Development of a vaccine against salmon louse (*Lepeophtheirus salmonis*)

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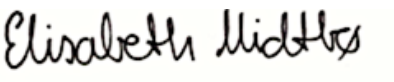
Preface

This thesis is written as a part of the Integrated Master's program in Aqua medicine at the University of Bergen, Faculty of Mathematics and Natural Sciences, Department of Biological Sciences. The work was carried out within the Sea Lice Research Centre (SLRC), a Centre for research-based Innovation appointed by the Research Council Norway. The laboratory work and experiments were conducted at Industrilaboratoriet (ILAB) and at the SLRC facilities at the University of Bergen.

The idea for the project was formulated by Sussie Dalvin, Associate professor II. I would like to thank her for being my supervisor and for letting me participate in a very diverse and interesting project. A special thanks to my co-supervisor Aina-Cathrine Øvergård for all the help and support she has given me throughout this period, and to both of them for reading and giving me feedback on my manuscript.

Further I would like to thank everybody at the SLRC for including me in their group, and for encouragement along the way.

It's been an interesting and challenging journey, both throughout the work with this thesis and for the 5 years that I have spent at the University. I have had the pleasure of meeting a lot of great people and would like to thank them for making my time in Bergen an adventure. I would especially like to give thanks to Jørgen for supporting me and taking me on many beautiful hikes.



Bergen, 29.05.2018

1. Contents

2. Abstract

Aquaculture is a growing industry whilst facing a major problem, the ectoparasite *Lepeophtheirus salmonis*. Parasite control in 2015 was estimated to cost 5 billion NOK. The challenges with the parasite are complex, and the infestations have previously been controlled with different kinds of chemical treatments. However, due to increasing resistance against existing treatments and the concern against harming non-target organisms, alternative treatment methods are needed. A vaccine against the parasite would be a cost-effective way to handle the infestation, though; several years of research have not uncovered an effective vaccine against the parasite. Currently, there is only one existing vaccine against ectoparasites. This vaccine is direct toward ticks, *Boophilus microplus*, in the cattle industry. A concealed antigen in the tick gut was used to develop a vaccine that resulted in lower fecundity.

The Mesh protein is a membrane protein involved in the formation of septate junctions in the gut of *Drosophila*. The protein was identified as potential vaccine candidate by the SLRC, where knockdown with RNAi resulted in a deformed gut in the louse and high rates of mortality. In the present study Mesh transcripts were confirmed to be localized in the intestine of *L. salmonis*. A recombinant Mesh protein was produced, incorporated into a vaccine and injected in Atlantic salmon. The trial fish were subjected to an infestation with copepodids, and the immune response was assessed at the end of the trial. No differences were detected in lice numbers post immunization, but an elevated immune response was detected in the vaccinated salmon.

3. Introduction

Aquaculture is a growing industry, and globally accounted for 76.6 million tons valued at 157.9 billion USD in 2015 (FAO). The industry is expected to contribute with a significant amount of protein for a future growing global population. In Norway, production of salmon and trout began in the 1960s, and today the production is estimated at 1.32 million tons valued at 63 618 million NOK (Statistics Norway). Although the production has risen gradually over the years, expansion of the industry was hampered in 1970-80 due to challenges with different bacterial diseases. Once vaccines against *Vibrio Anguillarum, Vibrio Salmonicida* and *Aeromonas Salmonicida* subspecies *salmonicida* were introduced in 1980-1990 this resulted in a major change for the industry. There was a rapid decline in antibiotics consumption and an increase in the biomass production of farmed fish (Brudeseth et al., 2013; Sommerset et al., 2005). Thus, vaccines have proven to be a sustainable solution in disease prevention, and an important factor for the growth of aquaculture in Norway. Today the aquaculture industry is facing new challenges. One of the major problems in Norwegian aquaculture today is an ectoparasite, *Lepeophtheirus salmonis*, also known as the salmon louse.

The first outbreaks of salmon lice infestations occurred at Norwegian Atlantic salmon farms during the 1960s, soon after cage culture was introduced (Pike and Wadsworth, 1999). The salmon louse is currently a big problem for the industry. It was estimated that the total lice costs in 1997 were 500 million NOK (Pike and Wadsworth, 1999). The amount has increased considerably in 16 years, and the costs were estimated to 5 billion NOK in 2015 (Audun Iversen et al., 2015). The costs in 2015 are estimated considering the prevention of lice, treatment of lice, loss in growth and increased feed conversion ratio. A salmon louse infestation can be treated and controlled by medicinal, mechanical and biological means, where chemical treatments through bath treatments and medicated feed have historically been the most common measure to prevent high abundance of salmon lice (Aaen et al., 2015; Denholm et al., 2002). In the later years, there has been a great focus on moving away from the traditional medicinal treatments against salmon louse, mainly because the louse has become resistant to most of the chemical treatments available (Aaen et al., 2015; Denholm et al., 2002). But also due to side effects of the chemical treatments, chitin synthesis inhibitors; diflubenzuron and teflubenzuron have shown to bind to organic material and affect non-target crustacea in the surrounding area of the cages (Samuelsen, 2008).

Non-chemical methods have also been utilized to reduce the number of lice on farmed fish. Among these methods: Laser, warm water, fresh water, and water pressure. Other management measures taken against the salmon louse are: producing larger smolts that will reduce the culture time in the ocean, cleaner fish, synchronized treatments, fallowing and plankton shielding skirts (Aaen et al., 2015). Amongst future methods currently being tested/developed are snorkel cages, enclosed cages and vaccines against lice.

3.1 *Lepeophtheirus salmonis*

Biology

Salmon lice are members of the copepod family Caligidae (Hamre et al., 2013). The lice of the genera *Caligus* and *Lepeophtheirus* are ectoparasites commonly found on wild and farmed salmonids. Two common species causing economical loss for the aquaculture industry is; *L. salmonis* (Krøyer 1837) and *Caligus elongatus* (Nordmann 1832) (Pike and Wadsworth, 1999). *L. salmonis* is more pathogenic and host specific towards salmonids and has been studied more than other species (Johnson et al., 2004; Mordue Luntz and Birkett, 2009; Nordi et al., 2016). The Atlantic form of salmon louse *(L. salmonis salmonis)* is distributed in the Atlantic ocean (Skern-Mauritzen et al., 2014). The pacific form of salmon louse can be observed on fish along the coast of Norway, Scotland, Ireland and North America (Mordue Luntz and Birkett, 2009). It has a wide salmonid host range as it can parasitizes migratory Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), residential sea trout (*Salmo trutta*) in addition to Arctic char (*Salvelinus alpinus*) (Mordue Luntz and Birkett, 2009; Pike and Wadsworth, 1999; Samsing et al., 2016).

In their natural environment the salmon louse were present in low number on wild stock and rarely caused severe pathological effects (Johnson and Albright, 1991). With the introduction of aquaculture, the parasite got a greater availability in number of hosts and they are present throughout the year (Ugelvik et al., 2017). Because of the high numbers of hosts in sea cages the density of lice larvae can be much greater than in farm-free areas (Browman et al., 2004; R. M. Serra-Llinares et al., 2014). This, again, leads to an increased lice infestation risk for local populations of wild salmonids, and threatens the remaining population of wild Atlantic salmon (Costello, 2009; R. M. Serra-Llinares et al., 2014). It has also been argued that the intensive salmon farming is selective for higher virulence in the lice, where the lice is more exploitative of the host (Ugelvik et al., 2017).

Lifecycle

The salmon louse has a direct lifecycle that includes eight developmental stages (Hamre et al., 2013). The female salmon louse carries her eggs in a pair of egg sacs, extending from her abdomen. The number of eggs per female is dependent on the temperature, salinity, size and host (Fast, 2014; Samsing et al., 2016). Lice on farmed host has been estimated to carry approximately 500 eggs per pair of egg strings, whereas females on wild host often carries around 1000 eggs (Costello, 2006), but Samsing et al. (2016) reported the reproductive output in lice on farmed host to vary from 140-300 eggs depending on temperature. Fertilized eggs are kept in two strings, which are extruded through the gonopores. Fertilization itself happens in the paired genial antra (Schram, 2000). The female normally carries the strings until all eggs are hatched, but when stressed the lice may release its strings (Schram, 2000). The female can produce as many as eleven sets of egg strings (Fast, 2014; Hamre et al., 2009). The first three stages in the lifecycle are planktonic larval stages. Two naupliar stages and the infective copepodid stage. These are followed by chalimus 1 and 2, preadult 1 and 2, and the adult stage (Hamre et al., 2013). Each life stage is separated by a molt. The developmental time from fertilization to adult is approximately 40 days for males and 52 days for females at 10 °C (Hamre et al., 2009).

The naupliar stages and the free living copepodid stage are lecithotrophic (Bron et al., 1993), relying on yolk stores as their energy source. It is therefore important for the copepodid to find a host before they have used up their energy. The planktonic stages drift mainly with the current and can potentially travel up to 100 km away from their origin. But Asplin et al. (2014) found that the majority of the copepodids keep close to their source and travels less than 40 km from their origin. Migration behaviour demonstrates that the copepodids are positively phototactic, where they gather near the surface during the day and sinks to the deeper layers at night (Mordue Luntz and Birkett, 2009). The copepodids also show preference to salinity and pressure, where they avoid areas with lower salinity and use mechanoreceptors in their antenna to respond to vibrations as the host approaches (Costello, 2006; Mordue Luntz and Birkett, 2009). Recent studies by Komisarczuk et al. (2017) uncovered that the copepodids use chemoreceptors in their antenna to determine host suitability. It has also been suggested that the copepodids respond visually to flashes from host scales (Costello, 2006; Mordue Luntz and Birkett, 2009).

The period of infectivity of the copepodids is time limited and temperature dependent (Pike and Wadsworth, 1999). But once a salmonid is encountered, the copepodid will attach and progress through their life stages on the host (Bui et al., 2016). Initial attachment to the host is achieved with the maxillipeds and by stabbing the second antenna into the epithelium (Bron et al., 1991; Pike and Wadsworth, 1999). This is followed by a more durable connection via a frontal filament, which anchors it to the host (Bron et al., 1991; Pike and Wadsworth, 1999). The frontal filament will persist throughout the larval development, where it is replaced at every molt (Gonzalez-Alanis et al., 2001; Pike and Wadsworth, 1999). The chalimus 1 and 2 stages are attached to the host via a frontal filament and graze the skin and mucus of the host around the attachment site. Preadult and adult stages can move around freely on the host, which increases the available feeding area. The free-moving stages are attached to the host by vacuum created under its cephalothorax (Fast, 2014). Males will develop to adults first and will exhibit precopula mate guarding. The females have internal fertilization, where polyandrous mating in the louse is possible (Todd et al., 2005). Adult females store sperm in a seminal receptacle (Todd et al., 2005), reducing the need for mating at low parasite densities. Concurrently, the female can continuously produce fertile egg strings without further mating.

3.2 Effects of salmon louse infestations on the host

The salmon louse is an ectoparasite which means that it is attached to the exterior surface of its host, like skin and fins. A parasite infestation is energetically costly for its host, and high abundance of parasites in the salmon can result in a reduced swimming performance. Current knowledge states that a parasite loads of >0.01 lice g^{-1} at any of the life stages can reduce the swimming performance of post-smolts (Bui et al., 2016). The fish will have problems with evading attacks, acquiring food, keeping up with a school or avoiding a suboptimal environment (Bui et al., 2016). Energy the fish would normally use for locomotion and metabolic activity has to redirected, and more energy will be used toward dealing with the parasite, through immune- and physiological responses (Bui et al., 2016). The damage caused by the lice can also leave the fish exposed to secondary infections with bacteria, virus or fungi (Bui et al., 2016; Costello, 2006).

The impact of an infestation with *L. salmonis* ranges from mild skin damage to stress induced mortality of individual fish (Costello, 2006). The salmon louse use their rasping mouthparts to graze on their host, they feed on mucus, epidermal, dermal or subcutaneous tissues (Bruno et

al., 2013; Costello, 2006), here excessive grazing can expose underlying muscle, fin rays and bone (Fast, 2014). Impacts on the hosts skin results in skin ulcerations, petechiae, hyperpigmentation, loss of epithelium and increased mucus discharge (Bruno et al., 2013; Costello, 2006). The erosion of the epidermis can have large effects on the osmoregulatory homeostasis of the fish, depending on the size of the fish and the number of lice. If the destruction of epidermis continues, the host will not be able to counteract the changes and will no longer be able to maintain homeostasis (Pike and Wadsworth, 1999). Epidermal erosion will allow the loss of body fluids such as; blood, lymph, protein and electrolytes (Pike and Wadsworth, 1999). This will result in anemia, reduction in lymphocytes, protein and ion imbalance (Costello, 2006). The changes also elicit hormonal responses, elevated cortisol level in response to the chronic stress. Expression of cortisol will induce immunosuppression, lowering the level of protection from the immune system (Costello, 2006; Pike and Wadsworth, 1999).

3.4 Available vaccines today

The use of vaccines against disease is favored in aquaculture due to the relatively low cost, ease of use and the preventive character of the treatment. Vaccinated fish have a reduced risk of disease development and even non-vaccinated fish can be protected due to herd immunity (Gudding, 2014). Fish vaccines are primarily used for control of bacterial diseases, and only a few exists against viral diseases. There are no commercially available parasite vaccines for fish today. Instead, there are large amounts of antiparasitic pharmaceuticals with potentially negative environmental effects (Sommerset et al., 2005).

The two main methods of vaccine delivery to fish today are immersion or injection into the body cavity, typically in the abdomen (Sommerset et al., 2005). Immersion vaccines gives good protection against many bacterial pathogens, it is less stressful for the fish and a cheaper alternative. Injection vaccines requires the fish to be over a certain size, is more stressful for the fish and more expensive. Injections, however, reduce the volume of the vaccine needed, and one is assured that every fish is vaccinated and injected with the same dosage (Sommerset et al., 2005). Another alternative is oral vaccination, this would be the ideal method of delivering the vaccine to the fish, but it has had little effect so far due to antigen destruction in the gut (Sommerset et al., 2005). The foregut of fish is acidic, and the antigens need proper protection to ensure passage to the hindgut (Berg et al., 2007).

There are four main vaccine types that are administered to fish today: inactivated vaccines, live/attenuated vaccines, subunit vaccines and DNA vaccines. Inactivated vaccines and live/attenuated vaccines are whole-cell formulations. Most vaccines are based on inactivated pathogens, but these must be combined with adjuvants to produce a long-term immunological response. They are also considered safer and more stable than live vaccines, since there is no chance that they will mutate back to the disease inducing pathogen (Munang'andu et al., 2014). Live/attenuated vaccines are weakened pathogens that still have the ability to replicate and induce a immune response in the host, but are not able to give the recipient the disease (Alexandersen, 1996). Subunit vaccines can be produced through cloning the protein molecules responsible for the protective immune response into vectors. The vectors can be transferred to bacteria culture where the protein can be cultivated in large amounts. These are safer for the fish, since there is no risk of transferring the disease to the recipient. DNA vaccines contains plasmid that are injected into the muscle of the fish. The muscle cells take up the plasmid and produce proteins through transcription and translation (Brudeseth, 2002). These antigens will be presented on the cell surface and the immune system will recognize them as non-self and elicit an immune response. Through this process the immune system will become activated and produce antibodies if the antigen is encountered again. But there is a concern with the use of DNA vaccines, regarding the pathogens DNA becoming incorporated in the chromosomal DNA of the host (Brudeseth, 2002).

Outside the aquaculture industry, the cattle industry has had a similar challenge, where they have been working for 30 years with the development of a vaccine against an ectoparasite. The tick *Rhipicephalus (Boophilus) microplus* is a blood sucking ectoparasite of cattle and has a significant economic impact on the cattle breeding industry worldwide (Parizi et al., 2012). However, in 1986 a protective protein from *R. microplus* named Bm86 was discovered. This antigen became the first tick antigen to compose a commercial vaccine against an ectoparasite (Parizi et al., 2012; Willadsen et al., 1995). The Bm86 protein is a "concealed" antigen, which is a component of the parasite that is capable of eliciting a protective immune response. It is normally not in direct contact with the host immune system during an infestation (Parizi et al., 2009; R. P. Lee and J. P. Opdebeeck, 1991). The antigen obtained from the tick gut is a glycoprotein present in the membrane of gut cells and plays a role in endocytosis (Parizi et al., 2009; R. P. Lee and J. P. Opdebeeck, 1991). The vaccine resulted in a lower tick infestation, but it has not been effective towards all *R. microplus*

populations. There is still ongoing work to enhance the efficacy of the available tick vaccines (Parizi et al., 2012).

3.5 Putative vaccine targets: Septate junctions

The intestinal epithelium plays an important role in forming barriers in the body that permits passage of water, ions and nutrients (Izumi et al., 2012; Resnik-Docampo et al., 2018). The intestinal epithelium also serves essential metabolic and innate immune functions (Resnik-Docampo et al., 2018). In vertebrates the cell-cell adhesion complexes that control transport across cell layers in all epithelia are tight junctions (Furuse and Izumi, 2017; Resnik-Docampo et al., 2018). Invertebrates lack tight junctions but possess a different type of occluding junction, the septate junctions. But the molecular architecture and mechanisms of functional regulations in the septate junctions are not yet fully understood (Furuse and Izumi, 2017). In arthropods two different types of septate junctions have been observed; pleated and smooth (Banerjee et al., 2006; Izumi et al., 2016). Where pleated septate junctions are found in ectodermally derived epithelia smooth septate junctions is mainly found in endodermally derived epithelia (Furuse and Izumi, 2017; Resnik-Docampo et al., 2018).

Mesh protein

In 2012, Izumi et al. (2012) identified a uncharacterized membrane protein, which they named Mesh. Mesh has a single-pass transmembrane domain and a large extracellular region containing a NIDO domain, an Ig-like E set domain, an AMOP domain, a vWD domain, and a sushi domain (Izumi et al., 2016, 2012). These extracellular domains are found in cell adhesions proteins, playing important roles in cell-cell adhesion. This ability was also found for Mesh (Izumi et al., 2012). It is proposed that Mesh is required for the formation and organization of smooth septate junctions, together with two other proteins Ssk and TsP2A (Furuse and Izumi, 2017; Izumi et al., 2016, 2012). These three septate junction components were reported to be mutually dependent of each other (Izumi et al., 2016).

Antigen candidates

The gut of the *L. salmonis* is an interesting research area for potential antigen candidates, since it is likely for antibodies to reach the gut. The idea behind using concealed antigens, is that when the louse would digest blood from its host, the antibodies would bind to the antigen in the parasites gut where they would impair gut function and result in mortality, or lower fecundity similar to the effect of the vaccine towards *B. microplus.*

4. Aims for this study

Previous work performed in the Sea Lice Research Centre identified the Mesh protein as a potential vaccine candidate. This thesis aims to assess this vaccine candidate through a vaccine trial where boost is included. A similar trial was performed in 2016, without vaccine boost.

The aims for this study is to:

1. Assess the presence of Mesh transcripts in the intestine of *L. salmonis.*
2. Produce recombinant Mesh proteins in *E. coli* cells.
3. Incorporate the recombinant protein into a vaccine against *L. salmonis.*
4. Administer the vaccine including a boost to Atlantic salmon in a pilot vaccine trial.
5. Conduct an infection trial with copepodids
6. Assess the effect of the vaccine on both the fish and lice.

5. Materials

5.1 Chemicals and reagents

Table 5.1: Chemicals and reagents used

Chemicals/reagents	*Producer/supplier*
Sybr Select Master Mix	ABI/Life technologies
AffinityScript qPCR cDNA synthesis kit	Agilent technologies
Deoxyribonuclease I (200U/μl)	Invitrogen
Q5 High-Fidelity 2X Mastermix kit	BioLabs
qScript cDNA SuperMix	Quanta Biosciences
GenElute PCR Clean-Up kit	Sigma-Aldrich
NI-NTA Fast start kit	Qiagen
Histoclear II	National diagnostics
DIG RNA Labelling kit	Roche
Proteinase K 2 mg/ml	Sigma-Aldrich
EDTA 0.2 M	Sigma-Aldrich
NBT 4-Nitro blue tetrazolium chloride, solution	Sigma-Aldrich
BCIP 4-Toluidine salt	Sigma-Aldrich
Anti-Digoxigenin-AP, Fab fragments from sheep.	Roche
Blocking solution 10%	Sigma-Aldrich
Paraformaldehyde	Sigma-Aldrich
Deionized Formamide	Sigma-Aldrich
RNase A	Sigma-Aldrich
LiCl 4M	Sigma-Aldrich
Sodiumcitrate∗2H_2O	Sigma-Aldrich
DNase I	Biolabs
LB Broth (lennox)	Sigma-Aldrich
Phosphate buffered saline tablet	Sigma-Aldrich
LB agar	Sigma-Aldrich
Ethanol absolute	VWR chemicals
GeneRuler, DNA ladder mix (0.1 μg/μl)	Thermo Scientific
6X DNA loading dye (1 ml)	Thermo Scientific

1X SDS-Page loading buffer	Premade
2X SDS-Page loading buffer	Premade
Tris-acetate-EDTA (TAE buffer)	Thermo Scientific
Precision Plus Protein dual standard	BIORAD
Tri reagent	Sigma-Aldrich
Chloroform	Sigma-Aldrich
Isopropanol	Sigma-Aldrich
Water, DEPC treated	Sigma-Aldrich
Shandon™ instant hematoxylin	Thermo Scientific
Erytrosin b	Certistain, Merck
Histomount	Invitrogen
Tween 20	Sigma-Aldrich
Skim milk powder	Sigma-Aldrich
Citric acid (monohydrate)	Merck
Sodium phosphate	Merck
Mouse anti-salmonid immunoglobulin	ImmunoPrecise
Sodium bicarbonate	Merck
Sodium carbonate	Fisher Scientific
Sulfuric acid	Sigma-Aldrich
BENZOAK VET	ACD Pharmaceuticals
METHOMIDATE	Western Chemical inc.
BL21(DE3) CHEMICALLY COMPETENT CELLS	Sigma-Aldrich
Dextran sulphate	Sigma-Aldrich
Triethanolamine (TEA)	Sigma-Aldrich
Acetic anhydride	Sigma-Aldrich
RNaseZAP	Sigma-Aldrich
Magnesium Chloride 1M	Sigma-Aldrich
Maleic acid	Sigma-Aldrich

5.2 Equipment

Table 5.2: Equipment used:

Equipment	*Supplier*
7500 Fast Real-Time PCR system	Applied Biosystems
Veriti 96 Well Thermal Cycler	Applied Biosystems
Tissuelyser II	Qiagen
Gel logic 212 pro	Carestream
Gel doc EZ imager	BIO RAD
Stain-Free sample tray	BIO RAD
Water bath VWB 12	VWR
Water bath SUB aqua 5 plus	GRANT
Centrifuge 5804 R	Eppendorf
Centrifuge Heraeus Fresco 21	Thermo scientific
30 MM Whatman paper 20 x 20 cm	VWR
Hybond N+ membrane	VWR
INCU-Line	VWR
Milli-Q Advantage purification system	Millipore
Thermomixer comfort 1.5 ml	Eppendorf
Avanti J-26 XP	Beckman Coulter
MicroAMP^{TM} Optical Adhesive Film	Applied Biosystems
MicroAMP Fast 96-well reaction plate	Applied Biosystems
Adhesive film for microplates	VWR
PCR Plate-Spinner Centrifuge	Axygen, VWR
Xplorer plus multi pipette (8 channels)	Eppendorf
25 ml Reagent Reservoir	VWR
ELISA-plate	Sarstedt
Mini-PROTEAN TGX Stain-Free Precast Gels	BIO RAD
Ultrospec 10	Amersham Biosciences
Superfrost objektglass for ISH sections	VWR
Mini-protean tetra vertical electrophoresis cell	BIO RAD
PAP pen	DAKO
Heraeus Pico 21 centrifuge	Thermo Scientific

ARKTIK Thermal cycler	Thermo Scientific
5 mm Stainless Steel Beads	Qiagen
UVC 500 UV crosslinker	Hoefer
Multitron Standard (horizontal shaker)	IFORS HT
AXIO Scope.A1 light microscope	ZEISS
Tecan microplate reader	SPARK

6. Methods

To confirm the Mesh protein as a potential vaccine candidate, this thesis will analyze the presence of Mesh transcripts in the gut of *L. salmonis*. To further validate the vaccine candidate, a recombinant vaccine will be produced and administered to *Salmo salar* twice. The fish will be exposed to *L. salmonis* and at the end of the trial the antibody response and infestation success will be assessed. In the analysis of antibody response, serum from a previous trial where the fish did not receive boost will be included for comparison.

6.1 Quantitative analysis of Mesh transcripts present in the salmon louse gut

6.1.1 Lice sampling

A laboratory strain of *Lepeophtheirus salmonis* (salmon louse) was used for all the experiments described in this thesis (Hamre et al., 2009). The sampling was performed in the Wet Lab of the Sea Lice Research Centre. One salmon was anesthetized (60 mg/l benzocaine and 5 mg/l methomidate mixed with water) and killed with a blow to the head. From the salmon, 10 lice were collected, 5 adult males and 5 adult females with egg strings (these were removed). With the use of a scalpel the gut of the lice was cut out (Figure 6.1) and sampled in RNA-later. The rest of the lice was collected in an additional RNA-later tube. The samples were stored at -20 °C until total RNA purification.

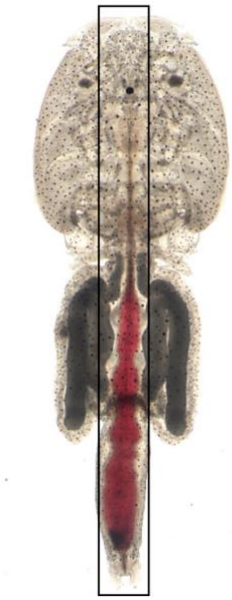


Figure 6.1: Illustration of the gut sampling. Photo: Lars Hamre

6.1.2 Total RNA purification

Total RNA was isolated from the samples using TRI reagent, according to the TRI reagent protocol (Sigma Aldrich). In short, the tissue samples were placed in 2 ml Microcentrifuge Safe-Lock tubes with a single 5 mm stainless steel bead. Each of the tubes were added 1000 µl TRI reagent and homogenized in a TissueLyser for 3 minutes at 60 Hz. The samples were incubated for 5 minutes at room temperature. Subsequently, the samples were added 2 µl chloroform, shaken for 20 seconds and left to incubate at room temperature for 10 minutes. After incubation, the samples were centrifuged at 21100 G for 15 minutes at 4 °C. Phase separation was then achieved, where the colorless aqueous phase containing total RNA was transferred to a fresh tube. The precipitation of RNA was induced by adding 500 µl of isopropanol, the solution was mixed and incubated at room temperature

for 10 minutes. The samples were centrifuged at 21100 G for 10 minutes at 4 °C. The supernatant was removed, and the pellet retained was washed twice with 1 ml 75% ethanol. The samples were centrifuged at 7500 G for 5 minutes at 4 °C. The supernatant was removed, and the pellet airdried and dissolved in diethylpyrocarbonate (DEPC) treated water. The adult female samples were dissolved in 50 µl and the male samples in 30 µl. The purity and concentration of total RNA was assessed by spectrophotometry (Nanodrop ND-1000). The samples were stored at -80 °C until further use. A negative control was included in the isolation. This sample was treated in the same way as the other samples, but without including lice tissue in the sample.

6.1.3 DNase treatment of RNA

The aim of the DNase treatment is to remove unwanted DNA in the samples that can contaminate the qPCR results. An aliquot of 1 µg of total RNA was used from each sample and mixed together with 1 U DNase I and 1X DNase I reaction buffer with DEPC water added up to 10 µl. The samples were incubated at room temperature for 15 minutes. The reaction was stopped by adding 1 µl EDTA (25 mM) to the samples and incubating them for 10 minutes at 65 °C. The DNase treated total RNA samples were stored in -80 °C until use.

6.1.4 cDNA synthesis

In this method, DNA was synthesized from total-RNA template via reverse transcription (RT). This gives complementary DNA (cDNA) which can be amplified through polymerase chain reaction (PCR). The advantage to this procedure is that the cDNA is more stable than the RNA and can be stored for a longer period. The AffinityScript qPCR cDNA Synthesis Kit was used as recommended by the supplier, with some minor adjustments as listed below. Mastermix for one reaction contained 1X First strand mastermix, 100 ng Oligo dT primer, 50 ng Random primers, 0.5 µl AffinityScript RT enzyme and 200 ng total RNA in 10 µl reactions. A sample without the RT enzyme (RT control) was also prepared as a no amplification control (NAC). This control will indicate the presence of contaminating DNA in the samples. cDNA was synthesized in an ARKTIK thermal cycler, 5 minutes at 25 °C to allow primer annealing, 15 minutes at 42 °C to allow cDNA synthesis and 5 minutes at 95 °C to terminate the cDNA synthesis. The samples were directly placed on ice, diluted 1:10 and stored at -20 °C until use.

6.1.5 Real time PCR analysis

Real time RT-PCR is used to determine the amount of a target gene transcripts that is present in a sample. This is done by amplifying a fragment of cDNA. The method relies on thermal cycling, which by heating and cooling the sample allows for separation of the DNA strand and an enzymatic replication of the target gene. The amount of PCR product in a RT-PCR analysis is measured after each round of amplification.

The 7500 Fast Real-Time PCR System was used, with a SYBR Green assay. The SYBR Green dye fluoresces when it binds to dsDNA and continues to bind to each new copy of DNA. This allows for a quantification of the product by the increase in fluorescence. EF1a (primer: b434-4345, table 6.1) was used as the reference gene, as it has been validated as a suited reference gene in the different life stages of *L. salmonis (Frost and Nilsen, 2003).* The target gene Mesh was detected with primers b2375-76 (table 6.1). In each assay, the samples were added in duplicates, including the control and RT control, with one NTC (no template control) used for each assay. A Fast 96-Well Reaction plate was used and each well received 1X SYBR Select Master Mix (Thermo Fisher Scientific), 0.5 μM Forward primer, 0.5 μM Reverse primer, 2 μl RNase free H_2O and 2 μl of each template (cDNA) were distributed to their assigned wells. The plate was sealed with film and centrifuged (Plate-Spinner) and run on the 7500 Fast machine. PCR program for SYBR Green was chosen (Table 6.2). The data obtained during Real-Time PCR was analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 6.1: primers used for qPCR.

Primers	Sequence 5' → 3'
EMLSA10881 q1_b2375	5-TTTCACCAATGAACTCCCAATGTGC-3
EMLSA10881q2_b2376	5-CCAATGGGAGAGGGAAGGGATCTTA-3
Elf_LPU1951_fpee_b434	5-GGTCGACAGACGTACTGGTAAATCC-3
Elf_LPU1951_rp_b435	5-TGCGGCCTTGGTGGTGGTTC-3

Table 6.2: Settings used for the qPCR.

PCR settings		
Initial denaturation	95 °C, 2 min.	1 cycle
Denaturation	95 °C, 2 min.	40 cycles
Annealing	95 °C, 15 sec.	
Extension	60 °C, 1 min.	
Melt Curve analysis	60-90 °C	

6.2 Qualitative analysis of Mesh transcripts present in the gut

In situ hybridization (ISH) is a method used to localize specific nucleic acid targets in fixed tissue. The method is performed by making an antisense RNA probe to your target (mRNA), allowing your target and probe to bind, and visualizing its location in the tissue. For this method to work it is necessary for the probes to send a signal. The probes that were used in this ISH is digoxigenin (DIG) labeled probes, this is a nonradioactive alternative and these probes are amplified using anti-DIG antibodies. These antibodies are conjugated with an enzyme that can bind to the substrate solution. Once the substrate is added, it will result in the color reaction. The production of the probes FT7 and RT7 will be described below, followed by a description of the *in situ* hybridization.

6.2.1 cDNA synthesis

The DNase treated samples from 6.1.3 were also used for the qualitative analysis of the Mesh transcripts. Two of the female gut samples with the highest concentrations were pooled together, 5 µl from each of the samples. The pooled sample was transformed into cDNA with the qScript cDNA SuperMix kit according to the supplier recommendations. Each tube received 6 µl RNase free water, 1X qScript cDNA SuperMix and 1000 ng total RNA. The cDNA synthesis was done in a Veriti 96 Well Thermal Cycler, 5 minutes at 22 °C to allow primer annealing, 30 minutes at 42 °C to allow cDNA synthesis and 5 minutes at 85 °C to terminate the cDNA synthesis. The samples were directly placed on ice.

6.2.2 PCR analysis

Traditional PCR analysis is similar to real time PCR, but the main difference is that traditional PCR measures the size of PCR product at the end point of amplification. Master mix was prepared for the cDNA sample from 6.2.1. The Q5 High-Fidelity 2X Master Mix was used according to the supplier recommendations, 1X Q5 reaction buffer, 200 µM dNTP, 9 µl Template DNA, 0.045 U Q5 high fidelity DNA polymerase and RNase free H_2O up to a 100 µl. Transferred 90 µl master mix to two reaction tubes and added 5 µl of the specific primers for the individual reactions, FT7 (primer: B2401-B2374) and RT7 (primer: B2373-B2402). The sense (FT7) and antisense (RT7) will now get a T7 promotor. Their sequences can be viewed in table 6.3. The two reactions were put through a pre-defined number of thermal cycles, see table 6.4 for the PCR settings, and was then stored at 4 °C until use.

Table 6.3: Primers used for PCR

Primers	Sequence 5' → 3'
EMLSA10881 Fw1_b2373	5-TGAATGGGTAAATCCTCGAACAGA-3
EMLSA10881 T7Fw1 b2401	5-TAATACGACTCACTATAGGGagaTGAATGGGTAAATCCTCGAACAGA-3
EMLSA10881 Rev1_b2374	5-GGGCAAATCTCTGATCAATGTTG-3
EMLSA10881 T7Rev1 b2402	5-TAATACGACTCACTATAGGGagaGGGCAAATCTCTGATCAATGTTG-3

Table 6.4: settings used for PCR

	PCR settings	
Initial denaturation	98 °C, 30 sec.	1 cycle
Denaturation	98 °C, 10 sec.	35 cycles
Annealing	55 °C, 30 sec.	
Extension	72 °C, 45 sec.	
Final extension	72 °C, 2 min.	1 cycle
Soak	4 °C, indefinite	1 cycle

To visualize PCR products, an agarose gel was run. A 1% agarose gel was made, applying 2 g agarose gel powder to 200 ml 1X Tris-acetate-EDTA (TAE Buffer), see table 6.5. In order to

visualize DNA bands, the fluorescent Gel Red Nucleic Acid Stain was added to the hot agarose solution, 1 µl Gel Red per 25 ml of gel solution. The solution was poured into a gel caster, and once the gel was stiff, the casting gates and the gel comb were removed. 1X TAE buffer was added until it covered the gel. The two reactions, 5 µl from each, were added 1 µl 6X loading dye. The gel was loaded and ran at 80 V for 45 minutes. Bands were visualized with the Carestream Gel Logic 212 PRO.

Table 6.5: Reagents used in PCR analysis:

Reagents	Recipe
0.5 M EDTA	Stir 93.05 g EDTA Add 9 g NaOH pellets until suspension of EDTA starts to clear. Use 4 M NaOH dropwise until EDTA dissolves. Adjust to pH 8
50X TAE buffer	Dissolve 246 g TRIS in 800 ml Milli-Q water. Add 57.1 ml concentrated acetic acid >99% Add 100 ml 0.5 M EDTA, pH 8. Add Milli-Q water up to 1 L

6.2.3 Purification of PCR product

The PCR products were purified with GenElute PCR Clean-Up Kit, which eliminates other components such as salts, excess primers, oils and nucleotides. The kit was used according to the supplier recommendations. The FT7 and RT7 PCR products were transferred to individual columns and eluted in 40 µl ddH_2O. The purity of the PCR products was assessed by spectrophotometry (Nanodrop ND-1000). The samples were stored at -20 °C until use.

6.2.4 Probe synthesis from PCR product

The purified PCR products were used to synthesize two single stranded DIG-labeled RNA probes (DIG RNA labeling kit). Transferred 1000 ng from the FT7 and RT7 PCR products to fresh tubes with master mix; 3 µl Nuclease free water, 2 µl 10X DIG label mix, 20 U RNase inhibitor, 2 µl 10X Transcription buffer and 40 U T7 polymerase. The Transcription buffer was preheated on a heating block at 37 °C to dissolve precipitation. The solutions were mixed and incubated at 37 °C (Thermomixer comfort) for 2 hours. The DNA template was removed

by adding 2 U DNase I to each of the solutions and incubated at 37 °C for 15 minutes. To deactivate the DNase, 2 µl 0.2 M EDTA was added. The probe was precipitated in 2.4 µl 4M LiCl and 60 µl cold 100% EtOH overnight at -20 °C to remove free nucleotides or other contaminants. The precipitated RNA pellets were washed with 1 ml chilled 70% EtOH. The solutions were centrifuged 16000 G at 4 °C for 15 minutes. The supernatant was removed and the pellet dried. The probes were dissolved with 49 µl DEPC water and 20 U RNase inhibitor. The concentration of FT7 and RT7 probes were assessed by spectrophotometry (Nanodrop ND-1000). The probes were stored at -80 °C until use.

6.2.5 Spot test of probe

A spot test was performed to determine probe labelling. A dilution series of the probes FT7 and RT7 (1X, 10X and 100X) were made. The dilutions were spotted, 1 µl of each dilution on a positively charged nylon membrane. To fixate the nucleic acids to the membrane, Crosslinker with UV light was used at 70.000 µJ/cm^2 for 1 minute. The membrane was rinsed in 10 ml washing buffer (Table 6.6) for 20 seconds. Removed the washing buffer and incubated the membrane for 30 minutes in 10 ml blocking solution while gently agitating. Antibody, Anti-Dig-Ap 2 µl was added to the solution (1:5000 dilution) and the solution was incubated for another 30 minutes. The antibody solution was removed, and the membrane was washed for 5 minutes with 10 ml washing buffer, while gently agitating. This step was repeated three times. The membrane was washed with 10 ml detection buffer for 1 minute, while gently agitating. The detection buffer was removed, and the membrane was incubated in 10 ml chromogen substrate, for 10 minutes. The membrane was allowed to develop in the dark. The probe dilutions were visualized through the BCIP and NBT colorimetric reaction with alkaline phosphates. The reaction was stopped by washing the membrane in distilled water. The membrane was left to dry and was preserved by putting see-through tape around it.

Table 6.6: Spot test solutions.

Solution	***Content***
Washing buffer	50 ml 1X Maleate buffer 150 µl Tween 20 (Sigma)
1% Blocking solution	10 ml 1X Maleate buffer 1000 µl 10X Block solution

Detection buffer	1 ml (1 M) Tris HCl, pH 9.11 1 ml (5 M) NaCl 8 ml DEPC water
Chromogen substrate	10 ml Detection buffer 4.5 ng/ml NBT 1.75 ng/ml BCIP

6.2.6 *In situ* hybridization

In addition to the two probes that were synthesized in 6.2.4, a premade *L. salmonis* Trypsin probe was used as the positive control. The LsTrypsin probe yields a strong signal after a relatively short incubation time. The sections that were used for the *in situ* hybridization were premade paraffin sections. All washes described in the method below are 50 ml solutions and the contents are described in Table 6.7. All the section treatments were performed in a coplin jar in the fume hood, if not otherwise mentioned. Before start, all surfaces were cleaned with RNaseZAP to ensure a RNase free working area.

Day 1:
The sections were baked at 65 °C for 20 minutes. The sections were washed with Histoclear 3 x 10 minutes to remove paraffin. The sections were rehydrated with alcohol, 2 x 100%,95%, 70% and 50% EtOH, each wash was 1 minute. Following a soak in 2X SSC buffer for 2 x 1 minute. The sections were treated with Proteinase K for 20 minutes to permeabilize the tissue in order for the probe to enter.

To maintain the tissue-structure, the sections were fixated for 5 minutes in cold 4% paraformaldehyde in 1X PBS. The sections were subsequently washed with PBS for 2 x 2 minutes. The sections were incubated in acetic anhydride treatment for 5 minutes, which inactivates endogenous phosphates and reduces the chance for unspecific coloring. The sections were subsequently washed with 2X SSC buffer for 2 minutes. The sections were dehydrated with alcohol; 50%,70%,95% and 2x 100% EtOH, each wash was 1 minute. Each section was dried with 3 mm Whatman filter paper, and a hydrophobic frame was drawn around the tissue using a PAP pen.

The hybridization solution for each section was prepared. The antisense and sense probe concentration for LsTrypsin was 2.5 ng/µl and 50 ng/µl for Mesh. The probes were added 80 µl Hybridization solution and ddH_2O to give a total volume of 100 µl. The hybridization solutions were further incubated at 95 °C for 5 minutes to denaturate secondary structures to ensure that the probes were single stranded and available for mRNA binding. The hybridization solutions were transferred directly to ice and 10 µl 10% blocking solution were added to each tube. Each slide received 100 µl of their assigned hybridization solution and was placed in a moist chamber with two layers of filter papers moistened with 2X SSC. The chamber was sealed on each side and incubated overnight at 65 °C (INCU-Line).

Day 2:

The heated solutions used during day 2 were preheated in the water baths and the coplin jar was kept in the water baths during the wash. The sections were flushed with 2X SSC to remove the hybridization solution. Subsequently the slides were washed with 2X SSC for 2 x 30 minutes. The slides were washed in 50% deionized formamide in 2X SSC for 30 minutes at 65 °C. The slides were washed with 2X SSC for 2 x 10 minutes at 37 °C. Subsequently the sections were submerged in 1 ng/ml RNase for 30 minutes at 37 °C.

The sections were washed three times with 1X Maleate buffer for 10 minutes. The sections were blocked for two hours with blocking buffer. The sections were subsequently washed twice with 1X Maleate buffer for 5 minutes. Anti-Dig-AP was diluted 1:2000 in blocking solution, and each slide was dried with filter paper and received 100 µl of the antibody solution. The slides were placed back in the moisture chamber and incubated overnight at room temperature.

Day 3:

The slides were washed twice with 1X Maleate buffer for 10 minutes. Subsequently the slides were submerged in Processing buffer for 10 minutes. Each section was now dried with filter paper and added 200 µl of the chromogen-substrate, a color-chamber was placed on top of the slide and they were allowed to develop in the dark. The slides were left to incubate for 48 hours, after 24 hours the processing solution for each of the slides were changed. In the positive control, the reaction was terminated after 1 hour, when a clear signal had been obtained.

Once the slides were developed the color-chamber was removed, and the slides were flushed with stop buffer, subsequently they were placed in stop buffer in the coplin jar. The slides were washed in water, dried with filter paper and sealed for storage with a couple drops of mounting solution (70% glycerol and 30% stop buffer). Once the cover glass was added, the sections were placed under a small weight-unit overnight to dry. Photos were taken with an axiocam 105 color camera mounted on a polarized light microscope (ZEISS Axio Scope.A1)

Table 6.7: In situ solutions

Solution	Content / recipe
SSC (20X), 1 liter	173.3 g NaCl 88.2 g Sodiumcitrate∗2H_2O (294 g/mol) 800 ml DEPC water Adjust pH with NaOH Adjust volume to 1 L with DEPC
Proteinase K solution	5 ml Tris, pH 8 (1 M) 5 ml EDTA (50 mM) 250 µl (2 mg/ml) Proteinase K 40 ml ddH_2O
4% Paraformaldehyde in PBS, 1 liter	40 g Paraformaldehyde and 500 ml DEPC treated water, heat to 65 °C. Add drops of 1M NaOH until dissolved (about 150 µl). Cool to room temperature. Add 100 ml 10X PBS / 250 ml 4X pH should be 7.4 Adjust volume to 100 ml and store at -20 °C in 50 ml aliquots.
1X PBS	Phosphate buffered saline tablets. 1 tablet/200 ml DEPC treated water.
Tris NaCl stock pH 9.5 (500 ml)	Dissolve 60.55 g Tris base in 350 ml Milli-Q water. Add 29 g NaCl Adjust pH with HCl Adjust volume to 500 ml with Milli-Q water.

Acetic anhydride treatment	50 ml DEPC treated water 665 µl (0.1 M) Triethanolamine (TEA) 125 µl 0.25% Acetic anhydride
Processing buffer, 1 liter	10 ml Tris-NaCl (1 M) pH 9.5 5 ml $MgCl_2$ (1 M) 85 ml DEPC treated water
Hybridization solution (20 ml)	2.5 g Dextran sulphate, add DEPC water up to 5 ml. Heat up to 70 °C to dissolve. Add: 250 µl (1 M) Tris HCl pH 7.5 50 µl (0.5 M) EDTA 1.5 ml (5 M) NaCl 0.7 ml DEPC water 12.5 ml deionized high-grade RNase free formamide. Freeze at -20 °C in 2.5 ml aliquots.
Blocking buffer	45 ml 1X Maleate buffer 5 ml 10 % blocking solution 25 µl Triton X-100
Chromogen substrate	10 ml processing buffer 4.5 ng/ml NBT 1.75 ng/ml BCIP
Maleate buffer (5X) 1 liter	58 g Maleic acid in 850 ml Milli-Q water. Adjust pH 7.5 using NaOH pellets (35-40 g to 1 L). Add 43.8 g NaCl Bring up to 1 L with Milli-Q water
Tris HCl stock pH 7.5 (1 liter)	Dissolve 121.1 g Tris base in 800 ml DEPC water Adjust pH with HCl and adjust volume to 1 liter with DEPC water.
4 mg/ml RNase A	100 mg RNase A 25 ml DEPC water Freeze at -20 °C in 2.5 ml aliquots.

Stop buffer	10 mM Tris-HCl pH 7.5 1 mM EDTA 150 mM NaCl

6.2.7 HE(S) staining

Hematoxylin Eosin Saffron (HES) is a standard staining method. But here the method was performed without the extra staining step with Saffron (stains connective tissue). The sections that were used for HES were the neighbour sections of the paraffin sections used for *in situ*. All washes described in the method below are 50 ml solutions, see Table 6.8 for contents. The sections were backed at 60 °C for 30 minutes. All the section treatments were performed in a coplin jar in the fume hood. The sections were washed with Histoclear 2 x 10 minutes to remove paraffin. The sections were rehydrated with alcohol; 2 x 100%, 95%, 70% and 50% EtOH, each wash was 5 minutes. It was followed with 5 minutes in fresh tap water.

The next step was the staining, there were two staining steps. Haematoxylin stains the basophile part of the cell. The sections were soaked in 50 ml Haematoxylin for 2.5 minutes, before being washed in running tap water for 4 minutes. Erythrosin stains the eosinophil part of the cell. The sections were washed with 1% Erythrosin for 1.5 minutes, before being washed in running tap water for 1 minute.

The sections were then dehydrated with alcohol, 95% and 2 x 100% EtOH, each wash was 1 minute. The last step were two washes with Histoclear. The sections were dried with 3 mm whatman filter paper and sealed for storage with a couple drops of Histomount. Once the coverglass was added, the sections were placed under a small weight-unit overnight to dry. Photos were taken with an axiocam 105 color camera mounted on a polarized light microscope (ZEISS Axio Scope.A1)

Table 6.8: Shows HE(S) solutions

Solution	Content / recipe
1% Erythrosin solution, pH 6.5	2.0 g Erythrosin B (Merck) mixed with 200 ml water

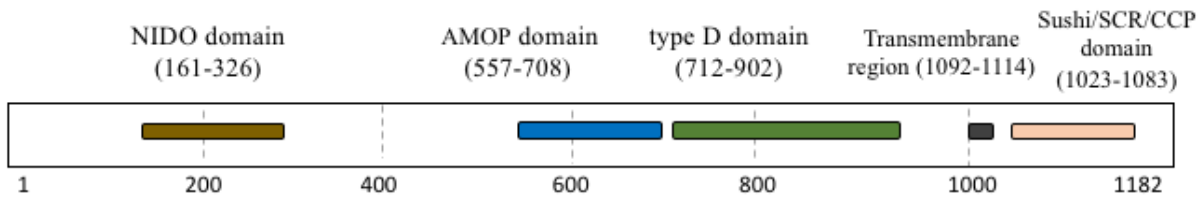
Shandon™ Instant Hematoxylin	Mix content of packet A and packet B (Thermo Scientific) Add 1-liter distilled water and mix until powder is in solution. Let the solution stand for 12 hours. Filtrate before use.

6.3 Vaccine preparation

6.3.1 Insert

A pET26b-F10881 vector containing the NIDO domain was received from Elanco, Canada. The expression vector also includes a n-terminal pelB signaling peptide, c-terminal His tag, lacI and T7 promoter. An *E. coli* BL21 bacteria was used to express the recombinant protein. An illustration of the Mesh protein is presented in figure 6.2, where the NIDO domain is located at basepair 161-326.

Figure 6.2: Mesh protein.



6.3.2 Transformation of expression vector by heat shock

The expression vector containing the protein sequence, must be introduced to *E. coli* BL21 cells in a process called transformation. The transformation was done according to the supplier's recommendations. The BL21 competent cells were thawed on ice, and 40 µl was added to pre-chilled 15 ml disposable polypropylene culture tube. To transform the pET26b-F10881 NIDO into the bacteria, 44.5 ng of the expression vector was added. Cells were incubated on ice for 30 minutes, and subsequently transferred to a 42 ºC water bath for 45 seconds. The tubes were then immediately transferred to ice for 2 minutes. After transformation, 960 µl room tempered Expression Recovery Medium was added to the tubes, before they were placed in a horizontal shaking incubator for 1 hour at 250 rpm and 37 °C.

Afterwards 50, 100, 150 and 200 µl was seeded out on LB-Lennox agar plates containing 25 µg/ml kanamycin to select for transformed bacteria, and incubated overnight at 37 °C.

6.3.3 Selecting bacteria stock

To further confirm that the bacterial colonies contained the plasmid construct, 16 bacteria colonies were screened with PCR and two primers. One gene specific primer (B4151) and one plasmid specific primer (T7 vector), see table 6.9 for details. The colonies were run in three parallels to test different annealing temperatures, since our gene specific primer was made for the original gene sequence in the salmon louse. In the construct, the gene sequence was codon optimized for prokaryote cells. This introduced 3 flaws in our primer. It was necessary to find the optimal temperature for binding to occur normally, see table 6.10 for the settings of the PCR. Each reaction contained 25 µl of the Master Mix (Table 6.11) and a sample of the colony. The colony sample was mixed with 5 µl water and sampled from here to produce three parallels. See Table 6.5 for solutions used in PCR analysis. After the samples were finished in the PCR they were stored at 4 ºC. To verify the PCR products, a 1% agarose gel was run as previously described in 6.2.2.

Table 6.9: Primers used in the PCR.

Primers	Sequence 5' → 3'
10881_seq_r_b4151	5-CACGTGGCCATTCCTGAATAGGA-3
T7	5-TAATACGACTCACTATAGGG-3

Table 6.10: Shows the setting of the PCR

PCR settings		
Initial denaturation	95 °C, 5 min.	1 cycle
Denaturation	95 °C, 30 sec.	30 cycles
Annealing	50 °C, 55 °C, 60 °C, 30 sec.	
Extension	72 °C, 1 min.	
Final extension	72 °C, 5 min.	1 cycle
Soak	4 °C, indefinite	1 cycle

Table 6.11: Reagents used in Master mix.

Reagents	Amount
5x Green Go Taq Buffer	5 µl
$MgCl_2$ (2.0 mM)	2 µl
dNTP (1.25 mM)	2 µl
Primer for T7 fwd (10 µM)	0.5 µl
Primer rev b4151 (10 µM)	0.5 µl
GoTaq DNA Polymerase (5 U/µl)	0.2 µl
Nuclease-free water	13.8 µl
Template DNA (<1000 ng)	1 µl

6.3.4 Preparation of bacteria stock

There were four colonies that contained the plasmid. These were used as stock in the following procedures. To produce bacterial stock, overnight cultures from the four colonies were prepared in 50 ml tubes. Each tube contained 5 ml LB medium (Table 6.12) and 25 µg/ml kanamycin. The tubes were incubated overnight in a horizontal shaker at 37 °C and 250 rpm. The following day, 850 µl of the overnight culture was mixed with 150 µl glycerol and the bacteria stock were frozen at -80 °C.

Table 6.12: Bacteria stock solutions:

Solutions	Content / recipe
LB medium (500 ml)	LB broth (Lennox)
	1 tablet + 50 ml ddH_2O
	Autoclave solution, store at 4 °C

6.3.5 Sample Induction

A small-scale production of proteins was performed first. To make sure that the culture has the optimal growth and expression condition for the protein of interest. A 24-well plate was used, the plate was divided into 2 sections, with 6 wells each (Figure 6.3). The bacteria stock from 6.3.3 was used to inoculate 5 ml LB medium. This culture was left overnight on the shaking incubator at 37 °C and 250 rpm. The next day, 100 µl of the overnight culture was mixed with 5 ml LB medium, which contained 25 µg/ml kanamycin. This mixture was added to three wells in the section below. These were incubated in horizontal shaker at 37 °C and 250 rpm for 6 hours. The wells were then divided in uninduced control and induced wells. They were induced with different concentrations of IPTG: 0.2 mM, 0.5 mM and 1 mM. The wells were sampled after 4 hours. The cultures were placed on ice for 10 minutes before they were harvested with centrifugation at 5000 G for 10 minutes at 4 °C. The supernatant was removed, and the cell pellets were stored at -20 °C.

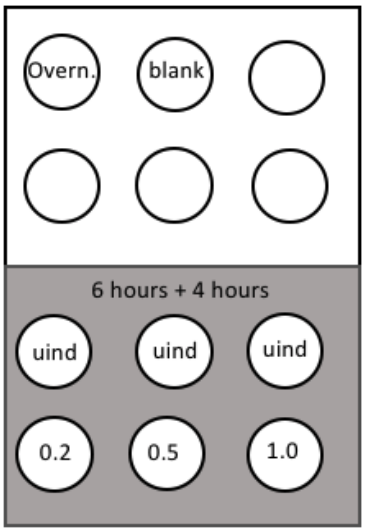


Figure 6.3: shows the induction plate.

The samples were analyzed with SDS-PAGE, using the Vertical Electrophoresis system with Stain-Free Precast Gels. Each sample was resuspended in 80 µl 1X SDS-PAGE loading buffer (premade) and heated for 5 minutes on 95 °C. The gel was loaded with 5 µl Precision plus protein dual standard ladder and the different samples. The gel ran for 40 minutes at 200 V. The bands were visualized with Gel Zoc EZ imager from BIO RAD

6.3.6 Large-scale production of harvesting cells

After confirming that the small-scale induction worked, a large-scale production was started. An overnight culture was made in a 50 ml tube with 10 ml LB medium with 25 µg/ml kanamycin. Using a pipette tip, the bacteria stock made in 6.3.3 was used to inoculate the LB medium. The solution was left to incubate overnight in a horizontal shaker **(**multitron standard) at 250 rpm and 37 °C.

The following day, 1500 ml LB medium with 25 µg/ml kanamycin was transferred to six 1000 ml Erlenmeyer flasks. The medium in each flask was pre-heated in a 37 °C water bath, before 5 ml of the overnight culture was added. The bacteria solutions were then incubated for

two hours in a horizontal shaker at 250 rpm and 37 °C. The optical density (OD) was measured during this time with a cell density meter (ultrospec 10 Amersham Biosciences). The instrument was first blanked by loading 2 ml of LB medium to a squared disposable cuvette. A 2 ml sample from the cell cultures were transferred to cuvettes and cell density measured. The OD is an approximation of the number of cells that are present in the culture. When OD was between 0.52 and 0.56, l mM IPTG (per beaker) was added. The cultures were further incubated for 4 hours.

After incubation, 500 µl from each culture was sampled. The samples were centrifuged at 4800 rpm for 30 seconds, the supernatant was removed and the tubes with the cell pellet were stored at -20 °C. SDS-PAGE analysis was performed the next day as described in section 6.3.5.

The remaining bacteria solution were transferred to 500 ml polypropylene bottles. The bottles were spun down, using the Avanti J-26 XP with the JA10 rotor at 6000 G for 15 minutes at 4 °C. The supernatant was removed from the flasks and the flasks with the cell pellet were stored at -20 °C overnight.

6.3.7 Purification of 6xHis-tagged proteins under denaturing conditions

The QIAexpress system is based on 6xHis tag, an affinity tag with six histidine residues. The use of such tags enables selective protein purification, since they have a high affinity for metal ions such as NI^{2+}. For fast his-tag purifications, Qiagen´s nickel-nitrilotriacetic acid (Ni-NTA) matrices were used. These are precharged with NI^{2+} and binds the 6xhis-tagged protein. With a low pH from the Elution buffer, the His-tagged protein can no longer bind to the nickel ions and will dissociate from the Ni-NTA resin in the columns.

The Ni-NTA Fast Start kit was used according to the supplier's recommendations, with a few modifications as follows; 40 mM imidazole was added to the Lysis buffer. Moreover, the supernatant ran through the columns twice to increase the amount of proteins to bind. The elution fractions were assessed by spectrophotometry (Nanodrop ND-1000), using the setting Protein A280. A 7 µl sample of each step was taken out and added 7 µl 2X SDS-PAGE sample buffer, the samples were stored at -20 °C while awaiting SDS-PAGE analysis. SDS-PAGE analysis was performed the next day as previously described.

6.3.8 Buffer exchange and concentration of protein

Thermo Scientific Pierce Protein Concentrators PES were used for protein retention. The tubes are divided in two parts with a polyethersulfone (PES) membrane. The membrane will prevent the proteins from passing through, based on their size. The old buffer will pass through, and new buffer can be added to the proteins.

The elution fractions from 6.3.6 were pooled and added to the 50 ml conical spin column. To maintain balance in the centrifuge there was an additional spin column with water. The tubes were centrifuged at room temperature in an Eppendorf Centrifuge 5804R (50 ml rotor) at 4500 G. The tubes were checked on regularly to see how fast the liquid passed through and to maintain balance in the centrifuge. The liquid that went through was discarded, and when the elution fraction had almost gone through, 10 ml PBS was added to the tube. This process was repeated three times for proper buffer exchange. Once most of the PBS had passed through the membrane, the protein concentration was assessed by spectrophotometry (Nanodrop ND-1000) using the setting Protein A280. The protein fraction was transported to NMBU Oslo where it was incorporated into an oil adjuvant vaccine. The concentration for the vaccine was 13.5 μg/ 0.1 ml.

6.4 Handling of fish

The fish that was used for the vaccine trial was Atlantic Salmon (*Salmo Salar*). The trial fish (Table 6.13) were kept in a common-garden, where they experienced identical environmental conditions. The fish were received as fry and were kept in a 3-meter, 7000 L tank at 12 °C, freshwater. The fish was tended to by the Industry laboratory (ILAB) in relation to the trial setup (Table 6.14). They were daily hand fed with commercial feed and maintained according to Norwegian animal welfare regulations.

Table 6.13: Details about the trial fish

Species	Atlantic Salmon (*Salmo Salar*)
Origin	ILAB
Batch no.	ILAB 16/500
Weight	Around 57 g at trial start
Number of fish	500

Feed	Commercial feed, 3 mm
Groups	Ctr (23 fish), recMesh (36 fish), pepMesh (29 fish)

Table 6.14: Overview of the environmental parameters and the timeline of the vaccine trial.
*The environmental parameters were logged on a daily basis by ILAB. *=Constant through the trial*

	Environmental parameters: Temperature, salinity, light, oxygen	Trial week
Climatizing	12 °C*, 0 ‰, 12:12, 70-100%*	0
Pit tagging	0 ‰, 12:12	3
Vaccination	0 ‰, 12:12	7
Boost vaccination	0 ‰, 12:12	13
New light regime 24:0	0 ‰, 24:0	14
Switched to saltwater	25 ‰, 24:0	19
Infection with copepodids	35 ‰, 24:0	21
Uninfected fish added	35 ‰, 24:0	22
Sampling of fish	35 ‰, 24:0	23

6.4.1 PIT-tagging of fish

The fish (on average 57 g) were individually marked with a 12 mm Pit Tag (i-Tag 162). They received the tag by needle injection into the abdomen. Prior to the tagging the fish had been starved and anesthetized. The fish was anesthetized with 60 mg/l benzocaine and 5 mg/l methomidate mixed with water (salinity 34.5 ppt and temperature 10±0.5 °C). The fish were also individually weighed and measured during the procedure. There were 24 fish chosen at random that were transferred to a smaller tank (500 l), these were later introduced back to the common garden once the fish were infected with copepodids and these had developed into the chalimus I stage.

6.4.2 Vaccination and boost vaccination of fish

Prior to vaccination, the fish (on average 80 g ± 30 g) were starved and bath anesthetized with 60 mg/l benzocaine and 5 mg/l methomidate (salinity 34.5 ppt and temperature 10±0.5 °C). Under the vaccination the fish were first identified by their pit tag. The fish were then measured, weighed and vaccinated. Each vaccine was given to 20 fish, before switching to a different vaccine. This was repeated throughout the vaccination. The vaccination was performed by intraperitoneal injection with a syringe (Figure 6.4). Once injected, the fish were transferred to a wakeup pool. They were returned to the common garden once all of them were vaccinated.

Figure 6.4: Intraperitoneal injection of vaccine Photo: Ingunn Wergeland

There were several groups in the vaccine trial, where three of the groups were part of this thesis. The NIDO recombinant (recMesh), which has been described in this thesis. The second group was the Mesh peptide (pepMesh) from the vaccine trial in 2016, to compare the two. The peptide Mesh vaccine was produced commercially. In addition, there was also a control group (ctr), which received the adjuvant mixed with 1X PBS instead of a vaccine. Each fish received a dosage of 100 µl.

The fish also received a second dosage of the vaccine they were registered with in December. With the exception of 12 fish in total (recMesh, pepMesh and control). It was not produced enough vaccine for these remaining fish to get the boost vaccination. The boost vaccination followed the same procedure as described above.

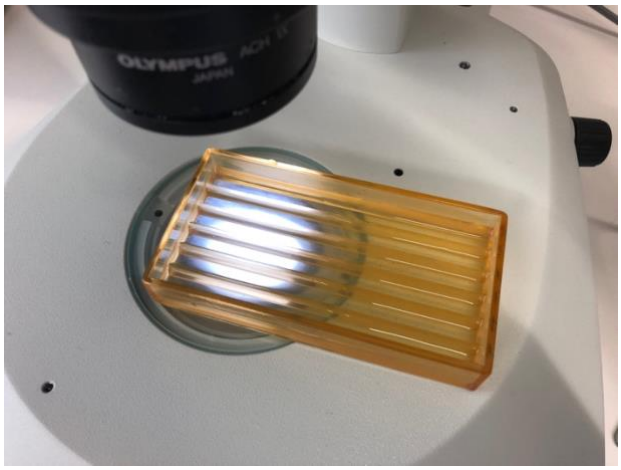
6.4.3 Infestation with copepodids

Prior to the infection, egg producers had been prepared in the wet lab. Their egg strings had been collected and incubated (Figure 6.5). Description of the incubation setup can be found in Hamre et al., 2009. Once the hatched egg strings developed into the copepodids stage they were prepared for infecting the fish in the common garden set-up.

Figure 6.5: Incubators (32 mm) used to hatch egg strings

The copepodids were transferred to a large jug. While constantly mixing, a sample from the known volume was transferred to a plankton counting chamber. The number of copepodids were counted with a stereo microscope (Figure 6.6). By using this method, it was possible to estimate the number of copepodids per ml. The 400 fish were infested with 60 copepodids/fish. The saltwater batch with copepodids was transferred to the common garden tank by slowly pouring them into the stream of the tank.

Figure 6.6: Plankton counting chamber used for counting copepodids



After one week, 24 non-infested fish were introduced to the common garden. In order for all the copepodids to develop to Chalimus I and be attached to their host. It is then possible to detect if *L. salmonis* transfer to a new host once they develop into the mobile stages.

6.4.5 Sampling

In order to keep individual fish and lice together, paper plates and small paper notes were prepared for each fish prior to the sampling. The fish were anesthetized and euthanized with a blow to the head once the lice were picked off. The sedation used for the fish was 60 mg/l benzocaine and 5 mg/l methomidate mixed with water (salinity 34.5 ppt and temperature 10±0.5 °C)

Figure 6.7: Shows how the lice were arranged on the paper notes. Photo: Ingunn Wergeland

Each louse was removed from the fish with tweezers and placed on the small paper notes. The note had different columns where the lice were placed based on stage and gender (Figure 6.7). Once all the lice were removed, blood samples were collected in vacutainers. The blood was allowed to clot on ice before being stored at 4 °C. The blood samples were centrifuged at 4500 G for 10 minutes the following day, and the serum were transferred to fresh tubes. The serum samples were stored at -20 °C until use.

Figure 6.8: Example of a pepMesh fish together with the PIT-tag reader, sampled lice and measurements for weight and length. Photo: Christiane Eichner

After the blood was taken from each fish the fish were registered by their pit tag, they were weighed and measured. Photos were taken of each individual fish and their lice count (Figure 6.8).

6.5 Antibody response

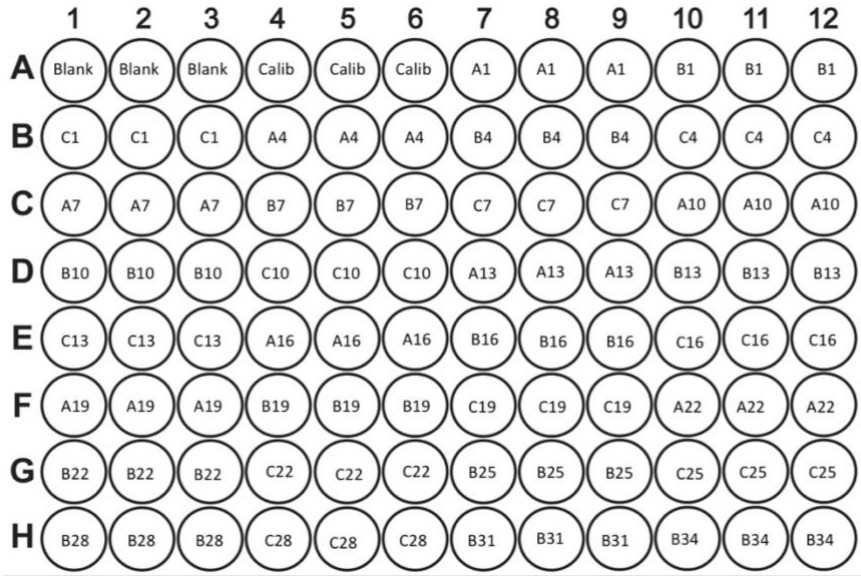
6.5.1 ELISA

Enzyme-linked immunosorbent assay (ELISA) is a plate-based method that can be used for detecting and quantifying antibodies. The plates in this method were coated with the same protein that was produced in 6.3.5. If there were antibodies present in the serum from the vaccinated fish, they would bind to the lice antigen. Each sample was run in triplicate and each plate had a blank sample and a calibrator (pool of samples with high absorbance values). The content of the reagents used in the procedure can viewed in Table 6.15.

Three ELISA plates were coated with 100 µl of salmon lice antigen (0.5 µg/µl) in each well and incubated overnight at 4 °C.

The next day, the plate was emptied and 250 µl washing buffer was added in each well. This was repeated three times. Once washed, the plates were banged against paper to remove residual liquid. The plates were added 250 µl blocking buffer to each well and incubated at room temperature for 2 hours.

Figure 6.9: Illustrates the setup for the one of the ELISA plates



While the plates were incubating, the serum-samples were organized by their vaccine groups, group A, B and C. From each serum sample, 8 µl was transferred to a new tube with 400 µl diluent buffer (1:50 dilution). The calibrator and blank samples were prepared together with the other samples. The setup for the plates were organized, so that sample A1, B2, C3 were together, the next plate had A2, B2, C2 and so forth until all the samples were divided between the three plates (Figure 6.9).

The plates were emptied and washed three times as described above. The plates were then added 100 µl triplicate of each sample as they were organized on the tray. When all the samples were added to the plates, the plates were left to incubate at 4 °C overnight.

The plates were emptied and washed three times as described earlier. Mouse anti-salmonid immunoglobulin 100 µl was added (1 µg/µl). This antibody is conjugated with an enzyme that can bind the substrate solution. The plates were left to incubate for an hour before washing three times as described earlier. The wells were added 100 µl of the substrate solution and the plates were incubated in the dark for 15 minutes. Subsequently each well was added 50 µl of stop solution and transferred to the Tecan microplate reader. The plates were analyzed at an absorbance of 492 nm.

Table 6.15: Reagents used in the ELISA procedure

Reagents:	Content:
Coating buffer (500ml)	Bicarbonate buffers (0.795 g Na_2CO_3 + 1.465 g $NaHCO_3$) Add distilled water up to a total of 500 ml
1X Phosphate buffered saline (PBS)	Phosphate buffered saline tablets 1 tablet/200 ml water.
Washing buffer (PBS/T)	500 ml 1X PBS 0.25 ml Tween 20 (0.05%)
Blocking buffer (5%)	4 g fat free dry milk 80 ml PBS/T
Diluent buffer (1%)	0.5 g fat free dry milk 50 ml PBS/T
Citrate phosphate buffer (1L)	10.30 g Citric Acid (monohydrate) 18.16 g Sodium Phosphate ($Na_2HPO_4 \bullet 2H_2O$) Dissolve salts in water, adjust to pH 5.0
Substrate solution	3 OPD tablets from Invitrogen were dissolved in 36 ml citrate phosphate buffer. Just before use: 15 µl 30% H_2O_2 was added.
Antibody solution	100 µg Mouse Anti-Salmonid immunoglobulin was restored with 100 µl deionized water.
Stop solution	27.5 ml of (95-97) 1M H_2SO_4 in 472.5 ml water.

6.6 Statistics

All statistical calculation was performed in Microsoft Excel 2018 or GraphPad Prism 7. A T-test was conducted to test the variance between the vaccine groups, and to see if there was a significant difference between them ($p < 0.05$).

7. Results

Before starting the production of the vaccine, the presence of Mesh transcripts in the intestine of *L. salmonis* was assessed both qualitative and quantitative, in order to see if the costs of a vaccine trial could be justified. The MESH protein was originally identified in the intestine of *Drosophila* (Izumi et al., 2012), and these analysis were done to confirm that the location was the same for *L. salmonis*

7.1 RNA isolation and real time PCR analysis of Mesh

The quantitative analysis of the lice samples containing gut only or whole lice with the gut removed, revealed a higher mRNA level of Mesh in the gut samples. While the samples with no gut had a low mRNA level of Mesh.

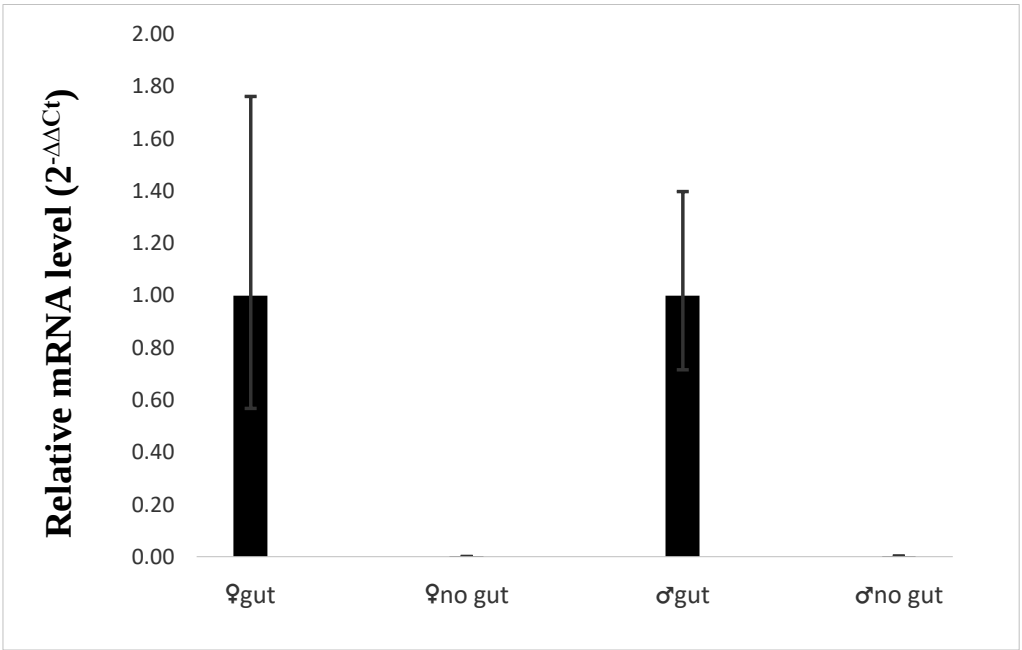


Figure 7.1: Female and male gut samples shows a higher mRNA level of Mesh. While the female and male samples without gut had a low mRNA level of Mesh.

7.2 *In situ* hybridization of Mesh.

The qualitative analysis used the DNase treated gut extractions as a template for cDNA synthesis. The cDNA was amplified with PCR and the products were used to produce the probes for *in situ* hybridization. The PCR products were visualized with 1% gel (Figure 7.2) and both the sense (FT7) and the antisense (RT7) PCR show one visible band of the expected size. After being synthesized into probes their labeling ability was assessed through a spot test (Figure 7.3) where the probes dilutions were positive, confirming that the substrate has bound to the anti-DIG antibodies.

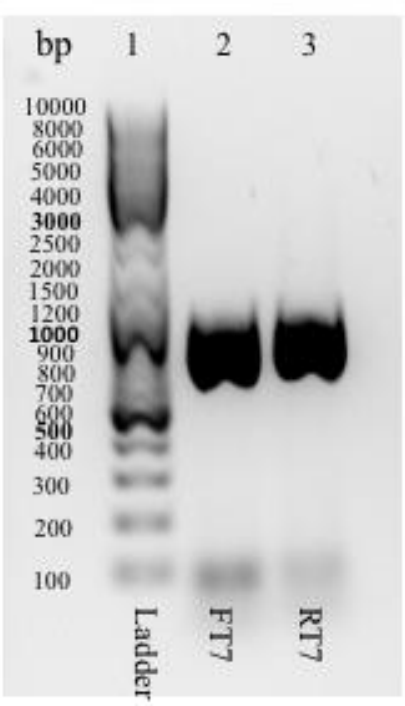


Figure 7.2: Bands from the PCR products (expected size 780 bp) used to produce the probes for in situ hybridization.

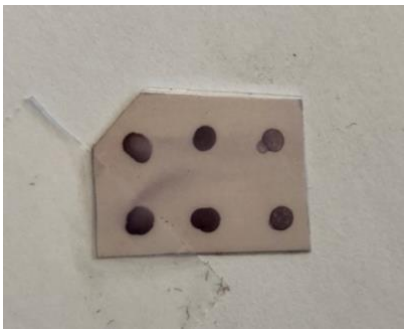


Figure 7.3: Spot test of sense (upper row) and antisense (bottom row) probes

After confirming the labelling of the sense and antisense probes, they were used in the *in situ* hybridization. The localization of Mesh transcripts on sections of adult female *L. salmonis* showed that the target mRNA is localized to the louse gut (Figure 7.4). The Mesh antisense probe was positive and had bound the gut epithelium. The sense probe was negative and had no specific binding (Figure 7.5).

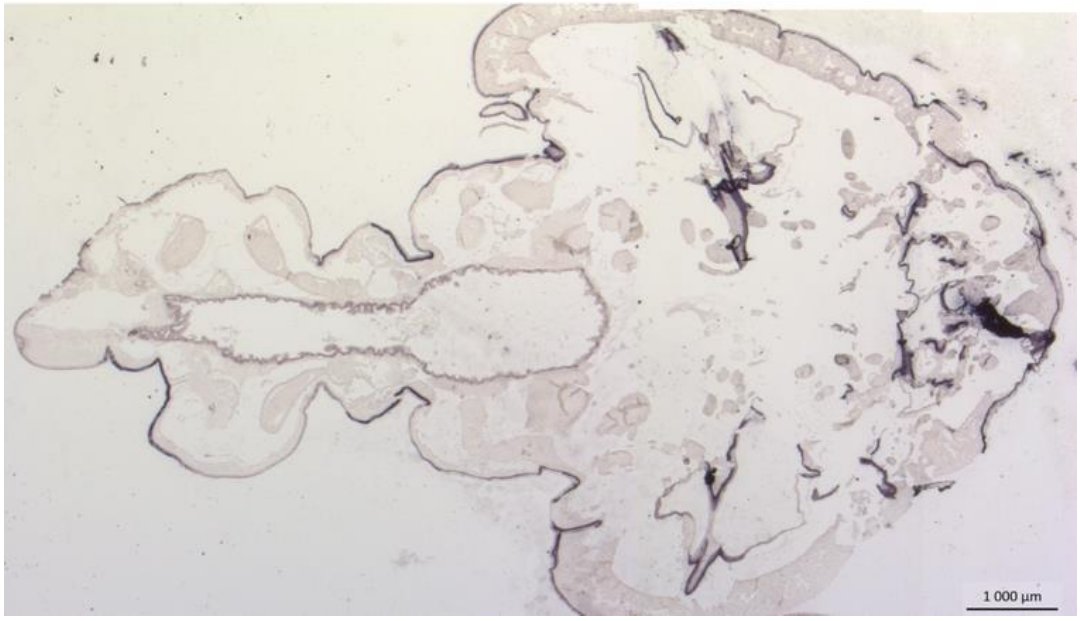


Figure 7.4: Expression of Mesh, antisense RNA probe. The probe is positive and shows staining around the gut. Scalebar 1000 µm.

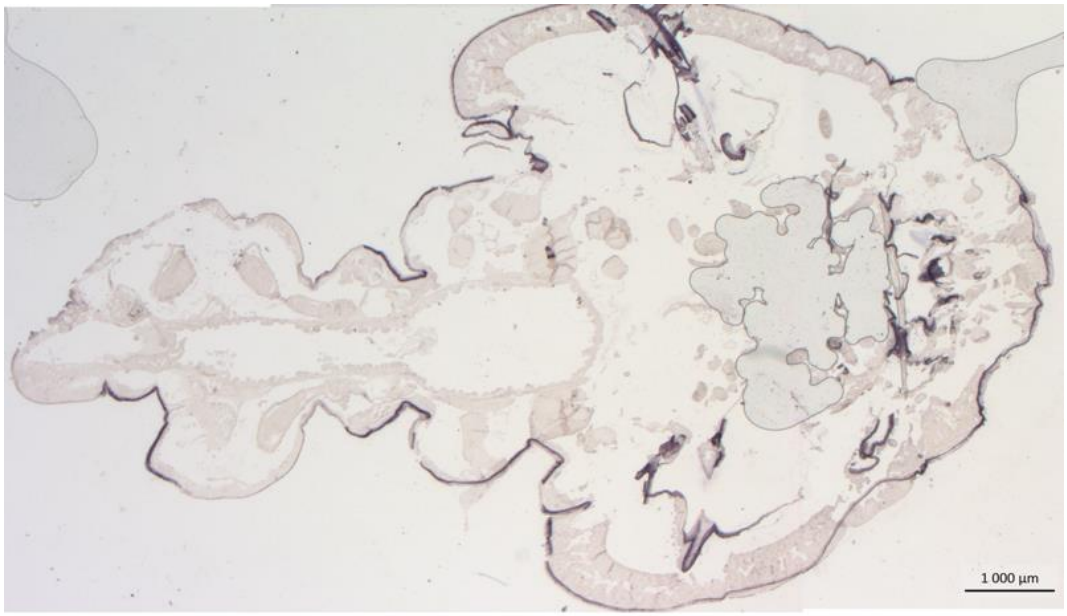


Figure 7.5: Expression of Mesh, sense RNA probe. The sense probe is negative with no specific binding. Scalebar 1000 µm.

The expression of LsTrypsin in the gut was used as the control. Where the sense RNA probe was used as a negative control (Figure 7.6) and the antisense probe (Figure 7.7) was used as the positive control. The negative control did not bind to any specific mRNA, while the positive control visualized the target mRNA with a bright stain around the gut.

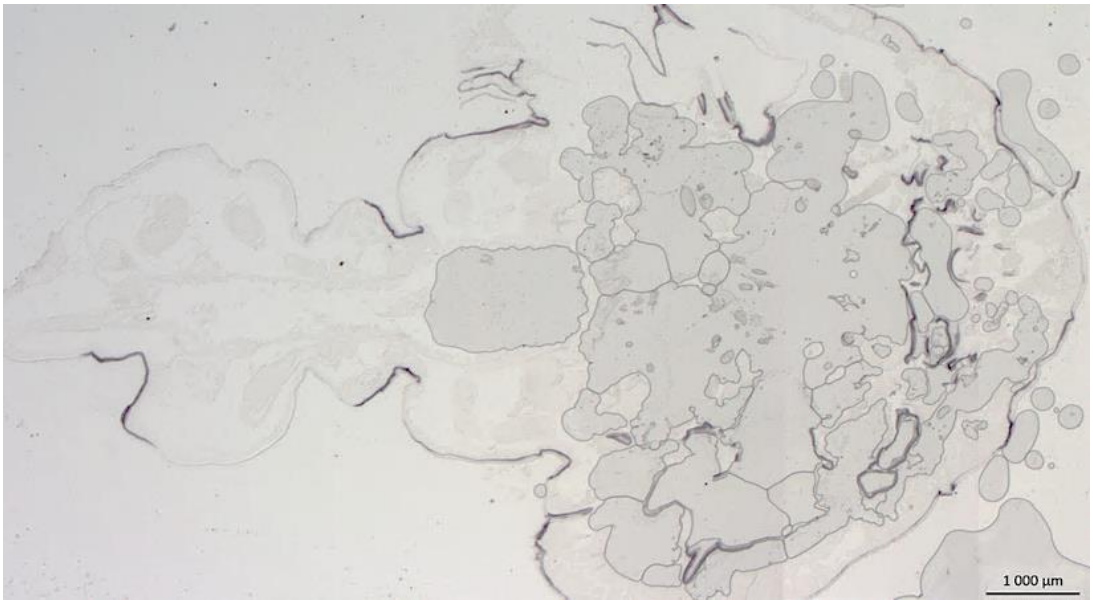


Figure 7.6: Expression of LsTrypsin, sense RNA probe. The sense probe is the negative control and has not bound to any specific mRNA. Scalebar 1000 µm.

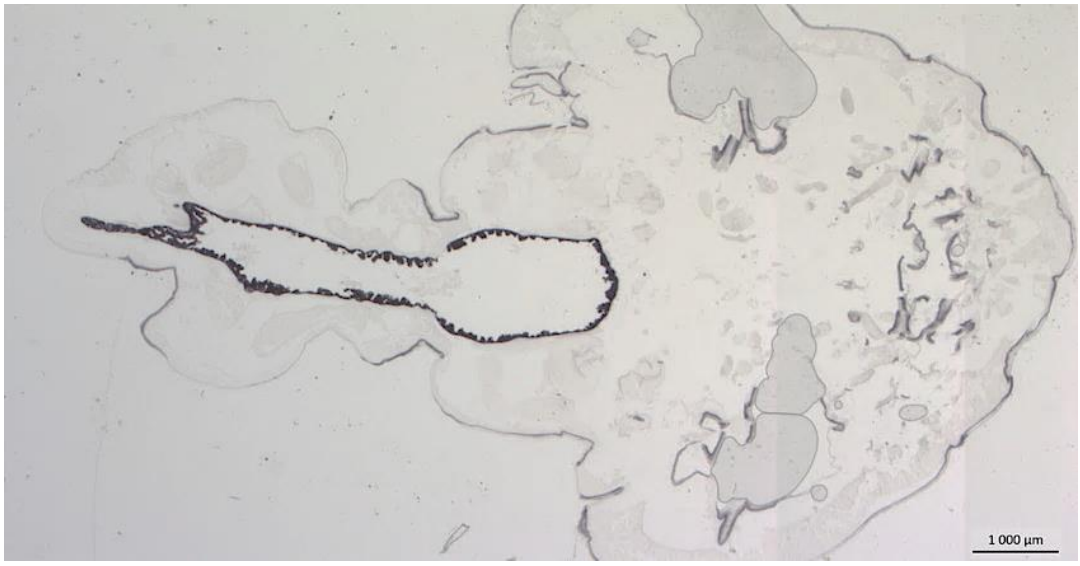


Figure 7.7: Expression of LsTrypsin, antisense RNA probe. The probe is a positive control and has visualized its complementary mRNA with a bright blue stain around the gut. Scalebar 1000 µm.

The color reaction between the antibodies enzyme and the substrate (Figure 7.4) is not as bright as with the LsTrypsin (Figure 7.7), but the LsMesh probe clearly gives a signal (Figure 7.8).

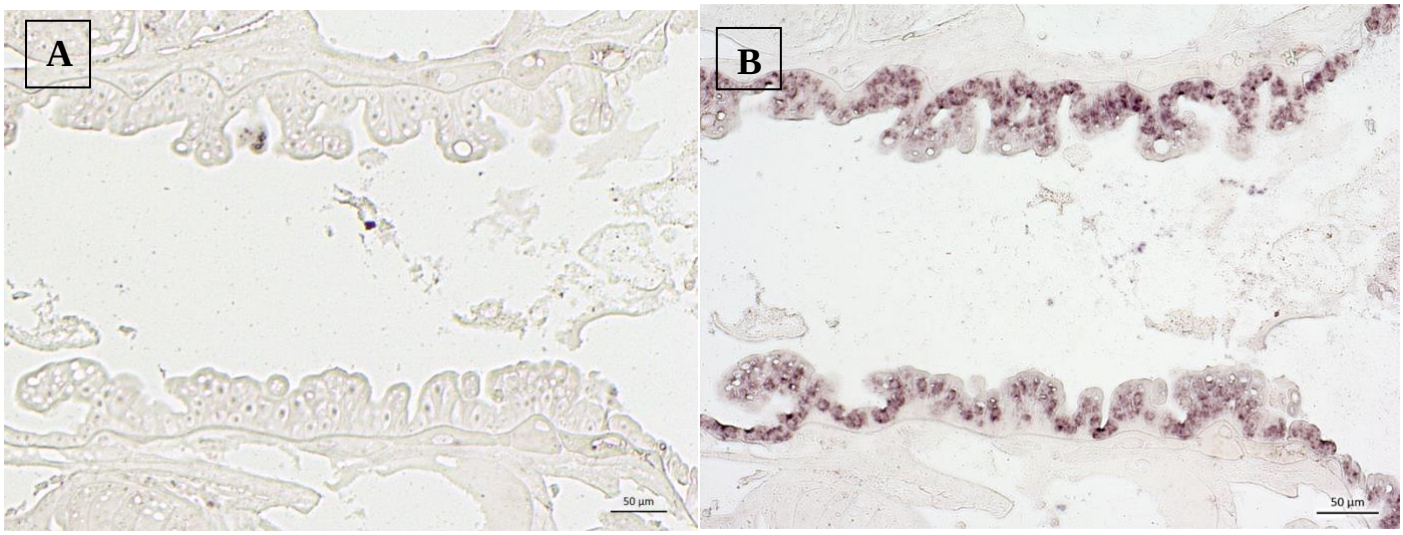


Figure 7.8: Comparison of the hybridization of mRNA Mesh in the gut, sense (Figure A) and antisense (Figure B) RNA probe. Scalebar 50 µm.

The same probes were also used to analyze the presence and localization of Mesh transcripts on sections with copepodids. Similar to the analysis in adults, the sense probe (Figure 7.9) did not bind to any specific mRNA, while the antisense probe has identified target mRNA localized to the gut.

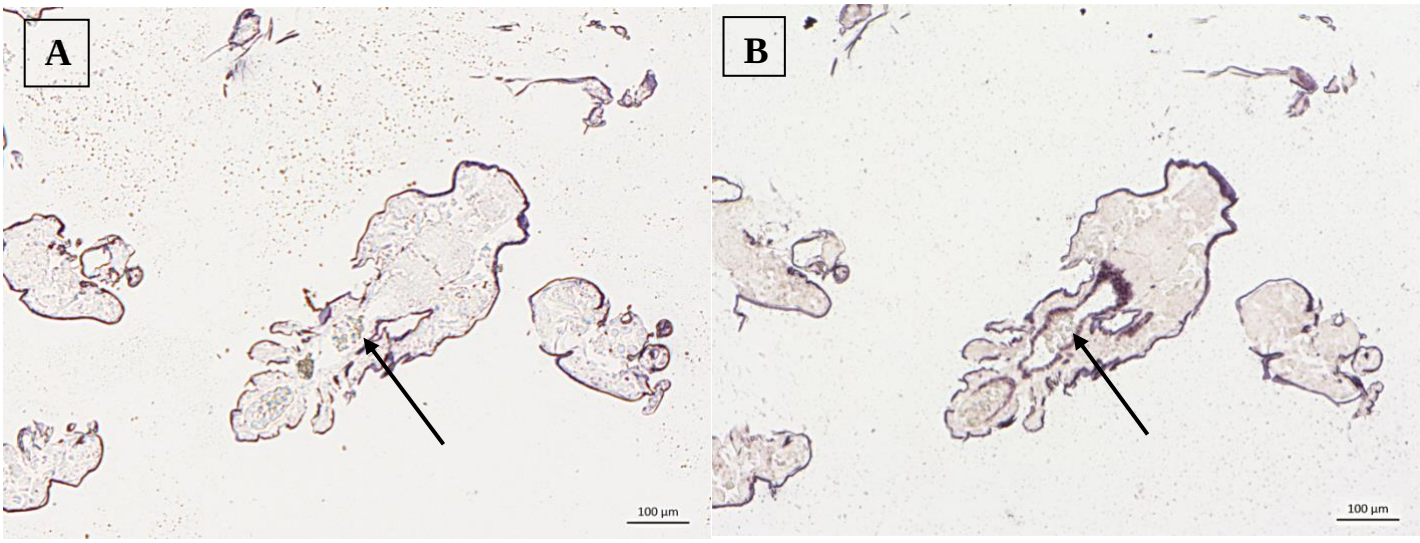


Figure 7.9: Expression of Mesh mRNA in the gut, sense (Figure A) and antisense (Figure B) RNA probe. The antisense probe has bound to its complementary mRNA. Gut indicated by arrow. Scalebar 100 µm

7.3 HES Staining

After confirming the Mesh transcripts in the lice, a different staining method was used on adjacent sections to assess the positive tissue and to confirm that it was gut epithelium. The section of the adult female was stained with Hematoxylin eosin saffron (HES) staining, excluding the extra straining step with saffron. Using previously published descriptions of the gut, (Nylund et al., 1992), the tissue was found to be gut (Figure 7.10 and 7.11).

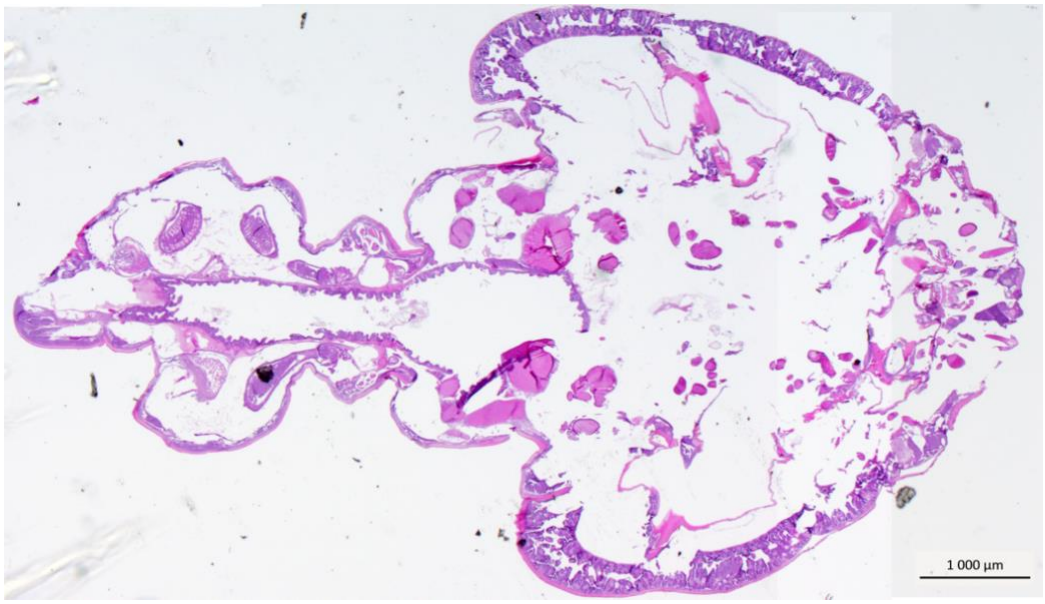


Figure 7.10: Overview of the adult female lice. Scalebar indicates 1000 µm.

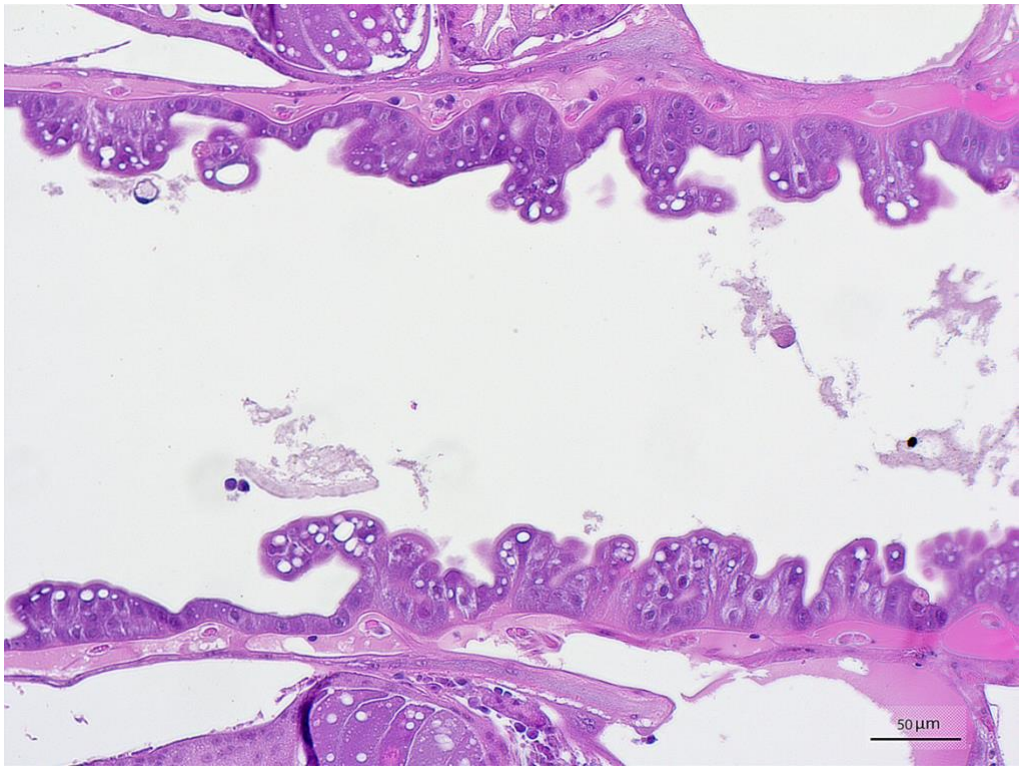


Figure 7.11: HES stained L. salmonis gut. Epithelium in the gut is visible. Scalebar indicates 50 μm

7.4 Selecting bacteria stock

Having confirmed the presence of the Mesh transcripts in the gut, the production of recombinant Mesh protein for the vaccine was started. BL21 cells was transformed with the expression vector, and PCR was used to confirm that the bacteria contained the NIDO insert. There were 4 colonies that contained the insert, Figure 7.12. These colonies were used further to produce bacteria stock for protein production.

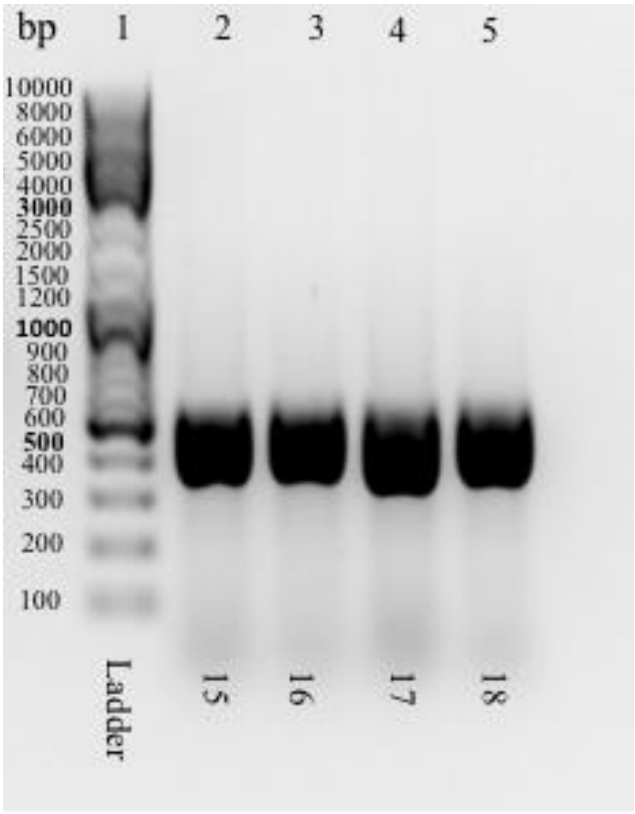


Figure 7.12: The gel shows PCR products from four bacterial colonies all containing the insert (expected size 524 bp).

7.5 Small scale production of the protein

A small-scale production was performed to make sure that the bacterial culture received the optimal growth and expression conditions for the recombinant protein. Different concentrations of IPTG was tested, but as figure 7.13 shows there was no difference in the amount of protein produced. The highest concentration of IPTG, 1mM was used for the large-scale protein production.

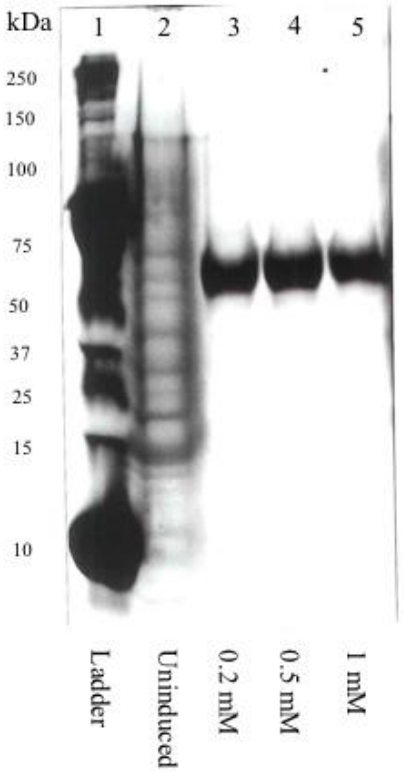


Figure 7.13: Tested different concentration of IPTG, all of them estimated to express the recombinant protein of the correct size of 65 kDa.

7.6 Purification of 6xHis-tagged proteins under denaturing condition

After adjustment of growth conditions, large-scale production of the protein was initiated. The bacteria cell pellets that were produced, were purified under denaturing conditions. Samples were taken from each step in the purification process and were analyzed with SDS-page (Fig. 7.14), to identify the recombinant proteins present. Six Ni-NTA matrices were used in total for the purification process all showing similar results (only one of the gels are shown Fig. 7.14). The gels revealed that most of the recombinant protein was eluted in Elution 2 and 3, but there was also protein present in Elution 4. The Elution fractions were measured with nanodrop, an overview of the concentration in relation to the bacteria cultures OD is presented in table 7.1 (Elution 1 is not presented, as no protein was present).

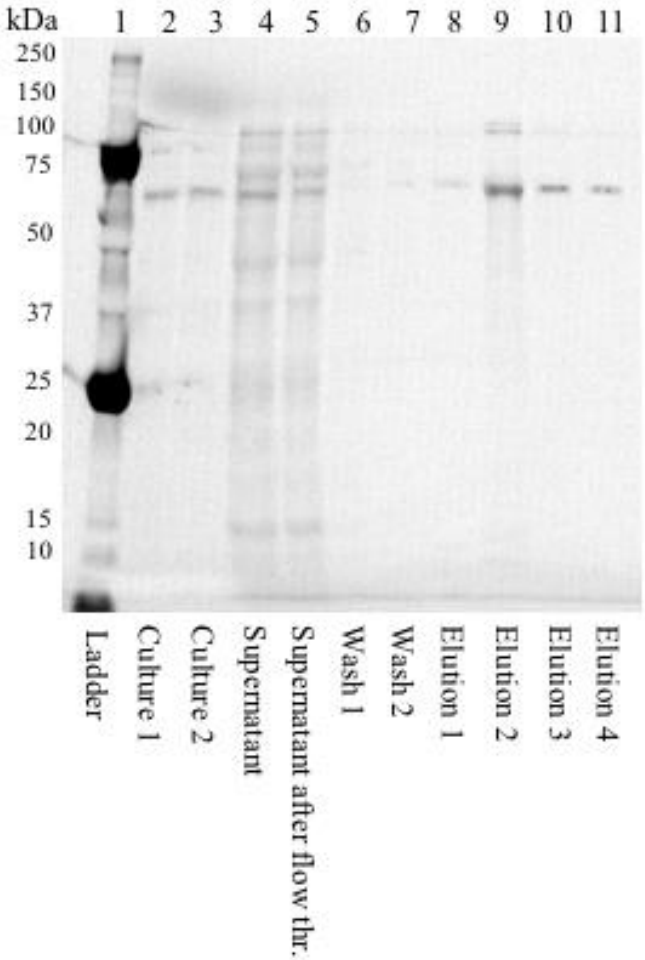


Figure 7.14: SDS-page analysis of samples from two bacteria cultures and the Ni-NTA matrices. Expected size of the recombinant protein 65 kDa.

*Table 7.1: OD in relation to concentration of purified protein. *=mg/ml*

Matrices	Elution 2*	Elution 3*	Elution 4*	OD
1	0.65	0.45	0.05	0.56
2	0.43	0.35	0.10	0.56
3	1.00	0.13	0.03	0.52
4	0.56	0.39	0.06	0.52
5	0.45	0.30	0.05	0.54
6	1.83	0.33	0.03	0.51

7.7 Vaccine trial

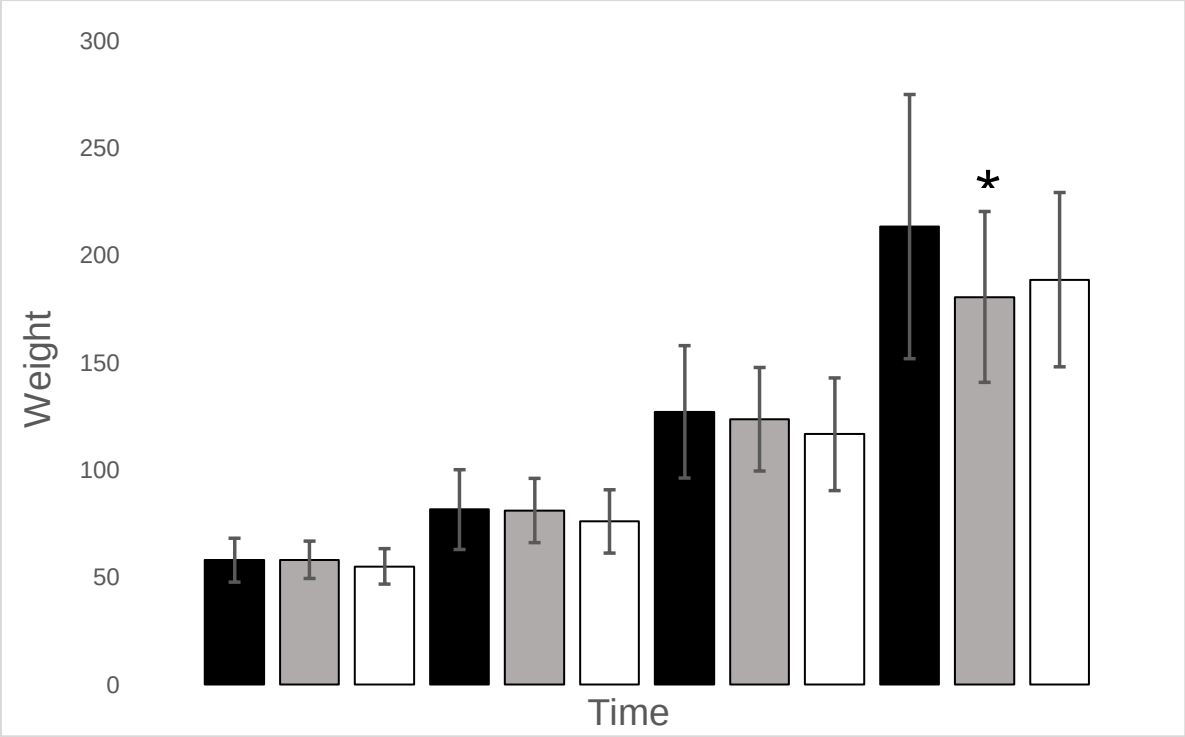
The recombinant protein was incorporated into an oil adjuvant vaccine. The recombinant Mesh (recMesh), peptide Mesh and control vaccine that contained the adjuvant mixed with 1X PBS were administered to 88 Atlantic salmon. The 2016 trial was repeated this year to see if there would be a different result if the fish received a boost vaccination. The previous trial was similar to this trial, but Table 7.2 sums up the differences.

During the vaccine trial no mortality was observed, and the fish had only minor damage due to grazing. The fish developed in a normal manner and all of them had smoltified at sampling. A T-test was done to see if there were any significant differences between the groups in length and weight. At the time of vaccination all fish groups were similar in weight and length, but at sampling there was a significant difference between control and the recMesh ($p < 0.05$) fish in weight. Similarly, the control group had a higher average length, but the difference was not significant (Figure 7.15 and 7.16). The groups were measured at specific timepoints, an overview of the average weight and length of the fishes are presented in Table 7.3. After boost vaccination the data has been divided into individuals with boost and individuals without boost.

Table 7.2: Differences between the 2016 and 2017 trial:

	Vaccine trial 2016	Vaccine trial 2017
Production of protein	Elanco, Canada	UiB, Norway
Incorporation of protein into vaccine	Elanco, Canada	NMBU, Norway
Vaccine groups	Ctr, recMesh, pepMesh	Ctr, recMesh, pepMesh
Number of fish in each group	29 (ctr), 20 (recMesh), 26 (pepMesh)	Ctr (23 fish), recMESH (36 fish), pepMESH (29 fish)
Adjuvant	Elanco's own adjuvant	MONTANIDE™ ISA 763 A VG from Seppic (non-mineral oil)
Vaccine boost	No	Yes

Concentration	recMesh: 55 μg/0.1ml pepMesh: 400 μg/0.1ml	recMesh: 13.5 μg/0.1ml pepMesh: 400 μg/0.1ml
Vaccine dosage	100 μl per fish	100 μl per fish



*The figure 7.15: Average weight development of the fish. Black=ctr, Grey=recMesh, White=pepMesh. *= p <0.05*

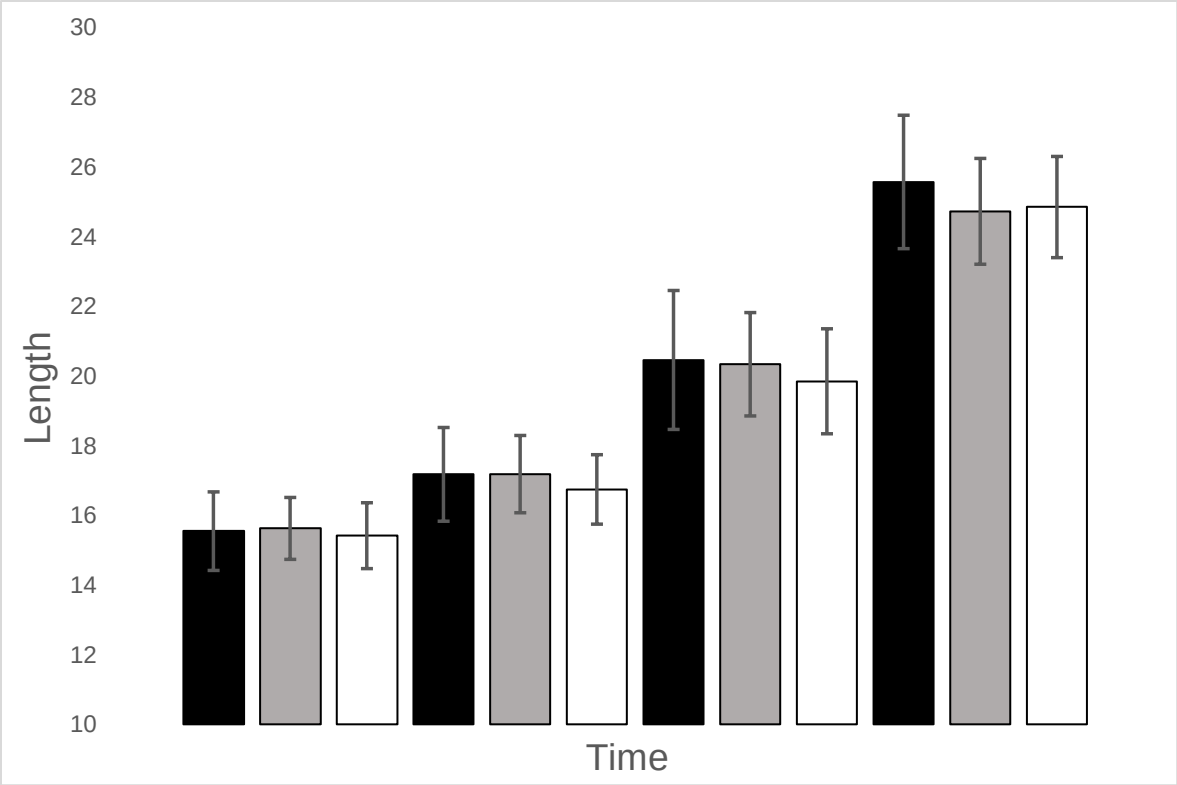


Figure 7.16: Average length development of the fish. Black=ctr, Grey=recMesh, White=pepMesh

*Table 7.3: Average weight and length for the groups, at the different measuring points. *= 1 fish did not receive boost in the control group. **= 5 fish did not receive boost in the recombinant Mesh group. ***= 6 fish did not receive boost in the peptide Mesh group.*

Group	**Ctr**		**recMesh**		**pepMesh**	
PIT-tag	57.91 g	15.54 cm	58.09 g	15.62 cm	55.03 g	15.41 cm
Vaccination	81.47 g	17.17 cm	81.05 g	17.18 cm	75.96 g	16.74 cm
Boost Vaccination	128.92 g	20.59 cm	123.10 g	20.27 cm	119.11 g	20.00 cm
No boost	85 g *	17.5 cm *	126.36 g **	20.7 cm **	106.8 g ***	19.25 cm ***
Sampling no boost	193.7 g *	25 cm *	173.28 g **	24.9 cm **	212.4 g ***	25.00 cm ***
Sampling boost	214.20 g	25.59 cm	181.71 g	24.69 cm	182.37 g	24.80 cm

The vaccine trial was ended once the lice had developed into chalimus 2 and preadult 1, but there were some males that had developed into preadult 2. All of the fish had lice, including the 24 non-infested fish that were introduced back to the common garden after a week. All visible lice were removed from the fish, and the fish was registered with the stages and gender of the lice. The lice were present in these life stages: Chalimus 1, Chalimus 2, Preadult 1 male, Preadult 1 female and Preadult 2 male. There was only 1 Chalimus 1 present, and 3 Preadult 2 males. The Preadult 1 stage was on average dominated by males, where all the groups showed a higher count in the presence of males (Table 7.4).

Table 7.4: Average life stage count of L. salmonis

Group	ch1	ch2	p1m	p1f	p2m	Tot lice
Ctr	0	4.78	18.57	12.17	0.09	35.61
recMesh	0.03	3.36	17.81	11.31	0.03	32.53
pepMesh	0	3.62	18.59	11.59	0	33.79

The previous trial (Figure 7.17) uncovered that there was a mean difference between the groups, where the recMesh had a lower number of lice compared to the other groups. But the T-test showed that there was no significant difference between the groups. The 2017 trial (Figure 7.18) showed a similar result, and no significant difference was uncovered in the lice numbers.

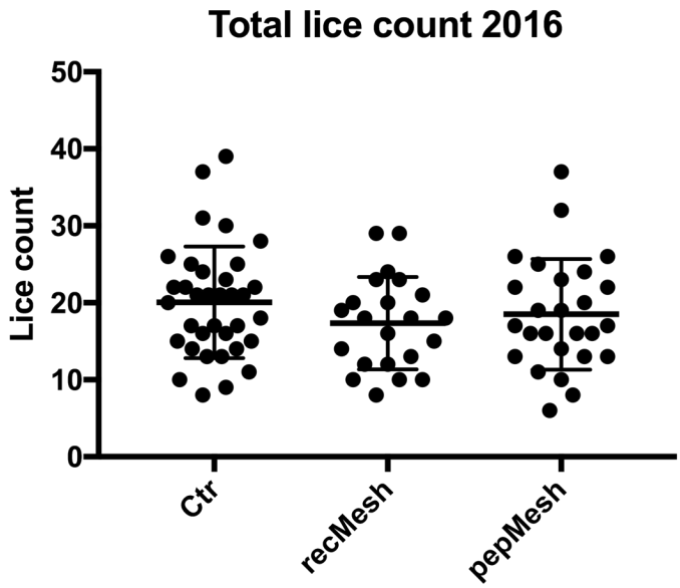


Figure 7.17 total lice count in vaccine trial 2016 for each of the groups.

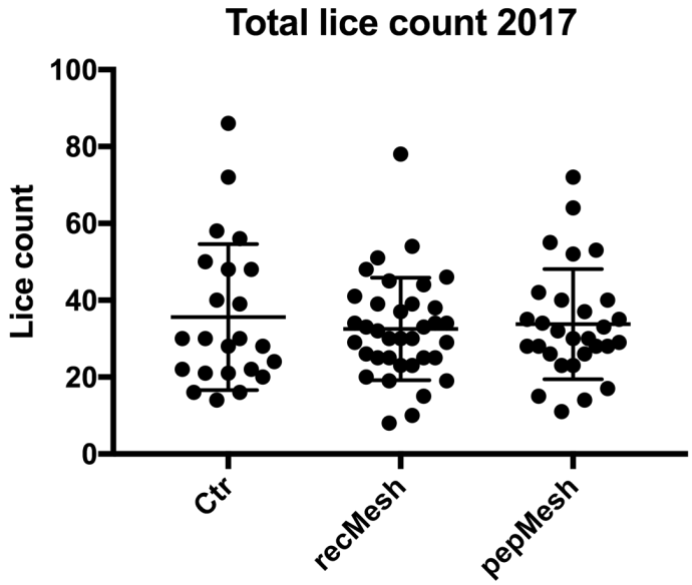


Figure 7.18: total lice count in vaccine trial 2017 for each of the groups.

7.8 ELISA

To assess the efficacy of the vaccines, we evaluated the immune response after immunization and challenge infection with lice, where ELISA was used to measure the antibody level in serum. The previous vaccine trial (Figure 7.19) uncovered that there was a mean difference between the groups, where recMesh had elevated antibody levels. While the ctr and pepMesh group had little antibody response. The T-test showed a significant difference between ctr and recMesh ($p < 0.05$). But no significant difference was detected between ctr and pepMesh. The analysis of the 2017 samples (Figure 7.20) revealed a similar response, with elevated antibody levels in the recMesh group. However, there were 5 individuals in this group that showed a low response, but the remaining fish in the group showed a high response. The control group and the peptide Mesh group showed little antibody response. A significant difference was detected between ctr and recMesh ($p < 0.05$). But no significant difference was detected between ctr and pepMesh. By comparing the antibody response in the two trials, its visible that the response from the 2017 trial is more dispersed than the 2016 trial. In relation to the lice numbers (Figure 7.21) it does not seem to be any correlation between the lice numbers and the elevated antibody response.

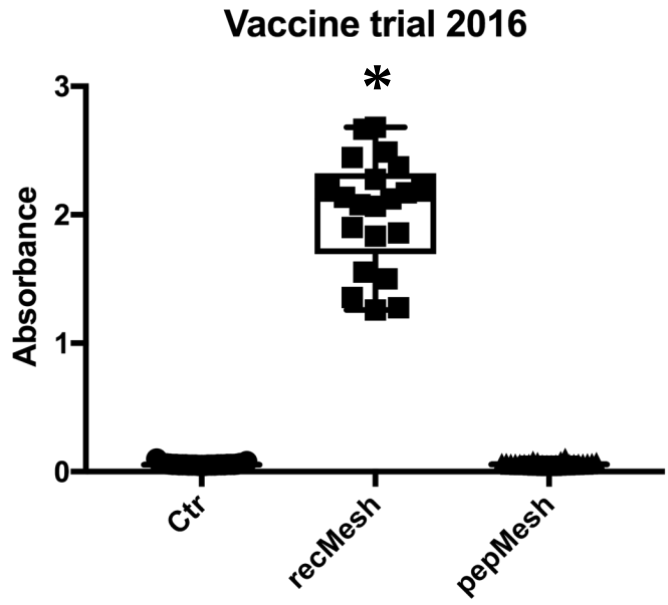


*Figure 7.19: Immune response after 2016 vaccine trial. Where the recMesh group shows the highest antibody response. *= p <0.05*

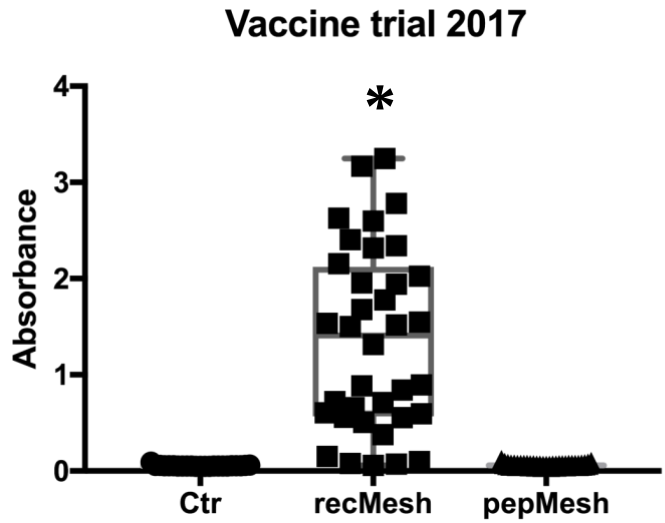


*Figure 7.20: Immune response after 2017 vaccine trial. Where the recMesh group shows the highest antibody response. *= p <0.05*

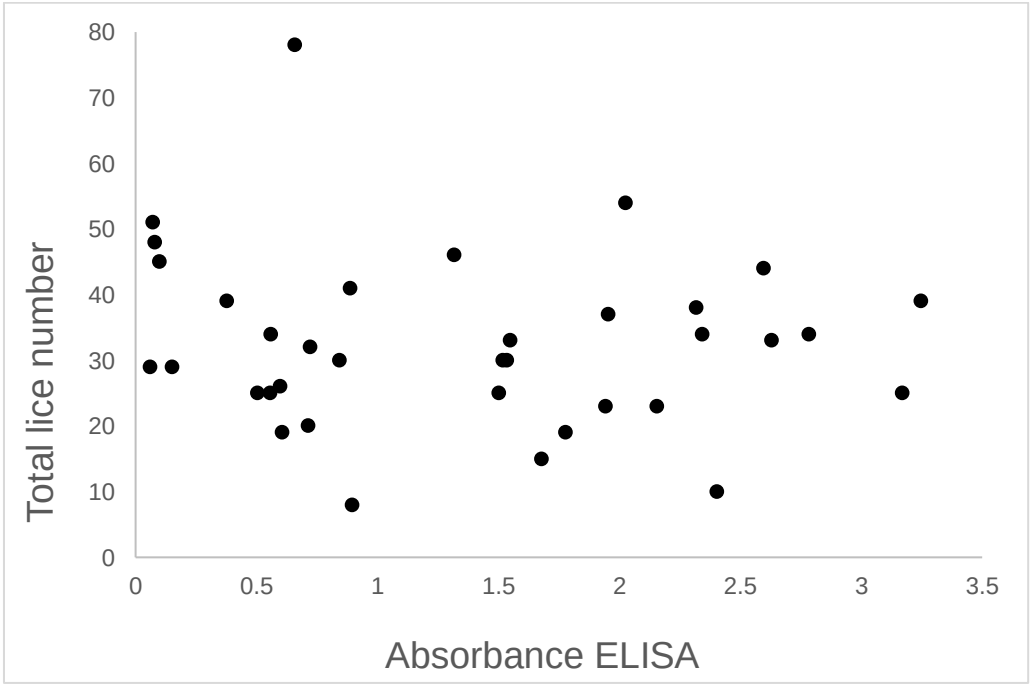


Figure 7.21: Total lice number of the recMesh group in relation to antibody response.

8. Discussion

8.1 Literature search on promising candidates

The aim of this study was to produce and test a vaccine candidate against *L. salmonis*. by expression of the recombinant Mesh protein and testing its efficacy in protecting the Atlantic salmon from *L. salmonis*. The Mesh protein was identified in 2012 by Izumi et al. (2012), where it was proposed that Mesh together with Ssk and TsP2A is required for the formation and organization of septate junctions in the gut of *Drosophila* (Izumi et al., 2016). The SLRC selected this candidate gene due to its location in the gut. Previous work in the SLRC tested the significance of the gene through RNA interference (RNAi) where the production of the Mesh protein was inhibited through denaturation of the mRNA. This resulted in a deformed gut in the lice and mortality. This result indicated the protein is significant for the lice and a vaccine trial was conducted in 2016. As mentioned earlier, this vaccine trial was similar to the one conducted in this thesis. But the trial was repeated to see if a boost vaccination would give a higher antibody response and a reduction in lice numbers.

8.2 Location of the transcripts

Before starting the production of the vaccine, the presence of Mesh proteins in the intestine of *L. salmonis* was assessed with real time RT-PCR and *in situ* hybridization. This in order to see if to confirm that the location was the same as in *Drosophila* and that the costs of a second vaccine trial could be justified.

We used the SYBR Green assay for the real time RT-PCR. A problem connected to this assay is that it can bind to nonspecific dsDNA products and give an overestimation of the target product. This problem was avoided by using DNase treated total RNA and melt curve analysis. The result from the real time RT-PCR indicated a large variation in the expression level of the gut samples. This could be due to the method used for the removal of the gut in the lice samples. A scalpel knife was used, and it is likely that more than gut tissue was included in these samples to a variable degree for each sample.

The *in situ* hybridization analysis revealed that the Mesh antisense probes were positive and localized their target mRNA. The transcripts were localized in the gut, as for *Drosophila*, which makes it a potential vaccine target. A previous attempt with *in situ* hybridization did

not manage to localize the transcripts. This is a low expressed gene and in this new attempt we optimized to get a successful attempt. The probes concentration was increased, and the slides were allowed to incubate with the processing solution for 48 hours, where the solution was changed after 24 hours. The colour reaction was not as bright as the control, but it is a visible colour change.

HES staining was used as a control to make sure that the tissue where the transcripts were localized was gut epithelium. The Saffron step was excluded due to costs and this staining step stains collagen fibres in connective tissue, which was not one of the target tissues. The staining was less blue than it should have been. This was due to the stock solution of Hematoxylin, which did no longer stain satisfyingly. There were no neighbour sections left to do a better HES stain, nevertheless, the tissue containing the transcripts were confirmed as gut epithelium.

8.3 Production of the recombinant protein

The goal in the production of recombinant proteins was to obtain high-quality pure protein samples, which in turn can produce high affinity antibodies once administered to the host. The major challenge with this method was finding the optimal growth conditions for the culture, and in turn optimal expression of the protein of interest. The optimal OD is normally 0.4-0.6, but it seemed like the ideal OD for high protein expression was 0.5. When the bacteria culture was induced with IPTG at OD 0.51 there was a higher concentration of the protein in the eluate with a concentration of 1.83 mg/ml. A problem occurred after the purification of the protein, with regards to the buffer exchange. Despite a high concentration of protein in the eluate, the protein precipitated when the buffer was changed to PBS lacking urea. As a consequence of this, it was hard to measure the concentration of the protein. We assumed that the concentration was higher than what was stated through the nanodrop measurements. Due to some of the challenges regarding the production of the vaccine and time limitations the concentration of the recombinant vaccine in 2017 was lower than the one in 2016. It is possible that a different buffer could have avoided this problem, but it needs to be a buffer that one can inject in fish.

8.4 Analysis of the trial

The vaccine was delivered to the fish through an intraperitoneal injection. The fish were starved in advance which reduces the risk for wrongly administering vaccine into the intestine. It also prevents remaining feed from lingering in the intestine and causing inflammation. It would have been an advantage to administer the vaccine to the fish with an automatic repeating syringe connected to a small vaccine bottle instead of an ordinary syringe. It would have been easier to control that the correct dosage is administered. But in both cases the size and length of the needle would have needed to be optimized to the size of the fish. We had some variation in fish size, ranging from 54-123 g at the vaccination and 75-186 g at the boost vaccination. The needle length was shortened by adding a stopper onto the needle (Figure 6.4 in methods). This prevented the needle for penetrating too deep, as it should only penetrate the abdominal wall (3-5 mm) (Lillehaug, 2014). Ideally, the fish should have been sorted based on the size before vaccination. This would have allowed the correct needle size to be used depending on the weight of the fish. This was not possible to accomplish in this pilot vaccine trial due to limited space and lack of equipment.

Without an internal control of the vaccination, there is a risk of administering the vaccine incorrectly. The recommended injection site is in the mid-line of the abdomen, one fin length anterior to the pelvic fin base (Lillehaug, 2014). Using the wrong angle or a too short needle can result in deposition of the vaccine in musculature. Injecting the vaccine too high or too low can result in deposition of the vaccine in the pyloric caeca, in the intestine or in the cartilage. Which in turn could give side effects in the fish or no effect since the vaccine is secreted with the feces.

The weight development of the fish indicated a significant difference in the weight of the recMesh group at sampling. But due to the set-up of the vaccine trial it is not possible to prove an effect. The groups seemed to separate in growth after vaccination. This could be due to short term side effects of the vaccine. After vaccination the fish can have a period of reduced appetite and lower growth (Berg et al., 2007). It is likely that the control fish had no loss of appetite and continued to grow. With a higher number of trial fish, it would have been possible to better assess this difference. A higher number of fish could also have included fish for internal control of the vaccination. But in trial experiments one also has to consider the ethical aspects where the number of trial fish should be kept at a minimum.

By comparing the vaccine trial in 2016 and 2017, the result is similar where the analysis of the fish serum revealed an elevated antibody response in the recMesh group. The control and pepMesh did not show a significant response. The 2016 trial had an elevated antibody response in all fish, whereas the 2017 trial had a more dispersed antibody response. Where 5 individuals showed the lowest response, being at a similar level to the control and the peptide group. These individuals included both fish which had or had not received boost. Low antibody response was thus not connected to non-boosted fish. It is possible that the concentration of the recMesh vaccine caused the dispersed response. In 2016 Elanco was responsible for the production of the recombinant protein, and incorporation of the vaccine. While for the 2017 trial we received the pET26b vector with the NIDO domain and were responsible for the production of the protein. The incorporation of the vaccine was done by a partner of SLRC. As previously mentioned a lot of work was put into finding optimal growth conditions and to achieve a high concentration of the protein. Due to time limitations, the concentration of the recombinant vaccine in 2017 was lower than in the 2016 trial. The 2017 trial had some individuals with a higher antibody response than in 2016. This could be due to the boost vaccination, as there was no boost vaccination in the 2016 trial.

The elevated antibody response was not reflected in the lice numbers. There was no significant difference in the lice numbers although there was a mean difference, it is therefore necessary to look at different explanations for this outcome. The gut of the salmon louse would be the ideal immunological target for a vaccine, due to the knowledge that the lice feeds of its host and the significance of gut in survival. As of now the nature and physiology of digestion in the *L. salmonis* is not fully understood, but there are two articles describing the intestine of the louse (Bron et al., 1993; Nylund et al., 1992). For the gut antigen approach to work, it is necessary to know more about digestion in the louse, and to compare it to the digestive physiology in mammalian parasites (Raynard et al., 2002). The ticks feeding and digestion is more researched, and studies have confirmed their blood meal digestion as a slow intracellular process (Tarnowski and Coons, 1989). Due to their consumption of large amounts of blood and digesting the blood over a slow period, it is likely for the ticks to accumulate a significant concentration of antibodies. The diet of *L. salmonis* in contrast consists of epithelial, mucous and blood cells. Where the adult female incorporates a larger proportion of blood into its meal (Brandal P.O. et al., 1976; Fast, 2014). It is not known how long the blood persist in the intestine of the louse and the overall amount of blood ingested in the salmon lice is lower than in ticks, relative to body size (Raynard et al., 2002). It is possible

that the antibodies taken up in the gut of the lice have a lower ability to target antigen, in comparison to tick, due to the belief that fish lack antibody affinity maturation (Magor, 2015).

Another possibility is that the antigen we have used in this trial is not an ideal vaccine candidate. For the vaccine to work it is necessary that the binding seat of the protein is available on the surface of the gut. As mentioned in the introduction, the molecular architecture and mechanisms of functional regulations in the septate junctions are not fully understood (Furuse and Izumi, 2017). We know that Mesh is involved in cell-cell adhesion in the gut epithelium, but it is not certain that the proteins are present on the surface of the gut and thus it is not possible for the antibodies to bind to the antigen.

9. Conclusion

In this study, the presence of Mesh transcript in the intestine was confirmed through real time RT-PCR and *in situ* hybridization. A recombinant protein including the NIDO domain from Mesh was produced and incorporated into a vaccine. A vaccine trial with an infection experiment was conducted. The trial revealed an elevated immune response in the salmon, but the vaccine did not protect against *L. salmonis*. Further studies are needed to determine if the lack of protection is due to the concentration of the vaccine, low concentration of high affinity antibodies in the louse, unavailability of binding seat or an unknown cause.

10. Future studies

In order for a clear indication of vaccinating Atlantic salmon with recombinant Mesh protein can protect against *L. salmonis*, a single fish tank trial should have been included. This method prevents lice from jumping between hosts and would have allowed a more detailed analysis of the lice development. The lice could have developed into adults, as adults eat more blood it is possible that the antibody response in the fish could have been different over time and that the adult lice could have accumulated a higher antibody concentration due to blood consumption. The egg strings could have been collected and incubated to see if they would have hatched in a normal manner. In addition to this, one could have conducted a re-infection trial to see if the hatched copepodids were able to survive and infect a host. These results will reveal if the antigen candidate has potential or if research should be focused elsewhere.

11. References

Aaen, S.M., Helgesen, K.O., Bakke, M.J., Kaur, K., Horsberg, T.E., 2015. Drug resistance in sea lice: a threat to salmonid aquaculture. Trends in Parasitology 31, 72–81.

Alexandersen, S., 1996. Advantages and disadvantages of using live vaccines risks and control measures. Acta Vet Scand Suppl 90, 89–100.

Asplin, L., Johnsen, I.A., Sandvik, A.D., Albretsen, J., Sundfjord, V., Aure, J., Boxaspen, K.K., 2014. Dispersion of salmon lice in the Hardangerfjord. Marine Biology Research 10, 216–225.

Audun Iversen, Øystein Hermansen, Otto Andreassen, Ruth Kongsvik Brandvik, Anders Marthinussen, Ragnar Nystøyl, 2015. Kostnadsdrivere i lakseoppdrett. Nofima. Page 37

Banerjee, S., Sousa, A.D., Bhat, M.A., 2006. Organization and function of septate junctions: an evolutionary perspective. Cell Biochem. Biophys. 46, 65–77.

Berg, A., Fjelldal, P.G., Hansen, T., 2007. Bivirkninger av vaksinasjon. Kyst og havbruk 161–163.

Brandal P.O., Egidius E., Romslo I., 1976. Host blood: A major food component for the parasitic copepod Lepeophtheirus salmonis Krøyer, 1838 (Crustacea: Caligdae). Norwegian Journal of zoology 24, 341–343.

Bron, J.E., Sommerville, C., Jones, M., Rae, G.H., 1991. The settlement and attachment of early stages of the salmon louse, Lepeophtheirus salmonis (Copepoda: Caligidae) on the salmon host, Salmo salar. J. Zool. 224, 201–212.

Bron, J., Sommerville, C., Rae, G., 1993. The Functional-Morphology of the Alimentary Canal of Larval Stages of the Parasitic Copepod Lepeophtheirus-Salmonis. J. Zool. 230, 207–220.

Browman, H.I., Boxaspen, K., Kuhn, P., 2004. The effect of light on the settlement of the salmon louse, Lepeophtheirus salmonis, on Atlantic salmon, Salmo salar L. J. Fish Dis. 27, 701–708.

Brudeseth, B.E., Wiulsrød, R., Fredriksen, B.N., Lindmo, K., Løkling, K.-E., Bordevik, M., Steine, N., Klevan, A., Gravningen, K., 2013. Status and future perspectives of vaccines for industrialised fin-fish farming. Fish Shellfish Immunol. 35, 1759–1768.

Brudeseth, B.E., 2002. Bruk av DNA-vaksiner mot virussykdommer hos fisk. Genialt.

Bruno, D.W., Noguera, P.A., Poppe, T.T., 2013. A Colour Atlas of Salmonid Diseases. Springer Netherlands, Dordrecht. Costello, M.J., 2006. Ecology of sea lice parasitic on farmed and wild fish. Trends Parasitol. 22, 475–483.

Bui, S., Dempster, T.D., Remen, M., Oppedal, F., 2016. Effect of ectoparasite infestation density and life-history stages on the swimming performance of Atlantic salmon Salmo salar. 387-395.

Costello, M.J., 2006. Ecology of sea lice parasitic on farmed and wild fish. Trends in Parasitology 22, 475–483.

Costello, M.J., 2009. The global economic cost of sea lice to the salmonid farming industry. Journal of Fish Diseases 32, 115–118.

Costello, M.J., 2009. How sea lice from salmon farms may cause wild salmonid declines in Europe and North America and be a threat to fishes elsewhere. Proceedings of the Royal Society of London B: Biological Sciences 276, 3385–3394.

Denholm, I., Devine, G.J., Horsberg, T.E., Sevatdal, S., Fallang, A., Nolan, D.V., Powell, R., 2002. Analysis and management of resistance to chemotherapeutants in salmon lice, Lepeophtheirus salmonis (Copepoda: Caligidae). Pest Management Science 58, 528–536.

Fast, M.D., 2014. Fish immune responses to parasitic copepod (namely sea lice) infection. Developmental & Comparative Immunology, Immunity to infectious diseases of fish 43, 300–312.

Frost, P., Nilsen, F., 2003. Validation of reference genes for transcription profiling in the salmon louse, Lepeophtheirus salmonis, by quantitative real-time PCR. Veterinary Parasitology 118, 169–174.

Furuse, M., Izumi, Y., 2017. Molecular dissection of smooth septate junctions: understanding their roles in arthropod physiology: Smooth septate junction-associated proteins. Annals of the New York Academy of Sciences 1397, 17–24.

Gonzalez-Alanis, P., Wright, G.M., Johnson, S.C., Burka, J.F., 2001. Frontal Filament Morphogenesis in the Salmon Louse Lepeophtheirus salmonis. Journal of Parasitology 87, 561–574.

Gudding, R., 2014. Vaccination as a Preventive Measure, in: Fish Vaccination. Wiley-Blackwell, pp. 12–21.

Hamre, L.A., Eichner, C., Caipang, C.M.A., Dalvin, S.T., Bron, J.E., Nilsen, F., Boxshall, G., Skern-Mauritzen, R., 2013. The Salmon Louse Lepeophtheirus salmonis (Copepoda: Caligidae) Life Cycle Has Only Two Chalimus Stages.

Hamre, L.A., Glover, K.A., Nilsen, F., 2009. Establishment and characterisation of salmon louse (Lepeophtheirus salmonis (Krøyer 1837)) laboratory strains. Parasitology International 58, 451–460.

Izumi, Y., Motoishi, M., Furuse, K., Furuse, M., 2016. A tetraspanin regulates septate junction formation in Drosophila midgut. J. Cell. Sci. 129, 1155–1164.

Izumi, Y., Yanagihashi, Y., Furuse, M., 2012. A novel protein complex, Mesh–Ssk, is required for septate junction formation in the Drosophila midgut. J Cell Sci 125, 4923–4933.

Johnson, S.C., Albright, L.J., 1991. The developmental stages of *Lepeophtheirus salmonis* (Krøyer, 1837) (Copepoda: Caligidae). Canadian Journal of Zoology 69, 929–950.

Johnson, S.C., Treasurer, J.W., Bravo, S., Nagasawa, K., Kabata, Z., 2004. A review of the impact of parasitic copepods on marine aquaculture. Zool. Stud. 43, 229–243.

Komisarczuk, A.Z., Grotmol, S., Nilsen, F., 2017. Ionotropic receptors signal host recognition in the salmon louse (Lepeophtheirus salmonis, Copepoda). PLoS One 12.

Lillehaug, A., 2014. Vaccination Strategies and Procedures, in: Fish Vaccination. Wiley-Blackwell, pp. 140–152.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.

Magor, B.G., 2015. Antibody Affinity Maturation in Fishes—Our Current Understanding. Biology (Basel) 4, 512–524.

Mordue Luntz, A.J., Birkett, M.A., 2009. A review of host finding behaviour in the parasitic sea louse, Lepeophtheirus salmonis (Caligidae: Copepoda). Journal of Fish Diseases 32, 3–13.

Munang'andu, H.M., Mutoloki, S., Evensen, Ø., 2014. Non-replicating Vaccines, in: Fish Vaccination. Wiley-Blackwell, pp. 22–32.

Nordi, G.A., Simonsen, K., Patursson, O., 2016. A method of estimating in situ salmon louse nauplii production at fish farms. Aquac. Environ. Interact. 8, 397–405.

Nylund, A., Økland, S., Bjørknes, B., 1992. Anatomy and Ultrastructure of the Alimentary Canal in Lepeophtheirus Salmonis (Copepoda: Siphonostomatoida). J Crustacean Biol 12, 423–437.

Parizi, L.F., Pohl, P.C., Masuda, A., da Silva Vaz Junior, I., 2009. New approaches toward anti-Rhipicephalus (Boophilus) microplus tick vaccine. Brazilian Journal of Veterinary Parasitology 18, 1–7.

Parizi, L.F., Reck, J., Oldiges, D.P., Guizzo, M.G., Seixas, A., Logullo, C., de Oliveira, P.L., Termignoni, C., Martins, J.R., da Silva Vaz, I., 2012. Multi-antigenic vaccine against the cattle tick Rhipicephalus (Boophilus) microplus: A field evaluation. Vaccine 30, 6912–6917.

Pike, A.W., Wadsworth, S.L., 1999. Sealice on Salmonids: Their Biology and Control, in: Baker, J.R., Muller, R., Rollinson, D. (Eds.), Advances in Parasitology. Academic Press, pp. 233–337.

R. M. Serra-Llinares, P.A. Bjørn, B. Finstad, R. Nilsen, A. Harbitz, M. Berg, L. Asplin, 2014. Salmon lice infection in wild salmonids in marine protected areas. Aquaculture Environment Interactions Vol. 5: 1–16, 2014.

R. P. Lee, J. P. Opdebeeck, 1991. Isolation of protective antigens from the gut of Boophilus microplus using monoclonal antibodies. Immunology 72, 121–126.

Raynard, R.S., Bricknell, I.R., Billingsley, P.F., Nisbet, A.J., Vigneau, A., Sommerville, C., 2002. Development of vaccines against sea lice. Pest Manag. Sci. 58, 569–575.

Resnik-Docampo, M., Sauer, V., Schinaman, J.M., Clark, R.I., Walker, D.W., Jones, D.L., 2018. Keeping it tight: The relationship between bacterial dysbiosis, septate junctions, and the intestinal barrier in Drosophila. Fly 12, 34–40.

Samsing, F., Oppedal, F., Dalvin, S., Johnsen, I., Vågseth, T., Dempster, T., 2016. Salmon lice (Lepeophtheirus salmonis) development times, body size, and reproductive outputs follow universal models of temperature dependence. Can. J. Fish. Aquat. Sci. 73, 1841–1851.

Samuelsen, O.B., 2008. Miljøeffekter av lakselusmidler. Kyst og havbruk 153–156.

Schram, T.A., 2000. The egg string attachment mechanism in salmon lice Lepeophtheirus salmonis (Copepoda: Caligidae). Contributions to Zoology 69, 21–29.

Skern-Mauritzen, R., Torrissen, O., Glover, K.A., 2014. Pacific and Atlantic Lepeophtheirus salmonis (Krøyer, 1838) are allopatric subspecies: Lepeophtheirus salmonis salmonis and L. salmonis oncorhynchi subspecies novo. BMC Genetics 15, 32.

Sommerset, I., Krossøy, B., Biering, E., Frost, P., 2005. Vaccines for fish in aquaculture. Expert Rev Vaccines 4, 89–101.

Tarnowski, B.I., Coons, L.B., 1989. Ultrastructure of the midgut and blood meal digestion in the adult tick *Dermacentor variabilis*. Exp Appl Acarol 6, 263–289.

Todd, C., J. Stevenson, R., Reinardy, H., G. Ritchie, M., 2005. Polyandry in an ectoparasitic copepod (Lepeophtheirus salmonis Krøyer) despite complex precopulatory and postcopulatory mate guarding. Marine Ecology-Progress Series 303, 225–234.

Ugelvik, M.S., Skorping, A., Moberg, O., Mennerat, A., 2017. Evolution of virulence under intensive farming: salmon lice increase skin lesions and reduce host growth in salmon farms. Journal of Evolutionary Biology 30, 1136–1142.

Willadsen, P., Bird, P., Cobon, G.S., Hungerford, J., 1995. Commercialisation of a recombinant vaccine against Boophilus microplus. Parasitology 110 Suppl, S43-50.