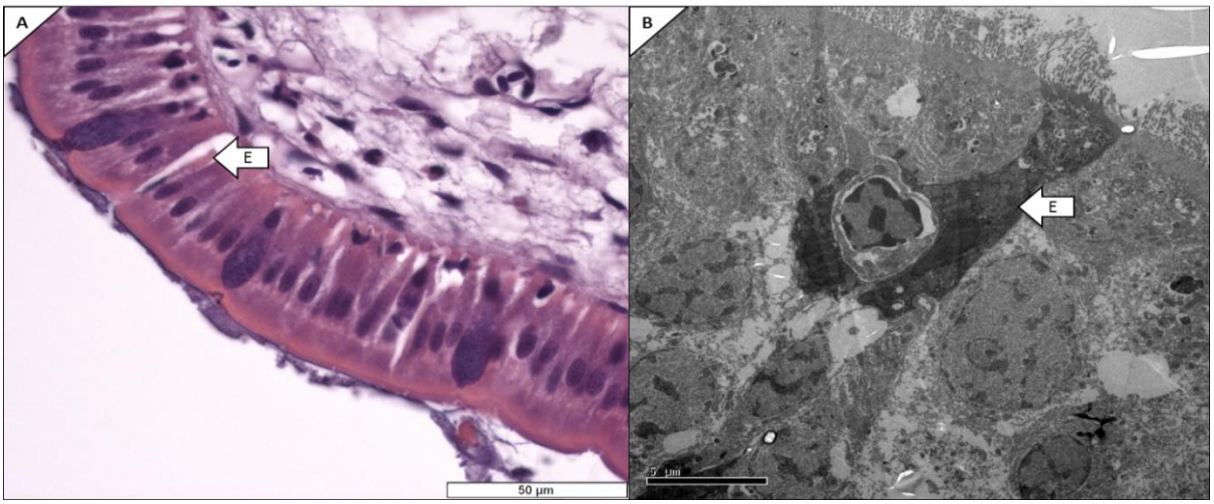

Figure 48. Enterocytes in pyloric caeca and mid intestine of Atlantic salmon. Enterocytes observed with light microscopy (Harris hematoxylin and eosin stained tissue) (A) and transmission electron microscopy (B). (**A**) Dead or dying enterocyte (E) leaving the mucosa layer in mid intestine (obj. x40). (**B**) Dead or dying enterocyte (E) in pyloric caeca mucosa (bar 5 µm), photo: Reidar Myklebust.

Cells, presumably intraepithelial lymphocytes (IELs), were observed between the roots of enterocytes towards the basal membrane during the histological analyses of the tissue sections in the three segments of the GI tract (Figure 49). IELs were not weighted in the scoring of the tissue samples.

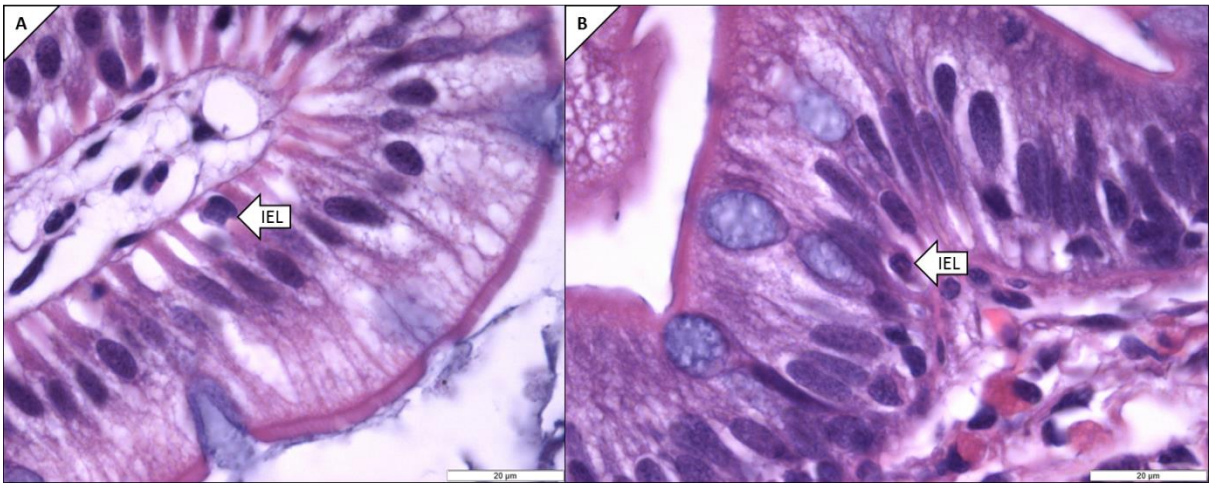

Figure 49. Atlantic salmon mid intestine showing intraepithelial lymphocytes (IELs) located basally around the enterocytes. (**A, B**) IELs in Harris hematoxylin and eosin stained mid intestine tissue, located in the basal parts of the epithelium near the basal membrane (obj. x100).

Eosinophilic granular cells (EGCs) were observed in varying numbers in the three gut segments. EGCs were located in submucosa and lamina propria, and were most numerous beneath stratum compactum (Figure 50). In cases of increased number of EGCs they were spread across stratum compactum and into lamina propria.

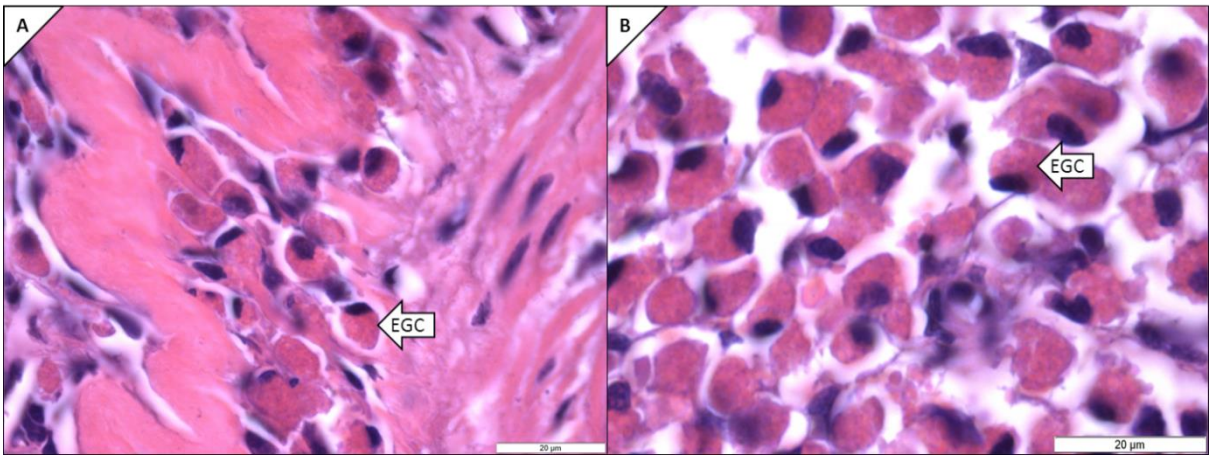

Figure 50. Eosinophilic granular cells (EGCs) in Atlantic salmon observed with light microscopy (A, B). (**A, B**) Mid intestine Harris hematoxylin and eosin stained tissue showing EGCs in stratum granulosum (obj. x100).

Rodlet cells (RCs) were observed in sections from both periods of the feeding trial and in the IPNV challenge trial. These cells were observed as encapsulated cells with arrow-like shapes in cytoplasm (Figure 51).

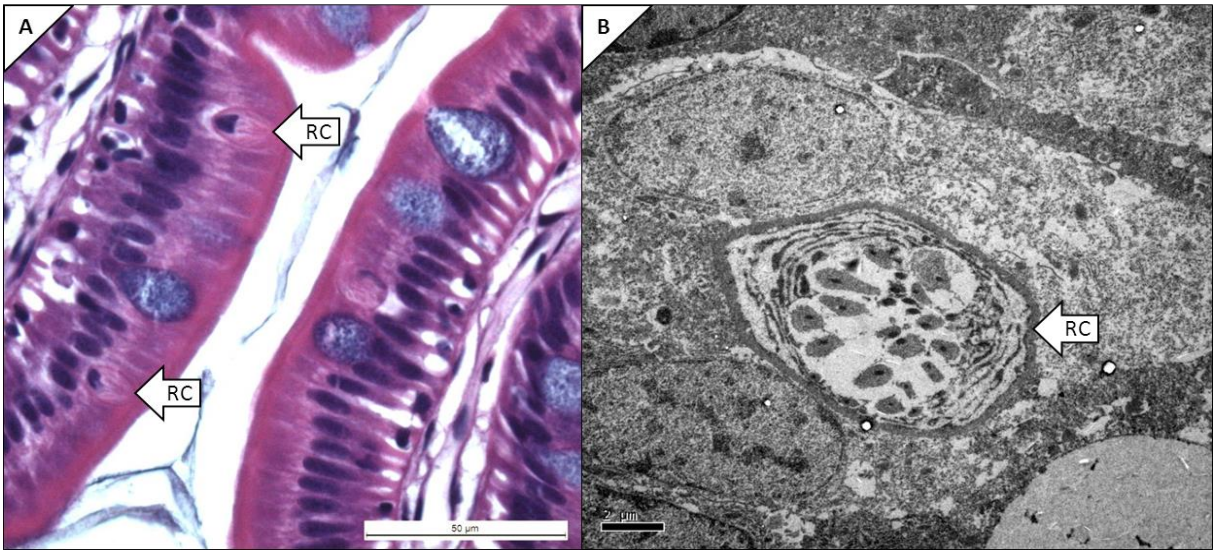

Figure 51. Rodlet cells (RCs) observed in tissue samples from Atlantic salmon using light microscope (A) and transmission electron microscope (B). RCs were observed in all gut segments, and were located in the apical part of the mucosa layer. (**A**) Mid intestine Harris hematoxylin and eosin stained tissue from the FW period showing two RCs in the mucosa layer (obj. x40). (**B**) RC in distal intestine of Infectious pancreatic necrosis virus (IPNV) challenged fish (bar 2µm), photo: Reidar Myklebust.

Goblet cells (GCs) are mucus (mucin glycoproteins) secreting cells located in mucosa (Jutfelt 2006). These cells were observed in all samples and gut segments from both the feed trial and the IPNV cohabitation challenge test (Figure 52).

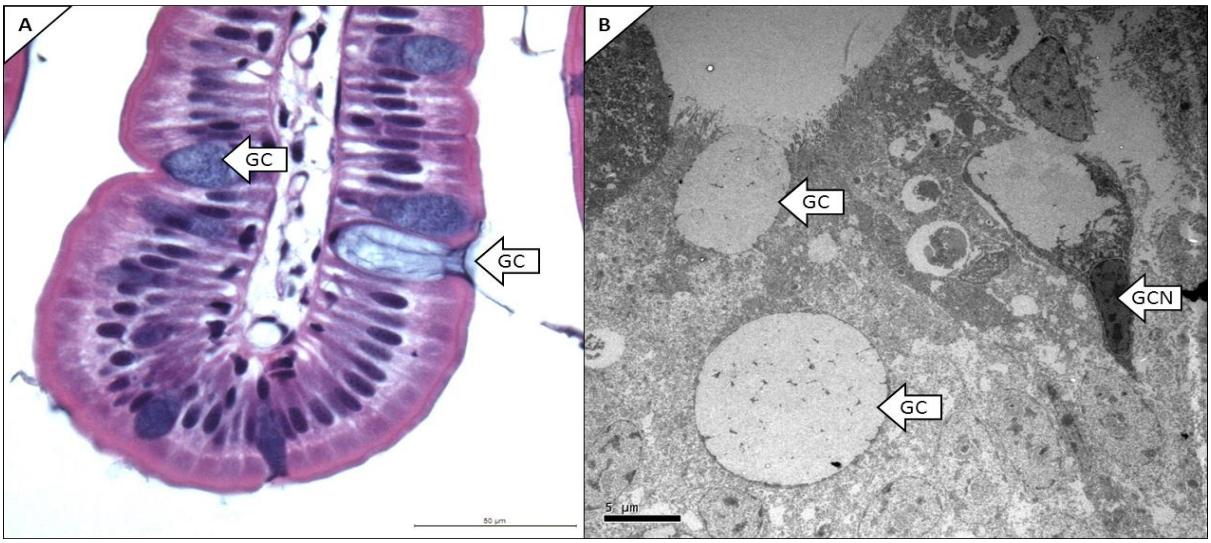

Figure 52. Atlantic salmon mucus producing goblet cells (GCs) observed with light microscope (A) and transmission electron microscope (B). (**A**) Mid intestine Harris hematoxylin and eosin stained tissue showing GCs emptying mucus into the gut lumen (obj. x40). (**B**) GCs and goblet cell nuclei (GCN) in distal intestine sample from the Infectious pancreatic necrosis virus (IPNV) challenge trial (bar 5 µm), photo: Reidar Myklebust.

3.9 Dietary effects on susceptibility to IPNV

An Infectious pancreatic necrosis virus (IPNV) challenge test was carried out after SW transfer to evaluate whether any of the feeds (FW1, FW2 and FW3) resulted in reduced mortality. The results are included in this study to show the complete effect of the three feeds. The fish fed FW2 before sea transfer had significantly ($p=0.04$) higher survival (17.4% mortality) 51 days after IPNV challenge than the groups fed FW1 (26.7% mortality) and FW3 (22.6% mortality) (Figure 53). A limited number of dying fish were sampled from the IPNV challenge, and were included in further histological analyses.

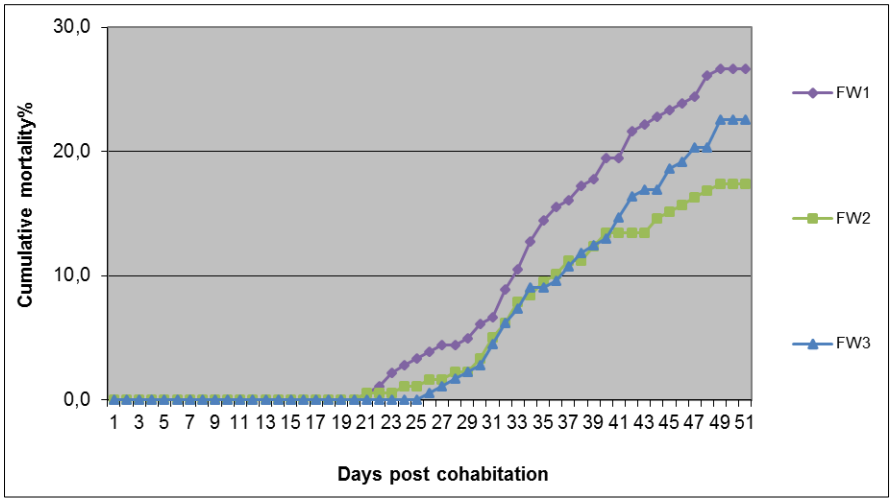

Figure 53. Cumulative mortality in groups of Atlantic salmon (n=180) fed freshwater (FW) feed 1, 2 and 3 post seawater (SW) transfer. The fish were challenged by cohabitation in SW. The fish were fed SW1 during the challenge period. Results kindly provided by Heidi E. Mikalsen.

3.10 IPN pathology

The PC, MI and DI were sampled from IPNV infected fish (n=6) from the feed groups FW1 and FW3. Morphological evaluations displayed similar changes; McKnight cells (MKCs) and eosinophilic casts were observed in all three gut segments. Pancreatic tissue surrounding the PC was also investigated, in addition pathological changes in PC, MI and DI. All six PC samples pancreatic tissue showed signs of IPN; focal necrosis in pancreas and rounding of pancreas acinar cells (Figure 54).

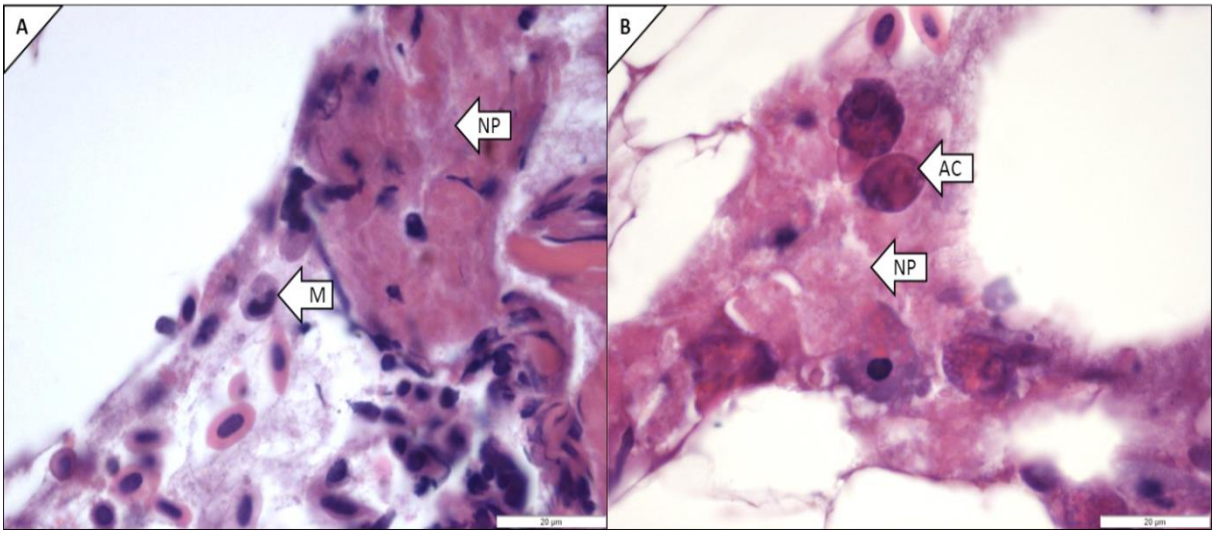

Figure 54. Atlantic salmon Harris hematoxylin and eosin stained pancreas tissue from Infectious pancreatic necrosis virus (IPNV) challenge. Necrosis of pancreas acinar cells was observed inn all fish sampled from the challenge trial. (**A**) Necrotic pancreas (NP) tissue and possible monocyte (M) (obj. x40). (**B**) Rounding of pancreas acinar cells (AC) and necrotic tissue (NP) (obj. x40).

3.10.1 Pathological effects of IPN on the GI tract

In addition to the changes observed in the pancreatic tissue, pathological changes were detected in the GI tract. MKCs were observed with light microscope and TEM in PC and DI. MKCs is described as degenerating enterocytes (McKnight and Roberts 1976) and manifested in terms of condensation of enterocytes nuclei surrounded by a clear halo (Figure 55 and 56). EGCs were observed in PC, MI and DI of IPNV infected fish and were different compared to EGCs in gut samples from both periods of the feeding trial. While almost all EGCs from the feed trial seemed to be intact, EGCs in IPNV infected fish showed signs of degranulation and emptying (Figure 57).

The PC, MI and DI showed varying numbers of MKCs in mucosa and eosinophilic casts from mucosa into the GI tract lumen. Severe pathological damage was observed in PC and DI, while MI showed a lower degree of pathological damage. Furthermore, the DI displayed a total loss of supranuclear vacuoles and a shortening of complex folds side branches. Figure 58 and 59 illustrate the pathological changes observed in PC and DI.

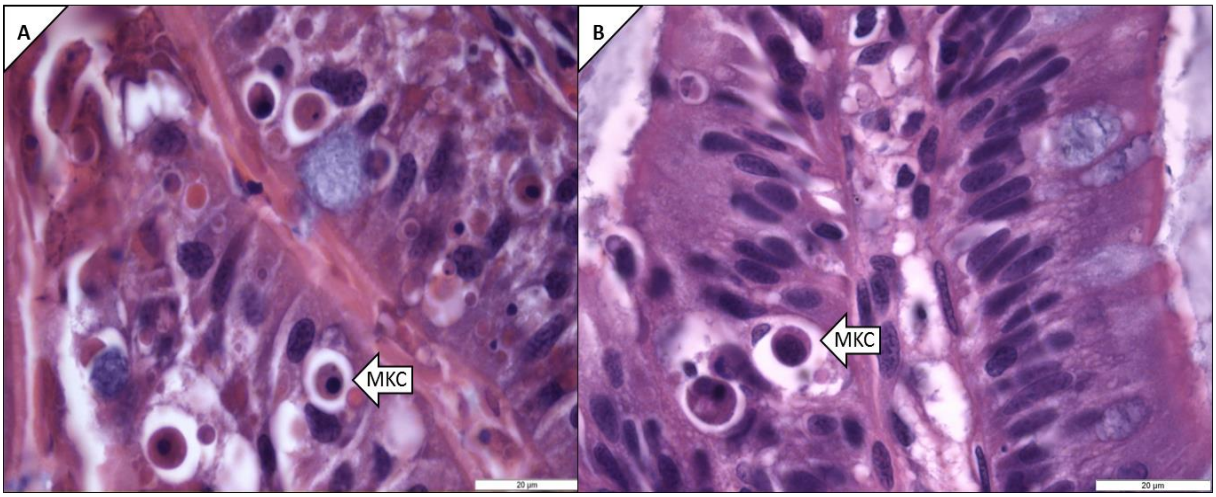

Figure 55. Infectious pancreatic necrosis virus (IPNV) infected Atlantic salmon showing several apoptotic enterocytes (McKnight cells, MKCs) in pyloric caeca (PC) and distal intestine (DI) mucosa layer observed by light microscopy. (**A**) Harris hematoxylin and eosin stained tissue showing McKnight cells (MKCs) in the PC mucosa layer of fish fed FW3 (obj. x100). (**B**) Mucosal layer in DI displaying MKCs from fish fed FW1 (obj. x100).

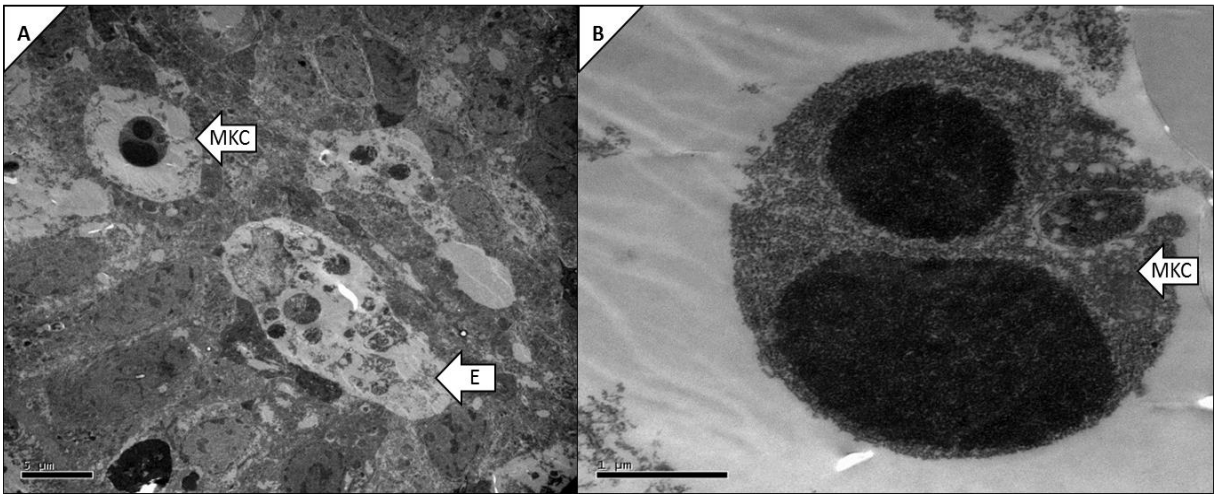

Figure 56. Infectious pancreatic necrosis virus (IPNV) infected Atlantic salmon showing several apoptotic enterocytes (McKnight cells, MKCs) in pyloric caeca (PC) (FW3) mucosa layer observed by transmission electron microscopy. (**A**) MKCs in Agar 100 Resin-embedded PC (bar 5 µm) (**B**) MKC showing condensation of chromatin (bar 1µm). Photo: Reidar Myklebust.

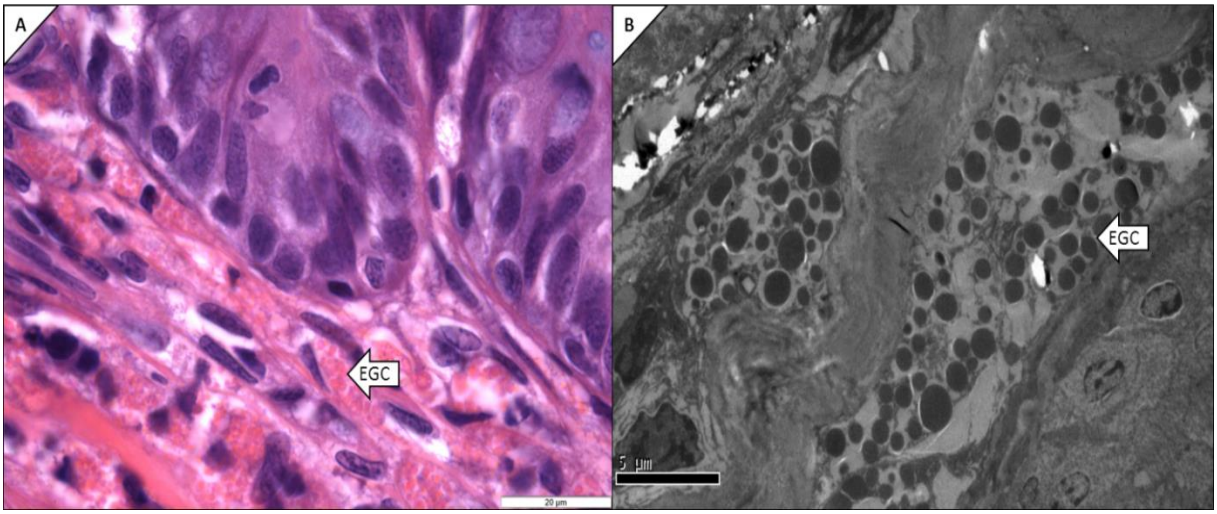

Figure 57. Eosinophilic granular cells (EGCs) between base of folds and stratum compactum in distal intestine (DI) of Infectious pancreatic necrosis virus (IPNV) challenged Atlantic salmon observed by light microscope (A) and transmission electron microscope (B). (**A**) DI Harris hematoxylin and eosin stained tissue with degranulating EGCs beneath base of folds and stratum compactum (obj. x100). (**B**) EGCs in lamina propria of DI (bar 5 µm). Photo: Reidar Myklebust.

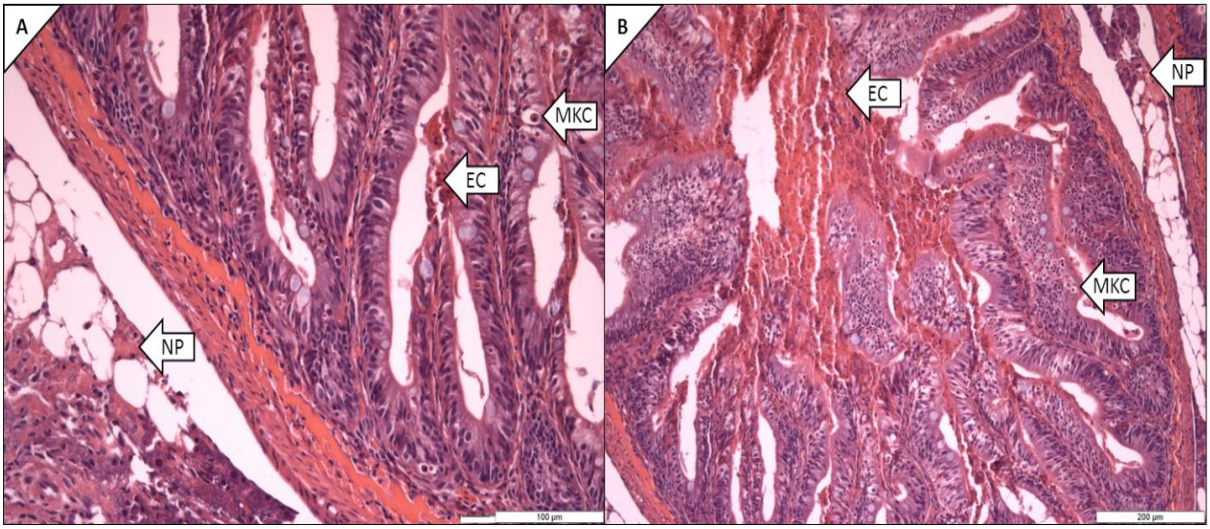

Figure 58. Morphological changes observed in pyloric caeca (PC); Harris hematoxylin and eosin stained samples from Infectious pancreatic necrosis virus (IPNV) infected Atlantic salmon. (**A**) Pockets with McKnight cells (MKCs) in mucosa and eosinophilic casts (EC) in lumen. Pancreatic tissue surrounding PC showed necrotic pancreas tissue (NP) (obj. x20). (**B**) MKCs, eosinophilic casts (EC) in lumen and necrotic pancreas tissue (NP) (obj. x10).

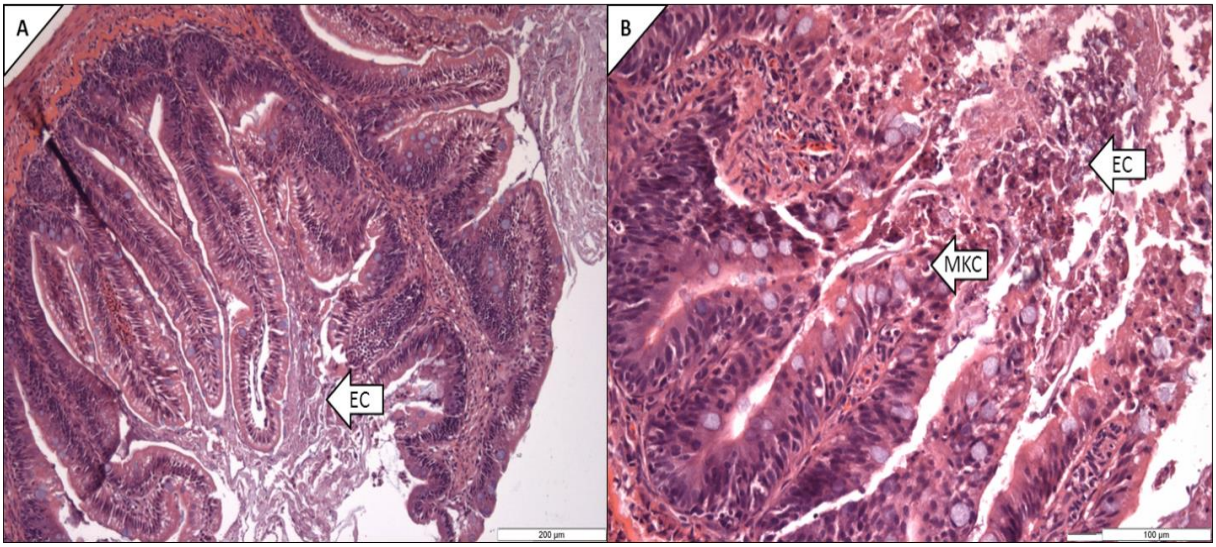

Figure 59. Morphological changes observed in distal intestine (DI); Harris hematoxylin and eosin stained samples from Infectious pancreatic necrosis virus (IPNV) infected Atlantic salmon. (**A**) Exfoliating enterocytes and eosinophilic casts (EC) in lumen (obj. x10). (**B**) Pockets with McKnight cells (MKCs) in DI mucosa and eosinophilic casts in lumen (EC) (obj. x20).

3.11 Detection of IPN virus in salmon intestine

One fish from feed group FW1 and one from feed group FW3 were collected and used for further analyses by TEM and immunolabeling on cryo-embedded tissue. The IPN virus was tagged with antibodies and gold particles (10 nm) to enable identification of the virus in the PC and DI using TEM. Low numbers of virus were detected in the PC and DI 19 days after the

start of the IPNV challenge (Figure 60). The virus was not detected in the negative control samples.

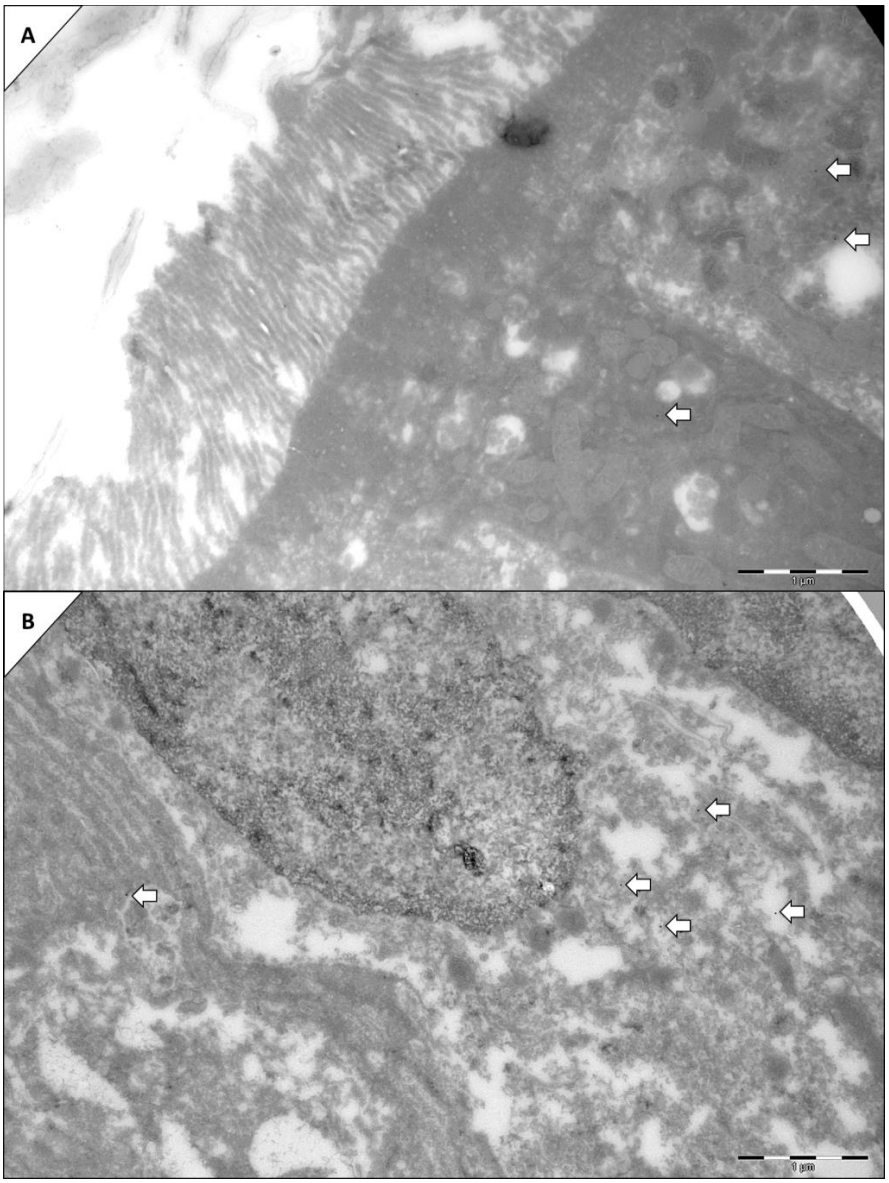

Figure 60. Infectious pancreatic necrosis (IPN) virus protein 3 (VP3) labeled with Protein-A gold (PAG_{10}, University of Utrecht, The Netherlands) in pyloric caeca (PC) and distal intestine (DI) from IPNV challenged Atlantic salmon. Gold particles were observed by transmission electron microscope (TEM). (**A**) PC of FW1 fed fish with virus (arrows) detected close to the brush border membrane (1µm bar). (**B**) DI of FW3 fed fish with virus (arrows) detected close to enterocyte nucleus (bar 1µm).

4 Discussion

Functional feeds are enhanced or enriched with certain nutrients for the purpose of increasing host health benefits (Mandel *et al.* 2005). The objectives of this study were to evaluate morphological and immunological effects on the GI tract of six feeds for Atlantic salmon during the parr-smolt transformation, in both FW and SW. The feeds had similar basic compositions of protein, oil, ash and water, but differed in the supplementation of vitamins, prebiotics, nucleotides, β-glucans and fatty acid composition of n-3 (eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA)) and n-6 (arachidonic acid (ARA)). The morphological changes of the different parts of the intestine of Atlantic salmon were studied by histological examinations (light microscopy and transmission electron microscopy (TEM)) and the expression of the innate immune genes *IL-1β*, *IL-10* and *TNF-α*. It is well known that different dietary ingredients could result in either positive or negative effects on the GI tract morphology (van den Ingh *et al.* 1991, Bakke-McKellep *et al.* 2000, Olsen *et al.* 2000, Olsen *et al.* 2001, Refstie *et al.* 2010, Penn *et al.* 2011, Navarrete *et al.* 2013, Romarheim *et al.* 2013).

To the author's knowledge only a few studies have revealed effects of functional feeds on shrimp (Olmos *et al.* 2011) and Atlantic salmon (Tacchi *et al.* 2011, Martinez-Rubio *et al.* 2012, Martinez-Rubio *et al.* 2013). Olmos *et al.* (2011) introduced *Bacillus subtilis* as probiotics in feeds with high levels of vegetable proteins for whiteleg shrimp (*Litopenaeus vannamei*) which produced better performance in terms of growth, food conversion ratio and stress tolerance. Tacchi *et al.* (2011) supplemented a salmon diet with prebiotics, nucleotides, vitamin C and E, which led to down-regulation of genes involved in the adaptive and innate immune system and enhanced growth. Martinez-Rubio *et al.* (2012) used functional feeds with lower lipid content and increased EPA and EPA/ARA ratio as clinical nutrition for salmon infected with Atlantic salmon reovirus (ASRV) which reduced the severity of heart and skeletal muscle inflammation (HSMI). Further, they found that LC-PUFA and genes involved in the eicosanoid pathway in the head kidney had important roles in this process (Martinez-Rubio *et al.* 2013). Several review studies (Mandel *et al.* 2005, Li and Gatlin III 2006, Merrifield *et al.* 2010, Ringø *et al.* 2010, Kiron 2012, Ringø *et al.* 2012) have

been conducted on the effect of different functional feed components, but to the author's knowledge no information is available on the effect of functional feeds on the GI tract morphology. This study is therefore the first to test combinations of functional ingredients effect on the morphology of the GI tract.

The GI tract functions as a physiological barrier between the organism and the external environment. The intestinal epithelium layer consists of a single cell layer that is responsible for the uptake of nutrients, water and ions as well as protection against harmful agents in the GI lumen. An intact mucosal layer is crucial for the health and growth of the animal (Jutfelt 2006). The functional feeds tested in this present study did not enhance the growth of the salmon compared to the control diet. In a study conducted by Tacchi *et al.* (2011) significant better growth was observed in SW adapted Atlantic salmon fed similar functional feeds as in this study. This present study is different from the latter in both the life stage of the salmon and the duration of feeding; indicating that differences may have occurred if the feed trial had been prolonged. In addition, the life stage of the salmon may have been a cause for the difference in specific growth rate (SGR) observed between the FW and SW period. Suppressed appetite and growth during the first days of SW residence have previously been demonstrated by Jorgensen and Jobling (1994), where salmon increasingly resumed feeding during the first two weeks in SW. Another cause of the temporary suppression of feed intake may be individual differences in the smolt population, as indicated by Usher *et al.* (1991).

One of the parameters for health status used in this study was the morphology of the GI tract. Previous studies (van den Ingh *et al.* 1991, Bæverfjord and Krogdahl 1996, Urán *et al.* 2004, Knudsen *et al.* 2007, Penn *et al.* 2011) revealed that dietary manipulations affect gut morphology. The results from the collected samples revealed tendencies towards increased morphological changes in samples from day 0 compared to samples from day 42 (just before SW transfer). From start feeding to the beginning of the feed trial the fish had been fed a commercial diet from another feed company. During the six days of acclimation the fish were fed FW1. Since the appearance of the gut health in the FW1 group seemed to have

improved during 42 days, one can speculate that the initial lower gut status may have been due to the commercial diet. For future trial design one control group feeding on the original diet should run parallel to the feed trial.

The majority of fish displayed normal morphology (defined as no- to mild changes) in the enterocytes, nuclei, lamina propria, submucosa and mucosal folds of PC, MI and DI in the FW and SW period. However, gut morphological changes detected from day 0 to day 56 included increased level of vacuolization and the number of pycnotic cell nuclei (PCN) in PC, increased vacuolization and amount of connective tissue in lamina propria and submucosa of MI and decreased level of supranuclear vacuoles and mucosal fold branching of DI. The observed increased vacuolization may be a result of dietary manipulations, as demonstrated with lipids and inulin (Olsen *et al.* 1999, Olsen *et al.* 2000, Olsen *et al.* 2001). Increased vacuolization of PC have previously been observed in SW adapted Atlantic salmon fed different levels of fish meal, soybean protein, corn gluten meal or pea protein concentrate (Penn *et al.* 2011) and rainbow trout fry fed a diet free of marine ingredients (Twibell *et al.* 2011). Samples with increased vacuolization also displayed increased levels of PCN. In an early study, Kerr *et al.* (1972) suggested that the number of PCN were characteristic with apoptosis, where the cell terminates itself. Whether or not the vacuolization of PC enterocytes that were revealed in the present study are pathological is to the author's knowledge not known, but the increased levels of PCN together with vacuolization indicate that this may be the case.

The influx of connective tissue in lamina propria and submucosa and loss of supranuclear vacuoles has been associated with soybean meal induced enteritis (van den Ingh *et al.* 1991, Urán *et al.* 2004, Knudsen *et al.* 2007). Due to the low degree of these changes observed in the present investigation a firm diagnosis of enteritis will not be made, rather indications on the possibility of these changes being the early stages of enteritis. Bæverfjord and Krogdahl (1996) showed regression of soybean-meal induced enteritis after changing to a fish meal based diet. In relation to this observation the changes observed in the present investigation can be temporary, and may not evolve in to enteritis.

The results of the present investigation showed that different feeds did not result in statistical differences in the overall morphological appearance during the FW period, but this was observed in the SW period. Differences were detected between feed groups criteria and the majority of differences were observed in tissue showing normal tissue to a few moderate changes. The significance of the detected differences in determining the effect of the feeds is low.

Further, speculations can be made that the changes observed in growth due to smoltification and SW transfer can be assigned to the morphological changes as well. During the parr-smolt transformation, a number of changes occur in salmon physiology, morphology and behavior (Folmar and Dickhoff 1980, Stefansson *et al.* 1991, Stefansson *et al.* 2008). As stated by Jutfelt (2006) the barrier function of salmon GI tract may be compromised by the large changes occurring during the parr-smolt transformation. Nonnotte *et al.* (1986) studied rainbow trout in the parr-smolt transformation using TEM analyses, and showed morphological changes in the first days post SW transfer. After one month of SW adaption the structure of the intestine was similar to that observed in FW. In correlation to the latter study, the morphological changes observed in this feed trial may have been induced by smoltification and SW adaption. In addition, the growth and morphological changes observed during this study may have been affected by the genetic variations in the fish population. This raises the question of the need for genetically standardized salmon, to be able to produce a stronger statistical material and reproducible results, as reviewed by Johansen *et al.* (2006).

Sub epithelial and intramuscular edema observed in the dietary groups SW1-1 and SW2-2 could have been caused by autolysis due to the duration of sampling or improper fixation due to tissue size. However, samples were taken successively, with a maximum of 3 minutes from euthanasia to fixation of the gut samples and tissue samples were only a few millimeters thick. Although Poppe, T. (personal communication, February 2014) has pointed

out freezing as a possible cause of sub epithelial and intramuscular edema, this could not be the case here since the samples had not been frozen during the process. Fat deposition may cause vacuoles, but then a thin veil should be observed inside (Poppe, T., personal communication, February 2014). No veil was observed, thus suggesting that the vacuoles were a result of water influx, hence edema. It remains unclear what the pathological impact, if any, may have been.

In this study the down-regulation of the relative expression of *IL-1β*, *IL-10* and *TNF-α* involved in the inflammatory response indicates that the feeds did not induce inflammation, which in turn indicates a healthy fish. This correlates with the low levels of morphological changes observed in the GI tract. Hynes (2011) showed the salmons ability to down-regulate the pro-inflammatory cytokine production one and two weeks post flagellin injection. Vitamin C has been shown to prevent activation of the pathway leading to the expression of *TNF-α* and subsequently inflammation in humans (Hartel *et al.* 2004). To the author's knowledge, similar studies have not been published in fish. However, the down-regulation of this gene, although not significant, may correlate with the vitamin-added diet. In correlation to enteritis, Urán *et al.* (2008) showed the up-regulation of TNF-α in common carp fed a diet without added vitamin C. However, the results in the SW2-2 group may have been caused by a combination of all the functional ingredients included in the diets. It seems that feeding salmon the functional feed ingredients of SW2-2 had a positive effect on the fish health and the down-regulation of *TNF-α* indicates that the feed do not induce an inflammatory reaction in the GI tract.

An IPNV cohabitation challenge was used to establish the dietary effects of susceptibility to diseases. A possible explanation of the enhanced resistance against IPNV of fish fed the functional diet FW2 may be the added nucleotides. In a previous study, Leonardi *et al.* (2003) showed that enriching diets for rainbow trout with nucleotides enhances the viral resistance by stimulating B lymphocytes and decreasing plasma cortisol levels, which may explain the significant better survival in the FW2 group in this study. Sundh *et al.* (2011) suggested that IPNV modulates the epithelial barrier and causes increased permeability during acute exposure. The decreased susceptibility to IPNV in the fish fed FW2 in this present study may

have been due to the added nucleotides in combination with the other functional ingredients, maintaining the GI tract barrier function. Feeding salmon functional diets included nucleotides showed to be positive for reducing the susceptibility of IPNV. For the future, the use of functional feeds in the challenging period of the parr-smolt transformation could be applied to reduce mortality post seawater transfer.

IPNV challenge revealed large numbers of McKnight cells (MKCs). Since these cells were not observed in the feed trial prior to the IPNV challenge this may indicate that the cells were a result of infection. This is in accordance with the findings of Lumsden (2006) in connection to IPNV infection in Atlantic salmon and calling them “nests of necrotic cells”. Poppe, T. (personal communication, February 2014) suggested that these cells were apoptotic epithelial cells, which fits with the anamnesis. All fish (n=6) sampled from the IPNV challenge showed necrosis of the pancreatic tissue, eosinophilic casts and MKCs. This verifies that the fish included in the IPNV challenge test were infected and showed the pathological changes associated with IPN.

EGCs observed in IPNV challenged salmon showed cell rupture with granule release or activation of the cells in response to the infection. Reite (1997) suggested that upon activation, eosinophilic granular cells (EGCs) release or produce substances involved in inflammatory reaction. This correlates with observations reported by Urán *et al.* (2009) where the granules content decreased during the trial in response to the soybean meal enteritis. Urán *et al.* (2008) also observed an increase in influx of basophilic granulocytes (here EGCs) in lamina propria and submucosa in cases of enteritis. Sundh *et al.* (2009) observed an increased infiltration of EGCs in response to IPNV infection. According to (Lumsden 2006) care should be exercised regarding the over-interpretation of the significance of EGCs in the construction of a diagnosis of enteritis. Although a lot is known of the EGCs role in the immune system, further research is required to establish the role of EGCs in enteritis.

TEM is a good tool for elucidating the aspects of the life-cycle of virus in an infected cell (Roingeard 2008). Detection of IPNV by TEM in this study was conducted to validate the presence of virus in GI tract enterocytes. The intestine as entry route for IPNV has previously been established by Sundh *et al.* (2011) using Ussing chamber and real time PCR analyses. The present study identified IPNV in PC and DI by TEM analyses. Low counts of IPNV in DI may be due to the long period between infection and sampling, thus the IPNV may have migrated to the liver or pancreas wich is the major target organ (Ellis *et al.* 2010). Low virus counts were also observed in PC and correlates with an immunohistochemistry study conducted by Ellis *et al.* (2010) where PC only showed one single cell positive for infection. In retrospect, a preferable method for screening of virus in TEM would be to conduct immunohistochemistry on the already prepared histological sections prior to the TEM analyses. When and if virus were detected, a small biopsy from the section could be brought through for TEM analyses, to ensure the presence of virus in the TEM sections.

Tacchi *et al.* (2011) have previously showed the positive effects of feeding Atlantic salmon a functional diet with similarities to the FW2 and the SW2 diets of the present study. The results of this master study showed that the functional diet SW2-2 produced lower morphological changes to the GI tract, compared to the control diet. Furthermore, a positive effect on mortality post seawater transfer of fish fed FW2 during the parr-smolt transformation in FW was shown. The use of feed as health promoter could be applied to other life stages of farmed salmon, however further research is needed.

5 Conclusion

- The overall impression of the GI tract of fish included in this study was good and normal tissue were primarily observed.
- The functional diet FW2 (added vitamins, prebiotics, nucleotides and β-glucans) fed during the parr-smolt transformation increased survival in the IPNV cohabitation challenge post seawater transfer.
- The functional feeds had low impact on the relative expression of the pro-inflammatory genes *TNF-α* and *IL-1β* and the anti-inflammatory gene *IL-10*, and produced mainly down-regulation of these genes.
- The functional diet SW2-2 (added vitamins, prebiotics, nucleotides and β-glucans) fed during the first period post seawater transfer produced less morphological changes to the GI tract compared to a non-functional diet (control diet).
- All fish sampled from the IPNV challenge showed pathological changes to the GI tract and pancreas associated with IPN.
- This study showed the presence of IPNV in GI tract enterocytes 19 days post cohabitation challenge, although in low counts.

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Appendix I

Chemicals

Histology - light microscopy	
Ethanol	Kemetyl
Paraformaldehyde	Aldrich
Paraffin wax	Sigma
Histo-Clear	National diagnostics
Harris hematoxylin	Sigma-Aldrich
Eosin Y solution (alcoholic)	Sigma-Aldrich
Histomount	National Diagnostics
Histology - transmission electron microscopy	
Xylene	Sigma-Aldrich
Ethanol	Sigma
Dimethylarsenic acid sodium salt trihydrate	Merck
Osmium tetraoxside	Electron Microscopy Sciences
Ascorbic acid	VWR BDH Prolab
Agar 100 Resin	Agar Scientific Ltd.
Propylene oxide	Electron Microscopy Sciences
Uranyl acetate	Merck
Lead nitrate	Merck
Immunocytochemistry - transmission electron microscopy	
Xylene	Sigma-Aldrich
Phosphate buffered saline	Sigma-Aldrich
Ethanol	Sigma-Aldrich
Gelatin from cold water fish skin	Sigma
Bovine serum Albumin	Calbiochem, EMD Millipore, Merck
monoclonal mice anti IPN antibody	Intervet Norbio, Bergen, Norway
Rat anti mouse, RαM	ICN Pharmaceuticals
Protein A-gold 10nm (PAG_{10})	University of Utrecht, The Netherlands
Glutaraldehyde	Merck
Methylcellulose	Sigma
Uranyl acetate	Fluka, Sigma-Aldrich
Immunology - real time PCR	
RNAlater solution	Ambion
Nucleic Acid Purification Lysis solution	Applied Biosystems
Phosphate buffered saline	Gibco, Life Technologies
AbsoluteRNA Wash Solution	Applied Biosystems
Purification Wash Solution 1 and 2	Applied Biosystems
Elution solution	Applied Biosystems
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems
nuclease free water	Ambion
Power SYBR Green Master PCR mix	Applied Biosystems

Appendix II

Equipment

Histology - light microscopy	
Histology cassettes	M490-Simport, Histonette I
Eppendorf tubes (2.0 ml)	Eppendorf AG
Heated paraffin embedding module	Leica EG1150 H
Cooling plate	Axel Johnson CP-4
Microtome	Leica MR2235
Water bath	Leica HI 1210
Microscope slides	Superfrost Plus, Menzel Gläser
Light microscope (Eclipse TE2000-S)	Nikon
Light microscope	Leica DM 6000B
Imaging software	Leica application suite imaging software
Light microscope	Leica DMLB
Digital camera	Leica DC 300
Imaging software	Analysis Image Processing (Leica)
Histology - transmission electron microscopy	
Ultramicrotome	Reichert ultracut S, Leica
Water bath	Diatome
Immunocytochemistry - transmission electron mincroscopy	
Cryo ultra microtome	Reichert FCS Ultracuts, Leica
Laboratory film	Parafilm, Bemis Flexible packaging
Electron microscope	Jeol JEM-1010
Digital camera	Morada – Soft Imaging System
Image analysis software	iTEM, Olympus
Immunology - real time PCR	
MagNaLyser Greenbeads tubes	Roche
MagNALyser instrument	Roche
ABI Prism 6100 Nucleic Acid Prep Station	Applied Biosystems
RNA purification tray	Applied Biosystems
96-well half skirt tray	Bioplastics
Vortex VF2	JK Janke & Kunkel IKA-Labortechnik
Centrifuge 5804	Eppendorf
NanoDrop 8000 Spectrophotometer	Thermo Scientific
96-well trays	Bioplastics
2720 Thermal cycler	Applied Biosystems
384 well plates	Applied Biosystems
7900 HT Fast Real-Time PCR System	Applied Biosystems

Appendix III

Histology procedures for Harris hematoxylin and eosin stained tissue

Tissue fixation

4% Paraformaldehyd, pH 7.4	Amount
Paraformaldehyd (95.0 – 100.5%)	40 g
dH_2O	750 ml (+), total 1000 ml
1 M NaOH	5 ml
10 x PBS	100 ml
6 M HCl	pH-adjustment (droplets)

Tissue processing

Shandon Citadel 1000 - solutions	Bath and time
96% ethanol	3 and 4, 2 hours each
100% ethanol	5 and 6, 2 hours each
Histo-Clear and 100% ethanol	7, 1 hour
Histo-Clear	8, 9 and 10, 1 hour each
Histo-Clear and paraffin wax	11, 1 hour
Paraffin wax	12, 3 hours

Tissue staining

De-paraffination and re-hydration of tissue samples	
Solution	**Time**
Histo-Clear	3 x 5 minutes
100% ethanol	5 minutes
96% ethanol	5 minutes
70% ethanol	5 minutes
50% ethanol	5 minutes
Distilled water	10 minutes

Staining of tissue samples	Time
Harris hematoxylin solution	2.5 minutes
Pouring water	10 minutes
Eosin Y Solution, alcoholic	2 minutes
96% ethanol	3 x 2 minutes
100% ethanol	2 minutes
Histo-Clear	3 x 2 minutes
Histomount	Mount the coverslide while the slide are wet

Appendix IV

Gene sequences

>Atlantic salmon *IL-1β* (NM_001123582.1)

ggattcacaagaactaaggactgaatacaagacactgttacctacctacaaactacaaaacaactcaagatctaactatacagacatggaatttgagtcaaactacagtttaataaagaacacctctgaaagtaaggcatggagctccaagctgcctcagggtctggatctggaggtatcccatcaccccatcaccatgcgtcacattgccaacctcatcatcgccatggagaggttaaagggtggcgagggggttaccatgggaaccgagttcaaggacaaggacctgctcaacttcttgctggagagtgctgtggaagaacatatagtgttggagttggagtcggcgccccctacgagcaggagggaagcagggttcagcagtacatcacagtacgagtgtagcgtcactgactctgagaacaagtgctgggtcctgatgagtgaggctatggagctgcacgccatgatgctccagggaggcagcggctaccacaaagtgcatttgaacctgtctacgtacgtcacgcccgtccccattgagactaaagccagacctgtagccctaggcataaagggatccaacctctacctgtcctgctccaaatcgggaggcaggccaaccctgcacctagaggaggttgcaaacaaagagcagctgaagtccatcagccagcagagcgacatggtgcgtttccttttctacagacggaacaccggggttgacatcagtaccctggagtctgccgggttcaggaactggttcatcagcacggacatgcagcaggacaacaccaaaccggtggacatgtgtcagaaggcagcccccaaccgcctcaccaccttcaccatccagcgccacaactaag

>Atlantic salmon *IL-1β* – Forward

CCCGTCCCCATTGAGACTAAA

>Atlantic salmon *IL-1β* - Reverse

AGCAGGACAGGTAGAGGTTGGATC

>Atlantic salmon *TNF-α* (NM_001123590.1)

ttgaagcagattgctggcaatgcaaaagtagccatccatttagagggtgaatacaatcctaatcttaccgctaacaccgtgcagtggagaaaggatgacggccaggccttttcccagggcgggttcaagctacaagggaaccaaattctcatcccacacactgggctcttcttcgtttacagccaggcttcgtttagggtcaagtgcaacagcccgggcgagcgtaccactcctctgagtcacgttatttggcgctattcggactccatcggggataatgctaatctacttagcggggtaaggtcagtttgtcaacaaaactatggtaattctgagtccaatatcggcgaggctggtacaatgcagtttaccttagtgcagtgtttcatctgaatgaaggggacaaactgtggactgagaccaatcgactgaccgaagtggagccagagcagggcaaaaatttcttcggtgtgtttgca

>Atlantic salmon *TNF-α* – Forward

CGTGCAGTGGAGAAAGGATGA

>Atlantic salmon *TNF-α* - Reverse

AAGAAGAGCCCAGTGTGTGGG

>Atlantic salmon *18S* (AJ427629)

tggttgattctgccagtagcatatgcttgtctcaaagattaagccatgcaagtctaagtacacacggccggtacagtgaaactgcgaa
tggctcattaaatcagttatggttcctttgatcgctccaacgttacttggataactgtggcaattctagagctaatacatgcagacgag
cgctgacctccggggatgcgtgcatttatcagacccaaaacccatgcgggccaatctcggttgccccggccgctttggtgactctaga
taacctcgagccgatcgcgcgccctttgtggcggtgacgtctcattcgaatgtctgccctatcaactttcgatggtactttctgtgccta
ccatggtgaccacgggtaacggggaatcagggttcgattccggagagggagcctgagaaacggctaccacatccaaagaaggcag
caggcgcccaaaattaccactccccactcggggaggtagtgaccaaaaataacaatacaggactctttcgaggccccgtaattgga
atgagtacactttaaatcctttaacgaggatccattggagggcaagtctggtgccagcagccgcggtaattccagctccaatagcgta
tcttaaagttgctgcagttaaaaagctcgtagttggatctcgggatcgagctggcggtccgccgcgaggcgagctaccgcctgtccca
gcccctgcctctcggcgcccccctcgatgctcttaactgagtgtcccgcggggtccgaagcgtttactttgaaaaaattagagtgttcaa
agcaggcccggtcgcctgaataccgcagctaggaataatggaataggactccggttctattttgtgggttttttcttctgaactggggcc
atgattaagagggacggccgggggcattcgtattgtgccgctagaggtgaaattcttggaccggcgcaagacggacgaaagcgaa
agcatttgccaagaatgttttcattaatcaagaacgaaagtcggaggttcgaagacgatcagataccgtcgtagttccgaccataaa
cgatgccaactagcgatccggcggcgttattcccatgacccgccgggcagcgtccgggaaaccaaagtctttgggttccgggggga
gtatggttgcaaagctgaaacttaaaggaattgacggaagggcaccaccaggagtggagcctgcggcttaatttgactcaacacgg
gaaacctcacccggcccggacacggaaaggattgacagattgatagctctttctcgattctgtgggtggtggtgcatggccgttctta
gttggtggagcgatttgtctggttaattccgataacgaacgagactccggcatgctaactagttatgcggccccgagcggtcggcgtc
caacttcttagagggacaagtggcgttcagccacacgagattgagcaataacaggtctgtgatgcccttagatgtccggggctgcac
gcgcgccacactgagcggatcagcgtgtgtctacccttcgccgagaggcgtgggtaacccgctgaaccccactcgtgatagggattg
gggattgcaattatttcccatgaacgaggaattcccagtaagcgcgggtcataagctcgcgttgattaagtccctgccctttgtacaca
ccgcccgtcgctactacc

>Atlantic salmon *18S* – Forward

TGTGCCGCTAGAGGTGAAATT

>Atlantic salmon *18S* – Reverse

GCAAATGCTTTCGCTTTCG

>Atlantic salmon *IL-10* (EF165028.1)

cccgtcagactgaaggaactccgcacatccttctccaccatcagagattactatgaggctaatgacgagctggagacttccctgttgg
acgaaggcattctacaccacttgaagagcccggtggggtgtcacgctatggacagcatcctgaagttctacctcgacacggtgttgc
ccaccgccatgaacaacagaacgcagaacaaccactttaaatctcccatcgactccatcggaaacatcttccacgagctgaagaaa
gagatcgtcctatgcaggaactacttctcctgtaagaaaccgtttgacatcaacgagttcatctcctcgtataagaagatgcagggta
agggc

>Atlantic salmon *IL-10* – Forward

GAACGCAGAACAACCACTTTAAATCT

>Atlantic salmon *IL-10* – Reverse

GATCTCTTTCTTCAGCTCGTGGAA

Appendix V

Statistics

Table A1: Results from t-test analyses on specific growth rate (SGR) in freshwater (FW) and seawater (SW).

	FW1	FW2	SW1-1	SW2-2	SW3-2	SW2-3	SW (all groups)
FW2	0.7615						
FW3	0.9773	0.7610					
SW2-2			0.7919				
SW3-2			0.8373	0.9322			
SW2-3			0.4900	0.7521	0.6172		
SW3-3			0.8043	0.5978	0.6100	0.2174	
FW (all groups)							**0.0004**

*n=18 per feed group

** FW1 = freshwater feed 1. FW2 = freshwater feed 2. FW3 = freshwater feed 3.

*** SW1-1 = seawater feed 1, originating from freshwater feed (FW) 1. SW2-2 = seawater feed 2, originating from FW2. SW3-2 = seawater feed 3, originating from FW2. SW2-3 = seawater feed 2, originating from FW3. SW3-3 = seawater feed 3, originating from FW3.

Table A2: Results from one way ANOVA analyses on weight of sampled fish from the freshwater (FW) and seawater (SW) period.

	Internal	Seawater period
Freshwater period	0.841	0.00001
Seawater period	0.672	

Table A3: Result from ANOVA analyses on relative expression of *IL-1β*, *IL-10* and *TNF-α*.

	Between all groups
IL-1β	0.106
IL-10	0.165
TNF-α	0.427

Table A4: Results of Chi-square analyses of freshwater (FW) dietary groups histology score.

	Day 0			FW1			FW2		
All criteria combined	*Sum of squares*		*Critical value*	*Sum of squares*		*Critical value*	*Sum of squares*		*Critical value*
FW1	5.249	<	9.488						
FW2	7.046	<	9.488	12.126	<	15.507			
FW3	8.436	<	9.488				1.253	<	7.815

* FW1 = freshwater feed 1. FW2 = freshwater feed 2. FW3 = freshwater feed 3.

Table A5: Results of Chi-square analyses of seawater (SW) dietary groups histology score.

	SW1-1			SW2-2			SW3-2			SW2-3		
All criteria combined	*Sum of squares*		*Critical value*	*Sum of squares*		*Critical value*	*Sum of squares*		*Critical value*	*Sum of squares*		*Critical value*
SW2-2	**26.774**	**>**	**26.296**									
SW3-2	12.225	<	21.026	3.916	<	7.815						
SW2-3				6.455	<	9.488	2.422	<	9.488			
SW3-3				9.298	<	9.488	4.310	<	9.488	5.899	<	9.488

* SW1-1 = seawater feed 1, originating from freshwater feed (FW) 1. SW2-2 = seawater feed 2, originating from FW2. SW3-2 = seawater feed 3, originating from FW2. SW2-3 = seawater feed 2, originating from FW3. SW3-3 = seawater feed 3, originating from FW3.

Table A6: Results of Chi-square analyses of pyloric caeca (PC) from freshwater (FW) dietary groups histology score of each criteria.

Pyloric caeca. freshwater		Day 0			FW1			FW2		
		Sum of squares		*Critical value*	*Sum of squares*		*Critical value*	*Sum of squares*		*Critical value*
Criteria 1 Vac.	**FW1**	4.354	<	9.488						
	FW2	4.086	<	9.488	3.500	<	15.507			
	FW3	5.507	<	9.488				1.652	<	9.488
Criteria 2 LP	**FW1**	2.758	<	3.481						
	FW2	0.720	<	3.481	1.257	<	5.991			
	FW3	**4.023**	**>**	**3.481**				1.250	<	3.841
Criteria 3 PCN	**FW1**	0.937	<	5.991						
	FW2	3.850	<	7.815	4.996	<	15.507			
	FW3	1.609	<	7.815				5.111	<	7.815
Criteria 4 MFs	**FW1**	**7.109**	**>**	**5.991**						
	FW2	4.455	<	5.991	5.500	<	5.991			
	FW3	2.919	<	5.991	**10.000**	**>**	**5.991**	4.000	<	5.991

* Vac. = vacuoles. LP = lamina propria. PCN = pycnotic cell nuclei. MFs = mucosal folds.

** FW1 = freshwater feed 1. FW2 = freshwater feed 2. FW3 = freshwater feed 3.

Table A7: Results of Chi-square analyses of mid intestine (MI) from freshwater (FW) dietary groups histology score of each criteria.

Mid intestine. freshwater		**Day 0**			**FW1**			**FW2**		
		Sum of squares		*Critical value*	*Sum of squares*		*Critical value*	*Sum of squares*		*Critical value*
Criteria 1 Vac.	**FW1**	1.257	<	3.841						
	FW2	3.850	<	5.991	**9.692**	**>**	**3.841**			
	FW3	2.895	<	5.991	4.308	<	3.841	0.253	<	3.481
Criteria 2 LP	**FW1**	**4.168**	**>**	**3.481**						
	FW2	4.746	<	5.991	2.154	<	5.991			
	FW3	4.746	<	5.991				0.000	<	3.841
Criteria 3 PCN	**FW1**	3.540	<	5.991						
	FW2	**6.926**	**>**	**5.991**	6.235	<	9.488			
	FW3	**15.174**	**>**	**5.991**	**13.098**	**>**	**9.488**	4.222	<	5.991
Criteria 4 MFs	**FW1**	1.650	<	5.991						
	FW2	1.273	<	5.991	1.167	<	5.991			
	FW3	1.061	<	5.991	**11.167**	**>**	**5.991**	2.267	<	5.991

* Vac. = vacuoles. LP = lamina propria. PCN = pycnotic cell nuclei. MFs = mucosal folds.

** FW1 = freshwater feed 1. FW2 = freshwater feed 2. FW3 = freshwater feed 3.

Table A8: Results of Chi-square analyses of distal intestine (DI) from freshwater (FW) dietary groups histology score of each criteria.

Distal intestine. freshwater		**Day 0**			**FW1**			**FW2**		
		Sum of squares		*Critical value*	*Sum of squares*		*Critical value*	*Sum of squares*		*Critical value*
Criteria 1 Vac.	**FW1**	0.687	<	3.841						
	FW2	1.564	<	5.991	4.463	<	9.488			
	FW3	1.385	<	5.991	4.463	<	9.488	2.329	<	5.991
Criteria 2 LP	**FW1**	**10.309**	**>**	**5.991**						
	FW2	**6.942**	**>**	**5.991**	2.179	<	5.991			
	FW3	**9.590**	**>**	**5.991**	2.179	<	5.991	0.667	<	3.841
Criteria 3 PCN	**FW1**	**3.850**	**>**	**3.841**						
	FW2	0.069	<	3.841	3.989	<	5.991			
	FW3	0.028	<	3.841	3.989	<	5.991	0.202	<	3.841
Criteria 4 MFs	**FW1**	**8.215**	**>**	**7.815**						
	FW2	5.587	<	7.815	7.625	<	9.488			
	FW3	3.984	<	7.815	7.625	<	9.488	1.778	<	5.991

* Vac. = vacuoles. LP = lamina propria. PCN = pycnotic cell nuclei. MFs = mucosal folds.

** FW1 = freshwater feed 1. FW2 = freshwater feed 2. FW3 = freshwater feed 3.

Table A9: Results of Chi-square analyses of pyloric caeca (PC) from seawater (SW) dietary groups histology score of each criteria.

Pyloric caeca. seawater		SW1-1			SW2-2			SW3-2			SW2-3		
		Sum of squares		*Critical value*	*Sum of squares*		*Critical value*	*Sum of squares*		*Critical value*	*Sum of squares*		*Critical value*
Criteria 1 Vac.	**SW2-2**	23.159	<	26.296									
	SW3-2				**8.022**	**>**	**7.815**						
	SW2-3				0.686	<	5.991	6.569	<	7.815			
	SW3-3				5.470	<	5.991	1.459	<	5.991	4.691	<	5.991
Criteria 2 LP	**SW2-2**	9.352	<	9.488									
	SW3-2				0.034	<	3.841						
	SW2-3				0.000	<	3.841	0.034	<	3.841			
	SW3-3				2.742	<	3.841	2.103	<	3.841	2.743	<	3.841
Criteria 3 PCN	**SW2-2**	**17.400**	**>**	**15.507**									
	SW3-2	11.000	<	12.592	1.559	<	5.991						
	SW2-3				0.917	<	5.991	1.703	<	5.991			
	SW3-3				1.858	<	5.991	2.390	<	5.991	5.867	<	5.991
Criteria 4 MFs	**SW2-2**	3.429	<	5.991									
	SW3-2	**8.231**	**>**	**7.815**	1.982	<	3.841						
	SW2-3	3.429	<	5.991	2.667	<	3.841	**7.987**	**>**	**3.841**			
	SW3-3	**8.231**	**>**	**7.815**	**4.195**	**>**	**3.841**	**10.145**	**>**	**3.841**	0.202	<	5.991

* Vac. = vacuoles. LP = lamina propria. PCN = pycnotic cell nuclei. MFs = mucosal folds.

** SW1-1 = seawater feed 1, originating from freshwater feed (FW) 1. SW2-2 = seawater feed 2, originating from FW2. SW3-2 = seawater feed 3, originating from FW2. SW2-3 = seawater feed 2, originating from FW3. SW3-3 = seawater feed 3, originating from FW3.

Table A10: Results of Chi-square analyses of mid intestine (MI) from seawater (SW) dietary groups histology score of each criteria.

Mid intestine. seawater		SW1-1			SW2-2			SW3-2			SW2-3		
		Sum of squares		*Critical value*	*Sum of squares*		*Critical value*	*Sum of squares*		*Critical value*	*Sum of squares*		*Critical value*
Criteria 1 Vac.	**SW2-2**	25.630	<	26.296									
	SW3-2				1.650	<	7.815						
	SW2-3				0.527	<	9.488	3.303	<	9.488			
	SW3-3				2.673	<	5.991	3.611	<	7.815	5.965	<	9.488
Criteria 2 LP	**SW2-2**	10.549	<	12.592									
	SW3-2	10.549	<	12.592	1.497	<	3.841						
	SW2-3	**16.754**	**>**	**15.507**	0.290	<	3.841	2.759	<	3.841			
	SW3-3	10.549	<	12.592	1.385	<	5.991	3.085	<	5.991	1.17727	<	5.991
Criteria 3 PCN	**SW2-2**	7.155	<	26.296									
	SW3-2				3.274	<	5.991						
	SW2-3				4.107	<	5.991	0.043	<	3.841			
	SW3-3				4.780	<	5.991	0.202	<	3.841	0.063	<	3.481
Criteria 4 MFs	**SW2-2**	10.780	<	15.507									
	SW3-2				1.944	<	5.991						
	SW2-3				2.695	<	5.991	1.635	<	5.991			
	SW3-3				0.104	<	3.841	1.351	<	5.991	2.702	<	5.991

* Vac. = vacuoles. LP = lamina propria. PCN = pycnotic cell nuclei. MFs = mucosal folds.

** SW1-1 = seawater feed 1, originating from freshwater feed (FW) 1. SW2-2 = seawater feed 2, originating from FW2. SW3-2 = seawater feed 3, originating from FW2. SW2-3 = seawater feed 2, originating from FW3. SW3-3 = seawater feed 3, originating from FW3.

Table A11: Results of Chi-square analyses of distal intestine (DI) from seawater (SW) dietary groups histology score of each criteria.

Distal intestine. seawater		**SW1-1**			**SW2-2**			**SW3-2**			**SW2-3**		
		Sum of squares		*Critical value*	*Sum of squares*		*Critical value*	*Sum of squares*		*Critical value*	*Sum of squares*		*Critical value*
Criteria 1 Vac.	**SW2-2**	14.033	<	26.296									
	SW3-2				3.048	<	5.991						
	SW2-3				2.222	<	5.991	2.807	<	5.991			
	SW3-3				4.952	<	7.815	**8.250**	**>**	**7.815**	3.311	<	7.815
Criteria 2 LP	**SW2-2**	10.500	<	15.507									
	SW3-2				2.667	<	3.841						
	SW2-3				**4.196**	**>**	**3.841**	0.202	<	3.841			
	SW3-3				2.666	<	3.841	0.000	<	3.841	0.202	<	3.841
Criteria 3 PCN	**SW2-2**	6.107	<	12.592									
	SW3-2	6.107	<	12.592	1.510	<	3.841						
	SW2-3	**15.643**	**>**	**15.507**	0.750	<	3.841	**4.196**	**>**	**3.841**			
	SW3-3	6.107	<	12.592	1.510	<	3.841	0.000	<	3.841	**4.196**	**>**	**3.841**
Criteria 4 MFs	**SW2-2**	10.333	<	7.815									
	SW3-2	**76.636**	**>**	**21.026**	4.743	<	7.815						
	SW2-3				7.543	<	7.815	1.193	<	7.815			
	SW3-3				**6.711**	**>**	**5.991**	4.565	<	7.815	2.909	<	5.991

* Vac. = vacuoles. LP = lamina propria. PCN = pycnotic cell nuclei. MFs = mucosal folds.

** SW1-1 = seawater feed 1, originating from freshwater feed (FW) 1. SW2-2 = seawater feed 2, originating from FW2. SW3-2 = seawater feed 3, originating from FW2. SW2-3 = seawater feed 2, originating from FW3. SW3-3 = seawater feed 3, originating from FW3.

Appendix VI

Score results

Table A12: Combined number of scores within each criteria of pyloric caeca, mid and distal intestine.

Gut segment	Sampling time	Criteria	All criteria					
		Score	1	2	3	4	5	
Pyloric caeca, mid intestine, distal intestine	Day 0	FW1	50	42	20	6	2	a
	Day 42	FW1	61	59	15	5	0	a
		FW2	47	70	20	7	0	a
		FW3	51	72	17	4	0	a
	Day 56	SW1	41	66	30	4	3	a
		SW2-2	45	81	15	3	0	b
		SW3-2	41	67	25	5	2	ab
		SW2-3	42	63	24	3	0	ab
		SW3-3	47	62	26	0	1	ab

* FW1 = freshwater feed 1. FW2 = freshwater feed 2. FW3 = freshwater feed 3. ** SW1-1 = seawater feed 1, originating from FW1. SW2-2 = seawater feed 2, originating from FW2. SW3-2 = seawater feed 3, originating from FW2. SW2-3 = seawater feed 2, originating from FW3. SW3-3 = seawater feed 3, originating from FW3. *** Day 0 = sampling from trial start. Day 42 = post 42 days in freshwater. Day 56 = Trial day no. 56, post 14 days in seawater. Different letters indicate significant difference, p<0.05.

Table A13: Score results of pyloric caeca, mid and distal intestine.

Gut segment	Sampling time	Criteria	Vacuoles						Lamina propria						Pycnotic cell nuclei						Mucosal folds					
		Score	1	2	3	4	5		1	2	3	4	5		1	2	3	4	5		1	2	3	4	5	
Pylorus caeca	Day 0	FW1	1	0	3	5	1	a	9	1	0	0	0	a	2	5	3	0	0	a	7	2	1	0	0	ac
	Day 42	FW1	0	2	5	5	0	a	7	5	0	0	0	ab	2	4	6	0	0	a	2	9	1	0	0	b
		FW2	0	2	4	6	0	a	6	2	0	0	0	ab	0	5	6	1	0	a	3	6	3	0	0	abc
		FW3	1	4	4	3	0	a	6	6	0	0	0	b	4	4	3	1	0	a	6	6	0	0	0	c
	Day 56	SW1	1	4	4	2	1	ab	4	8	0	0	0	a	5	5	2	0	0	a	5	7	0	0	0	a
		SW2-2	0	8	2	2	0	a	7	5	0	0	0	a	9	1	2	0	0	b	4	8	0	0	0	ab
		SW3-2	1	3	7	0	0	b	6	5	0	0	0	a	6	3	2	0	0	ab	1	10	0	0	0	b
		SW2-3	0	6	3	3	0	ab	7	5	0	0	0	a	7	1	4	0	0	ab	8	4	0	0	0	ac
		SW3-3	0	5	7	0	0	ab	3	9	0	0	0	a	8	4	0	0	0	ab	9	3	0	0	0	c
Mid intestine	Day 0	FW1	9	0	0	0	1	ab	0	7	3	0	0	a	8	2	0	0	0	a	4	5	1	0	0	ab
	Day 42	FW1	12	0	0	0	0	a	0	12	0	0	0	b	5	6	1	0	0	ab	7	5	0	0	0	a
		FW2	9	3	0	0	0	b	1	11	0	0	0	ab	3	7	2	0	0	bc	5	7	0	0	0	ab
		FW3	10	2	0	0	0	ab	1	11	0	0	0	ab	0	11	1	0	0	c	3	6	3	0	0	b
	Day 56	SW1	7	4	0	0	1	a	0	6	6	0	0	a	1	7	4	0	0	a	2	6	4	0	0	a
		SW2-2	7	3	2	0	0	a	0	10	2	0	0	ab	0	9	2	1	0	a	4	8	0	0	0	a
		SW3-2	4	3	2	1	0	a	0	6	4	0	0	ab	0	5	5	0	0	a	4	4	1	0	0	a
		SW2-3	3	3	3	0	2	a	0	10	1	0	0	b	0	5	6	0	0	a	2	7	2	0	0	a
		SW3-3	5	5	0	0	0	a	1	8	1	0	0	ab	0	4	6	0	0	a	4	6	0	0	0	a
Distal intestine	Day 0	FW1	2	8	0	0	0	a	0	8	2	0	0	a	7	3	0	0	0	a	1	1	7	1	0	a
	Day 42	FW1	4	7	0	0	0	a	7	4	0	0	0	b	11	0	0	0	0	b	4	5	2	0	0	b
		FW2	4	7	1	0	0	a	5	7	0	0	0	b	9	3	0	0	0	ab	2	6	4	0	0	ab
		FW3	1	10	1	0	0	a	7	5	0	0	0	b	8	4	0	0	0	ab	4	3	5	0	0	ab
	Day 56	SW1	0	9	2	0	1	ab	6	5	1	0	0	ab	7	4	1	0	0	a	3	1	6	2	0	a
		SW2-2	0	10	2	0	0	ab	4	8	0	0	0	a	5	7	0	0	0	ba	5	4	3	0	0	ac
		SW3-2	1	11	0	0	0	a	8	4	0	0	0	ab	8	4	0	0	0	a	1	5	3	2	0	b
		SW2-3	2	8	2	0	0	ab	9	3	0	0	0	b	3	9	0	0	0	b	0	6	4	2	0	bc
		SW3-3	1	5	5	0	1	b	8	4	0	0	0	ab	8	4	0	0	0	a	0	5	7	0	0	bc

* FW1 = freshwater feed 1. FW2 = freshwater feed 2. FW3 = freshwater feed 3. ** SW1-1 = seawater feed 1, originating from FW1. SW2-2 = seawater feed 2, originating from FW2. SW3-2 = seawater feed 3, originating from FW2. SW2-3 = seawater feed 2, originating from FW3. SW3-3 = seawater feed 3, originating from FW3. *** Day 0 = sampling from trial start. Day 42 = post 42 days in freshwater. Day 56 = Trial day no. 56, post 14 days in seawater. Different letters indicate significant difference, p<0.05.