Can fresh water diets influence recovery in Atlantic salmon post-smolts after SAV3 challenge in sea water?

Master's degree in Aqua medicine

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Abstract

Pancreas disease (PD), caused by the salmonid alphavirus (SAV), is a significant challenge in Norwegian aquaculture. Examining how differences in nutritional status prior to sea water (SW) transfer could affect disease progression and recovery is interesting both from a fish welfare and economic perspective.

The objective of this Master's project was to determine whether optimization of feed during the fresh water (FW) phase could increase robustness and or recovery of Atlantic salmon challenged with SAV3 after seawater SW transfer. Three groups of Atlantic salmon (*Salmo salar*) were fed diets with different levels of fatty acid (FA), amino acid (AA) and a commercially relevant experimental (SR) diet for a period of 10 weeks prior to SW transfer. Two weeks after SW transfer the fish were exposed to SAV 3. Growth, disease progression and histological changes were monitored during the trial period of 56 days post exposure (dpe) at 5 sampling points (7, 14, 21, 35 and 56 dpe) to ascertain any differences in disease progression between the groups. At the final sampling point, gene transcription of muscle tissue was examined to investigate potential differences in the expression of genes coding for selected antiviral proteins and inflammatory markers between diets.

The results suggest that the diet with a relatively higher content of selected amino acids had a favourable effect on growth, viral load and muscle pathology. A second implication is that the FA-diet appears to have led to undesirable development in growth. The test did not uncover any significant differences between the different diets with regards to transcription of inflammatory and antiviral genes in muscle samples.

The results indicate that although gene transcription in muscle did not vary significantly at the tested time point the overall results show that diet likely influenced growth and pathology. Further studies could for example investigate whether the effect was due to a specific amino acid in the fish diet.

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Sammendrag

Pankreas sykdom (PD) forårsaket av salmonid alfavirus (SAV) er en betydelig utfordring i norsk oppdrettsnæring. Å undersøke om forskjeller i fôrsammensetning før overføring til sjøvann kan påvirke sykdommens progresjon og utvikling er interessant både fra et fiskevelferds- og økonomisk perspektiv.

Målet med dette masterprosjektet var å undersøke om optimalisering av fôr i ferskvannsfasen kunne øke robustheten og eller motstandsdyktighet hos atlantisk laks som ble smittet med SAV3 etter overføring til sjøvann. De tre gruppene av atlantisk laks (Salmo salar) ble fôret dietter med ulike nivåer av fettsyrer (FA) og aminosyrer, i tillegg til et kommersielt relevant fôr i en periode på 10 uker før overføring til sjøvann. To uker etter overføring til sjøvann ble fisken badsmittet med salmonid alfavirus subtype 3. Vekst, infeksjonsstatus og histologiske forandringer ble overvåket i prøveperioden på 56 dager etter eksponering (dpe) ved 5 prøvetakingspunkter (7, 14, 21, 35 og 56 dpe) for å konstatere eventuelle forskjeller i immunrespons mellom gruppene. Ved det endelige prøvetakingspunktet ble gentranskripsjon av muskelvev også undersøkt for å kartlegge potensielle forskjeller mellom gruppene.

Resultatene antyder at kostholdet med et høyere innhold av aminosyrer (AA) hadde en gunstig effekt på vekst, mengde virus i hjertet og muskelpatologi. En annen implikasjon er at FA-dietten ser ut til å ha ført til uønsket utvikling i vekst. Testen avdekket ingen signifikante forskjeller mellom de forskjellige diettene med hensyn til transkripsjon av inflammatoriske og antivirale genuttrykk i muskelprøver.

Resultatene indikerer at selv om gentranskripsjon i muskel ikke varierte betydelig på det testede tidspunktet, viser de samlede resultatene at kosthold sannsynligvis påvirket vekst og patologi. Ytterligere studier kan for eksempel undersøke om effekten skyldtes en spesifikk aminosyre i dietten.

Denne masteroppgaven var en del av det FHF-finansierte prosjektet OptiNutr (nr. 901431), arbeidspakke nummer fire.

Abbreviations

AA	Amino Acid
ANOVA	Analysis of variance
ARA	Arachidonic acid
BC	Bath-challenged
CAA	crystalline amino acids
cDNA	Complementary DNA
CHH-1	Chum salmon heart cells 1
CMS	Cardiomyopathy syndrome
CT	Cycle threshold
CTL	Cytotoxic T Lymphocytes
DAMPs	Damage-associated molecular patterns
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DPE	Days past exposure
DPI	Days past injection
EPA	Eicosapentaenoic acid
FA	Fatty acid
FAO	Food and Agriculture Organization
FBS	Fetal Bovine Serum
FW	Fresh water
HSI	Hepatosomatic index
HSMI	Heart- and skeletal inflammation
HUFA	Highly unsaturated fatty acids
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Intramuscular
IP	Intraperitoneal
IPN	Infectious pancreatic necrosis
ISA	Infectious salmon anaemia
LC	Long-chain
MHC	Major histocompatibility complex
MNE	Mean normalised expression
mRNA	Messenger ribonucleic acid
MUFA	monounsaturated fatty acids
n-3	omega-3
n-6	omega-6
NE	Non-exposed
NE	Normalised expression
NFSA	Norwegian Food Safety Authority
NK-cells	Natural killer cells
NOS2	nitric oxide synthase 2
nsP	non-structural polyprotein
NVI	Norwegian Veterinary Institute
OIE	World Organization for Animal Health
PAMPs	Pathogen-associated molecular patterns
PD	Pancreas disease
PL	Phospholipid
PRR	Pattern recognition receptors
PRV	Piscine reovirus
PUFA	Polyunsaturated fatty acids
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription PCR
SAV	Salmonid Alphavirus
SD	Standard deviation
SFA	saturated fatty acid
SGR	Specific Growth Rate
SSB	Norwegian national statistics institute
SW	Sea water
TCID50	Median Tissue Culture Infectious Dose
TNF	Tumour-Necrosis Factors
VSI	Visceral somatic index

Contents

1. Introduction

1.1 Introduction

Atlantic salmon (*Salmo salar*) post-smolts are typically reared in open sea cages. They are hence exposed to a number of environmental factors including but not limited to viruses, bacteria, algae, fungi and parasites. Diseases and infections in the fish can have significant impacts on their welfare and mortality rates. This in turn can have important financial and reputational consequences for the Norwegian aquaculture industry (Aunsmo et al., 2010, Gudding, 2014). According to a questionnaire the NVI (Norwegian Veterinary Institute) conducts with fish health personnel and employees from the Norwegian Food Safety Authority (NFSA) pancreas disease (PD) is considered one of the most important viral diseases in Norwegian aquaculture. The only other viral disease that is considered more severe than PD is CMS (Cardiomyopathy syndrome) (Sommerset et al., 2020).

The Norwegian aquaculture industry is significant in economic terms. According to the Norwegian national statistics institute (SSB), 1.282.003 tons of Atlantic salmon and 68.345 tons of rainbow trout (*Oncorhynchus mykiss*) were produced in 2018, updated 24.10.2019 (SSB, 2019). This amounts to a total value of approximately 64.5 billion NOK. It is difficult to fully assess the negative impacts of PD infection for the industry. This is partially due to the nature of the clinical signs and the low mortality rates, which makes it difficult to record every occurrence of the infection. Several studies attempt to estimate the economic losses associated with PD (Aunsmo et al., 2010, Pettersen et al., 2015, Pettersen et al., 2016). In Aunsmo et al. (2010) the costs was estimated to be 6.0 NOK/kg. These estimations are sensitive to pricing of feed, fish pricing and services at the time of estimation.

Considering that fish are now reared longer on land to produce larger and more robust smolts, perhaps optimizing the diet at this life-stage could help in strengthening fish prior to SW transfer. This could in turn reduce economic losses due PD and other pathogens facing Atlantic salmon in the field. To potentially reduce economic losses and increase fish welfare through nutritional immunomodulation has been shown to be promising. Several published reviews highlight the potential advantages of nutritional immunomodulation (see for example Waagbø (2006), Trichet (2010) and Kiron (2012)). In addition, many studies discuss the effects of single macro- and micronutrients (Espe et al., 2010, Lopez-Jimena et al., 2015, Espe and Holen, 2013).

This paper will briefly review the background research and current knowledge about the Salmonid alphavirus (SAV) causing PD. This will be followed by a brief overview of fish immunology and a more in-depth discussion on how modification of feed components can affect a fish's response to viral infection. General feed composition will be introduced, in addition to a thorough review of how fatty acids and amino acids in fish nutrition can influence disease susceptibility, progression and recovery.

1.2 Aims of the study

The overall aim of this thesis was to examine whether feeds of different nutritional composition, given in the FW stage could affect susceptibility and or disease progression when challenging post-smolts with SAV3.

This included:

- Examine whether susceptibility to SAV can be influenced by the present diets
- Observe effects of SAV on weight, condition factor, SGR and somatic indexes, to investigate potential differences between diets
- Examine viral load in heart and muscle tissue
- Examine potential differences in pathological changes, and to investigate if there are differences in pathology between diets

2.0 Background

2.1 Pancreas disease in Norwegian aquaculture

PD was first observed in Scotland in 1976 (Munro et al., 1984), and first reported in Norway in 1989 in farmed Atlantic salmon (Poppe et al., 1989). Both Atlantic salmon and rainbow trout can be infected by SAV during the SW phase (Munro et al., 1984). PD is caused by SAV, of which there are currently six known subtypes. In Norway subtypes SAV2 and SAV3 have been detected. According to the Norwegian Veterinary Institute's Fish health report chapter on PD, there were 176 new cases of PD in 2018, which is a significant increase from the previous year of 138 cases (Sommerset et al., 2020). This development can be partially explained by increased screening due to new regulations from the NFSA. In 2019, 152 outbreaks were recorded. Figure

1 illustrates that the reported cases of PD have increased steadily (Sindre and Jensen, 2020, Sommerset et al., 2020).

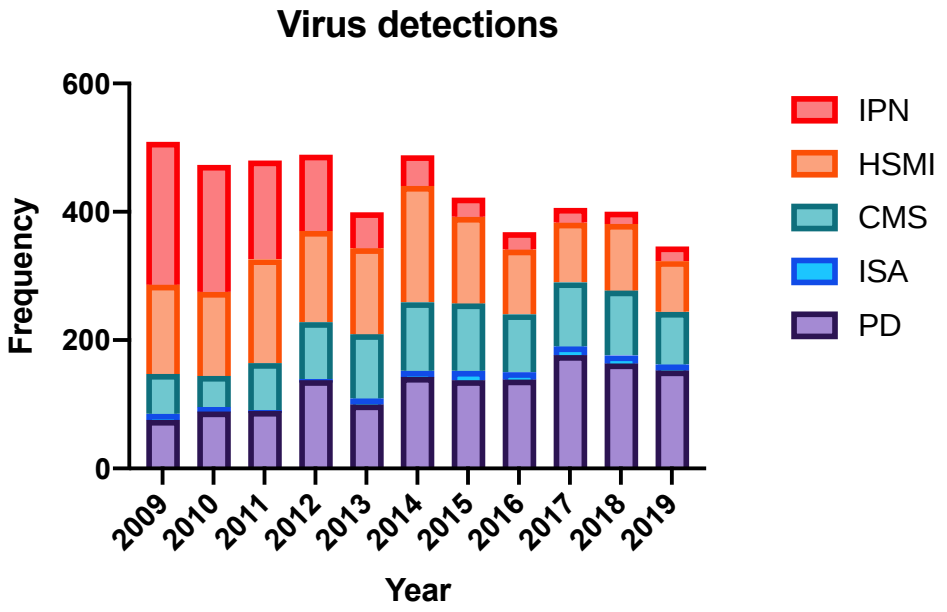


Figure 1 - Reported incidences of viral diseases in salmonids 2009-2019 shown in percent. The values for diseases that are not mandatory to report are based on samples from the Veterinary Institute. Data from The Fish Health report 2019 (Sommerset et al., 2020).

In Norway, PD is a List 3 disease, meaning that outbreaks are legally required to be reported to the NFSA and the World Organization for Animal Health (OIE) as of May 2014. PD must be confirmed by qPCR and supported by histopathogical observations.

There have been monovalent and multivalent vaccines available on the market that have had some effects in reducing the severity of outbreaks and mortality (Jensen et al., 2012). A more efficient vaccine could possibly prevent and or reduce PD-outbreaks. Recently (in 2017), Elanco received marketing permission for the first DNA vaccine "Clynav" available for animals in Europe. The vaccine targets SAV subtype 3, which is considered endemic in Vestland county. This is an on-going process and field experience is being acquired.

The NFSA have created zones in order to combat the spread of the virus along the Norwegian coastline. The zones were the counties Rogaland, Vestland, in addition to parts of Møre and

Romsdal. An overview of outbreaks in 2019 is found in **Error! Reference source not found.**. There are also precautionary routines with regards to hygiene measures and regular fallowing of fish-farm locations (Nelson et al., 1995). As of July 1st 2020, the NFSA will require that all Atlantic salmon and rainbow trout from Taskneset (Fræna) in the south to Langøya by Kvaøya (Sømna) in the north must be vaccinated against PD (Sommerset et al., 2020).

2.1.1 Clinical signs, pathology and growth/welfare indicators

As the name may suggest PD primarily affects the pancreas through the loss of exocrine pancreas, however, severe changes in both heart and skeletal muscle are frequently observed (McLoughlin et al., 2002, Poppe et al., 1989). Clinical signs of PD are lethargic fish; swimming closely to the surface and often observed accumulating along the edges of the sea cage (McLoughlin and Graham, 2007). During a PD outbreak a common observation is that the fish stop eating. This is likely due an inability to digest the pellets (Pringle et al., 1992). This results in a reduced weight gain, growth rate, condition factor and changes in organ somatic indexes (Larsson et al., 2012, Lerfall et al., 2012, Aunsmo et al., 2010). Condition factor is the relationships between bodyweight and length, and is a commonly accepted tool to assess a fish's nutritional status (Bolger and Connolly, 1989). It has been documented that there is a correlation between higher lipid content and condition factor, therefore higher condition factor indicates a "rounder" fish (Noble et al., 2018). As illustrated in **Error! Reference source not found.** the healthy individual is rounder compared to the anorexic SAV-affected individual below. Condition factor can therefore be a useful tool in supporting a PD-diagnosis, although loss of appetite can be a sign of many other afflictions.

Organ somatic indexes such as the hepatosomatic index (HSI) and visceral somatic index (VSI) are lethal because organs have to be removed in order to record the body weight in proportion to liver or viscera. These indexes can be used to nuance the nutritional status of the fish (Noble et al., 2018).

Figure 2 - The fish above is a healthy individual and below a typical "runt" as a result and PD (pancreas disease) is shown. The fish below is clearly a thinner individual. / photo: Trygve Poppe (Pharmaq, previously Veterinary Institute and NMBU)

There have also been reports of PD leading to increased yellow-white faecal casts and darkening of the skin (McLoughlin et al., 2002). In fish suffering from PD there might be petechial bleeding in the fat tissue surrounding the pyloric caeca. Additionally, ascetic fluids in the visceral cavity, pale/yellow liver and or haemorrhages on the viscera can be observed (McLoughlin and Graham, 2007). The histological changes that can be observed include necrosis or loss of exocrine pancreatic tissue, inflammation and necrosis in of the epicardium, necrosis in somatic muscle tissue and oesophageal muscle lesions (Munro et al., 1984, McLoughlin et al., 2002). Changes in heart and acinar pancreas tissue are generally found approximately 2 weeks after infection and precede those in muscle tissue (Braceland et al., 2013). Although most fish survive the infection, not all fish recover completely. The fish that fail to recover are known as runts (McVicar, 1987).

2.1.2 Salmonid Alphavirus Characteristics and replication

The SAV virions are approximately 55-65 nm in diameter and consist of single stranded positive RNA packaged into an icosahedron surrounded by a lipid membrane created from the host cell (Villoing et al., 2000, Nelson et al., 1995). The membrane is perforated by two virus-encoded glycoproteins; E1 and E2 as illustrated in figure 2. SAV belongs to the family Togaviridae, genus Alphavirus.

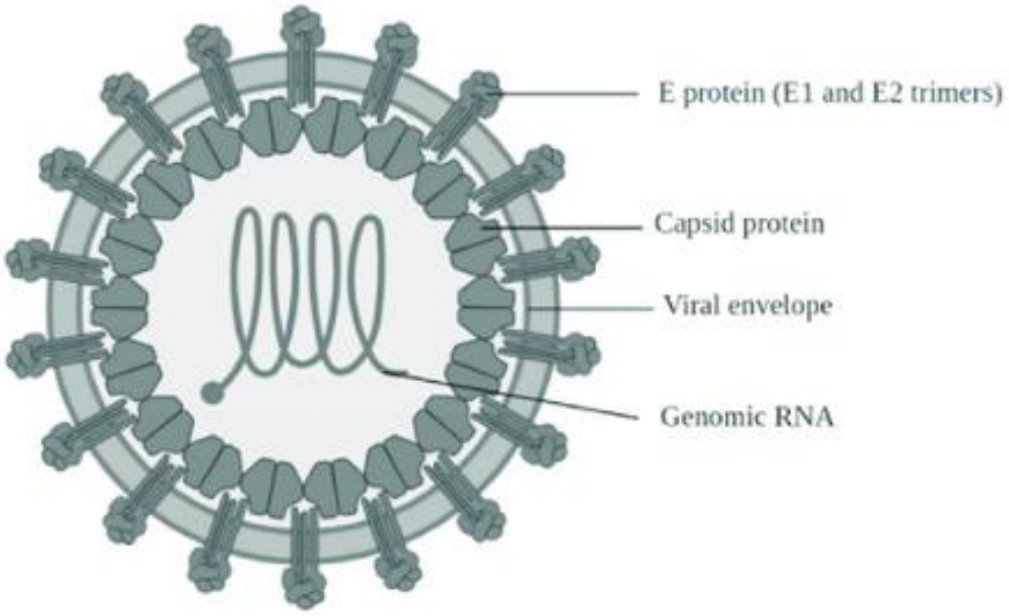


Figure 3 – Alphavirus structure showing E-proteins, capsid protein and genomic RNA positioning. Illustration taken from Mandary et al. (2019)

Pathway of entry, replication and budding are similar to that of other known alphaviruses (Karlsen et al., 2010). Detailed description of alphavirus replication is discussed in Andersen (2012). Briefly summarized, the virus enters the host through receptor-mediated endocytosis by attaching to an unknown receptor mediated by the E2 protein. Within the endosome pH drops, causing glycoproteins E1 and E2 to dissociate, this exposes a fusion peptide on E1. The fusion peptide mixes with the endosomal membrane and creates a fusion pore through which the nucleocapsid enters the cytoplasm. Replication occurs in the cytoplasm as shown in step 1 – 3 in the figure below.

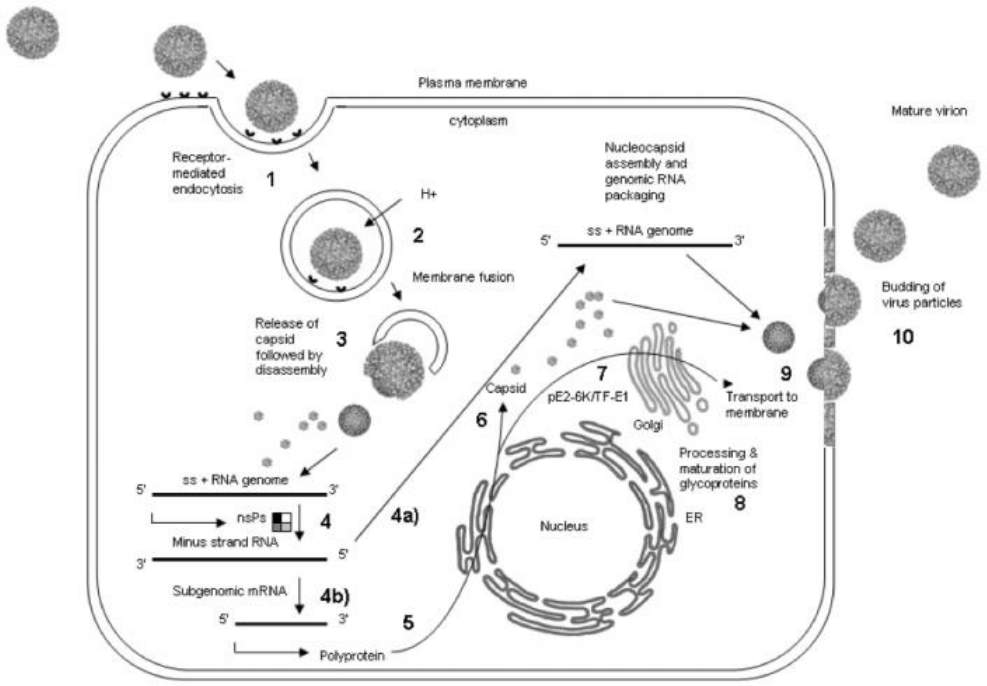


Figure 4 - Schematic overview of terrestrial alphavirus replication. Figure from Andersen (2012)

Inside the cytoplasm the nucleocapsid dissociates and releases the single stranded genome which functions as mRNA (messenger RNA) and as a template for synthesis of the complementary negative strand. Ribosomes then translate the non-structural polyprotein (nsP) P1234. This protein is cleaved and becomes the negative strand replicase complex P123 + nsP4 due to the virus-encoded proteinase located within nsP2 (Hodneland et al., 2005).

Figure 5 - Genomic organization of SAV showing the two ORFs encoding the non-structural and structural genes from (McLoughlin and Graham 2007)

P123 accumulates in the cytoplasm and eventually becomes unstable resulting in it being cleaved into nsP1, nsP2 and nsP3. nsP1-4 form a new replicase complex that synthesizes positive strand sub-genomic mRNA and new genomic RNA using the negative strand as a template. The new full-length positive sense RNA genomes are made and packaged into new virus particles. These particles are in turn released from the host cell by budding through the plasma membrane. During the budding process they acquire a lipid envelope from the host cell

containing the E1 and E2 glycoproteins (Andersen, 2012, Fringuelli et al., 2008, Hodneland et al., 2005).

2.1.3 Transmission

SAV is one of two known marine Alphaviruses (Andersen, 2012). An average outbreak has been reported to last 2.8 months and is typically observed during the summer months although outbreaks can occur all year round. After mandatory screening outbreaks seem to occur all year round. It is believed that the main route of transmission is horizontally between neighbouring farms (Aldrin et al., 2010, McLoughlin et al., 1996). Other studies have also emphasized horizontal transmission as the main route of infection (Bratland and Nylund, 2009, McLoughlin and Graham, 2007, McVicar, 1987, Fringuelli et al., 2008). However, vertical transmission has also been suggested as the virus has been found in eggs from broodfish companies (Bratland and Nylund, 2009). A study examining pathways of infection in the field suggested that distance between neighbouring farms was significant as a route of transmission (80%) while unspecified routes of infection were not likely to be as significant (5%) (Aldrin et al., 2010). Survival studies of SAV in SW confirms that horizontal transmission is possible (Graham et al., 2007). Infected fish can remain viraemic after the clinical signs have subsided, and in chronic cases, SAV RNA can be detected in the host as long as 6 months after infection making them one of the main reservoirs for SAV (Andersen et al., 2007, Graham et al., 2012, Stene et al., 2016). It has been shown that SAV can be cultivated from infected fish 4-6 months after the first confirmed case. In addition, SAV has been found in fish ready for slaughter that had the first diagnosis early in the production cycle (Jansen et al., 2010a, Jansen et al., 2010b).

Infected fish release virus particles through mucus and excrements in the water (Jarungsriapisit et al., 2016b, Graham et al., 2012, Hellebø et al., 2014) . Other fish then absorb these virions through their gills and/or oesophagus (OIE, 2018). Shedding from infected hosts is detected 4-13 days after infection and incubation period is estimated to be 7-10 days after exposure (Andersen et al., 2007, McLoughlin and Graham, 2007). Experimental studies have shown that SAV is easily transmitted when distance between Atlantic salmon is short such as within a tank or sea cage environment (Graham et al., 2007, Jansen et al., 2010a, Jarungsriapisit et al., 2016b). A bath challenge model for SAV3 in SW was established to investigate the natural route of infection for Atlantic salmon post-smolts Jarungsriapisit et al. (2016b).

2.2 Fish immunology

2.2.1 Overview of fish immunology

As in higher vertebrates, the immune system within fish can be divided into two parts: the innate and the acquired immune system. In later years, these systems have been proven to function together in combination (Magnadóttir, 2006). The innate immune system can be divided into three parts: the physical barriers, humoral and cellular part. The physical barriers are considered to be part of the humoral and cellular defence.

The innate immune system uses germ line pattern-recognizing proteins/receptors known as PRPs/PRRs to differentiate between foreign and its own cells. These recognize pathogen-associated molecular patterns (PAMPs) such as glycoproteins, lipopolysaccharides and intracellular components that are released by damage or infection (Magnadóttir, 2006). In addition, fish are also able to discover damage in its own tissues through damage-associated molecular patterns (DAMPs) that active downstream inflammatory response (Zou and Secombes, 2016).

The humoral response is the production of antibodies, complement proteins and antimicrobial peptides (Magnadóttir, 2006). The complement system consists of 35 soluble membrane-bound proteins and cell receptors that has a key role in the hosts' defence both in the innate and acquired immune systems. The proteins in the complement system are numbered C1-C9, some of which are referred to with a lower-case letter (i.e. C3a and C3b) which refers to the fragment size (a; smaller fragment, b; larger fragment). These fragments combine to form functioning complexes through 3 pathways (Boshra et al., 2006).

The cellular response consist of specialized cells i.e. monocytes/macrophages, granulocytes and cytotoxic cells (Zhu et al., 2013). Cytokines are signal molecules derived from macrophages, lymphocytes, granulocytes, dendritic cells, etc., and can be categorized as interferons (IFN), interleukins (ILS), tumour-necrotic factors (TNF), colony-stimulating factors and chemokines (Magnadóttir, 2006).

Interferons are proteins and glycoproteins produced in virus-infected cells as a response to infection. Interferons can be divided into three types type I (IFNa, IFNb, IFNk, IFNε, IFNk, IFNu), II (IFNγ) and III (IFNλ1, IFNλ2, IFNλ3) in mammals. In teleost's, type I and II have been found so far. IFNγ is produced by $CD4^+$T helper 1 (Th1) and $CD8^+$ cytotoxic T lymphocytes (CTL) in response to MHC-presented antigens (Robertsen, 2006, Robertsen,

2018). IFNγ is critical to the adaptive cell mediated immunity, but can be activated as a product of both the innate and adaptive. IL-12 and IL-18 activate NK-cells that again activate IFNγ through the innate, while the adaptive is described above. IFNγ binds its' receptor and signals through a JAK-STAT pathway. This allows for transcription and upregulation of MHC class I molecules and antiviral proteins (PKR, OAS). Additionally, IFNγ activates macrophages through NOS2 (nitric oxide synthase 2) production and other MHC class II molecules (Robertsen, 2006).

Type I IFN and partly IFNγ can induce the expression of several antiviral proteins such as Mx and Viperin (Sun et al., 2011, McBeath et al., 2007, Moore et al., 2017). Mx proteins can trap nucleocapsids involved in virus replication and degrade virus RNA, inhibits translation and inhibit exit of viruses (Haller and Kochs, 2002, Robertsen, 2018). Viperin has been shown to inhibit budding of influenza A virus (Wang et al., 2007) therefore interrupting virus replication and the spread of infection to other cells or tissues within a host.

There are two types if Interleukin-I (IL-1); IL-1α and IL-1β. These are prototypic pro-inflammatory cytokines and is often observed connected to another pro-inflammatory cytokine TNF (Dinarello, 1997). IL-1β is a conserved gene (Secombes et al., 2011). IL-1β indicate an acute response and stimulate production of adhesion molecules allowing neutrophils to migrate toward target tissue. It functions as a chemoattractant for leucocytes in fish. In higher vertebrates, it is theorized that muscle atrophy can be mediated by proinflammatory cytokines such as IL-1β and TNFα (Pooley et al., 2013) citing others. In (Pooley et al., 2013) they stimulated primary muscle cell cultures from Atlantic salmon with IL-1β for 24 hours to induce an acute immune response. Conclusions were that IL-1β modulates the expression of immune related genes and genes involved in growth and metabolism looking at the potential negative effects of inflammation on muscle protein and growth. TNFα-receptors induce genes normally involved in protein degradation and has some overlapping functions with IL-1β (Zou and Secombes, 2016). TNFα is associated with tissue damage, and has in humans been found in connection to rheumatoid arthritis and inflammatory bowel disease (Calder, 2006).

An overly active inflammatory response can potentially do more harm than good. IL-10 helps by inhibiting inflammation in fish, and is associated with the specific immune response in a later phase of inflammation (Zou and Secombes, 2016). IL-10 has been found to be induced in parallel with IFNγ (Xu et al., 2012).

Because the fishes' immune system has been shown to be influenced by diet the next section will give a brief summary on the composition of fish feed for the past three decades and later explore how modifying the composition of fatty acids and content of amino acids may modulate the immune response.

2.3 Fish nutrition

2.3.1 Commercial diets in Norwegian Salmonid production

With the rapid growth of the aquaculture industry the need for alternative protein and fat sources to fishmeal and fish oil has increased. Simultaneously, the global supply has reached a plateau resulting in this protein source becoming more expensive (Nunes et al., 2014, Mai et al., 2006). Due to the existing strain on the worlds' fish resources (FAO, 2018), the aquaculture industry must look elsewhere for their protein supply as the production has increased. According to the FAO (2018) aquaculture's contribution to the amount of fish available for human consumption overtook that of wild-caught (fisheries) in 2013 and has since increased. In 2016 it accounted for 53%, compared to 41% in 2006 (FAO, 2018).

In light of this the traditional feed composition has changed dramatically over time. In the 1990s (Figure 6), 90% of ingredients used in Norwegian salmon feed had marine origin. This figure stood at 25% in 2016 (Aas et al., 2019). In the past two decades, plant-oils and plant proteins have been have been introduced (Figure 7). With these developments new risks and opportunities have arisen with regards to the impact of several macro- and micronutrients. For example, plant-oils do not have the same fatty acid content as fish-oil. This sparked research into exactly how the fatty acid ratios and composition affect fish health. This will be explored further in the next section.

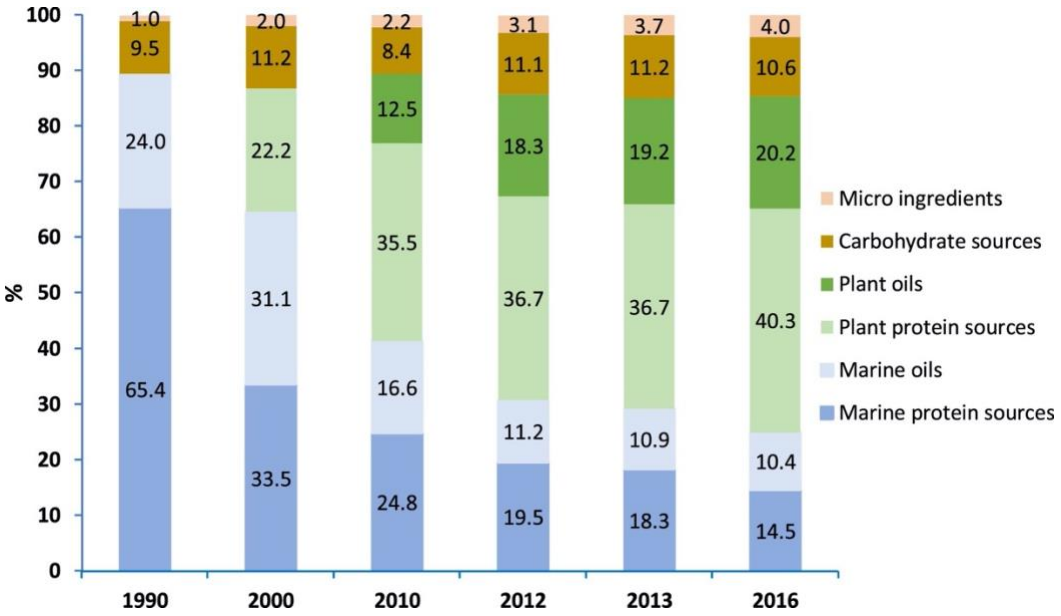


Figure 6 - Ingredient sources for fish feed from 1990 - 2016 showing an increased use of micro ingredients and plant proteins graph from Aas et al. (2019).

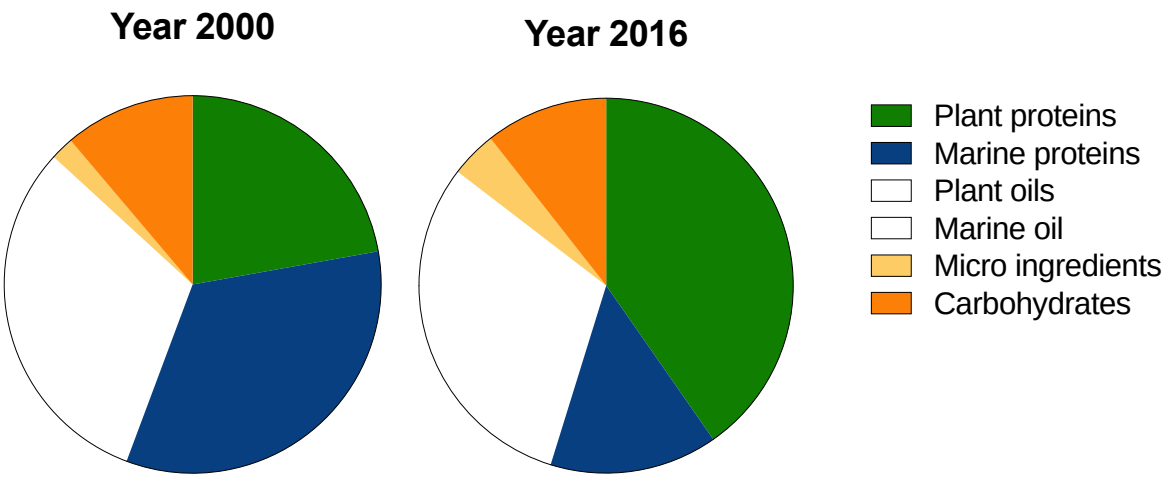


Figure 7 - Diagram showing the composition of typical salmonid diet in year 2000 as opposed to 2016. The blue parts (marine resources) of a whole are largely replaced by green plant-based sources in the right-hand diagram. Numbers are from (Aas et al., 2019)

2.3.2 Fatty acids in fish nutrition

Fatty acids (FA) are hydrocarbon chains with a varying number of carbon atoms. If the carbon chain is saturated with hydrogen molecules is known as a saturated fatty acid (SFA). When there is one less hydrogen molecule a double bond between carbon atoms is formed and it is now an unsaturated FA. FAs generally consist of a hydrocarbon chain with a carboxyl- and methyl group. FAs nomenclature is determined by the number of carbon atoms in the carbon

chain, number of double bonds and the position of the first double bond has from the methyl end of the carbon chain. For example, Eicosapentaenoic acid (EPA) an omega-3 FA has 20 carbon atoms, 5 double bonds where the first double bond is in the 3 position from the methyl side thus 20:5n-3 as shown in figure 6.

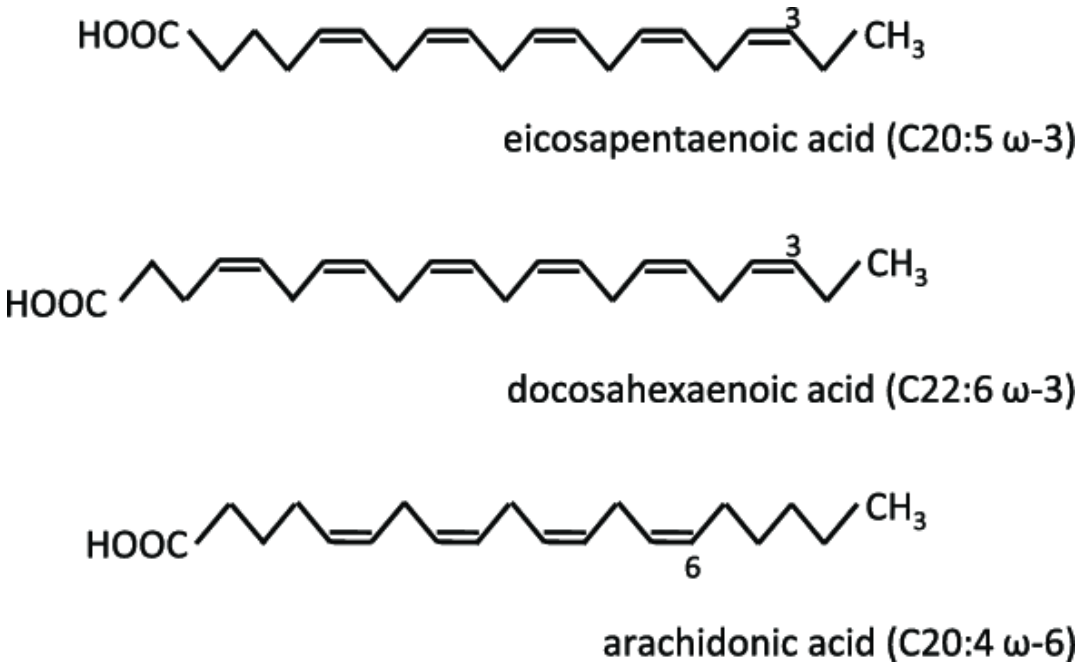


Figure 8 - Structure and nomenclature of PUFAs. Cx:y u-z refers to the chemical structure where x = number of carbon atoms, y=carbon - carbon double bonds, z=position of the first carbon - carbon double bond away from the methyl (u) end of the hydrocarbon chain. Figure from (Cockbain et al., 2011)

An FA is also classified based upon the number of double bonds. A FA without double bonds is unsaturated, one double bond is a monounsaturated fatty acid (MUFA), with more than one double bond are known as polyunsaturated fatty acids (PUFA), and lastly FAs with more than four double bonds are known are highly unsaturated fatty acids (HUFA) (Waagbø, 2001).

As previously touched upon in the section about commercial feeds in the aquaculture industry the inclusion of plant based raw materials have been necessary to keep up with the limitations of global fisheries as a source of marine protein and fats. High costs and resource scarcity have driven the aquaculture industry to explore different avenues to meets the nutritional demands with regards to growth, sustainability and welfare (Turchini et al., 2009). The requirement of long chain n-3 PUFA (LC-PUFA; i.e., EPA; 20:5n-3 and DHA 22:6n-3) has been a challenge as there are lower proportions of this in plant oil and higher concentrations of n-6 PUFA, giving a higher n-6/n-3 ratio (Waagbø, 2006).

In the review by Turchini et al. (2009) numerous papers are cited confirming that the replacement or partial replacement of fish oil with vegetable oil changes the composition of both the edible fillet and is mirrored in the fishes' organs and lipid storages. To what extent the tissues are influenced by dietary fatty acids depends on the proportions of neutral lipids and polar lipids of the tissue in question (Jobling et al., 2002)..

Dietary lipid composition can affect the mechanisms in the innate immune system, the specific immune system and resistance to diseases in fish (Waagbø, 2006). The health impacts of single or classes of FAs seem to be connected to different mechanisms and therefore changes in dietary lipids and tissue FA composition can affect both the physiology and immunology of fish. In addition, fish are ectothermic meaning that their body temperature is adjusted according to their environment. The cell membranes respond to temperatures by changing lipid and FA composition in the phospholipid layer of cell membranes (Waagbø, 2006).

Waagbø et al (1993) showed that the membrane phospholipid (PL) FA composition in lymphoid tissues (spleen and head kidney) and circulating blood cells was influenced by the dietary lipid sources. This is reflected in the hypothesis that the fish's nutritional status influences the survival rates when challenged. The differences in survival are likely related to the cell membrane composition, as lipids are essential to their structure. PUFAs have been found to have an essential role in the development and control of inflammatory responses. PUFAs are components of plasma membranes involved in controlling membrane-signalling pathways in addition affect the composition of immune cell membranes (Montero and Izquierdo, 2010). Montero and Izguierdo (2010) showed that n-3 LC-PUFAs are required to promote bactericidal activity of macrophages and that fish oil substitution by n-3 FA rich oil (soya bean oil) decreased basal and post-infection levels of serum bactericidal activity.

These are locally active and are part of a variety of activities including but not limited to inflammatory response and immune responses which are of interest in this thesis (Tocher et al., 2003). ARA is known to be the preferred substrate for enzymes involved in eicosanoid pathways in fish so altering the ratio of ARA in the feed may alter the production of eicosanoids changing the inflammatory response in fish (Kiron, 2012). Eicosanoids derived from ARA promote pro-inflammatory cytokines and adhesion molecules, whilst those from EPA and DHA have the opposite function by reducing the inflammatory process or even stopping it (Calder, 2007, Calder et al., 2009, Martinez-Rubio et al., 2012).

In previous studies, it has been shown that nutritional status can influence inflammation response, immune response and regeneration of tissues after SAV challenge. In particular the dietary omega-6/omega-3 ratio and level of saturated FAs the time of infection (Thompson et al., 1996, Martinez-Rubio et al., 2013, Holen et al., 2018)(S.C. Remø – personal communication). A study comparing how different dietary vegetable oils n-6/n-3, found that pro-inflammatory genes might be linked to the transcription of selected FA genes. The study examined the gene responses involved in inflammation, signalling pathways, FA synthesis and oxidation, apoptosis and eicosanoid production in Atlantic salmon head kidney tissues (Holen et al., 2018). It is important to achieve a balanced level of dietary n-3 and n-6 PUFAs in order to achieve increased immuno-competence and disease resistance (Kiron, 2012)

In Lopez-Jimena et al. (2015) rainbow trout was challenged with SAV1 where the fish were fed two diets differing in n-3/n-6 fatty acid ratio four weeks prior to i.p. injection challenge with SAV1. Diet one contained a high n-3/low n-6 PUFA ratio (high omega-3, 3.08 n-3/n-/6 PUFA) and the other a low n-3/high n-6 PUFA ratio (high omega-6, 0.87 n-3/n-6 PUFA ratio). Targeting the viral E1 and E2 glycoprotein genes in several sampled organs revealed a lower concentration of virus in tissues originating from the fish fed the high omega-6 diet. Although this study did not find any significant differences in histological lesions between diets the finding of lower viral concentrations could indicate that the fatty acid content affects viral replication or response to viral infection by SAV1.

Today's diets high in fish meal and fish oil fed to Atlantic salmon in the FW life stage differ from what is seen in the composition of their diet in the wild. Bell et al. (1994) investigated 10 species which were considered to be part of the natural diet for Atlantic salmon parr (FW) and compared the fatty acid composition of these to that found in the commercial diets at the time. Findings were that the natural diet (insects) had a higher amount of EPA and ARA, and a considerably lower DHA (22:6n-3). These findings were consistent with those in Ackman and Takeuchi (1986) where the lipid content in wild Atlantic salmon was higher than of that in farmed salmon in the parr life-stage, this is likely due to the natural diet increasing the n-6 content.

Altering the FA composition is not the only way to influence immune responses and growth. In the next a short overview of the amino acids altered in the trial diet will be presented.

2.3.3 Amino Acids in fish nutrition

AAs are known as the building blocks for protein and have traditionally been classified as either essential or nonessential. Those that are defined as essential cannot be synthesized or are inadequately synthesized *de novo* relative to the needs of that organism. Conditionally essential AAs refers to those whose synthesis that under certain pathophysiological conditions are limited (Li et al., 2009). An overview of AAs and their classification is shown in Table 1 below.

Table 1 - Overview of nutritionally essential and nonessential amino acids for fish and other aquatic animals. Table from Li, Mai et al. (2009)

Essential AA	Nonessential AA	Conditionally essential AA
Arginine	Alanine	Cysteine
Histidine	Asparagine	Glutamine
Isoleucine	Aspartate	Hydroxyproline
Leucine	Glutamate	Proline
Lysine	Glycine	Taurine
Methionine	Serine	
Phenylalanine	Tyrosine	
Threonine		
Tryptophan		
Valine		

In terms of chemistry an AA is an organic molecule composed of a basic “amino” group (-NH2), an acidic carboxyl group (-COOH) and an organic “R” group/side chain (the functional group), the general structure is illustrated in figure below (fig. 6).

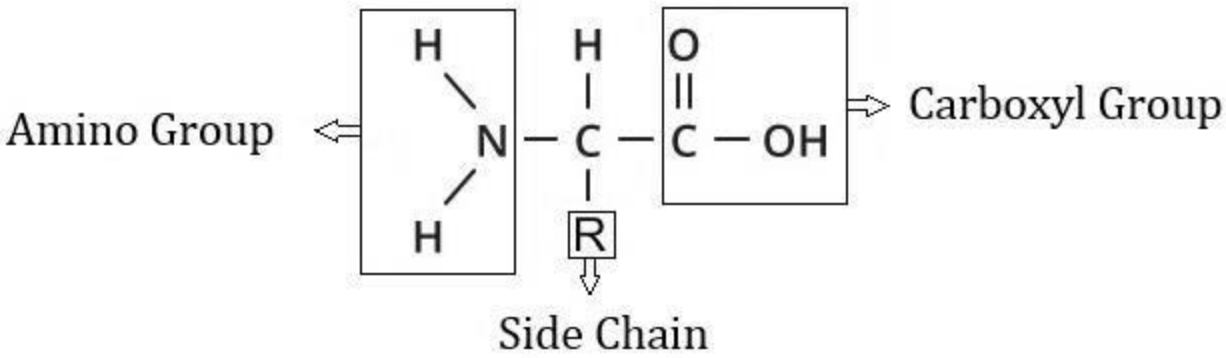


Figure 9 - The generalized structure of an amino acid. Illustration from Nasrolahpour (2017)

It has been established that proteins are the most expensive macronutrient in fish diets and supplies of amino acids (AA) for growth, energy and key metabolic pathways, in salmonid diets protein makes up 35-55% of the diet (Andersen et al., 2016, Kaushik and Seiliez, 2010). The replacement of fishmeal with cheaper and more sustainable protein sources, such as plant proteins, bi-products from fisheries and terrestrial animal production, has increased the need to supplement feeds with AAs (Nunes et al., 2014). For instance more than half of the AAs consumed by fish may be deposited as body protein (Andersen et al., 2016). Increasing the percentage of the protein content in the diet to ensure enough EAAs is a costly solution, and may lead to excessive amounts of protein and/or other nutrients rendering the feed less sustainable and more expensive. Feeds have been supplemented with crystalline amino acids (CAA) as a less costly and more sustainable solution to “fine tune” feeds in recent years (Nunes et al., 2014).

The amounts of micro ingredients such as crystalline AAs (and many more) have increased gradually from 1.0% in the 1990s to 4.0% in 2016 (Aas et al., 2019). This has also been illustrated in the section on commercial diets. With the replacement of marine proteins with plant proteins the nutritional composition has to be examined closer to achieve a beneficial balance.

Looking closer at AAs in fish feed show that they can influence growth and health in a variety of ways. According to a review by Li et al. (2009) AAs and their metabolites can influence a several important metabolic pathways that regulate growth, feed intake, nutrient utilization, immunity, behaviour, larval metamorphosis and reproduction in addition to resistance to environmental stressors and pathogenic organisms in various fish species. Kiron (2012) states that AAs have a central role in the defence mechanisms due to their involvement in the synthesis an assortment of proteins i.e. antibodies and in the control of key immune regulatory pathways.

Methionine (Met) and Lysine (Lys) are the first limiting essential amino acids (EAA). As mentioned Methionine is the most limiting EAA in many fish diets, especially those containing high levels of plant proteins sources (i.e. soybean meal, peanut meal and copra[1] meal) (Mai et al., 2006). Methionine is an EAA that can be used to synthesize cysteine, and together these constitute the sulphur amino acids (SAA[2]) (Andersen et al., 2016). Methionine also provides the methyl group for the methylation of DNA and proteins and is therefore very important in the production of proteins and regulation of gene expression. (Li et al., 2007).

In an experiment with chickens where the methionine content was increased from 0,35% to 1,2% results showed that the chickens fed higher amounts of methionine had enhanced immune responses and T-cell proliferation in response to mitogen stimulation when challenged with the Newcastle virus. However, too much methionine (>1.45%) was been found to negatively affect the chickens with regard to growth and immune responses (Li et al., 2007).

Both taurine and methionine supplementation have been proven to be indispensable to plant protein based diets (Gaylord et al., 2007). Taurine is known as a free abundant AA in lymphocytes and is a potent antioxidant. When taurine reacts with hypochlorous acid it becomes a microbial agent and is produced by activated monocytes and neutrophils known as taurine chloramine (oxidant) which reduces production of pro-inflammatory cytokines (IL-1, IL-6 and TNFα) and prostaglandin E2 (Li et al., 2007). Taurine is derived from methionine therefore, methionine levels have an impact on the amounts of taurine (Andersen et al., 2016). Taurine protects cells against oxidation by protecting mitochondrial integrity and respiration. In a trial with liver cells isolated from Atlantic salmon grown in taurine supplemented and non-

[1] **Copra meal**, or coconut meal, is an important feed ingredient and the by-product of the oil extraction from dried coconut kernels (copra)

[2] Sulfur Amino Acids refers to methionine, cysteine, homocysteine and taurine which all contain sulfur.

supplemented media, apoptosis was higher in liver cells grown in the non-supplemented medium (Espe and Holen, 2013).

Lysine is another of the limiting AAs in fish feeds and is known for its antiviral properties. In chickens lysine deficiency limits protein synthesis, proliferation of lymphocytes and impairs immune responses (Li et al., 2009). Lysine is a limiting factor for growth performance and health. Dietary lysine supplementation is effective in the enhancement of immune responses and gastrointestinal development of agastric fish. Lysine functions as a substrate for carnitine synthesis that is required for the transport of long chain FAs from the cytosol into mitochondria for oxidation (Li et al., 2009).

Threonine is a major component of intestinal mucin and plasma y-globulin and has been proven an indispensable AA for optimal growth of fish (Jobling, 2012). The intestinal mucosa is one of the physical barriers to pathogens. Threonine has been found to prevent apoptosis in cell culture mediums, stimulate cell growth and promote antibody production in lymphocytes through protein synthesis and cellular signalling mechanisms (Li et al., 2007). In challenge studies with both chickens and pigs, increased antibody production amongst other immunological benefits (Li et al., 2007).

3.0 Materials & Methods

3.1 Fish husbandry and dietary history

The following experimental procedure was approved by the Norwegian National Animal Research Authority (NARA) Fots ID 15465.

The fish used in this study were unvaccinated pit-tagged Atlantic salmon post-smolts (Aquagen), which were produced by The Institute of Marine Research (IMR) Matre Research station. During the ten weeks prior to transport to IMR Bergen, the fish were given three diets differing in FA and AA composition, and a standard reference diet (

Table 2). The standard reference diet is based on IMRs feed surveillance programme and is composed based on average nutritional content in commercial FW salmon feeds. All feeds were produced by Skretting ARC.

Fish were transported in closed-tanks by truck to IMR. After transportation the fish from each feeding group were acclimatized in 400 L tanks for two weeks where the salinity was gradually increased to full salinity. The fish population was screened for SAV, PRV, IPNV and PMCV prior to the start of this trial. Prior to bath challenge weight and length was recorded for all individuals.

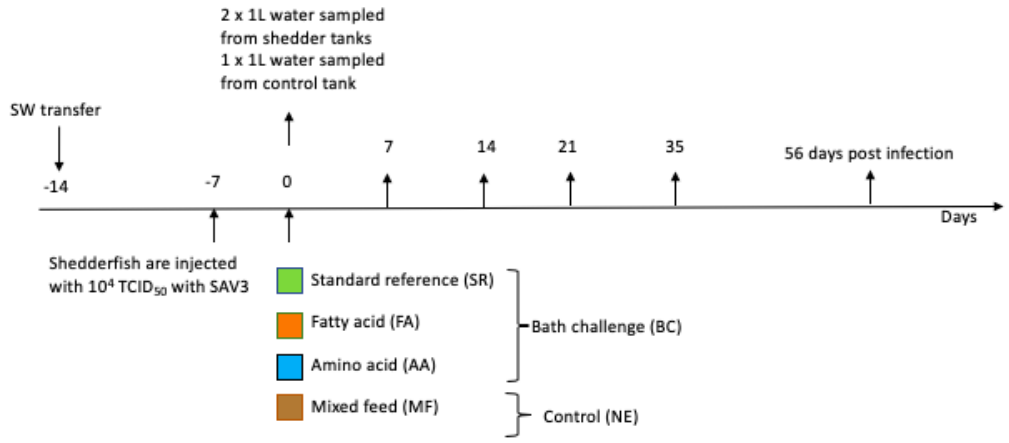


Figure 10 - Experimental set up. The time-line shows start point, sampling points and progression.

Seawater during the experiment was filtered through a 20-µm filter, UV-sterilized and maintained at 12 °C throughout the experiment. The seawater flow rate and oxygen saturation were kept at 300 L/h and above 80% oxygen saturation. The tanks were 200 L with 45 fish per tank, estimated density was 23 kg/m3 at the beginning of the trial.

After transfer to IMR and for the duration of the trial the fish were fed a commercial feed (Skretting spirit supreme). No mortality was detected during the experiment.

Table 2 - Table shows proximate composition of the three diets fed in the FW phase in this study. The AA and FA diet are identical to the SR diet with the where the table is blank for each column. Two different pellet sizes have been used throughout the 10 weeks the fish were fed the trial diets. MUFA=Monounsaturated fatty acids, SFA=Saturated fatty acids, ARA=Arachidonic acid, EPA=Eicosapentaenoic acid, DHA=Docosahexaenoic acid, PUFA=Polyunsaturated fatty acids. Courtesy of Nini H. Sissener

	Standard Reference (SR)		***Fatty Acid (FA)***		***Amino Acid (AA)***	
						Proximate composition
Pellet size:	*2mm*	*3mm*	*2mm*	*3mm*	*2mm*	*3mm*
Protein	*46*	*46*	*46*	*44*	*46*	*46*
Lipid	*19.0*	*19.0*	*18.3*	*18.8*	*18.8*	*18.6*
Energy kJ/g	*21.8*	*21.7*	*21.7*	*21.3*	*21.7*	*21.6*
Ash	*6.2*	*6.1*	*6.2*	*5.8*	*6.1*	*6.1*
Dry matter	*93*	*93*	*93*	*91*	*92*	*92*
						Fatty acids % of total fatty acids:
16:00	*13.1*	*12.7*	*11.3*	*11.0*	-	-
SUM SFA	*19.7*	*19.7*	*16.3*	*16.3*	-	-
18:1n-9	*27.4*	*27.3*	*24.4*	*24.3*	-	-
22:1n-11	*6.2*	*6.9*	*2.1*	*1.9*	-	-
Sum MUFA	*46.9*	*45.6*	*32.3*	*31.2*	-	-
18:2n-6	*10.1*	*10.0*	*11.7*	*11.7*	-	-
20:4n-6 (ARA)	*0.4*	*0.4*	*3.9*	*4.2*	-	-
Sum n-6	*10.9*	*10.8*	*16.5*	*16.9*	-	-
18:3n-3	*4.0*	*4.1*	*10.2*	*10.3*	-	-
20:5n-3 (EPA)	*5.7*	*5.6*	*15.8*	*15.9*	-	-
22:6n-3 (DHA)	*8.3*	*7.8*	*5.5*	*5.5*	-	-
Sum n-3	*20.7*	*21.1*	*33.9*	*34.6*	-	-
SUM PUFA	*31.8*	*32.2*	*50.3*	*51.4*	-	-
						Amino acids mg/g
Taurine	*3.5*	*3.2*	-	-	*5.2*	*5.0*
Methionine	*8.5*	*8.1*	-	-	*14.6*	*14.0*
Lysine	*32*	*33*	-	-	*38*	*37*
Threonine	*18.2*	*17.6*	-	-	*22.7*	*21.8*

3.2 Bath challenge

The control fish, in total 45 fish from each of the 3 feed groups were moved to 3 x 200 L experimental control tanks prior to bath challenge in the 400L tanks, hereafter known as mixed feed (MF).The remainder of the fish were distributed into 9 x 200 L tanks according to the feed they received during the FW phase as illustrated in *Figure 11* (n=3 tanks per FW feed group).

The bath challenge of the experimental fish was performed as previously described in Jarungsriapisit et al. (2016b) with modifications. Shedder fish were given i.p. injections of 10^4 $TCID_{50}$ with SAV3 (in a saline vehicle) per fish, 7 days prior to the bath challenge. This timepoint was chosen as previous studies have shown that the amount of shedded virions is at its' peak between 6 – 13 days after infection (Andersen et al., 2010).

The SAV3 isolate used was provided by Øystein Evensen (NMBU). The virus was cultivated in CHH-1 cells in L-15 medium with 2% fetal bovine serum (FBS) at 15 °C. Cultivation by Craig Morton (IMR). Titration at the Veterinary Institute in Oslo.

On the day of the bath challenge, the water flow in the shedder tank was stopped for 2 hours. Subsequently, the shedder fish were removed and the water containing SAV virions was distributed amongst the three challenge tanks manually with buckets. The fish were exposed for 6 hours before water flow was started again. During which time the tanks were aerated and the oxygen levels were monitored to maintain adequate O_2 and CO_2 levels. After the bath challenge, the fish were distributed; 45 fish per tank according to the feed they received during the FW phase. Randomization of individuals by pit tag reading to mitigate any differences in distribution. Tanks were also randomly assigned with the exception of strategic placement of control groups at the beginning of each row. Water samples were taken from the shedder tank to determine the dose the fish were exposed to and analysed using RT-qPCR and TCID50 titration (see below).

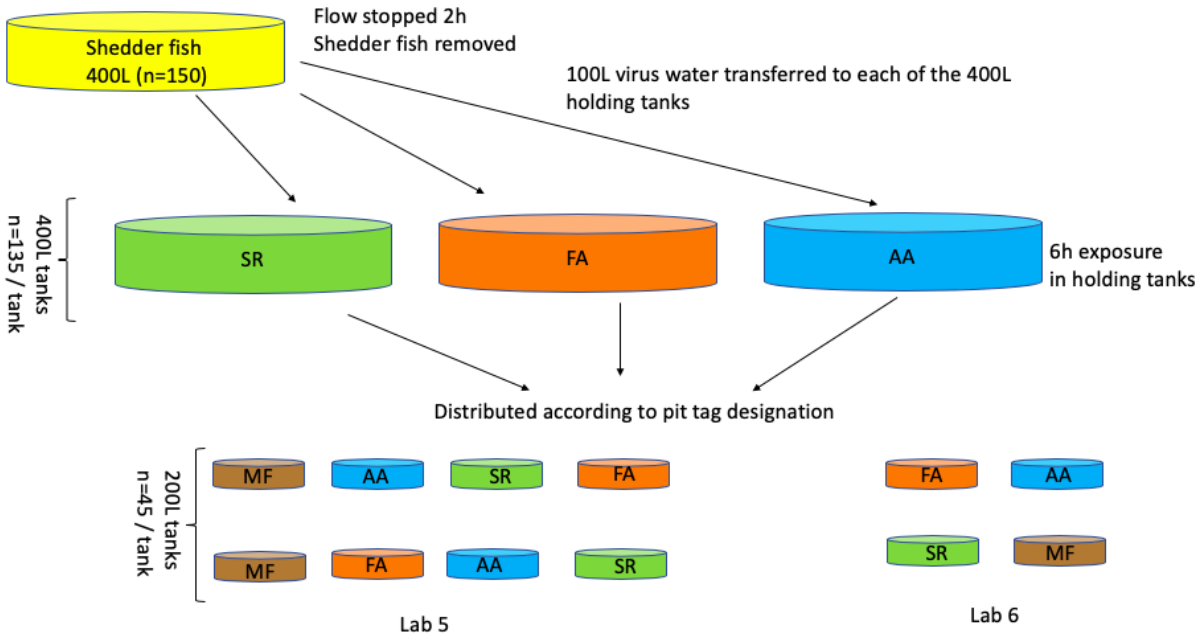


Figure 11 - Bath immersion set-up. Control fish were distributed into three common garden tanks (MF) prior to bath challenge. SR=standard reference, FA=fatty acid and AA=amino acid modified diet.

Standard reference (SR), Fatty Acid modified (FA) and Amino Acid modified (AA). Those exposed to SAV3 will be referred to as bath challenged (BC) and those that are not exposed as non-exposed (NE). The control fish tank is shown as mixed feed (MF) in the image above.

3.2.1 Water sampling

Water samples 2 x 1 L were taken from the shedder tank and 1 L from 1 of the control tanks after the flow had been stopped for 2 hours. One of the samples from the shedder tank and the sample from the control tank was spiked with 5 µL Nodavirus suspension as an exogenous control (Korsnes et al., 2005).

The samples were vacuum filtered through electropositive Zeta plus ® Virosorb® 1 MDS filters (Cuno Inc., USA) using Millipore® sintered glass filters and funnels (Moore, Nilsen et al. 2017). Viral particles were eluted from these filters in 1.2 mL of L-15 medium containing 10% FBS (Jarungsriapisit, Moore et al. 2016). Filters were then placed in secured petri dishes that were shaken for 15min at 500 rpm. The eluent was removed using syringe and concentrated samples were transferred to 1,5 mL microtube. 100 µL was dispended into 350 µl lysis buffer for RT-qPCR analysis and the remainder samples for end-point dilution sent to NVI for titration to determine $TCID_{50}$.

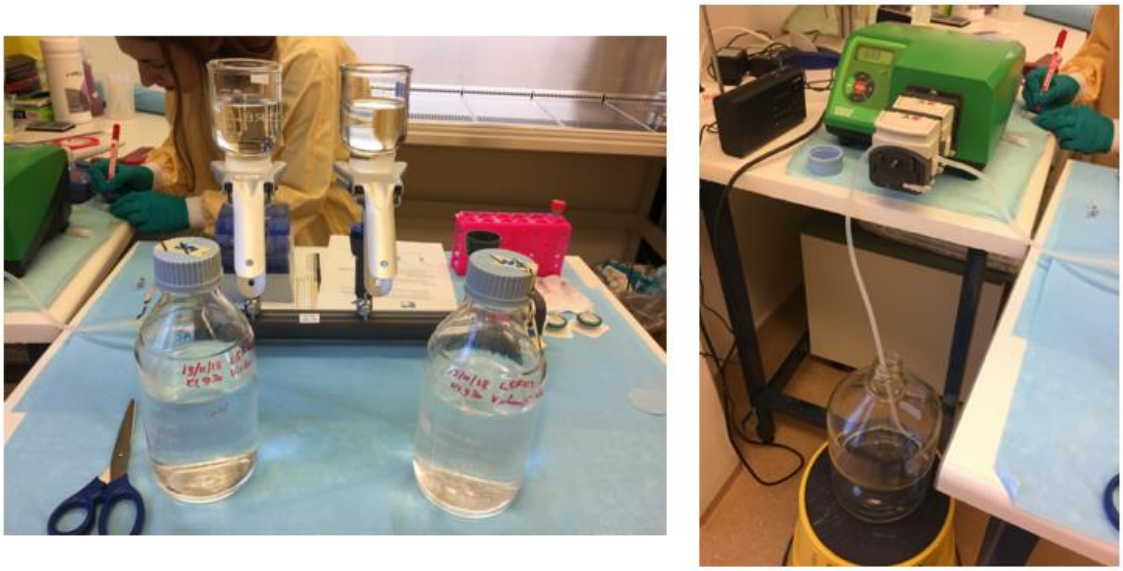


Figure 12 – Photo from the filtration process. The bottles in front are 1L samples from two of the tanks that are being poured into the filtration device in the background. Photo on the right-hand side shows the pump.

Protocol from IMR.

Heart and blood samples from 5 of the 150-shedder fish were taken for RT-qPCR to confirm infection prior to bath challenge by the same methods as described in the section below.

3.2.2 Infective dose

The Veterinary institute was sent a water sample for titration; however, it was not possible to estimate dosage based on results.

Filtered water samples spiked with Nodavirus suspension tested positively indicating that the filters worked to a satisfactory degree. RT-qPCR of the Nodavirus suspension yielded a CT of 15.8 while the spiked samples indicated an average CT 27.1. The discrepancy in CT (-11.3) suggests that the method did not intercept all virions in the filtered water samples, and that the actual dose of SAV was presumably higher than estimated. The water samples were positive for SAV3. RT-qPCR was used to quantify the amount of virus copies. The average of four samples indicated that there was $7.2 \cdot 10_6$ SAV virions/L.

3.3 Sampling

3.3.1 Shedder fish sampling

All sampled shedder fish were positive for SAV in both heart and head kidney. RNA extraction from blood samples was also attempted, however, unsuccessful due to sample viscosity – the samples resulted in clogging up the robot used for extraction.

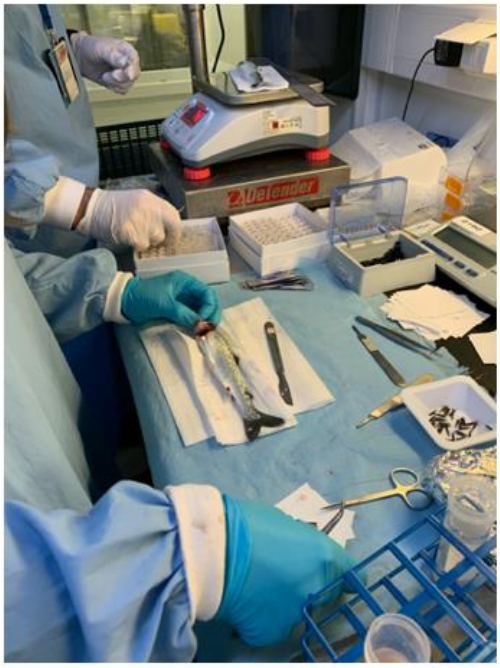
3.3.2 Tissue sampling

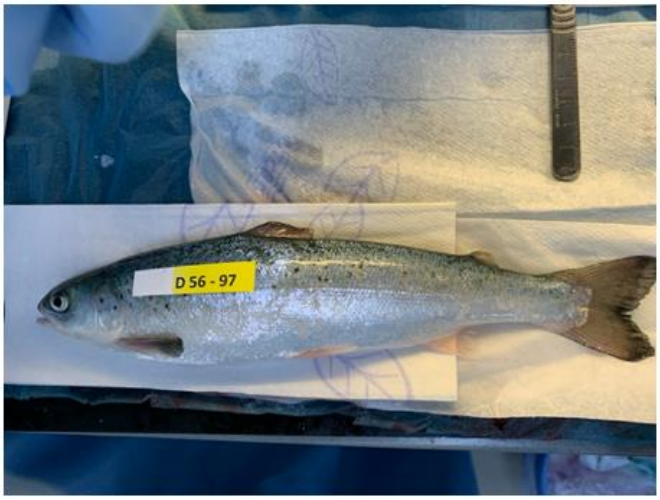
Fish were sampled at start and 7, 14, 21, 35, and 56 days post exposure. At each sampling point, six fish were sampled from each tank. Tricaine mesylate 1g/L (Finquel Vet., MSD Animal Health) was used to euthanise fish prior to sampling.

During sampling individuals were identified by pit tag, then weight and length was recorded. Any macroscopic lesions or deformities were noted.

Tissue sampling was extensive therefore samples taken for different studies or not used in this thesis are not described here. Blood was drawn immediately using a heparinized syringe. Viscera with associated pancreatic and pyloric caeca and liver were weighed separately and recorded. Gut content was recorded by checking stomach for pellets and intestine for faeces as a rough estimate for appetite.

At all samplings heart (the apical tip of the ventricle) was taken for RNA extraction in tubes containing a homogenization solution consisting of homogenization buffer and 1-thioglycerol. These were stored on ice until homogenization. Simultaneously samples for histology were taken (heart and pancreas tissue associated with pyloric ceca) and red and white muscle tissue were fixed in 10% neutral buffered formalin. Backup samples of heart and a sample of white muscle were flash frozen in N_2 and stored at -80°C.





Weight of viscera, liver, body weight and length were used for the following calculations:

VSI (Viscera Somatic Index):

$$VSI = \frac{Weight\ viserca}{Body\ weight} \times 100$$

HSI (Hepatosomatic Index):

$$HSI = \frac{Weight\ liver}{Body\ weight} \times 100$$

Fulton's Condition Factor (CF):

$$CF = \frac{Fish\ weight\ (g) \times 100}{Fork\ length\ (cm)^3}$$

SGR (Specific Growth Rate)

$$SGR = \frac{(\ln(final\ weight) - In\ (Initial\ body\ weight))}{Days} * 100$$

3.4 Quantitative real-time PCR

3.4.1 RNA extraction

RNA isolation was performed using Promega Reliaprep simplyRNA HT 384, art nr X9601(Nerliens) in a Biomek 4000 Laboratory Automated Workstation in a 96-well format (2.2mL, square-well deep well plate 96, VWR). Reagents were manually measured and distributed into sterile Biomek reservoir trays (Nerliens) of either 25mL or 50mL.

Homogenization was achieved using a FastPrep 96 instrument (MP Biomedicals) with a speed of 1600rpm (Rounds per minute) for a total of 1 minute. The homogenized solution was then centrifuged at 1000g for 5 minutes at 4°C and then stored at -80°C.

Samples taken for RNA extraction were transferred to tubes containing a homogenization solution consisting of homogenization buffer and 1-thioglycerol and stored on ice until homogenized.

RNA isolation was conducted according to the manufacturer's standard protocol and the isolated RNA eluted in 50 ul nuclease free water in a 96-well Axygen PCR plate (VWR). The RNA samples were then quantified by NanoDrop™-1000 spectrophotometer (Thermofisher Scientific) and normalised to the concentration of 25 ng/µL using the Biomek 4000 Laboratory Automated Workstation (Beckman Coulter).

3.4.2 Virus Quantification

The extracted RNA was used to determine the relative amounts of SAV3 RNA. This was measured by using AgPath-ID one-step RT-PCR reagents (Thermofisher Scientific) according to the manufacturer's instructions. RNA was diluted into 4 µL and mixed with 400nM forward primer, 600 nM reverse primer and 160nM probe – a total volume of 10 µl on a 384 well-plate.

Elongation factor α 1 gene (ELF) was used as an endogenous control and quantification of virus was done using the nsp1 sequence of the SAV3 genome. Primers and probes (Taqman) are listed in Table 3.

Table 3 Overview of sequences for primers and probes used in SAV3 virus quantification

Name	Sequence 5'- 3'	Reference
nsP1 Forward	CCG GCC CTG AAC CAG TT	(Hodneland and Endresen 2006)
nsP1 Reverse	GTA GCC AAG TGG GAG AAA GCT	
nsP1 Probe	6FAM-TCG AAG TGG TGG CCA G-MGBNFQ	(Andersen et al., 2007)
ELA Forward	CCC CTC CAG GAC GTT TAC AAA	(Olsvik, Lie et al. 2005)
ELA Reverse	CAC ACG GCC CAC AGG TAC A	
ELA Probe	6FAM-ATC GGT GGT ATT GGA AC -MGBNFQ	(Moore, Somamoto et al. 2005)

For the qPCR assay, amplification and fluorescence detection were performed by a QuantStudio™ 5 Real-Time PCR System (Thermofisher Scientific) for 40 cycles according to the manufacturer's conditions (Table 3). The standard curve and quantification of SAV3 RNA copies in samples were constructed automatically with the QuantStudio™ Design & Analysis Software. Results are presented as N-fold log2 separately for heart and muscle (Andersen et al., 2010) in the results section.

Table 3 - qPCR amplification conditions

	cDNA synthesis	**Activation**	**qPCR**		
			Denaturation	Anneal/extend	
Temperature	45 °C	95 °C	95 °C	60 °C	RT
Time	10 min	10 min	15 s	45 s	∞
Cycles	1	1	40		1

3.5 Histology

Formalin fixed (24 h) samples of heart, muscle and pancreas tissue was processed and imbedded in paraffin wax before being sectioned (3.5-5 µm) and stained with Haematoxylin Erythrosine Saffron (HES). The samples were then examined for signs of histopathological tissue changes

that are characteristic for PD. Each sample was scored according to the level of severity. Degrees of severity are determined based on standardized scoring systems as shown in table below (Table 4) (McLoughlin et al., 2002, Taksdal et al., 2007, McLoughlin et al., 2006). When in doubt half scores have been employed to mark a middle point between two scores. This has been done in conference an experienced histologist.

Table 4 - Semi-quantitative lesion score system used to compare lesion severity from McLoughlin et al. (2006).

Score	Pancreas	Heart	Muscle
0	Normal appearance	Normal appearance	Normal appearance
1	Focal pancreatic acinar cell necrosis	Focal myocardial degeneration ± inflammation (<50 fibres affected)	Focal myocytic degeneration ± inflammation
2	Significant multifocal necrosis/atrophy of pancreatic acinar tissue, plus some normal tissue remaining	Multifocal myocardial degeneration ± inflammation (50-100 fibres affected)	Multifocal myocytic degeneration ± inflammation
3	Total absence of pancreatic acinar tissue	Severe diffuse myocardial degeneration ± inflammation (>100 fibres affected)	Severe diffuse myocytic degeneration ± inflammation
R	Recovery of pancreas	Repair and/or regeneration	Repair and/or regeneration

3.6 Gene expression - qPCR to selected gene transcript:

3.6.1 cDNA Synthesis

Total RNA from selected samples were normalised to the concentration of 100 ng/µL using the Biomek 4000 Laboratory Automated Workstation (Beckman Coulter). Reverse transcription was carried out using SuperScript® VILO™ cDNA Synthesis kit (Thermofisher Scientific) according to the manufacturer's instructions, and a total RNA input was 500 ng in each reaction in a total volum of 10 µL.

3.6.2 Quantitative real-time RT-qPCR

The PCR primers used to quantify the selected genes are shown the table below (Table 5). The qPCR assay was run using Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Matriks) according to the manufacturer's instructions, with 2 µL of cDNA diluted 1 to 20 in a reaction

mix containing 400 nM of forward primer, 400 nM of reverse primer in a total volume of 7 µl on a 384 well-plate. For the qPCR assay, amplification and fluorescence detection were performed by a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems) for 40 cycles. Quality controls "no template controls" (ntc) and "no amplification controls" (RT neg) were run for quality assessment for each PCR assay.

Mean normalized expression of the target genes was calculated based on a normalization factor using geNorm software (Vandesompele et al., 2002).

The Mx assay used in this trial detects Mx1, 2 and 3.

Table 5 - Primers used in the analysis of immune genes and the reference for the assays.

Target gene	Forward primer 5'-3'	Reverse primer 5'-3'	E*	Reference / GenBank accession no.
IFNy	GGTCCACTATAAGATCTCCAAGGA	CTGGCAAGATACTCCGATACAC	2.00	AY795563
IL-1B	GAGAGGTTAAAGGGTGGCGA	TGCTTCCCTCCTGCTCGTAG	1.89	NM_001123582
IL-10	CGACTCCATCGGAAACATCTT	GCCCTTACCCTGCATCTTCTTA	1.99	EF165028
MX	GGTGGTTGTGCCATGCAA	TGGTCAGGATGCCTAATGTC	2.02	U66475
TNFa III	CACGGCAAGAAACAAGATCCCA	GATCCACTGGGGTTGTATTCACCTTCTA	1.91	EF079662
Viperin	AGCAATGGCAGCATGATCAG	TGGTTGGTGTCCTCGTCAAAG	2.03	(Grove et al., 2013)
EF1a	CAAGGATATCCGTCGTGGCA	ACAGCGAAACGACCAAGAGG	2.02	(Olsvik et al., 2005)
RPL1	ACTATGGCTGTCGAGAAGGTGCT	TGTACTCGAACAGTCGTGGGTCA	1.96	CB516726

*Efficiencies from Moore et al. (2017), except Type III TNFa (Chance et al., 2018), IL-10 and RPL1 (Jorgensen et al., 2006)

4.0 Data analysis

Statistical analyses were performed using Microsoft Excel and PRISM 8. Potential outliers were identified by Prisms' ROUT test (Q=1%). Some values were identified but considered to be the result of biological variation and therefore kept in the data sets.

Normality and lognormality were tested by Shapiro-Wilk and Kolmogorov-Smirnov test. Data that passed normality tests have been analysed with One-Way ANOVA, followed by Tukey multiple comparisons test when $p<0.05$. When data has failed normality tests Kruskal-Wallis (KW) has been used and followed with Dunn's multiple comparisons test when $p<0.05$.

Two-way ANOVA has been run at trial endpoint (56 dpe) on growth and gene expression data with factors being diet and treatment (SAV-exposure). Where data was significant Sidak's or Tukey's multiple comparisons test has been used.

When examining data such as viral load over time the confidence interval has been adjusted so that $p<0.01$ are considered significant due to the fact that the data did not consistently pass normality tests (Glass et al., 1972). This conservative approach should counteract that the underlying assumptions are not met for all included data. This also applies to data sets used for both two-way and one-way ANOVAs. In addition to ANOVAs Spearman's rank correlation test is used on non-parametric data to assess how the relationship between two variables.

5.0 Results

5.1 Prevalence

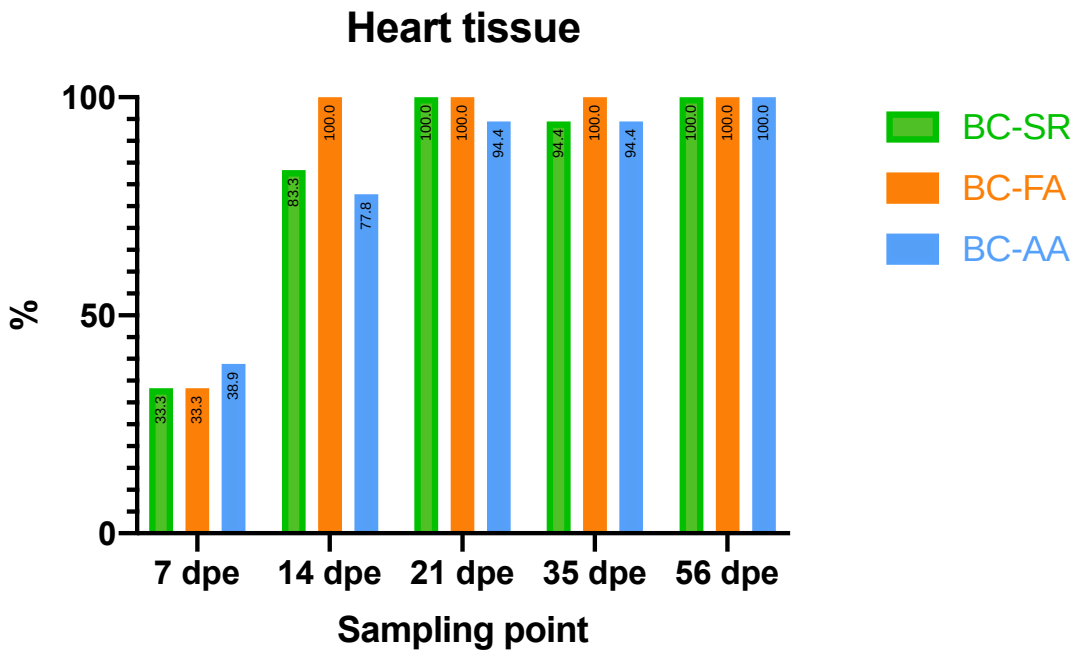


Figure 13 - Shows prevalence of SAV-positive heart samples in percent (%) for each feed group and each sampling point. Non-exposed fish were not included. SR=standard reference diet, FA=fatty acid modified, AA=amino acid modified

At the first sampling point individuals from each diet in the bath challenged (BC) group tested positively for SAV (n=18 pr. diet in BC-group). The proportion of fish testing positive for SAV increased substantially from 7 to 14 dpe with the BC-FA group being 100% prevalent from this point and throughout the trial (**Error! Reference source not found.**).

Sampled fish from the BC-AA group did not reach 100% prevalence until sampling at 56 dpe. Fish sampled from the BC-SR group varied in prevalence ranging between 94.4 – 100% from 21 dpe until end of trial. At 56 dpe all sampled fish from the BC-group were SAV-positive.

5.2 Viral load

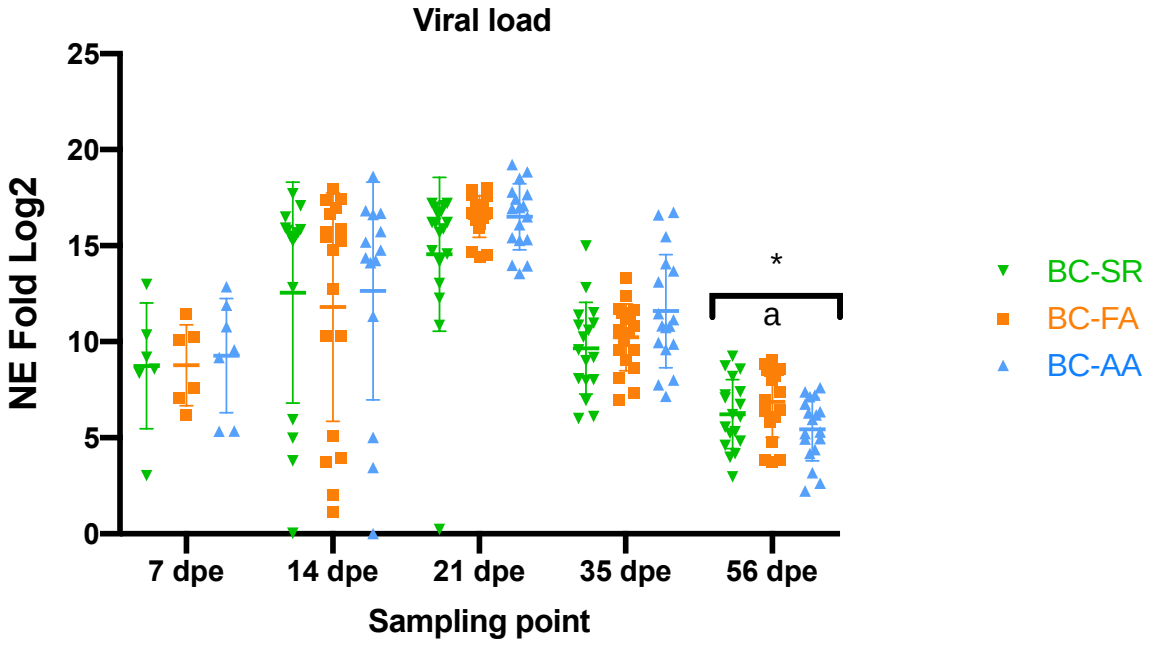


*Figure 14 – NE Fold Log2 of heart samples from the SAV-positive fish (from BC-groups) throughout the trial at all sampling point. Prevalence is superimposed, with bars annotated at the bottom with prevalence. Significant differences are indicated with * (p<0.05). BC=bath challenged, SR=standard reference diet, FA=fatty acid modified diet, AA=amino acid modified diet.*

Viral load in heart in all diets peaked at 21 dpe in all diets and gradually decreased toward the end of the trial. Looking closer at the viral load at the trial endpoint with One-way ANOVA yielded no statistically significant difference ($F(2, 51) = 2.973$, $p= 0.0601$). Although this was not a significant difference post hoc testing with Tukeys' multiple comparisons was done, this revealed that the BC-FA group had a significantly higher viral load than BC-AA group ($p=0.0478$).

The same analysis was run for viral load in muscle (*Figure 15*), only samples from 56 dpe were examined in the current study. No significant differences between diets were found when viral

load in muscle was analysed (p=0.9550, F (2, 45) = 0.04610). When testing correlation between viral load in heart and muscle using Pearson test with a conservative confidence interval of 99% (p<0.01) showed that there was a significant correlation between the viral load found in heart tissue and that in muscle tissue.

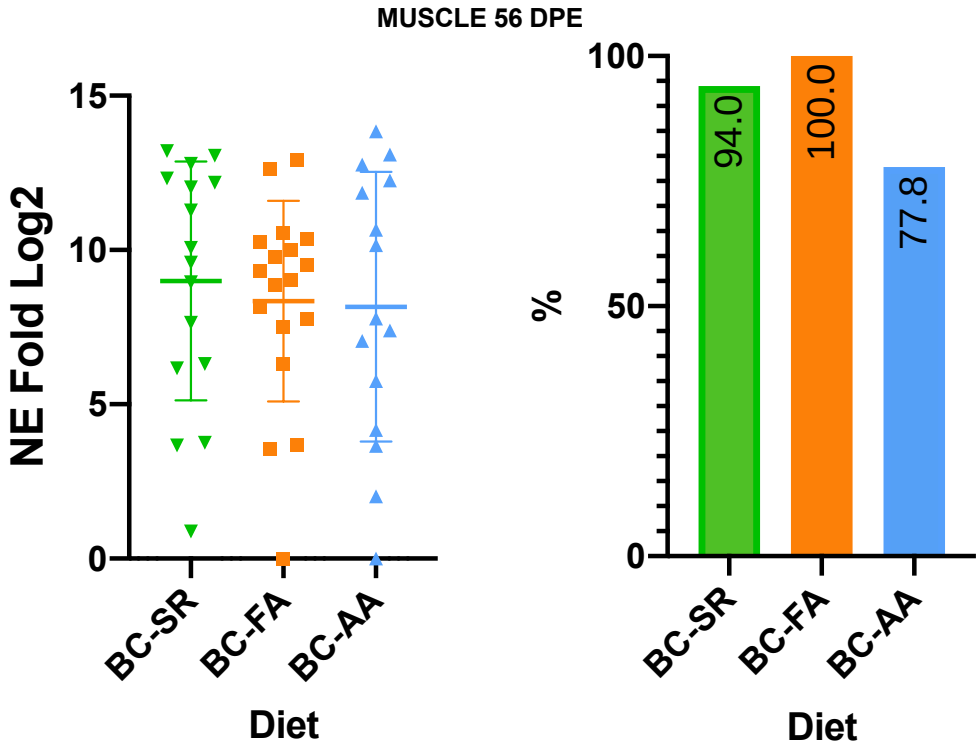


Figure 15 – Viral load found in muscle tissue at 56 dpe with mean and standard deviation on the left-hand side. On the right-hand side prevalence (%) in muscle tissue is shown and annotated in the bars. BC=bath challenged, NE=non-exposed, SR=standard reference diet, FA=fatty acid modified diet, AA=amino acid modified diet.

5.3 Growth

5.3.1 Weight and length development

One-Way ANOVA at day 0 revealed that there was already a significant difference in weight between diets groups (p=0.0012, F (2, 259) = 6,86). Investigated by Tukey's multiple comparisons test revealed that the average weight in the AA-diet group (n=86) was significantly heavier than in the FA-diet group (p=0.0009, n=88). This was not reproduced in fish that were in the non-exposed group, where no significant differences between diet groups were found (n=30 pr. diet).

This difference in weight was also found at 56 dpe with Kruskal-Wallis test. It indicated a significant difference in weight (p=0.0109, KW statistic=9.035) in bath-challenged fish. Post hoc testing with Dunn's multiple comparisons test revealed that the AA-diet group was significantly heavier than the FA-diet group (p=0.0084, n=18 pr. diet).

Table 6 - Average weight and length at the beginning of the trial shown with standard deviation (SD) SR=standard reference, FA=fatty acid, AA=amino acid. Data from day 0 is prior to challenge by SAV but collated after trial termination. Weight and length data for day 0 is based on SR (n=86), FA (n=88) and AA (n=88) for the bath-challenge group, the non-exposed n=30 per diet. At day 56 n=6 per diet for non-exposed, n=18 per diet for bath-challenged.

Average weight and length

Treatment	*Diet*	*Length±SD (cm)*		*Weight±SD (g)*	
		Day 0	*Day 56*	*Day 0*	*Day 56*
Non-exposed	*SR*	*19.5±0.8*	*24.9±1.0*	*84±10*	*190±24*
	FA	*19.2±0.8*	*23.8±0.8*	*79±9*	*165±15*
	AA	*19.7±0.8*	*25.3±1.0*	*85±11*	*187±24*
Bath-challenged	*SR*	*19.7±0.8*	*21.4±1.0*	*84±10*	*102±23*
	FA	*19.2±1.0*	*20.5±1.3*	*81±11*	*91±25*
	AA	*19.7±0.7*	*21.8±0.9*	*87±11*	*110±18*

There were significant variations in length at day 0 ($p<0.0001$) indicated by Kruskal-Wallis test (Kruskal-Wallis statistic=18.53). Where the FA-diet group (n=88) was significantly shorter than both SR (p=0.006, n=86) and AA (p=0.005, n=88). The same differences were not found in the NE-group (n=30 per diet) with a p-value of 0.1144.

At day 56 a significant difference was found in the BC-group p=0.0011. Similarly, to findings at day 0 the FA-diet group was significantly shorter than both the SR (p=0.0355) and AA-diet (p=0.0009). Interestingly the FA-diet group was also significantly shorter than fish in the AA-diet group (p=0.0291) when the same analysis was run for the NE-group at day 56 (p=0.0307, F (2, 15) = 4,435).

5.3.1 Fulton's condition factor (K-factor)

Condition factor calculated as the relationship between weight and length (formula found on page 30). At day 0 condition factor varied significantly in the BC-group (p=0.0058, Kruskal-Wallis statistic=10,29) where both the FA (n=88) and AA (n=88) diet groups were superior compared to the SR-diet group (n=86) with p-value of 0.0114 and p-value 0.0237 respectively. No significant variations in the fish allocated the NE-group.

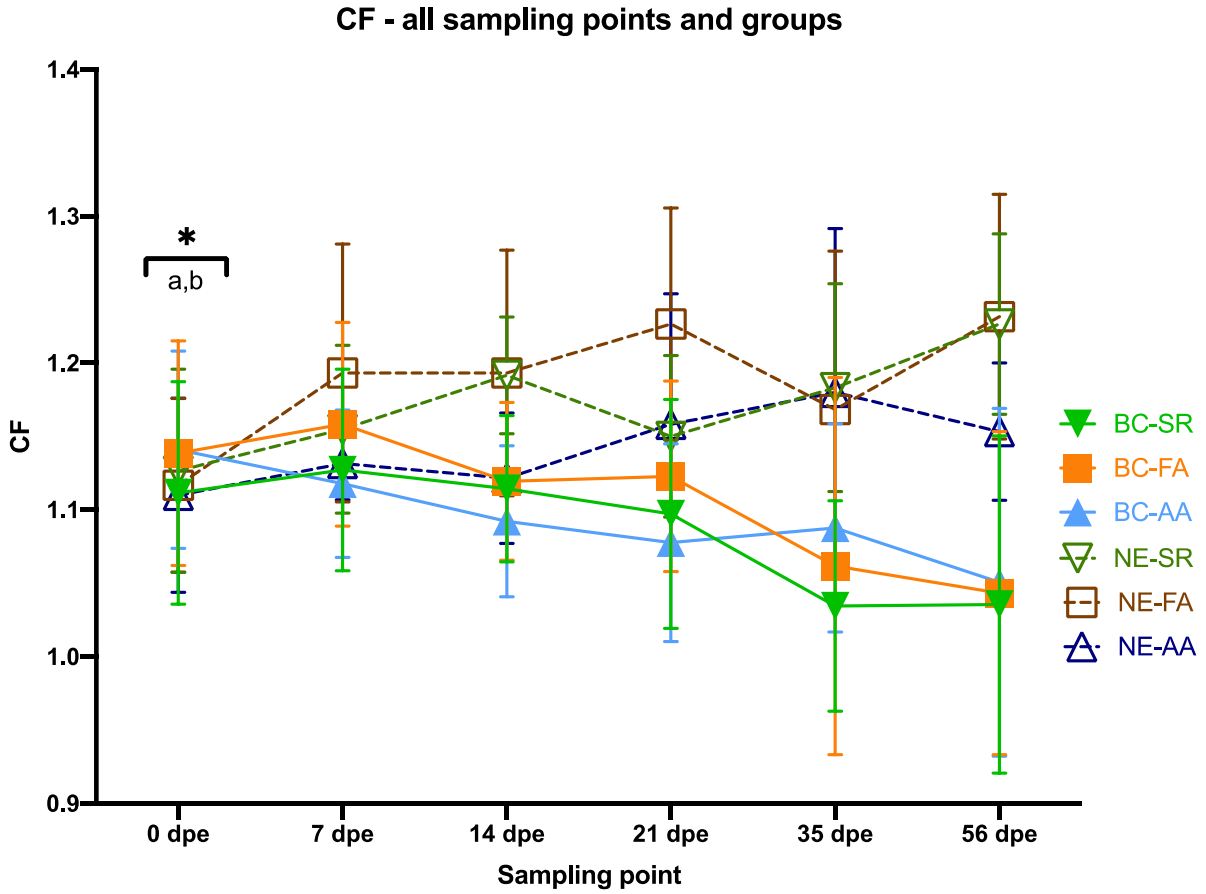


Figure 16 – Condition factor from all samplings with mean and standard deviation (SD). BC=bath challenged, NE=non-exposed, SR=standard reference diet, FA=fatty acid modified diet, AA=amino acid modified diet.

Condition factor varied significantly between BC- and NE-groups. SAV-exposure significantly affected the fish accounting for 31.5% of variance with a p-value of <0.001 (F (1, 66) = 31.37). Diet did not significantly influence condition factor (p=0.5618). As shown in Figure 16, the condition factor for BC-groups declined, and remained stable for NE-groups throughout the trial. One-way ANOVA with data collected at day 56 did not indicate significant differences between the different diets in the BC-group.

Two-way ANOVA of condition factor examining diet and sampling point of the NE-group indicated that diet significantly affected condition (p=0.0217 F (2, 75) = 4.034) and accounted for 8.7% of variation. The same analysis in the BC-group did not indicate significant difference between diets (p=0.2434 F (2, 255) = 1.421).

5.3.2 SGR

Two-Way ANOVA with data from 56 dpe confirms that SAV-exposure significantly affected the growth rate accounting for near 71% of variance with a p-value of <0.0001 (F (1, 66) = 171.0), whereas diet did not have any significant influence. Growth rate in the BC-group was reduced while growth rate in the NE-group increased slightly (Figure 17).

Examining data from all time points with the same analysis, factors being diet and sampling point showed that diet significantly affected growth rate the growth rate of fish in the BC-group with p-value of <0.0001 F (2, 247) = 10.6). Post hoc testing with Sidak's multiple comparisons test indicated a significant difference at 35 dpe between the BC-FA group and the BC-AA group yielding a p-value of 0.0235. No significant effects of diet were found in the NE-group (p=0.5123 F (2, 75) = 0.6748).

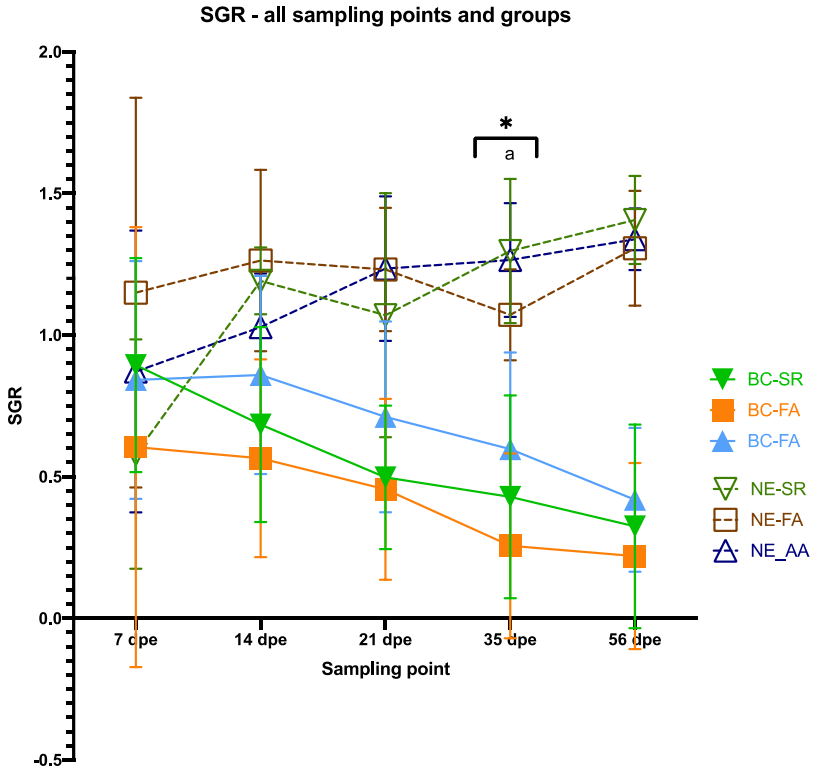


Figure 17 – SGR for all feed groups with mean and standard deviation. Significant differences are indicated for p<0.05 (*), different letters=significant differences between diets or treatments, p<0.05) BC=bath challenged, NE=non-exposed, SR=standard reference diet, FA=fatty acid modified diet, AA=amino acid modified diet.

5.3.3 VSI

Two-way ANOVA indicated that SAV-exposure affected weight of viscera (p= 0.0482 F (1, 43) = 4.137) and accounted for 8.4% of variance.at 56 dpe. No significant difference was found when looking at data from all sampling points in regard to diet for neither the BC- or NE-group.

5.3.4 HSI

Two-way ANOVA indicated that SAV-exposure significantly influenced the body/liver ratio at day 56 with a p-value of <0.0001 (F (1, 44) = 38.54).

HSI increased for diets in the BC-group, while body size was relatively larger in the fish in the NE-group (Figure 18). No significant differences were found when data from all sampling point were tested by two-way ANOVA. For diets in the BC-group p-value was 0.0598 (F (2, 255) = 2.848), while the NE-group had a p-value of 0.0446 (F (2, 75) = 0.815).

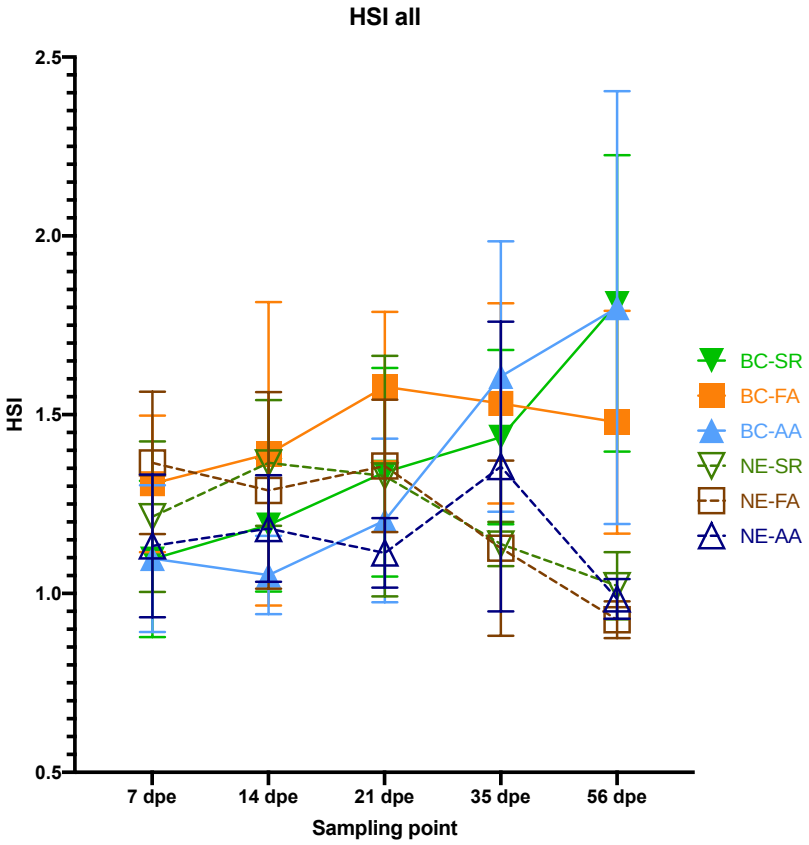


Figure 18 - Shows mean HSI with standard error for all diets at all sampling points. HSI for all NE-diets declined while BC-diets increased. BC=bath challenged, NE=non-exposed, SR=standard reference diet, FA=fatty acid modified diet, AA=amino acid modified diet.

5.3.5 Gut content

Gut content declined from 14 to 21 dpe when viral load in heart was at its peak. Prevalence of fish with gut content increased toward the end of the trial, the inverse of Figure 14.

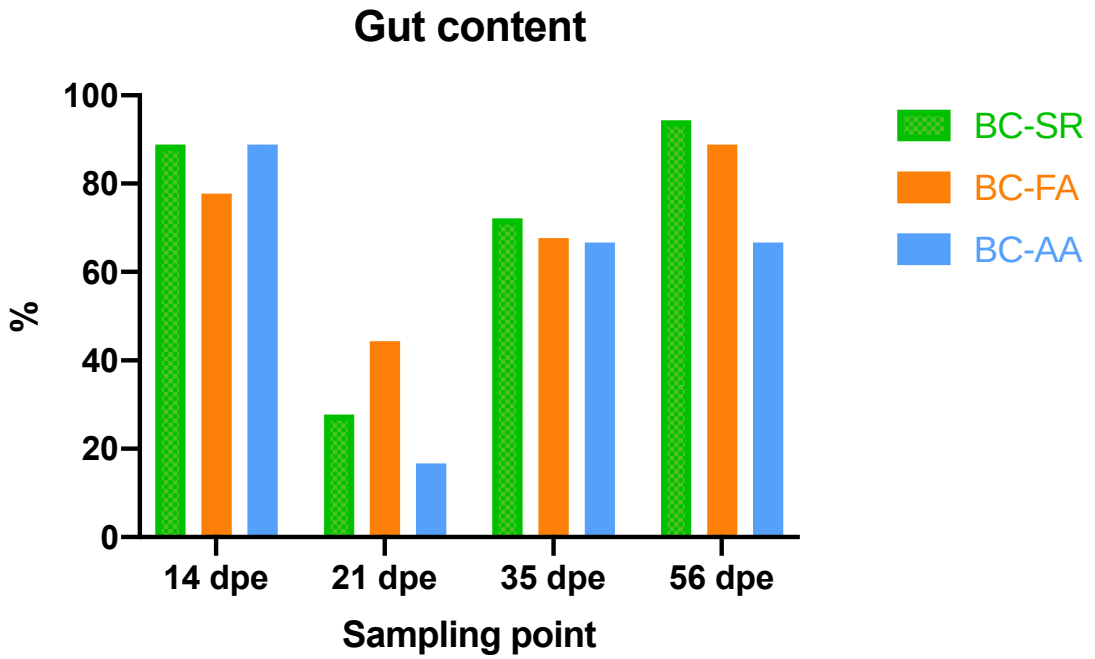


Figure 19 - Graph showing the percentage of fish that had either pellets in stomach and or faces in the intestine. BC=bath challenged, NE=non-exposed, SR=standard reference diet, FA=fatty acid modified diet, AA=amino acid modified diet.

5.4 Histopathology

5.4.1 Histopathological changes in heart

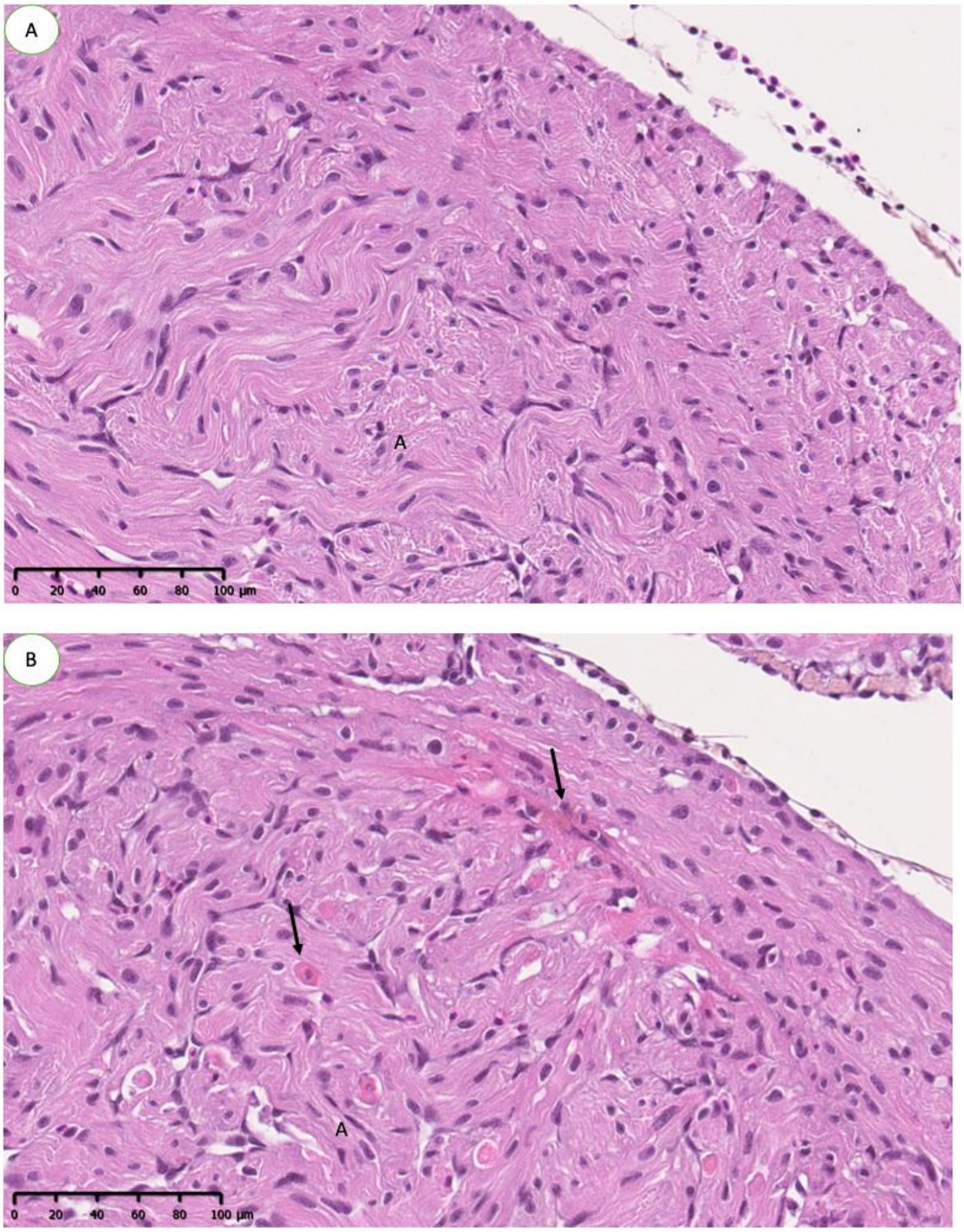


Figure 20 - Histological changes in heart tissue. Healthy tissue (A) from control fish and inflamed cells (B) below from one SAV-positive fish sampled at 14 dpe. Arrows indicate a multifocal eosinophilic degeneration and a necrotic myocyte.

Cardiac pathology almost absent at 56 dpe, with the exception of one individual with 0.5 in the BC-SR group. There was, however, some epicarditis (data not shown) although no visible inflamed fibres or any signs of tissue regeneration that could be determined.

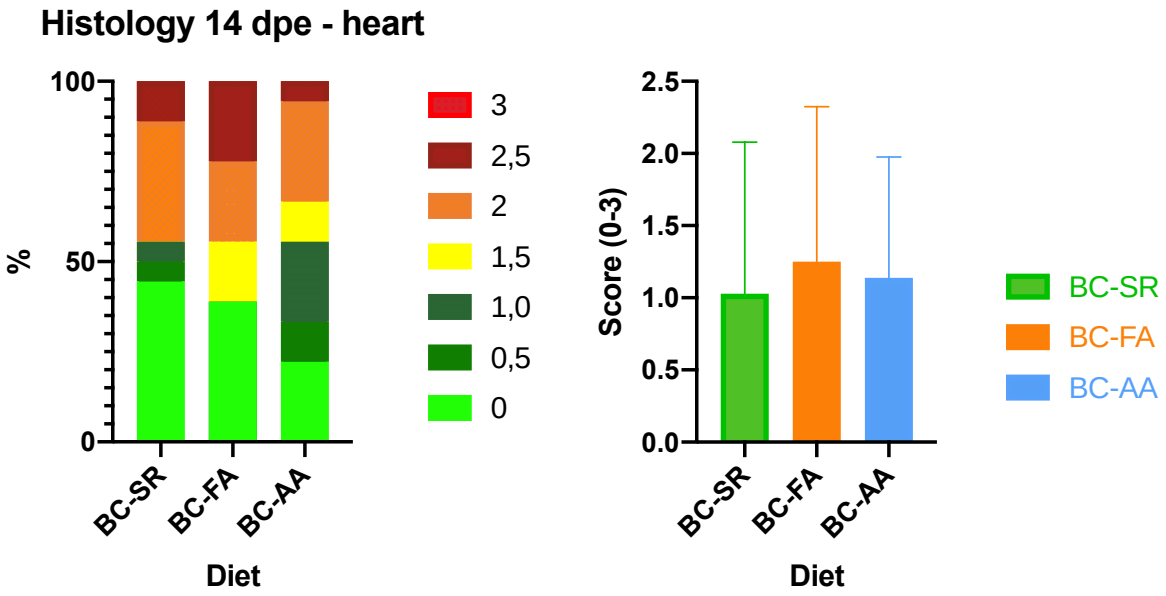


Figure 21 - Histology scores (0-3) for heart samples from 14 dpe. Left-hand side shows sorted by diet and color-coded in reference to severity. Right-hand side shows mean score with standard deviation sorted by diet. BC=bath challenged, SR=standard reference diet, FA=fatty acid, AA=amino acid.

Kruskal-Wallis test showed no significant difference between diets when comparing heart scores at 14 dpe, resulting in a p-value of 0.8272. Total sample size 54, n=18 for each diet. In **Error! Reference source not found.** the severity of histopathological changes colour-coded, the fraction of severe (≥2) scores is lower in BC-AA group, although mean score is lowest in BC-SR group as shown in the right-hand figure.

5.4.2 Histopathological changes in pancreas

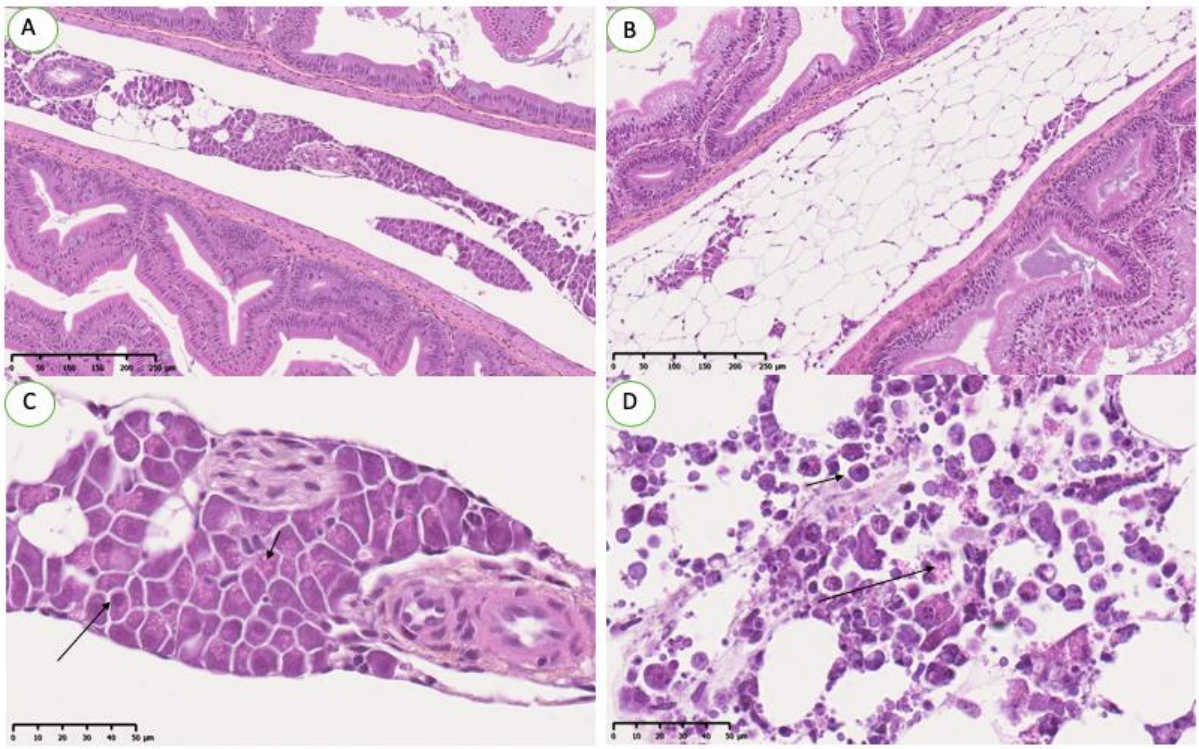


Figure 22- Histology from pancreatic tissue at 14 days post exposure (dpe). In (A) we can see healthy pancreatic tissue with pylorus tubules at either side. In (B) we can see a lack of exocrine pancreatic tissue and the necrosis of the visible pancreatic tissue is shown more closely in (D). In (C) we see healthy pancreas tissue from fish short arrow indicating zymogen granules and long arrow endobasal reticulum. In (D) pancreatic tissue from a SAV-positive fish is show long arrow indicating necrosis and short arrow showing a rounded acinar cell with pyknotic cell nucleus.

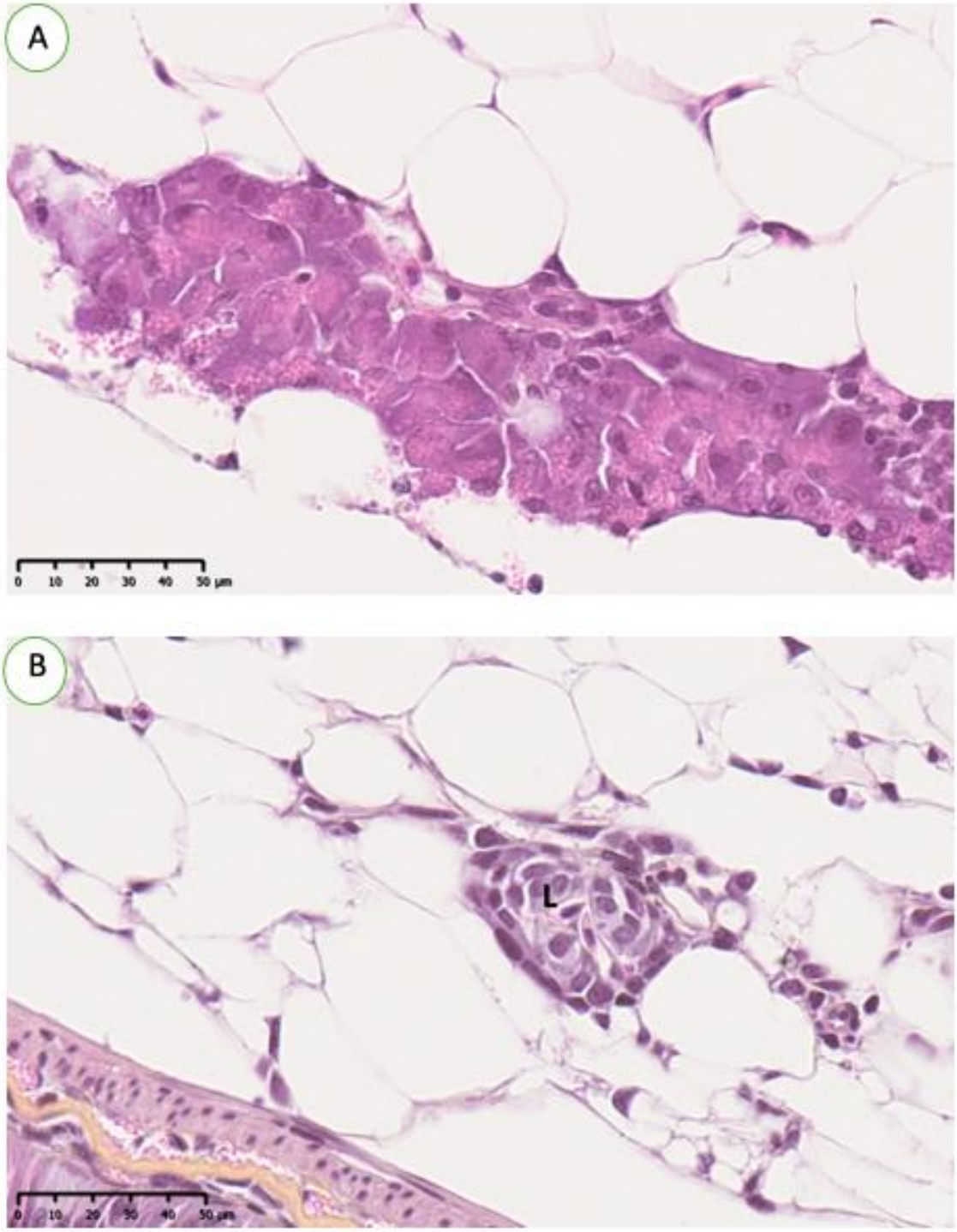


Figure 23 - Pancreatic tissue from sampling point 56 dpe. Healthy tissue is shown in (A). Below in (B) a complete absence of exocrine pancreatic tissue is shown, a Langerhans islet without surrounding exocrine pancreatic tissue is indicated with "L".

Exocrine pancreatic tissue was visibly affected in the majority of individuals from BC-groups at 14 dpe, and when comparing scores from 14 and 56 dpe the severity of tissue damage had increased in roughly the same percentage of sampled fish that were affected at 14 dpe (Figure 24). At 14 dpe there was still exocrine pancreas tissue left, however, this was inflamed and

necrotic, while at 56 dpe most of the individuals whose pancreas was affected there was a complete absence of exocrine pancreas tissue.

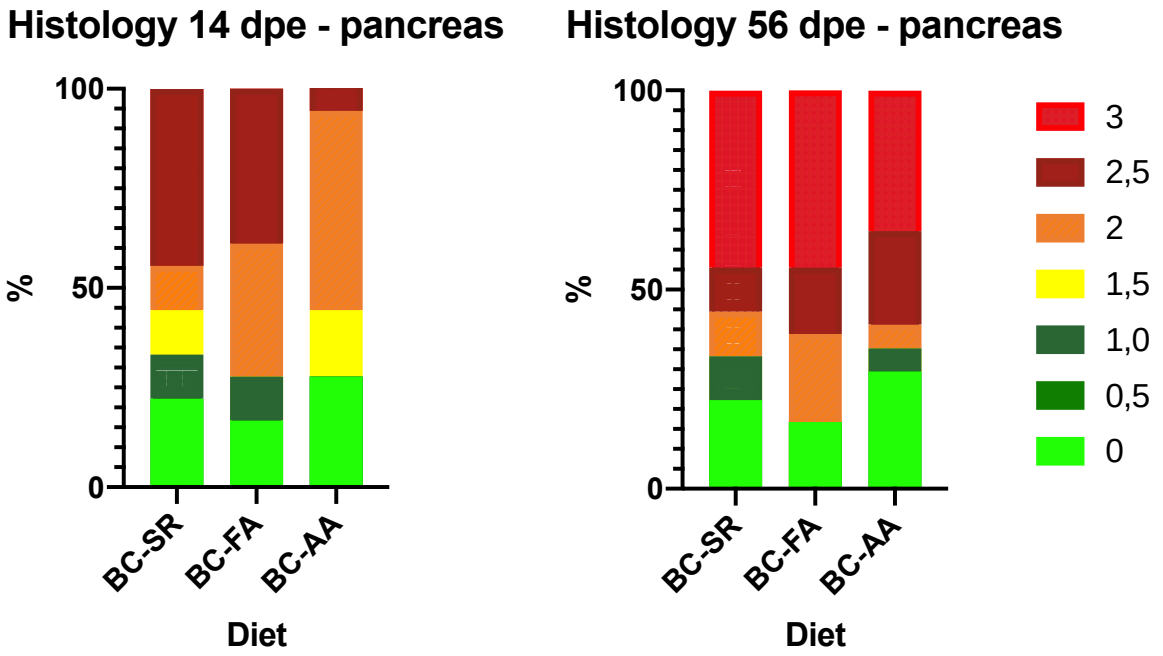


Figure 24 – Histology scores (0-3) from 14 and 56 dpe pancreas sorted by feed group and color-coded in reference to severity. BC=bath challenged, SR=standard reference diet, FA=fatty acid modified diet, AA=amino acid modified diet.

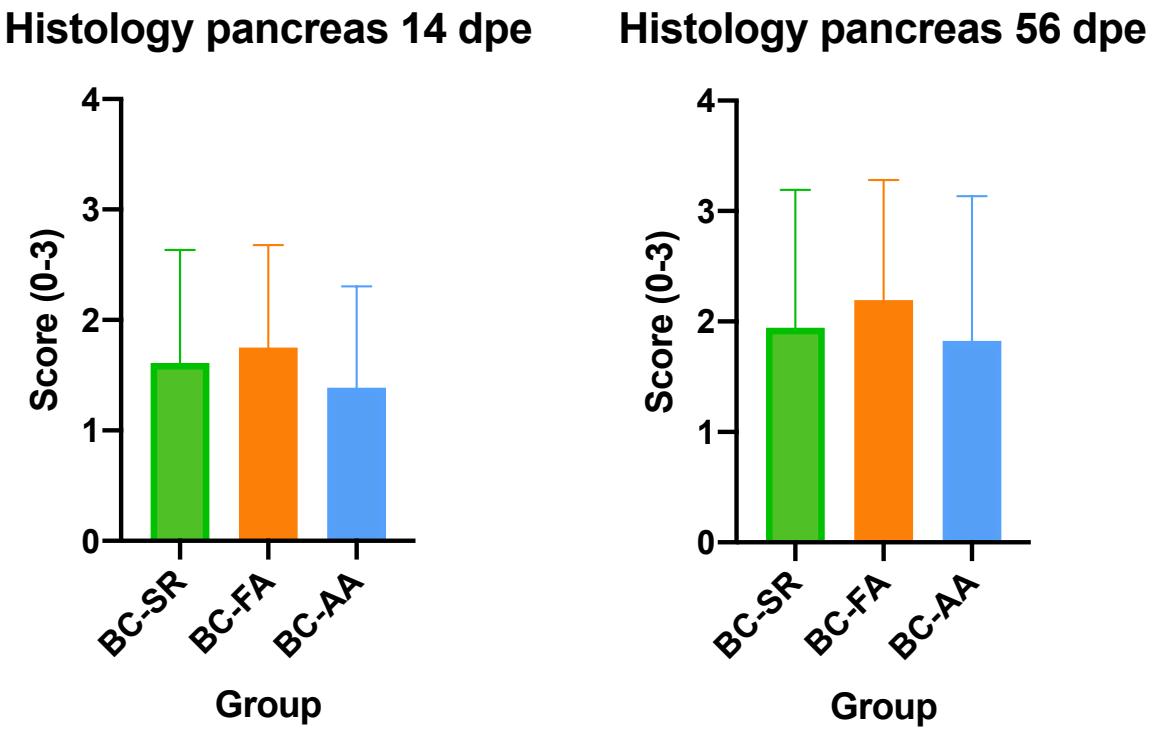


Figure 25 - Pancreas score (0-3) from 14 and 56 dpe shown with mean and standard deviation. BC=bath challenged, SR=standard reference diet, FA=fatty acid modified diet, AA=amino acid modified diet.

Statistical analysis of scoring data illustrated in the figures above (Figure 24 and **Error! Reference source not found.**) did not reveal any significant differences between diets at 14 dpe resulting in a p-value of 0.2269 (Kruskal-Wallis statistic= 2.966) or 56 dpe where the p-value was 0.7826 (Kruskal-Wallis statistic=0.4903). Running a two-way ANOVA with scores from both sampling points (14 and 56 dpe) did not indicate that diet influenced histopathological severity (p=0.3727, F (2, 101) = 0.9966)

5.4.3 Histopathological changes in muscle fibres

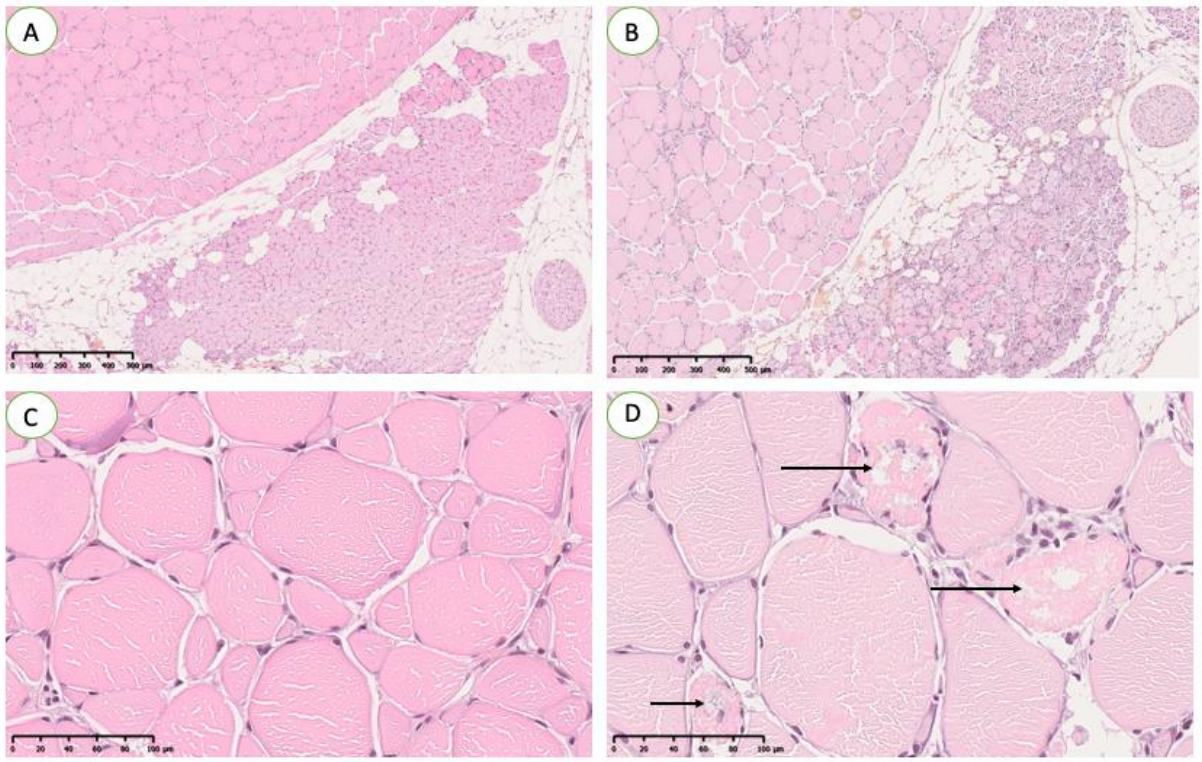


Figure 26 – Images (A) and (C) are from a healthy individual, the first showing an overview of both white and red muscle tissue, and (C) showing a closer look at healthy white muscle tissue for comparison to the images on the right-hand side. In (B) overview of muscle pathology and regeneration from a SAV-exposed fish is shown, image (D) being a closeup of degradation in white muscle tissue indicated with arrows. Near the degraded white muscle fibres regeneration is active. All fish are from 56 days post exposure.

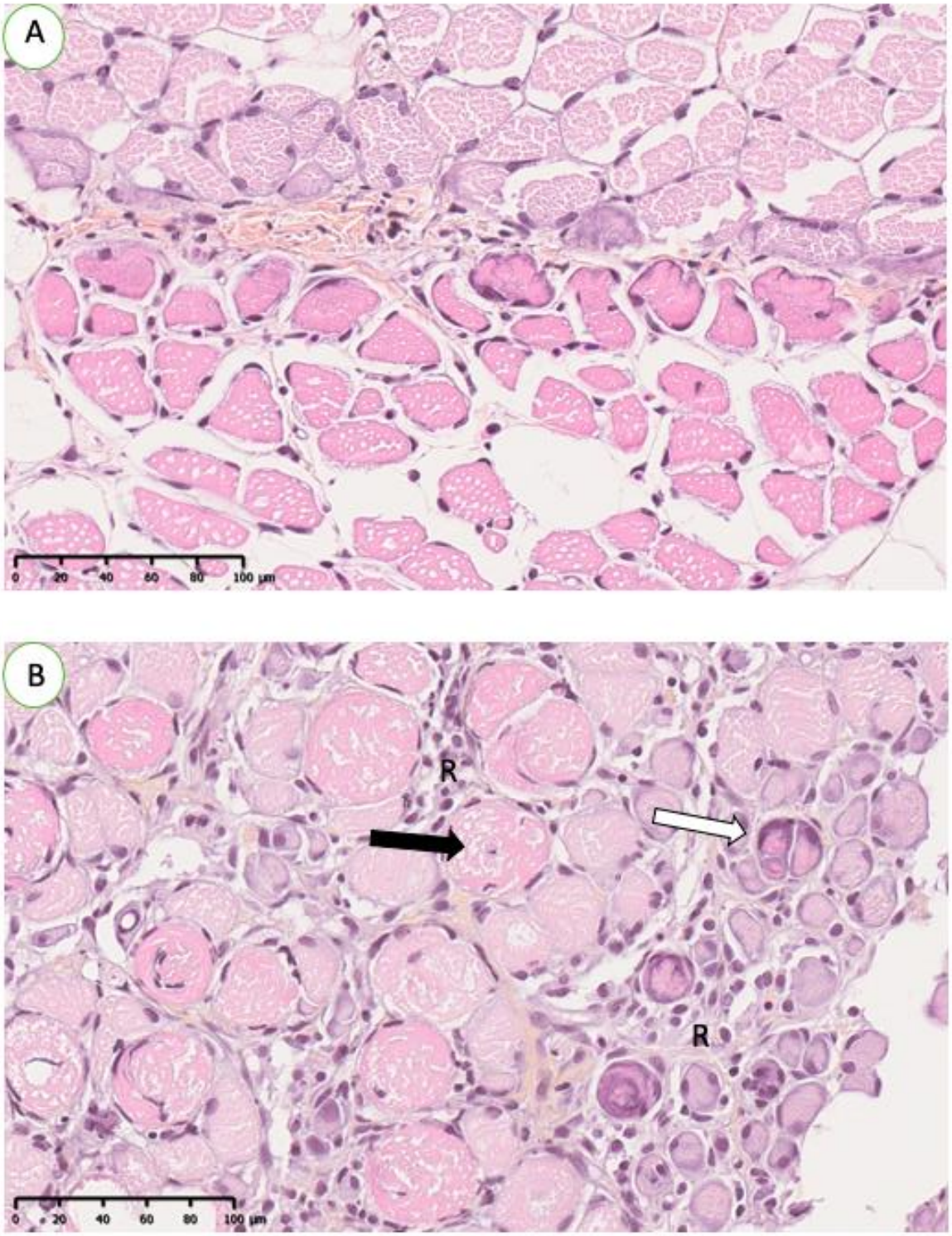


Figure 27- In (A) the black arrow marks degeneration of a white muscle cell while the white arrow shows red muscle cells. In (B) we can see degenerated red muscle cells with proliferation of cells and increased basophilia indicating attempted regeneration. Regeneration is indicated with "R" in the image.

The only muscle samples examined in this study were those from 56 dpe. The majority of these had active regeneration of red and white muscle cells in addition to some degeneration and

inflammation in both red and white muscle cells. To examine if viral load in muscle correlated with the histopathological score a Spearman's rank correlation test was run, results confirmed a significant correlation with p-value of 0.0001 (r=0.5302).

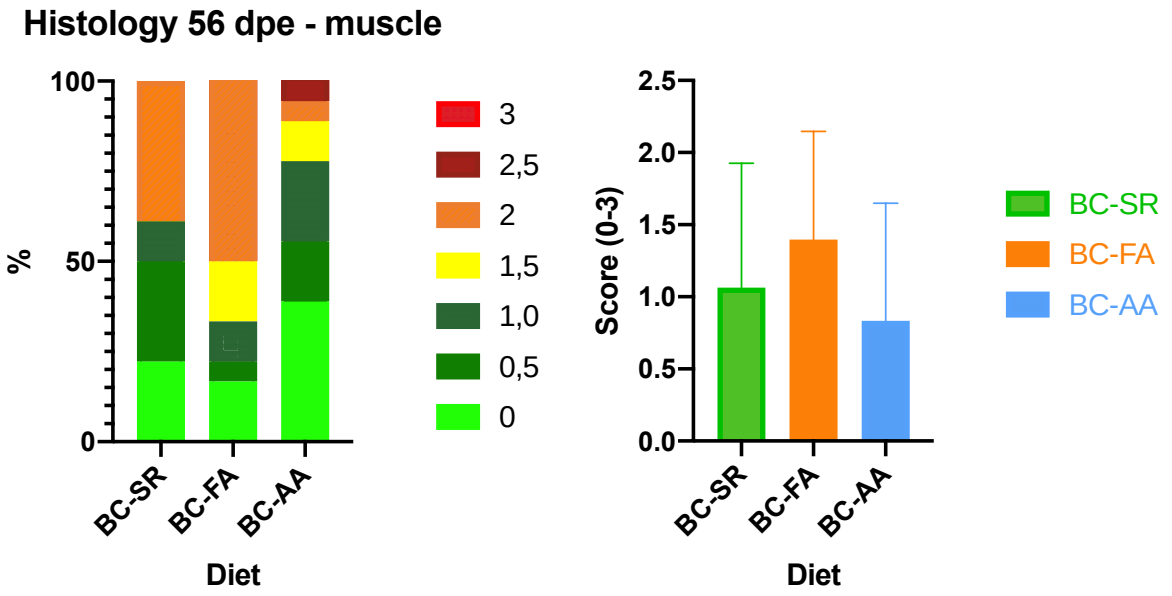


Figure 28 - Left-hand side shows histology score (0-3) for muscle samples from 56 dpe color-coded in reference to severity. Right-hand side shows mean histological score with standard deviation for each diet. BC=bath challenged, SR=standard reference diet, FA=fatty acid, AA=amino acid.

Kruskal-Wallis test did not indicate any significant differences BC diet groups in regard to histological score indicating p-value of 0.0784 (Kruskal-Wallis statistic=5.091). Although not significant, post hoc testing by Dunn's multiple comparisons test was done. This revealed that the AA-diet group had a lower score than the FA-diet group with a p-value of 0.0727.

5.5 Transcriptional analysis of muscle

SAV challenge induced a higher expression level of genes coding the antiviral proteins *IFNγ* (p=0.01), *mx* (p=0.0009) and *viperin* (p=0.001) compared to the NE-group, while no differences were seen for the expression level of *IL-1β* and *TNFα*. For *IL-10*, no expression was detected

in the muscle from NE-fish. No differences were seen in expression level in fish given different diets in FW (Figure 29).

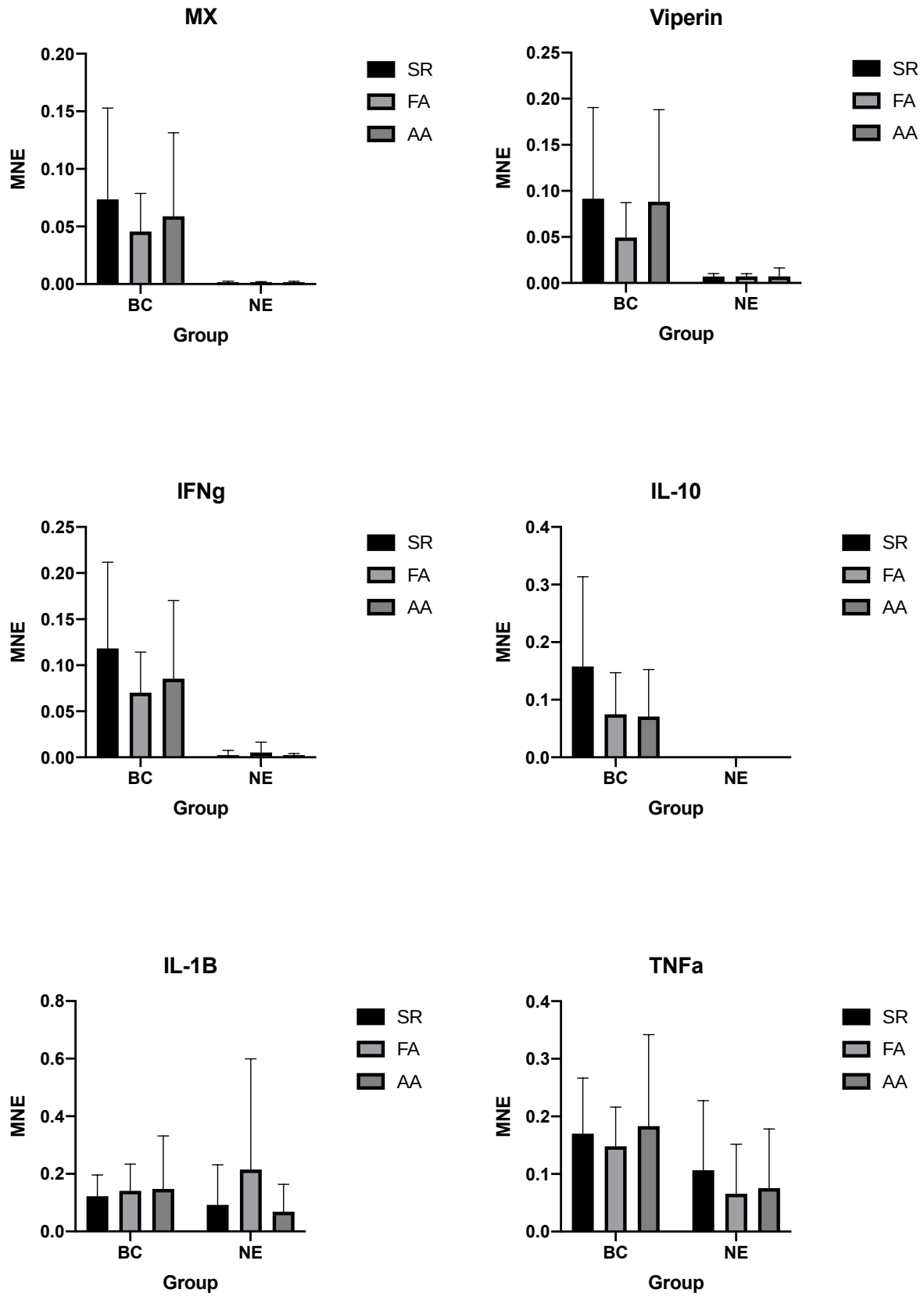


Figure 29 – Overview of mean expression with standard deviation (SD) of target gene with both BC (bath-challenged) and NE (non-exposed) from each diet. No significant differences were found between diets in expression levels within BC groups. There

was significant variation between BC and NE-groups coding for antiviral proteins.SR=standard reference, FA=fatty acid, AA=amino acid modified.

A significant correlation was seen between muscle score and expression level of mx, viperin, IFNγ, IL-10, TNFα and viral load (Table 7) in the BC-group. Similarly, muscle viral load correlated significantly with mx, viperin, IFNγ, IL-10, IL-1β, and TNFα as indicated in the table below.

Table 7 -Correlation between viral load, muscle score and transcription of antiviral and inflammatory genes in muscle. Significant values are indicated by **< 0.05** *whilst insignificant* >0.05*. All samples correlated are from the trial endpoint (56 dpe).*

Spearman Rank Order Correlations (Spreadsheet in Workbook1) MD pairwise deleted Marked correlations are significant at p <.05000								
Variable	MNE MX	MNE Viperin	MNE IFNγ	MNE IL-10	MNE IL-1β	MNE TNFα	Muscle score	Viral load heart
MNE MX		**0.859653**	**0.837929**	**0.676392**	0.139601	**0.664328**	**0.520636**	**0.900696**
MNE Viperin	**0.859653**		**0.756249**	**0.734461**	0.128962	**0.743267**	**0.351343**	**0.768647**
MNE IFNγ	**0.837929**	**0.756249**		**0.771106**	0.098676	**0.735251**	**0.451440**	**0.824312**
MNE IL-10	**0.676392**	**0.734461**	**0.771106**		0.152905	**0.712927**	**0.316703**	**0.712474**
MNE IL-1β	0.139601	0.128962	0.098676	0.152905		0.281046	0.118268	0.124416
MNE TNFα	**0.664328**	**0.743267**	**0.735251**	**0.712927**	0.281046		**0.282669**	**0.673147**
Muscle score	**0.520636**	**0.351343**	**0.451440**	**0.316703**	0.118268	**0.282669**		**0.579508**
Viral load heart	**0.900696**	**0.768647**	**0.824312**	**0.712474**	0.124416	**0.673147**	**0.579508**	

6.0 Discussion

The overall aim of this thesis was to examine whether feeds of different nutritional composition, given in the FW stage could affect susceptibility and or disease progression when challenging post-smolts with SAV3.

In order to evaluate any connections between nutritional status and disease progression factors such as SGR, condition factor, HSI, VSI, and viral load in both muscle and heart tissue were examined at selected time points after experimental infection. Additionally, pancreas, heart and muscle were histology examined from 14 and 56 dpe to provide indicators of the overall health status of the fish. Muscle tissue has previously been shown to be highly affected at 56 dpe (Braceland et al., 2013, Braceland et al., 2017). The possibility of the different diets affecting

the initial immune responses were also examined, based upon previous studies with SAV (Moore et al., 2017, Heidari et al., 2015, Herath et al., 2012).

In this study, bath immersion was chosen in order to most closely mimic the assumed natural route of transmission (Jarungsriapisit et al., 2016b). This was done to examine whether there was a discernible difference in susceptibility to SAV between diets. The established prevalence of SAV in heart tissue supports the efficiency of this bath model, although the exact dosage could not be determined. This challenge model does however entail some uncertainty with regards to when each individual became infected. Although it is likely that the majority became infected during the bath challenge some individuals may also have become infected at a later stage due to a "second wave" effect from viral shedding beginning 4-13 days after infection (Andersen et al., 2010). There might also be individual differences between fish, and viral load might not yet have reached a detectable level for RT-qPCR at the first sampling time points (Jarungsriapisit et al., 2016a, Jarungsriapisit et al., 2016b). However, due to the considerable increase in prevalence in the beginning of the trial it is likely that most individuals were infected at day 0. Additionally, we can speculate at what stage of disease progression an individual was at based on pathology and development of other growth and welfare markers (i.e. condition factor, SGR) as whole. Choosing to infect the fish by either i.p. or i.m. injection would have eliminated the possibility to assess potential differences in susceptibility, in addition bath immersion more closely resembles the route of transmission found in field conditions. Bath challenge gave us the opportunity track possibly increased resistance through natural barriers.

6.1 Prevalence

SAV infection persisted throughout the trial with SAV positive hearts and histopathological changes either in muscle and or pancreas. Therefore, the bath challenge was considered successful. Heart tissue has previously been identified as the most suitable tissue for SAV RNA detection (Andersen et al., 2007).

At the end of the trial all sampled fish from the bath-challenged group were SAV-positive. This is supported by findings in Braceland et al. (2013) that it takes 10-12 weeks to clear a SAV infection. A second wave infection 4-13 days after infection is a possibility (Andersen et al., 2007, McLoughlin and Graham, 2007). As expected there were no mortalities during the trial. In aquaculture, mortality due to PD has been reported to range from 80% to negligible, however the economic losses are mainly due to reduced growth (Aunsmo et al., 2010, Jansen et al., 2015). There have been difficulties replicating mortality observed in the field in SAV-trials under laboratory conditions. This could perhaps be due to lack of other environmental stressors and pathogens found in the field (Weston et al., 2002, Christie et al., 2007). Some also hypothesize that SAV-isolates used in trials have mutated into less lethal versions due to growth in cell cultures rather than the organisms from which the strain was originally isolated from. This could impact viral replication ability, thus becoming less virulent (Andersen et al. (2007) citing others). In Taksdal et al. (2015) various SAV2 and SAV3 isolates were tested *in vivo* confirming their hypothesis that difference in virulence exists between different isolates of the SAV-subtype. In this thesis the goal was to track how diet could affect growth and disease progression based on the feed in FW phase, but discrepancies in pathology and growth could also be attributed to differences in SAV isolates used when comparing results to other trials. There was not detected any differences in prevalence between diets suggesting that there was no notable difference in susceptibility to SAV which was one of the aims in this study to examine. It is however worth mentioning that the group fed the AA-diet had a lower prevalence consistently, and was the only group not 100% prevalent until the final sampling and that trends in growth and viral load seem to favour the AA-diet as opposed to the FA-diet. Specifically, one hypothesis was that the AA-diet may influence the barrier tissues, based on previous studies showing that threonine supplementation could influence mucus components (Li et al., 2009), and therefore possibly provide stronger barriers against pathogens. Threonine may also have played a role in protection against SAV as threonine is a component of mucin and thus the physical barrier against pathogens (Li et al., 2007).

6.2 Viral load

SAV-infection was confirmed by RT-qPCR of heart tissue from all sampling points, and muscle samples the trial endpoint. Results were used to assess viral load in the tissues. At the final sampling point it was discovered that the fish from the FA-diet had a statistically higher viral load with a p-value of 0.0478 when compared to the AA-diet which had the lowest viral load.

Findings in Lopez-Jimena et al. (2015) were opposite. In the mentioned study, rainbow trout were challenged with SAV-1 and differences between dietary n-3/n-6 PUFAs were examined. Higher n-6 ratio gave a lower viral load at 5 and 30 dpi (days past injection). The inconsistencies between the present trial and that of Lopez-Jimena et al. (2015) could be due to discrepancies between species and subtype of alphavirus used in the trials. There was also no difference in n-6/n-3 ratio of the diets used in the current study. Statistically there were negligible differences between the SR- and FA-diet in the current study suggesting that the difference in viral load can be attributed to effects of the AA-diet. The modifications in the AA-diet, increased threonine levels could have increased antibody production allowing for more efficient immune response (Li et al., 2007).

Overall pattern of viral load in heart was similar to that found in other studies (Andersen et al., 2007, McLoughlin and Graham, 2007). Viral load increased from 7 – 21 dpe before declining toward the end of the trial. Viral load in muscle tissue was tested only at 56 dpe and therefore the progression could not be examined in this thesis. The sampling point at which muscle was chosen based on what was expected to be the peak in viral load and histopathological changes as observed in other studies (Braceland et al., 2013). Previous studies have found that pathology and viral load in muscle «lags» behind that of heart tissue (McLoughlin and Graham, 2007, Andersen et al., 2007, Braceland et al., 2013). No significant differences were found between diets when viral load in muscle was examined.

6.3 Performance

Bath challenged fish had significantly lower condition factor and SGR compared to non-exposed fish at the end of this trial. The effects of PD on growth and condition have been well examined and overall results are as expected. Loss of appetite and reduced growth are clinical signs of a PD-outbreak (McLoughlin and Graham, 2007, McLoughlin et al., 2002, McVicar, 1987).

For the purpose of the present study it is important to note that the process of smoltification tends to reduce condition factor, since the population had recently smoltified prior to the start of the bath challenge (Farmer et al., 1978). It is difficult to define an exact "golden standard" for condition factor as it will vary depending on a myriad of factors in addition to pathogens such as life-stage (e.g. smoltification), environmental stressors, season (temperature) etc., however, Stien et al. (2013) suggested that a condition factor of 1.0 at the beginning of SW

production to 1.6 near slaughter are to be considered normal, and that <0.9 could be considered emaciated depending on life-stage.

Consistent with findings in e.g. Heidari et al. (2015) growth (SGR) of challenged fish was severely reduced at 14 dpe and continued to decline throughout the trial. Conversely the non-exposed group had increased growth throughout the trial and therefore the assumption is that SAV-exposure was the causation. As tank effects were taken into consideration when distributing fish and there were equal amounts of handlings/samplings in both NE- and BC-groups it is unlikely that differences are due to anything other than SAV exposure. In parallel to reduced growth, the condition factor was reduced in BC-groups. Decreasing condition factor suggests that fishes' energy reserves are diminishing as energy is being redistributed to fight off infection. Analysis of HSI and VSI-data throughout the trial confirmed that SAV-exposure significantly affected the development of these indexes. A starving fish will have decreasing VSI, while HSI increases as the liver weight is reduced when fat deposits are utilized elsewhere.

Starvation or malnutrition is a consequence of PD, and some fish that survive PD do not regain the ability to digest feed due to lack of digestive enzymes that are normally produced by pancreas in healthy fish (Larsson et al., 2012). In Lerfall et al. (2012) fish with slim body shape were found 6 months after the PD outbreak. Periods of starvation will influence the immune system and physiological performance, making the fish more susceptible to other pathogens as summarized by Waagbø (2006). For the purposes of this thesis, replication of potential comorbidity would have complicated our analysis further. It is common practice and often advised to reduce feeding during outbreaks of infectious disease to reduce metabolic activity, however this can be debated from both a fish health and economic perspective.

The trial did not last long enough to identify "runts". A 20 week long study has estimated >40% reduced weight gain in SAV1 and SAV3-infected fish compared to control (Christie et al., 2007), hence SGR and condition factor could have declined even further in this study. The reduced growth might be due to reduction in digestive enzymes as a result of damage or loss of pancreatic tissue (Pringle et al., 1992). Gut content was examined to gauge appetite, as loss of appetite is seen as an indication of PD in the field. The results indicate that appetite and viral load are linked, as appetite drops with rising viral load in tissues.

Differences in starting weight and length differed significantly between diets with the FA-group being shorter and weighing less than the two other groups at day 0 in the BC-group prior to challenge. We were not able to examine this prior to the trial as we distributed the fish randomly

and the overall weight and length of *all* received fish did not differ significantly. This statistic is based on the fish that were actually sampled throughout the course of this study. The same statistical differences were not found in the fish that were distributed into the NE-group at day 0. Sampled fish at day 56 in the BC-group reflected that there was a statistically significant difference in weight and length where the FA-group was still lighter and shorter than both the AA- and SR-group. Having been differences prior to challenge with SAV could potentially influence the outcome of growth analysis and should be taken into consideration.

In other studies such as McLoughlin and Graham (2007) recovery of pancreatic tissue has been shown from 35 dpe. In this study, regeneration was not examined due to the resolution of slides (these were electronically scanned at a given resolution), however severity of pathology increased from 14 to 56 as in Braceland et al. (2013). Results are similar to those in Taksdal et al. (2015).

Due to the nature of alphavirus replication, using the hosts' cell membrane when budding one hypothesis would be that influencing the host organisms' membranes by adjusting fatty acid content would in turn influence the replication of the virus. As there was no apparent advantage or disadvantage compared to the fish in the SR-diet group the assumption is that the increased amounts of n-6 in the trial diet did not affect SAV-replication.

6.4 Tissue damage

Only one fish showed pathological changes in the heart (score 0.5) at 56 dpe, whilst 64.8% of all BC fish showed pathological changes in heart tissue at 14 dpe – a mean score of 1.14. These findings are similar to that of the group injected with SAV3 isolate in Christie et al. (2007) with a mean score of 1.3. In the mentioned study, severity of heart pathology increased until 21 dpe and decreased from 35 dpe. At 70 dpe, pathological changes were nearly absent. Peak in heart pathology is typically found at 14-28 dpe (Heidari et al., 2015, Braceland et al., 2013, McLoughlin and Graham, 2007). In the current study, changes in heart tissue were examined at 14 and 56 dpe. In Taksdal et al. (2015) where Atlantic salmon were i.p. injected with different SAV subtype 2 and 3 isolates heart pathology was still present at 56 dpe, however in the current study only one individual had a score of 0.5. The fish used in said study were of similar weight (79.3g) as those bath-challenged in the present study (83.3±10.3 all diets compiled). In McLoughlin and Graham (2007) it is suggested that younger fish have the ability to replace

damaged cardiomyocytes by cell division in a way that is not present in older fish, resulting in different pathogenesis and impact of PD infection in the heart of Atlantic salmon if infected at different life stages; smolt versus grower. In zebra fish *in vivo* studies suggest that naturally occurring heart regeneration in lower vertebrates is based on cardiomyocyte proliferation (van Amerongen and Engel, 2008). In Taksdal et al. (2007) fish without significant heart lesions but with skeletal muscle lesions and loss of exocrine pancreatic tissue were found. Another reason could be differences in virulence of SAV isolates as previously discussed. As heart samples were taken at all sampling points future studies could examine the remainder of the samples, seeing a more complete overview of disease progression in the heart tissue and or be verified by transcriptomic tests.
There was not found any significant differences in regard to histopathology in pancreas tissue between diet groups. A theory was that there would be increased damage in the pancreatic tissue in the FA-diet group compared to the two other diets due to the increased amount of omega-6 fatty acids.

In the present trial the FA-diet was designed to mimic the fatty acids found in the natural diet of Atlantic salmon in FW phase (Bell et al., 1994), to investigate if a dietary composition that more closely resembles what the fish eat in the wild may be beneficial. The n-3/n-6 ratio was similar in the feeds, but the total amount of PUFAs was higher in the FA diet, in part due to the increased amount of ARA and EPA in the diets. The present results suggest that the fatty acid composition in the FW feeds did not influence disease progression or tissue damage during SAV3 challenge. Similarly, Lopez-Jimena did not find differences in tissue damage in rainbow trout fed diets with different n-3/n-6 ratios challenged with SAV1. However, previous studies have shown that altering the ratio between n-3 and n-6 fatty acids may influence tissue damage after other viral infections. In Martinez-Rubio et al. (2012) it was shown that diets containing 2.45 and 3.59 n-3/n-6 PUFA ratio reduced heart pathology and inflammation as a result of PRV compared to the ratio in a standard commercial diet. High omega-6 diets have previously been associated with increased inflammation (Thompson et al., 1996).

Based on the present results, it appears that a dietary FA composition closer to what the fish eat in nature does not influence susceptibility to SAV3 or tissue damage. There was however a non-significant indication of a higher number of severe scores in heart tissue at 14 dpe compared to both the SR and AA that should be followed up in future studies.

This trial differs from many other trials featuring functional feeds in the sense that the fish are not fed different diets *during* challenge, only prior to. Only changing diet prior to SW transfer allows testing of possible "long-term" effects. In addition, fish infected with PD have reduced appetite as one of the clinical signs therefore treating this disease through dietary means becomes challenging, and probably less efficient than potentially being able to achieve a prophylactic effect through change in FW diet. For instance, Dessen et al. (2019) showed that increasing the ratio of protein-to-lipid (P/L) content reduced mortality of Atlantic salmon during a natural PD outbreak and delousing treatments. The number of so-called "runts" decreased, and the amount of superior grade quality fish increased in the group fed increased P/L-ratio. Considering that fish do not reach slaughter weight of 4-5kg before 12-18 months after SW, the findings in this study would have to be adjusted to relevant time frame and fish size.

Damage to muscle tissue is of special interest from a commercial stance as this constitutes the final "product" that is sold. Certain parameters (i.e. firmness, colour, melanin etc.) are used to grade filets with prices adjusted thereafter, a comprehensive review on these methods is written by Sigurgisladottir et al. (1997). One of the aims of the present study was to investigate whether the freshwater diets could influence disease progression and recovery after SAV infection. There are few studies that have followed muscle pathology until 56 dpe or later with exceptions such as Taksdal et al. (2015), Christie et al. (2007), Heidari et al. (2015), (Braceland et al., 2013) and Braceland et al. (2017). Pathology in muscle appears 3-4 weeks after changes in heart and pancreatic tissue (McLoughlin and Graham, 2007).

When histological scores from the final sampling point were analysed no significant differences were found between diets. However, the AA-group had lower severity of muscle lesions compared to FA but not significant, yielding a p-value of 0.0727. Placing this into the context of overall lower prevalence and lower viral load in heart tissue found in the BC-AA group indicates a positive trend for the AA-group. The AA-diet in the present study has increased amounts of taurine, lysine, threonine and methionine. Which as presented in the introductory section on amino acids in fish nutrition has been shown to have major effects on both the innate and acquired immune system. For instance, taurine supplementation has been shown to reduce production of pro-inflammatory cytokines and prevent apoptosis in liver cells *in vitro* (Li et al., 2007, Espe and Holen, 2013), and it may be hypothesised that this played a role in the present study reducing muscle pathology in BC-AA group. Threonine may also have played role in

protection against SAV being a component of mucin and thus the physical barrier against pathogens, in addition threonine has been to aid in preventing cell apoptosis and promote antibody production (Li et al., 2007).

The AA-diet can have short-term benefits versus an FA-diet during a certain stage of the disease trajectory. In Heidari et al. (2015) it is hypothesized that during the maximal innate immune response the fish will reallocate amino acids and energy reserves from muscle protein to continue enabling an efficient immune response. This hypothesis is based on the upregulation of genes involved in protein degradation at 28 dpe.

Silvery-black porgy (*Sparidentex hasta*) juveniles fed amongst others threonine-deficient diets have been shown to have lowered immunological competence, the fish fed the threonine-deficient diet were also found to have the lowest survival rates (Yaghoubi et al., 2017). Threonine has been reported to maintain intestinal integrity and immune functions especially in innate immunity in the humoral and cellular mechanisms. In previous studies such as Habte-Tsion et al. (2015) and Li et al. (2009), threonine has been found to affect gene expression of amongst them TNFα however, in the present study no significant differences have been detected in neither the bath challenged nor the non-exposed fish. The interpretation of these results was complicated by uncertainty as to what phase of infection the fish were in. It is however likely that fish were in late stages of disease progression and beginning to recover considering results from muscle histology and heart pathology.

Higher survival rates were also found in European seabass (*Dicentrachus labrax*) fed a methionine supplemented diet after challenge with *Photobacterium damselae* subsp. piscicida (Machado et al., 2018). Results from the mentioned study observed an enhancement of immune status where higher cellular proliferation led to improved immune response to the infection. Observations were improved plasma humoral immune parameters and modulation of key immune-related genes.

6.5 Transcriptional responses

In the present study transcriptional responses did not indicate any significant differences between diets. Regulation of antiviral proteins and inflammatory genes were upregulated in response to SAV-infection in the BC-group. NE-group showed little to no expression of target genes, this confirms that there is upregulation of target genes in the BC-group as a response to SAV. In Heidari et al. (2015) target genes in both innate and adaptive immune system peaked at 4 weeks past challenge and then declined. These results are based on a cohabitation challenge model, and similarly to bath challenge it is difficult to pin-point exact time of infection.

Proinflammatory genes such as TNFα and Il-1β were examined in muscle tissue however interpretation of the results is complicated by the uncertainty as to what phase of disease progression the fish were in. The overall trend that the AA-diet performed favourably in growth and pathological development should be followed up in future studies.

6.6 Summary

The overall aim of this thesis was to examine whether feeds of different nutritional composition, given in the FW stage could affect susceptibility and or disease progression when challenging post-smolts with SAV3.

No significant differences were found in regard to susceptibility to SAV3 due to the diets used in the present study. All growth and welfare indicators were significantly influenced by SAV-exposure. In regard to growth in the bath-challenged group the AA-group had a higher weight and length toward the end of the trial compared to the FA-group. Additionally, the bath-challenged fish fed the FA-diet had a slightly higher mean score in heart pathology at 14 dpe, in addition to significantly higher viral load in heart tissue at 56 dpe, which warrants future studies. In regard to viral load and muscle pathology a slight albeit insignificant advantage was detected in the bath-challenged fish fed the AA-diet. These indications should both be followed up in future studies.

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