Co-infection of Atlantic salmon (*Salmo salar*) by *Lepeophtheirus salmonis* and *Moritella viscosa*

Effect on growth, disease development and expression of immune genes

Hege Sørvåg Hauge

Thesis for the degree

Master of science in Aquamedicine

Department of Biology
University of Bergen

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II

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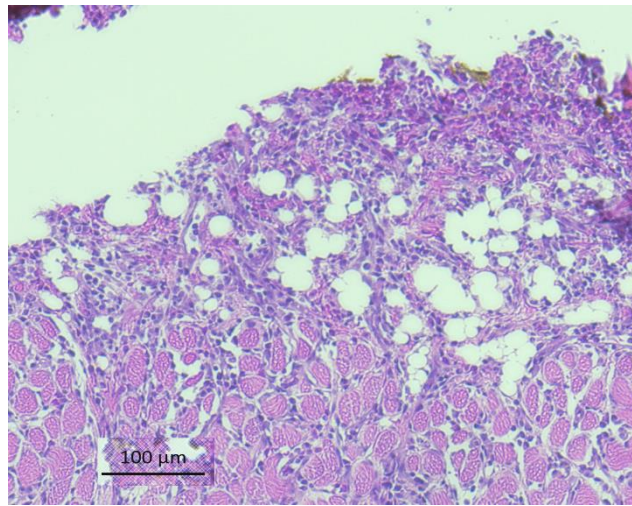Department of Biology
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03.06.1

Co-infection of Atlantic Salmon (Salmo salar by *Lepeophtheirus salmonis* and *Moritella viscosa*)

Hege S. Hauge

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Abstract

A co-infection is defined as an infection with two or more different pathogens, where the pathogens can either have a synergistic or antagonistic effect or alternatively not affect each other at all. *Lepeophtheirus salmonis* are the single largest problem found in salmonid farming today. It is a marine ectoparasitic copepod (*Caligidae*) occurring on both wild and farmed salmonids in cold temperate waters in the North Atlantic and North Pacific Ocean. They feed on mucus, skin and blood of their host leading to mild skin lesions that can disturb the osmotic balance. *Moritella viscosa* is a gram-negative bacterium thought to be the main agent causing winter ulcer disease in salmonids and occurs when water temperature is under 10 ºC. Clinical signs are necrosis of the skin, which advance to skin lesions on the flank, ranging from raised scales to larger lesions with exposed muscle tissue. This study looks at the co-infection of *L. salmonis* and *M. viscosa* and how these two pathogens affect growth, disease development (lice and ulcer number, size and severity) and transcription of immune genes. Study fish were sampled at four time points: (A) 5 days prior to infection, (B) 6 days post infection (dpi) *L. salmonis*; 7 dpi *L. salmonis* and 2 dpi *M. viscosa* [co-infection] dpi. (C) 26 dpi *L. salmonis*, 23 dpi *M. viscosa* [co-infection]. (D) 40 dpi *L. salmonis*, 37pi *M. viscosa* [co-infection]. During sampling, weight, length, condition factor (K-factor) was measured; lesions were counted, sized and graded; Lice were counted and staged. Transcription of immune genes Interleukin 1b (IL-1b), Interleukin 4 (IL-4), Interleukin 8 (IL-8), Interleukin 10 (IL-10), complement protein 3 (C3) and Immunoglobulin M (IgM). $2^{-\Delta\Delta Ct}$ were analyzed from sampling B and C in skin and head kidney with Elongation factor α (EFα) as a reference gene. Results showed that a *M. viscosa* infection (both single and co-infected) did affect weight and k-factor compared to control and *L. salmonis*., but no differences between a M. viscosa single and co-infection. *M. viscosa* were to be able to develop or infect faster under a single infection but over time there were more ulcers on co-infected fish. Also, a larger area of the fish was covered in ulcers for co-infected fish. No difference was observed in grade/severity of lesions. Similarly, there were more *L. salmonis* in a single infection compared to co-infection. The co-infection did not affect developmental rate of *L. salmonis*. There was a higher expression of pro-inflammatory genes (IL-1β and IL-8) for co-infected fish at time B. And a lower expression of anti-inflammatory genes (IL-4/13A and IL-10). IL-4/13A had a lower expression in almost all skin sites, and co-infected head kidney. IL-10 B was significantly lower for all co-infected sites at time B. There were no clear differences in expression of C3 and IgM for co-infected fish.

Acknowledgements

Bergen, 3. June 2019

Hege S. Hauge

List of abbreviations

APC	Antigen presenting cell
AVC	Atlantic Veterinary College
BSA	Bovine serum albumin
C3	Complement component 3
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming units
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
Ddpi	Degree days post infection
Dpi	Days post infection
EFα	Elongation factor 1α
FAO	Food and aquaculture organization
gDNA	Genomic deoxyribonucleic acid
HES	Hematoxylin, Eosin, Saffron (stain)
Ig	Immunoglobulin
IgM	Immunoglobulin M
IL	Interleukin
IL-1β	Interleukin 1β
IL-4	Interleukin 4
IL-8	Interleukin 8
IL-10	Interleukin 10
IPMC	Integrated Pathogen Management of Co-infections in salmon
K-factor	Condition factor
LPS	Lipopolysaccharide
NTC	Non-template control
PAMP	Pathogen associated molecule pattern
PCR	Polymerase chain reaction
RAS	Recirculating aquaculture system
RNA	Ribonucleic acid
R.T.	Room temperature
-RT	No reverse transcriptase
SLRC	Sea lice research center
TB	Toludine blue (stain)
TBST	Tris-buffered saline tween
Th1	Type 1 helper T cell
Th2	Type 2 helper T cell
TSA	Tryptic soy agar
TSB	Tryptone soya broth
UIB	University of Bergen

Glossary

Anadromous fish: Fish that live in saltwater and migrate to freshwater rivers to spawn (Folmar & Dickhoff, 1980).

Antagonistic effect: (of pathogens) Two or more pathogens that interact and suppress the effect/virulence of each other (Kotob *et al*., 2016).

B cell: Lymphocytes that secrete antibodies, play an important part in the adaptive immune system (Magnadòttir, 2006).

Barophilic: Organisms capable of growth and reproduction at high pressure (Zobell & Morita, 1957).

Co-infection: Infection of two or more pathogens (Kotob *et al*., 2016).

LPS: A component in the cell wall of gram-negative bacteria (Warr & Simon, 1983).

Macrophages: White blood cells that detect, phagocytes and kill harmful bacteria and other organisms (Magnadòttir, 2006).

PAMP: Patterns of molecules that are commonly associated with pathogens (Magnadòttir, 2006).

Prevalence: Percentage of the population infected.

Psychrophilic: Organisms capable of growth and reproduction at low temperatures (Morita, 1975).

Synergistic effect: (of pathogens) Two or more pathogens that interact and increases the effect/virulence of each other (Kotob *et al*., 2016).

T cell: Lymphocytes with many different functions as there are several types of t- cells that function in different ways. Are especially important in intracellular infections (Magnadòttir, 2006).

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1   Introduction

Aquaculture is a growing industry, and in 2016 more than 80 million tons of food was produced from aquaculture worldwide according to Food and Agricultural Organization (FAO). Norway's aquaculture fish production was 1,326 thousand tons, which was 1.7% of the worlds production. From 2000 to 2016 aquaculture grew with over 5% (FAO, 2018). With a growing world population there will be a need for more food. As fish are one of the most resource effective meats and has a lower carbon footprint compared to land-based meat production, it is assumed that aquaculture will play an essential role in the future (Winther *et al.,* 2009). For aquaculture to grow we must deal with pressing issues limiting growth, fish welfare, and the economic aspects. Diseases have been a problem since the beginning of aquaculture, and some have been easier to overcome than others. Right now, the single largest problem affecting aquaculture in Norway, and many other parts of the world, is the salmon louse, *Lepeophtheirus salmonis,* creating significant economic losses and fish welfare in attempts of controlling it (Bruno *et al.,* 2013). *Moritella viscosa* has also been a persistent pathogen which, despite routine vaccinations and antibiotic treatments, is still not entirely under control (Coyne *et al.,* 2004)**.**

1.1 Atlantic salmon (*Salmo salar*)

The Atlantic salmon is an anadromous fish, they spawn, hatch, and spend the first part of their lives in fresh water, and migrate to the ocean for foraging (Folmar & Dickhoff, 1980). The eggs are deposited in the gravel of freshwater rivers. After hatching, the fry remains in the river until they are fully smoltified. This can take anywhere from one to eight years, depending on river characteristics, two to three years being the most common. After smoltification, the salmon migrates to the sea (Metcalfe & Thrope, 1990). The smoltification is a process that alters the physiological, behavioral, and morphological characteristics enabling the salmon to live in a pelagic and salty environment. A parr is bottom-dwelling, territorial, and has distinctive parr marks, which are vertically dark pigmented lines allowing for salmon to blend into the riverine background. During smoltification salmon turns silvery, losing the parr marks, and develops a more streamlined body (Folmar & Dickhoff, 1980). Smoltification also increases the seawater adaptability of the salmon. This is mainly due to increased Na^+/K^--ATPase activity in the gills, increasing hypo-osmoregulatory capacity. There are also observed changes in hormones like

thyroid, growth hormone, cortisol, and more (Purnet *et al.,* 1989). Migration occurs in late spring, and after a period of one to four years in seawater, the salmon will return to their nursery river to spawn (Metcalfe & Thrope 1990; Jonsson *et al.,* 1991).

Cultivation of Atlantic salmon in aquaculture starts with the fertilization of eggs and hatching in freshwater. Hatched salmon are usually placed in tanks on land where the water flow contains treated freshwater or a recirculating system. After smoltification, the fish are ready to be placed in sea cages where they stay until slaughter. There are several challenges at all salmon life stages linked to disease and welfare (Hansen, 1998). In that last stage, pathogens like bacteria, viruses, and parasites all pose a threat. *Vibrio anguillarum* and *Aeromonas salmonicida* are two bacteria that have posed as big threats to aquaculture but have been kept under control thanks to vaccinations, but for the bacteria *M. viscosa* vaccinations and antibiotics have not been as effective. Common and problematic parasites include Amoebic gill disease (AGD) and *L. salmonis,* which despite treatment and constant surveillance, are still not under control. Pancreas disease (salmonid alphavirus, SAV) is a virus that has not been stopped, despite several new vaccines against SAV on the market. Cardiomyopathy syndrome (Piscine myocarditis virus, PMCV) is another virus where there are no current prophylaxis methods except general fish welfare. There are many more diseases that are problematic to varying degrees based on season, temperature, water quality, location, and more. All these diseases can affect welfare, slaughter quality, and potential for growth of the industry in the future (Hjeltnes *et al.,* 2018).

1.2 Co-infections

In experiments under controlled lab conditions, one single pathogen is often studied at a time to understand various mechanisms of the pathogen; however, in nature co-infections are common. A single infection, therefore, does not simulate a realistic host response in what is expected to see during the lifetime of salmon in aquaculture. A co-infection is defined as an infection with two or more different pathogens, where the pathogens can either have a synergistic or antagonistic effect or alternatively not affect each other at all (Cox, 2001). A pathogen causes harm to the host, and its virulence could be modified depending on other pathogens the host is carrying. Synergistic effects can include immunosuppression or physical damages to the skin caused by one pathogen, making way for the second pathogen and resulting in increased severity of pathogenic impacts on the host (Telfer *et al.,* 2008; Bradley & Jackson, 2008). Antagonistic effects will give a reduced infection of the second pathogen; this could be due to the first pathogen activating the host's immune system, making it prepared so it can easier fight an infection that uses similar entry mechanisms (Andrews *et al.,* 1982). Lastly, two pathogens can also not affect each other, possibly due to specific tissue tropism. Results that indicate no interactions could also be due to focusing on the wrong aspects of the disease. This study looks at a heterologous co-infection with a parasite and bacterium. Parasitic infections are known to increase the risk of secondary bacterial diseases, and most co-infection studies on fish these two types of pathogens show a synergistic interaction (Kotob *et al.,* 2016).

1.3 Salmon lice (Lepeophtheirus salmonis)

L. salmonis are the single largest problem found in salmonid farming today (Costello, 2009). It is a marine ectoparasitic copepod (*Caligidae*) occurring on both wild and farmed salmonids in cold temperate waters in the North Atlantic and North Pacific Ocean (Bruno *et al.,* 2013). There are two subspecies of *L. salmonis salmonis* and *L. salmonis oncorhynchi,* which are reproductively compatible, but there are some biological and genetic differences (Skern-Mauritzen *et al.,* 2014).
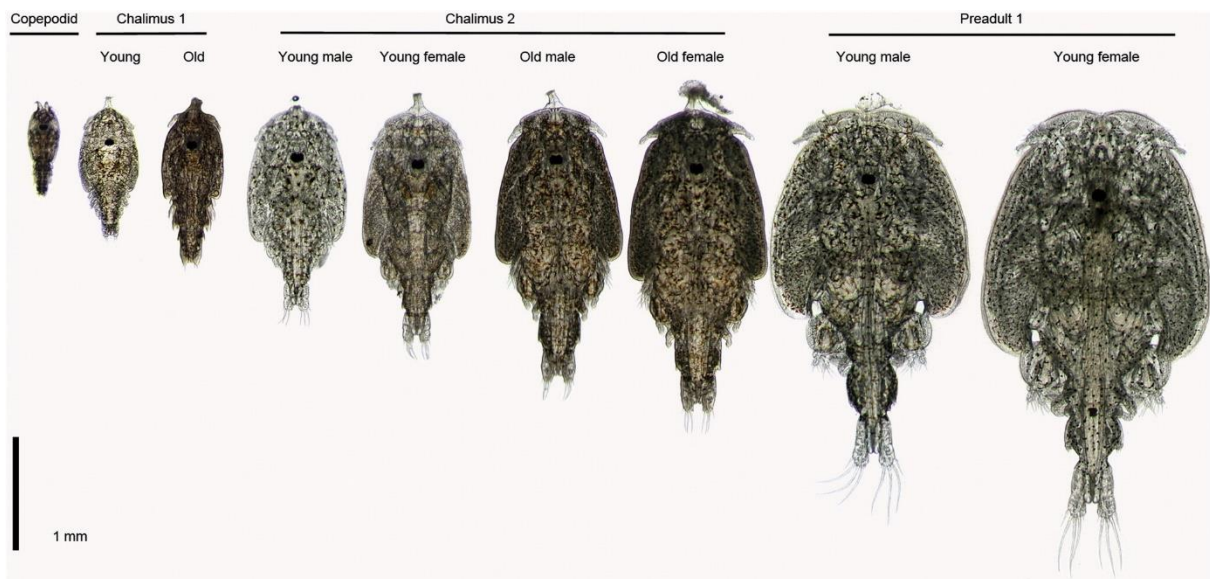
Fig. 1: Photographs of developmental stages of *L. salmonis* from copepodid to pre-adult 1 (Eichner *et al.,* 2015)

1.3.1 Life cycle

Lepeophtheirus salmonis life cycle consists of a total of 8 stages all separated by a molt. Two stages are free-living, one infective, and five parasitic stages (Johnson & Albright 1991b, Hamre *et al.,* 2013). The nauplii (nauplii 1 and 2) are planktonic and become infective after reaching the copepodid stage, which is when it starts searching for a host (Johnson & Albright, 1991a). A copepodid responds to disturbances in the water (pressure waves) and swims towards whatever is making these disturbances. It finds a potential host, and before attachment, chemosensory mechanisms are used to determine if it is on a salmonid (Bron *et al.,* 1991; Komisarczuk *et al.,* 2017). Attachment is only possible after molting to chalimus 1 when the frontal filaments have developed. Initial attachment of chalimus usually occurs on fins, especially the dorsal fin (Pike *et al.,* 1993; Tully *et al.,* 1993). The chalimus stages (chalimus 1 and 2) are attached to the skin through their frontal filament and is fixed to this location. The pre-adult stages (Preadult 1 and 2) and adult stage are attached through suction from the

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cephalothorax and are consequently mobile and can move around on the fish and in-between individuals (Johnson & Albright, 1991a). The mobile stages are mostly found on the lower half on the body (Bui *et al.,* 2017).

The sex ratio is approximately 1:1 in both laboratory and field (Ritchie *et al.,* 1996). The male louse develops quicker than the female louse (Costello, 1993). *L. salmonis* exhibits sexual dimorphism, as the genital complex of females is bigger and has protruding posterolateral lobes compared to the males (Eichner *et al.,* 2015). A female can produce and carry from 100-1200 eggs deposited into two paired egg string and produce up to 11 pairs in a lifetime (Costello, 1993).

1.3.2 **Temperature and salinity**

Growth and development of salmon lice are dependent on water temperature, as this controls the metabolic rate. Temperature is especially crucial for the planktonic larvae as they are non-feeding and only have a limited energy reserve (Samsing *et al.,* 2016). The success of infestation and growth rate is also positively correlated with temperature (Costello, 2006). At 10 °C, it takes 40 days from fertilization to adult for a male and 52 days for a female (Johnson & Albright, 1991a). Salinity levels below 29 ppt are harmful to free-living copepodids, and they are absent at salinity levels under 27 ppt (Bricknell *et al.,* 2006; Sutherland. *et al.,* 2012)

1.3.3 **Clinical signs and pathology**

The salmon lice feed on mucus, skin, and blood of their host. This can lead to mild skin lesions that can disturb the osmotic balance (Grimnes & Jakobsen, 1996). Damage to the skin increases the risk of secondary bacterial infections (Llewellyn *et al.,* 2017). High amounts of lice also lead to stress and possibly immunomodulation (Fast *et al.,* 2006), which again weakens the hosts' immune response, increasing susceptibility to other diseases (Tully & Nolan, 2002).

Moderate inflammations are often detected at the attachment site as well as the increased transcription of genes important in tissue repair and extracellular killing. This immune response, however, is not sufficient to clear infections, possibly due to immune regulatory substances secreted at the feeding area by *L. salmonis* (Fast *et al.,* 2014; Øvergård *at al*. 2018).

1.3.4 **Significance and economic importance**

Reduced biomass due to a loss in appetite and growth is estimated to result in a production loss of 3.62-16.55% in salmon culture annually. In Norway, the parasite was responsible for a loss of 436 million US dollars in 2011 (Abolofia *et al.,* 2017). Salmon lice are the single largest problem in salmonid farming, affecting fish health both in farmed and wild salmon. Treatments like chemotherapeutic intervention can have environmental costs, affect welfare as well as influence public perception of aquaculture. Despite research efforts and the development of new treatment methods, sea lice remain a huge problem (Costello, 2009).

1.4 Winter ulcer (*Moritella viscosa*)

M. viscosa is the bacteria thought to be the causative agent of winter ulcer disease in salmonids (Bruno *et al.,* 2013). It belongs to the Moritellaceae family, which are mainly psychrophilic and barophilic species found in marine sediments and the deep-sea (Hjerde *et al.,* 2015). The genus Moritella consists of seven psychrophilic species, where only *M. viscosa* is pathogenic to fish (Karlsen *et al.,* 2017a). *M. viscosa* is a gram-negative, curved rod, and motile bacterium (Bruno *et al.,* 2013).

1.4.1 Genetic diversity and demography

There are two clades, a typical and variant clade. These are separated both by phenotype and genotype. The typical clade has been isolated from farmed Atlantic salmon in Norway, Faroe Islands, and Scotland (Grove *et al.,* 2000). High acute mortality has been observed in Atlantic salmon, whereas in rainbow trout (*Oncorhynchus mykiss*) lower mortality and a more chronic ulcerative infection are common, indicating host-specificity (Karlsen *et al.,* 2014). The variant clade has been isolated from farmed Atlantic salmon in Canada, farmed rainbow trout in Norway and Iceland, and lumpfish (*Cyclopterus lumpus*) in Iceland (Grove *et al.,* 2000). Fish infected with the variant clade show lower levels of mortality (Karlsen *et al.,* 2014). The bacterium has also been isolated from other fish such as Atlantic Cod (*Gadus morhua*), Atlantic halibut (*Hippoglossus hippoglossus*) as well as cleaner wrasse species among others (Grove *et al.,* 2008, Karlsen *et al.,* 2014; Colquhoun & Olsen, 2018).

1.4.2 Growth and colony morphology

The growth of the bacterium is temperature and salinity dependent (Lunder *et al.,* 1995). *M. viscosa* can be cultivated on blood agar with 1-4% NaCl at temperatures between 4 to 25 ºC (Lunder *et al.,* 2000). For diagnostics *M. viscosa* it is commonly grown on agar with 2% NaCl, however, as it is slow growing this method can be unreliable, giving false negative results (Grove *et al.,* 2008). It is therefore recommended to use blood agar with NaCl and a vibriostat to inhibit faster-growing vibrio species (Colquhoun & Olsen, 2018). Colonies grown on 2% NaCl blood agar for 48h at 15 ºC are easy to distinguish from other bacteria as they have a unique viscosity and are thread-forming. The colonies are also round, translucent, grey, hemolytic, non-swarming, and non-luminescent (Lunder *et al.,* 2000). Very little is known about virulence and how these factors contribute to ulcer development. Extracellular products

have been shown to be cytotoxic to fish cells. At lower temperatures, the bacteria show increased adhesion and secrete products that break down cytoskeleton leading to cell lysis (Tunsjø *et al.,* 2011).

1.4.3 Pathology

Initial sites of infection include the gills and skin, suggesting that ulcer formation is a direct result from surface colonization (Karlsen *et al.,* 2012). Early clinical signs are necrosis of the skin, which advances to skin lesions on the flank, ranging from raised scales to larger lesions with exposed muscle tissue (Lunder *et al.,* 1995). The lesions are typically round or oval with a

Fig. 2: Winter ulcer after *M. viscosa* infection (marinhelse.no)

white demarcation zone towards the unaffected skin. Other signs can include reduced appetite, gill pallor (pale gills), and fin rot. The infection can become systemic, infecting the organs which can be followed by terminal septicemia, associated with higher mortality rates (Bruno *et al.,* 2013). Outbreaks of ulcers are often observed in connection with handling, e.g., treatment of salmon lice, which cause stress to the fish. When water temperatures increase over 10-12ºC or salinity decreases to under 12-15‰, the infection process is halted, the mortality rate decreases, and lesions start to heal, leaving scar tissue (Lunder *et al.,* 1995).

1.4.4 Interactions with other pathogens

Vibrio wodanis and *Tenacibaculum spp*. are also often co-isolated from ulcers together with *M. viscosa*, but how these are involved in ulcer development is still somewhat unknown (Karlsen *et al.,* 2014). *V. wodanis* belongs to the Vibrionaceae family and is like *M. viscosa* gram-negative gammaproteobacteria and cytotoxic to fish cells (Lunder *et al.,* 2000; Hjerde *et al.,* 2015). *V. wodanis* has also been the only bacteria isolated from a few winter ulcers in the field, but during experimental trials with only *V. wodanis* has not created ulcers. *A. wodanis* adhere to the fish cells, causing them to vacuolate, round up and detach from the surface as well as rearrange actin filaments *in vitro* (Karlsen *et al.,* 2014). Even though interactions between them are somewhat unknown, some studies of co-cultivation have shown that *A. wodanis* inhibit *M.*

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viscosa growth. This is thought to be due to competition over either nutritional resources or that *A. wodanis* produce bacteriocin-like agents as the growth inhibition is not contact-dependent (Hjerde *et al.,* 2015). *Tenacibaculum* sp. are gram-negative, rod-shaped bacteria. It has been proven to be challenging to isolate, leading to underdiagnoses of the pathogen, however, it is frequently co-isolated with *M. viscosa* from winter ulcers. It has also been the only bacterium isolated from winter ulcers from field research. During laboratory trials, it has only been shown to create ulcers and not induce systemic infections like *M. viscosa* (Olsen *et al.,* 2011).

1.4.5 Prophylaxis and treatment

In 1993 a vaccine for *M. viscosa* was introduced to the industry, and now almost all farmed Norwegian salmon are vaccinated (Grove *et al.,* 2008; Gismervik *et al.,* 2018). It is a part of a multivalent vaccine together with *V. anguillarum*, *V. salmonicida*, *Aeromonas salmonicida,* and infectious pancreatic necrosis virus. The vaccines are composed of inactivated bacteria and an oil adjuvant, which is administrated intraperitoneally (Gismervik *et al.,* 2018). Despite these vaccinations, winter ulcers are still seen at low but consistent prevalence during colder months, especially in Northern Norway and other parts of the world like Iceland, Faroe Islands, Scotland and Canada (Bruno *et al.,* 2013; Karlsen *et al.,* 2015). Over one-third of all antibiotics administrated in aquaculture from 1997-2000 were used to control winter ulcer disease. However, a study by Coyne (2004) concluded that antibiotics are not effective against winter ulcer disease.

1.4.6 Significance and economic importance

As vaccination, antibiotics or management measures have not eliminated winter ulcer disease, the infection reduces animal welfare, growth, and osmoregulatory capacity as well as an increased risk of secondary infections (Løvoll *et al.,* 2009; Bruno *et al.,* 2013). Even though the mortality rate is typically less than 10%, studies have shown a clear correlation with weight and health. Infected fish, therefore, have reduced growth and slaughter weight, creating a significant economic loss (Løvoll *et al.,* 2009; Coyne *et al.,* 2006).

1.5 The immune system

Studying *L. salmonis* and *M. viscosa* together is of importance as they both mainly infect the skin, and comparisons of single vs. co-infection may elucidate shared pathways and mechanisms by which these pathogens infect and impact their hosts. The skin works as a first line of defense, a physical barrier, inhibiting pathogenic organisms from entering the fish, as well as an immune organ. The head kidney is one of the major lympho- and hematopoietic tissues in fish and a site of isolation for systemic bacterial infection (Esteban, 2012). It has been shown that pathogens affecting the skin can increase the risk of secondary bacterial diseases, creating a synergistic effect by giving the bacteria an entry port (Kotob *et al.,* 2016).

1.5.1 Teleost skin

The skin is divided into two layers, epidermis and dermis. The epidermis layer contains mucus cells which produce and secrete mucus molecules (Kryvi & Poppe, 2016) which create a semipermeable barrier, allowing water and nutrients in, but also work as a mechanical barrier for pathogens. Many pathogens are immobilized by the mucus and therefore, unable to stick to the skin (Esteban, 2012). Mucus is also a part of the humoral function of the innate immune system contains; lectins, cytokines, complement proteins, immunoglobulins (Ig) and more. The dermis is under the epidermis and consists of mostly connective tissue and blood vessels (Magnadòttir, 2004). Maintaining good skin quality is essential in fish farming both in quality and welfare (Jensen *et al.,* 2015).
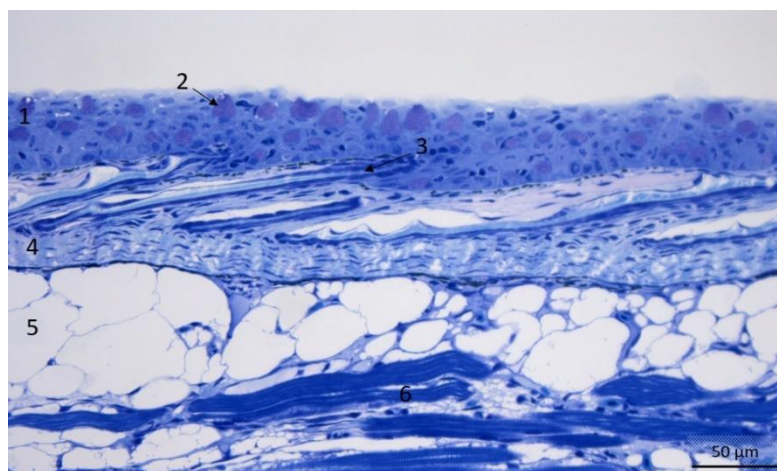
Fig. 3: Histological section of Atlantic salmon skin stained with toluidine blue (TB). 1- Epidermis, 2- Mucus cell, 3 – Scale, 4 -Dermis, 5 – Hypodermis, 6 – Muscle (Histology slide and picture provided by H. Kryvi)

1.5.2 **Head kidney**

The head kidney is a unique organ for teleost fish, and it is important in hematopoiesis and immunity. The kidney is found dorsally up to and along the spine, with the most anterior part being the head kidney. It consists of lymphoid cells and endocrine cells. The lymphoid cells produce

Fig. 4: Drawing of Atlantic salmon, arrow indicating location of head kidney

cytokines, and the endocrine produces cortisol, catecholamines, and thyroid hormones. It is therefore imperative in the immune system, along with the thymus, spleen, liver, and skin (Geven & Klaren, 2017).
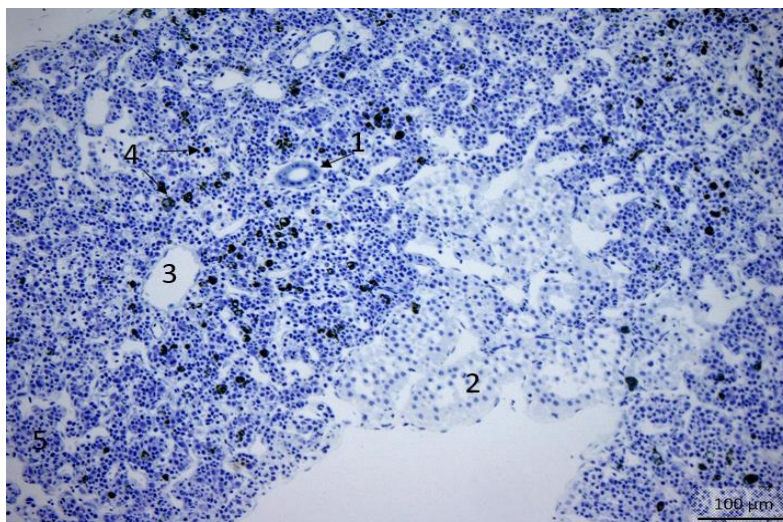
Fig. 5 Histological section of Atlantic Salmon head kidney stained with TB. 1-Tubuli (nephron), 2- Endocrine tissue, 3- Blood vessel, 4 - Melanomacrophages, 5-Hematopoietic tissue. (Histology slide and picture provided by H. Kryvi)

1.5.3 **Immune response**

The immune response is crucial for fighting infections and is divided into two main responses, the innate and adaptive immune response. The innate immune response is both physical, chemical and cellular. It works fast to alert the body to prevent further spread of pathogens; however, it is non-specific. Primary cells include natural killer cells (NK-cells) macrophages, neutrophils, dendritic cells, and more. The adaptive immune system is unique to vertebrates and can generate a response specific to the pathogen it is challenged with but works much slower, primary cells are T- and B-cells (Magnadottir, 2004). The complement system is fundamental in fighting infections and, depending on the pathway, a part of both the innate and adapted

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immune system, consisting of over 30 proteins (Dunkelberger & Song, 2010). It can lysate pathogens, remove immunocomplexes, and bind to immunoglobulins (Magnadottir, 2004).

Immunoglobulins (Ig)

Immunoglobulins, often called antibodies, are glycoproteins produced by B-lymphocytes. Its structure has two heavy and two light polypeptide chains that create a Y-shape. The outer part, antigen binding site, binds to antigens and is important in the adaptive immune response. The inner part, the Fc receptor, is important in the innate immune system as it activates the complement system and can bind to monocytes, macrophages, granulocytes, and NK-cells. In fish there are three known types of Ig isotypes; IgD, IgM, and IgT/IgZ (Mashoof & Criscitello, 2016). Ig can agglutinate to bacteria or toxins and thereby neutralize and potentially stop further spread of a bacterium but do not kill the bacteria itself (Lea, 2000). Ig bind to pathogen associated molecular patterns (PAMPs) identifying the pathogen for phagocytosis and lysis. Highly polymorphic regions in the antigen binding site of Ig allows for binding to specific epitopes on invading pathogen. Once Ig has bound to its specific ligand, B-cells expressing that unique binding site are clonally expanded and allows for faster, and stronger antibody responses upon rechallenge with the same pathogen (Magnadottir, 2004).

Cytokines

Cytokines are signal molecules, often glycoproteins, secreted by activated macrophages or dead and dying cells as danger signals, and are central to the immune response. There are different groups: chemokines, tumor necrosis factor, interferons, and colony stimulating factors (Zhu *et al.,* 2013). These signals act through binding to specific membrane receptors on target cells in mostly an autocrine (on producing cell) or paracrine (on neighboring cell) fashion (Lea, 2000).

Pro-inflammatory mediators like, IL-1β and IL-8, are mainly released by antigen presenting cells (APC) and epithelial cells. IL-1β increases the production of substance P and prostaglandin E_2 (PGE_2) in neuronal and glial cells. IL-8 is a chemokine that induces chemotaxis for neutrophils and T-cells. IL-10 and IL4/13A are anti-inflammatory cytokines and control the pro-inflammatory response. IL-10 represses expression of inflammatory cytokines (TNF-α, IL-6, and IL-1) as well as down-regulate pro-inflammatory cytokine receptors and up-regulate endogenous anti-cytokines (Zhang & An, 2009). IL-4 activates naive $CD4^+$ T cells

which in turn starts to produce and secrete cytokines important in autocrine growth and differentiation of naive T cells to type 1 helper cell (Th1) and type 2 helper cell (Th2) cells. Th1 produces cytokines like IL-2, interferon-gamma (IFN-γ), and tumor necrosis factor (TNF) and is vital for cell-mediated immunity. Th2 cells produce cytokines like IL-4, IL-5, IL-6, and IL-13 and help B-cells and class switching of immunoglobulins (Choi & Reiser, 1998). In Table 1, all immune genes that were analyzed for both skin and head kidney are listed with produces cell and function.

Table 1: List of immune genes that were analyzed in this thesis with producer cell and function

Name	Type	Producer cells	Action	References
IL-1β	Cytokine	Macrophages, Epithelial cells	Inflammation T-cell activation Macrophage activation	(Murphy & Weaver, 2017)
IL-4/13A	Cytokine	T-cells Mast cells ILC2 cells	B-cell activation Induce differentiation into Th1 and Th2 cells	(Murphy & Weaver, 2017)
IL-8	Chemokine	Macrophages Epithelial cells	Recruits granulocytes Recruits phagocytes Induce phagocytosis	(Murphy & Weaver, 2017)
IL-10	Cytokine	Macrophages Dendritic cells T-cells B-cells	Suppress macrophage function	(Murphy & Weaver, 2017)
C3	Acute-phase protein		Complement protein- Classical complement system Alternative complement system	(Erdei *et al*, 1991)
IgM	Immunoglobulin		Recruits phagocytes Induce phagocytosis Inflammation response Agglutinate to microorganisms	(Lea, 2000)

Expression of these immune genes was chosen due to previous research indicating a higher expression after infection of these pathogens. Both pathogens show an increased expression of IL-1β at attachment/lesion site (Øvergård *et al.,* 2018; Tadiso *et al.,* 2011; Løvoll *et al.,* 2009; Ingeselv *et al.,* 2010). Øvergård *et al.,* (2018) found higher transcription of Il-1β in *L. salmonis* attachment site compared to non-attachment. Ingeselv *et al*., (2010) found higher transcription in muscle tissue and Løvoll *et al.,* (2009) in skin.

L. salmonis also show a higher transcription of IL-4/13A, IL-8, and IgM (Øvergård *et al.,* 2018; Tadiso *et al.,* 2011). Lesions after *M. viscosa* infections have an increased expression of IL-8 at and IL-10 ulcerated muscle tissue and C3 in skin (Ingerselv *et al.,* 2010; Løvoll *et al.,* 2009).

Other genes known to be associated with *L. salmonis* infection include IL-1 receptor type 1, CD4, IL-12β, CD8α and much more in skin attachment site along with an increase in Ig in the head kidney (Skugor *et al.,* 2008). There is limited research on immune response from an *M. viscosa* infection, but other immune geneses known to be affected include TLR5, TLR22, MMP-2, myostatin-1αβ, collagen-1α CTGF, TGF-β, TLR5 in ulcerated muscle tissue and ISG15 in skin (Ingerselv *et al.,* 2010; Løvoll *et al.,* 2009). Expression of many genes has been linked to resistance against *L. salmonis* as a more resistant salmon can avoid immunosuppression and fight the infection more efficiently. Especially expression of genes involved in Th1 and Th2 response has been linked to this resistance (Fast *et al.,* 2014 Holm *et al.,* 2015).

1.5.4 **Stress**

Homeostasis is when an organism is in a steady state, both physically and chemically. Stress is one factor that can affect an individual's ability to maintain homeostasis (Stott, 1981). The stress hormones, corticosteroids, and catecholamines are released from the head kidney and into the bloodstream (Barton, 2002). Catecholamines are released immediately as a response to stress and decreases quickly within minutes. Cortisol is released after a few minutes, but elevated levels can often be observed over extended periods of time, in terms of hours to days depending on the acute versus the chronic nature of the stress (Thomas *et al.,* 1991; Sundh *et al.,* 2010). Salmonids in aquaculture are known to be stressed due to handling, transport, treatments, and disease (Barton and Iwama, 1991). Stress can lead to secondary metabolic, osmoregulatory, and cellular changes leading to tertiary impacts such as increased disease susceptibility. Increased susceptibility is a result of chronically elevated stress hormones like cortisol, which suppress the natural inflammatory response (Barton, 2002, Pickering *et al.,* 1989; Johnson and Albright, 1992). In cases of chronic stress (i.e. chronic cortisol elevation), immunosuppression can lead to higher mortality as has been shown in studies on brown trout (*Salmo trutta*) infected with bacterial and fungal diseases (Pickering and Pottinger, 1989).

1.6 Aim of the project

The aim of the project is to experimentally co-infect Atlantic salmon (*Salmo salar*) with *L. salmonis* and *M. viscosa* and see how these two pathogens affect fish growth, disease development as well as expression of immune genes skin and head kidney of Atlantic salmon. Disease development includes the outcome of the infection influencing the lice and severity of skin lesions.

There have been no published studies on co-infections of *L. salmonis* and *M. viscosa*. As mentioned, both these pathogens are a problem in the farming of Atlantic salmon where treatment and vaccinations have presented limited results. By studying pathogens together through co-infection, it is anticipated we can better understand field conditions salmon are exposed to, which will result in better management strategies and treatment decisions.

Hypothesis: A co-infection of *L. salmonis* and *M. viscosa* does not affect the growth but does affect disease development, mortality, and immune response of Atlantic salmon compared to the two pathogens on their own.

Research questions:

1) Does the co-infection of *L. salmonis* and *M. viscosa* influence the location of *M. viscosa* in the tissue?

2) Does the co-infection of *L. salmonis* and *M. viscosa* influence growth (weight, length, k-factor)?

3) Does the co-infection of *L. salmonis* and *M. viscosa* lead to higher mortality?

4) Does the co-infection of *L. salmonis* and *M. viscosa* lead to higher cortisol levels?

5) Does the co-infection of *L. salmonis* and *M. viscosa* influence lesion count, size, and severity?

6) Does the co-infection of *L. salmonis* and *M. viscosa* influence the lice count and developmental stages?

7) Does the co-infection of *L. salmonis* and *M. viscosa* affect the expression of immune genes in the skin and head kidney?

2 Material and Methods

The co-infection experiment was conducted as a collaboration between the University of Bergen (UIB) and the Atlantic Veterinary College (AVC), University of Prince Edward Island (UPEI), in Charlottetown, Canada. The challenge of fish was part of an on-going Genome Canada project (Integrated Pathogen Management of Co-infections in salmon; IPMC) and was planned and conducted at AVC with an industry partner, Cargill Inc. I took part in sampling A and B, so data and tissue samples taken later were sent to the University of Bergen after sampling.

Histology and tissue samples were processed at UIB at Høyteknologisenteret unless stated otherwise.

2.1 Fish conditions

Post-smolt Atlantic salmon (Saint John River strain) were placed in 300 L tanks with approximately 40 fish in each tank early in November 2018 and were on average 257 ± 47.8 g. 14h light and 10h dark photoperiod. The tanks were divided into three rooms and biofiltration units; 1, 2 and 3. Biofiltration unit 1 had 12 tanks, while unit 2 and 3 had nine tanks. Water was recirculated through a recirculating aquaculture system (RAS) and was 10 ± 1 ℃ and 33 ± 1 ppt saltwater (salt was added to get desired salinity) prior to exposure of *M. viscosa* which was conducted at 8 ± 1 ℃, and that temperature maintained in all RAS systems thereafter. The infection started on December 12th and was terminated on January 28th, 48 days later. Fish were fed at 1% body weight/day and observed a minimum of twice a day. Mortalities were removed, necropsied, and plated on Tryptic Soy Agar (TSA + 2% NaCl).

2.2 Copepodids

Ovigerous female *L. salmonis* were collected from New Brunswick salmon farms in the Bay of Fundy Canada. Eggs were hatched at the Huntsman Marine Science Centre on ambient SW (9 ± 2 ℃ and 33 ± 3 ppt). When the lice had reached the infectious copepodid stage, they were transported to AVC and used in the infection trial as described in 2.4.2.

2.3 *M. viscosa*

M. viscosa was provided by the Research Productivity Council of New Brunswick. An active culture of Stock FFa-371 (Pacific Ocean isolate from British Columbia, variant clade) was prepared at 8±1 °C in flasks containing Tryptone Soya Broth (TSB-2, 2% NaCl).

2.4 Infection trials

2.4.1 *M. viscosa* infection

Unit 2 was infected on the 12th of December (challenge 1) and unit 1 on the 18th of December (challenge 2). The water temperature was decreased from 10 ± 1 ºC to 8 ± 1 ºC the day before infection. The fish were transferred to five tanks (1x 500 L, 2x 250 L, and 2x 200 L tanks) where the bath infection took place. After a period of 1h, the fish were returned to their original tanks. Challenge 1 had a dose of 1.02e+05 cfu/ml and was incubated at 8 °C. Challenge 2 had a dose of 1.04e+05 cfu/ml, and the main culture was inoculated on site. The culture was incubated overnight on an orbital shaker at 8±3 ºC. The cultures were kept at 7.5± 1ºC two hours prior to infection.

2.4.2 *L. salmonis* infection

Unit 1 and 3 were infected on the 13th of December. The waterflow to the tanks were turned off and slightly reduced (just below the outflow) before adding 50 infective copepodids fish[-1]tank[-1]. Eight tanks were infected with *L. salmonis,* and the last tank remained uninfected as a control group. After 1h, the waterflow was turned on again. The water temperature was maintained at 10 ± 1 ºC for five days after infection before reduced to 8 ± 1 ºC. To ensure that lice would not infect control tanks (in the same recirculation system) a filter (100 µm) sleeve was added over the incoming water to avoid contamination.

2.4.3 Sampling

There were four sampling points throughout the experiment, the first being on day 1 (4 weeks after acclimation to experimental groups), and the last on day 48. Weight, length, ulcers and lice from sampling B-D, and cortisol (stress) and immune response from sampling B and C were analyzed in this thesis.
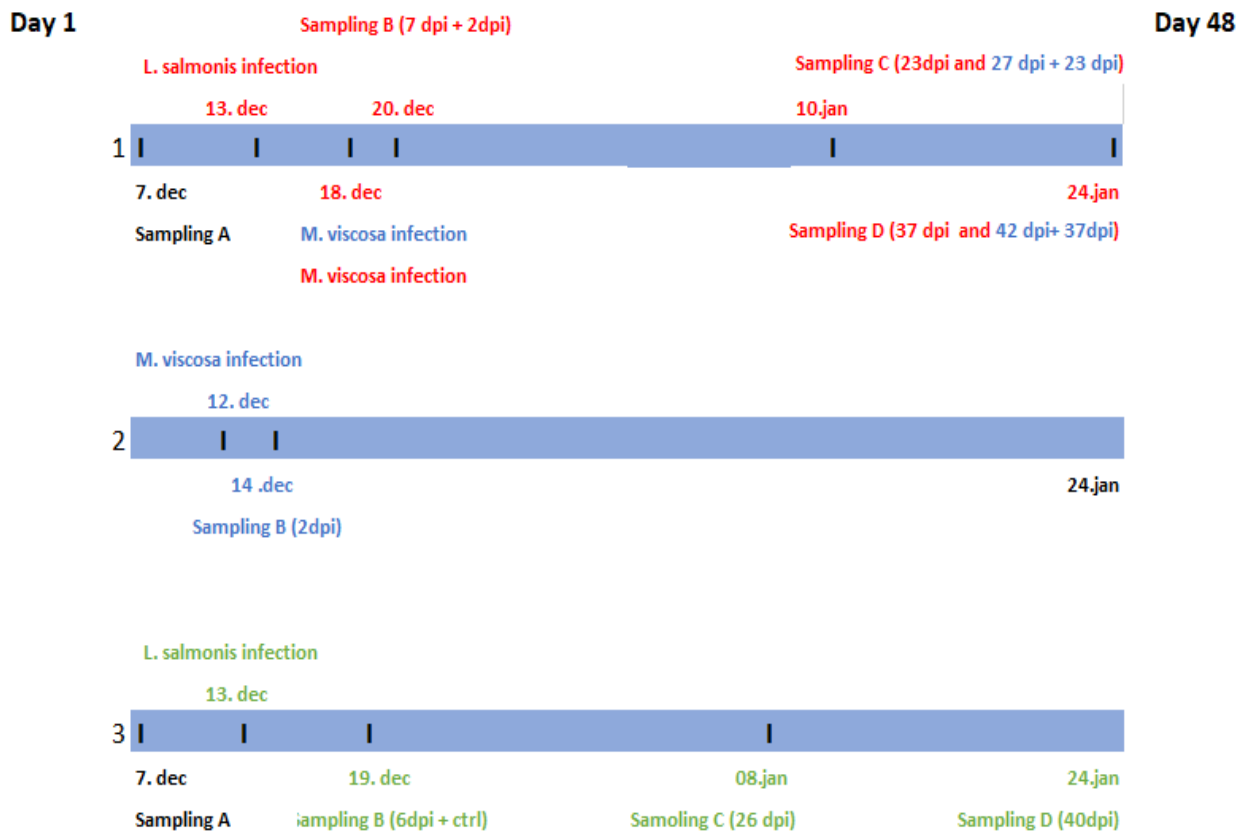
Fig. 6 Timeline of infection trials for the different RAS units (1, 2 and 3). Blue is *M. viscosa*, green is *L. salmonis* and red is Co-infection. All timelines start at 7th of December and end on 24th of January.

Prior to all sampling, the fish were starved for 24 h. On sampling A two fish from each tank were sampled. For sampling B and C 10 fish from each tank were sampled, while for D the remainder of fish were sampled (Table 2) Fish were euthanized with MS-222 - tricaine methosulphate (250 mg/L) in separate tanks. The fish were weighed (g), measured fork length (cm), and blood was taken for serum cortisol analysis. Lesions were counted, sized, and graded according to table 3. Lice were counted and staged. Fish with *M. viscosa* lesions or lice had skin samples taken at affected skin, and the unaffected control site was taken directly beside on healthy skin. Only one of each sample site was taken pr. fish. Fish without lesions or lice were sampled as seen in fig. 7. Other tissue samples included head kidney and gills (from

Fig. 7: Point of skin sampling for reference locations of fish without lice (A) or lesions (B)

first gill arch). All tissue samples for gene expression ware stored on RNAlater (Ambion) as described in 2.6; only skin and head kidney were analyzed in this thesis. Tissue samples of lesions and lice attachment for histology were put in 10% neutral buffered formalin.

Table 2: Fish sampled for each group in the different samplings

	Sampling B	Sampling C	Sampling D
M. viscosa	40	20	21
Co-infection	40	40	13
L. salmonis	30	30	30
Control	10	10	10

Table 3: Size classes and the corresponding size in cm

Size class/Grade	Size (cm)	Grade description
1	< 0.5	Discolouration/scale loss
2	0.5-2	Ulceration
3	< 2	Muscle exposure

Fig. 8: Picture of lesion grading scheme (Made by L. Carvalho, used with permission.)

The total size was calculated by multiplying the number of ulcers with size score, while the average size was calculated using the total size score divided by the number of ulcers. The same was done for total and average grade. From weight and length, the condition factor (K-factor) was calculated using this formula:

$$K = \frac{10^n W}{L^3}$$

K= Condition factor

W= Weight (g),

L=length (mm)

The four different sampling points are listed in table 4 which shows the sampling points with days and degree days after infection.

Table 4: Days and degree days after infection at each sampling point

| | *L. salmonis* | | *M. viscosa* | | *L. salmonis + M. viscosa* | |
	dpi	ddpi	dpi	ddpi	dpi	ddpi
Sampling A			5 days prior to infection			
Sampling B	6	58	2	16	7+2	66 + 16
Sampling C	26	218	23	184	28+23	234 + 184
Sampling D	40	346	37	296	42+37	362 + 296

2.5 Histology

During sampling, skin tissue samples from *M. viscosa* lesions, lice attachment and unaffected areas were preserved in 10% neutral buffered formalin, until dehydration and embedding in paraffin which was done at AVC following standard protocols. Two replicates/fish of each infection site at sampling C and only at one sampling point B that were used for histopathology and immunohistochemistry.

The paraffin embedded tissues were sectioned in 3 μm from *M. viscosa* lesion, *L. salmonis* attachment, and unaffected areas. Mounted on Superfrost Plus microscope slides (Thermo Scientific) and left at 60 ℃ for 24h. Prior to staining and immunohistochemistry, the sections were first left at 60 ℃ for 30min and then dewaxed with Histo-Clear II (National Diagnostics) 2x10 min, following rehydration with ethanol 100% 2x5 min and 5 min x 96%, 80%, and 50%. Sections were then rinsed in MilliQ H_2O for at least 5 min.

2.5.1 **HE(S) staining**

Following rehydration, the sections were stained in Hematoxylin for 2.5 min followed by tap water for 4 min. The samples were then placed in 1% Erythrosine (pH 6.5) for 1.5 min and rinsed in tap water for 1 minute. Sections were then dehydrated again with ethanol, 96 % for 1min and then 2x2min in 100 %. Lastly, they were placed in Histo-Clear II 2x5 min before mounted with Histomount (Life Technologies).

2.5.2 **Immunohistochemistry**

Polyclonal antibodies which were antiserum raised in rabbit against *M. viscosa* strain NVI88/478 and K230 (Løvoll *et al.,* (2009); Grove *et al.,* (2010)), were kindly provided by Dr. S. Grove. Following the previously described rehydration steps, the sections were washed with Tris-buffered saline Tween (TBST tablets in MilliQ H_2O, EDM) 2x2min. Blocking was done with 5% bovine serum albumin (BSA) (Sigma Aldrich) in TBST for 2h at room temperature (R.T.). Sections were washed 2x2min with TBST. Primary antibodies rabbit anti-*M. viscosa* were diluted 1:10.000 in 2.5 % BSA in TBST and the sections were then incubated for 1h in a humidity chamber. Sections were washed 2x2min TBST before incubated with secondary antibody (goat anti-rabbit IgG, Sigma-Aldrich), diluted 1:1000 in 2.5% BSA for 30 min in a humidity chamber. Sections were washed rinsed 2x2 min with TBST followed by flushing with processing buffer (100mM Tris-NaCl, 50 mM $MgCl_2$, pH 9.5) and incubation for 10 min. 1-stepNBT/BCIP plus suppressor (Thermo Scientific) was used for 2 min for staining incubated protected from light before stopping the reaction with stop buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl) following addition MilliQ H_2O. The slides were then counterstained with only hematoxylin for 20 sec and mounted as described in 2.5.1. Images were captured using Axio Scope A1 light microscope with an Axiocam 105 (Zeiss). Two negative controls were run by not adding primary antibodies and not counterstained. To determine the optimal concentration of primary antibody, dilutions of 1:2000, 1:5000, and 1:10.000 were tested, with 1:10.000 giving the best results.

2.6 RNA extraction

Tissue samples from sampling B were stored in RNAlater (Ambion) at 4ºC for one day and -20 ºC until isolation (1 month). Samples from sampling C were stored at 4ºC for 3-4 days, followed by the removal of RNA later and then storage at -80 ºC before isolation (3 months). Most samples were rather large, so a smaller piece (0.5 cm^2) was cut out. RNA isolation was done with TRI reagent (Sigma-Aldrich) according to the suppliers' protocol with some modifications. The samples were first homogenized by adding 600 μL trizol with 5mm beads, and shaken in TissueLyser II (Qiagen) for 3 min at a frequency of 30.0 1/s. An additional 400 μL trizol was added, and the samples were kept at R.T. for 5 min. Before 200 μL chloroform was added and shaken for 15 sec. After 2 min, the samples were centrifuged at 21,100 x g for 15 min at 4 ºC Herafus fresco centrifuge (Thermo Scientific). About 450 μl of the upper aqueous phase RNA was transferred to new tubes with 500μL isopropanol. After 5 min at R.T., the solution was centrifuged at 21,100 x g for 10 min at 4 ºC. The supernatant was removed, and the pellet washed with 750 μL 75% ethanol (EtOH) and centrifuged at 21,100 x g for 10 min at 4 ºC twice. EtOH was removed and the pellet dried before adding 100μL nuclease-free water. The quality and quantity of RNA of the first 24 skin samples were checked by using Nanodrop1000 spectrophotometer (Thermo Scientific). 9 of these samples were not at an ideal purity so all samples were further purified. This was done through an extra precipitation which was completed by adding 10 μL 3M NaAc pH 5.2 and 250 μL EtOH which was placed at -20 ºC overnight. The solution was centrifuged at 21,100 x g for 30 min at 4 ºC before being washed with 1 ml EtOH and centrifuged at 21,100 x g for 15 min at 4 ºC, dried and resuspended in 25-50 μL nuclease-free water depending on pellet size. RNA was stored at -80 ºC until DNase and cDNA synthesis.

The quality and quantity of RNA in the samples was checked by using NanoDrop1000 spectrophotometer (Thermo Scientific) and Bioanalyzer. All samples were checked with Nanodrop. Agilent RNA 6000 Nano Kit Guide (Agilent Technologies) was used on 24 representative samples. Six skin and six HK samples from sampling B and six samples from control and six skin samples from sampling C (appendix A). The settings of analysis were set to mRNA (not RNA) by mistake and therefore did not provide a RIN number.

Tables 5 and 6 show the number of samples analyzed for the transcription of immune genes. Samples analyzed were from the same fish with lice and/or lesion and unaffected control site

and were chosen randomly among replicate tanks. A few fish only had just the unaffected control site. A sample set of at least 8 for each group was planned to be analyzed, but for some samples, the RNA concentration after extraction was not sufficient, so these were removed. Also, some co-infection *L. salmonis* samples from sampling B was mislabeled and contained fins and not skin and were therefore not analyzed.

Table 5: Number of samples analyzed for each group in the different samplings for transcriptional response in skin

	Sampling B	Sampling C
Control	10	10
M. viscosa lesion	13	8
M. viscosa unaffected control	12	8
L. salmonis attachment	11	8
L. salmonis unaffected control	12	8
Co-infection: *M. viscosa* lesion	8	7
Co-infection: *M. viscosa* unaffected control	10	8
Co-infection: *L. salmonis attachment*	5	7
Co-infection: *L. salmonis* unaffected control	5	7

Table 6: Number of samples analyzed for each group in the different samplings for transcriptional response in head kidney

	Sampling B	Sampling C
Control	9	9
M. viscosa	12	8
L. salmonis	10	8
Co-infection	11	7

2.7 DNase and cDNA synthesis

TURBO DNA-free (Ambion) was used to remove potential genomic DNA (gDNA). It was done following the manufacturer's recommendations adding 4 µg RNA, resulting in a concentration of 160 ng/µl. Most samples went directly to cDNA synthesis; however, leftovers were stored at -80 ºC and used if needed. cDNA synthesis was carried out using Superscript® IV Reverse Transcriptase kit (Invitrogen) following the manufacturer's recommendations divided by two to get a total volume of 10µL. Using 5.5 µL DNase treated RNA (880 ng). The 10 µL solution was diluted with 30 µL (1:4) nuclease-free water resulting in a total concentration of 22 ng/µL. Samples were stored at -20 ºC until further use. Affinity Script, Superscript III and IV were tested and compared to determine which was most suitable to use for the samples, with Superscript IV giving the best results for RT-qPCR.

2.8 Quantitative reverse transcription PCR (RT-qPCR)

Quantitative RT-PCR performed on a QuantStudio 3 and 7500 Fast (Applied Biosystems) RT-qPCR machine. Genes investigated were interleukin-1β (IL-1β), interleukin-4 (IL-4/13A) interleukin-8 (IL-8), interleukin-10 (IL-10), complement component 3 (C3), immunoglobulin M (IgM). Elongation factor 1α (EF 1α) was used as a reference gene. (Lie *et al.,* 2005) Primer sequences are listed in table 7. Assays were run using SYBR Green (Sigma–Aldrich). Duplicate wells were run of each gene for each sample with a difference in Ct-values <0.45 (See table 8 and 9 for ingredients and program). The expression of immune genes was analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Baseline was set to 0.2, and samples were run with a -RT and negative transcription control (NTC). The verification of primers was performed through a two-fold dilution series (six dilutions) with three parallels for all genes to confirm the efficiency of close to 100% (91.9-101,2%). A primer for immunoglobulin T (IgT) was also tested, but efficiency and parallels were bad and therefore not used.

Table 7: Sequences of primers (Forward=F, Reverse=R) for Atlantic salmon used for RT-qPCR analysis for skin and head kidney samples for sampling B and C.

Gene	Abbrevation	Primer	Sequence	Ref
Elongation factor 1α	ELFα	F	CACCACCGGCCATCTGATCTACAA	Øvergård *et al*, 2018)
		R	TCAGCAGCCTCCTTCTCGAACTTC	
Interleukin 1β	IL-1β	F	GCTGGAGAGTGCTGTGGAAGA	Øvergård *et al*, 2018)
		R	TGCTTCCCTCCTGCTCGTAG	
Interleukin 4/13A	IL-4/13A	F	CGTACCGGCAGCATAAAAATCACCATTCC	Øvergård *et al*, 2018)
		R	CCTTGCATTTTGTGGTGGTCCCA	
Interleukin 8	IL-8	F	GCATCAGAATGTCAGCCAGCC	Øvergård *et al*, 2018)
		R	ACGCCTCTCAGACTCATCCC	
Interleukin 10	IL-10	F	ATGAGGCTAATGACGAGCTGGAGA	SLRC (L. Sandlund)
		R	GGTGTAGAATGCCTTCGTCCAACA	
Complement protein 3	C3	F	ATTCTTCCCCTCCACTCCCTCG	SLRC (L. Sandlund)
		R	CGATTTGGTCGTCAAGCCAGG	
Immunoglobulin M	IgM	F	TGAGGAGAACTGTGGGCTACACT	SLRC A. Øvergård)
		R	TGTTAATGACCACTGAATGTGCAT	

Table 8: Ingredients and volume for RT-qPCR

Ingredients	Volume
PowerUpSYBR Green PCR mastermix	5 µl
Forward primer (10 µM)	0.5 µl
Reverse primer (10 µM)	0.5 µl
H_2O	2 µl
cDNA	2 µl (44 µg)
Total volume	10 µl

Table 9: Program for RT-qPCR

Phase	Temperature	Time
Pre-PCR read	50ºC	2 min
Holding stage	95 ºC	2 min
Cycling stage x40	95 ºC	15 sec
	60 ºC	1 min
Melting curve stage	95 ºC	15 sec
	60 ºC	1min
	95 ºC	30 sec

2.9 Statistical Analysis

Two-tailed T-test was performed on all data with a significant threshold of 0.05 for p-values. For immune genes when comparing different fish, the t-test was unpaired while comparing lesion/attachment site and unaffected control site in the same fish it was paired. Microsoft Excel was used to do statistical analysis and make graphs. All results for gene expression in the skin were compared to each other using a t-test, however only the factors which were noteworthy are shown in the figures. The rest are listed in appendix B. Figures with immune response that had no significant differences are found in appendix C.

Bar charts were used when the differences between individuals weren't too large, and the average and standard deviation was representative (used in size, ulcers and lice). Box and whiskers were used when there were substantial differences between individuals. A box and whiskers plot were therefore more suitable to show the considerable variation as well as average and median (used in cortisol and immune response)

2.10 Other methods done at AVC

As the project was done at AVC, I did not participate in all parts that are mentioned in this thesis. All tanks were controlled daily for mortalities and were removed. Bacteria were cultured from mortalities, and all colonies were confirmed to be *M. viscosa* as well as some were sequenced with specific primers to support the claim that *M. viscosa* was the cause of the ulcers. Blood samples were centrifuged, and the serum of some fish was sent in for analysis of serum cortisol. The number of fish serum cortisol analyzed is listed in table 10, the fish were selected randomly among the replicate tanks.

Table 10: Number of fish samples analyzed for each group in the different samplings for serum cortisol

	Sampling B	Sampling C
Control	5	5
M. viscosa	10	10
L. salmonis	10	10
Co-infection	10	10

3   Results

An experimental infection of Atlantic salmon with the two pathogens *M. viscosa* and *L. salmonis* was performed to investigate the effects of single infections compared to co-infections. The experiment contained four experimental groups: One uninfected control, one group infected with *M. viscosa*, one group infected with *L. salmonis* and one co-infected group first infected with *L. salmonis* followed by *M. viscosa* five days later. At the beginning of the experiment fish were randomly distributed among the different tanks, with approx. 40 fish per tank. All tanks were sampled prior to infection, and post-infection there was one tank for control, three tanks with *L. salmonis* and four tanks with co-infection. *M. viscosa* was sampled from 4 tanks in RAS unit 2 for time B, and from 2 tanks in RAS unit 3 for time C and D.

3.1 Histopathology and immunohistochemistry

Histological analysis was performed on skin samples from lesions (Fish 10, tank 8 and fish 8, tank 14) and lice attachment (Fish 1, tank 10) and unaffected control sites in the same fish. The slides were stained with HE(S) to assess histopathology in the lesions, attachment sites, and the nonaffected skin. Immunohistochemistry was performed to see it was possible to determine the presence and location of *M. viscosa* in the lesions.

Histology of unaffected areas of the fish looked healthy and was not affected by the *M. viscosa* infection with intact epidermis and no signs of inflammation or hemorrhaging (Fig. 9).
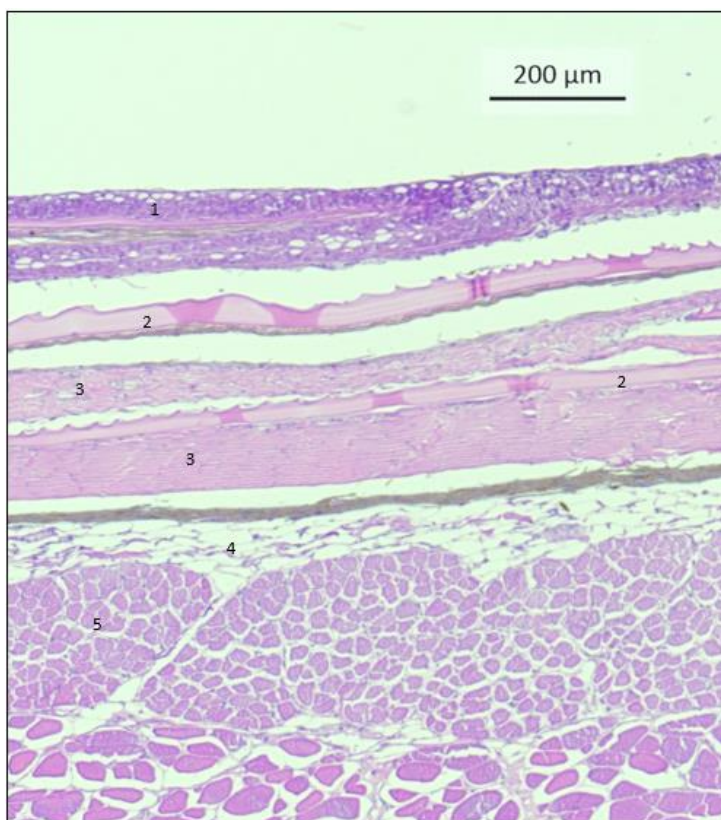
Fig. 9: Histology of unaffected skin from time C of fish infected with *M. viscosa*. 1 - Epidermis, 2- Scale, 3-Dermis, 4-Connective tissue, 5-Muscle tissue

Histopathological characteristics found for lesions included numerous erythrocytes indicating hemorrhaging. Epidermis and dermis were varied from necrotic to completely removed depending on location in the ulcer. There was also heavy inflammation in epidermis and muscle cells (Fig. 10).
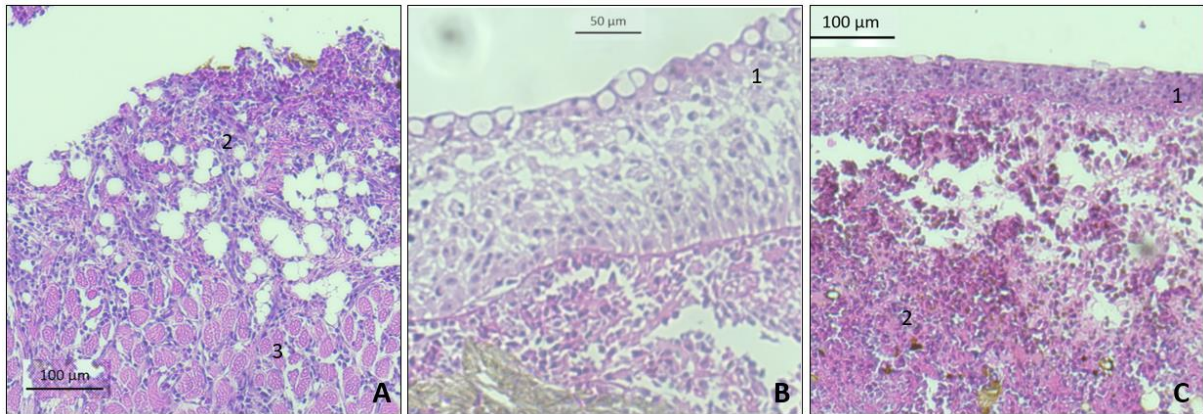
Fig. 10: HE(S) stained histology from ulcers from sampling C. 1- Epidermis, 2- Inflammation, 3- Muscle tissue. A (Fish 8, tank 14)- The entire epidermis and dermis is gone, leaving exposed inflamed muscle tissue. B – (Fish 10, tank 8)- epidermis is necrotic with blood in dermis. C – (Fish 8, tank 14)- Massive hemorrhaging and inflammation.

The attachment sites of *L. salmonis* showed loss mucus cells and epidermis. A moderate inflammation could also be observed in the top layer of dermis (Fig. 11).
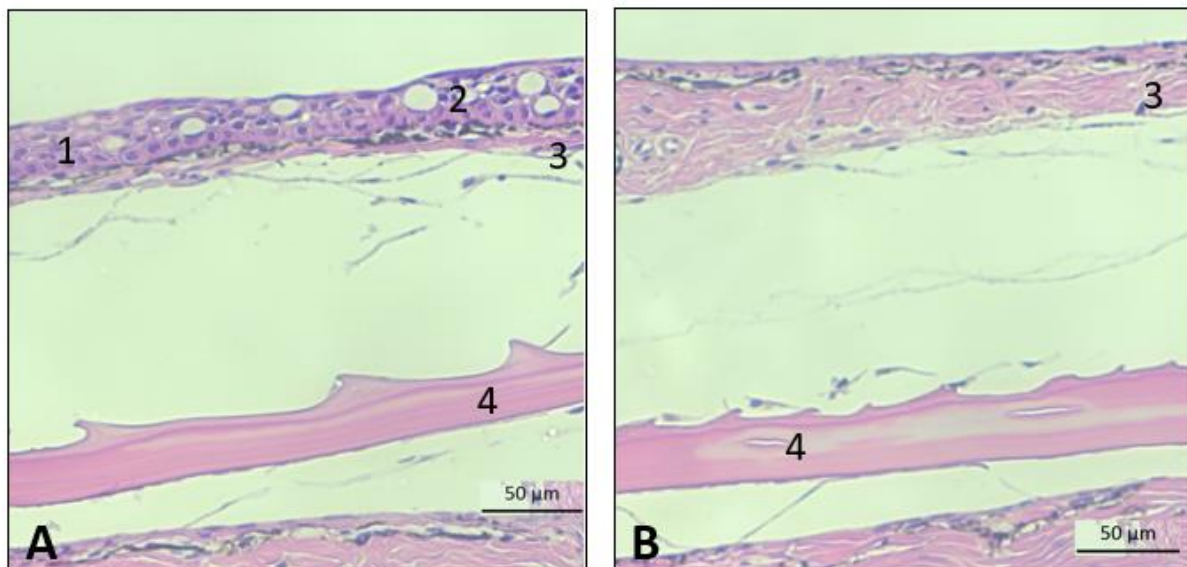
Fig. 11: HE(S) stained histology of skin after *L. salmonis* infection at sampling C (Fish 1, tank 10). 1- Epidermis, 2- Mucus cells, 3- Dermis, 4- Scale. Picture A - Normal intact skin directly adjacent to picture B. Picture B - Attachment site of a lice with little epidermis left and a moderate inflammation.

After immunohistochemically staining *M. viscosa* and H(ES) staining, there were a few darker spots in the slides about the same size as macrophages in dermis and muscle tissue. However, these spots were few and far between. (Fig. 12) These spots could not be observed in tissue from time B or control tissue only incubated with the secondary antibody (Fig. 13).
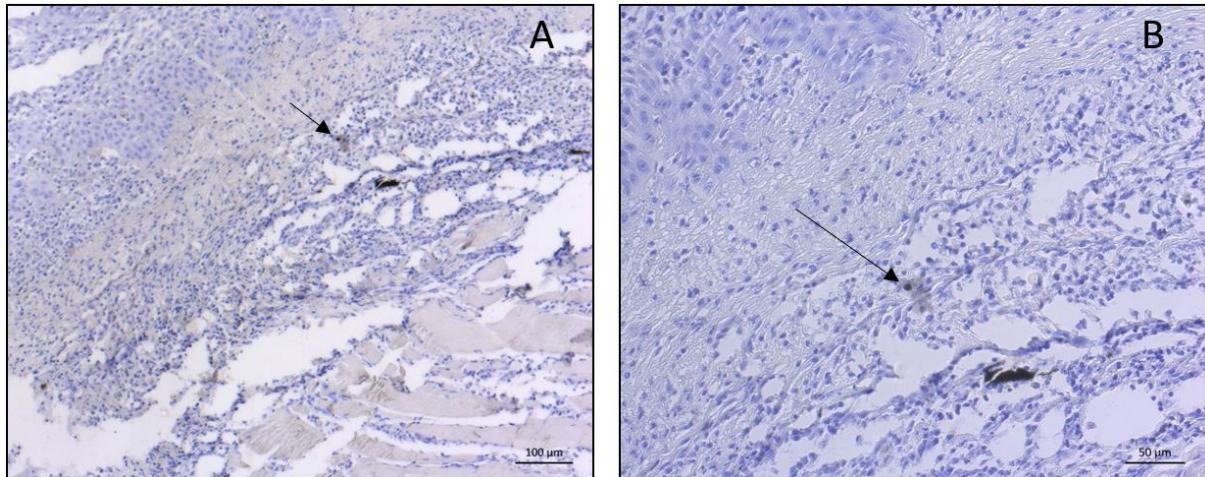
Fig. 12: Hematoxylin and immunohistochemically stained for *M. viscosa* skin after *M. viscosa* infection at sampling C (Fish 8, tank 14). The arrow shows a darker spot that has been stained.
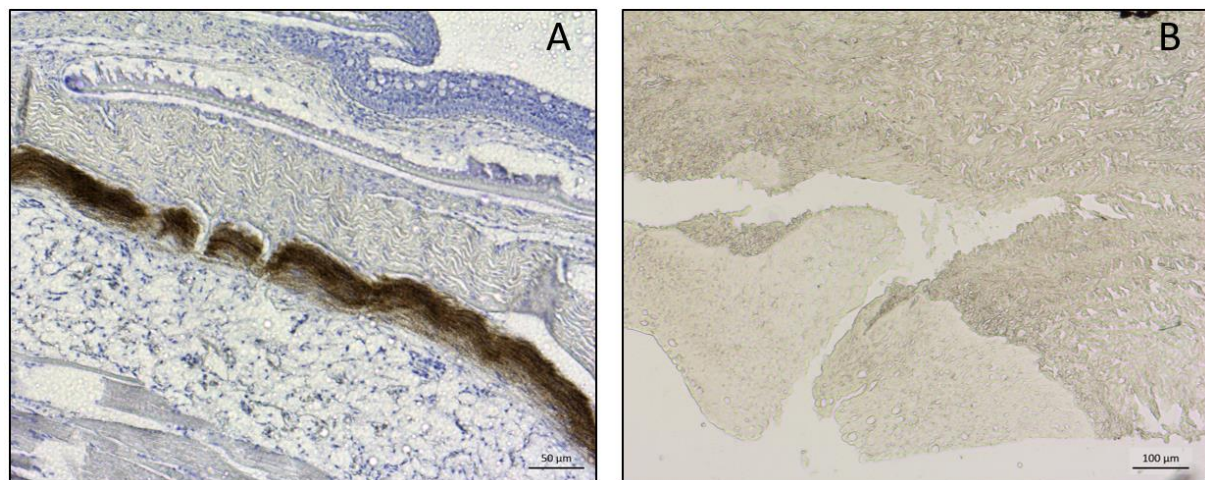
Fig. 13: A- H Hematoxylin and immunohistochemically stained for *M. viscosa* skin after *M. viscosa* infection at sampling B (Fish 3, tank 1). B – Control for immunohistochemistry, not incubated with primary antibody.

3.2 Size: Weight, Length and K-factor

3.2.1 Weight

At time B, the weight of the co-infected infected fish was significantly lower than the control fish, whereas infection with *L. salmonis* had no significant impact compared to control but significantly higher than the co-infection group. At time C no control fish were analyzed, weight was significantly higher for *L. salmonis* infected fish compared to *M. viscosa* single infection and co-infection. Time D, weight was again significantly higher for control compared to *M. viscosa* infection and co-infection. The *L. salmonis* fish weight was also significantly higher than *M. viscosa* and co-infection fish. There was an overall increase in the average weight of the fish during the experiment from time B to D in all groups. Control fish gained an average 67 g whereas *M. viscosa* infected fish gained 26 g, *L. salmonis* infected fish gained 88 g for *L. salmonis*, and co-infected fish gained 45 g (Fig. 14).
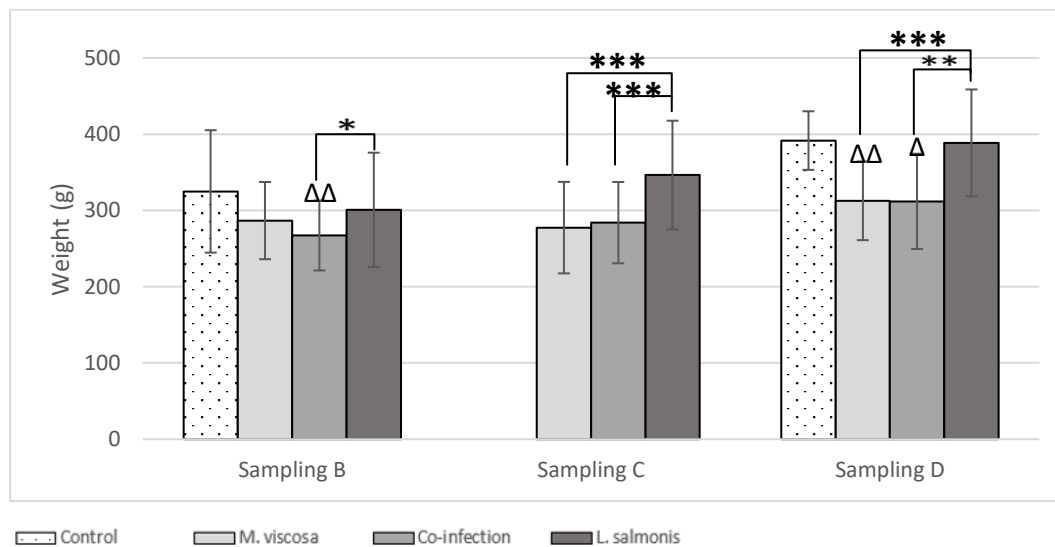
Fig. 14: Average weight ± SD of fish at sampling points B, C and D. Control (n: B=10, C=10, D=10), *M. viscosa* (n: B=40, C=20, D=21), *L. salmonis* (n: B=30, C=30, D=30), and co-infection (n: B=40, C=40, D=13),. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.

3.2.2 **Length**

At time B, *M. viscosa* group was significantly higher for compared to co-infection group. Time C length *L. salmonis* group was significantly higher than both *M. viscosa* and co-infection groups. At time D *L. salmonis* group was significantly higher than *M. viscosa*. From sampling B to sampling D there was an average growth of 1.3cm for control group, -0.01cm for *M. viscosa* group, 1.2cm for *L. salmonis* group, and 1.7cm for co-infection group (Fig. 15).
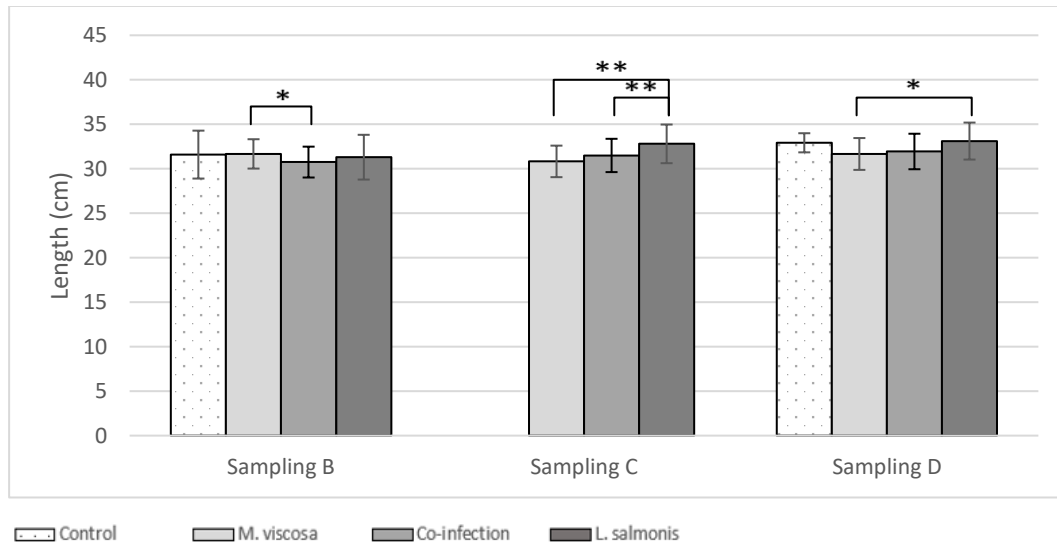
Fig. 15: Average fork length ± SD of fish at sampling points B, C and D. Control, *M. viscosa*, *L. salmonis* and co-infection (n= as indicated in fig 10). Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.

3.2.3 **Condition factor (K-factor)**

At time B, the k-factor for the control group was significantly higher than the co-infection group. The *L. salmonis* group was significantly higher than the *M. viscosa* group. Time C the *L. salmonis* group was significantly higher than the co-infection group. Time D Control was significantly higher than *M. viscosa* and co-infection groups. *L. salmonis* group was significantly higher than *M. viscosa* and co-infection groups (Fig. 16).
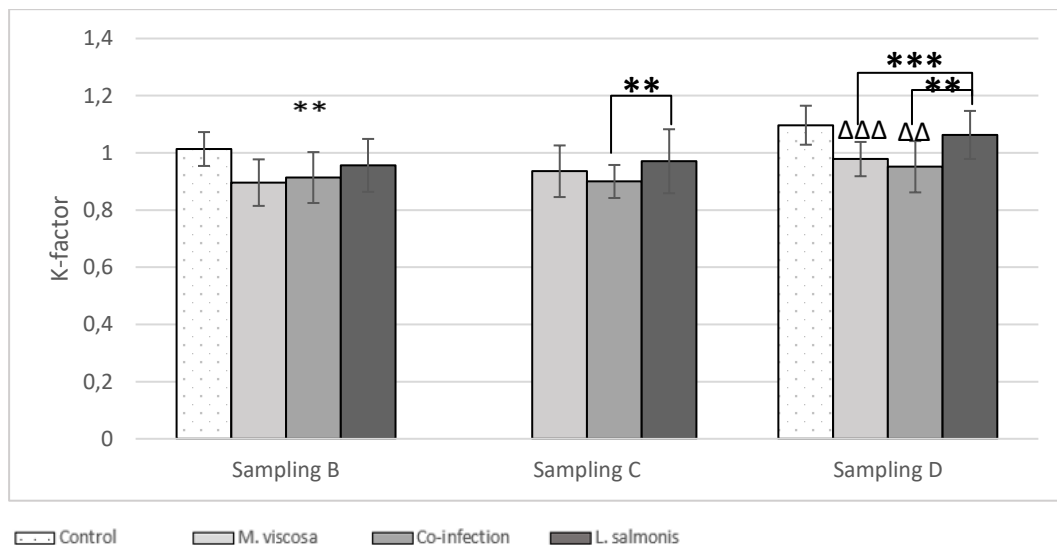
Fig. 16: Average k-factor ± SD of fish at sampling points B, C and D. Control, *M. viscosa*, *L. salmonis* and co-infection (n= as indicated in fig 10). Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.

3.3 Survival

Survival of infected fish was followed from the start of the experiment for over 40 days from the *L. salmonis* infections. The graph was made based on percentage survival on estimated remaining fish after sampling. Each tank had 40 fish after sampling A, and ten fish were removed from both B, and C. Fish were infected with *M. viscosa* five days after *L. salmonis*. The *L. salmonis* group had the highest survival with 97% followed by control group survival of 85%. *M. viscosa* group had a survival of 58% while the co-infection group had the lowest survival of only 44% (Fig. 17).
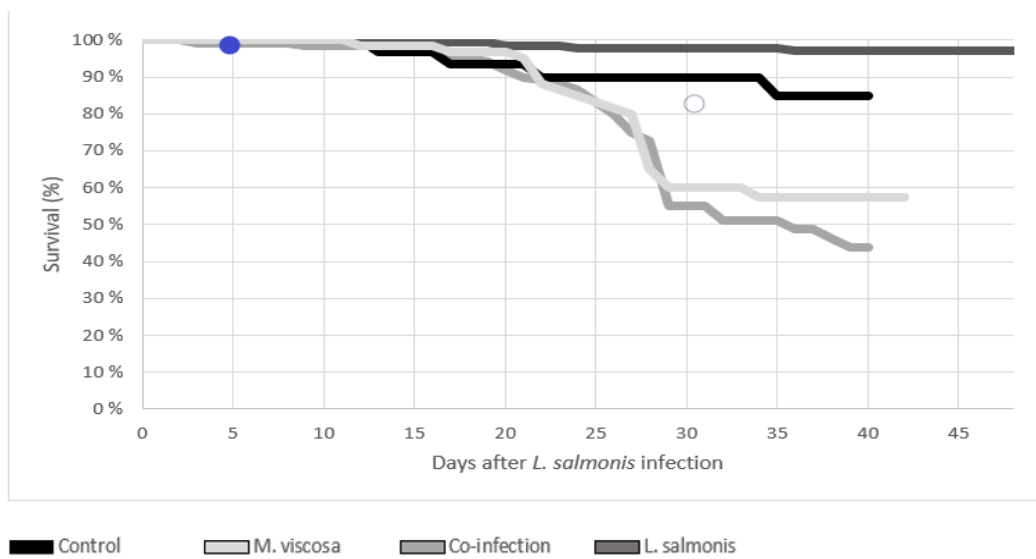
Fig. 17: Survival for all groups, *M. viscosa* (n=38), *L. salmonis* (n=140), co-infection (60) and control (n=60) during the experiment from *L. salmonis* infections were completed. *M. viscosa* infections are indicated as a blue dot.

3.4 Serum cortisol - Stress

There were no statistically significant differences in serum cortisol in time B (Fig. 18). *L. salmonis* group had a statistically higher serum cortisol than control in time C (Fig. 19).
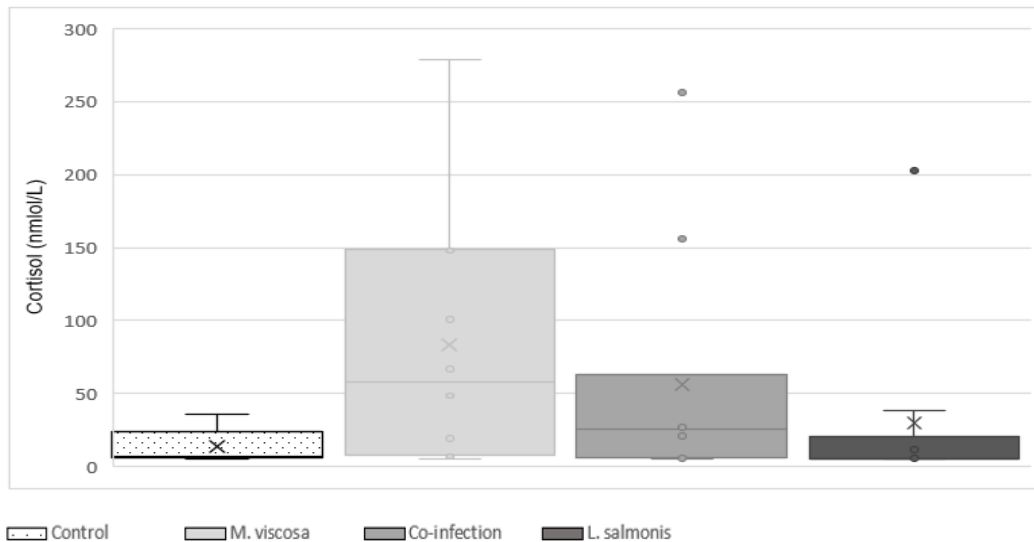
Fig. 18: Average serum cortisol ± SD of fish at sampling point B. Control (n=5), *M. viscosa* (n=10), *L. salmonis* (n=10) and co-infection(n=10). Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.
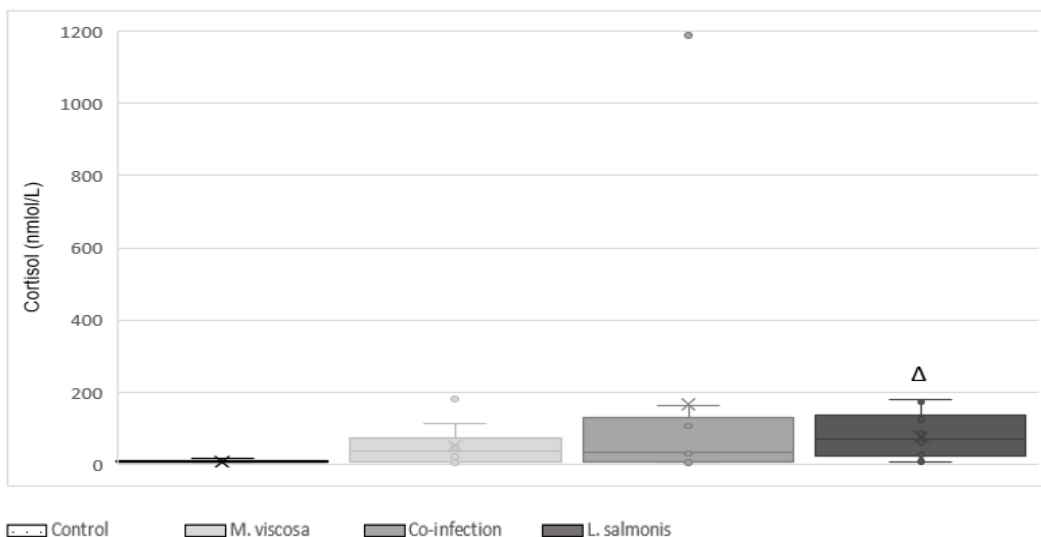
Fig. 19: Average serum cortisol ± SD of fish at sampling point C. Control (n=5), *M. viscosa* (n=10), *L. salmonis* (n=10) and co-infection(n=10). Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.

3.5 *M. viscosa* ulcers

Bacteria were re-cultured and (some) sequenced from ulcers on fish (from mortalities) to confirm the presence of *M. viscosa* in ulcers. This analysis was done by personnel at AVC Ulcers had a prevalence of 70%, 95% and 85% in the single infected group at time B, C, and D while co-infected fish had 62.5%, 87.5% and 92.3%. The average number ulcers detected at time C was significantly higher in fish infected with only *M. viscosa* compared to fish that were co-infected. However, this was not observed at time D (Fig. 20).
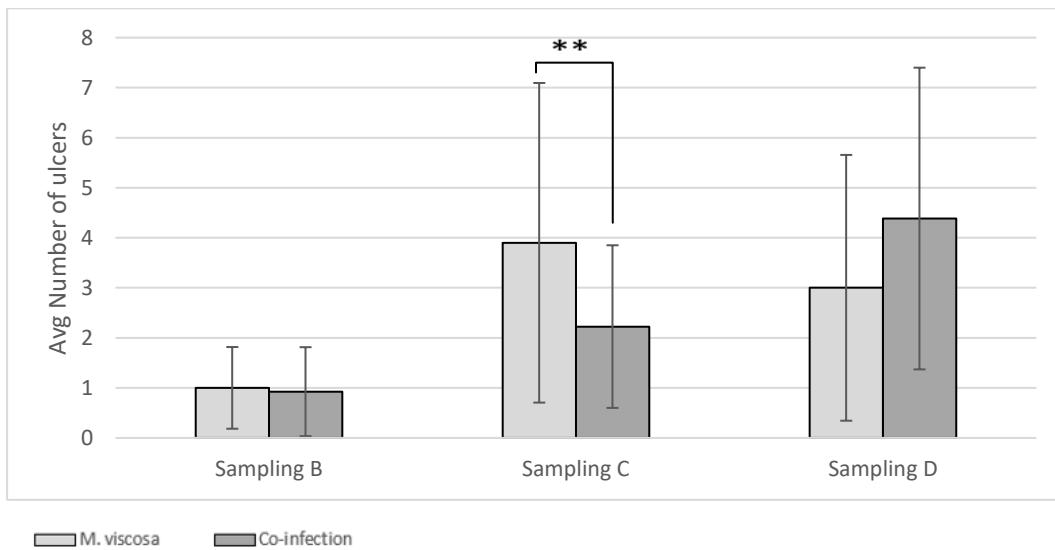
Fig. 20: Number of lesions ± SD at sampling points B, C and D for *M. viscosa* and co-infection. (n= as indicated in fig 10). Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.

The average sizes of ulcers were not significantly different from each other in any samplings, however, there was a trend for the co-infection group of larger ulcers at time D (fig. 21). The total size of ulcers for time D was significantly higher for co-infection group than *M. viscosa* group (fig. 22).
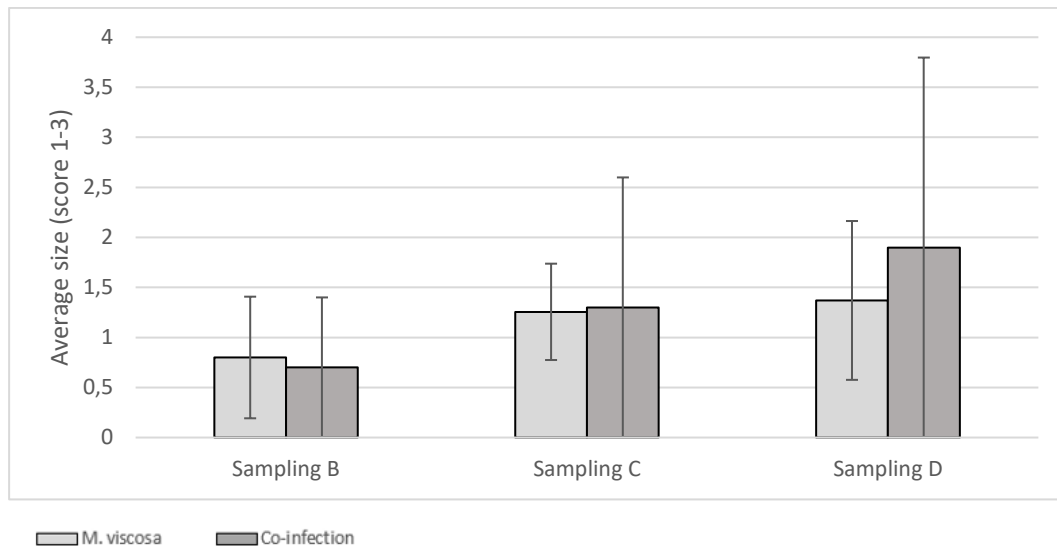
Fig. 21: Number of lesions ± SD at sampling points B, C and D for *M. viscosa*, and co-infection (n= as indicated in fig 10). Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.
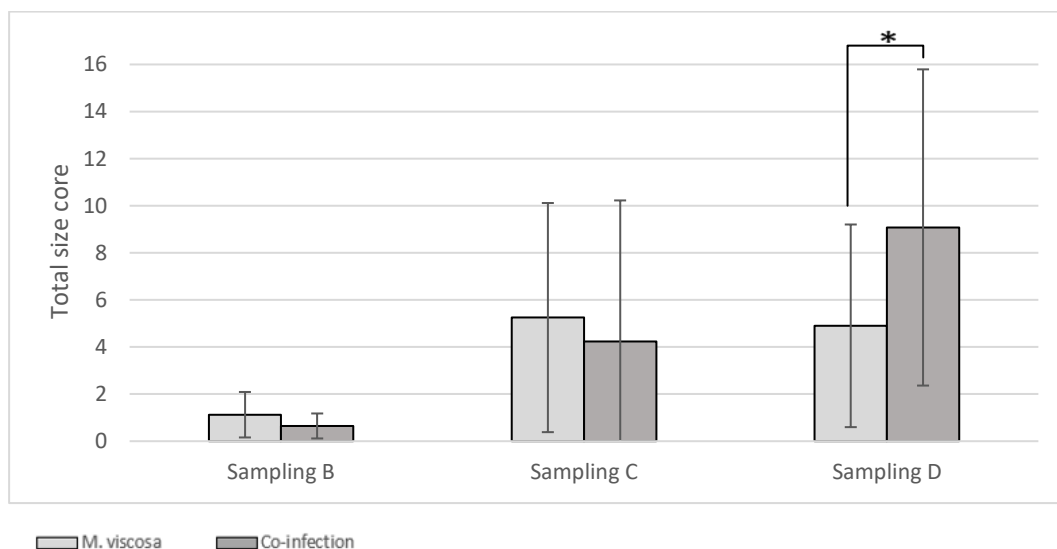
Fig. 22: Total size of ulcers ± SD at sampling points B, C and D for *M. viscosa*, and co-infection (n= as indicated in fig 10). Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.

The average grade of ulcers was not significantly different from each other (Fig.23).

Total ulcer grade was significantly higher for *M. viscosa* group than the co-infection group at time C (Fig. 24).
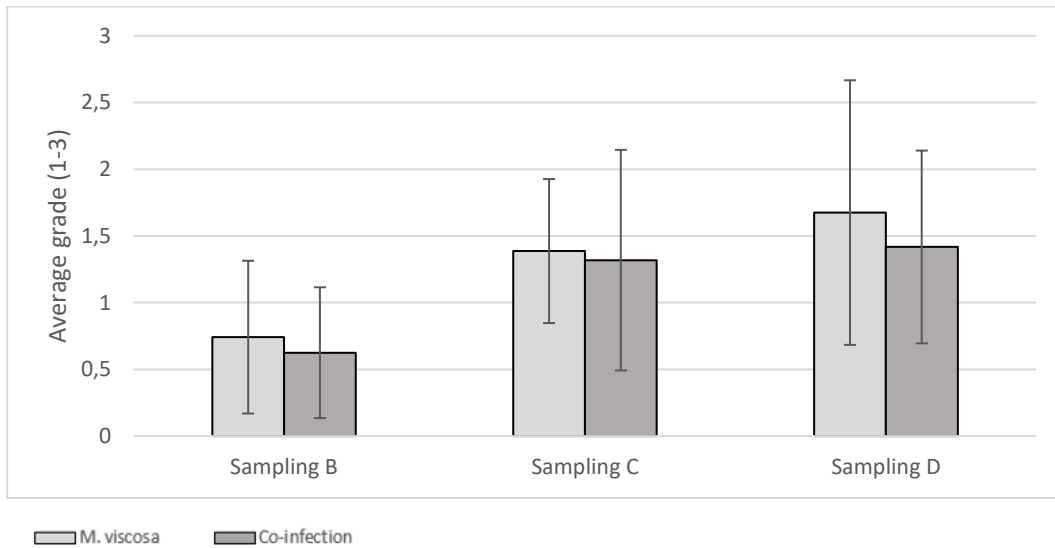
Fig. 23: Average ulcer grade ± SD at sampling points B, C and D for *M. viscosa*, and co-infection (n= as indicated in fig 10). Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.
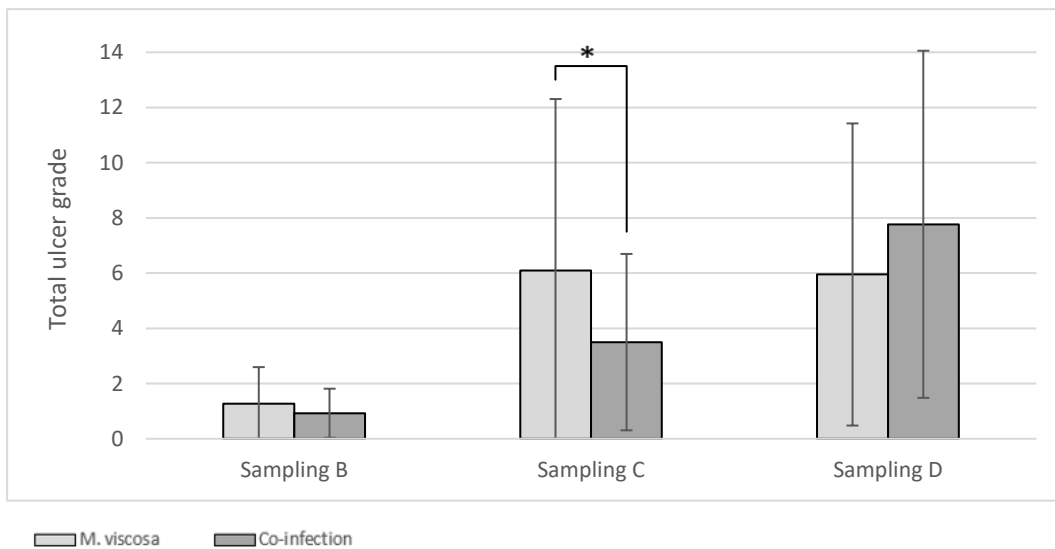
Fig. 24: Total ulcer grade ± SD at sampling points B, C and D for *M. viscosa*, and co-infection (n= as indicated in fig 10). Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.

At time C, there was a significantly higher grade 1 lesions and significantly higher grade 2 lesions at time B in co-infection compared to single infection. (Fig.25).
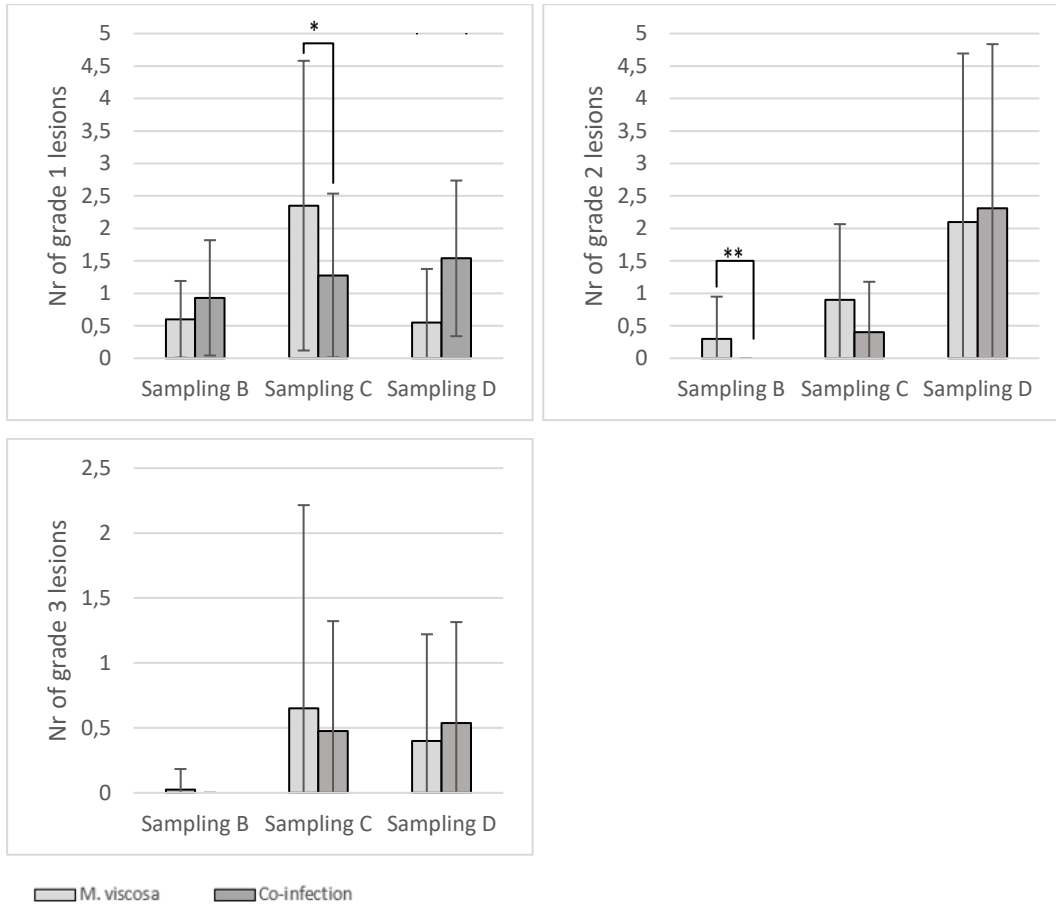
Fig. 25: Average amount of ulcers of each grade ± SD at sampling points B, C and D for *M. viscosa*, and co-infection (n= as indicated in fig 10). Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.

3.6 *L. salmonis*

L. salmonis had a prevalence of 96.6% for single infected fish and 97.5% for co-infected fish at sampling B. All other samplings had a prevalence of 100%. Atlantic salmon were infected with 50 copepodids/fish resulting in the largest average count for the co-infected fish being 5.6 (time C) and 8.03 for *L. salmonis* group (time D); the infection success was 11.2% and 16.1% respectively. There was a trend (not significant) of more lice found on fish infected with just *L. salmonis* at time C, and there was a significant difference in D of more lice in a single infection (Fig. 26). There was a significantly higher amount of chalimus and pre-adult female in *L. salmonis* group for time C and pre-adult female and adult male for time D (Fig. 27).
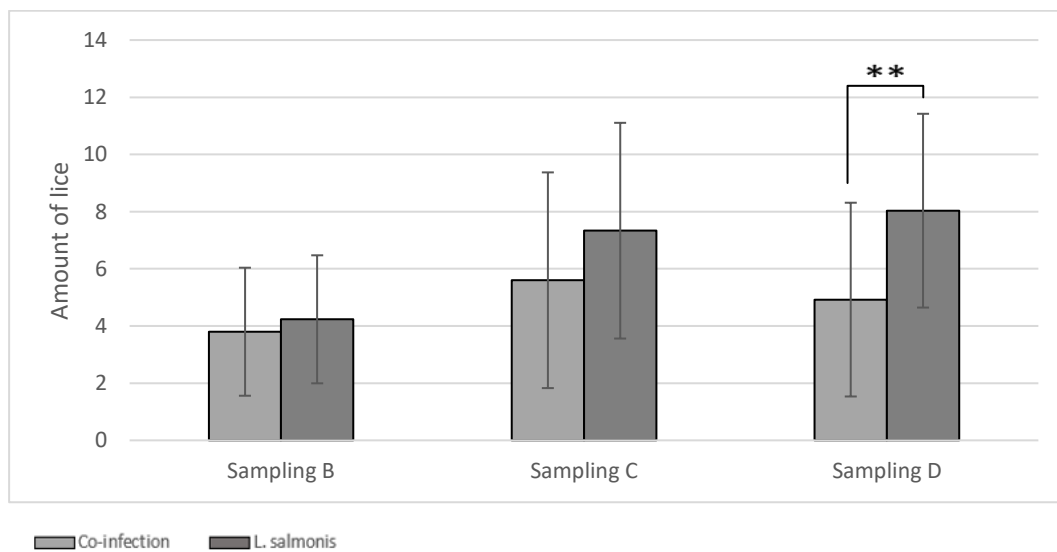
Fig. 26 Lice count ± SD at sampling points B, C and D for *L. salmonis* and co-infection. (n= as indicated in fig 10). Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.
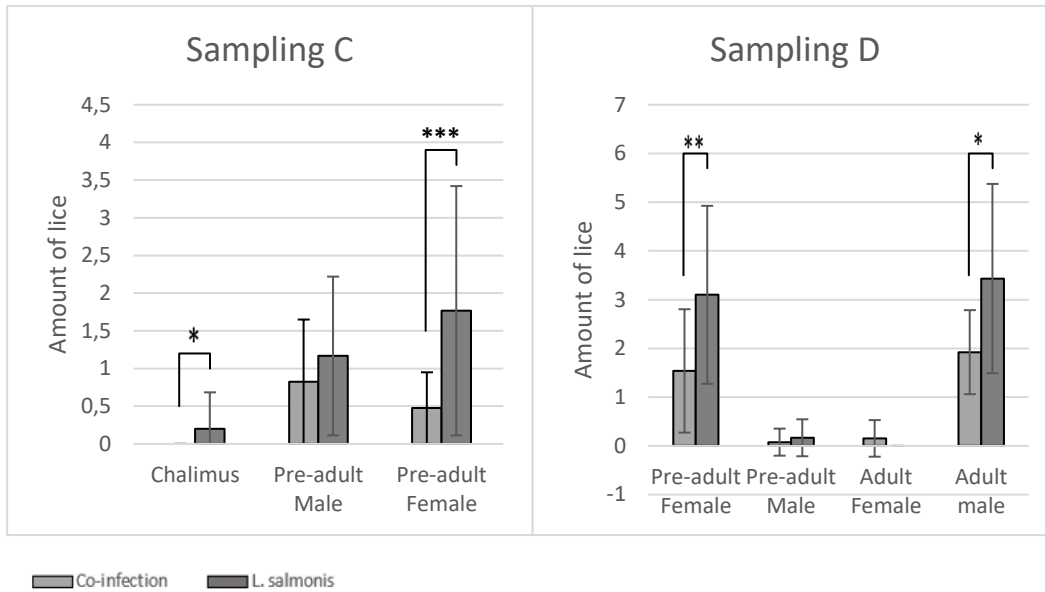
Fig. 27. Growth stage and gender of L. salmonis ± SD at sampling points C and D for *L. salmonis* and co-infection. (n= as indicated in fig 10). Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.

3.7 Tank effects

As there where several tanks for the different groups the tanks were compared to determine if there were any tank effects. Any statistically significant differences between replicate tanks are shown in table 11, 12 and 13 for size, ulcers and lice. Two tanks for time D co-infection contained only 1 fish and was therefore not possible to perform a t-test while the remaining two (11 and 13) contained three and eight fish. All other samplings had ten or more fish from each tank. Tank effects for immune response was not checked, as there were only 1-4 samples from replicate tanks.

Table 11: P-values of statistically significant differences in replicate tanks at sampling B

Group	Tank ID	Avg Size of ulcers	Total lice count	Total/avg ulcer grade
M. viscosa	4-5	-	-	0,039
	1-8	-	0,010	-
	4 -8	-	0,023	-
L. salmonis	10-11	-	0,046	-
Co-infection	10-13	0,014	-	-

Table 12: P-values of statistically significant differences in replicate tanks at sampling C

Group	Tanks	Avg size of ulcers	Preadult ♀	Total lice
M. viscosa	8-9	0,021		-
L. salmonis	1-4	-	-	0,050
	10-11	-	0,015	-
	11-12	-	0,004	-
Co-infection	11-13	-	-	0,032
	12-13	-	-	0,014

Table 13: P-values of statistically significant differences in replicate tanks at sampling D

Group	Tanks	Length	Ulcer gr2	Preadult lice	Preadult ♀	Adult ♂	Lice count
M. viscosa	8-9	-	0,027	-	0,0076	0,0005	-
L. salmonis	1-4	-	-	-	-	-	0,039
	1-8	-	-	0,025	-	-	0,009
	4-8	-	-	-	0,0175	0,0189	-
Co-infection	11-13	0,035	-	-	-	-	-

3.8 Expression of immune genes

To study the differences between the immune response in Atlantic salmon during *M. viscosa* infection, *L. salmonis* infection and co-infection, transcription of six immune genes were evaluated in the skin and head kidney. Tissue samples were analyzed from sampling B, and sampling C. Immune genes were chosen according to previous research. The analysis included IL-1β, IL-4/13A, IL-8, IL-10, C3, and IgM. Transcript levels were calculated in relation to control fish at the same timepoint. Infected skin sites (lice attachment/*M. viscosa* ulcer) are compared to control (uninfected fish) and unaffected skin of infected fish. Unaffected skin was taken directly beside the lice attachment or ulcer. A t-test was performed to test for significant differences between all groups, but the figures only show significant differences of sites that were appropriate to compare. All p-values are listed in appendix B, with significant differences in bold. e. Figures of gene expression in head kidneys that had no significant differences are shown in appendix C.

3.8.1 Pro-inflammatory genes

IL-1β expression in skin

Time B Skin from co-infected fish away from site of infection (lice attachment/*M. viscosa* lesion) had a significantly higher expression of IL-1β than control (uninfected) fish. Skin from co-infected fish at the site of the lice infection also had a significantly higher expression of IL-1β (fig. 28). The t-test was also run by removing the outlier for co-infected *M. viscosa* lesion, which resulted in a significantly higher expression compared to control similar to the pattern observed in all other samples from co-infected fish (result not shown).

Time C Co-infected fish had a significantly higher expression of IL-1β at *M. viscosa* lesion and both *M. viscosa* unaffected control site. The expression was also significantly higher in *M. viscosa* lesion compared to unaffected control site in single infected fish (fig 29). All samples affected and non-affected by *L. salmonis*, had no significant changes compared to controls.

Fig. 28: Expression of IL-1β in skin samples from sampling B. Relative RNA ($2^{-\Delta\Delta ct}$) of IL-1β in fish skin samples from control (n=10), *M. viscosa* infection (lesion n=13, control n=12), *L. salmonis* infection (attachment n=11, control n=12) and co-infection (lesion n=8, *M. viscosa* control n=10, attachment n=5 *L. salmonis* control n=5). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.

Fig. 29: Expression of IL-1β in skin samples from sampling C. Relative RNA ($2^{-\Delta\Delta ct}$) of IL-1β in fish skin samples from control (n=10), *M. viscosa* infection (lesion n=8, control n=8), *L. salmonis* infection (attachment n=8, control n=8) and co-infection (lesion n=7, *M. viscosa* control n=8, attachment n=7 *L. salmonis* control n=7). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001

IL-1β expression in head kidney

Time B IL-1β was significantly higher expressed in head kidneys from co-infected fish compared to the control group (Fig. 30), *Time C* whereas IL-1β was significantly lower expressed in co-infected fish compared to the control group and *L. salmonis* group (Fig. 31).

Fig. 30: Expression of IL-1β in head kidney samples from sampling B. Relative RNA ($2^{-\Delta\Delta ct}$) of IL-1β in head kidney samples from control (n=9) *M. viscosa* infection (n=12), *L. salmonis* infection(n=10) and co-infection(n=11). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.

Fig. 31: Expression of IL-1β in head kidney samples from sampling C. Relative RNA ($2^{-\Delta\Delta ct}$) of IL-1β in head kidney samples from control (n=9) *M. viscosa* infection (n=8), *L. salmonis* infection(n=8) and co-infection(n=7). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.

IL-8 expression in skin

Time B All skin sites from fish infected with *L. salmonis* had a significantly higher expression of IL-1β than control (uninfected) fish. Skin from co-infected *M. viscosa* unaffected control site also had a significantly higher expression of IL- β (Fig. 32). The t-test was also run by removing the outlier for co-infected *M. viscosa* lesion, which resulted in a significantly higher expression compared to control similar to the pattern observed in all other samples from co-infected fish (result not shown).

Time C Co-infected fish had a significantly higher expression of IL-8 at co-infected *M. viscosa* lesion and single infected *M. viscosa* unaffected control site. Expression of IL-8 was also significantly higher in co-infected fish *M. viscosa* lesion than co-infected *M. viscosa* unaffected control (Fig. 33).

Fig. 32: Expression of IL-8 in skin samples from sampling B. Relative RNA ($2^{-\Delta\Delta ct}$) of IL-8 in fish skin samples from control (n=10), *M. viscosa* infection (lesion n=13, control n=12), *L. salmonis* infection (attachment n=11, control n=12) and co-infection (lesion n=8, *M. viscosa* control n=10, attachment n=5 *L. salmonis* control n=5). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.

Fig. 33: Expression of IL-8 in skin samples from sampling C. Relative RNA ($2^{-\Delta\Delta ct}$) of IL-8 in fish skin samples from control (n=10), *M. viscosa* infection (lesion n=8, control n=8), *L. salmonis* infection (attachment n=8, control n=8) and co-infection (lesion n=7, *M. viscosa* control n=8, attachment n=7 *L. salmonis* control n=7). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001

IL-8 expression in head kidney

There were no significant differences in expression if IL-8 at time B or C (Fig. 46 and 47, appendix C).

3.8.2 Anti-inflammatory genes

IL-4/13A expression in skin

Time B Skin from co-infected fish at both *M. viscosa* sites had a significantly lower expression of IL-4/13A than control (uninfected) fish. Skin from single infected fish at *M. viscosa* lesion also had a significantly lower expression of IL-4/13A (Fig. 35).

Time C All sites except for single infected *L. salmonis* unaffected control site had a significantly lower expression of IL-4/13A than control (uninfected) fish (Fig. 36).
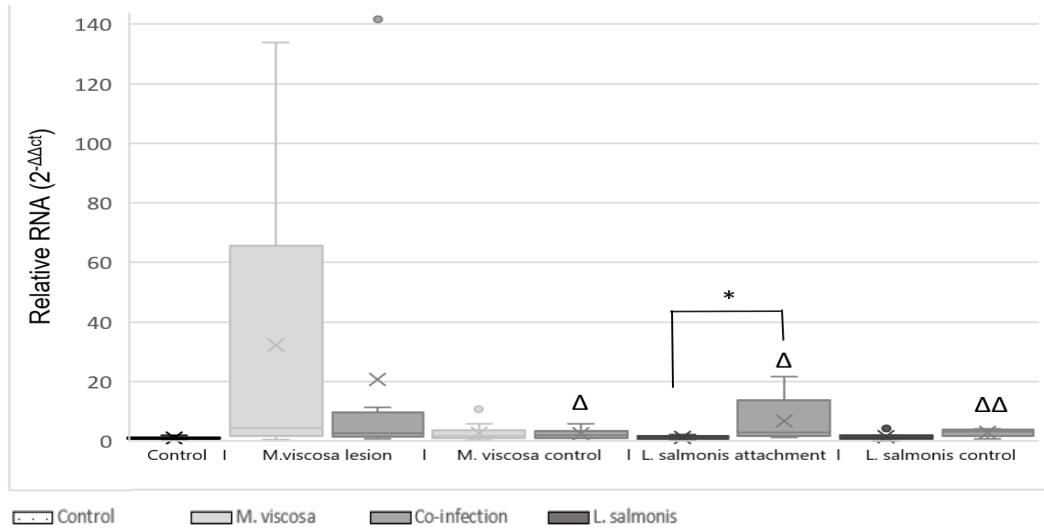

Fig. 34: Expression of IL-4/13A in skin samples from sampling B. Relative RNA ($2^{-\Delta\Delta ct}$) of IL-4/13A in fish skin samples from control (n=10), *M. viscosa* infection (lesion n=13, control n=12), *L. salmonis* infection (attachment n=11, control n=12) and co-infection (lesion n=8, *M. viscosa* control n=10, attachment n=5 *L. salmonis* control n=5). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values P values */Δ =0.05, **/ $\Delta\Delta$=0.01, ***/ $\Delta\Delta\Delta$=0.001.
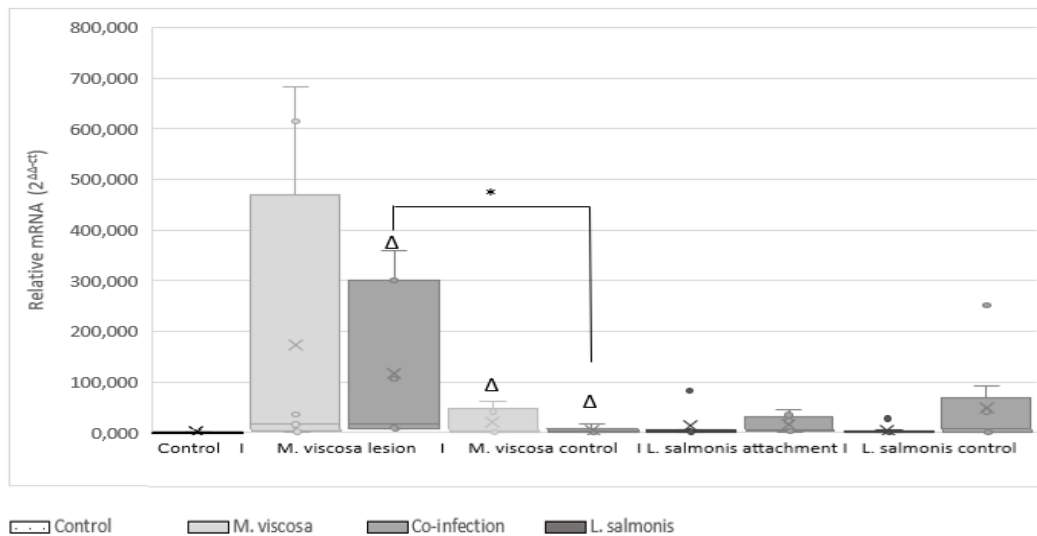
Fig. 35: Expression of IL-4/13A in skin samples from sampling C. Relative RNA (2-ΔΔct) of IL-4/13A in fish skin samples from control (n=10), *M. viscosa* infection (lesion n=8, control n=8), *L. salmonis* infection (attachment n=8, control n=8) and co-infection (lesion n=7, *M. viscosa* control n=8, attachment n=7 *L. salmonis* control n=7). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001

IL-4/13A expression in head kidney

Time B IL-4/13A was significantly higher expressed in co-infected fish than only infected with *M. viscosa* (Fig. 36).

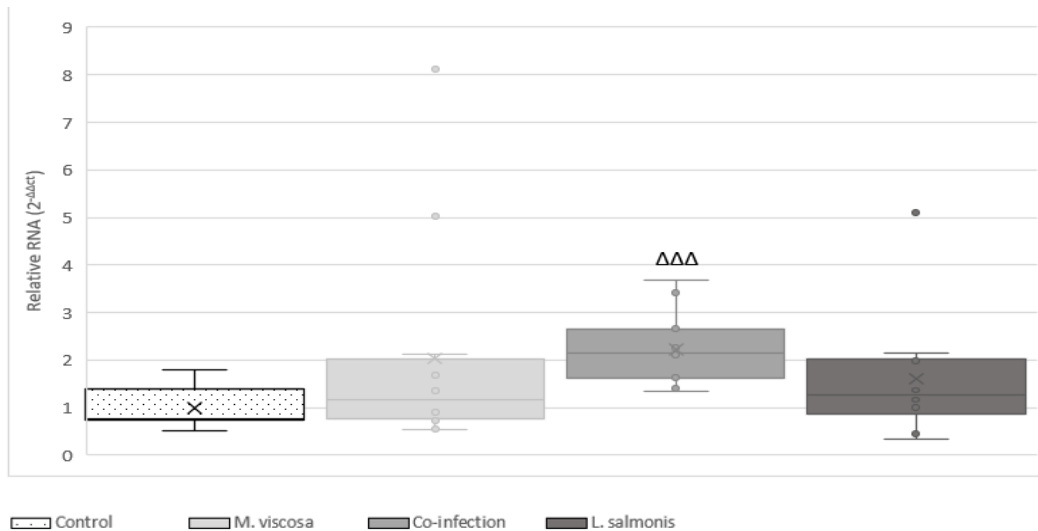*Time C* IL-4/13A was significantly lower expressed in co-infected fish than both single infections and control (Fig. 37).

Fig. 36: Expression of IL-4/13A in head kidney samples from sampling B. Relative RNA ($2^{-\Delta\Delta ct}$) of IL-4/13A in head kidney samples from control (n=9) *M. viscosa* infection (n=12), *L. salmonis* infection(n=10) and co-infection(n=11). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.
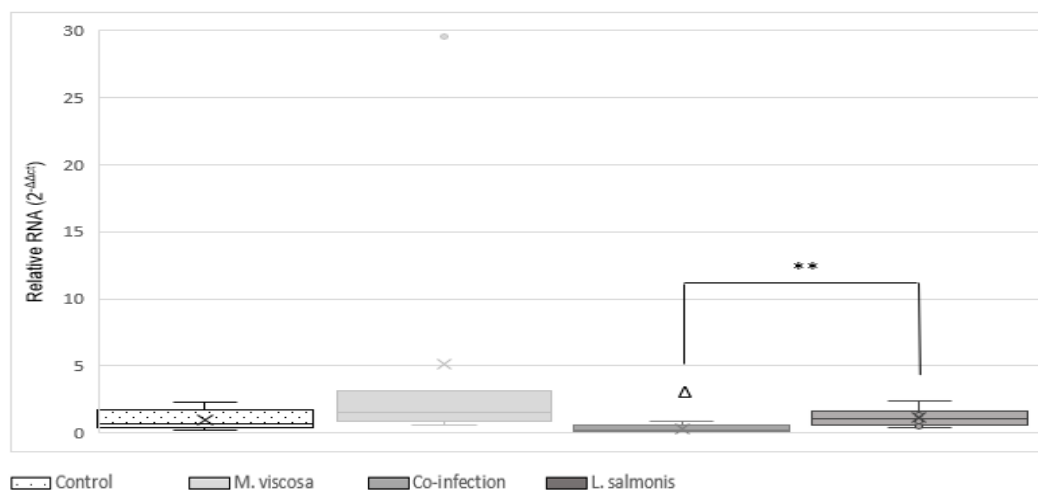

Fig. 37: Expression of IL4/13A in head kidney samples from sampling C. Relative RNA ($2^{-\Delta\Delta ct}$) of IL4/13A in head kidney samples from control (n=9) *M. viscosa* infection (n=8), *L. salmonis* infection(n=8) and co-infection(n=7). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.

IL-10 expression in skin

Time B Skin from co-infected fish all skin had a significantly lower expression of IL-10 than control (uninfected) fish as well as their single infected counterpart (Fig. 38).

Time C Skin from single infected fish away from site of *L. salmonis* infection had a significantly higher expression of IL-10 than control (uninfected) fish. Skin from *M. viscosa* unaffected control site was significantly higher expression of IL-10 in single infection compared to co-infection. Skin from *L. salmonis* unaffected control site was significantly higher expression of IL-10 in co-infection compared to single infection (Fig. 39).

Fig. 38: Expression of IL-10 in skin samples from sampling B. Relative RNA ($2^{-\Delta\Delta ct}$) of IL-10 in fish skin samples from control (n=10), *M. viscosa* infection (lesion n=13, control n=12), *L. salmonis* infection (attachment n=11, control n=12) and co-infection (lesion n=8, *M. viscosa* control n=10, attachment n=5 *L. salmonis* control n=5). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values P values */Δ =0.05, **/ $\Delta\Delta$=0.01, ***/ $\Delta\Delta\Delta$=0.001.
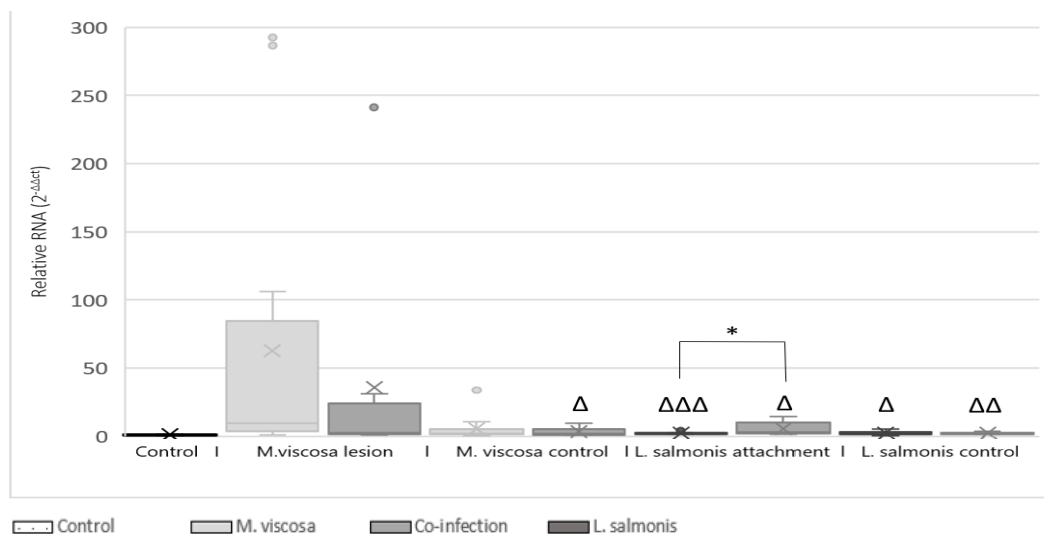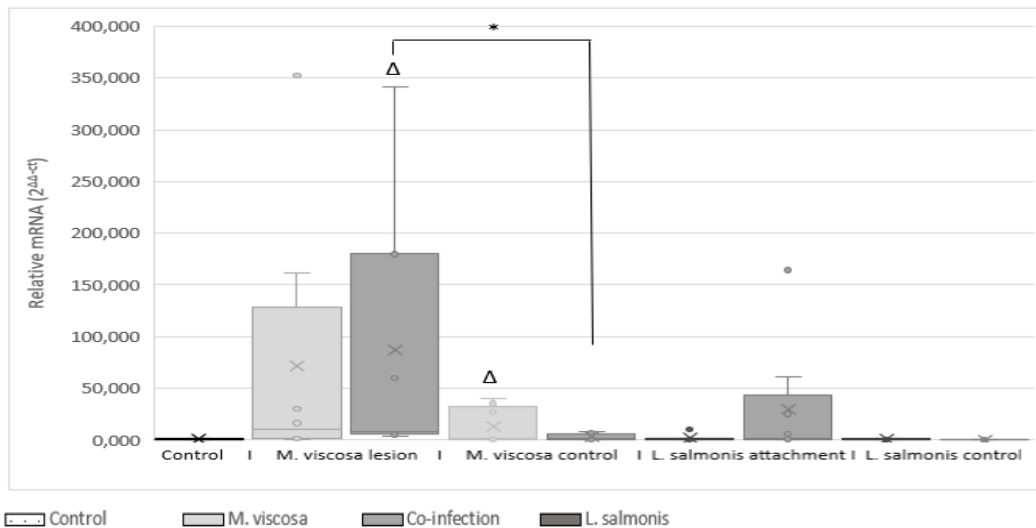
Fig. 39: Expression of IL-10 in skin samples from sampling C. Relative RNA ($2^{-\Delta\Delta ct}$) of Il-10 in fish skin samples from control (n=10), *M. viscosa* infection (lesion n=8, control n=8), *L. salmonis* infection (attachment n=8, control n=8) and co-infection (lesion n=7, *M. viscosa* control n=8, attachment n=7 *L. salmonis* control n=7). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001

IL-10 expression in head kidney

There were no significant differences in expression if IL-10 at time B and C (Fig. 48 and 49, appendix C).

3.8.3 **Acute-phase protein: C3**

C3 expression in skin

Time B Skin from single infected *M. viscosa* lesion and both co-infected *M. viscosa* sites had a significantly higher expression of C3 than control (uninfected) fish. Skin from single infected *M. viscosa* lesion had also a significantly higher expression of C3 than single infected *L. salmonis* attachment site (Fig. 40).

Time C Skin from both *M. viscosa* lesion sites and single infected *M. viscosa* unaffected control site had a significantly higher expression C3 than control (uninfected) fish. Skin from single infected *L. salmonis* unaffected control also had a significantly higher expression C3 than control (uninfected) fish. C3 in skin from single infected *M. viscosa* lesion was significantly higher expressed than single infected *L. salmonis* attachment (Fig. 41).
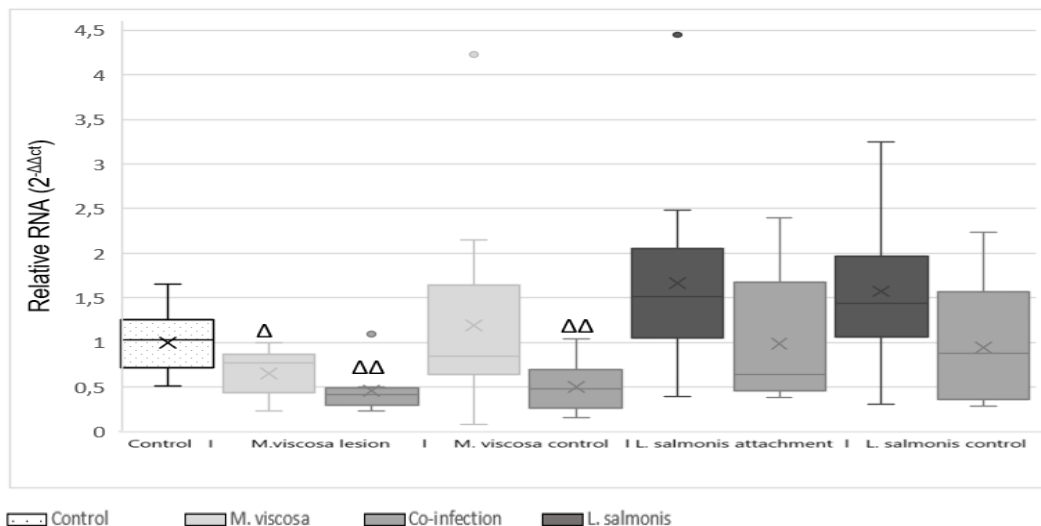

Fig. 40: Expression of C3 in skin samples from sampling B. Relative RNA ($2^{-\Delta\Delta ct}$) of C3 in fish skin samples from control (n=10), *M. viscosa* infection (lesion n=13, control n=12), *L. salmonis* infection (attachment n=11, control n=12) and co-infection (lesion n=8, *M. viscosa* control n=10, attachment n=5 *L. salmonis* control n=5). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.
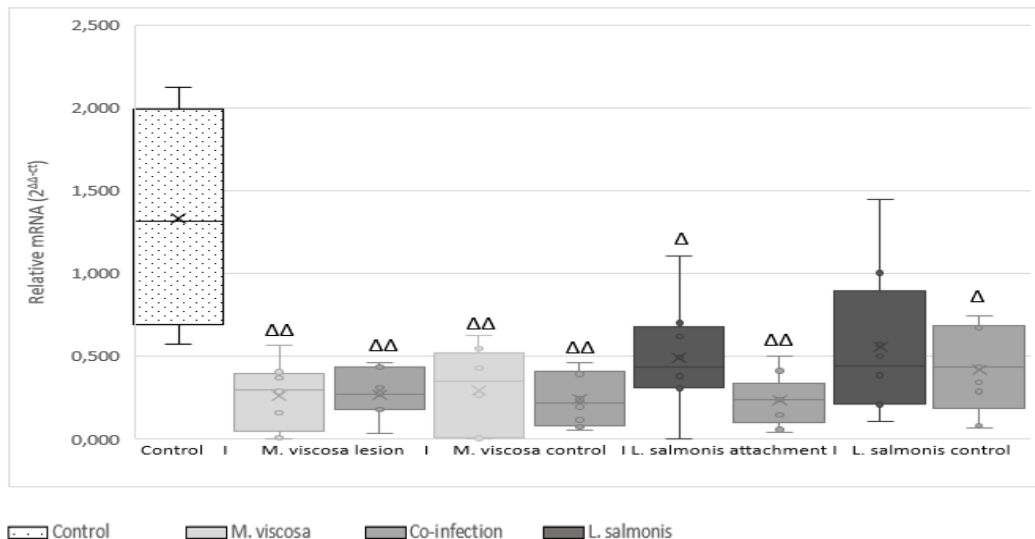
Fig. 41: Expression of C3 in skin samples from sampling C. Relative RNA ($2^{-\Delta\Delta ct}$) of C3 in fish skin samples from control (n=10), *M. viscosa* infection (lesion n=8, control n=8), *L. salmonis* infection (attachment n=8, control n=8) and co-infection (lesion n=7, *M. viscosa* control n=8, attachment n=7 *L. salmonis* control n=7). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001

C3 expression in head kidney

Time B There was a significantly higher expression of C3 in co-infected compared group to *M. viscosa* group at time B (Fig. 42). There were no significant differences in expression if C3 at time C (Fig. 50, appendix C).
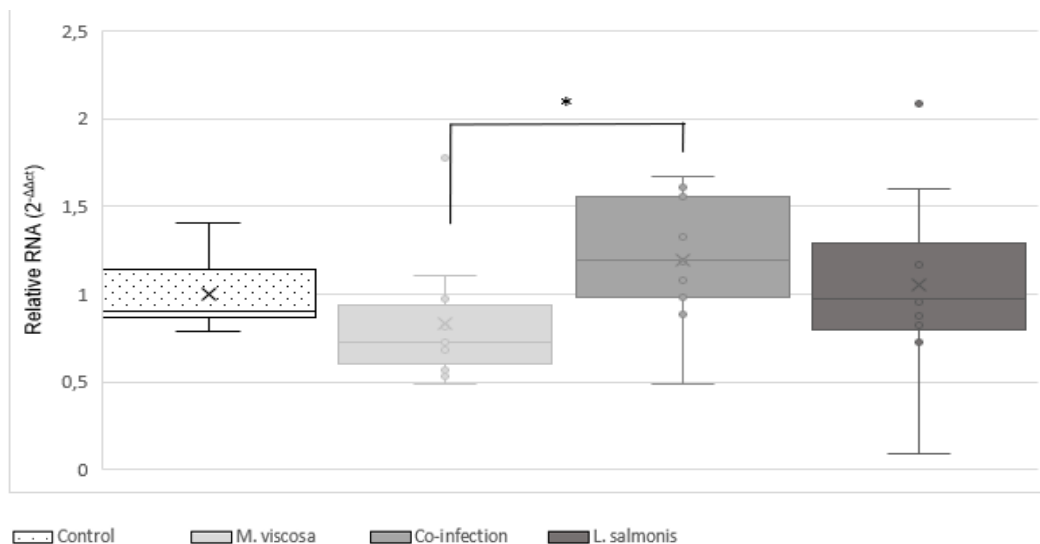

Fig. 42: Expression of C3 in head kidney samples from sampling B. Relative RNA ($2^{-\Delta\Delta ct}$) of C3 in head kidney samples from control (n=9) *M. viscosa* infection (n=12), *L. salmonis* infection(n=10) and co-infection(n=11). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.

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3.8.4 **Immunoglobulin: IgM**

IgM expression in skin

Time B Skin from single infected *M. viscosa* unaffected control site and co-infected *L. salmonis* attachment sites had a significantly higher expression of IL-1β than control (uninfected) fish

Time C Skin from single infected *M. viscosa* lesion site and *L. salmonis* attachment sites had a significantly higher expression of IL-1β than control (uninfected) fish (Fig. 43).
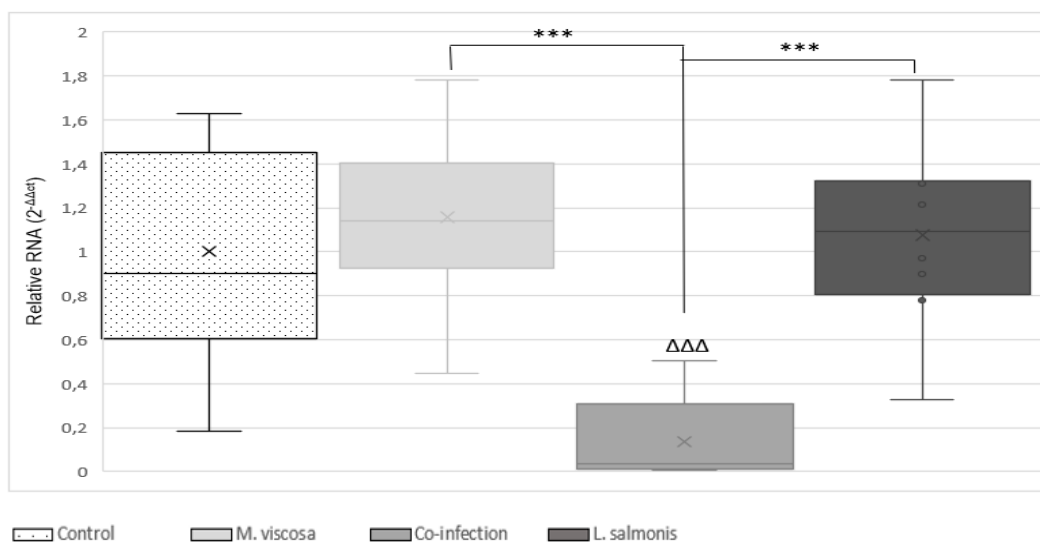

Fig. 43: Expression of IgM in skin samples from sampling B. Relative RNA ($2^{-\Delta\Delta ct}$) of IgM in fish skin samples from control (n=10), *M. viscosa* infection (lesion n=13, control n=12), *L. salmonis* infection (attachment n=11, control n=12) and co-infection (lesion n=8, *M. viscosa* control n=10, attachment n=5 *L. salmonis* control n=5). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.
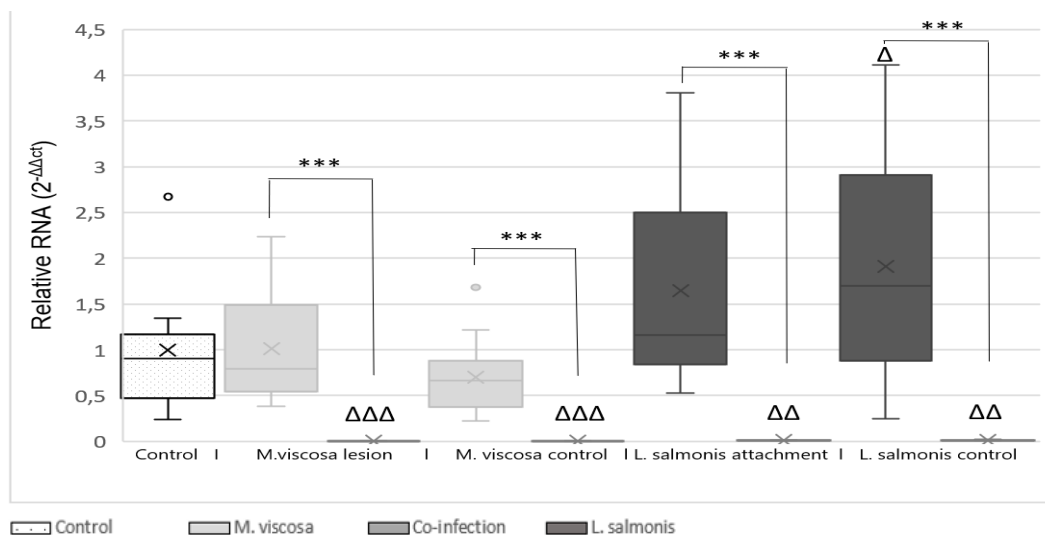
Fig. 44: Expression of IgM in skin samples from sampling C. Relative RNA ($2^{-\Delta\Delta ct}$) of IgM in fish skin samples from control (n=10), *M. viscosa* infection (lesion n=8, control n=8), *L. salmonis* infection (attachment n=8, control n=8) and co-infection (lesion n=7, *M. viscosa* control n=8, attachment n=7 *L. salmonis* control n=7). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values P values */Δ =0.05, **/ $\Delta\Delta$=0.01, ***/ $\Delta\Delta\Delta$=0.001

IgM expression in head kidney

No significant differences of IgM at time B (Fig. 51, appendix C). Time C Expression of IgM in co-infection group was significantly lower than *M. viscosa* group at time C (Fig. 45).

Fig. 45: Expression of IgM in head kidney samples from sampling C. Relative RNA ($2^{-\Delta\Delta ct}$) of IgM in head kidney samples from control (n=9) *M. viscosa* infection (n=8), *L. salmonis* infection(n=8) and co-infection(n=7). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ $\Delta\Delta$=0.01, ***/ $\Delta\Delta\Delta$=0.001.
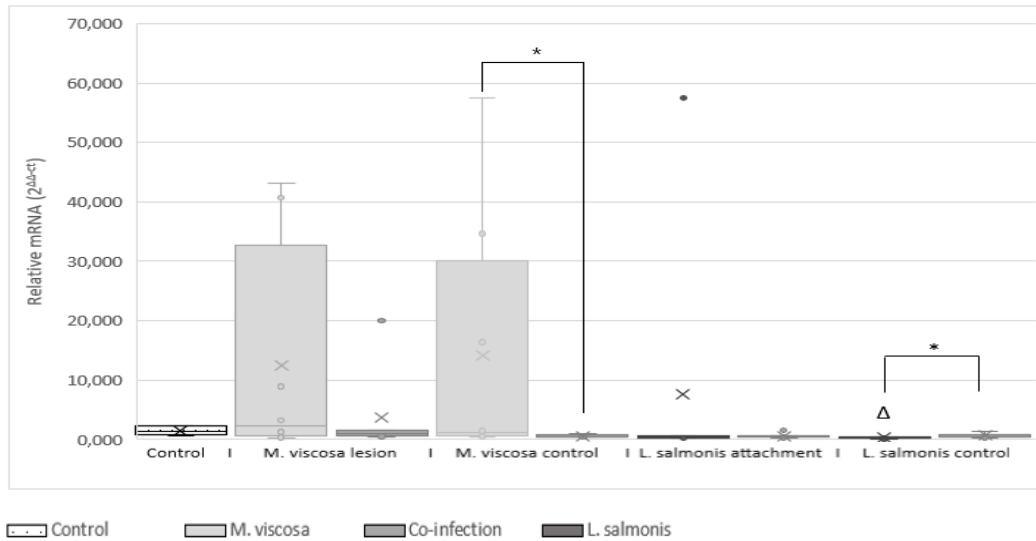
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4 Discussion

This study gives an understanding of how the pathogens *M. viscosa*, and *L. salmonis* affect the Atlantic salmon both separately and as a co-infection. In this study, several factors including weight, length, frequency, the severity of ulcers, number of lice, and expression of immune genes were analyzed. Despite large differences between individuals in most of these factors, especially in the expression of immune genes, some significant differences between the groups were detected.

4.1 Methodological limitations

In this section the limitations of the general experimental setup and infection is discussed as these apply to all parts. Other limitations are mentioned in the appropriate section.

4.1.1 Experimental setup

The experiment was a part of a large experiment with different feeds, and a total of 29 tanks with approx. 40 fish/tank, equaling to over 1100 fish. The experiment was scheduled to start in September, but due to slow growth and delays regarding feed, it did not start until December. A sudden drop in water temperature further delayed delivery of copepods, and due to weather conditions, the *M. viscosa* had to be delivered the day before and stored overnight at AVC for the second bath challenge. The infection plans therefore had to be rearranged to carry out the infections and get sampling A and B done before Christmas.

The number of fish tanks for each group was different with one control tank, four *L. salmonis* tanks, four co-infection tanks. *M. viscosa* at sampling B had four tanks (from first infection challenge) in RAS unit 1, however these tanks were used in the *M. viscosa + L. salmonis* infection, so two different tanks (from the second challenge) in RAS unit 2 were used for sampling at timepoint C and D. Because of individual differences between tanks, this makes a direct comparison of fish infected with *M. viscosa* between time B to time C problematic.

Due to a large number of fish, sampling stretched over several days after infection for different groups. This made it harder to compare as one group has been infected longer than the other. e.g., in sampling C, fish infected with *L. salmonis* (26 dpi) was compared to co-infected fish (lice 28dpi +23dpi *M. viscosa*). This may have affected the immune response where

transcriptional changes can occur fast. On the other hand, fish size, lice numbers and ulcer development are a result of slower processes and were unlikely to have been affected by this sampling regime.

4.1.2 **Infection**

As mentioned, the culture for the second challenge had to be stored overnight at AVC, even though it was set to 8± 1 ℃ the temperature had risen to 11± 1 ℃ the following morning. However, the higher temperature should not affect the bath challenge given that the water temperature during infection and after was satisfactory.

The fish were divided into tanks which again were connected to three separate RAS units. All RAS units, but not all tanks, were infected with *L. salmonis*, while units 1 and 2 had *M. viscosa*. The control group was in RAS unit 3. The use of RAS implies an added risk of pathogens being spread between tanks. Therefore, filters were added to the waterflow to stop lice from entering the control tanks or tanks only containing *M. viscosa*. In unit 1 and 2, all tanks were infected with *M. viscosa* as it is difficult to be certain the water is not contaminated. As no control fish or single infected fish showed signs of the other pathogen, it was effective in stopping unwanted spread of pathogens.

4.2 **Histology**

Sørum *et al.* (2010) described frequent histopathological observations of lesions form a *M. viscosa* infection as subepidermal edema, inflammation in the dermis, subcutis, and red muscle, hemorrhage and necrosis of white muscle fibers. Through histology of our samples, some of these characteristics like hemorrhaging, inflammation in dermis and muscle tissue and complete loss of epidermis and some dermis were observed.

Bacteria were re-cultured and (some) sequenced from ulcers on fish (from mortalities) to confirm the presence of *M. viscosa* in ulcers. To further confirm the identity of *M. viscosa* and location, immunohistochemistry using antibodies raised against *M. viscosa* was performed on skin samples. The antibodies used were raised against different strains of *M. viscosa,* which may limit the efficacy of the antibodies. One of the antibodies had unacceptable levels of non-specific binding(K230), but the other antibody looked as if to bind more specifically (NVI88/478). By using immunohistochemistry, there were some spots stained at about the same

size as macrophages around at time C. These spots were not found in a lesiond from time B and the negative control. This could indicate that *M. viscosa* that has been phagocytosed and was stained inside these macrophages. However, these spots were few and far between, so it is not possible to conclude whether the staining was successful. As the antibody was not specific for the strain it is most likely the reason for these limited results. Another reason could have been that the lesions samples were not from the *M. viscosa* infection, but this is not likely due to successful re-culturing of ulcers.

L. salmonis study from Jónsdóttir *et al.,* (1992) found common histopathology to be edema, hyperplasia, inflammation, lesions, scale loss, and hemorrhaging from pre-adult and adult lice. As the histopathology of *L. salmonis* sites showed loss of epidermis and moderate inflammation.

There were only two replicates per fish of skin from each infection site and only at one time point (C) that were used for histopathology due to limited time. Three replicates were used for immunohistochemistry, one from time B and two from time C. Ideally more replicates should have been made to look at the effects on more fish and see if the same staining was present in more ulcers.

Histopathological characteristics were found like hemorrhaging, inflammation in the dermis and muscle tissue in lesions and showed loss of epidermis and a moderate inflammation for *L. salmonis* attachment site. Immunohistochemistry could have been used to see the location of the bacteria in tissue and see if the co-infection leads to a deeper infection, but the results were inconclusive.

4.3 Size: Weight, Length and K-factor

Factors such as weight, and length as well as K-factor were used to see the effects of *M. viscosa* and *L. salmonis* on salmon growth. It is important to know how these are affected, especially to understand how these infections potentially can affect slaughter weight, or how long it takes to reach an optimal weight when farming. Sampling A was done only with two fish per tank in two units. This was not adequate to calculate average weight and length for the different groups. There are several significant differences between weight and length with different treatments for time B. These differences at time B has not been regarded as significant differences due to disease, as most likely not enough time had passed for the pathogens to affect the fish. These

differences are more likely tank effects that should have been detected in sampling A. This to some extent limits how time C and D can be interpreted. As well as large differences between individuals, there were also observed a few significant differences between tanks. This is not ideal, but because the sample size is large, the average across replicate tanks should represent the actual outcome of the infections.

At time C, the *L. salmonis* infected fish were significantly heavier than both *M. viscosa* and co-infection groups. This was also what was observed during the last sampling (D), along with the control group being significantly heavier than *M. viscosa* and co-infection groups. This indicates that *M. viscosa* significantly affects weight; however, the co-infection together with *L. salmonis* did not change the impact of *M. viscosa*. This is further supported by the slower growth of *M. viscosa* and co-infection groups compared to control and *L. salmonis* groups. The *L. salmonis* group was significantly longer than the *M. viscosa* group and co-infection at the end, which was similar results as for weight. Looking at the average growth from time B compared to time D we observed that the growth had halted for the *M. viscosa* group while the co-infection and control group were similar and *L. salmonis* a bit higher.

K-factor is interesting to look at, as this tells us more about the weight to length ratio. Factors that influence a fish's k-factor include species, maturation, fat reserve, muscular development, and more. A higher k-factor is expected in larger fish, but it should at least be around 1. Results for k-factor was the same for as seen in weight, with *M. viscosa* influencing K-factor but the co-infection did not have an impact beyond the effects of *M. viscosa.*

There is limited research done on growth during *L. salmonis* infection, but Wells *et al.,* (2006) found 13 lice per fish at 19-70 g to be a cutoff point for many physiological measures, including weight, in wild brown trout (*salmo trutta*). Coyne (2006) found moribund and dead fish with signs of a *M. viscosa* infection were 50% lighter than healthy fish in a field research. Thus, these findings further support that *M. viscosa* affects growth but cannot thoroughly compare effect as the *M. viscosa* infection depends on dose, strain, temperature, water quality, and more. Coyne (2006) was done in Norway and can therefore be assumed that it is the typical clade creating the disease, which has a higher virulence, and not the variant clade used in this study (Grove *et al.,* 2000).

The infection of *M. viscosa* result in reduced growth, but *L. salmonis* did not. The co-infection of *M. viscosa* and *L. salmonis* did not influence growth from what was observed under the *M. viscosa* infection alone.

4.4 Survival

The infection dose for *M. viscosa* had been tested prior to this experiment to determine optimal concentration. Co-infected fish had a mortality of almost 60%. This affected number of fish sampled for time D, resulting in less fish than for time B and C. A LD_{50} is often considered to be necessary to determine differences in between the groups, so slightly more fish in the experiment could be beneficial to have a larger sampling at the end. The dose for *L. salmonis* was chosen to be a low to medium infection, with the expectation of no mortality.

As there was a limited number of replicate tanks and fish were removed at sampling B and C, statistical analysis was not performed. There look as if *L. salmonis* did not induce mortality as *L. salmonis* survival was higher than the control there is some variation between tanks and that *L. salmonis* at this infection dose and up to 49 days after infection does not increase mortality. Noland *et al.* (1999) found no mortality of at infection levels of 3.6 and 10 lice per fish for fish a 200-250 g, which was very similar to this study. Ross *et al.* (2000) experienced 100% mortality after infection of 178±67 lice per fish of 55.5 g large fish. This supports that the infection levels used in this study did indeed not induce mortality.

M. viscosa and co-infection groups were quite lower than the control group; this indicated that the *M. viscosa* infection does affect survival. There was a small difference between the *M. viscosa* and co-infection groups, but no statistical tests were done so it is tough to say for certain if there were a significant difference, so further research with adequate replicate tanks would have to be done to draw any conclusions.

Karlsen *et al.* (2014) observed survival of 50% at 7 dpi and Løvoll *et al.* (2009) had a survival of 72 % after 7 days after infection (temp 8.9 to day 4 °C followed by 13.4 °C). Both had a mortality that occurred earlier than observed in this thesis, with most mortality observed 15 days after *M. viscosa* infection. These two studies used the typical clade, which is known to be more virulent than the variant clade used here (Grove *et al.,* 2000). Mortality after *M. viscosa* infection depends on dose, strain, temperature, water quality, and more and can therefore be

difficult to compare with other studies in observed survival. But it was clear that *M. viscosa* did lead to a higher mortality.

A heterologous co-infection study on Rainbow trout with *Argulus coregoni* (freshwater fish louse) and *Flavobacterium columnare* (bacteria) found a significantly higher mortality under the co-infection (Bandilla *et al.,* 2006). The same was found for a co-infection of *Caligus rogercresseyi* and *Piscirickettsia salmoni* in Atlantic salmon (Lohorente *et al.,* 2014). These results were similar to our results.

L. salmonis does not impact mortality at these infection levels. The infection of *M. viscosa* and the co-infection of *L. salmonis* and *M. viscosa* has a higher mortality. Further research has to be done to determine the significance of the co-infection.

4.5 Serum cortisol – stress

Serum cortisol can be an indicator of how stressed a fish is, which in turn can greatly affect susceptibility to disease and immune response. The only significant difference found in serum cortisol was for *L. salmonis* group compared to control at time C, at which the average lice count was 7.3 lice perfish. There were trends of higher cortisol in most groups compared to control, but there were quite large differences between individuals. The sampling process is known to be stressful for fish, but as the fish were euthanized within less than 4 min from the removal of tanks it should not affect the results (M. Fast personal communication).

Research with cortisol levels and *L. salmonis* agrees with our findings, Finstad *et al.* (2000) found elevated plasma cortisol in higher *L. salmonis* infestation levels after 25dpi (at a slightly higher intensity, 36 ± 17), and when the lice had molted into pre-adults. Ross *et al.* (2000) and found elevated plasma cortisol at 3dpi and 10 dpi and Fast *et al.,* (2006) at 25,6, 33 and 40 dpi, however this was at a very high infection (178 ± 67 and 142.8 ± 12.8). Little research has been done on *M. viscosa* and cortisol levels, but a study on *Aeromonas salmonicida*, showed elevated water cortisol concentrations 5 to 7 dpi. *A. salmonicida* is a gram-negative bacterium and known to be a very aggressive disease (Ellis *et al.,* 2007). It can be difficult to compare these two bacteria as the diseases are quite different, and based on pathology alone, it could be probable that *A. salmonicida* would cause more stress on the host compared to *M. viscosa*.

4.6 *M. viscosa* ulcers

M. viscosa infections are characterized by formations of ulcers in the skin of the fish. They vary in amount, size, and depth, all of which can impact have adverse effects on fish welfare. *M. viscosa* lesions were graded (1-3) according to severity/depth and area by converting a size into a score; it was easier and quicker than having to measure the exact size. The downside to this is the large differences of an ulcer within a score, limiting our understanding of the actual size of the ulcers, so ideally it should have been measured in actual size however not feasible considering the sample size. But by using size scores, it did give us an indication of ulcer development, which is the most important in regards of fish health. Grading of ulcers was necessary, and a score of one to three was an effective way of defining the severity of the ulcer. No studies have been done on the actual development of ulcers (number, size, and severity), making it hard to compare to current literature.

Ulcer development is detrimental to fish welfare, where many and deep ulcers will put a large amount of strain on the fish and can affect osmotic regulation. Also, by breaking the skin barrier, new pathogens can more easily infect the fish. The average number of ulcers were significantly higher during time C for *M. viscosa* than co-infection, however during time D there was an opposite trend but not significant. Ulcers found on *M. viscosa* infected fish were more at time C than D while to the opposite is observed for co-infected fish. This can indicate that ulcers can develop/infect faster for a single infection, but over time, co-infected fish are not able to fight further spread of the bacteria.

The size of the ulcers showed how much of the skin surface area was covered in ulcers. The skin is important both as a mechanical barrier against pathogens and osmoregulation. An intact epidermis is therefore crucial for a fish to stay healthy. So, with larger ulcers the fish will be more exposed.

Average sizes of ulcers were not significantly different from each other, but there was a trend for co-infection having larger ulcers in the last sampling. When looking at the total size of ulcers at the last sampling, it was almost double the size in co-infection compared to *M. viscosa* (4.90 and 9.08) and statistically significant. The total size of ulcers gives a better understanding of how much of the surface area that is covered in lesions compared to average size. This is particularly important for factors like osmotic stress. *L. salmonis* attachment might create an

entry point for *M. viscosa*, enabling them to cover a larger area. Lice saliva is also thought to be an immunosuppressant, which also could be connected to more ulcers for co-infected fish (Fast *et al.,* 2006, Øvregård *et al.,* 2018). The study on *C. rogercresseyi* and *P. salmonis* found the parasite to reduce resistance to the bacterium, which was what was seen here aswell (Lhorente *et al.,* 2014).

There was a significant difference with a higher total ulcer grade for *M. viscosa* at time B. As in ulcer size there is little difference from time C to time D for *M. viscosa,* however for co-infection the total ulcer grade has more than doubled (from 3.5 to 7.8). So, for time C single infected fish both had a larger number of ulcers as well as a total ulcer grade. This can most likely be linked to number of ulcers, as the total grade also will go up and the data for average ulcer grade is for both groups were similar to each other. Thus, ulcers were more unlikely to be more severe in single infected group at time C. This is further supported by the ulcer grades. The only noteworthy significant difference is grade 1 ulcers for time C which again can be explained by the higher number of ulcers.

It looks as if *M. viscosa* were able to develop or infect faster under a single infection, but over time there are more ulcers for co-infected fish. At the last sampling, there was a much larger area of the fish covered in ulcers for co-infected fish. No difference observed in grade/severity of lesions.

4.7 *L. salmonis*

L. salmonis infection was done by stopping water flow for 1h to give the coepepodids a possibility to attach before being flushed out of the tank. The prevalence was close to 100% throughout the experiment. Lice counting is challenging, especially for smaller stages like chalimus I and II. Time B had about 3.8, and 8 lice per fish for time D. As there were only lice added at the start of the experiment, and there are no ovigerous females there can't be more lice at the end than the early in the experiment. This is likely due to the difficulty of finding the smaller stages on the fish or lice fallen off through scale loss.

There was a clear difference where fish infected with lice harbored more lice than fish co-infected with *M. viscosa* at time D. A similar but not significant trend was observed at time C. As mentioned lice eat mucus and skin, which is removed when *M. viscosa* creates ulcers. For time D co-infected fish had an average of total ulcer size score of 9.1. This might indicate that there is some competition of resources of food, which *M. viscosa* looked as if to be slightly outcompeting *L. salmonis*. Another explanation could be that *L. salmonis* (might) immunomodulate, reducing the immune response at the attachment site, (Fast *et al.,* 2014; Øvergård *et al.,* 2018) which in turn decreases the natural immune response which could normally fight of or slow down ulcer development.

There was some significant difference between stage and genders between the two groups. As there was a higher average on single infected fish, these differences were due to differences in lice counts between the two groups. For time C, most salmon lice had molted into pre-adults, except for a few in single infection. At time D, most males are adults and females still pre-adults for both groups. This indicates that the co-infection does not affect the developmental rate of *L. salmonis,* and this agrees with studies on developmental rates (Johnson & Albright, 1991a). Gender distribution was approximately 1:1, which was expected.

There were found more *L. salmonis* in a single infection compared to co-infection at the last sampling, possibly due to competition between the two pathogens. The co-infection did not affect the development of *L. salmonis*.

4.8 Expression of immune genes

Immune gene expression provides an insightful profile of the overall health and response of the host fish to individual and in this case, co-infecting pathogens. Furthermore, in the current study, by analyzing host gene expression in single and co-infections we can determine the individual and interactive impacts of the pathogens themselves. In the current examination, we analyzed both early (acute; time B) and late (long-term; time C) times post infection. Transcriptional responses are more sensitive, responsive, and easily measured compared to phenotypic changes like growth, lesion development, and survival which take much longer and/or only occur in extreme infections. The immune response towards *L. salmonis* is known to be more local, so the response is often more distinct at attachment sites in skin compared to immune response in head kidney (A-C. Øvergård personal communication, on-going studies at SLRC). As mentioned *L. salmonis* is also thought to produce saliva/glandular secretions with immunomodulating properties (Fast *et al*., 2014; Øvergård *et al*., 2018). Studies in Atlantic salmon with higher resistance to lice have shown a higher immune response compared to low resistant lines (Holm *et al*., 2015). *M. viscosa* infects the skin but can become systemic over time so it could be expected to see a difference in immune response in the head kidney for fish infected with *M. viscosa* (Bruno *et al.,* 2013). To my knowledge, there has been no studies done on the immune response in head kidney against *M. viscosa* making the current investigation novel but difficult to contextualize with the lack of other literature.

Skin tissue samples taken for RNA extraction were large. Large samples increase potential RNA degradation due to RNAlater not penetrating the whole sample in time. All tissue samples for time C were also sent per mail to Norway on ice, but when they arrived, the samples had thawed. Control skin samples were isolated RNA, as they were isolated in Canada, and from the other samples, excess RNAlater had been removed, which could have resulted in RNA degradation. RNA extraction, DNase treatment, cDNA, and RT-qPCR all also increases the risk of degradation of RNA. 24 of the samples were analyzed in a bioanalyzer, 12 from sampling B, (six skin and six head kidney) and 12 from sampling C (six skin isolated in Canada and six skin) which is >10% of the sample size. Some samples of the larger samples were slightly degraded, but to a small degree which is unlikely to have greatly affected the results of RT-qPCR. Another problem with the larger samples was that they were much larger than the actual attachment site of the louse, which could affect results in gene expression as it is very local (A-C. Øvergård personal communication). Putative tank effects could not be analyzed in samples

taken for transitional analysis as only one to five samples were taken from replicate tanks. Analyzing the same amount of fish from each replicate tank would have improved the study.

Sampling B was done two days post infection with *M. viscosa*. Small scale loss, that did not look like mechanical damage, was assumed to be early *M. viscosa* lesion. As it was so early in the infection, there might have been other reasons for this scale loss. This could potentially affect the expression of immune genes. This early in the infection, there was a prevalence of 70% (single) and 62.5% (co-infected) for *M. viscosa* ulcers. Therefore, this early in the infection, not all fish had both signs of a co-infection, so some samples are uncertain whether the fish at that stage was infected with both pathogens.

As expected, there were many large differences in expression between individuals. Larger differences with this sample size may affect potential significant differences. Ideally a larger sample set could have been analyzed in this thesis.

One fish from the co-infection group, time C was removed as the results from all skin sites and head kidney were drastically higher than the other fish in the same group (for example the average IL-1β for attachment was under 1 while this sample was over 200). Even though there had not been any notes on abnormalities on this individual, as this was such a substantial deviation in two tissues and several immune genes it was assumed to not be representative for the group.

4.8.1 **Pro-inflammatory genes**

IL-1β expression

IL-1β is important in inflammation, T-cell, and macrophage activation (Murphy & Weaver, 2017). At time B, co-infected fish had a significantly higher expression of IL-1β at *M. viscosa* unaffected control and both *L. salmonis* sites. Since these two unaffected control sites had a higher expression, this could indicate a systemic skin response (epithelial mucosal response) (Salinas, 2015). IL-1β was also higher for *L. salmonis* attachment for co-infection than single infection, which could suggest that the co-infection leads to a higher expression IL-1β in attachment site. *M. viscosa* lesion single infection had the highest average of IL-1β expression, due to the considerable variation this result is however, not statistically significant. A t-test was also run by removing the outlier for co-infected *M. viscosa* lesion, which resulted in a significantly higher expression compared to control, but as there was no suitable reason to remove the outlier, these results have not been discussed further.

At time C co-infected fish had a significantly higher expression of IL-1β at *M. viscosa* lesion and *M. viscosa* unaffected control site. The expression was also significantly higher in *M. viscosa* lesion compared to unaffected control site in single infected fish. These results indicate that the immune response increases with disease progression and that the immune response towards the *M. viscosa* infection was not as local as the *L. salmonis* infection*,* which could be due to spread of infection that is not yet visible (no ulcers). As in time B, *M. viscosa* has the highest average and looked as if there was a trend of a higher expression. Results like these would be expected as the bacterial colonization of the skin is not at local as the attachment site as the louse.

Salmon lice copepodites are attached to the host by hooking its second antenna into the skin while mobile *L. salmonis* (pre-adult and adult) are attached through suction of their cephalothorax. Damage from attachment is greater from the copepodid than a mobile louse, but the mobile lice cause more damage due to feeding (Jones *et al.,* 1990; Kabata. 1981). The higher expression at time B (for co-infected *L. salmonis* attachment site) could therefore be due to the attachment stimulating a different immune response or that the pre-adult and adult stages have a greater capacity of immunomodulation. (Fast *et al*., 2014)

Øvergård *et al.* (2018) found increased expression if IL-1β at 24, 36, 48, and 72 ddpi in *L. salmonis* attachment sites. Time B was at 58ddpi so according to this it could be assumed that at least single infected *L. salmonis* attachment sites should have been significantly higher than the control for time B. The difference could be due to a slightly higher level of infection in the latter study. The study by Løvoll *et al.,* (2009) showed higher expression in *M. viscosa* ulcer skin 7 dpi. In this study, expression was assessed at 2 dpi (B) and 23 dpi (C) making it difficult to compare. But it can indicate that the co-infection did, in fact, result in a higher expression of IL-1β at time B. Ingerselv *et al*. (2010) showed higher expression at 7 and 14 dpi in muscle, as it is a different tissue it could be difficult to compare but might give an indicator of what could be expected in skin. And these results seem to line up as Time C showed significantly higher expression in all *M. viscosa* sites except *M. viscosa* lesion single infection.

Results for the head kidney from time B showed that co-infected fish had significantly higher expression of IL-1β than control while for time C it was significantly lower, indicating that the co-infection can affect expression in head kidney both up and down depending on timing and progression of disease. Fast *et al.* (2006) found a higher expression after *L. salmonis* infection in the head kidney at 9 and 40 dpi, but no differences in-between. According to these results, the *L. salmonis* group would be expected to have a higher expression at time B (6dpi), but this was not the case, again possibly because the infection level in the current study was <50% of Fast *et al.* (2006). Chettri *et al*. (2011) found higher expression in the head kidney after exposing rainbow trout to PAMPs at 1, 4, and 12 h after infection. As our first time is 2 dpi it might indicate that a higher expression of IL-1β could be expected after a bacterial infection, but it is tough to compare as a lot happens this early in the infection Fast *et al.* (2007) looked at lipopolysaccharides (LPS, a type of PAMP) and stress, finding a higher expression in at 7 dpi and lower 14 and 21 dpi. LPS is a component in the cell wall of gram-negative bacteria and is known to stimulate the proliferation of lymphocytes and macrophage activity (Warr & Simon, 1983; Dalmo & Seljelind, 1995). These results are similar to what was found here, indicating that even though the co-infected fish were not significantly more stressed by *L. salmonis* but in a combination of the bacterial infection lead to the same results

IL-8 expression

IL-8 is a chemokine produced by macrophages and epithelial cells and plays a role in inflammation attracting neutrophil granulocytes (Murphy & Weaver, 2017). All co-infected sites except for *M. viscosa* lesion had a significantly higher expression of IL- 8 compared to control in time B. All *L. salmonis* sites also had higher expression than control. This, like IL-1β, indicates a systemic skin response, but as there were no significant differences for IL-8 in the head kidney at either of the samplings indicating no internal systemic response. There was a significantly higher expression in co-infected *L. salmonis* attachment compared to single infection, which indicates that the co-infection also induces higher expression of IL-8 compared to *L. salmonis* alone.

At time C, IL-8 was significantly higher expressed in the co-infected *M. viscosa* lesion and single infected control compared to control. Expression in co-infection *M. viscosa* lesion was also significantly higher compared to *M. viscosa* unaffected control. This shows that the immune response against *M. viscosa* increased as the ulcers had developed while the *L. salmonis* infection response had stopped. This could again be linked to what was mentioned about IL-1β about attachment methods of the louse or potential immunomodulation.

 Øvergård *et al*. (2018) found higher IL-8 24, 48 and 72 ddpi which corresponds to findings in time B. Ingerselv *et al*. (2010) found higher expression at 7 and 14 dpi in muscle tissue, which is also what was found in skin for co-infected *M. viscosa* lesion during time C (23dpi).

The results for pro-inflammatory genes showed a higher systemic IL-1β response early in the infection for co-infected fish (B) and reduced at time C, IL-8 was not affected. *L. salmonis* attachment sites had a higher expression of IL-1β at time B while *M. viscosa* lesions were higher at time C. The immune response after *L. salmonis* infection was also more local than *M. viscosa* infection, with *M. viscosa* leading to a heightened systemic response of epithelial mucosal immune response.

4.8.2 **Anti-inflammatory genes**

IL-4/13A expression

IL-4 and IL-13 are anti-inflammatory cytokines and known to be important in Th cell response and important in parasite immune response in mammals (Mosmann and Coffman. 1989, Zhu. 2015). IL-4/13A, found in teleosts, is closely related to these genes and is commonly expressed in the skin under parasitic infections (Chettri *et al.,* 2014, Wang *et al.,* 2016). At time B, both *M. viscosa* lesion sites and co-infection *M. viscosa* unaffected control site had a lower expression of IL-4/13A than the control. Looking at the graph, it gave the impression of a trend where all single infection sites were slightly higher expressed than the co-infected counterpart; however, none of these were significant. All sites were downregulated except for *L. salmonis* unaffected control site (single infection) at time C. With the decrease at time C, it could indicate that IL-4 /13A expression was suppressed under both pathogens, and this response was seen faster during the co-infection for time B. As all *M. viscosa* sites are affected, there also seems like there was a systemic skin response after the *M. viscosa* infection, both single and co-infected. As the anti-inflammatory cytokines can have the opposite effect as inflammatory genes they could also be suppressed for the inflammation to work as efficiently as possible. This is supported by the increase in pro-inflammatory, and accordance with the decrease in anti-inflammatory responses.

Comparing the trends of IL-4/13A to the pro-inflammatory cytokines and IL-10 it is slightly different. Expression of IL-1β, IL-8 and IL-10 showed a higher initial response (time B) of co-infected and *L. salmonis* with a more persistent response in *M. viscosa*. But for IL-4/13A the most distinctive effects were seen at time C, indicating a delayed repressive response which could have been from the as they pathogens have developed or suppression from the immune system to induce more pro-inflammatory responses. There was a significantly lower expression in the co-infected group compared to all other groups in the head kidney at time C, indicating that the suppression was systemic. In the study by Øvergård *et al.* (2018), there was a higher expression of IL-4/13A 48, 36, and 72 ddpi, which is not observed in any cases in this study. A reason for this could have been the differences in lice intensity or differences in resistance, among others. Holm *et al*. (2016) found a lower expression (not significant) in the skin near lice attachment site at 21 dpi in Atlantic salmon that were less resistant to lice. Holm at al saw a trend for higher expressions of IL-4/13A for their highly resistant salmon.

IL-10 expression

IL-10 is a cytokine produced by macrophages as well as dendritic-, T- and B-cells and is a suppressant of macrophages. As it is an anti-inflammatory, it can be important in controlling the inflammatory response thus reducing damage from inflammation (Murphy & Weaver, 2017). At time B, all co-infected sites were significantly less than the control, indicating again as with IL-4/13A that the pro-inflammatory cytokines were the focus of the immune response to fight the infections. However, there was also a possibility that the co-infection downregulated IL10 expression. All single infections were significantly higher than their co-infection counterpart, which again indicates that it was the two infections together that caused these responses. In general, the results of time B was the opposite of what is observed in the pro-inflammatory genes were most co-infected sites were upregulated.

At time C, there was a different trend where only single infection *M. viscosa* had a higher (not significant) expression and all other sites had a lower expression. The response of co-infected with decreased expression was acute and transient, mostly occurring at time B. This further supports that it was there was no need for anti-inflammatory cytokines as it was trying to fight the infection.

Skugor *et al.* (2008) found higher expression in lice attachment sites 33 dpi in IL-10, which contradicts this theory, but as this may have been caused by different timepoints and a higher level of infection. Braden *et al.* (2015) did not find upregulation of IL-10 in fish after *L. salmonis* infection levels but did see it in Coho salmon (*Oncorhynchus kisutch*), indicating that expression IL-10 can be important its resistance against *L. salmonis*. Ingerselv *et al.* (2010) found a higher expression of IL-10 at7, and 14 dpi in *M. viscosa* ulcerated muscle tissue, which appears to be similar to the trend observed here as well; however, it was not significant.

No significant differences for IL-10 in head kidney was found at either of the samplings. This suggests that the IL-10 response was limited to the systemic skin immune response.

Overall results of anti-inflammatory cytokines showed a lower expression of IL-4/13A in *M. viscosa* lesions at time B, and in most sites at time C. IL-10 had a lower expression in all co-infected sites at time B and a lower expression in *L. salmonis* attachment single infection at time C. IL-4/13A in co-infected fish head kidney at time C was lower expressed than single infected fish. No differences IL-10 head kidney.

4.8.3 **Acute-phase protein: C3**

C3 plays a role in activating the complement system and activates both B- and T-cells (Erdei *et al.*, 1991). *M. viscosa* lesion single infection and both *M. viscosa* unaffected controls were significantly higher than the control. *M. viscosa* lesion also has significantly higher expression of C3 than *L. salmonis* attachment in time B. Similar results in time C, with higher expression in both *M. viscosa* lesions and single infection *M. viscosa* unaffected control site. These results indicate that C3 expression was mostly associated with *M. viscosa* and not with *L. salmonis*. The complement system is often considered to be vial in the antibacterial response with its cell lysing properties, and thus often observed in response to bacterial infections and not parasites (Amara *et al.*, 2010). Higher expression *M. viscosa* un-affected control sites could indicate that the response was higher close to an ulcer, as seen with IL-1β and IL-8. It was therefore expected to see a higher response in the *M. viscosa* infection compared to *L. salmonis*. Løvoll *et al.* (2009) did not find a higher expression in ulcers until 7 dpi even though we found at 2dpi, which might have been due to the strain used which could have induced different immune responses.

In the head kidney from time B, the co-infection group was significantly higher than *M. viscosa*, which indicates a quicker or stronger systemic response when co-infected. There were no differences in the expression of C3 at time C, and therefore no systemic response.

In both time B and C, sites associated with *M. viscosa*, both lesions, and control sites, were upregulated indicating a less local C3 response for *M. viscosa* ulcers.

4.8.4 **Immunoglobulin: IgM**

IgM is an immunoglobulin and is important in activating the classic pathway of the complement system as well as having agglutinating properties and is therefore important in the early immune response (Lea, 2000). *M. viscosa* unaffected control and *L. salmonis* attachment had significantly higher expression of IgM than the control for time B. The trend for all sites appears to be a higher expression of IgM. At Time C, single infection *M. viscosa* lesion and *L. salmonis* attachment was significantly lower with a general trend for all sites appeared to be a lower expression of IgM. Tadiso *et al*. (2011) found higher expression of IgM 5 and 15 dpi louse attachment site, but none at 1 and 10dpi, indicating a variable response of IgM throughout an infection.

Co-infection has a significantly higher expression of IgM than *M. viscosa* infection at time c.

Expression of IgM in head kidney at time C is significantly lower in co-infection compared to *M. viscosa* infected fish. Skugor *et al.* (2008) found expression of immunoglobulins in the head kidney at 22dpi after *L. salmonis* infection to be lower, which complement our results.

The general trends for IgM appear to be a slight upregulation at time B and a slight downregulation at time C, but no clear significant trends.

5 Conclusions

1. The co-infection did not affect weight, length or K-factor, however the *M. viscosa* infection (both single and co-infected) did affect weight and K-factor compared to control and *L. salmonis*. Length was also smaller; however, the differences were variable throughout the experiment.

2. *L. salmonis* did not impact mortality at this low infection level. The infection of *M. viscosa* and the co-infection of *L. salmonis* and *M. viscosa* had higher mortality. Further research must be done to determine significance of the co-infection.

3. The co-infected fish did not have higher levels of serum cortisol than control fish, but a single infection of *L. salmonis* after reaching pre-adult did.

4. *M. viscosa* ulcers were able to develop or infect faster under a single infection but over time there were more ulcers for co-infected fish. At the last sampling there was a much larger area of the fish covered in ulcers for co-infected fish. No difference observed in grade/severity of lesions.

5. There were more *L. salmonis* in a single infection compared to co-infection. The co-infection did not affect development of *L. salmonis*.

6. There was a higher expression of pro-inflammatory genes (IL-1β and IL-8) for co-infected fish within time B (7 dpi *L. salmonis* and 2 dpi *M. viscosa* [co-infection] dpi). *L. salmonis* attachment sites were higher at this time as well, while expression of these genes increased with increasing disease severity and mortality in *M. viscosa* lesions and unaffected skin at time C (26 dpi *L. salmonis*, 23 dpi *M. viscosa* [co-infection]).

There was a lower expression of anti-inflammatory genes (IL-4/13A and IL-10). IL-4/13A for *M. viscosa* lesion at time B and almost all skin sites, both co-infected and single infected, and head kidney for time C. IL-10 at time B was significantly lower for all co-infected sites at time B.

C3 was found to be associated on and around *M. viscosa* ulcers at both sampling points. IgM had trends indicating a slightly higher expression at sampling B and slight lower expression at sampling C.

6 Further research

In further research within this type of co-infection could be interesting to look at:

- Effect of co-infection on mucus quality/quantity and potentially connect this to lower lice counts at co-infected fish compared to single infected, especially interesting since there is a higher systemic skin response from *M. viscosa* and co-infections.

- Look at location of lesions and lice and see if it affected by a co-infection

- Higher infection of *L. salmonis* where it is expected to see mortality to see if this affects mortality of co-infected fish

- Compare the two different clades of *M. viscosa* in a co-infection

- Look at gutted weight to further support that these weight differences are in muscle and not organs.

- Longer trial with more samplings throughout, could show even further how much *M. viscosa* affects growth.

- Further investigate immunohistochemistry and through successful staining look at location of *M. viscosa* in the tissue.

Other types of co-infections would also be interesting to investigate. Studies on co-infections are important to further our knowledge in how diseases develop in a fish farm. There are few co-infections done with *L. salmonis*, and as this parasite is found all year round it is typically found in fish with many other diseases. As it also is such a significant problem most types of co-infections with *L. salmonis* could be very beneficial in further understanding a *L. salmonis* infection outside of the lab and therefore also help in increasing fish welfare.

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Appendix A – Bioanalyzer results

Agilent RNA 6000 Nano Kit Guide (Agilent Technologies) was used on 24 representative RNA samples. Six skin and six HK samples from sampling B and six samples from control and six skin samples from sampling C.

Samples:

35,37,39, 42,43,45	- Skin, sampling B, *L. salmonis* infected
101- 106	- Head kidney, sampling B, Control
1-5	- Skin, sampling C, control (Control RNA samples sent from Canada)
201-207	- Skin, sampling C, *M. viscosa* infected

Assay Class: mRNA Nano
Data Path: C:\...09\2100 expert_mRNA Nano_DE72901192_2019-04-09_10-02-59.xad

Created: 09.04.2019 10:02:59
Modified: 09.04.2019 10:30:02

Electrophoresis File Run Summary
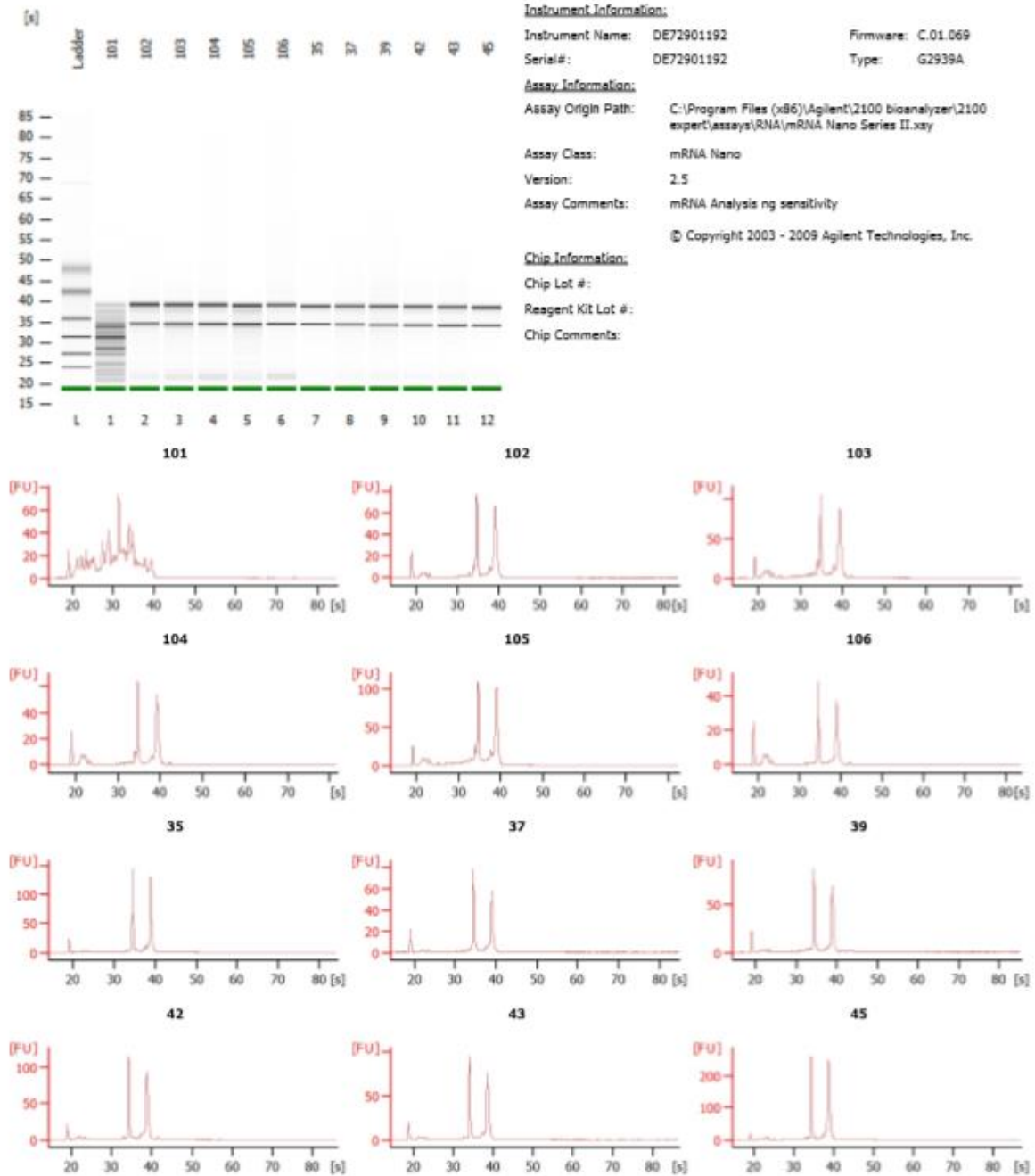
Instrument Information:

| Instrument Name: | DE72901192 | Firmware: | C.01.069 |
| Serial#: | DE72901192 | Type: | G2939A |

Assay Information:

Assay Origin Path:	C:\Program Files (x86)\Agilent\2100 bioanalyzer\2100 expert\assays\RNA\mRNA Nano Series II.xsy
Assay Class:	mRNA Nano
Version:	2.5
Assay Comments:	mRNA Analysis ng sensitivity

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Chip Information:

Chip Lot #:

Reagent Kit Lot #:

Chip Comments:

101

102

103

104

105

106

35

37

39

42

43

45

2100 Expert (B.02.08.SI648)   © Copyright 2003 - 2015 Agilent Technologies, Inc.   Printed: 09.04.2019 11:31:47

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Modified: 09.04.2019 11:20:52

Electrophoresis File Run Summary

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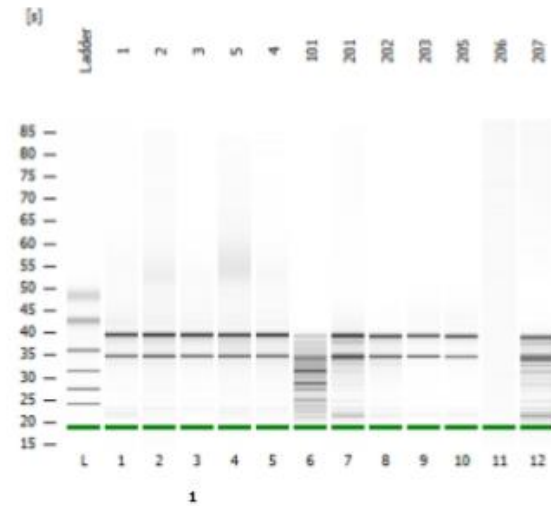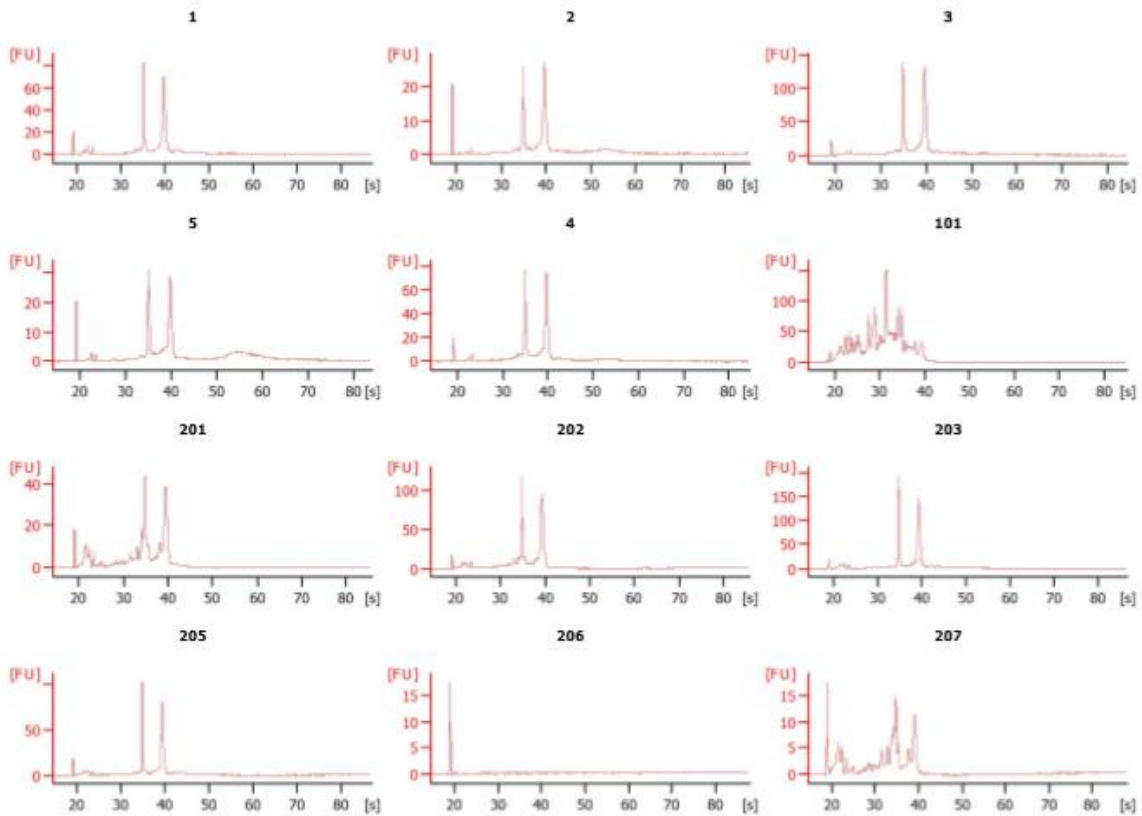
Assay Class: mRNA Nano

Version: 2.5

Assay Comments: mRNA Analysis ng sensitivity

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Chip Information:

Chip Lot #:

Reagent Kit Lot #:

Chip Comments:

Appendix B – Significant values of immune response in skin samples

All p-values of immune response in skin samples from sampling B and C, with significant differences in bold.

Table 14: P-values of IL-1β in skin samples from sampling B

	Control	M. viscosa lesion	M. viscosa control	L. salmonis attachement	L. salmonis control	Co-infection: M. viscosa lesion	Co-infection: M. viscosa control	Co-infection: L. salmonis attachment
M. viscosa lesion	0,114							
M. viscosa control	0,067	0,107						
L. salmonis attachement	0,243	0,101	0,116					
L. salmonis control	0,254	0,090	0,221	0,536				
Co-infection: M. viscosa lesion	**0,045**	0,400	0,156	**0,043**	**0,049**			
Co-infection: M. viscosa control	**0,001**	0,306	0,933	**0,003**	0,099	0,375		
Co-infection: L. salmonis attachment	0,215	0,855	0,212	0,198	0,184	0,545	0,437	
Co-infection: L. salmonis control	**0,011**	0,136	0,743	0,031	0,194	0,127	0,574	0,248

Table 15: P-values of C3 in skin samples from sampling B

	Control	M. viscosa lesion	M. viscosa control	L. salmonis attachement	L. salmonis control	Co-infection: M. viscosa lesion	Co-infection: M. viscosa control	Co-infection: L. salmonis attachment
M. viscosa lesion	**0,005**							
M. viscosa control	**0,048**	**0,032**						
L. salmonis attachement	0,089	**0,010**	0,275					
L. salmonis control	0,147	**0,044**	0,983	0,392				
Co-infection: M. viscosa lesion	0,826	**0,044**	0,161	0,177	0,290			
Co-infection: M. viscosa control	0,369	0,061	0,307	0,602	0,436	0,224		
Co-infection: L. salmonis attachment	**0,019**	0,140	0,539	0,093	0,625	0,089	0,168	
Co-infection: L. salmonis control	**0,029**	0,103	0,464	0,222	0,220	0,334	0,402	0,239

Table 16: P-values of IL-8 in skin samples from sampling B

	Control	*M. viscosa* lesion	*M. viscosa* control	*L. salmonis* attachement	*L. salmonis* control	Co-infection: *M. viscosa* lesion	Co-infection: *M. viscosa* control	Co-infection: *L. salmonis* attachment
M. viscosa lesion	0,091							
M. viscosa control	0,118	0,085						
L. salmonis attachement	**0,000**	0,081	0,227					
L. salmonis control	**0,028**	0,068	0,204	0,921				
Co-infection: *M. viscosa* lesion	**0,014**	0,268	0,965	**0,046**	**0,046**			
Co-infection: *M. viscosa* control	**0,003**	0,242	0,418	0,938	0,983	0,236		
Co-infection: *L. salmonis* attachment	0,207	0,557	0,230	0,199	0,178	0,447	0,399	
Co-infection: *L. salmonis* control	**0,029**	0,103	0,464	0,222	0,220	0,334	0,402	0,239

Table 17: P-values of IgM in skin samples from sampling B

	Control	*M. viscosa* lesion	*M. viscosa* control	*L. salmonis* attachement	*L. salmonis* control	Co-infection: *M. viscosa* lesion	Co-infection: *M. viscosa* control	Co-infection: *L. salmonis* attachment
M. viscosa lesion	0,087							
M. viscosa control	**0,033**	0,639						
L. salmonis attachement	0,056	0,932	0,560					
L. salmonis control	0,068	0,806	0,847	0,730				
Co-infection: *M. viscosa* lesion	**0,034**	0,663	0,434	0,660	0,542			
Co-infection: *M. viscosa* control	0,306	0,414	0,268	0,372	0,346	0,501		
Co-infection: *L. salmonis* attachment	0,080	0,755	0,924	0,682	0,937	0,534	0,359	
Co-infection: *L. salmonis* control	0,136	0,552	0,790	0,504	0,705	0,507	0,389	0,767

Table 18: P-values of IL-10 in skin samples from sampling B

	Control	*M. viscosa* lesion	*M. viscosa* control	*L. salmonis* attachement	*L. salmonis* control	Co-infection: *M. viscosa* lesion	Co-infection: *M. viscosa* control	Co-infection: *L. salmonis* attachment
M. viscosa lesion	0,928							
M. viscosa control	0,203	0,167						
L. salmonis attachement	0,128	0,114	**0,012**					
L. salmonis control	0,052	**0,041**	**0,004**	0,603				
Co-infection: *M. viscosa* lesion	**0,007**	**0,004**	**0,003**	**0,006**	**0,004**			
Co-infection: *M. viscosa* control	**0,007**	**0,004**	**0,003**	**0,006**	**0,004**	0,973		
Co-infection: *L. salmonis* attachment	**0,001**	**0,000**	**0,000**	**0,001**	**0,000**	**0,037**	0,202	
Co-infection: *L. salmonis* control	**0,000**	**0,000**	**0,000**	**0,000**	**0,000**	**0,007**	0,113	0,422

Table 19: P-values of IL-4 in skin samples from sampling B

	Control	*M. viscosa* lesion	*M. viscosa* control	*L. salmonis* attachement	*L. salmonis* control	Co-infection: *M. viscosa* lesion	Co-infection: *M. viscosa* control	Co-infection: *L. salmonis* attachment
M. viscosa lesion	**0,022**							
M. viscosa control	0,585	0,135						
L. salmonis attachement	0,084	**0,007**	0,322					
L. salmonis control	0,071	**0,003**	0,367	0,835				
Co-infection: *M. viscosa* lesion	0,960	0,252	0,710	0,241	0,225			
Co-infection: *M. viscosa* control	0,852	0,293	0,657	0,212	0,192	0,674		
Co-infection: *L. salmonis* attachment	**0,002**	0,068	0,086	**0,008**	**0,003**	0,117	0,125	
Co-infection: *L. salmonis* control	**0,002**	0,110	0,069	**0,004**	**0,002**	0,107	0,118	0,775

Table 20: P-values of IL-1β in skin samples from sampling C

	Control	*M. viscosa* lesion	*M. viscosa* control	*L. salmonis* attachement	*L. salmonis* control	Co-infection: *M. viscosa* lesion	Co-infection: *M. viscosa* control	Co-infection: *L. salmonis* attachment
M. viscosa lesion	0,082							
M. viscosa control	**0,030**	0,084						
L. salmonis attachement	0,199	0,149	0,292					
L. salmonis control	0,212	0,129	0,065	0,227				
Co-infection: *M. viscosa* lesion	**0,028**	0,654	0,051	0,080	0,057			
Co-infection: *M. viscosa* control	**0,037**	0,130	0,063	0,459	0,932	**0,029**		
Co-infection: *L. salmonis* attachment	0,080	0,208	0,227	0,886	0,400	0,121	0,380	
Co-infection: *L. salmonis* control	0,135	0,203	0,181	0,767	0,553	0,115	0,544	0,420

Table 21: P-values of C3 in skin samples from sampling C

	Control	*M. viscosa* lesion	*M. viscosa* control	*L. salmonis* attachement	*L. salmonis* control	Co-infection: *M. viscosa* lesion	Co-infection: *M. viscosa* control	Co-infection: *L. salmonis* attachment
M. viscosa lesion	**0,014**							
M. viscosa control	**0,021**	0,321						
L. salmonis attachement	0,243	**0,024**	**0,016**					
L. salmonis control	**0,028**	**0,022**	**0,014**	0,252				
Co-infection: *M. viscosa* lesion	**0,005**	0,397	0,339	**0,008**	**0,006**			
Co-infection: *M. viscosa* control	0,136	**0,045**	**0,034**	0,069	**0,031**	**0,016**		
Co-infection: *L. salmonis* attachment	0,932	0,060	**0,038**	0,342	0,080	**0,029**	0,272	
Co-infection: *L. salmonis* control	0,420	0,053	**0,033**	0,848	0,403	**0,022**	0,138	0,253

Table 22: P-values of IL-8 in skin samples from sampling C

	Control	*M. viscosa* lesion	*M. viscosa* control	*L. salmonis* attachement	*L. salmonis* control	Co-infection: *M. viscosa* lesion	Co-infection: *M. viscosa* control	Co-infection: *L. salmonis* attachment
M. viscosa lesion	0,094							
M. viscosa control	**0,041**	0,108						
L. salmonis attachement	0,358	0,142	**0,049**					
L. salmonis control	0,267	0,135	**0,031**	0,148				
Co-infection: *M. viscosa* lesion	0,051	0,820	0,067	0,086	0,082			
Co-infection: *M. viscosa* control	0,113	0,146	0,060	0,675	0,104	**0,044**		
Co-infection: *L. salmonis* attachment	0,238	0,206	0,072	0,903	0,165	0,139	0,562	
Co-infection: *L. salmonis* control	0,122	0,208	0,079	0,888	0,097	0,141	0,768	0,373

Table 23: P-values of IgM in skin samples from sampling C

	Control	*M. viscosa* lesion	*M. viscosa* control	*L. salmonis* attachement	*L. salmonis* control	Co-infection: *M. viscosa* lesion	Co-infection: *M. viscosa* control	Co-infection: *L. salmonis* attachment
M. viscosa lesion	**0,039**							
M. viscosa control	0,052	0,375						
L. salmonis attachement	**0,019**	0,504	0,142					
L. salmonis control	0,062	0,760	0,485	0,189				
Co-infection: *M. viscosa* lesion	0,091	0,478	0,344	0,109	0,771			
Co-infection: *M. viscosa* control	0,117	0,368	0,259	0,128	0,589	0,376		
Co-infection: *L. salmonis* attachment	0,689	0,237	0,129	0,200	0,266	0,321	0,322	
Co-infection: *L. salmonis* control	0,127	0,543	0,363	0,201	0,790	0,983	0,801	0,183

Table 24: P-values of IL-10 in skin samples from sampling C

	Control	*M. viscosa* lesion	*M. viscosa* control	*L. salmonis* attachement	*L. salmonis* control	Co-infection: *M. viscosa* lesion	Co-infection: *M. viscosa* control	Co-infection: *L. salmonis* attachment
M. viscosa lesion	0,067							
M. viscosa control	0,069	0,436						
L. salmonis attachement	0,318	0,622	0,270					
L. salmonis control	**0,009**	0,084	**0,045**	0,164				
Co-infection: *M. viscosa* lesion	0,263	0,261	0,121	0,638	0,210			
Co-infection: *M. viscosa* control	0,115	0,092	**0,048**	0,348	**0,003**	0,126		
Co-infection: *L. salmonis* attachment	0,079	0,143	0,075	0,413	**0,022**	0,310	0,256	
Co-infection: *L. salmonis* control	0,255	0,149	0,077	0,426	**0,001**	0,339	0,538	0,052

Table 25: P-values of IL-4 in skin samples from sampling C

	Control	*M. viscosa* lesion	*M. viscosa* control	*L. salmonis* attachement	*L. salmonis* control	Co-infection: *M. viscosa* lesion	Co-infection: *M. viscosa* control	Co-infection: *L. salmonis* attachment
M. viscosa lesion	**0,003**							
M. viscosa control	**0,005**	0,407						
L. salmonis attachement	**0,039**	0,111	0,095					
L. salmonis control	0,090	0,119	0,087	0,378				
Co-infection: *M. viscosa* lesion	**0,005**	0,949	0,424	0,121	0,138			
Co-infection: *M. viscosa* control	**0,002**	0,819	0,329	0,072	0,089	0,372		
Co-infection: *L. salmonis* attachment	**0,008**	0,960	0,428	0,153	0,171	0,993	0,773	
Co-infection: *L. salmonis* control	0,059	0,081	0,084	0,991	0,775	0,075	**0,044**	0,051

Table 26: P-values of IL-1β in head kidney samples from sampling B

	Control	M. viscosa	L. salmonis
M. viscosa	0,196	-	
L. salmonis	0,220	0,607	-
Co-infection	**0,001**	0,795	0,210

Table 27: P-values of C3 in head kidney samples from sampling B

	Control	M. viscosa	L. salmonis
M. viscosa	0,239	-	
L. salmonis	0,355	0,179	-
Co-infection	0,102	**0,026**	0,719

Table 28: P-values of IL-8 in head kidney samples from sampling B

	Control	M. viscosa	L. salmonis
M. viscosa	0,379	-	
L. salmonis	0,219	0,581	-
Co-infection	0,055	0,564	0,862

Table 29: P-values of IgM in head kidney samples from sampling B

	Control	M. viscosa	L. salmonis
M. viscosa	0,590	-	
L. salmonis	0,427	0,193	-
Co-infection	0,990	0,526	0,307

Table 30: P-values of IL-10 in head kidney samples from sampling B

	Control	M. viscosa	L. salmonis
M. viscosa	0,999	-	
L. salmonis	0,387	0,321	-
Co-infection	0,546	0,538	0,248

X

Table 31: P-values of IL-4 in head kidney samples from sampling B

	Control	M. viscosa	L. salmonis
M. viscosa	0,204	-	
L. salmonis	0,798	0,254	-
Co-infection	0,152	0,019	0,455

Table 32: P-values of IL-1β in head kidney samples from sampling C

	Control	M. viscosa	L. salmonis
M. viscosa	0,225	-	
L. salmonis	0,683	0,270	-
Co-infection	**0,037**	0,218	**0,008**

Table 33: P-values of C3 in head kidney samples from sampling C

	Control	M. viscosa	L. salmonis
M. viscosa	0,199	-	
L. salmonis	0,275	0,297	-
Co-infection	0,796	0,273	0,459

Table 34: P-values of IL-8 in head kidney samples from sampling C

	Control	M. viscosa	L. salmonis
M. viscosa	0,351	-	
L. salmonis	0,842	0,355	-
Co-infection	0,155	0,264	0,260

Table 35: P-values of IgM in head kidney samples from sampling C

	Control	M. viscosa	L. salmonis
M. viscosa	0,558	-	
L. salmonis	0,718	0,868	-
Co-infection	0,070	**0,018**	0,059

Table 36: P-values of IL-10 in head kidney samples from sampling C

	Control	M. viscosa	L. salmonis
M. viscosa	0,723	-	
L. salmonis	0,102	0,224	-
Co-infection	0,430	0,431	0,527

Table 37: P-values of IL-4 in head kidney samples from sampling C

	Control	M. viscosa	L. salmonis
M. viscosa	0,485	-	
L. salmonis	0,745	0,701	-
Co-infection	**0,000**	**0,000**	**0,000**

Appendix C – Immune response figures with no significant differences

Figures with immune response that had no significant differences.

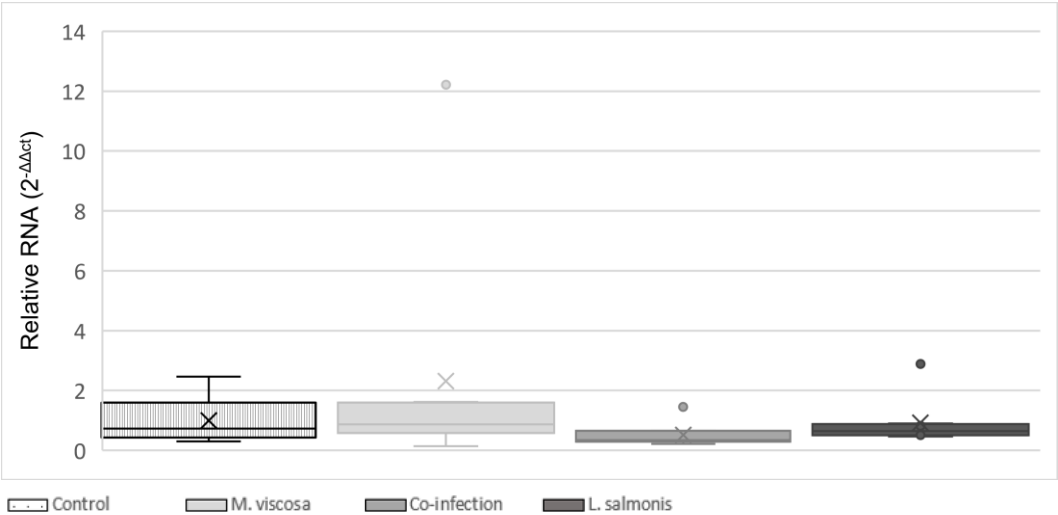Fig. 46: Expression of IL-8 in head kidney samples from sampling B. Relative RNA ($2^{-\Delta\Delta ct}$) of IL-8 in head kidney samples from control (n=9) *M. viscosa* infection (n=12), *L. salmonis* infection(n=10) and co-infection(n=11). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ $\Delta\Delta$=0.01, ***/ $\Delta\Delta\Delta$=0.001.
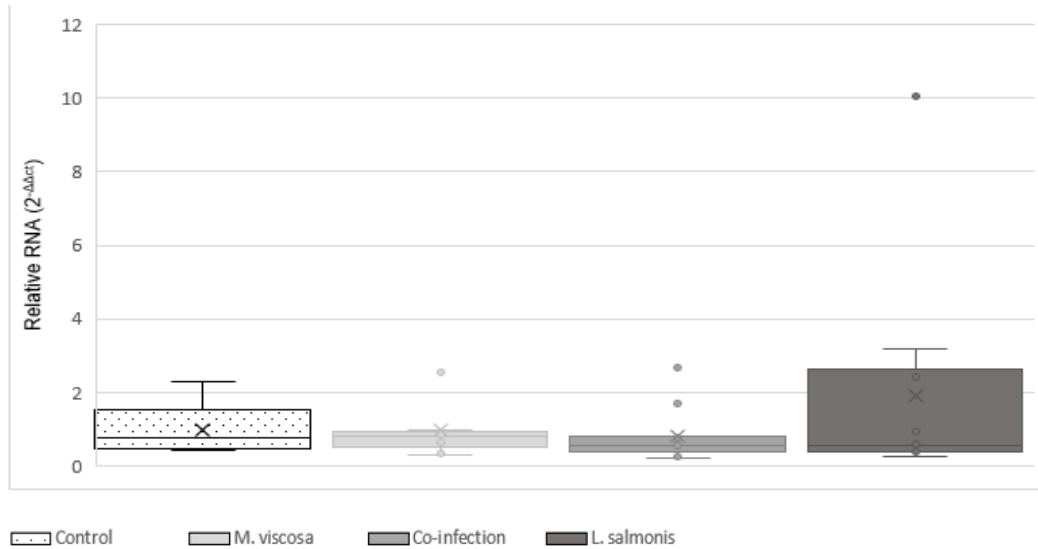

Fig. 47: Expression of IL-8 in head kidney samples from sampling C. Relative RNA ($2^{-\Delta\Delta ct}$) of IL-8 in head kidney samples from control (n=9) *M. viscosa* infection (n=8), *L. salmonis* infection(n=8) and co-infection(n=7). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ $\Delta\Delta$=0.01, ***/ $\Delta\Delta\Delta$=0.001.
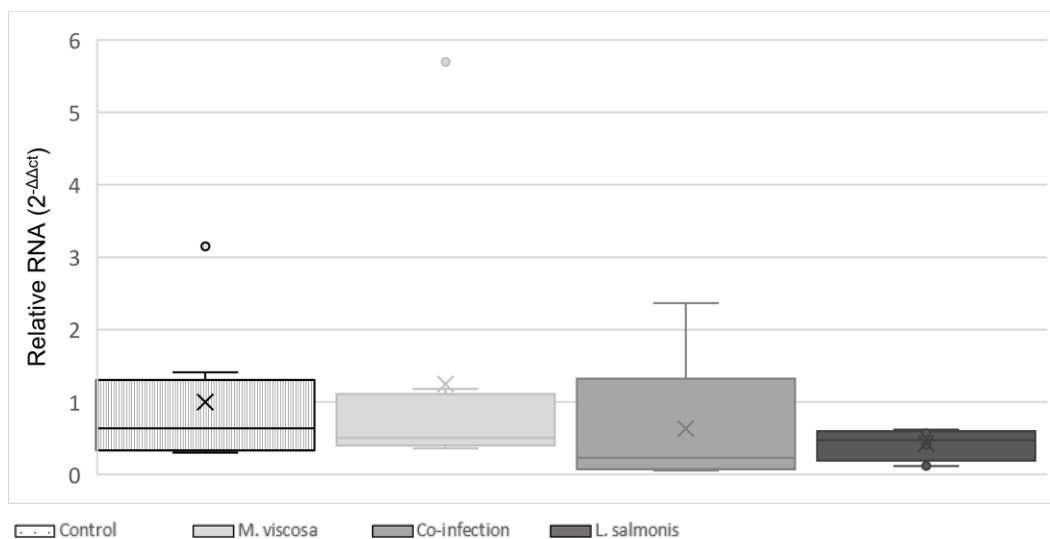
Fig. 48: Expression of IL-10 in head kidney samples from sampling B. Relative RNA ($2^{-\Delta\Delta ct}$) of IL-10 in head kidney samples from control (n=9) *M. viscosa* infection (n=12), *L. salmonis* infection(n=10) and co-infection(n=11). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.

Fig. 49: Expression of IL-10 in head kidney samples from sampling C. Relative RNA ($2^{-\Delta\Delta ct}$) of IL-10 in head kidney samples from control (n=9) *M. viscosa* infection (n=12), *L. salmonis* infection(n=10) and co-infection(n=11). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.

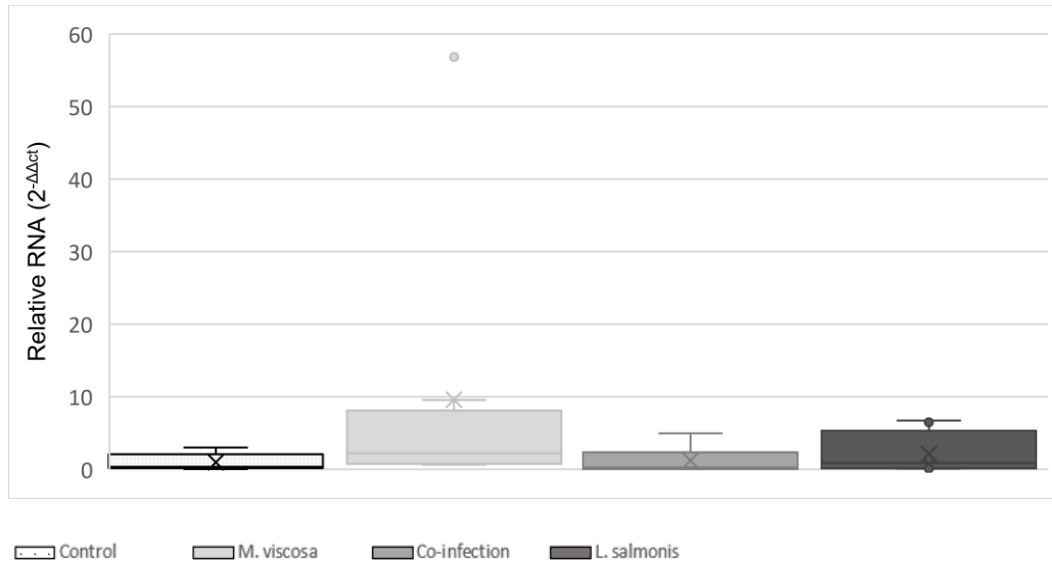Fig. 50: Expression of C3 in head kidney samples from sampling C. Relative RNA ($2^{-\Delta\Delta ct}$) of C3 in head kidney samples from control (n=9) *M. viscosa* infection (n=8), *L. salmonis* infection(n=8) and co-infection(n=7). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.
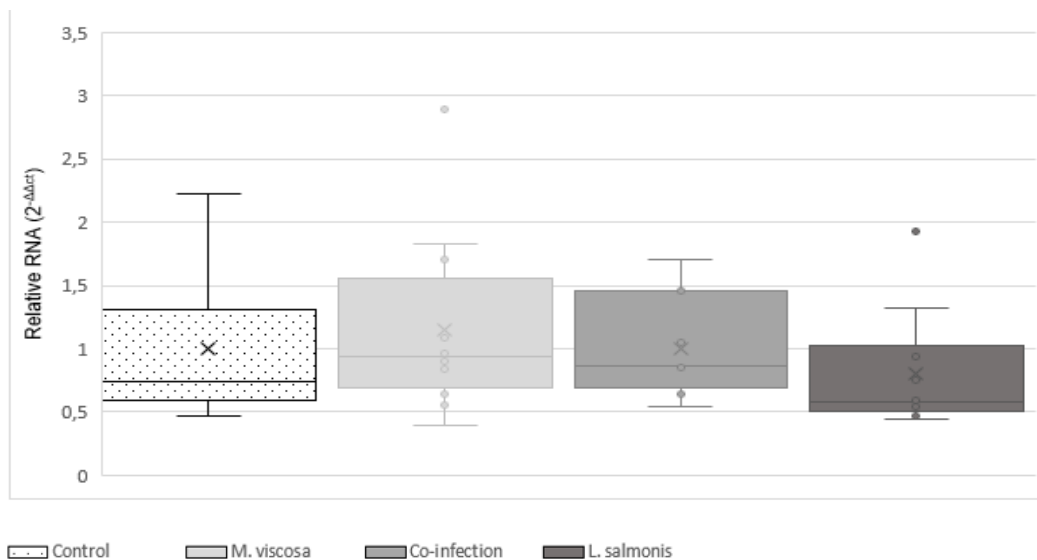

Fig. 51: Expression of IgM in head kidney samples from sampling B. Relative RNA ($2^{-\Delta\Delta ct}$) of IgM in head kidney samples from control (n=9) *M. viscosa* infection (n=12), *L. salmonis* infection(n=10) and co-infection(n=11). In all infected groups, skin was sampled in site of infection and in adjacent unaffected control sites. Box and whisker chart: box=first to third quartile, x=average, line=median, bars=minimum and maximum values, dots=outliers. Δ indicate significant difference compared to control, brackets with * indicate significant difference between two treatment groups. P values */Δ =0.05, **/ ΔΔ=0.01, ***/ ΔΔΔ=0.001.