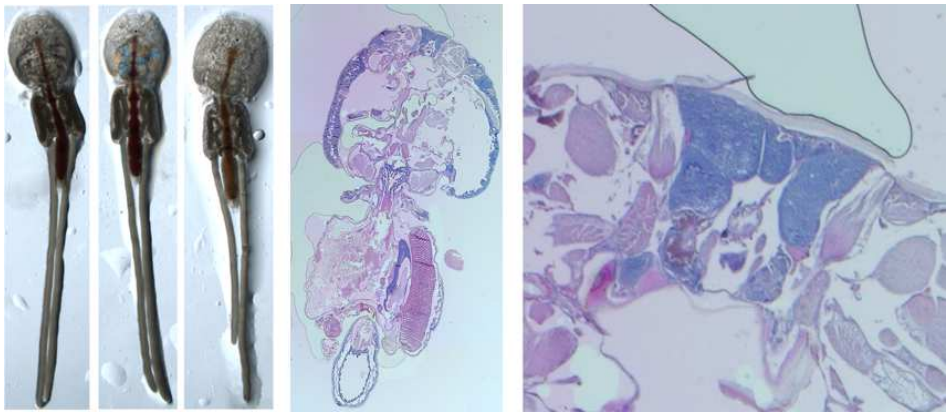
Characterization of Some Kunitz Domain Containing Genes – Possible Link to Salivary Gland



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Sérgio Rocha

Abstract

As a hematophagous parasite, anticoagulants are crucial for *L. salmonis*. In hematophagous animals specific anticoagulants are produced by salivary gland in order to keep the blood liquid and to allow the parasite to process it properly. Such proteins are unknown in *L. salmonis* as well as its site of expression. At the same time the function of the salivary gland as production of anticoagulant factors has not been confirmed in *L. salmonis*.

Genes with Kunitz domain are typically proteinase inhibitors and some are involved in anticoagulation. They are present in *L. salmonis* but with unknown function and site of expression. This studied demonstrated the presence of two salivary gland specific genes belonging to the Kunitz family and other highly expressed in the intestine.

The silencing of these genes did not give any distinct phenotypes in adults or larvae stages. The present study could not conclude if the three investigated genes are involved in anticoagulation in the salmon louse. However, the lack of detectable phenotypes in the RNAi experiments indicates that could be other compensating molecules in the lice for the processes that LsKunitz1-3 are involved in.

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1. Introduction

1.1 *L.salmonis* in aquaculture

Norway is the leading producer of salmonids. Its breakthrough was during the 70's and the production of Atlantic salmon makes Norway the second largest exporter, exporting more farmed than wild catch fish (FAO, 2011).

Salmon is exposed to several water conditions and interactions with other wild animals such as some parasites. The interaction with parasites can damage the fish in the cage, leading to possible economic losses. According to Costello (2009), *Lepeophtheirus salmonis* is responsible for commercial losses above 1.5 billions NOK in the northern hemisphere and between 200 and 500 million NOK in Norway (Hanssen and Ditlefsen, 2012).

There is obvious interference between farmed fish with the wild stocks (Heuch et al., 2005; Torrisen et al., 2013). *L. salmonis* is a natural occurring parasite of wild salmon in sea water but the conditions at intensive farming are more propitious to parasite growth, leading to problem in farmed salmon and eventually to wild stock too. Lice can attach to any part of the host body. At adult stage it is found more often in the head and operculum regions. (Costello, 2006). After the attachment, louse hold itself using the second pair of antennae and maxillipeds and then it rasps the skin of the host using the mouthparts in order to remove mucus, skin, and underlying tissues (Costello, 2006). This grazing leads to epithelium loss, bleeding, increased mucus discharge, altered mucus biochemistry, tissue necrosis and consequent loss of physical and microbial protective function (Johnson et al., 2004). The same author also observed a reduction in appetite, growth and food conservation efficiency in the host. Furthermore, stress and exposure of wounds leads to secondary infections (Costello, 1993). Changes in the host blood composition are also observed, such as reduced lymphocytes and proteins, host anemia, reduced ion balance and elevated cortisol (Johnson et al., 2004). Then, it was observed reduced osmoregulatory and respiratory ability, impaired immunocompetence and even influence in swimming performance (Wagner and McKinley, 2004).

1.2. Biology of *L. salmonis*

L. salmonis is an obligate ectoparasite, belonging to the subphylum Crustacea, subclass Copepoda, order Siphonostomatoida, family Caligidae and genus *Lepeophtheirus*. Sea louse has bilateral symmetry, hard exoskeleton, segmentation and jointed appendages. It is consider to be host specific on Salmonidae, contrasting for instance with *Caligus elongates*, which has been found in more than 70 different host species (Kabata, 1979). The same author proposed that specificity of *L. salmonis* is due to its nutritional requirements and/or the capacity to cope with the innate immunological defense mechanism of other species.

1.3. Sea lice life cycle

L. salmonis has eight developmental stages in their life cycle, consisting of two nauplius, one copepodid, two chalimus, two pre-adult and one adult stage (Fig.1.1) (Schram, 1993; Hamre et al. 2013). Each stage is separated by ecdysis and its growth rate is temperature and salinity dependent. The time from the hatching until mature adult male is 40 days and 52 days for females lice at a temperature of 10 ºC (Johnson, 1991).

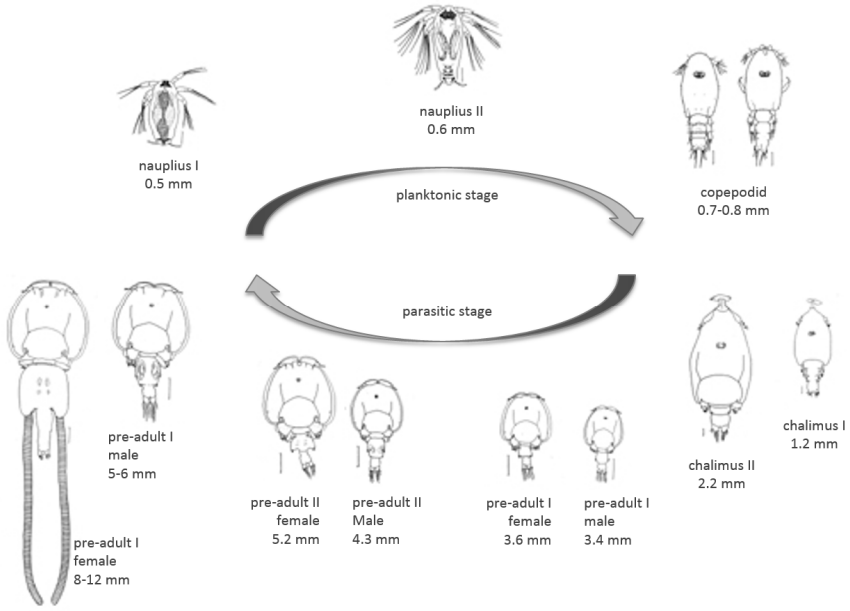


Fig. 1.1 - The life-cycle of sea lice. Illustration by T. A. Schram, 1993; adapted according with Hamre et al 2013; Scales bars: Nauplius – chalimus: 0,1 mm, Pre adult and adult: 1m; Illustration by T. A. Schram, 1993; adaptation according Hamre, L. et al 2013

Hatching occurs directly from the eggstrings while they are attached to the female louse. Both nauplius I and II are free-living larvae and they are not prepared to infect the host, using the yolk as energy source. It is at the copepodid stage the lice can attach to the host via second antenna and stay there until adult stage, if the conditions are satisfactory (Costello, 2006; Costello 1993).

Later, copepodid moults into chalimus I which are attached to the host by the frontal filament (Pike and Wadsworth, 1999) and later to chalimus II. At this stage they are sessile and they feed on the skin of the fish, around the point of attachment (Costello, 2006).

The last three stages are the pre-adult I and II and adults. They are mobile and attached to the host by the second antenna. They are able to move on the host surface where they feed. After all these stages, they reach sexual maturation and become adult with completely developed genital segments (Johnson and Albright, 1991).

1.4 Sea lice control

Different methods and compounds have been used to control the salmon lice. The development of lice resistance has increased the difficulty to achieve a proper medical treatment. The high host density in the cages facilitates the horizontal transmission.

Since 2013 the Norwegian Government declared a new legal limit to the presence of lice on the salmon. "*Luseforskriften*" states that a treatment is required at all time when there is more than an average of 0.5 adult female lice per fish. It also says that *Mattilsynet* can set their own limits for lice in specific zones and grant permission for a higher limit for lice for broodstock in the last six months at the sea (§8 of *Forskrift om bekjempelse av lakselus i akvakulturanlegg*. 2012).

The methods used to lice treatment can be divided into chemical, mechanical and biological. Some mechanical methods are still in experimental phase, but most of the treatments are based in water flushes, temperate water, electrical pulses or ever lasers. The biological control can be made by using cleaner fish, such as *Labrus bergylta* and *Cycloperus lumpus* which starts removing the bigger lice without stressing the salmon (Costello, 2004). The chemical methods can be applied as in-feed additives or into bath-treatment. Compounds such as organophosphates, hydrogen peroxide and synthetic

pyrethroids are used in bath treatment. On the other hand, emamectin benzonate and chitin synthesis inhibitor are used as in-feed additives (Costello, 2006).

Organophosphates acts in the nervous system and leads to paralysis by blocking the neurotransmitter acetylcholine esterase (Corbett, 1974). Hydrogenperoxid mechanism is not well understood but Grant (2002) suggest that there is an induction of paralysis by oxygen release to the gut and hemolymph. Pyretroides also acts in nervous system, more specifically in the sodium channels. There is a disturbance in the depolarization and in repolarization of the nervous cells, leading to problems in movements or even death (Burridge *et al*, 2010).

Due to the resistance of the methods above, in 2000 emamectin benzonate started be to be used as an in-feed medicine. It is a semi-synthetic product, which opens the glutamate gated chloride channels, leading to an increase of chloride concentrations, hyperpolarization of muscle and nerve tissue and inhibition of neural transmission (Grant, 2002).

1.5 Salmon lice – a blood feeding parasite

Lice infection leads to a cortisol production by the salmon to increase the metabolism, but that also can suppress the immune function. On the other side, lice secrete prostanglandin E2 and other immunomudulatory molecules (Wagner et al., 2008). These compounds are released in order to down regulate the inflammatory gene expression and might increases the availability of blood since they are also potent vasodilators (Fast et al., 2004, Wagner et al., 2008).

L. salmonis consistently consume blood visualized by the red gut seen in adult females (Brandal et al., 1976, Boxaspen, 2006). Hematophagous parasites can have several anticoagulant proteins in their saliva that specifically target blood coagulation proteinases in order to keep the blood liquid (Ciprandi et al., 2003). Hematophagy is a polyphyletic evolutionary strategy (Ciprandi et al., 2003). Different species can use different target molecules in order to avoid coagulation of ingested blood. The molecules are typically produced in the salivary gland and they are introduced in the host through their saliva during feeding (Koh and Kini, 2008).

Thrombin and factor Xa are common targets for preventing coagulation (Ciprandi et al., 2003). Thrombin is a part of the intrinsic and extrinsic blood coagulation

pathways in teleost fish and important for the production of fibrin. Thrombin can also reinforce the thrombocyte plug when fish suffer from injury (Tavares-Dias and Oliveira, 2009) and it is inhibited by, e.g., TTI (tsetse thrombin inhibitor) in the fly *Glossina morsitans* (Cappello et al., 1996), hirudin in the leech *Hirudino medicinalis* (Salzet, 2001) and also by ixin in the tick *Ixodes ricinus* (Markwardt, 1994). Serine protease Factor Xa is an enzyme also present in the coagulation cascade process and it is inhibited by, for example, draculin in the bat *Desmodus rotundus* (Fernandez et al., 1998), antistasin in the leech *Haementeria officinalis* (Tuszynski et al., 1987) and also by TAP (tick anticoagulant peptide) by the tick *Ornithodoros moubata* (Waxman et al., 1990).

TAP is a peptid found in soft tick's saliva, *Ornithodoros moubata*, and it is specific for factor Xa (Lim-Wilby et al., 1995). Its amino acid sequence has close homology to the Kunitz-type domain inhibitors (Waxman et al., 1990), which inhibits the protein degradation. They have a relatively small molecular weight of 6 kDa and a length of about 50 to 60 amino acids (Waxman et al., 1990).

The pharmaceutical companies use Kunitz domains as a framework for the development of new antithrombotic drugs inspired by blood-sucking animals (Keating, 2013). Bovine pancreatic trypsin inhibitor is an extensively studied model structure similar to TAP. Some molecules from this family are also present in the *L. salmonis* genome and they are possible good candidates to function as anticoagulants.

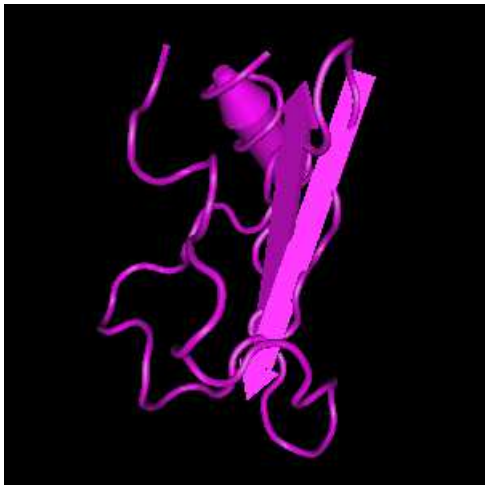


Fig. 1.2 - NMR Structure Determination of Tick Anticoagulant Peptide (TAP), based in Lim-Wilby et al (1995), and Madej et al. (20014)

2. Aims of study

Genes with the Kunitz domains have been shown to be involved in anticoagulation process in some hematophagous parasites. They are also present in *L. salmonis* but it is not known if they are involved in anticoagulation processes.

Then, the aims of this study are:

1. Identify candidate genes with Kunitz domains that could be involved in anticoagulation based on expression properties (in situ hybridization and RNA seq).

2. RNAi on some of these genes to assess the significance of knock-down.

3. Confirm silencing of candidate genes by means of Q-PCR and some fitness measurements.

3. Materials

Table 3.1 Chemicals and reagents used.

Product name	Supplier
100% Ethanol	Kemetyl Norge AS, Norway
10X DNase I reaction buffer	Invitrogen, USA
2-propanol	Sigma-Aldrich, USA
Agarose	Merck, Germany
BCIP (5-bromo-4-chloro-3'-indolyphosphate)	Roche Diagnostics, Germany
Benzocaine	Statens legemiddelverk
Blocking powder	Roche Diagnostics, Germany
Bromphenol blue	Roche Diagnostics, Germany
Chloroform	Sigma-Aldrich, USA
Deoxyribonucleotide triphosfate (dNTP)	Promega, USA
Diethylpyrocarbonate (DECP)	Merek, Germany
DNase I (1U/µl)	Invitrogen, USA
Erythrosine	Merek, Germany
Ethylene-diamine-tetra-acteic acid (EDTA)	Sigma-Aldrich, USA
Formamide	Merek, Germany
GelRed 10000X	Biotium, Inc., USA
GenElutet-LPA	Sigma-Aldrich, USA
Glacial acetic acid	Merck, Germany
Haematoxylin	Shandon Inc., USA
Histoclear	VWR International Ltd., England
Hybond N+ membrane	GE Healthcare, Netherland
ImmunoHistoMount	Sigma-Aldrich, USA
KCl	Merck, Germany
LiCl	Merek, Germany
Maleic acid	Fluka Chemie, Germany
MassRuler DNA Ladder Mix (SM0403)	Thermo Scientific, USA
Metamidate	Aquacalm
Na_2HPO_4	Merck, Germany
NaCl	Merek, Germany
NaOH	Merek, Germany
NBT (4-nitro blue tetrazolium)	Roche Diagnostics, Germany
Paraformaldehyde	Merek, Germany
Proteinase K	Sigma-Aldrich, USA
RNAlater	Qiagen, USA
TaqMan Fast Universal PCR mastermix (2x)	Applied Biosystems, USA
Triethanolamine (TEA)	Sigma-Aldrich, USA
Tris base (Tris-(hydroxymethyl)-aminomethan	Merck, Germany
Triton X-100	VWR International Ltd., England
Trizol Reagent	Invitrogen, USA
Tween 20 (Polyxyethylenesorbitan)	Sigma-Aldrich, USA

Table 3.2 Molecular biology kits used.

Product name	Supplier
AffinityScript cDNA kit	Matriks, Norway
Deoxyribonuclease I, Amplification Grade	Invitrogen, USA
DNase treatment	Invitrogen, USA
GenElute™ PCR Clean-Up kit	Sigma-Aldrich, USA
GoTaq® Flexi DNA polymerase kit	Promega, USA
MEGAscript RNAi kit	Life Technologies Corporation, USA
qScript™ Flex cDNA Kit	Quanta Bioscience, USA
RNeasy Micro kit	Qiagen, Netherlands
SMARTer™ RACE, cDNA amplification kit	Clontech, USA
UltraClean® 15, DNA purification kit	Mo Bio, USA

Table 3.3 Equipment used.

Equipment	Supplier
7900 Real-Time PCR system	Applied Biosystems, USA
Dialux 20 Microscope	Leitz, USA
Gel Logic 212 PRO	Fisher Scientific
GenAmp PCR system 9700	Applied Biosystems, UK
Heraeus Fresco 21 Centrifuge	Thermo Scientific, USA
Microamp 96-well reaction Plate	Applied Biosystems, USA
NanoDrop ND-1000	Thermo Scientific, USA
Thermal Cycler, Veriti 96 Well	Applied systems
Thermomixer Confort	Eppendorf. Germany
Tissue Lyser LT	Qiagen, Netherlands
UVC 500 Crosslinker	Hoefer. USA

4. Methods

4.1 Sampling

Salivary glands were extracted from adult females of *Lepeophtheirus salmonis*. To proceed with the salivary gland extraction we used adult female salmon lice for their bigger size. Between 30-40 animals are necessary to have enough biologic material to proceed with RNA extraction. Due to the small size of the specie and the even smaller size of the gland, a square around the mouth pore was isolated and preserved in RNAlater. It was located between second antenna and first maxilla as we can see in figure 4.1a and b. In the figure 4.1b we can observe other close structures to the salivary gland, including other glands. We believe that other genetic materials will be extracted besides the salivary gland but at least the front gland complex (Bell, 2000) will be sorted out from the rest of the digestive tract.

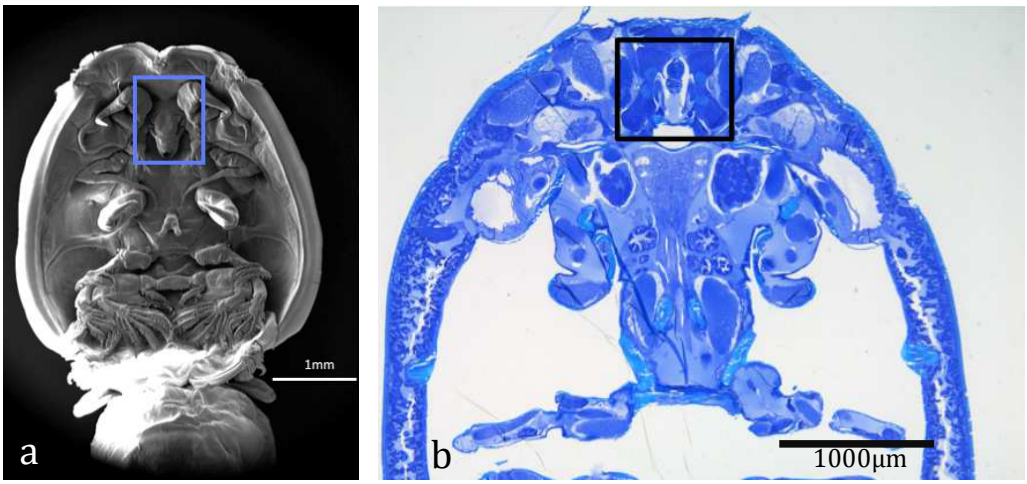


Fig. 4.1) Representation of the cut section. a) Picture from the ventral side of a Lepeophtheirus salmonis. *Electron microscopy. Bar = 1 mm; b) Histologic slide of* Lepeophtheirus salmonis.

4.2 Molecular Analysis

To collect RNA from our samples, we used Trizol reagent. To evaluate the RNA concentration and evaluate its purity, the NanoDrop 1000 was used. To be able to work with DNA molecule it was necessary to convert RNA into cDNA by the reverse transcriptase reaction and then compare gene expression thought PCR.

Molecular analyses were performed at the laboratories of SLRC.

4.2.1 RNA isolation

To isolate RNA from bigger stages we followed the protocol provided by Sigma-Aldrich (TRI reagent, catalog number T9424). One adult female was placed into an eppendorf tube together with one 5mm steel ball and 1ml of trizol. This allowed the dissociation of nucleoproteins complexes after two minutes of homogenization processes and incubation at room temperature for five minutes. DNA, RNA and protein integrity were preserved during the lysis and homogenization. 0.2ml of chloroform was added and the homogenate was mixed vigorously for 15 seconds, followed by incubation for 15 minutes at room temperature. The mixture was centrifuged at maximum speed for 20 minutes at 4 °C. Then, we observed stratification into three phases, according with the content: an aqueous and upper phase containing the RNA, a white interphase with DNA and a pinkish organic phase containing proteins. 450µl of the supernatant was extracted and mixed with 0.5ml of isopropanol in a new eppendorf. Samples were incubated for five minutes at room temperature in order to dissolve RNA. Later, they were centrifuged again at maximum speed for 10 minutes and at 4 °C. A precipitation was observed and the supernatant was discarded. The pellet was washed with 1ml of 75% ethanol twice and air-dried. Finally, it was eluted in 50µl of nuclease-free water and stored at -80 °C.

In order to isolate RNA from smaller stages, such as nauplii and copepodids, we followed the protocol of the RNeasy Micro kit. The homogenization process was the same of the one described above but to the isolation we used spin columns provided in the kit. 450µl of the supernatant was extracted and mixed with a same volume of 70% ethanol in a new eppendorf tube. The mixture was placed in the column and centrifuged at maximum speed for one minute. The flow-through was discarded and 700µl of buffer RW1 was added to wash the column during a centrifugation at maximum speed during one minute. The column was placed in a new collection tube and 500µl of buffer RPE was added and centrifuged again at maximum speed for one minute. The flow-through was discarded and 500µl of 80% ethanol was added. After a centrifugation at maximum speed for two minutes, the column was placed in a new collection tube. Then, the column was spined at maximum speed during five minutes with open lid. The column was transferred to a new 1.5ml collection tube and 14µl of RNA-free water was added directly into the center of the spin column. After one minute of centrifugation at maximum speed, the flow-through was placed directly into the center of the spin

column and centrifuged once again. Although there is a 20% volume lost, the final RNA concentration obtained was higher.

The RNA concentration was determined by Nanodrop Spectrophometer and also its purity and integrity. These analyses were based in the absorbance at 230, 260 and 280nm (A_{260}/A_{280} and A_{260}/A_{280} ratios). The A_{260}/A_{280} ration should be around 2 (+/- 0.20) and it measures its purity. Lower ration than that indicates contamination of protein, phenol or others contaminants which strongly absorb at or near of 280nm. The A_{260}/A_{280} should have similar values and it measures RNA integrity. Samples with a ratio lower than 1.8 might indicate contamination by proteins, chaotropic salts or phenol. When values are not satisfactory they might be precipitated again or discarded, since they are not suitable to further analysis.

4.2.2 Reverse transcription reaction

RNA molecules were converted in cDNA by the reverse transcriptase enzyme. To standard polymerase chain reaction (PCR), cDNA synthesis was based in the qScript Flex cDNA Kit (table 4.1). Reverse transcriptase enzyme was already present in the provided SuperMix. A first-strand synthesis occurs during an incubation thermal cycling (table 4.2). cDNA products were stored at -20 ºC.

Table 4.1) Master mix used to the cDNA synthesis, per reaction.

Component	**Amount**
qScript cDNA SuperMix (5x)	4 µl
Template RNA	1 ng
Nuclease-free water	Up to 20 µl

Table 4.2) Thermal cycling conditions of reverse transcription reaction.

Step	**Time**	**Temperature**
Incubation	5 min	25 ºC
Reverse transcriptase reaction	30 min	42 ºC
Inactivation of reverse transcriptase	5 min	85 ºC
		Hold in 4 ºC

To real-time PCR (RT-PCR) or quantitative PCR (Q-PCR) the reverse transcription reaction includes additional steps. First, the purified RNA went through a DNase treatment (table 4.3) and incubated at room temperature for 15 minutes, where single and double strands of DNA were digested. After the incubation the treatment was inactivated for 1µl for 25mM EDTA (pH 8.0) and incubated at 65 ºC for 10 minutes.

Table 4.3) Reaction for DNase treatment

Component	**Amount**
Total RNA	max. 1 ng
10X DNase treatment I reaction buffer	1 µl
DNase I, 1U/µl	1 ng
Nuclease-free water	Up to 10 µl

Then, the RNA was purified and ready to be converted until cDNA to be later used in Q-PCR. 2µl of RNA per reaction was mixed according with the protocol AffinityScript cDNA kit (table 4.4) and incubated (table 4.5).

Table 4.4) Reaction for cDNA synthesis. AffinityScript cDNA kit.

Component	**Volume**
First strand mastermix (2X)	5 µl
Oligo dT (100 ng/µl)	1 µl
Random primers (100 ng/µl)	0.5 µl
AffinityScript RT enzyme mixture	0.5 µl
Nuclease-free water	1 µl
DNase treated total RNA (0.3pg-1.5 µg)	2 µl

Table 4.5) Thermal cycling conditions of reverse transcription reaction to Q-PCR.

Step	**Time**	**Temperature**
Annealing	5 min	25 ºC
Reverse transcriptase reaction	15 min	42 ºC
Inactivation of reverse transcriptase	5 min	95 ºC

A control reaction was created, with no enzyme and with 0.5µl extra of nuclease-free water to achieve a final volume of 10µl (section 4.2.5). The reactions were diluted in 90µl and stored at -20 ºC

4.2.3 Polymerase Chain Reaction

The principle of the PCR is the generation of a large number of copies of a precise cDNA sequence from a complex mixture of starting material - the template. A master mix was created with provided buffers and reagents (table 4.6) and they were placed together with specific pair-bases in PCR thermal cycles (table 4.7). The cycles are dived in three different steps. The denaturation is the first step and the increase of temperature denaturize the template, what means the opening of the double strand. During the annealing step there is a drop in the temperature and it allows the annealing of the primers. It is dependent in the lowest primer melting temperature. In the last step, the extension, the increase of temperature makes the elongation possible. The duration of the extension depends on the number of bases in PCR product (1min/1000bp) (Wilson, 2010).

Table 4.6) Master mix per reaction used to the PCR.

Component	Volume
5X green GoTaq flexi buffer	5 µl
$MgCl_2$ solution [25nm]	2 µl
dNTP (Deoxyribonucleotide triphosfate) [1.25nM]	2 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
GoTaq DNA polimesare (5u/µl)	0.2 µl
Template DNA	1 µl
Nuclease-free water	13.8 µl

Table 4.7) Thermal cycling conditions of PCR.

Step	Time	Cycles	Temperature
Initial denaturation	2 min	1	94 ºC
Denaturation	30 sec	35	94 ºC
Annealing	15 sec	35	Variable
Extension	1 min / 1kb	35	72 ºC
Final Extension	5 min	1	72 ºC
		Hold in 4ºC	

4.2.4 Agarose Gel

The presence of the concerned sequences was verified in 1% agarose gel electrophoresis in 1x Triethanolamine (TAE). Gel Red was added to the agar gel in order to track the progress of the PCR products in the gel according with their size and conductivity.

4.2.5 Real Time PCR (RT-PCR) or Quantitative PCR (Q-PCR)

This is the most sensitive method for detection of mRNA abundance present in the samples. The principle of Q-PCR is to generate a large number of copies of cDNA sequences and measure theirs expression. A fluorescence marker, SYBR green, was used and it bounds to the major groove of double-stranded DNA. As the PCR product is more amplified, the signal gets stronger. The absolute quantification of stained amplified DNA was relatively measured with a linear standard curve after each cycle. Relative quantification through the algorithm ΔΔCt ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001) was used to determine the changes in gene expression compared to a reference gene (EF1α) previous validated as a reference gene (Frost and Nilsen, 2003). The endpoint of Q-PCR is when the Ct value reaches the threshold line. The Ct-value is inversely proportional to replicated nucleic acid present from the original sample. In a clean room, a master mix was prepared (table 4.8) and 2 µl of the specific template was later added. The plate was then incubated in a thermal cycle (table 4.9).

Two control wells were prepared. No amplification control (NAC) well was deprived of enzyme during the reverse transcriptase reaction and no template control (NTC) was deprived of DNA template.

Table 4.8) Master mix design to Q-PCR, per well.

Component	Volume
2X SYBR Select Master Mix	5 µl
Primer F (10 µM)	0.5 µl
Primer R (10 µM)	0.5 µl
RNase free water	2 ml

Table 4.9) Thermal cycling conditions of Q-PCR.

Cycle	Time	Cycles	Temperature
Initial denaturation	2 min	1	50 ºC
Denaturation	10 min	1	95 ºC
Annealing	15 sec	35-45	95 ºC
Extension	1 min	35-45	60 ºC
Final Extension	Melt Curve	1	60 - 95 ºC

Samples error and efficiency were also measured. An acceptable error should be below than 0.04 and the efficiency between 1.8 and 2.2. Efficiency above 2.2 means an inhibition in the transcription from RNA to cDNA and below 1.8 means and inhibition from cDNA to DNA. The technical replicates should have a standard deviation below 0.35.

4.2.6 PCR products purification

The purification of PCR products allows the exclusion of excess primers, nucleotides, DNA polymerase, oil and salts. We followed the protocol GenElute PCR Clean-Up kit (Sigma-Aldrich, USA, catalog number NA1020). This kit bases in the binding of DNA and a silica membrane within the spin column. The PCR product is mixed with a binding solution with a ratio 1:5 and the solution is transferred to the column. After a centrifugation during a minute at maximum speed, the flow-through is discarded. 0.5ml of diluted washing solution was added to the column and centrifuged at maximum speed twice, 1 and 2 minutes per each time, respectively. The column was transferred to a new collection tube and then the DNA was eluted in 40µl of nuclease-free water after being one minute at room temperature and centrifuged at maximum speed for one minute.

Other process to PCR product purification was used, the UltraClean 15 DNA purification kit (Mo Bio, USA, catalog number 12100-300). After determine the volume of the DNA product, three times of that volume was added of Ultra salt. 6µl of Ultra bind was added to the mix and incubated at room temperature for five minutes, while mixing several times. During this process the DNA binds to the silica and after a centrifugation of five seconds, the DNA and silica were moved to the bottom of the eppendorf tube. The supernatant was discarded and 12µl of water was added and mixed by pipetting. Another incubation at room temperature took place for five minutes followed by a centrifugation at maximum speed for one minute. The supernatant was removed and transferred to a new tube. The DNA was then ready to be used.

The DNA concentration, quality and integrity was determined by Nanodrop Spectrophometer and stored at -20 ºC.

4.3 *In situ* hybridization

In situ hybridization (ISH) allows us to determine gene expression in tissues section. Labeled RNA or DNA probe identify the expression of a specific DNA or RNA sequence to which it is complementary. Sequences that are not complementary are washed out and through a light microscope it is possible to localize where the expression takes place (Wilson, 2010).

In the current study, antisense RNA probes were used to identify the location of transcription in adult female lice and sense RNA probes were used as a negative control.

4.3.1 Single stranded RNA (ssRNA) probe synthesis

The probes were synthesizes using the primers with and without T7 promotor (Appendix II, table XX). DNA sequences were produced and its products were verified in 1% agarose gel and purified using Gen elute PCR Clean up kit. RNA probes were synthetized and labeled by DIG RNA Labeling Kit, according with table 4.10 and incubated at 37 ºC during two hours.

Table 4.10) Probe synthesis set-up to in situ *hybridization, per probe.*

Component	Volume
PCR product	10 µl
Nuclease free water	3 µl
DIG label mix	2 µl
RNase inhibitor	1 µl
Transcription buffer	2 µl
T7 polymerase	2 µl

Then, probes went through a DNase treatment, where 2µl of DNase were added. After 15 minutes at 37 ºC, 2µl of 0.2M EDTA was added to inactivate the DNase treatment. The probes were precipitated (table 4.11) and incubated during over night at -20 ºC.

Table 4.11) Probe precipitation set-up to in situ *hybridization, per probe.*

Component	Volume
0.2M EDTA (stops the synthesis)	2 µl
GenElute LPA	1 µl
LiCl	2 µl
EtOH 96 %, ice cold	66 µl

After incubation, RNA pellet was washed with 1ml of 100% ethanol and eluted in 40 µl of DEPC water. Probe yield was measured and exanimated by Nanodrop.

In order to verify its quality, probes were subjected to a spot test. First, three solutions were made: washing solution A, 1% blocking solution and detection buffer (Appendix I, table V). Six dilution series were made with a ratio of 1:400 to the first spot and 1:2 in the next five spots. 1µl of each series was placed on a positively charged nylon transfer membrane (hybond N+ membrane) and exposed during one minute to UV-light. Then, the membrane was washed in a 10ml of washing buffer A during 20 seconds. 10ml of blocking solution A was added and incubated during 30 minutes while gently agitating to prevent unspecific binding. Afterwards, 2µl of antibodies (Anti-Dig-AP) was added and incubated during 30 minutes while gently agitating. After the blocking, the membrane was washed with 10 ml of washing three times with buffer A during five minutes, each time. Then, the membrane was washed with detection buffer A for one minute while gently agitating. To proceed with the detection, NBT and BCIP were added (table 4.12) and gently agitating during three minutes.

Table 4.12) Chromogen substrate to spot-test.

Component	**Volume**
NBT	45 µl
BCIP	35 µl
Processing buffer	10 ml

To visualize the probes, the membrane was washed in distilled water for 3-10 minutes while gently agitating.

4.3.2 Hybridization in paraffin slides

Horizontal sections of salmon lice (3µm thick) were pretreated before ISH. The slides were baked for 20 minutes at 60 ºC and after washed in histoclear three times for 10 minutes each, in order to remove the paraffin. Then, the sections were rehydrated with a decreasing gradient of ethanol in DEPC until 50% concentration and after soaked in 2X SSC. In order to increase the permeability of the tissue and to allow probes to enter, the sections were digested by proteinase K for 15 minutes. Then, the slides were fixed, to keep the histological structure, in 4% paraformaldehyde in 1X PBS for five minutes and washed in 1X PBS twice for two minutes each. In order to inactivate endogenous phosphatase, tissues were treated with acetic anhydride during five

minutes. Afterwards, the slides were soaked in 2X SCC twice for two minutes each and dehydrated by an increasing gradient of ethanol in DEPC until 100% ethanol. Later, the slides were left to dry for at least one hour. Finally for the hybridization, 3-15µg of each probe was boiled with the 40 µl of hybridization solution for 5 minutes and cooled down on ice. 5 µl of 10% blocking solution was added and DEPC until a final volume of 50 µl. The probes were added to the slides and incubated overnight at 65 ºC in a moister chamber and airtight.

Next day, slides were flushed and after washed with 2X SSC for 30 minutes twice. Later, the slides were washed in 25 ml of deionized formamide in 25 ml 2XSSC for 30 minutes at 65 ºC. After that, they were washed in 2X SSC for 10 minutes at 37ºC twice. When washed, the slices went through a RNA digestion, exposed to 250µl of 4mg/ml RNase A in 50 ml of RNase buffer, for 30 minutes at 37 ºC. To finished the RNA digestion, the slides were washed in 1X maleate buffer there times during 10 minutes each. The followed process was the blocking, where the slides were blocked during between one and two hours with a mix presented at table 4.13 (1 ml of this mixed was removed to be used later).

Table 4.13) Blocking solution to in situ *hybridization.*

Component	**Volume**
1% blocking solution	5 ml
Triton X-100	25 µl
Maleate buffer	45 ml

The slides were washed with 1X maleate buffer during five minutes twice. To the 1ml of the mix described above, 0.5 µl of Anti-Dig-AP-FAB fragment was added. 100µl of the solution was placed on the tissue and incubated at room temperature overnight.

In the third day of the process the slides were washed in 1X maleate for 10 minutes twice, followed by a wash with processing buffer, also during 10 minutes. A chromogen substrate was prepared according with table 4.14 in dark conditions (the final volume and concentration can be adjusted to a smaller number of sections). 200µl of the substrate was placed on the slides and incubated in dark conditions.

Table 4.14) Chromogen substrate to in situ *hybridization, enough for 50 sections.*

Component	**Volume**
NBT	45 µl
BCIP	35 µl
Processing buffer	10 ml

The incubation is considered finished when a development is observed. That can vary considerably between different probes, depending in the gene expression. After a sign being observed the sections were exposed to a stop buffer and washed in water. The slide could then be sealed with ImmunoHistoMount and a cover glass.

Several chemicals used were toxics. Then, some procedures needed to be performed at the fume hood and collected in specific waste containers. All the buffers and solutions are present in Appendix I.

4.3.3 Hematoxylin and Erythrosine staining (H&E)

This method was performed in order to see the anatomy of lice and at same time as a control to check if the gene expression was in the organs where it was expected to be expressed.

Hematoxylin stains the basophile parts of a cell in blue, such as the nucleus. On the other hand, erythrosine stains the acidophil parts of a cell in red, for example the cytoplasm.

Before the staining the tissues need to be hydrated, in other words, the paraffin needs to be replaced by water through several infiltration baths. To do that, the sections were incubated at 65 ºC for 30 minutes and soaking in histoclear twice for 10 minutes each time. Then, a decreasing percent of ethanol bath and water from 100% ethanol twice, during five minutes each, and then 96%, 80% and 50%, for five minutes each bath. Finally, slices were placed in a water bath for five minutes. The slides were then ready to be stained in hematoxylin for 2.5 minutes, followed four minutes in water. After we placed the slides in 1% erythrosine for 1.5 minutes and again in water, but this time for only one minute. The slides needed then to be dehydrated by a crescent percent bath of ethanol during one minute at 96% ethanol bath followed by 100% twice, one minute each. To finishing the staining, sections are washed with histoclear during five minutes twice. To mount the sections, they were dried and covered with histomount and a cover slip on.

4.4 SMARTer RACE – cDNA Amplification Kit

This kit allows a 5'- and 3'-rapid amplification for cDNA amplitication. Oligos with terminal stretch of modified bases anneal to the extended cDNA tail and then they serve as a template for the reverse transcriptase. Primers were designed to bind as close as possible of specific cDNA ends.

This method facilitates the amplification of a full-length transcript of genes, which can be sequenced entirely.

A PCR Master Mix was prepared (table 4.16) for both 5'- and 3'-RACE reactions and then used in the RACE reaction together with stocked cDNA gene specific primers (table 4.17). Then, the samples went through thermal cycles of different temperatures to ensure an efficient extension of each gene.

Table 4.16) PCR master mix to RACE, per reaction.

Component	Volume
PCR-grade water	17.25 µl
10X Advantage 2 PCR buffer	2.5 µl
dNTP mix	0.5 µl
50X Advantage 2 Polymerase Mix	0.5 µl

Table 4.17) RACE reaction, per primer.

Component	Volume
cDNA	1.25 µl
Universal Primer A mix (10X)	2.5 µl
Specific primer	0.5 µl
Master mix	20.75 µl

The sequencing was performed at the sequencing facility at the University of Bergen.

4.5 RNA interference

RNAi is a powerful method to study the function of a specific gene through the suppression of the target gene expression. To do that, a double-stranded RNA molecule (dsRNA) was synthetized. In the cell, dsRNA is cleaved in small interfering RNA (siRNA) fragments of 21-23bp by dicer. siRNA are characterized by two nucleotide long 3-prime over-hangings. Together with ribonucleoprotein particles (RNP), siRNA form the RNA-induced silencing complex (RISC), which turns the siRNA in single strands. The antisense siRNA that is coupled to RISC binds to mRNA sequence

specific region and cleavage it. The cleaved mRMA is recognized by the cell and destroyed. This prevents translation from occurring, silencing the expression of the gene from which mRNA was transcribed (Wilson, 2010).

4.5.1 dsRNA synthesis

Six PCR products were produced using primers with and without a 5'T7 promoter extension (5'-TAATACGACTCACTATAGGGAGA-3'). Then, the PCR products were purified mixed with the ribonucleotides (table 4.18) and incubated over night at 37 ºC, following the protocol MEGAscript RNAi kit (Life Corporation, USA, part number AM1626).

Table 4.18) Transcription reaction per product to RNAi.

Component	Volume
Linear DNA template (1 µg of sense template)	4 µl
Linear DNA template (1 µg of anti-sense template)	4 µl
10X T7 Reaction Buffer	2 µl
ATP Solution	2 µl
CTP Solution	2 µl
GTP Solution	2 µl
UTP Solution	2 µl
T7 Enzyme Mix	2 µl

In the next day, the samples were incubated at 75 ºC during five minutes to allow the annealing and cooled down at room temperature to form the dsRNA. Then, the dsRNA went through a nuclease digestion, where DNA and ssRNA were removed (table 4.19).

Table 4.19) RNase digestion reaction per product to RNAi.

Component	Volume
dsRNA	20 µl
Nuclease-free Water	21 µl
10X Digestion Buffer	5 µl
DNase I	2 µl
RNase	2 µl

After an incubation of one hour at 37 ºC, the dsRNA was purified and washed by adding a binding buffer, nuclease-free water and ethanol (table 4.20). The entire 500 µl were placed in a filter cartilage and centrifuged at maximum speed during two minutes.

Table 4.20) dsRNA binding mix per product to RNAi.

Component	Volume
dsRNA	50 µl
10X Binding Buffer	50 µl
Nuclease-free Water	150 µl
100 % Ethanol	250 µl

The flow-through was discarded and 500µl of washing solution was added onto the filter. After other centrifugation, the flow-though was discarded and the washing process was repeated. Finally, the dsRNA was eluted and analyzed by spectrophotometry.

4.5.2 Injection of dsRNA in adult females

After an adjustment of concentration to 600 ng/µl, 1 µl of bromophenolblue was added to 50 µl of dsRNA. Bromophenolblue works as a dye to make it possible to follow the dsRNA during the injection. Pre-adult females were removed from the host with forceps. An incision was created on an agar gel in order to support the lice during the injection of 1 µl dsRNA. Between nine and ten females received a specific gene, as well as a control group, injected with the control fragment of cod trypsin gene (CPY185). Then, females were injected dorsally to the haemocoel of the cephalothorax. Before infect the salmon with the injected females, they were kept in running water for three hours.

4.5.3 Sampling of adult lice

After 36 days after the injection, the experiment was terminated. The lice were removed from the host and the females were photographed. Some lice were placed in 4% paraformaldehyde in PBS and the majority were placed in RNA later. PBS allows us to conserve lice tissues to be used later *in situ* hybridization and the RNA later preserve the RNA content for verification of genetic knockdown, through Q-PCR.

4.5.4 Host and experimental design

The RNAi experiment in pre-adult lice was conducted at IMR (Institute of Marine Research) in Bergen, according to Norwegian animal-welfare regulations. The host, *Salmon salar*, was kept individually in tanks of seawater, with an average salinity of 34‰ and temperature between 8-10ºC.

Before the infection, the hosts were placed in a mixture of benzocaine and metomidate until they become sedate and suitable for the handling. 10 females injected with the dsRNA and 10 males were place upside down in a wet paper. Then, a sedated fish were carefully placed on the paper, the lice could infect the salmon by itself and the salmon were placed back to the tank.

4.5.5 RNAi in larvae

In order to get a significant down-regulation of the candidate genes, RNAi must to be performed in the right time-point (Eichner et al. 2014). During the molting between nauplii I and II there is a water uptake by the larvae and that is desired time-window to expose nauplii to the dsRNA. Just for reference, at 10 ºC nauplii I take about 24 hours to molt into nauplii II.

Egg-strings pairs were incubated in individual hatching wells and the hatching time was register. Between 20-60 nauplii I were incubated in 150µl of seawater and exposed to 1.5 µg of dsRNA (figure 4.3a). Five parallels were made for each of the three studied genes. In addition, a control group was created with a control fragment of cod trypsin gene (CPY185). After 24h the molting into nauplii II was confirmed by the number of exuvia being the same as the number of animals. Then, nauplii II were transferred to incubation wells (figure 4.3b) and kept there until molt until copepodids.

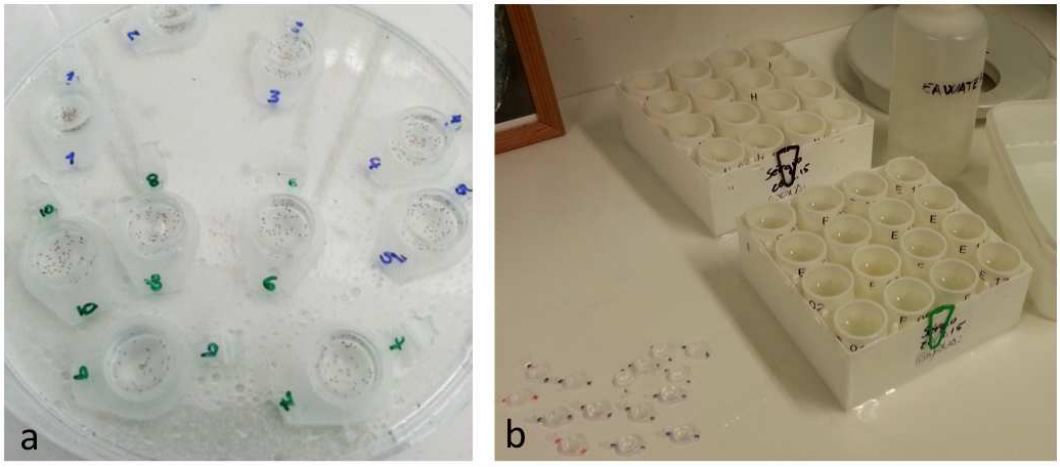


figure 4.2) Exposure of larvae to dsRNA and incubation wells used to RNAi

4.5.6 Sampling of larvae

After molting into copepodids, larvae were collected, photographed and preserved in RNAlater for later analysis of gene suppression.

4.6 Software, Statistical Analyses and Calculations

NCBI BLAST (The Basic Local Alignment Search Tool) was used in order to designe primers, identify open reading frames and putative conserved domains.

Microsoft Excel 2011 and StatPlus:mac LE, a free edition of StatPlus:mac Professional developed by AnalystSoft, was used to calculate the mean and standard deviation of data, as well as all statistical calculations.

In order to analyze the differences between the groups, an analysis of variance (ANOVA) was performed. With this test we can observe or not the difference between group means and its origin of difference. The homogeneity of variance within the Groups (normality) was also tested as a pre-request to the analysis of variance.

A significance level of 0.05 was considered in all statistics, giving a confidence level of 95%.

ImageJ version 1.47 (National Institutes of Health, USA) was used to measure the length of adult female lice, egg strings and copepods after RNA interference experiment.

5. Results

5.1 Assessment of Target Genes

A selection of genes containing Kunitz-domain (PF00014) was identified based on annotation from the salmon louse genome. To assess if any of these candidates are expressed in the *L. salmonis* salivary gland a simple PCR-based test was set-up. Nine candidate genes of the Kunitz domain family were tested in this assay. All of them were expressed in the tissue from the total animal. From all the nine tested sequences only two showed to be specific of salivary gland in salmon lice (LsKunitz1 and LsKunitz2, see fig. 5.1e).

A gland specific gene be expressed in the total lice (TL) and also be expressed in the salivary gland (SG). At same time it should have a reduced or no expression in the samples of salmon lice where the gland was removed (TL-SG). Most of the genes tested were not specifically connected to the salivary gland (fig 5.1, LsKunitzA-C) or not specific of the isolated area (fig. 5.1D-F). When the sequence of the gene was relatively long, it was divided in different fragments (e.g. LsKunitz3).

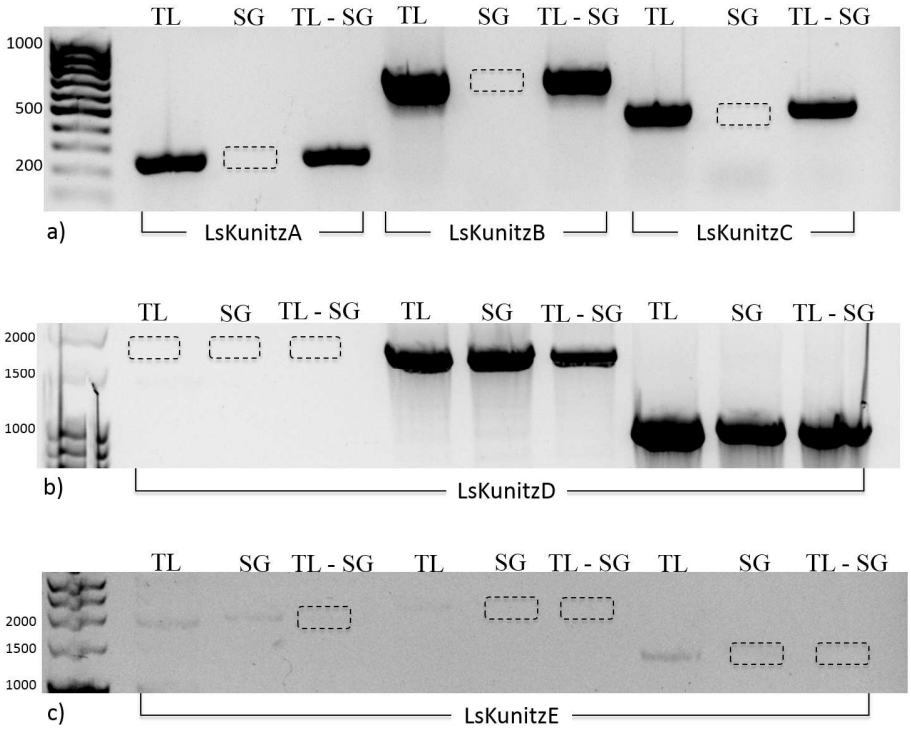


Figure 5.1 (continuing in the next page)

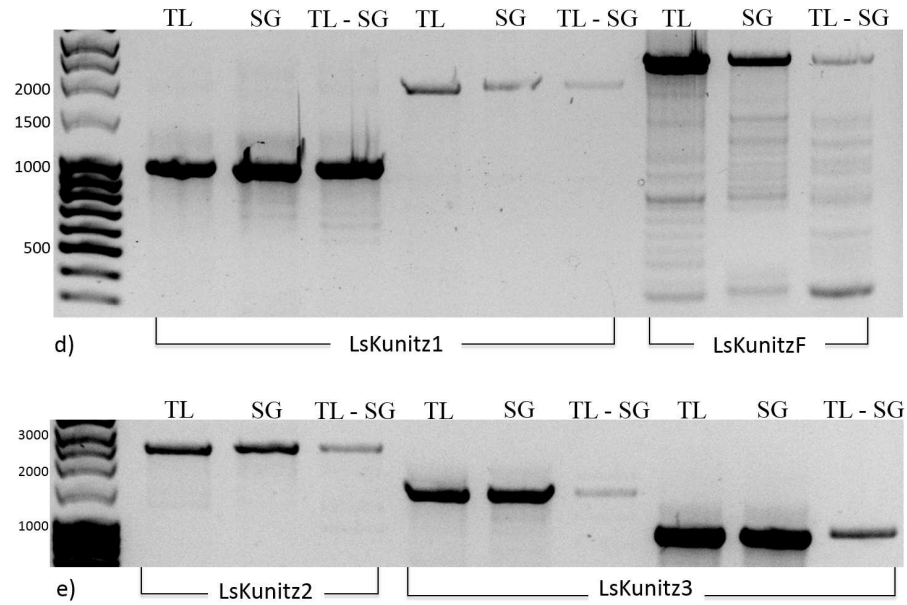


Figure 5.1. PCR products in 1% Agarose gel of candidate genes. TL stands to Total Lice; SG stands to Salivary Gland; TL - SG stands to Total Lice without Salivary Gland. Numbers on the left side of each picture and close to the MassRuller indicate the number of base pairs of the PCR products.

The PCR-screening indicates that the gene LsKunitz2 and LsKunitz3 are good candidates for genes expressed in the salivary gland (see figure 5.1). Although not a salivary gland specific gene, we also selected LsKunitz1 to be further studied due to its high expression (see figure 5.2).

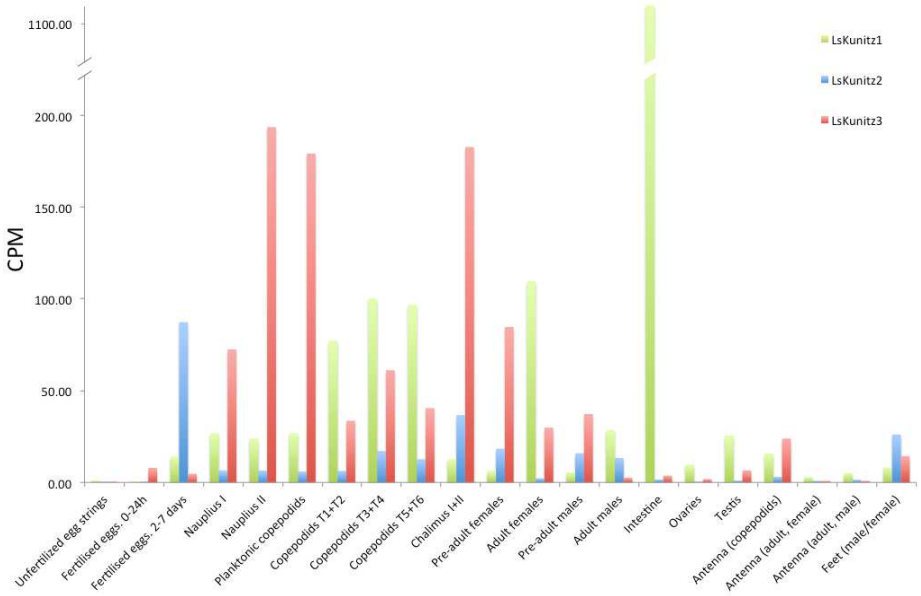


Figure 5.2. Relative measurement of gene expression in the different stages of L. salmonis. CPM stands for counts per million. RNAseq Data obtained from LiceBase.

LsKunitz1 is clearly more expressed in the intestine of sea lice, as expected. Besides fertilized eggs, LsKunitz2 has a higher expression at the chalimus stages. Excluding the intestine, LsKunitz2 is the gene with largest relative expression at the stage of nauplius II and Chalimus I and II.

5.2 RACE and Sequencing

SMARTer RACE reaction was performed using the primers presented at appendix II table XXI. Thermal cycles were optimized to each gene in order to obtain a well define and strong PCR band. Most of the reactions presented satisfactory bands but after the sequencing none of the terminal areas were expressed (appendix V). Sequences in the appendix III were obtained in the Lice Base. LsKunitz1, LsKunitz2 and LsKunitz3 have 2542, 2725 and 7371 bases pares, respectively. Additionally, NCBI blast showed theirs open reading frame are 431, 772 and 2346 amino acids, in the same order than above, and the protein sequence hits the conserved domain Kunitz_BPTI (pfam00014), which is the Kunitz/Bovine pancreatic trypsin inhibitor domain(KU/Kunitz_) (see Apendix IV).

5.3 *in situ* Hybridization

In situ hybridization was performed in adult females. Hematoxylin and erythrosine staining was used for proper identification of the different tissues (figure 5.3). The *in situ* hybridization did not show any expression of LsKunitz2 and LsKunitz3 (figure 5.4a and 5.4b).

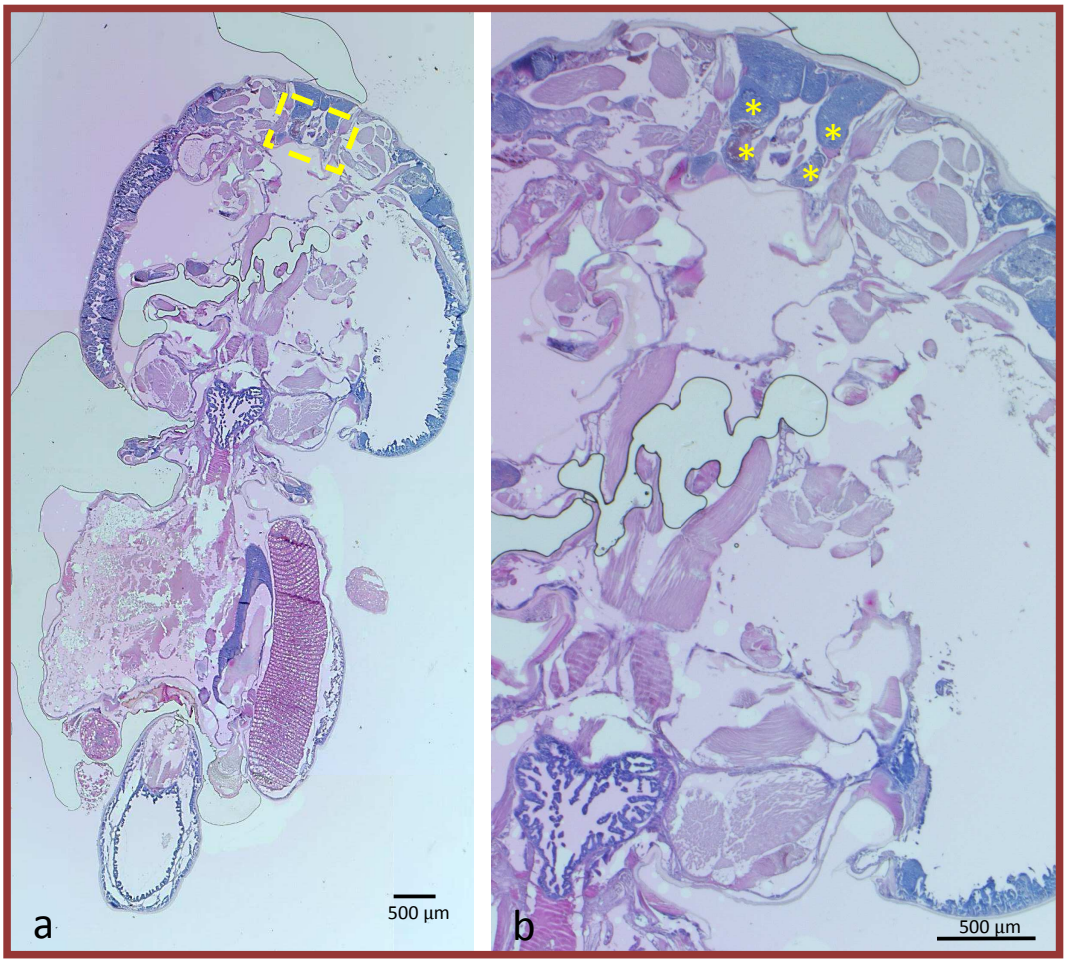


Figure 5.3) Microscopy pictures of paraffin-embedded sections of a female salmon lice. The dashed square (a) represents the sectioned part to isolate the salivary gland. The stars (b) indicate the lobes of the salivary gland. The samples were stained with H&E.

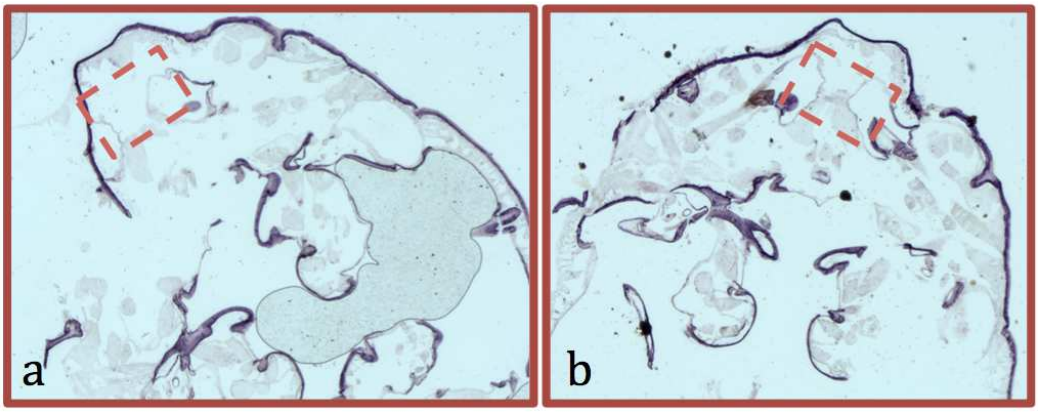


Figure 5.4) Microscopy pictures of in *situ hybridization sections of salmon lice. a) Section exposed to antisense probe of the fragment LsKunitz2 b) Section exposed to antisense probe of the fragment LsKunitz3. In both figures the dashed squares indicate the place where the signal from salivary gland should be expressed.*

On the other hand, LsKunitz1 presented a stronger signal in the intestine as expected (figure 5.5a and 5.5b). The sense probe (used as a control) did not show any signal.

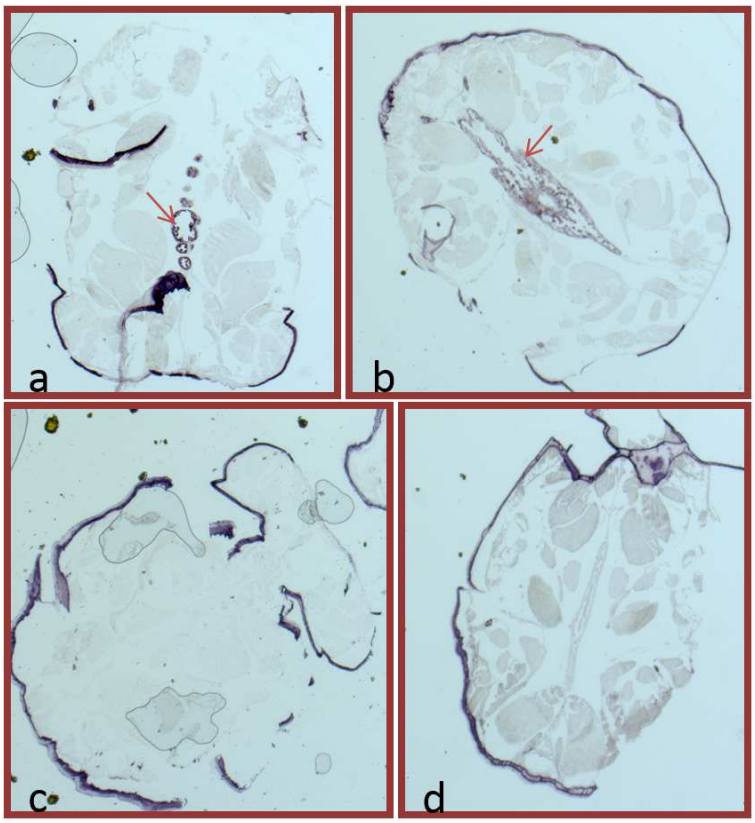


Figure 5.5) Microscopy pictures of in *situ hybridization sections of a female salmon louse. a) Longitudinal section exposed to antisense probe of the fragment LsKunitz1; b) transversal section exposed to antisense probe of the fragment LsKunitz1; c) longitudinal section exposed to sense probe of the fragment LsKunitz1 d) transversal section exposed to sense probe of the fragment LsKunitz1. Arrows (a and b) indicates expressed signal of the gene LsKunitz1 in the intestine.*

5.4 RNA interference

5.4.1 RNA interference in adults

RNAi was done by injecting dsRNA for the three selected genes and a control into pre-adult female.

Table 5.1 Number of injected lice versus recovered lice

	Injected female lice	Recovered female lice	Recovered male lice
Control	30	9 (30.0%)	4 (13.3%)
LsKunitz1	30	6 (20.0%)	8 (26.7%)
LsKunitz2	28	10 (35.7%)	7 (35.0%)
LsKunitz3	20	9 (45.0%)	4 (20.0%)

We recovered between 6 and 10 female lice after the RNAi experiment (table 5.1). 10 male lice were placed on each fish and between 4 and 8, per group, were recovered. One of the fish of LsKunitz3 died during the experiment, leading to a lower number of considered injected female and male lice applied on the fish.

5.4.1.1 Evaluation Gene silencing

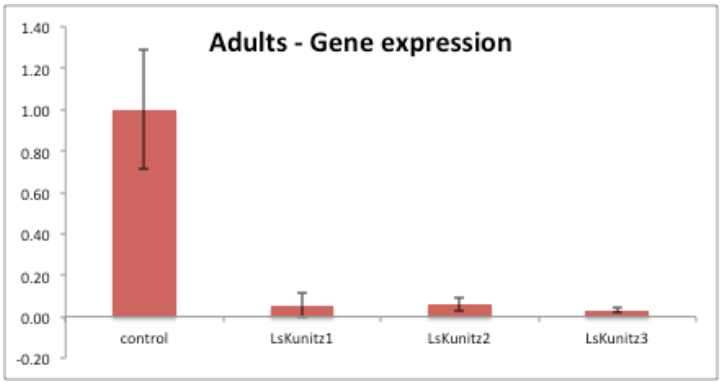


Figure 5.6) Transcript levels for LsKunitz1, LsKunitz2 and LsKunitz3 in L. salmonis after RNAi experiment. The values are normalized to EF1α.

In order to assess the effect of the RNAi in adult females, the transcription levels of LsKunitz1, LsKunitz2 and LsKunitz3 were measured by quantitative PCR, with eEF1α as a reference gene (fig. 5.6). The results show a significant down regulation of LsKunitz1, LsKunitz2 and LsKunitz3 after the RNAi. The silencing of these genes was highly successful with knockdown percentages of 94.5%, 94.3% and 96.9%, respectively. There is a highly statistical significant difference (p-value < 0.01).

5.4.1.2 Length measurements

Table 5.2 – Female length and egg strings length of the adult females collected in the termination of the RNA interference experiment.

	Female length	Egg string length
Control	11.34 (±0.98)	15.51 (±4.14)
LsKunitz1	11.18 (±0.69)	16.14 (±2.48)
LsKunitz2	11.61 (±0.49)	15.38 (±3.56)
LsKunitz3	11.44 (±0.35)	15.64 (±2.46)

The female average length of collected samples was 11.42mm (±0.65mm), range between 9.69mm and 12.39mm. The control group was the one with the highest standard deviation (0.98mm). There was no statistically significance between the different groups (p-value = 0.63)

The egg strings average length from the collected samples was 15.69mm (±3.17mm), range between 5.29mm and 20.07mm. The control group was the one with the highest standard deviation (4.17mm) and the animals injected with the fragment LsKunitz2 presented the shorter egg strings, 15.38mm. Although is possible to observe a small difference between the groups it is not statistically significant different (p-value = 0.96).

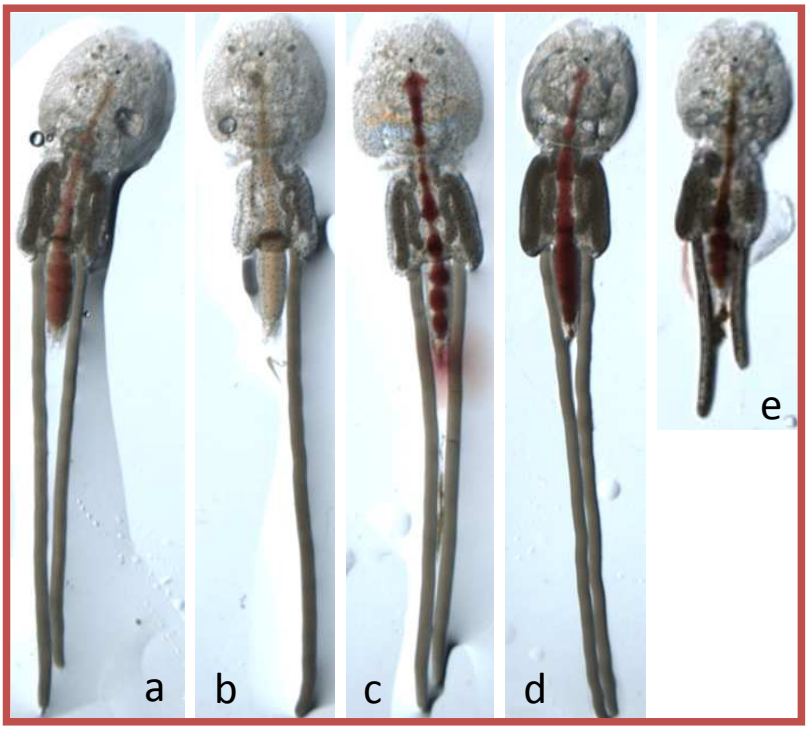


Figure 5.7) Five representativ female lice from the control group after the RNAi experiment.

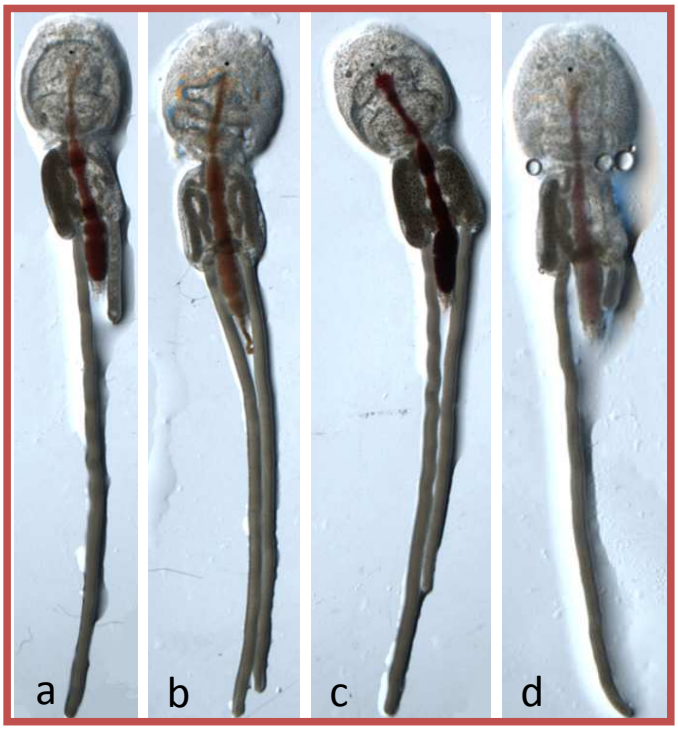


Figure 5.8) Four representative female lice from the LsKunitz1 group after the RNAi experiment.

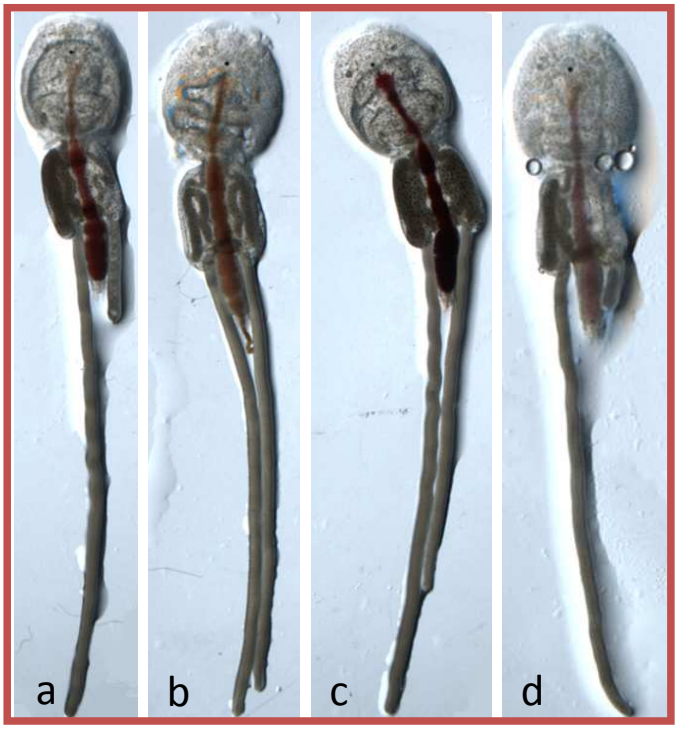


Figure 5.9) Four representative female lice from the LsKunitz2 group after the RNAi experiment.

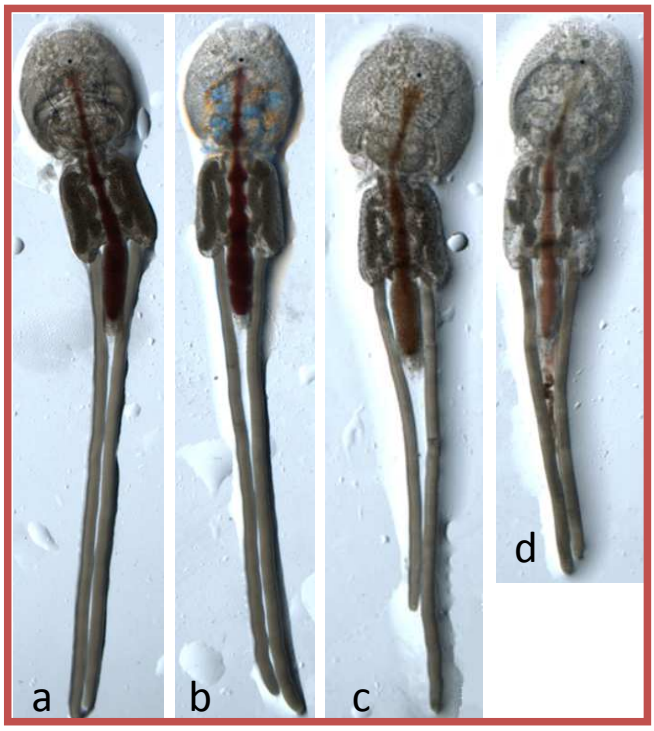


Figure 5.10) Four representative female lice from the LsKunitz3 group after the RNAi experiment.

5.4.1.3 Hatching success

Table 5.3 Hatching success of egg strings collected from RNA interference experiment, average of eggs by female per group.

	Incubated egg strings / group	Mean of expected egg / female	Mean of collected copepodids / female
Control	8	482	398
LsKunitz1	4	489	318
LsKunitz2	8	482	362
LsKunitz3	9	507	340

The egg strings in the present study had an average of 16 eggs per millimeter. Based on the egg string length of the egg strings (see table 5.2) it was possible to calculate an expected number of eggs per egg string pair. After molting into copepodids, the number of lice was determined and percentage copepodids was calculated (fig. 5.11). Only the double egg strings in good conditions were incubated.

The success values were between 80.1% (control) and 64.8% (LsKunitz1) but no statistical significant difference was observed between the groups (p-value = 0,17).

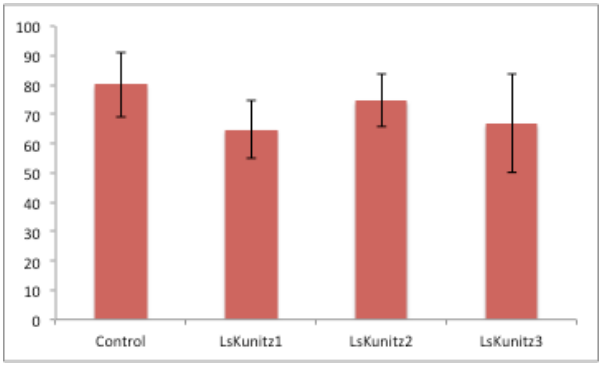


Figure 5.11 – Hatching success of egg string collected from RNAi experiment, in percentage. 100% would mean as much copepodids observed as expected, while 0% success would mean no observed copepodids after observed eggs in the egg strings.

5.4.2 RNA interference in larvae

RNAi was conducted with the three candidate genes and the control in salmon louse larvae.

The transcription levels of the LsKunitz1, LsKunitz2 and LsKunitz3 genes were also analyzed in the copepodids after a RNA interference experiment during the ecdysis from Nauplius I to Nauplius II.

5.4.2.1 Gene silencing

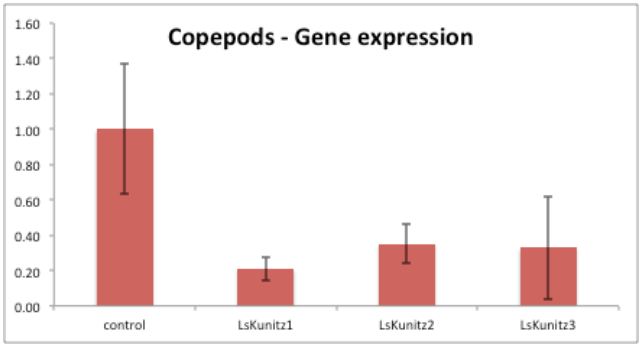


Figure 5.12) Transcript levels for LsKunitz1, LsKunitz2 and LsKunitz3 in L. salmonis copepodids after RNAi experiment. The values are normalized to EF1α.

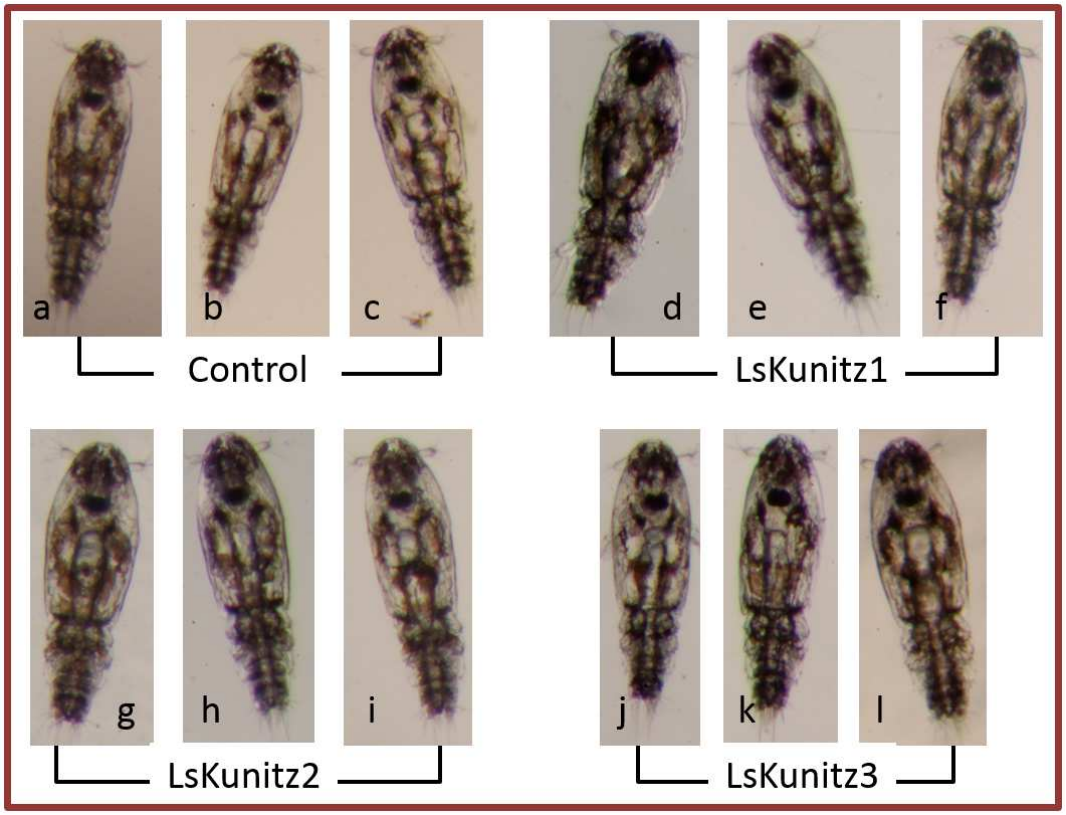
To evaluate the effect of the RNAi experiment of the concerned genes in copepodids, the transcription levels of LsKunitz1, LsKunitz2 and LsKunitz3 were measured by quantitative PCR, with eEF1α as a reference gene (fig. 5.12). The results show a considerable down regulation of LsKunitz1, LsKunitz2 and LsKunitz3 after the

RNAi experiment. The silencing of these genes was successful with knockdown percentages of 79.1%, 64.9% and 67.2%, respectively.

5.4.2.2 Length measurements

The average length of collected copepodids was 0.73mm (±0.004mm). It was not observed any statistical significant difference between the groups at this stage (p-value = 0.79). Furthermore, no obvious phenotype was observed in animals exposed to the dsRNA fragments (figure 5.13).

The measurement of the copepodids length was performed using pictures and with as much accuracy as possible. Only extended louse was measured and also when in a favorable angle. Contracted lice or blurry specimens were excluded. Although probably not so significant, when lice is swimming or standing in different vertical points in the water drop, it may influence in the measurement. In order to avoid this influencing factor, the pictures for the measurements were taken with as little water as possible.



5.13) Three examples of copepodids from each group after the RNAi experiment. No distinguished phenotype was observed.

6. Discussion

A total of 9 candidate genes were selected from the lice genome annotation based on presence of putative Kunitz domain. They were used in the PCR assay in order to observe their transcription or absence in the salivary glad of *L. salmonis*. Two of the nine tested genes showed indications of salivary gland expression. One other candidate gene showed very high expression in the intestine. These three genes were used in the further studies.

Blast search with these candidates showed the presence of the conservative domains of Kunitz family. Ciprandi et al. (2003) presents a list of several anticoagulants used by different hematophagous animals. Among them, TAP (tick anticoagulant peptide), which is produced in the salivary gland of *O. moubata.* TAP also belong to the Kunitz-type domain inhibitors family and it is specific in the inhibition of factor Xa (Lim-Wilby et al. 1995; Waxman, et al. 1990).

The expression of the studied genes is considerably different between them (figure 5.2). *In situ hybridization* also presented differences in the expression of the studied genes, which is in accordance with the expression from RNA sequences. LsKunitz1 presented a strong signal in the intestine where as LsKunitz2 and LsKunitz3 did not present any signal. LsKunitz1 showed to be produced in the intestine. This is not unexpected since there are a large number of proteases transcribed in the intestine that LsKunitz1 and other protease inhibitors could interact with. Although we observed expression of LsKunitz2 and LsKunitz3 in the frontal part of the lice in the preliminary PCR test, most likely in salivary gland, it was not detected by ISH. This lack of signal could be explained by the low expression of these genes in the adult stage or even by the absence of the desired tissue/cells on the slides.

These results also point towards issues related to isolation of salivary gland tissue. LsKunitz1 showed expression in the PCR-assay, but ISH proved no expression of such gene in the frontal part of the lice. The reason for this might be the presence of some digestive gut tissue in the samples of isolated salivary gland or very low or local expression in a few cells in the gland that easily could be absent in tissue sections.

The lack of favorable results after the sequencing also leaded to a poor knowledge about the studied genes. Due to the lack of time it was not possible repeat the experiment, try new primers or new tissues. Then, we were not able to obtain a proper characterization of the studied genes.

Regarding to RNAi experiment, all the recovered lice were photographed and although with a gene silencing up to 96.7% no obvious phenotype was observed (see figures 5.7-5.10). Since *L. salmonis* is a hematophagous parasite and need to keep the host blood liquid for proper digestion. It was expected some influence on the parasite development due to a possible lack of nutrients or the absence of blood in the lice intestine. If the studied genes were key players in anticoagulation we expected some negative effect on the lice after successful RNAi. It could also be expected a higher loss of lice or some physical phenotype. Lice did not present a significant statistical difference in size and in the egg strings neither (see table 5.2). Almost all the adult females were observed still with blood in their digestive gut.

At same time, the absence distinct phenotype cannot indicate by itself the non-relation between the studied genes and anticoagulation process in *L. salmonis.* There is the possibility for compensation by other proteins or some undetected phenotypes at the cellular level that was not investigated here. In addition, we did not measure the protein levels of the silenced genes and to obtain a phenotype the protein level need to drop below a threshold level. It is possible that the life time of the proteins for the investigated genes are long and that this can explain the lack of phenotypes. Egg strings were collected and incubated. From the egg string length and the average number of eggs per millimeter, we calculated an expected number of eggs per group in order to calculate the hatching success. No statistical significant difference was observed in the hatching success. According to Hamre et al. (2009) the hatching success for salmon lice in a small incubator system is between 65 and 85%, which support the normality of our results.

There is also the possible presence of others anticoagulants, maybe belonging to a completely different family than those containing the Kunitz domain. One example of that is *O. moubata,* which has three anticoagulants acting in different steps of the coagulation process (Ciprandi, 2003). The new anticoagulant could have a more

significant role at the anticoagulation process in compensation process, in order to still allow the lice cope with the blood.

As observed in the gene expression (see figure 5.2) LsKunitz2 and LsKunitz3 have higher expression in larvae stages, which could indicate different function in different stages. Then we exposed *L. salmonis* larvae to dsRNA probes and the copepodids were analyzed. Q-PCR results showed low expression of the concerned genes. These values indicate a successful knock-down (see figure 5.12), but once more, no distinguished phenotype was observed for the larvae. However, larvae were not used to infect the fish and it is possible that the effects would be evident when they entered the fish and started to feed. It would be interested to see how these copepodids developed after infection. This was not done due to time limitation.

These present results did not revealed any large significant phenotypes in adults or larvae, reducing survival or fitness of the lice.

7. Conclusion

The present study demonstrated the presence of two salivary gland specific genes belonging to the Kunitz family in *L. salmonis*, LsKunitz2 and LsKunitz3. Other gene from the same family, LsKunitz1 is highly expressed in the digestive gut and it was also showed by *in situ hybridization*. We did not observe any positive signal of LsKunitz2 and LsKunitz3 in ISH. The lack of signal might be due their low expression in adult stage.

All the studied genes were successfully silenced in adults and larvae trough RNAi. However, none of the groups presented a distinct phenotype. There is the possibility of existence of other anticoagulants in *L. salmonis* in order to allow it to cope with the host blood or a compensatory mechanism.

With this, we can conclude that LsKunitz1, LsKunitz2 and LsKunitz3 have no significant role in the anticoagulation in *L. salmonis* in adults and larvae stages. As far as we know, the specific place to anticoagulants production in *L. salmonis* is still unknown.

More studies are needed in order to understand better the anticoagulation mechanism in *L. salmonis*, which genes are involved in the production of anticoagulants and where they are produced.

8. Future studies

In order to get a better understanding of the anticoagulation process in *L. salmonis*, further studies are necessary. Since the three included genes with the Kunitz domain did not presented any distinct phenotype after RNAi, different anticoagulants should be tested.

LsKunitz2 and LsKunitz3 could also be analyzed again in ISH with higher probe concentrations in order to observe their expression on the salivary gland. They could also be tested in chalimus I and II. In case of no signal, new probes should be designed.

Analysis regarding other proteins related to these genes should also be take in consideration in order to conclude about the successful gene knock-down or observe some possible compensation.

New primers for RACE reaction should also be designed in order to obtain a better characterization of the genes.

It could also be interesting to observe the results of an RNAi assay where both genes are knocked down at same time. It could show if these two specific genes have a synergistic action. If this RNAi experiment is repeated, it could also be interesting to collect the lost lice in order to observe if the silencing of the genes were successful or if it was due to fish behavior. Histology could be applied to both lost lice and lice still present on the host until the terminartion of the experiment. Larvae subtimed to RNAi could be also used to infect the fish.

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Appendix I

Buffers and solutions

Table I) Agarose Gel 1%.

Component	**Amount**
Agarose	0,25 g
1x TAE buffer	Up to 25ml

Table II) 50 X TAE (Tris-Acetat-EDTA) buffer

Component	**Volume**
Glacial acetic acid	57 ml
EDTA pH 8,0 0,5M	100 ml
ddH2O	Up to 1000 ml

Table III) Washing buffer

Component	**Volume**
Maleate buffer, 5x	10 ml
DEPC	40 ml
Tween 20	150 µl

Table IV) Detection buffer

Component	**Volume**
Tris HCl, 1M	5 ml
NaCl, 5M	5 ml
DEPC	40 ml
Ajust pH to 9.5	

Table V) 1% Blocking solution

Component	**Volume**
Maleate buffer (5x)	10 ml
DEPC	40 ml
Blocking powder	5 ml

Table VI) Deionized formamide

Component	**Volume**
Formamide	Desired amount
Resin	0,1g/ml formamide
Stir formamide with resin for 60 minutes at room temperature. Filter and freeze.	

Table VII) DEPC solution

Component	Volume
DEPC 1 ml	1 ml
MilliQ water	1000 ml
Incubate at 37 °C overnight. Autoclave.	

Table VIII) 4% Paraformaldehyde in PBS (1 liter)

Component	Amount
Paraformaldehyde	4g
Deionized H_2O	50 ml
NaOH, 1M	1 ml
Heat to app. 65 °C until the paraformaldehyde is dissolved	
PBS, 10x	10 ml
Cool to room temperature	
Adjust pH to 7,4	
Adjust to 100 ml and filter solution through 0,45 µm membrane filter. Store at -20°C.	

Table IX) Maleate buffer (5X)

Component	Amount
Maleic acid	58g
Milli-Q water	850 ml
NaOH pellets	Adjust pH to 7,5 (app. 35 g to 1 L)
NaCl	43,8g

Table X) Hybridization solution

Component	Amount
Dextran sulphate	2,5 g
DEPC water	Up to 5 ml
Dissolve the dextran sulphate by heating to app. 70 °C	
Tris HCl pH 7,5 (1M)	250 µl
NaCl, 5M	1,5 ml
DEPC H_2O	0,7 ml
Deionized high grade RNase free formamide	12,5 ml

Table XI) EDTA stock 0,5 M

Component	Amount
EDTA	14,6 g
DEPC	100 ml

Table XII) RNase buffer

Component	Amount
NaCl	29,23g
1M Tris HCl pH 7,5	10 ml
0,5M EDTA	2 ml

Table XIII) Tris NaCl pH 9.5 10x

Component	Amount
Tris base	60,55g
MilliQ water	350 ml
NaCl	29g
Adjust pH to 9,5 with NaOH pellets Bring volume to 500 ml	

Table XIV) Tris HCl pH 7.5 (1 liter)

Component	Amount
Tris base	121,1 g
DEPC water	800 ml
Adjust pH to 7,5 with HCl Bring volume to 1 L	

Table XV) $MgCl_2$ (0.5M)

Component	Amount
$MgCl_2$	4,65 g
DEPC	100 ml

Table XVI) Processing buffer

Component	Amount
Tris NaCl pH 9,5	100 mM, 100 ml of 10x stock
$MgCl_2$	50 mM, 100 ml of 0.5M stock
Adjust to 1 litre.	

Table XVII) SSC (20X)

Component	Amount
NaCl	175,3 g
Trisodiumcitrat	88,2 g
DEPC dH_2O	800 ml
Adjust pH to 7,0 with NaOH and volume to 1 L with DEPC	

Table XVIII) Stop buffer

Component	Amount
Tris HCl pH 7,5	10 mM
EDTA	1 mM
NaCl	150 mM

Appendix II

Listo of Primers

Table XIX. Primers used for PCR

Gene/fragment	Sequence
LsKunitzD_f1	GCACGACCTGGAGATTCATCTGTGA
LsKunitzD_r1	GTAGTCTATGCTTGTCAGCCCCACA
LsKunitzD _f2	GGTAGCTATTGCGCCAGAGAAGAGG
LsKunitzD _r2	AACTTGTTGCCGTTTCCAGCACATC
LsKunitzD _f3	CAAGAACTCTGGACTCTGCAAGGCA
LsKunitzD _r3	CCTTCAGTGTTGACGGGTGTGATGA
LsKunitz1_f1	GGGACTATGCTGGGTTAGGAGTCTT
LsKunitz1_r1	AAAGCCATATCTGGGAAGGGAAGCC
LsKunitz1_f2	AGAGGGCACTTGTGTTATCCGCAAT
LsKunitz1_r2	TGCTTAGGCATGCAAGTAAGGATTA
LsKunitzA_f1	ATTTATTCCATGGCCATTACTGCTG
LsKunitzA_r1	GGAACATTTTTCTTTGCAGAGTTCC
LsKunitzE_f1	CTCTCAGGATCTCATCCAATTTCCA
LsKunitzE _r1	TGACACTGAGGAGATCAAATCCTTT
LsKunitzE _f2	CCCATGGAATTTGTAACCCTCAAAA
LsKunitzE _r2	GTTTCGGATACAATCCTCCATTGTC
LsKunitzE _f3	CAATGGAGGATTGTATCCGAAACTG
LsKunitzE _r3	TCTAGGGAGATCAGTCTTAAAGGGT
LsKunitzB_f1	CTATCCGTGATCCTTCACCCA
LsKunitzB_r1	AGGATCCACTTGAACCAAACCAG
LsKunitzC_f1	TCCTTGTAGGCATTTCTTCTGGAG
LsKunitzC_r1	GAGCTGTGTCTTTCTCATGTCTG
LsKunitzC_f1	CACCACTGGTTCTGAGGACG
LsKunitzC_r2	CAGGGAGAAATCCAGCCTCC
LsKunitz2_f1	TGAAAAAGTCGGTCAGTGAGGT
LsKunitz2_r1	TACTCGACCTACATGCGGGA
LsKunitz3_f1	GCAAAAACATTTGTCAACTTCCCAG
LsKunitz3_r1	CCATATCATCTCCAGAGGACTCAAG
LsKunitz3_f2	GCAGTCATGTAATGAAAATTCGTGC
LsKunitz3_r2	TTTTACTTCTGGAAGAACACATGCC
LsKunitz3_f3	TCCTGATAACTACACTCCAGCAAAA
LsKunitz3_r3	TTGCTCACAACTTGAGTACACATTC
LsKunitz3_f4	GAATGTGTACTCAAGTTGTGAGCAA
LsKunitz3_r4	GCATTCAATTTGGATATACTGCCCA

Table XX. Primers used for *in situ* Hybridization and RNA Interference.

Gene/fragment	**Sequence (5'-3')**
LsKunitz1_f1	TGTGAAACATTCATCTTTGGAGGC
LsKunitz1_r1	ACGCCATCAATGTGTTCGTTG
LsKunitz1f1T7	TAATACGACTCACTATAGGGAGATGTGAAACATTCA TCTTTGGAGGC
LsKunitz1r1T7	TAATACGACTCACTATAGGGAGAACGCCATCAATGT GTTCGTTG
LsKunitz2_f1	TCCTCCGGACAAGAGTGTCA
LsKunitz2_r1	AACCTCACACGAGGCTTGAG
LsKunitz2_f1T7	TAATACGACTCACTATAGGGAGATCCTCCGGACAAG AGTGTCA
LsKunitz2_r1T7	TAATACGACTCACTATAGGGAGAAACCTCACACGAG GCTTGAG
7907f1_b3291	CAGCGCATGGTCCATTGAAG
7907r1_b3292	GCCCGACCAGTAGGATTGAC
LsKunitz3_f1T7	TAATACGACTCACTATAGGGAGACAGCGCATGGTCC ATTGAAG
LsKunitz3_r1T7	TAATACGACTCACTATAGGGAGAGCCCGACCAGTAG GATTGAC

Table XXI. Primers used for RACE.

Gene/fragment	**Sequence**
LsKunitz1_r	ACGCCGTTAATTATACTACGGGCTGCCT
LsKunitz1_f	GGGTTATTGTGGTGACTTCCTCCAGTGT
LsKunitz2_r	TTGTGACCGGCTTTCCTAAGCCCCA
LsKunitz2_f	GCCTGAAAAGTTGTCTTCGTTCTCCCC
LsKunitz3_r	GGGCCGAGGCAACGGATAGAGGAACG
LsKunitz3_f	TCGTCATTGGGGACTGGAGAGCGGT

Table XXII. Primers used for Q-PCR

Gene/fragment	**Sequence**
LsKunitz1_r_SY	GCCCAAGAACTGCAAAGGAA
LsKunitz1_f_SY	TCCTGGGTCAGGAGCTAGAG
LsKunitz2_r_SY	CTGGTCCATGCAATGGCTAC
LsKunitz2f_SY	CGAGGCTTCCGACATTGTTT
LsKunitz3_r_SY	ATACTCGCCCTCACGTCTAC
LsKunitz3_f_SY	CGAGGAAGCCTGGATCACTA

APPENDIX III

cDNA sequences

LsKunitz1 | 2502 bp

GTGAAAGTGAACAATTGCGCCGCGGGACTATGCTGGGTTAGGAGTCTTATTGATGGGTCTAATTATACAAAAATC
CAGTAAAAGGAAGGCGGTTCATCTTCTAAAGAATGAGTCAAAAAACGTACAGACAAATCCTCAACACTGGAGGA
AGTCACCACAATAACCCTTTTCACAAGGAGGAAGAATTGATATATCTTGAACAGGGAAAGTATAATGCACGATGC
CGAAGATCCGATATATCCAAATTCGTAGCACTTCTTTTCTCTTTAATTTTCGTATTTGTCGTCCTCTCCGTTTACTGG
GTATCCGTGAAGAATTTGACCGAACAAAATAAAGATGATCTTGAACGATTACTCGAGGAAAAAGAATTAAATAGC
ACACGAAGAGCATATATAAATTTAACGCCAGGAATTAGTTCACACAACGGAAAATGTAAACTGCCCAAGAACTGC
AAAGGAACAAATTCTCAATGGTATTTTGCCGAGGAAGAGGGCACTTGTGTTATCCGCAATCTTTCCGAATGCGATT
CTTCAACTACTCCTTTCGAATCTGTTGAAGATTGTAACTCTCAATGCATATCACCAACATATGCTGTGAATAAGAA
TCCCTGTACTCTAGCTCCTGACCCAGGACCATGTCGTGGCTCAATGGTCCGCTTTCACTATAATCACGAAAGTCGT
TTATGTGACAAATTCATCTATGGAGGTTGTGTAGGGAATGAAAATAATTTTCGCACAGTGGAGAGTTGTCAGAAG
AAATGTGATGTGCCACTACCAATTTCACTGGAAGATCCCCCGTGTTCTATTCCACCGTACCGAGGACCATGCCGAG
GAAATATTCAGAGATGGTATTTTAACAATGTTAAGAAAATATGTGAAACATTCATCTTTGGAGGCTGCCTCTCCAA
TAAAAACAATTTTTTAAGCTTAGAGGACTGTAACGATAAATGCGACCAGAGGTCAGGATCACCTTCTACAGCCAA
CAAGTGCTTCCTCCCCATTGATGTAGGAAACTGCAAGGCTTCCCTTCCCAGATATGGCTTTGATGTAGGCAGCAAA
AAATGTATCGTCTTTAATTATTCAGGCTGTGAAGGGAATAATAATCGATTTGAGAGTTTAGAAGATTGTAAGAGG
AATTGCTCAGGAAGTTTTTTAGATACGGAAGGAGACAATCAGGATGATGACGAGGAAGAGAAGAAAGTAGGTGA
AGAGATCACTTCTAAGACTTTAGTGGATTGTACATCAGATGCAGACTCAGGAAGTTGCAAAGGGATGTTCATGAG
ATATTATTTTGATGGCAAGCAATGTAGTACTTTCATATATGGAGGCTGTCTTGGAAATAATAACAACTACAAAAGT
GCAGAAAAGTGCAAGGAAGCTTGTAGTGGACGACCTACTAAATAAACTGTAACTCCACGAATTTAAATTATACTG
GGTTTAGAAAAAGTATTGATACCTACCTTGTATTAAATATTGAAATGCTTGTAATTGATCAGAATGAAGTTGTATT
TAGAAAATTTTATTTTATAATTATTTATACTACGATAAAGAACATAATTTGTAAATTTCGGGTTTTATCAGCCTCAA
TGACAGCCTCCACTCCCCTTACGGAATCCCTTCAACGAACACATTGATGGCGTAATCCTTCCTCATGATCCTCCAC
TGCTGGTTGACAGAGTTCTCCAGACCATCAATAATATTTGGGTCACGGACTTTGAAAGCCTTCTTTTCAATTTACC
AGCAAATGGAGTAATCCAATGGATTCAAACCCAGCCTCGGGGGGGGGGGGGAKGTCCTTGGAAARAAAMTTCATG
TTMCCGCGCATCCACTCTAGTACAATTTTCGCAGTCTGGGGTGGAGACTGTATATTAGATGATTTATTAAGAATAA
AATTTTGAGTTCTGATTTACGTCGTTACCTTTATTGTATCTCGCCACATTTCCATCAAAAAGACATAAATGAAGACC
CAAAATCCGTGCGTAGTAAACCAATTCTTCGCAACTACGGAATAATTATAAAAACATTATTGGAGCTTGTTCACAC
CTTTAGAAGACTAAATTTGAATGGACCCACTTAACGTATATAACATTGATAATAGGGAAAGAAGCAGAAAAATCG
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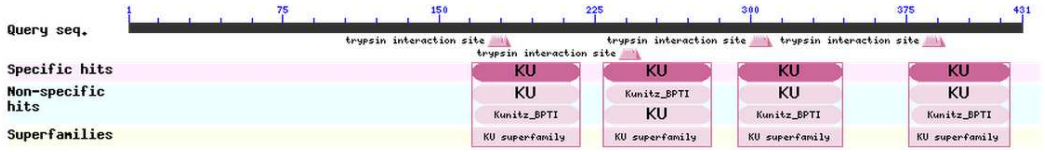
APPENDIX IV

NCBI Blast



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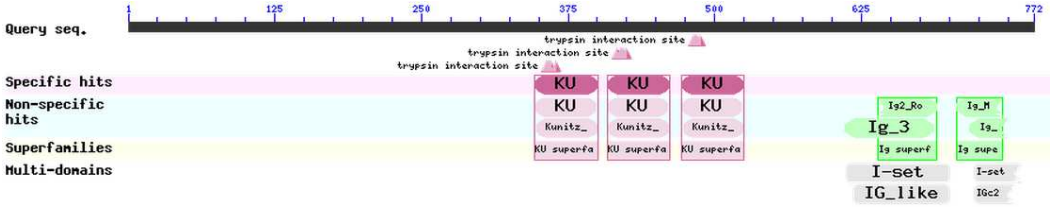

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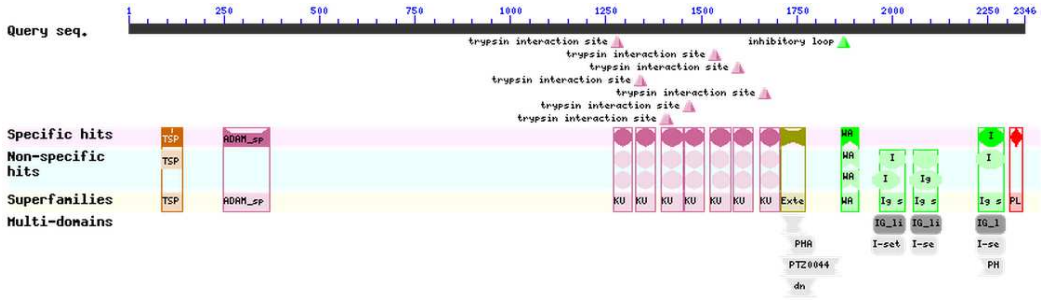
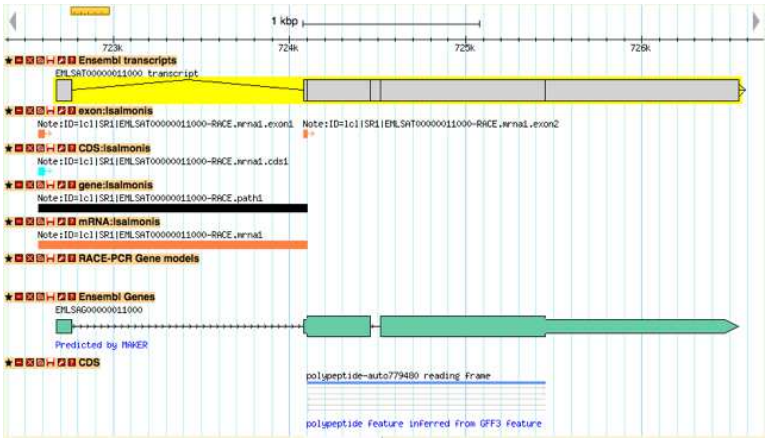


LsKunitz2) KU - Kunitz/Bovine pancreatic trypsin inhibitor domain

APPENDIX V

Alignments of RACE reaction sequencing using Gmap.



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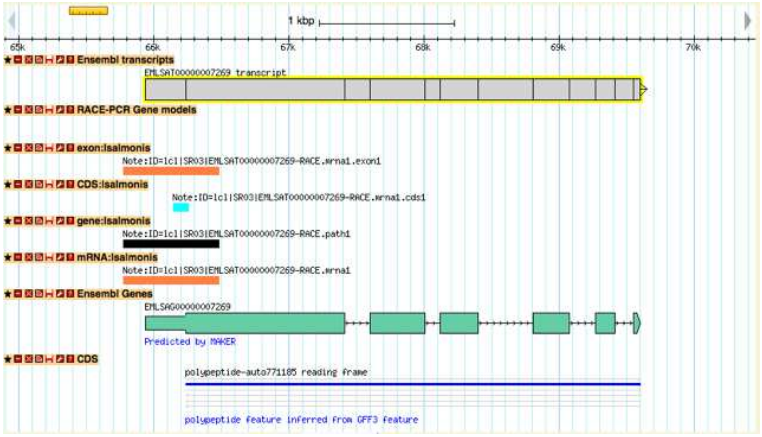
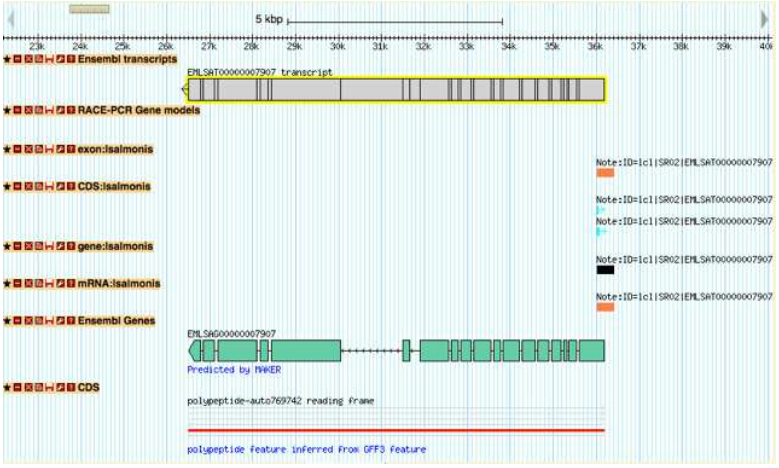


LsKunitz2



LsKunitz3