# Different delivery routes of double stranded RNA to obtain RNA Interference in the salmon louse (*Lepeophtheirus salmonis)*

**Thesis for the degree of Master of Science in Biology**

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**Preface**

This thesis was written as a part of the Master's Programme in Biology - specialization in Aquaculture Biology at the University of Bergen, Faculty of Mathematics and Natural Sciences, Department of Biology. The laboratory work and experiments were performed at the Sea Lice Research Centre, a Centre for Research-based Innovation appointed by the Research Council of Norway.

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## 1. Contents

## 2. Abstract

*Lepeophtheirus salmonis* (Krøyer 1837), commonly known as the salmon louse, is an ectoparasitic copepod specific to wild and farmed salmonids (Boxaspen, 2006), and probably the most serious problem that the Norwegian Aquaculture Industry faces in modern times. It is estimated that costs related to salmon louse control have reached 5 billion NOK in 2015 (Frank Nilsen, 2016, personal communication). The extraordinary increase in production of salmonids by Norwegian Aquaculture Industry since the 1970s (reached 1.5 million tonnes in 2009) (Torrissen et al. 2011) coupled with the limited amount of chemotherapeutants available to treat salmon louse infestation have led to a rise in parasite resistance and reduced sensitivity to available treatments (Denholm, 2002; Hosberg, 2012). This trend has created a demand for alternative treatment methods, of which the most coveted is an efficient vaccine. Two studies have been published in which candidate antigens were tested on Atlantic salmon (Grayson et al., 1995; Carpio et al., 2011) but none of them produced the desired outcome of drastic reduction in the number of lice and lice fitness. The sequencing, assembly and ongoing annotation of the salmon louse genome has provided researchers with a wide range of possible new treatment targets and potential vaccine antigens. The Sea Lice Research Centre (SLRC), where this work is being pursued, has already started the evaluation of several of those targets (Dalvin et al. 2009, 2011; Eichner et al. 2014, 2015a, 2015b, 2015c; Sandlund et al., 2015). A very relevant molecular technique used in this exploration process has been RNA interference (RNAi), a phenomenon by which gene transcription downregulation is achieved upon introducing of dsRNA molecules homologous to the target mRNA. There is, however, little information on how RNAi is achieved in the louse, namely which proteins are involved in the transport of dsRNA into the cytosol. In *C. elegans,* the model organism for RNAi, the SID-1 protein has been identified as a selective importer of extracellular dsRNA. The present study attempted to induce the transcriptional knock-down via RNAi of LsSID1a and LsSID1b, two genes that encode two putative SID-1 like proteins in *L. salmonis,* in order to verify their effect in the systemic gene knock-down of other known genes. RNAi in the salmon louse was attempted via intestine injection, cephalothorax injection and by mixing Atlantic salmon blood with dsRNA (blood+dsRNA) and feeding it to pre-adult II male lice. Results show that intestine micro injection is not a viable dsRNA delivery method in the salmon louse but show promise when it comes to blood+dsRNA feeding. LsSID genes knock-down was not achieved via cephalothorax injection, although other target genes were successfully knocked-down using that same dsRNA delivery method. Additionally, results from this study show it is possible to

detect dsRNA in the louse after it is fed blood+dsRNA and there is also a potential downregulation of LsSID1b at 192h post-feeding using this method. Further investigation is nonetheless required as the gathered evidence is not strong enough to unequivocally confirm this hypothesis.

## 3. Introduction

Sea lice (Copepoda, Caligidae) are parasites of wild and farmed fish in the marine environment. Lice of the genera *Lepeophtheirus* and *Caligus* are commonly found on wild salmonids. *Lepeophtheirus salmonis* (Krøyer 1837) is commonly known as the 'salmon louse' due to its specificity to salmonids (Boxaspen, 2006) and is the dominant species found on farmed and wild salmonids in Northern Europe (Whelan, 2010). This ectoparasitic copepod infects and lives on salmonid hosts and occurs naturally on Atlantic Salmon of the genus Salmo and on all species of Pacific Salmon (Tully & Nolan, 2002). It is present in open ocean, coastal and estuarine locations (Kabata, 1979) and it is responsible for many outbreaks of disease in intensive salmonid aquaculture (Johnson et al., 2004; Boxaspen, 2006) as well as mortality in wild Atlantic and Pacific salmon (Johnson et al. 1996; Whelan & Poole, 1996). In Norway, according to Pike and Wadsworth (1999), costs related to sea lice infestations were as high as 500 million NOK in the distant year of 1997 and have been raising swiftly reaching the 5 billion NOK mark in 2015 (Frank Nilsen, 2016, personal communication). In fact, since the nineteen seventies establishment of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) farming in Norway, salmon lice has been considered a major problem. Norway's long and sheltered coastline together with a stable and reliable water temperature (courtesy of the Gulf stream) (Trygve, 1993), provides various locations suitable for intensive salmon farming. Nevertheless, that same intensive salmon production creates a high host density and holds fish in sea pens for several months in the same location, thus creating ideal living conditions for salmon lice and facilitating disease and parasite transmission within the fish farm (Murray & Peeler, 2005). This problem also extends to the wild stocks of salmonids as pelagic larvae are prone to disperse through the net mesh of sea pens and into the surrounding environment (Johnsen, 2001) and, depending on water temperature and current, can travel distances as high as 25 kilometres in 48 hours (Asplin, 2014). Norway, Ireland, Scotland, and Canada all currently consider dense infestations of salmon lice a major challenge in Atlantic salmon aquaculture (Torrisen et al., 2013).

**Treatment with chemotherapeutants and the problem of resistance** Norwegian salmon farms are under constant scrutiny when it comes to lice levels. The Salmon Lice Directive (2009) defines some of the compulsive management requirements to minimize lice impact not only in the produced salmon but also on wild salmon and trout. One crucial point of the directive involves regulation of the drugs used in treatments and the appearance of drug

resistance. Farms are compelled to take action when average lice levels reach 0.5 mature female or three mobile lice - from 1 January to 31 August - and one mature female or five mobile during the rest of the year. Cleaner fish (several species of wrass) and chemotherapeutants are the most common means of treatment. While the use of cleaner fish started in the late 1980s, the use of chemotherapeutants dates back to the 1970s. The initial formaldehyde baths were rapidly substituted by the organophosphate metrifonate (oral and bath treatments) and later dichlorvos, which was the preferred solution until the first cases of resistance started appearing in the early 1990s (Torrissen et al., 2013). At that point in time, other options became available, namely natural pyrethrins and hydrogen peroxide (bath application) and the macrocyclic lactone ivermectin (oral treatment). During the 1990s other promising drugs appeared: the chitin synthesis inhibitors diflubenzuron and teflubenzuron (target the early developmental stages of the sea louse), the organophosphate azamethiphos, the synthetic pyrethroids cypermethrin and deltamethrin (oral treatment; target all stages), and the macrocyclic lactone emamectin benzoate (oral treatment; targets all stages). By 2012 the most used treatment was, by far, hydrogen peroxide (3144 tonnes) followed by azamethiphos (2.437 tonnes), diflubenzuron (704 kg) and emmamectin benzoate (105 kg) (The Norwegian Institute of Public Health, 2012).

During this period, the aquaculture production of Atlantic Salmon in Norway increased markedly and reached 1.5 million tonnes in 2009 (Torrissen et al. 2011). Such intensification was not accompanied by the arrival of new treatments and, consequently, there was a large-scale reliance on very few chemotherapeutants. The theoretical risk of selection for resistance in the salmon louse became a reality and by the mid 1990s a huge loss of effect of organophosphates was reported in several regions of Norway (Denholm et al. 2002). Treatment failure with pyretrhoids followed and, more recently, emamectin benzoate. For the last, a study by Horsberg (2012) found that more than 50% of the salmon lice strains analysed showed reduced sensitivity to emamectin benzoate.

In this context, with an ever smaller number of naive parasites in circulation (farmed salmon greatly outnumbers wild salmon), there is a rising need for new and non-pharmaceutical treatment methods.

**Cleaner fish** The species most commonly used in Norway are Goldsinny wrasse (*Ctenolabrus rupestris*), Ballan wrasse (*Labrus bergylta*), Corkwing wrasse (*Symphodus melops*) and Rock cook (*Centrolabrus exoletus*) (Torrisen et al., 2013). These animals are able to remove and eat the lice from Atlantic salmon and have been stocked together with good results. Although the use of cleaner fish has increased since first introduced in the 1980s

(estimated use of wrasse surpassed 10 million fish in 2010) (Skiftesvik et al., 2015), several challenges to their use are still present. High maintenance requirements (frequent cage cleaning required; need shelter inside their cages), the concerns of overexploitation (the industry is highly dependent on wild catches), the lack of knowledge in cleaner fish biology, ecology and population dynamics are some of the persistent problems. Nonetheless, some advances have been made in the last years regarding the farming of ballan wrasse (Skiftesvik et al., 2013). It is yet highly unlikely that cleaner fish are able to substitute chemotherapeutants and are seen as a complementary treatment.

**Vaccination** Contrary to antiviral and antibacterial vaccines, parasite vaccines are still at an early stage of development (Alvarez-Pellitero, 2008). Two studies were published in which vaccine candidate antigens were tested on Atlantic salmon against salmon lice but, despite the promising results, none of them produced the desired outcome of drastic reduction in the number of lice and lice fitness. Grayson et al. (1995) used proteins from adult *L.salmonis* and while Carpio et al. (2011) used a recombinant antigene from the novel my32 gene obtained from C. rogercresseyi. Nevertheless, one big step towards the discovery of new vaccine targets was the sequencing, assembly and ongoing annotation of the salmon louse genome. Ensembl Metazoa release 31 includes the *L. salmonis* genome and has made it publicly available. LiceBase (https://licebase.org/), a project developed at the Sea Lice Research Centre (SLRC), provides the genome annotation, a genome browser, Blast functionality and access to related high-throughput genomics data. These tools will provide the basis for the identification of all potential treatment targets and a fraction of these will surely be recognized as useful vaccine antigens. In this context, systemic RNA interference methods are already playing an important part in facilitating research aimed at developing new treatment methods.

**Salmon louse life cycle.** Costello (2006) reviewed in detail the known ecology of the salmon louse. Hamre et al. (2013) proposed that the *Lepeophtheirus salmonis* life cycle has only two chalimus stages. The female salmon louse carries her eggs in a pair of egg sacs extruding from her abdomen. Egg number per female is dependent on the time of year, host species and both the louse size and age. For modelling louse populations, it is considered that sea lice on farmed salmon carry an average of 500 ova while those on wild salmon carry 1000 ova. It has been suggested that this estimation is very conservative (Heuch & Mo, 2001; Costello, 2006). Hatching of the eggs is sequential and females can produce several broods during their lifetime (Pike & Wadsworth, 1999). Free-living nauplii hatch from the external egg

strings and after two free-living, non-feeding nauplius stages (5–15 days, temperature dependent) develop into the infectious copepodid stage. At this point, the lice, which had been using the yolk mass as their main source of energy (Pike and Wadsworth, 1999), attach themselves to the host salmonid by their antennae (Costello, 1993) and start feeding on the host skin. Soon after, the lice subsequently molt into two (2) chalimus stages (I and II), while attached to the host by a special frontal filament (Pike & Wadsworth, 1999). During the ensuing pre-adult stages (I and II) and the adult stage, the lice are able to move freely on the host's surface. This increases the feeding area and, consequently, the damage they induce on the host. Timely female maturation will lead to a new cycle of egg production. Mature female *L. salmonis* stop growing when egg production is initiated and commit to the continuous production of eggs for life (Eichner et al., 2008).

**Feeding methods and impacts on the host** Up to the infective copepodid stage, the salmon louse is dependent on yolk mass as the main source of energy (Pike and Wadsworth, 1999). Copepodids first attach to their hosts via a modified second antennae and maxillae (Fast, 2014). Early reports suggested that upon infection of Atlantic salmon by copepodids, tissue erosion, with little to no inflammation at attachment site occurred (Johnson and Albright, 1992), but recent microarray analyses suggest rapid and sizeable transcriptomic responses to lice with induction of genes involved in innate immune reactions as soon as one (1) day post infection (dpi) (Tadiso et al., 2011). The authors of this study observed a gradual increase of immunoglobulin transcripts in skin and spleen during the period from from 1 to 15 dpi, which could be interpreted as a mounting of adaptive immunity. However, from 5 to 10 dpi, the period when lice molts from copepodids to chalimus abrupt changes in gene expression were registered. Tadisso et al. (2011) was not able to discern if this change is linked to the metamorphosis of copepodids, host immune modulation by the parasite or the already mentioned transition from innate to adaptive immunity. What could be concluded without doubt was that the Atlantic salmon immune response was insufficient as the lice load per fish remained very high at the end of the study (58.4 ± 9.48 lice per fish 15 dpi from 100 copepodids per fish of initial infection).

Chalimi, attached by a special frontal filament, feed on the skin around their point of attachment (Costello, 2006). The epithelial layers covering the scale are removed and the eosinophilic filament is secured directly to the scale below (Fast, 2014). In the adult stage the filament disappears and the parasites maintain adherence to the host by suction involving the ventral regions of a dorsoventrally flattened body (Fast, 2014). This adaptation allows the pre-

adult and adult stages to actively graze over the entire surface of the host, use rasping mouthparts to graze and remove mucus, skin and underlying tissues (Costello, 1993) and select preferred areas on the host to mate, reduce the current drag or find ideal attachment (Fast, 2014; Pike and Wadsworth, 1999).

Histopathological analyses of Atlantic salmon upon maturation of the parasite to the pre-adult life stage describe mild inflammation (Johnson and Albright, 1992). In line with the preciously described findings of Tadiso et al., 2011 (up to 15 dpi), Skugor et al. (2008) described a second innate inflammatory spike following the moult to pre-adult and adult stages of lice (around 33 dpi). The author further describes a transition from acute to chronic wound healing. The increased leaping behaviour described by Costello (1993), which takes places within minutes of the host's exposure to lice and ceases a few days post infection, can therefore be connected to the rapid transcriptomic responses of Atlantic salmon, measured at 1 dpi and extending to 5 dpi by Tadisso et al. (2011). Similarly, the observations of Skugor et al. (2008) following the 33 dpi mark, when the adult life stages of the parasite are present, correlates with the often observed external lesion formation, which progress to larger and deeper wounds that may ultimately result in host mortality (Fast, 2014).

The adult female louse is thought to predominantly ingest blood, whereas the adult male louse (and earlier developmental stages) feed primarily on skin and mucus (Fast, 2014). Several factors dictate the impact of the lice infection on the host: lice developmental stage, number of lice and the fish's general health status. The process starts with the successive removal of mucus and skin, exposing the underlying muscle and, in the case of heavy infestations, can lead to severe lesions with particular incidence on the head region and can go as far as to expose the fish's cranium (Fast, 2014). Stress (e.g., elevated cortisol) and open wounds expose fish to osmoregulatory stress and opportunistic secondary infections. It is also believed that heavy lice infestation is the cause of the sea trout's premature return to fresh water (Birkeland, 1996). The associated drop in salinity may reduce this osmotic stress and/or displace the attached lice (low salinity compromises lice survival and attachment).

**Reproductive anatomy and physiology of the salmon louse.** The anatomy and histology of the sea louse reproductive system has been described by Ritchie et al. (1996). The reproductive structures of both sexes are visible at the preadult stage, but only mature at the adult stage. The female reproductive system consists of a pair of ovaries, oviducts, cement glands, and a single receptaculum seminis. Oogenesis is discernible within the ovaries and oviducts of preadult II and adult females. The male reproductive system consists of a pair of

testes, vasa deferentia, spermatophore sacs, and cement glands. Spermatogenesis is discernible within the testes of preadult and adult males (Ritchie et al., 1996).

The life span of the louse is difficult to measure but adults can over-winter on wild salmon (Jacobsen & Gaard, 1997) and survive for over five and a half months (Hamre et al., 2009). The earlier perception that *L. salmonis* stopped growing and reproducing in the winter was contradicted by studies which showed that although biological processes slow down, *L. salmonis* can still develop to the infectious stage in temperatures as low as 4˚C (Boxaspen and Naess, 2000). Lower temperatures allow sea lice to live longer and grow larger (Pike and Wadsworth, 1999; Heuch et al., 2000). This strong relationship between growth and temperature also creates a visible difference in the size of the eggs produced by the larger over-wintering females (bigger and richer eggs) and the summer brooders (Pike and Wadsworth, 1999). A similar relation is seen in egg number, with the over-wintering females producing more eggs in the spring than the summer-brooders (Costello, 2006).

Several studies have focused on reproductive output of *L. salmonis* and various steps in the mechanism have been clarified. Heuch et al. (2000) found that the female louse can produce up to 11 sets of egg-strings from a single fertilisation. Eichner et al. (2008) observed that the adult female salmon louse is not fully developed immediately following the last molt to adult. In fact, prior to egg production "(…) *the animal matures in a process that includes a large increase of the genital segment and the abdomen, whereas the frontal cephalothorax appears unchanged*." This same study analysed EST sequences from pre adult and adult stages of *L. salmonis* and was able to find a large proportion of novel transcripts. Microarray analysis revealed several highly abundant transcripts induced prior to the release of mature eggs and a 2.5 kb mRNA encoding a novel protein containing three Fasciclin I (FAS 1) domains was further characterised in a subsequent study by Dalvin et al. (2009). This novel protein (denoted as L. salmonis yolk-associated protein, LsYAP) is *"transcribed and translated in subcuticular cells, released into the haemocoel, incorporated into the maturing oocytes, and utilised during embryonic and larval growth"*. Further assessment of the protein's function showed that LsYAP silencing in adult females prior to egg deposition had no maternal consequences but led to the formation of strongly deformed larvae or embryos unable to hatch (Dalvin et al., 2009). In this publication, LsYAP silencing in *L. salmonis* adult females was achieved using an RNA interference (RNAi) procedure.

Posterior studies increased the knowledge of reproductive processes in the salmon louse. Dalvin et al. (2011) characterised two vitellogenins in the salmon louse (LsVit1 and LsVit2), purported to be precursors of salmon-louse egg-yolk glycoprotein. Their production in the

subcuticular tissue of the adult female lice was demonstrated. LsVit1 and LsVit2 (processed into 2 smaller fragments) were, furthermore, detected in maturing oocytes and developing embryos and early larval stages. Their degradation profile through embryogenesis and the early non-feeding larval stages confirmed their role as providers of embryonic and larval nutrition.

Other studies focused on nuclear receptors in the salmon louse, for which the information is still wanting. Sandlund et al. (2015) used the RNAi technique to study the salmon louse ecdysone receptor (LsEcR) and was able to terminate egg production upon knock-down of the gene in pre-adult II females which suggests an important role of LsEcR in reproduction and oocyte maturation. Eichner et al. (2015c) identified an RXR (Retinoid X Receptor) type of nuclear receptor in the salmon louse (LsRXR) and, after knock-down of transcription by RNAi in adult reproducing female lice, observed a strong reduction in transcription of the major yolk proteins as well as a down regulation of genes involved in lipid metabolism and transport.

**Studies in *Caenorhabditis elegans (C.elegans)* have provided mechanistic insights into RNA transport between cells and the process of RNA interference**. The current model of RNAi in *C.elegans* postulates that long dsRNA (>100 bp) is recruited by the dsRNA-binding protein RDE-4 and is processed into double-stranded short interfering RNA (ds-siRNA) by the conserved endonuclease Dicer. The Argonaute protein RDE-1 cleaves one strand of this ds-siRNA producing a single-stranded short interfering RNA (ss-siRNA). This ss-siRNA is then used by RDE-1 as a guide RNA to find mRNAs with complementary sequences. Upon target identification, the RNA-directed RNA polymerase (RdRP) RRF-1 is recruited to generate a large number of short single-stranded secondary siRNAs. In turn, the newly generated secondary siRNAs are bound by additional Argonaute proteins and will cause posttranscriptional silencing by degrading mRNA in the cytoplasm or, as an alternative, initiate co-transcriptional silencing through binding of nascent pre-mRNA in the nucleus. (Jose, 2015)

**Mobile RNAs: biogenesis, stability and signalling.** Jose (2015) reviewed the current knowledge on movement of regulatory RNA between animal cells. According to the author, recent studies suggest that RNA can move from donor cells through cell boundaries and into recipient cells where it is able to regulate genes through sequence-specific interactions with other RNA. Mobile RNAs act therefore in a similar way to steroid hormones, with the main difference being that the hormones rely on binding-specificity of the hormone receptor while the sequence regulated by a mobile RNA is commanded by the base-pairing ability of the mobile RNA itself. Although the reason for its evolutionary conservation and the details of the process of signaling by these so called mobile RNAs are still unclear, there is some agreement

on the steps an RNA must go through to produce gene regulation in recipient cells (Figure 1.1).

As the author reports, a mobile RNA could possibly skip the first two steps of this process if it is present in the extracellular space but has not been generated or secreted by a donor cell. ss-siRNAs were identified by Fire et al. (1998) as the first and most promising mobile RNA candidates due to the systemic and potent RNA silencing they induced. Winston et al. (2002), based on observations made by Parish and Fire (2001) that systemic RNAi was possible in *C.elegans* rde-4 mutants, suggested that siRNAs are not required for systemic RNA and, therefore, should not be regarded as mobile RNA candidates. Currently, biogenesis of mobile RNAs is largely unknown but genetic mosaic analyses suggest that long dsRNA and ds-siRNA, potentially modified by a nucleotidyltransferase, may be exported from donor cells as mobile RNAs (Jose et al., 2011).

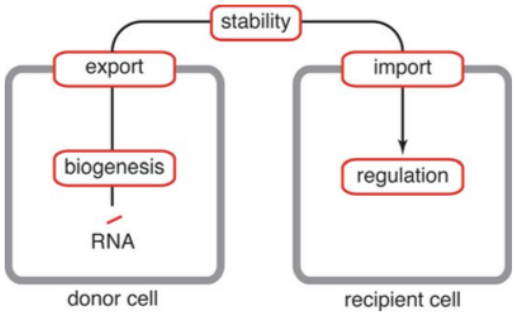


*Figure 3.1 Adapted from Jose (2015). The five steps of gene regulation by RNA: RNA is either produced or recruited from intracellular RNA (biogenesis); secreted (export), protected from degradation in the extracellular space (stability), imported into recipient cells (import) and induces gene regulation (regulation).*

**A stable method for gene knock-down by RNA interference in *L. salmonis*.** Advances in larval production systems and infection procedures (Hamre et al., 2009) made it possible to establish laboratory strains with different properties and have been crucial in creating stable and predictable production conditions that were of use in the first RNAi experiment in *L. salmonis*. In a study conducted by Dalvin et al. (2009) dsRNA was injected into the haemocoel of the cephalothorax of immature adult females and gene knockdown induced by RNAi was found to be stable for up to one month. One other study by Campbell et al. (2009) performed dsRNA mediated knockdown using a putative prostaglandin E synthase type-2 (PGES2) as a target gene. Different life stages of L. salmonis were tested and RNAi was achieved by administering dsRNA either by injection to the haemocoel (adult and pre-adult lice) or soaking in a dsRNA-seawater solution (nauplii and copepodids), depending on the size of the parasite. Downregulation of PGES2 was detected within 24 h, regardless the delivery method or life stage analysed (Lima et al., 2013).

Eichner et al. (2014), after failing to achieve knockdown of genes using the soaking protocol proposed by Campbell et al. (2009), developed a new and robust method for RNAi in nauplii

of the salmon louse. This protocol involves soaking of the nauplius larvae in seawater plus dsRNA and induces a fast acting and significant knock-down effect that is stable up to the copepodid stage. Lower lice mortality was registered when compared to the lice mortality reported in Campbell et al. (2009). Nevertheless, this method did not induce down-regulation of gene expression in dsRNA treated copepodids (unlike Campbell et al, 2009) and the longevity of the gene knock-down was not determined.

The KDEL receptor and COPB2 gene, which are involved in the retrograde transport of proteins from the endoplasmic reticulum to the Golgi, were next targeted for silencing. LsCOPB2 knockdown lice had a significantly higher mortality and failed to develop normally, while both LsCOPB2 and LsKDELR knockdown caused disturbed digestion and the absence of egg strings (Trösse et al., 2014). The RNAi experiment was carried out as previously described by Dalvin et al. (2009) by injecting dsRNA into the haemocoel of preadult II female lice. In this way, LsKDELR and LsCOPB2 were identified as suitable target candidates for additional studies.

**The SID-1 protein: function and role in RNA interference.** As mentioned before, *C.elegans* has been the model organism when it comes to RNAi and mobile RNA. Table 3.2 lists some of the *C.elegans* proteins with roles in RNA transport. SID-1 is a conserved transmembrane domain protein with homologs present in all sequenced vertebrates (Jose, 2015; Winston et al., 2002) and is required in *C.elegans* cells for the import of extracellular dsRNA into the cytosol (Winston et al., 2002). Studies have shown that although *C.elegans* cells that overexpress SID-1 near the source of mobile RNAs showed increased gene silencing, the SID-1 protein is not required for the export of mobile RNAs produced from dsRNA expressed in neurons, muscles or intestinal cells (Jose, 2009). Furthermore, ingested dsRNA can be transported across intestinal cells, even if it does not enter the cytosol of these cells, and is able to generate gene silencing in other tissues that express the SID-1 protein (Jose et al., 2009; Jose 2015). Feinberg and Hunter (2003) also showed that dsRNA entry into the cytosol is not highly dependent on endocytosis and energy as expression of SID-1 in *Drosophila melanogaster* S2 cells with low membrane fluidity and low ATP levels still allowed substantial import of dsRNA.

Another study by Shih and Hunter (2011) suggested that the pore formed by SID-1 in order to transport dsRNA across the cytoplasmic membrane can be selectively opened by dsRNA but not dsDNA or RNA/DNA hybrids. Nonetheless, the SID-1 dependent mechanism for entry of dsRNA into the cytosol must not be the only way that cells internalize dsRNA. As an example, *Drosophila melanogaster* possesses robust cell-autonomous RNAi but lacks homologous to the

*C.elegans* sid-1 gene and systemic RNAi (Feinberg and Hunter, 2003). Additionally, the presence of SID-1 like genes in an organism is not by itself a guarantee that the uptake of dsRNA is done by a SID-1 dependent mechanism. As an example, no defect in systemic RNAi is observed upon knockdown of the SID-1-like proteins present in the migratory locust *Locusta migratoria* (Luo et al., 2012), in the diamondback moth *Plutella xylostella* (Wang et al., 2014) or in the red flour beetle *Tribolium castaneum* (Miller et al., 2012).

*Table 3.1 L. salmonis genes targeted by RNAi experiments and delivery method of double-stranded RNA (dsRNA), adapted from Marr et al. (2014)*

| **Target gene(s)** | **dsRNA delivery** | **Phenotype** | **Reference(s)** |
|---|---|---|---|
| LsPGES2, LsCOPB2, LsRXR, LsPAD1, LsETHR, LsChi2, LsChi4 | Immersion | Reduced mRNA level | Eichner et al., 2014 |
| PGES2 | Immersion | Reduced mRNA level | Campbell et al., 2009 |
| LsYAP | Microinjection | Increased mortality, deformity in offspring | Dalvin et al., 2009 |
| LsKDELR, LsCOPB2 | Microinjection | Lethality, abnormal development, disturbed digestion and absence of egg strings | Tröße et al., 2014 |
| LsPGES2 | Immersion + Microinjection | Knock-down did not affect any essential functions of the salmon louse, neither in the free-living nor the parasitic stages | Eichner et al., 2015a |
| LsChi2 | Immersion | Deformed copepodids with highly reduced infection success | Eichner et al., 2015b |
| LsRXR | Microinjection | Close to zero viable offspring, Transcription of the major yolk proteins was strongly reduced | Eichner et al., 2015c |
| LsEcR | Microinjection | Knock-down of LsEcR terminated egg production | Sandlund et al., 2015 |

*Table 3.2 C.elegans proteins with roles in RNA transport (adapted from Jose, 2015)*

| Protein | Function | Human homologs |
|---|---|---|
| SID-1 | dsRNA selective importer | SIDT1, SIDT2 |
| SID-2 | dsRNA receptor | TLR3? |
| SID-3 | Tyrosine kinase | ACK |
| SID-5 | Endosomal trafficking | Unknown |
| MUT-2 | Nucleotidyltransferase | TUT2/GLD2/PAPD4 |
| RSD-3 | Endocytosis | CLINT1 |

**The sid-1 gene (LsSID1) in *Lepeophtheirus salmonis*** Following the sequencing of the sea louse genome, two genes encoding two putative SID-1 like proteins have been predicted: SID1a and SID1b. Both proteins have domains that include them in the SID-1 transmembrane family. This protein family (PF13965) is defined by the Pfam database (http://pfam.xfam.org/) as a family of transmembrane dsRNA-gated channels which passively transport dsRNA into cells and do not act as ATP-dependent pumps (Feinberg and Hunter, 2003). They are required for systemic RNA interference (Dong et al., 2005; Winston et al., 2002).

**The sea louse intestine** The intestine of the sea louse is a simple tube composed by a short, tubular foregut, a large midgut running from the anterior part of the cephalothorax and into the abdomen, and a short, tubular hindgut (Nylund et al., 1992). The wide midgut constitutes the major part of the alimentary canal. The foregut and hindgut are covered with a thin cuticle and the long, undifferentiated midgut has a thin wall and its anatomy includes ridges, folds and fingerlike projections (Nylund et al., 1992). Peristaltic movements are present and the gut content is pumped back and forth. It is possible to see these movements with a naked eye once the louse has ingested blood from its host. This characteristic will also be explored during the experiments of this study.

## 4. Aims of the study

Previous work performed in the Sea Lice Research Centre has led to the development of RNAi methods targeting the salmon louse nauplii (soaking/immersion in a dsRNA and seawater solution) (Eichner et al., 2014) and the pre-adult and adult stages (microinjection of dsRNA into the haemocoel of the cephalothorax) (Dalvin et al., 2009). There has been, however, no clarifications to how the systemic interference mechanism works and which are the *L. salmonis* equivalents of the RNAi machinery identified in *C. elegans*. The recent sequencing of the salmon louse genome, its subsequent assembly and annotation allowed the identification of two genes encoding two putative SID-1 like proteins (LsSID1a and LsSID1b). The SID-1 protein plays a pivotal role in the RNAi mechanism in *C.elegans* and has been identified as a selective importer of extracellular dsRNA into the cytosol (Winston et al., 2002).

The salmon louse's intestine is of vital importance in the relationship it establishes with the host, Atlantic salmon. After a successful parasite attachment, the fish mounts an immune response but is unable to displace the parasite (Fast et al., 2014; Skugor et al. 2008; Tadiso et al., 2011). Blood is eventually drawn out by the parasite through suction and a direct contact is established between the host's blood and the parasite's intestine, making this organ an interesting target for vaccines or, potentially, salmon blood solutes (e.g. vectors expressing dsRNA, ds-siRNA, ss-siRNA).

The aims of this study are:

1) To assess the viability of intestinal microinjection as a dsRNA delivery method in the salmon louse;
2) To investigate the occurrence of systemic RNAi upon injection of dsRNA into the louse's intestine;
3) To use this model to evaluate the relevance of the *L.salmonis* sid-1 like genes in the process of systemic RNAi through a comparative double gene knock-down experiment targeting, in a first phase, the transcription of LsSID1a and LsSID1b and, in a second phase, of LsTryp1, LsSub and LsYAP (transcribed in the intestine, the ovaries and subcuticular tissue, respectively);

The additional aims are:

4) To evaluate the relevance of the *L.salmonis* sid-1 like genes in the process of systemic RNAi through a comparative double gene knock-down experiment using the microinjection technique described by Dalvin et al., (2009) and the previously mentioned targets;

5) To assess the ability of the salmon louse to ingest dsRNA dissolved in Atlantic salmon's blood;
6) To evaluate the occurrence of systemic RNAi upon ingestion of dsRNA dissolved in Atlantic salmon's blood.

## 5. Material and Methods

### 5.1 Animals

A laboratory strain of *Lepeophtheirus salmonis* (*L.salmonis*, salmon louse) (Hamre et al., 2009) was used for all the experiments described henceforth. Two different procedures were followed to maintain the lice after the different experiments. In the case of the intestine injection trials 1, 2, 3 and the blood feeding experiment, and after the experimental procedures, the lice were maintained in individual cylinders inside flow-through incubator boxes. All boxes were supplied with particle filtered full salinity seawater (salinity 34.5 ppt and temperature 10±0.5°C) (Hamre et al., 2013). Total water volume and water exchange for each cylinder was approximately 31 ml and 34 ml/min respectively. In all the other experiments, the lice were either left to rest for a couple of hours or an overnight period (O/N) in 2 litre boxes filled with particle filtered full salinity seawater (salinity 34.5 ppt and temperature 10±0.5°C) before being placed on farmed Atlantic salmon (*Salmo salar*, average fish weight between 400 g and 1000 g). The fish were kept in single-fish tanks with full salinity seawater (salinity 34.5 ppt and temperature 10±0.5°C) as described by Hamre and Nilsen (2011). These animals were daily hand fed a commercial diet (about 1–2% of their own weight) and maintained according to Norwegian animal welfare regulations.

### 5.2. Molecular Analysis

#### 5.2.1 RNA Isolation

Animals collected for RNA isolation were placed in 1.5 mL tubes containing RNAlater (Ambion) following termination of the experiments. Total RNA was isolated from whole animals using TRI Reagent (Sigma–Aldrich) or Ribozol™ RNA extraction reagent (Amresco). Sample homogenisation was carried out inside 2 mL tubes to which a single 5 mm stainless steel bead had been added. TissueLyser LT (Qiagen) was used for 2 to 3 minutes at 50 Hz to achieve homogenisation. Samples were visually inspected for complete homogenisation. Samples that were not completely homogenised were exposed to an additional minute of homogenisation after which they were, once again, visually inspected. Phase separation was achieved by the addition of 0.2 mL of Chloroform (≥ 99.5%, Sigma-Aldrich) per mL of TRI Reagent used and a subsequent centrifugation at 14800 rpm (21100 x G) for 15 minutes (Haraeus Fresco 21 Centrifuge, Thermo Scientific). The resulting aqueous phase (≅ 450 µL) was transferred to a fresh tube where RNA precipitation was induced by addition of 2-Propanol

(Isopropanol, Kemetyl) and centrifugation at 14800g (21100 x G) for 15 minutes. Resulting pellet was washed twice: first wash using 1 mL of 75% ethanol and a second wash using 0.5 mL of 75% ethanol. Pellet was briefly air-dried and dissolved in a pellet size dependent volume of diethylpyrocarbonate (DEPC, inactivates RNases) treated water. The amount and purity of the isolated RNA was ascertained by spectrophotometry (Nanodrop ND-1000, Thermo Fisher Scientific).

An aliquot corresponding to 1 µg of total RNA went through a DNase treatment using the Deoxyribonuclease I Kit (Invitrogen) in order to remove traces of genomic DNA. Digestion mixture consisted of 1 µg of total RNA, 1 µL of DNase I (1 U/µL) and 1 µL of 10X DNaseI reaction buffer. DEPC treated water was added up to a final volume of 10 µL (Table 5.1). Samples were then incubated for 15 minutes at room temperature after which the reaction was stopped by the addition of 1 µL of a 25mM EDTA solution. Samples were then heat-inactivated by exposure to a temperature of 65°C for 10 minutes. The remainder of the isolated total RNA that did not go through DNase treatment was stored at −80 °C without further dilution as total RNA is better preserved at higher concentration.

*Table 5.1 Reaction for DNase I treatment.*

| Component | Amount |
|---|---|
| Total RNA | 1 µg |
| 10X DNase I reaction Buffer | 1 µL |
| DNase I (1 U/µL) | 1µL |
| DEPC-treated water up to 10 µL | variable |

A negative RNA extraction control (NEC) was included for each RNA isolation procedure. This control consisted of 1mL of TRI reagent that was treated as all the other samples. This control was also subsequently converted to cDNA along with the other samples and was used as a negative control in the Q-PCR assays.

### 5.2.2 cDNA synthesis

cDNA was synthesised using the Reverse Transcriptase enzyme plus the purified and DNase

treated total RNA as template. AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies) was used with slight modifications to the manufacturer's protocol. Master Mix for one (1) reaction was composed of 5 $\mu$L of 2x cDNA synthesis master mix, 1 $\mu$L of Oligo(dT) primers (100 ng/$\mu$L), 0.5$\mu$L of Random primers (100 ng/$\mu$L) and 1 $\mu$L of DEPC treated water. 0.5 $\mu$L of Affinity Script RT/RNase Block enzyme mixture was added per reaction after preparation of the Negative Reverse Transcriptase control (no amplification control, NAC). 8$\mu$L of this Master Mix were pipetted into each reaction tube. Finally, 2 $\mu$L of purified RNA template (corresponding to approximately 200 ng of total RNA) was added to each reaction tube bringing the reaction volume up to 10$\mu$L.

*Table 5.2 Reaction for cDNA synthesis using the AffinityScript QPCR cDNA Synthesis Kit.*

| **Component** | **Amount** |
|---|---|
| First strand master mix (2×) | 5 µL |
| Oligo(dT) primer (100 ng/$\mu$L) | 1 µL |
| Random primers (100 ng/$\mu$L) | 0.5 µL |
| AffinityScript RT/ RNase Block enzyme mixture | 0.5 µL |
| DNase I digested total RNA (100ng/µL → 200 ng) | 2 µL |
| DEPC-treated water to 10 µL | 1 µL |

Negative Reverse Transcriptase control was prepared alongside the other samples and consisted of 8 $\mu$L of the Master Mix (without the Affinity Script RT/RNase Block enzyme mixture). 2 $\mu$L aliquots of three random samples were pooled together and 2 $\mu$L of that mixture was pipetted into the Negative Reverse transcriptase control to serve as template. This control, which lacks the Reverse Transcriptase enzyme, verifies that the signal detected in the subsequent Q-PCR is not due to genomic DNA contamination. All samples were treated for 5 minutes at 25°C, 15 minutes at 42°C and, 5 minutes at 95°C in an Arktik Thermal Cycler (Thermo Scientific).

The final product of this reaction was diluted 10-fold in order to achieve maximum PCR efficiency and facilitate pipetting for the subsequent Q-PCR. cDNA products were stored at -20°C.

*Table 5.3 Thermal cycling conditions of the reverse transcription reaction.*

| Step | Time | Temperature |
|---|---|---|
| Annealing | 5 min. | 25˚C |
| Reverse transcriptase reaction | 15 min. | 42˚C |
| Inactivation of reverse transcriptase | 5 min. | 95˚C |

### 5.2.3 DNA Isolation

Animals used for DNA isolation were conserved in 1.5 mL tubes containing 70% Ethanol following termination of the experiments. Genomic DNA was isolated using GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). Sections of the animals used for genomic DNA isolation were cut into smaller pieces using a sterile scalpel in order to facilitate tissue digestion, and placed inside 1.5 mL tubes. 180 µL of Lysis Solution T, followed by 20 µL of a chilled 20 mg/mL solution of Proteinase K were added and this mixture was vortexed using a vortex mixer (VWR International) and subsequently incubated for several hours at 55˚C. Tubes were regularly vortexed and observed in order to evaluate the degree of completion of tissue digestion. The time necessary to achieve complete tissue digestion is variable but digestion was normally complete after 5 hours. Residual RNA was removed by incubation at room temperature for 2 minutes with 20 µL of RNase A Solution. Lysis was performed until a homogenous mixture was obtained by addition of Lysis Solution C and 15 seconds of thorough vortexing followed by sample incubation at 70˚C for 10 minutes. Lysis solutions provided with the kit are chaotrophic salt containing solutions which insure the complete denaturation of macromolecules. Chaotrophic agents are molecules in water solution that disrupt the hydrogen bonds between water molecules affecting the stability of other macro molecules (proteins and nucleic acids) in solution by weakening the hydrophobic effect.

DNA extraction from the lysate was attained by its selective binding to the silica membrane present in the binding columns provided with this kit. In order to do so, 500 µL of Column Preparation Solution were added to each pre-assembled GenElute Miniprep Binding Column and centrifuged (Heraeus Fresco 21, Thermo Scientific) at 9100 rpm (8000 x G) for 1 minute after which flow-through was discarded. 200 µL of (95-100%) ethanol (Sigma-Aldrich) was added and mixed with the lysate and an homogenous solution was formed. This solution was

then transferred into the treated binding column and centrifuged at 9100 rpm (8000 x G). The collection tube with the resulting flow through was discarded and the column was placed in a new 2 mL collection tube. The following step entailed the washing of the column bound DNA using the ethanol diluted Wash Solution Concentrate (70 prep package; 80 mL 95-100% ethanol added according to kit instructions). First wash was performed by addition of 500 µL Wash Solution and centrifugation at at 9100 rpm (8000 x G) and the collection tube with the flow through was discarded. After transferring the column to a new 2 mL collection tube, a second wash was performed using the same volume of Wash Solution followed by a maximum speed centrifugation (14800 rpm; 21100 x G) for 3 minutes. The collection tube was then emptied and reused for an additional maximum speed centrifugation for 1 minute in order to remove any traces of ethanol. The collection tube was then discarded and the column transferred to a new 2 mL collection tube. Elution of the column bound DNA was performed by pipetting 200 µL of the Elution Solution (10 mM Tris-HCl; 0.5 mM EDTA; pH=9.0) directly into the centre of the binding column. According to the manufacturer's recommendation, the binding column was allowed to stand at room temperature for 5 minutes after the addition of the Elution Solution. The column was subsequently centrifuged for 1 minute at 9100 rpm (8000 x G). After transfer to a new collection tube, the Elution step was repeated using an additional 200 µL of the Elution Solution in order to obtain a second elution with the remaining traces of column bound DNA. Genomic DNA concentrations were measured by spectrophotometry (Nanodrop ND-1000, Thermo Fisher Scientific). Eluted genomic DNA was considered to be of good quality if the ratio of absorbance at 260 nm to 280 nm ($A_{260}/A_{280}$) was between 1.6 and 1.9.

### 5.2.4 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a molecular biology technique used to amplify a single or a few copies of a fragment of DNA. Developed in 1983 and later patented (Mullis et al., 1990) it became a common technique in medical and biological research (Saiki et al., 1985). This method relies on thermal cycling, which by heating and cooling the sample allows for DNA strand separation (melting) and its enzymatic replication. Short DNA fragments known as primers, that contain complementary nucleotide sequences to the target DNA region, are mixed with the template DNA molecules, a mixture of the four essencial deoxyribonucluotides (deoxyadenylate [A], deoxyguanylate [G], deoxycytidylate [C], deoxythymidylate [T]), a thermostable DNA polymerase as well as the appropriate buffer (which includes the necessary

stabilizing salts) and $MgCL_2$ solution (a divalent cation like $Mg^{2+}$ is necessary for optimal polymerase activity). The enzyme DNA polymerase is not able to produce a new DNA chain (*de novo*) but it can extend the annealed primers by adding free nucleotides to their 3' end. By means of repeated thermo cycling that comprises DNA denaturation, primer annealing, and fragment extension, the DNA sequence between the primer pair (forward primer and reverse primer) can be exponentially amplified, as the newly generated DNA fragments can themselves be used as templates for replication.

For each amplified target DNA, a master mix was prepared using the necessary reagents and buffers (Table 5.4). The template DNA and specific primer pairs for each individual reaction were added and this mixture was then put through a pre-defined number of thermal cycles inside a thermocycler (Veriti 96 well Thermal Cycler, Applied Biosystems). When not already available, specific primer pairs were newly designed in order to obtain amplification of the desired region of DNA.

Each cycle of amplification is divided in three steps. The first step is denaturation which entails the separation of the double stranded DNA strands. This step is followed by a drop in temperature which reduces the entropy and allows the specific annealing of the primers. It should be noted that the temperature in this step is dependent on the lowest primer annealing temperature. Finally, during the extension step, the temperature is raised to a value (≅ 72˚C) close to optimum activity temperature for the enzyme Taq Polymerase (75–80 °C) and elongation of the annealed primers is made possible (Chien et al. 1976, Lawyer et al. 1993). The duration of the extension step is dependent on the number of base pairs of the desired PCR product. As a rule-of-thumb, the DNA polymerase polymerizes a thousand bases per minute (1000 bp/min), when operating at its optimum temperature.

In addition to the amplification cycle, two single steps are also performed during a PCR. The first one occurs before the start of the amplification cycle and is therefore called an initialization step. It consists of heating the reaction up to a temperature of 94-96˚C and holding it for 1-3 minutes. This step is called initial denaturation and it is aimed at achieving the complete denaturation of the DNA template at the start of the PCR reaction, which is essential to obtain a good yield of PCR product. Incomplete denaturation of DNA during this step results in the inefficient utilization of template in the first amplification cycle and, consequently, in a poor yield of PCR product. The second of these steps is the final elongation step which takes place after the amplification cycles are completed. This step generally occurs at a temperature of 70-74˚C and lasts between 5 and 15 minutes. This ensures that the remaining single stranded DNA

will be fully extended. When the PCR is completed there is also a final hold step at 4°C which is added in order to preserve the PCR products in the period between the end of the PCR and the time the operator collects the reaction tubes from the thermocycler. After being removed from the thermocycler the PCR products are then stored at 4°C.

*Table 5.4 Master Mix per reaction used for the Polymerase Chain Reaction.*

| Component | Volume | Final concentration |
|---|---|---|
| 5X Green GoTaq flexi buffer (Promega) | 5 µL | 1X |
| $MgCl_2$ solution (25 mM) | 2 µL | 2 mM |
| dNTPs (Deoxyribonucleotides triphosfate) [1.25 mM] | 2 µL | 0.1 mM |
| Forward primer (10 µM) | 0.5 µL | 0.2 µM |
| Reverse primer (10 µM) | 0.5 µL | 0.2 µM |
| GoTaq DNA polymerase (5 U/µL) | 0.2 µL | 1 U per sample |
| Template DNA | 1 µL | __ |
| Nuclease-free water | 13.8 µL | __ |
| Total volume | 25 µL | __ |

*Table 5.5 Thermal cycling conditions for the Polymerase Chain Reaction.*

| Step | Time | Temperature | Number of cycles |
|---|---|---|---|
| Initial denaturation | 2:00 | 94°C | 1 |
| Denaturation | 0:30 | 94°C | Variable |
| Annealing | 0:15 | Variable | |
| Extension | Variable | 72°C | |
| Final extension | 5:00 | 72°C | 1 |
| Final hold | ∞ | 4°C | 1 |

#### 5.2.4.1 Measuring *L.salmonis* bacterial content using the Polymerase Chain Reaction

16S ribosomal RNA (rRNA), a component of the 30S small subunit (SSU) of the prokaryotic ribosomes is coded by a region of DNA (rDNA) that is known to have slow rates of evolution and to be interspersed and highly conserved among the prokaryotes (Frank et al., 2008). For this reason, the genes coding for 16S rRNA have been targeted in the investigation of microbial communities, where small differences allow for the distinction of phylogenetic groups (Frank et al., 2008; Jiang et al., 2006). On the other hand, the highly conserved SSU rRNA gene regions provide close to universal sequences for the identification of bacteria. These studies rely on a set of primers known as 27f (bacterial forward primer) and 1492r (universal reverse primer), which amplify the DNA between positions 27 and 1492 of the bacterial 16S rRNA gene (Jiang et al., 2006; Weisburg et al., 1991; Wilson et al., 1990).

### 5.2.5 Agarose Gel

Identity and size of the newly synthesised PCR products was verified in 1% agarose gel electrophoresis in 1x TAE buffer (Tris-Acetate-EDTA). In order to visualize the products and track their progress in the agarose gel, the fluorescent Gel Red Nucleic Acid Stain (Biotium) was added to the hot agarose solution (1 μL of a 25X solution per 25 μL of gel; final Gel Red concentration: 1X).

MassRuler DNA Ladder Mix (Thermo Scientific) was run side by side with the samples to allow an approximate quantification and sizing of the DNA fragments.

### 5.2.6 Real Time PCR (RT-PCR) or Quantitative PCR (Q-PCR)

Quantitative PCR is similar to traditional PCR, the major difference being that with Q-PCR, the amount of PCR product is measured after each round of amplification, in contrast with normal PCR which only measures the amount of PCR product at the end point of amplification. In Q-PCR the amplification products are measured as they are produced by means of a fluorescent dye that binds directly or indirectly (via a labeled hybridising probe), to the accumulating DNA molecules. Fluorescence values are recorded during each cycle of the amplification process. There are two main types of chemistries to detect PCR products using real-time PCR instruments: SYBR® Green-based detection or TaqMan®-based detection. The former uses SYBR® Green dye (a dsDNA binding dye) to detect PCR product as it accumulates

during PCR while the latter uses a fluorogenic probe specific to a target gene. The SYBR® Green dye fluoresces when bound to dsDNA and, as the polymerisation cycles continues, the dye binds to the double stranded product and allows its quantification by a net increase in fluorescence. (qPCR Technical Guide, 2008).

The basis for real-time quantitative PCR (Q-PCR) were laid down by Higuchi, *et al*. (1993), whose experiments revealed that the relationship between the amount of target DNA and the amount of PCR product generated after a specific number of amplification cycles is linear. A researcher can therefore derive initial sample concentrations from the number of amplification cycles needed to reach a threshold. This threshold, set above the amplification baseline and within the exponential increase phase, is chosen by the researcher himself. Quantitative gene expression can be presented using two methods: absolute and relative quantification. Absolute quantification relates the number of copies of a gene to a standard curve. Relative quantification infers the expression of a gene of interest by relating it to a constitutively and uniformly expressed standard gene or an untreated control (Livak and Schmittgen, 2001). The point at which fluorescence is first detected as statistically significant above the baseline or background, is called the threshold cycle or Ct Value. This value is inversely correlated to the logarithm of the initial copy number and is determined from a log-linear plot of the PCR signal versus the number of cycles. Following this logic, the higher the initial amount of sample DNA, the sooner the accumulated product is detected in the fluorescence plot, and the lower the Ct value.

Real-Time Quantitative PCR was run on an CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) using SYBR® Select Master Mix (Thermo Fisher Scientific) with slight modifications to the manufacturer's instructions. Table 5.6 lists the components and volumes of the several reagents used per well. A Master mix was prepared according to the number of used wells and 8µL of this Master mix were distributed per well in a Hard-Shell® Low-Profile Thin-Wall 96-Well Skirted PCR Plates (Bio-Rad). 2 µL of each of the templates (cDNA) were then distributed to their assigned wells in a separate and clean room, bringing the reaction volume to 10 µL per well. Negative Reverse Transcriptase control (NAC), no template control (no template added to master mix, NTC) and negative RNA extraction control (NEC) were included as negative controls in every 96 well plate.

Relative gene expression data obtained during Real-Time Quantitative PCR was analysed using the ΔΔCt method (Livak and Schmittgen, 2001) and used to calculate fold changes ($2^{-\Delta\Delta Ct}$) relative to the uniformly expressed housekeeping gene translation elongation factor 1 α (eEF1α). This gene has previously been validated as a reference gene for the various life stages of *L.salmonis* (Frost and Nilsen, 2003) and was used to normalise target gene Ct values. The

endpoint of the Q-PCR is achieved when the Ct value reaches the threshold line. This value is inversely proportional to the amount of nucleic acid present in the sample. Baseline and threshold values were set automatically by the CFX Manager™ Software (Bio-Rad) and the threshold value was corrected manually to be equal in all runs.

*Table 5.6 Real-Time Quantitative PCR Master mix design per well.*

| Component | Volume | Final concentrations |
|---|---|---|
| 2X SYBR Select Master Mix | 5 µL | 1X |
| Forward primer (10 µM) | 0.5 µL | 0.5 µM |
| Reverse primer (10 µM) | 0.5 µL | 0.5 µM |
| RNase free water | 2 µL | __ |
| Total volume | 8 µL | __ |

*Table 5.7 Thermal cycling conditions for Real-Time Quantitative PCR.*

| Step | Time | Temperature | Number of cycles |
|---|---|---|---|
| Heat-labile Uracil-DNA Glycosylase (UDG) Activation | 2:00 | 50˚C | 1 |
| AmpliTaq DNA Polymerase Activation | 2:00 | 95˚C | 1 |
| Melting | 0:15 | 95˚C | 40 |
| Annealing/Extension | 1:00 | 60˚C | |
| Dissociation Step | Melt Curve | 60-95˚C | 1 |

#### 5.2.6.1 SYBRGreen assay design

Groups were formed depending on RNAi target gene. As mentioned above, gene expression fold changes (ΔΔCt method) were measured relative to the uniformly expressed housekeeping gene translation elongation factor 1 α (eEF1α) which has been previously validated as a reference gene for the various life stages of *L.salmonis* (Frost and Nilsen, 2003).

*Table 5.8 SYBRGreen assay targets*

| Target Gene | Reference |
|---|---|
| LsSID1a | Systemic RNA interference–deficiency gene (Predicted from the annotated sea louse genome, LiceBase.org) |
| LsSID1b | Systemic RNA interference–deficiency gene (Predicted from the annotated sea louse genome, LiceBase.org) |
| LsYAP | Yolk-associated protein (Dalvin et al., 2009) |
| LsSub | Subolesin (Ls4D8 gene) (Birkeland, 2010) |
| LsTryp1 | Intestinal Trypsin gene (Kvamme et al., 2004) |
| EF1α | Translation Elongation Factor 1α (Frost and Nilsen, 2003) |

The assays were designed in order to evaluate the effect of the SID genes knock-down on systemic RNAi silencing of three (3) other genes that are transcribed in different locations in the salmon louse: the yolk-associated protein gene (LsYAP) transcribed in the subcuticular tissue, the subolesin gene (LsSub) transcribed in the ovaries and an intestinal trypsin gene (LsTryp1) transcribed in the intestine. In order to achieve this goal, double knock-downs of the SID genes and one other gene were produced by dsRNA injection into the cephalothorax of pre-adult II and adult females. Assays for the blood feeding experiment focused on SID genes and ΔCt variations were evaluated using the housekeeping gene EF1α.

### 5.2.7 Primers used in the experiments

*Table 5.9 List of the primers used*

| Primer code | Sequence (5´-3´) | Supplier |
|---|---|---|
| b3472 | GCGATATGACCAAACTTAGAGCTACA | Sigma-Aldrich |
| b3473 | GTCCTGGATTTCTGATTCCTGAA | Sigma-Aldrich |
| b3476 | TAATACGACTCACTATAGGGGCGATATGACCAAACTTAGAGCTACA | Sigma-Aldrich |
| b3477 | TAATACGACTCACTATAGGGGTCCTGGATTTCTGATTCCTGAA | Sigma-Aldrich |
| b1137 | GGCTTTAGGCTCTGATGTAAGCA | Sigma-Aldrich |
| b1138 | TGTTCACAAGTAAAAGCAGTGTCATTT | Sigma-Aldrich |
| a179 | GTCTCCTTTGTCAGCTGGCGAAAT | Sigma-Aldrich |
| a201 | GGAGTCGTTAAATCGCTGCTGAATT | Sigma-Aldrich |
| a180 | TAATACGACTCACTATAGGGGTCTCCTTTGTCAGCTGGCGAAAT | Sigma-Aldrich |
| a202 | TAATACGACTCACTATAGGGGGAGTCGTTAAATCGCTGCTGAATT | Sigma-Aldrich |
| b1454 | CCAGGGAGAAGCCCATCTT | Sigma-Aldrich |
| b1455 | GCGTCGTATTGTTCAGTTAACTTTTGA | Sigma-Aldrich |
| b3470 | GCTGTTCCCCCTCAGATCAAA | Sigma-Aldrich |
| b3471 | GGTCCTTTTCTGGGAGAGCAA | Sigma-Aldrich |
| b3474 | TAATACGACTCACTATAGGGGCTGTTCCCCCTCAGATCAAA | Sigma-Aldrich |
| b3475 | TAATACGACTCACTATAGGGGGTCCTTTTCTGGGAGAGCAA | Sigma-Aldrich |
| b1261 | CACCTTCTCCAGTTCTTAAAGCTGTT | Sigma-Aldrich |
| b1262 | AGATCATGGTCTCATCAATAGATCCA | Sigma-Aldrich |
| b1482 | GATCCACTCGAGACGGATGCGGATACTT | Sigma-Aldrich |

| | | |
|---|---|---|
| b1481 | TGATCGCATCTTCTGTGTTGACCGTGTG | Sigma-Aldrich |
| b3725 | TAATACGACTCACTATAGGGGATCCACTCGAGACGGATGCGGATACTT | Sigma-Aldrich |
| b3726 | TAATACGACTCACTATAGGGTGATCGCATCTTCTGTGTTGACCGTGTG | Sigma-Aldrich |
| b3821 | TGCCATTTGGCACGAAACAC | Sigma-Aldrich |
| b3822 | TCTCGAGTGGATCCCCCATT | Sigma-Aldrich |
| b1633 | GGCATCACTACGACCGTCACAAAGA | Sigma-Aldrich |
| b1483 | GGGAGATGATCCCTCACTCACATATGCCTT | Sigma-Aldrich |
| b3727 | TAATACGACTCACTATAGGGGGCATCACTACGACCGTCACAAAGA | Sigma-Aldrich |
| b3728 | TAATACGACTCACTATAGGGGGGAGATGATCCCTCACTCACATATGCCTT | Sigma-Aldrich |
| b3823 | CCTTCAGAGGCAGCTACGAC | Sigma-Aldrich |
| b3824 | GCTCGTACCCGCTATCCTTC | Sigma-Aldrich |
| b3630 | ATAGGCAAGTAAGTTTGCTGCT | Sigma-Aldrich |
| b1640 | TGATCGGATAATTGGACGGCTT | Sigma-Aldrich |
| a205 | TAATACGACTCACTATAGGGATAGGGCGAATTGGGTACCG | Sigma-Aldrich |
| a206 | TAATACGACTCACTATAGGGAAAGGGAACAAAAGCTGGAGC | Sigma-Aldrich |
| 27f | AGAGTTTGGATCMTGGCTCAG | Sigma-Aldrich |
| 1492r | CGGTTACCTTGTTACGACTT | Sigma-Aldrich |
| k39f1 | GCTTTAAGAACTGGAGAAGGTGGAC | Sigma-Aldrich |
| k39f4 | GTTGCAGCCAATGCTGTTCCCC | Sigma-Aldrich |

#### 5.2.7.1 Confirmation of LsSID SYBR green assay optimization by the evaluation of newly designed primer pairs effect on amplification efficiency

Real-time PCR quantification is based on the relationship between initial template concentration and the Ct value obtained during the amplification cycle. In order to perform an accurate and reproducible quantification of the sample, an optimal Q-PCR assay is of unsurmountable importance. Literature defines an optimized Q-PCR assay as one in which the obtained Ct value plotted against the log of the starting quantity of template for a dilution series generates a linear standard curve ($R^2 > 0.980$) with high amplification efficiency (90-105%) and consistency across replicate reactions.

In order to evaluate the efficiency of the real-time amplification of a template using the newly synthesized SID1b set of primers (b3821+b3822; b3630+b1640), SID1a set of primers (b3823+b3824) and the previously tested EL1α set of primers (b434+b435), a dilution series of a template with known concentration was prepared. Q-PCR reaction conditions were previously optimized in the Sea Lice Research Centre, are considered highly efficient for the existing sets of primers and have been used as a standard for several years (Heidi Kongshaug, senior engineer, 2015, personal communication). Therefore, the master mix design per well as well as thermal cycling conditions were kept the same as previously described. The Ct values obtained for each dilution in the series were plotted against the log of the starting quantity of template. The equation of the linear regression line, along with the coefficient of determination ($R^2$) was used to evaluate Q-PCR optimization. The following equations were used to calculate Efficiency (E):

$$E = 10^{-1/slope}$$

$$\%\, Efficiency = (E - 1) * 100\%$$

#### 5.2.7.2 Note regarding primer pairs targeting LsSID1b in the SYBR green assays.

As previously described, transcription regulation of LsSID1b was quantified via SYBR green assay using two different set of primers: b3821+b3822 and b3630+b1640. This strategy was used for LsSID1b as there were two sets of primers available and, after analysis of the fragments each primer pair produced, it was concluded that primer pair b3821+b3822 annealed to a region of LsSID1b cDNA that was located within the region of LsSID1b cDNA that was

used for the synthesis of LsSID1b dsRNA via T7 primers (see section 5.3.1). However, the other primer pair (b3630+b1640) did not share this characteristic and annealed to a region of the LsSID1b cDNA that was completely different and did not overlap whatsoever with the cDNA region used for synthesis of LsSID1b dsRNA. As there were previous reports of SYBR green assay results being compromised when a situation of overlap between cDNA sequence used for dsRNA production and SYBR green amplification occurred (Heidi Kongshaug, senior engineer, 2016, personal communication), it was decided to use both LsSID1b SYBR green primer pairs available.

Later emerged that by using these two sets of primers, one was able to infer the presence of LsSID1b dsRNA inside the salmon louse, particularly in the blood+dsRNA feeding experiment.

### 5.3 RNA Interference

RNA interference (RNAi) is one several closely related RNA silencing phenomena and, like posttranscriptional and transcriptional gene silencing, is an ancestral conserved process. In fact, RNA silencing is found in a large variety of eukaryotic organisms and can be classified as a highly conserved pathway. RNA silencing is therefore a general term for a number of phenomena that occur when the presence of short RNA molecules triggers the repression of homologous gene sequences (Almeida and Allshire, 2005).

Work performed by Napoli et al (1990) and Fire et al. (1998) identified this phenomena in plants and *C.elegans,* respectively. Fire et al. (1998) went as far as to identify double-stranded RNA (dsRNA) of different length and origin as a precursor to the short RNA sequences and, in fact, the primary trigger of the cascade. Later publications (Bernstein et al. 2001, Hammond et al 2000, Zamore et al. 2000) made it clear that the dsRNA molecules, which can be of endogenous or exogenous origin, were converted to shorter intermediates (21-28 bp) by the enzyme Dicer. These intermediates were named small interfering RNAs (siRNAs) and were found to serve as guide sequences that initiate a process by which a multicomponent nuclease named RISC (RNA-induced silencing complex) destroys specific messenger RNAs. In fact, RNAi inhibits gene expression in a sequence-specific manner and one of the features of this phenomenon is the reduction in the level of mRNAs that are homologous to the uncleaved dsRNA (Hammond et al., 2000). The last piece of the RNAi puzzle was the identification of the Argonaute protein as the catalytic engine of RNAi. Argonaute enables siRNA directed cleavage of mRNA's by RISC (Liu et al., 2004, Song et al., 2004). Therefore, the RNAi

pathway consists of two phases: the initiator phase (dsRNA converted to siRNA) and the effector phase (RISC-mediated cleavage of target mRNA).

### 5.3.1 Synthesis of dsRNA for the genes of interest (LsSID1a, LsSID1b, LsSub, LsYAP, LsTryp1)

dsRNA for the genes of interest was synthesised using the MEGAscript® RNAi Kit from Applied Biosystems/Ambion. Manufacturer's protocol was followed used with slight modifications. PCR templates were produced via a single PCR with T7 promoters appended to both primers. Sea louse adult female cDNA was used as template for the synthesis of dsRNA for LsSID1a, LsSID1b, LsSub, LsYAP and LsTryp1. A plasmid with a CPY cDNA insert was used as template for the synthesis of CPY dsRNA. Table 5.10 lists the final dsRNA fragment sizes as well as the T7 primers used to produce the starting PCR products.

*Table 5.10 T7 primers used to produce starting PCR products per target gene and final dsRNA fragment length*

| Target gene | Set of T7 primers used (code) | Final dsRNA fragment length |
|---|---|---|
| LsSID1a | b3725+b3726 | 449 bp |
| LsSID1b | b3727+3728 | 607 bp |
| LsYAP | b3476+b3477 | 916 bp |
| LsSub | a180+a202 | 356 bp |
| LsTryp1a | b3474+b3475 | 431 bp |
| CPY | a205+a206 | 860 bp |

T7 PCR products were produced as previously described with an annealing temperature of 55˚C and an extension step of 1:00. Products were verified on 1% agarose gel to check uniqueness and size. Table 5.11 lists the components of the Transcription reaction per sample (final volume: 20 μL). Reagents were thawed at room temperature and micro centrifuged. Transcription reaction assembly was performed at room temperature according to manufacturer's recommendations. Tubes were thoroughly mixed and incubated O/N at 37˚C. The following day, a second incubation step (5 minutes at 75˚C) was performed after which the tubes were left to cool down at room temperature to allow RNA annealing, forming dsRNA. Nuclease digestion was subsequently performed in order to remove template DNA and any

ssRNA that did not anneal (Nuclease digestion reaction components and amounts listed in table 5.12). Tubes were incubated at 37°C for one (1) hour.

*Table 5.11 Components of a single 20 µL transcription reaction. Amount of linear template DNA was increased (in relation to manufacturer's protocol) due to the fact that the T7 PCR products were not purified*

| Component | Amount |
|---|---|
| Linear template DNA (T7 PCR product) | 8 µL (of a 10 µL PCR) |
| 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 |

*Table 5.12 Components of a single 50 µl nuclease digestion reaction.*

| Component | Amount |
|---|---|
| dsRNA | 20 µL |
| Nuclease free water | 21 µL |
| 10X Digestion Buffer | 5 µL |
| DNase I | 2 µL |
| RNase | 2 µL |

The next step was the purification of dsRNA to remove proteins, free nucleotides and nucleic acid degradation products. dsRNA Binding mix was prepared as described in table 5.13.

*Table 5.13 Components of a single 500 µl dsRNA binding mix.*

| Component | Amount |
|---|---|
| Nuclease treated dsRNA | 50 µL |
| 10X Binding Buffer | 50 µL |
| Nuclease free water | 150 µL |
| 100% Ethanol | 250 µL |

The entire 500 µL of the dsRNA Binding mix was pipetted into the Filter Cartridge provided by the manufacturer, placed inside a collection tube (also provided with the kit) and centrifuged at 14800 rpm (21100 x G) for two (2) minutes (Haraeus Fresco 21 Centrifuge, Thermo Scientific). Flow through was discarded and the filter transferred to a new collection tube. The filter was subsequently washed two times with 500 µL of 2X Wash Solution which was drawn through the filter as in the previous step. This liquid was then discarded and an extra thirty (30) seconds of maximum speed centrifugation (14800 rpm) was performed in order to remove any traces of 2X Wash Solution. Finally, the dsRNA was eluted from the filter by applying 80 µL of hot (95˚C) Elution Solution (10 mM Tris-HCl pH 7; 1 mM EDTA) and centrifuging the new collection tube holding the filter at 14800 rpm for two (2) minutes (first elution). After transferring the filter to a new collection tube, a second elution was performed with 60 µL of hot (95˚C) Elution Solution following the exact same procedure. dsRNA concentration was measured by spectrophotometry (Nanodrop ND-1000, Thermo Fisher Scientific). Upon completion of the procedure, dsRNA was stored at -20˚C.

### 5.4 Micro Injection of dsRNA in *L.salmonis* pre-adult II and adult females

Two types of injection were performed on sea lice: cephalothorax micro injection and intestine micro injection. In both cases the lice were injected with a solution of 600 ng/$\mu$L dsRNA under a dissecting microscope (Olympus SZX9). Prior to the injection procedure, 1$\mu$L of saturated and filtered bromophenol blue was added per 50 $\mu$L of dsRNA solution. In order to provide support for the injection, lice were placed inside a Petri dish on top of a circular sheet of absorbent paper moistened with seawater. This procedure restricts the lice's movement facilitating the injection and, in addition, creates a humid environment which partially shields the specimen from the increase in temperature caused by the optical microscope's illumination (which is transmitted through the specimen).

The injection mechanism was composed by custom glass tips (0.5 mm interior diameter) coupled to a micro injector controlled by blowing air into a tube. Lice were injected with 0.2 – 0.5 $\mu$L of the dsRNA solution. Although the exact volume of dsRNA solution is impossible to measure due to the nature of the equipment used, care was taken by the operator in order to inject a similar volume of dsRNA solution in each louse.

### 5.4.1 *L.salmonis* Intestine Micro Injection

To the best of this author's knowledge, this is one of the first attempts (if not the first) to perform a direct injection into the louse's intestine. As such, several trials were performed in order to evaluate the ideal location for the injection. Early trials were aimed at hitting the intestine by placing the needle dorsally between the plates of the cephalothorax and releasing the dsRNA solution as soon as the intestinal perforation was sensed by the operator. Successful injections were identified by the appearance of a single blue line following the length of the intestine. High mortalities caused by these injections highlighted the need for different injection entry points on the lice. Therefore, direct injections into the intestine were attempted in two additional locations: (1) the junction between the cephalothorax and the genital segment and (2) the abdominal area. For both these techniques, the needle was placed dorsally in a 45° to 90° angle relative to the lice and the exoskeleton was perforated in order to hit the intestine. Again, successful injections were identified by the appearance of a single blue line following the length of the intestine. As control dsRNA, an unrelated 860bp fragment of cod trypsin gene (CPY) was also injected and its concentration adjusted to 600ng/$\mu$L.

### 5.4.2 *L.salmonis* Cephalothorax Micro Injection

For the cephalothorax injections, the needle was placed dorsally into the haemocoel of the cephalothorax as previously described (Dalvin et al., 2009). The dispersal of blue colour made it possible to see dispersion of the injected solution inside the haemocoel of the cephalothorax. Successful injections were visually identified by this phenomenon.

#### 5.4.2.1 The double gene knock-down experiment

Following the unsuccessful intestine injection trials, a new experiment was designed in order to evaluate the influence of the LsSID genes in the process of RNA interference in *L.salmonis*. Figure 5.1 illustrates this experiment. One hundred and thirty-three (133) pre-adult II female lice were divided in two groups which were injected (cephalothorax injection) with different solutions of dsRNA. Group X was injected with an equimolecular mixture of one (1) LsSID1a dsRNA fragment and one (1) LsSID1b dsRNA fragment with the objective of knocking down this pair of genes (henceforth referred to as LsSID). Group Y was injected with

one (1) fragment of an unrelated cod trypsin (CPY) (Figure 5.1). dsRNA fragments identity, design and concentration as well as lice cephalothorax injection method as previously described.

After the injection procedure, the lice were left to rest for a period of two hours in 2 litre boxes filled with particle filtered full salinity seawater (salinity 34.5 ppt and temperature 10±0.5°C). No mortalities were registered in this period of time and the two groups of lice were transferred to Atlantic salmon (procedure described in section 5.4.3) and maintained for 13 days (312h). The lice in each group were transferred to different fish which were then kept in single-fish tanks as described by Hamre and Nilsen (2011). Lice losses were registered daily. Upon completion of this 13-day period on fish, five (5) animals from the X group were terminated by individually transferring each animal into a 1.5 mL tube containing RNAlater (Ambion). These animals, which were destined for LsSID gene knock-down confirmation were then preserved at 4˚C while awaiting further processing. If animal processing was not possible in the forty-eight hours after the termination procedure, the 1.5 mL tubes were preserved at -20˚C until processing could begin, as indicated in the manufacturer's protocol.

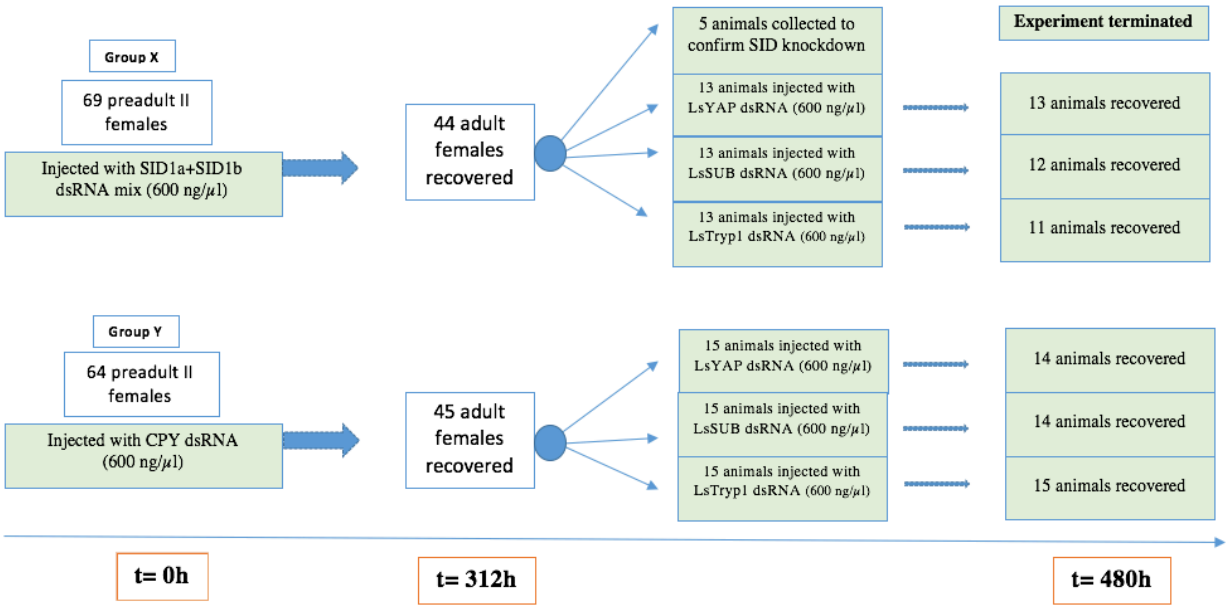


*Figure 5.1 Schematic representation of the double gene knock-down experiment. Subgroups of 5 animals from each group were processed (RNA extraction, DNase I treatment, cDNA synthesis) and analysed by Q-PCR*

The remaining animals in groups X were equally divided in three (3) groups and a second dsRNA cephalothorax injection was performed with the objective of knocking down a second gene of interest. Each group was injected with either a LsYAP dsRNA fragment, a LsSub

dsRNA fragment or a LsTryp1 dsRNA fragment. The same procedure was repeated with the animals in group Y. Following a rest period of two hours the animals were again transferred to Atlantic salmon. This was executed as previously described. The experiment was terminated 7 days (168h) later. At this point, the animals were individually transferred to a 1.5 mL tubes containing RNAlater (Ambion) and preserved as previously described.

### 5.4.3 Lice and host handling during RNAi experiments

All RNAi experiments were performed in the Wet Lab of the Sea Lice Research Centre (SLRC). After the injection procedure, the circular sheet of absorbent paper on which the lice were placed was transferred to a 2 litre box filled with sea water (salinity 34.5 ppt and temperature 10 °C). This procedure was used in order to avoid individual handling of the lice after the injection procedure and therefore reduce handling stress on the lice. Lice were grouped in boxes according to injection type and target gene. Different groups were kept in separate boxes. As described above, the lice were allowed to rest inside the boxes for either a couple of hours or an overnight period before being placed on their host, farmed Atlantic salmon (*Salmo salar*, average fish weight between 400 g and 1000 g). Fish were sedated before being infected with the newly injected lice. Sedation was performed according to Norwegian Animal Welfare regulations. Sedation bath was prepared by addition of benzocaine (final concentration 60 mg/l) and methomidate (final concentration 5 mg/l) to 10 litres of water (salinity 34,5 ppt and temperature 10±0.5°C).

Fish were transferred from individual tanks into this solution and were allowed to stand in it for up to 3 minutes. The operator was present during the complete procedure and fish sedation was carefully assessed in order to avoid overexposing the fish to the sedation bath. When sedation was achieved the fish were carefully rinsed with water in order to remove traces of the chemicals from its scales. This procedure was followed to avoid exposing the lice to the chemicals used in the sedation procedure. Groups of lice were then placed “belly up” in a small rectangular moistened piece of paper which was placed in the fish’s lateral line, between the pelvic and the dorsal fin and against the fish’s scales for 3-5 seconds. The paper was then removed and lice attachment was visually confirmed. Following this procedure, the fish were again transferred to single-fish tanks as described by Hamre and Nilsen (2011). Nets with appropriate mesh size were placed in all the tanks outflow to recover lost lice. The nets were checked daily and losses were registered for the duration of the experiment.

### 5.5 Blood+dsRNA feeding experiment

Sixty-six (56) *L.salmonis* preadult II males were left to starve for a period of thirty-four (34) hours in two (2) litre boxes filled with sea water (salinity 34.5 ppt and temperature 10±0.5°C). Care was taken not to overpopulate the boxes in order to reduce stress induced by overcrowding. Following this 34h period, the sixty-six (56) lice were divided in two groups: one group of fifty-nine (49) animals (group A) and a group of seven (7) animals (Group B). Group B (control group) was terminated at t=0 by individually transferring each animal into a 1.5 mL tube containing RNAlater (Ambion). Animals were then preserved at 4˚C while awaiting further processing.

Animals in groups A were individually placed inside a Petri dish, on their ventral side, on top of a drop (≅15 µL) of freshly collected Atlantic salmon blood onto which an equimolecular mixture of LsSID1a and LsSID1b dsRNA fragments were dissolved to a final concentration 50ng/µL. Each louse was allowed to stand for two (2) minutes on top the blood+dsRNA solution. Due to the nature of this experimental procedure and the need to minimize exposure of the lice to room temperature, each Petri dish was placed inside an ice filled Styrofoam box for the duration of the procedure. Atlantic salmon's blood was collected using a Green Vacutainer ® Blood Collection Tube (BD) which includes the anticoagulants sodium heparin and lithium heparin as additives to avoid blood coagulation. The tube was inverted eight (8) times according to manufacturer's protocol to achieve mixing of the anticoagulant (heparin) with the gathered blood. Blood collection procedure followed Norwegian animal welfare regulations.

Following this experiment, forty-three (43) lice were transferred to individual cylinders inside flow-through incubator boxes (previously described). Six (6) lice were terminated following completion of the experimental procedure (t=0) by individually transferring each animal into a 1.5 mL tube containing RNAlater (Ambion).

## 6. Results

### 6.1 Micro injection of dsRNA in pre-adult II and adult females to obtain RNA interference mediated knockdown of target genes

#### 6.1.1 Intestine injection viability trials

Several trials were performed in order to test the viability of this type of injection in the salmon louse. The salmon louse intestinal injection is a delicate and meticulous procedure so several trials were planned and executed whenever lice were available. Some of the trials consist of groups formed by few animals due to their limited and somewhat unpredictable availability.

**Injection trial 1:** 41 pre-adult II female lice were injected with dsRNA corresponding to 3 genes of interest (LsSub, LsTryp1 and LsYAP) in addition to an unrelated dsRNA fragment of cod trypsin gene (CPY). Concentration of all dsRNA's was adjusted to 600ng/$\mu$l. For the cephalothorax injections (CT), the needle was placed dorsally into the haemocoel of the cephalothorax. For the intestine injections (INT) the needle was placed dorsally between the plates of the cephalothorax and the dsRNA solution was injected when the intestinal perforation

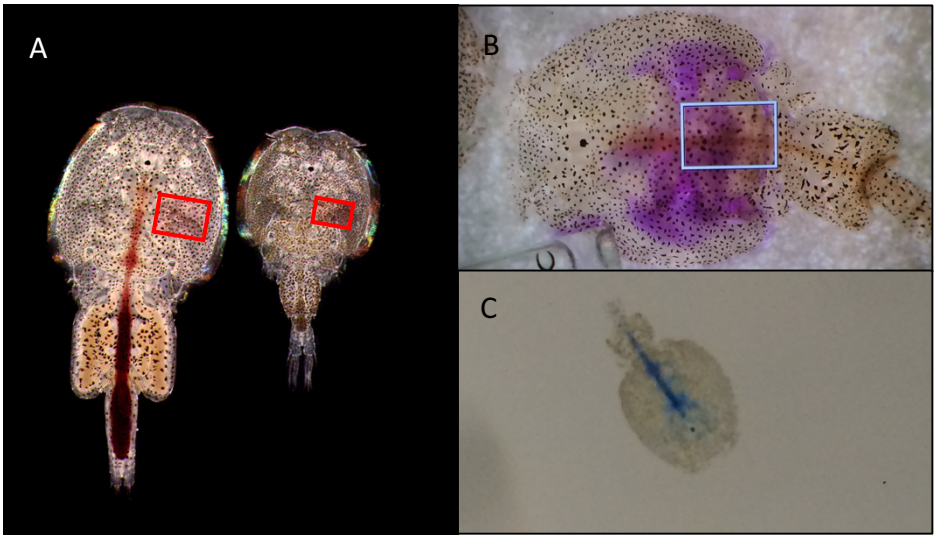


*Figure 6.1 (A) Red boxes mark the point of needle penetration during cephalothorax micro injection in the adult female salmon louse (left) and adult male salmon louse (right). Same point of penetration was used for intestine injections trials 1-4 (B) Cephalothorax [CT] injected female louse; (C) Intestine [INT] injected male louse.*

was sensed by the operator (Figure 6.1). Criteria defined in the material and methods section were used to evaluate successful injections. Following the injection procedure, the lice were

kept in hatching cylinders (Hamre et al., 2013) with running sea water (salinity 34.5 ppt and temperature 10±0.5°C) for the duration of the experiment.

*Table 6.1 dsRNA intestine injection trial 1. CT stands for Cephalothorax injection and INT stands for Intestine injection*

| Targeted gene - dsRNA | Type of injection | Number of injected animals (t=0) | Surviving animals at experiment termination (t=120h) | Mortality |
|---|---|---|---|---|
| CPY | CT | 5 | 5 | 0% |
| LsSub | CT | 4 | 2 | 50% |
| LsTryp1 | CT | 5 | 5 | 0% |
| LsYAP | CT | 5 | 5 | 0% |
| | | | | |
| CPY | INT | 5 | 5 | 0% |
| LsSub | INT | 7 | 4 | 43% |
| LsTryp1 | INT | 5 | 5 | 0% |
| LsYAP | INT | 5 | 5 | 0% |

**Injection trial 2:** 9 adult females were injected with dsRNA corresponding to one of the genes of interest (LsYAP). dsRNA concentration, injection technique and criteria for successful injection were the same as in injection trial 1. Lice were kept in hatching wells for the duration of the experiment (7 days, 168h), as previously described.

*Table 6.2 dsRNA intestine injection trial 2. CT stands for Cephalothorax injection and INT stands for Intestine injection*

| Targeted gene - dsRNA | Type of injection | Number of injected animals (t=0) | Surviving animals at experiment termination (t=168h) | Mortality |
|---|---|---|---|---|
| LsYAP | CT | 4 | 4 | 0% |
| LsYAP | INT | 5 | 5 | 0% |

**Injection trial 3:** Due to the promising results in terms of lice mortality in the previous trials, a more complex experiment was designed. 39 adult female lice were injected with dsRNA corresponding to 3 genes of interest (LsSub, LsTryp1 and LsYAP) in addition to an unrelated dsRNA fragment of cod trypsin gene (CPY). dsRNA concentration, injection technique and

criteria for successful injection were the same as in injection trial 1. Eight (8) groups of lice were formed according to the injected dsRNA and the type of injection (CT or INT). Following the injection procedure, the lice were kept in hatching cylinders for 5 days (120h; same condition as previously described) after which the surviving lice were transferred to Atlantic salmon (as previously described) and maintained for 10 days (240h). The fish were kept in single-fish tanks as described by Hamre and Nilsen (2011). Nets with appropriate mesh size were placed in all the tanks outlet to recover lost lice. Daily net checks were performed and losses registered for the duration of the experiment.

*Table 6.3 dsRNA intestine injection trial 3. CT stands for Cephalothorax injection and INT stands for Intestine injection*

| Targeted gene - dsRNA | Type of injection | Number of injected animals (t=0) | Surviving animals at t=120h | Surviving animals at t=360h |
|---|---|---|---|---|
| CPY | CT | 6 | 6 | 2 |
| LsSub | CT | 5 | 3 | 1 |
| LsTryp1 | CT | 5 | 3 | 3 |
| LsYAP | CT | 6 | 6 | 2 |
| | | | | |
| CPY | INT | 3 | 3 | 2 |
| LsSub | INT | 5 | 4 | 2 |
| LsTryp1 | INT | 4 | 2 | 0 |
| LsYAP | INT | 5 | 5 | 0 |

*Table 6.4 Registered mortalities during injection trial 3. CT stands for Cephalothorax injection and INT stands for Intestine injection*

| Targeted gene - dsRNA | Type of injection | Mortality at t=120h | Mortality between t=120h and t=360h | Total Mortality | Mortality according to injection type |
|---|---|---|---|---|---|
| CPY | CT | 0% | 67% | 67% | |
| LsSub | CT | 40% | 67% | 80% | |
| LsTryp1 | CT | 40% | 0% | 40% | |
| LsYAP | CT | 0% | 67% | 67% | **64%** |
| | | | | | |
| CPY | INT | 0% | 33% | 33% | |
| LsSub | INT | 20% | 50% | 60% | |
| LsTryp1 | INT | 50% | 100% | 100% | |
| LsYAP | INT | 0% | 100% | 100% | **76%** |

**Injection trial 4:** The high mortalities registered in injection trial 3 led to some modifications in experimental design in order to increase lice survival. 78 adult female lice were injected with dsRNA corresponding to 1 gene of interest (LsYAP) in addition to an unrelated dsRNA fragment of cod trypsin gene (CPY). Four (4) groups of lice were formed according to the injected dsRNA and the type of injection (CT or INT). Following the injection procedure, each group of lice was kept in a different 2 litre box filled with particle filtered full salinity seawater (salinity 34.5 ppt and temperature 10±0.5°C) for one overnight period (O/N, 12h). The surviving lice were then transferred to Atlantic salmon (as previously described) and maintained for 9 days (216h) with filters on the outlet. dsRNA concentration, injection technique and criteria for successful injection were the same as in injection trial 1.

*Table 6.5 dsRNA intestine injection trial 4. CT stands for Cephalothorax injection and INT stands for Intestine injection*

| Targeted gene - dsRNA | Type of injection | Number of injected animals (t=0) | Surviving animals at t=12h (O/N) | Surviving animals at t=216h |
|---|---|---|---|---|
| CPY | CT | 15 | 15 | 4 |
| LsYAP | CT | 20 | 15 | 3 |
| | | | | |
| CPY | INT | 16 | 15 | 3 |
| LsYAP | INT | 27 | 22 | 1 |

*Table 6.6 Registered mortalities during injection trial 4. CT stands for Cephalothorax injection and INT stands for Intestine injection*

| Targeted gene - dsRNA | Type of injection | Mortality at t=12h (O/N) | Mortality between t=12h and t=216h | Total Mortality | Mortality according to injection type |
|---|---|---|---|---|---|
| CPY | CT | 0% | 73% | 73% | **80%** |
| LsYAP | CT | 25% | 80% | 85% | |
| | | | | | |
| CPY | INT | 6% | 80% | 81% | **91%** |
| LsYAP | INT | 18% | 95% | 96% | |

**Injection trial 5:** Due to the high mortalities registered in injection trials 3 and 4, an experiment was designed in order to evaluate the possibility of incorrect handling of the animals. The objective of this experiment was to assess the author's technique and to exclude incorrect lice handling as the source of the high mortalities. With this objective in mind, 25 adult female lice were divided in two groups. Cephalothorax (CT) injections were performed

on the 25 animals by the author (operator A, 12 animals injected) and an engineer (operator B, 13 animals injected) with several years of experience in this technique. The injection mixture was composed of 0.5$\mu$l of saturated and filtered bromophenol blue per 50$\mu$l of DEPC treated water. As with the dsRNA and bromophenol blue mixture, this injection mixture enabled the operator to visualise successful injections through the dispersion of the solution inside the haemocoel of the cephalothorax. Following the injection procedure, each group of lice was kept in a different 2 litre box filled with particle filtered full salinity seawater (salinity 34.5 ppt and temperature 10±0.5°C) for a period of 3h. The surviving lice were then transferred to Atlantic salmon (as previously described) and maintained for 8 days (192h) as previously described.

*Table 6.7 Injection trial 5 - lice handling test. CT stands for Cephalothorax injection*

| Operator | Type of injection | Number of injected animals (t=0) | Surviving animals at t=3h | Surviving animals at t=192h | Mortality at t=192h |
|---|---|---|---|---|---|
| A | CT | 12 | 12 | 10 | 17% |
| | | | | | |
| B | CT | 13 | 13 | 11 | 15% |

**Injection trial 6:** After injection trial 5, incorrect lice handling was excluded as the source of the elevated mortalities registered in injection trials 3 and 4. Consequently, a new experiment was devised in order to evaluate different points of entry in the the intestine of the adult female louse. Figure 6.2 depicts the two different locations selected for the tentative injections. Location A corresponds to the junction of the cephalothorax and the genital segment and location B corresponds to the louse's abdominal area. Injections were performed as described in the material and methods section. 50 adult female lice were injected with dsRNA corresponding to a *L.salmonis* unrelated dsRNA fragment of cod trypsin gene (CPY). Two (2) groups of lice were formed according to the location of the injection. Criteria defined in the material and methods section (section 5.4.1) were used to evaluate successful injections. Following the injection procedure, each group of lice was kept in a different 2 litre box filled with particle filtered full salinity seawater (salinity 34.5 ppt and temperature 10±0.5°C) for one overnight period (O/N, 12h). The surviving lice were then transferred to Atlantic salmon (as previously described) and maintained for 7 days (168h). Lice losses were registered daily as previously described.

*Table 6.8 dsRNA intestine injection trial 6. INT stands for Intestine injection*

| Targeted gene - dsRNA | Type of injection | Location of the injection | Number of injected animals (t=0) | Surviving animals at t=12h (O/N) | Surviving animals at t=168h |
|---|---|---|---|---|---|
| CPY | INT | **A** | 20 | 16 | 3 |
| CPY | INT | **B** | 30 | 19 | 2 |

*Table 6.9 Registered mortalities during injection trial 6. INT stands for Intestine injection*

| Targeted gene - dsRNA | Type of injection | Location of the injection | Mortality at t=12h (O/N) | Mortality between t=12h and t=168h | Total Mortality |
|---|---|---|---|---|---|
| CPY | INT | **A** | 20% | 81% | 85% |
| CPY | INT | **B** | 37% | 89% | 93% |


*Figure 6.2 Points of entry for the tentative adult female lice intestine injections.*


*Figure 6.3 Adult female louse. Red lines mark the sectioning locations. DNA was extracted from fragments 1 and 2.*

### 6.1.2 Evaluation of bacterial content of lice that survived injection trial 6

In order to get some insight into the consequences of the intestinal injection in adult female lice, DNA was extracted from a group of lice recovered from injection trial 6. This group (treated group) is comprised of 3 lice injected in the junction of the cephalothorax and the genital segment (type A injection) and 2 lice injected in the abdominal area (type B injection). In order to correctly evaluate the bacterial content of this group of animals, two sections destined for DNA isolation were cut from the cephalothorax region, avoiding the intestine, and the rest of animal was discarded. Figure 6.3 depicts the locations where the lice were sectioned.

The procedure was repeated for a different group of 5 adult female lice (untreated group) which had not been injected or treated in any way. Following DNA extraction, sample DNA concentration was normalized (10 ng/µL) and a PCR was run using 2,5 µL of each template, annealing temperature of 55˚C, extension time of 90 seconds and 30 cycles. Two reactions were run in parallel each using a different set of primers: primer pair k39 f1 + k39 f4 (targets a highly expressed louse Trypsin; 550 bp fragment) and primer pair 27f + 1492r (targets the bacterial 16S rRNA gene; 1500 bp fragment). Figure 6.4 correspond to a 1% Agarose gel of the PCR products of these reactions.

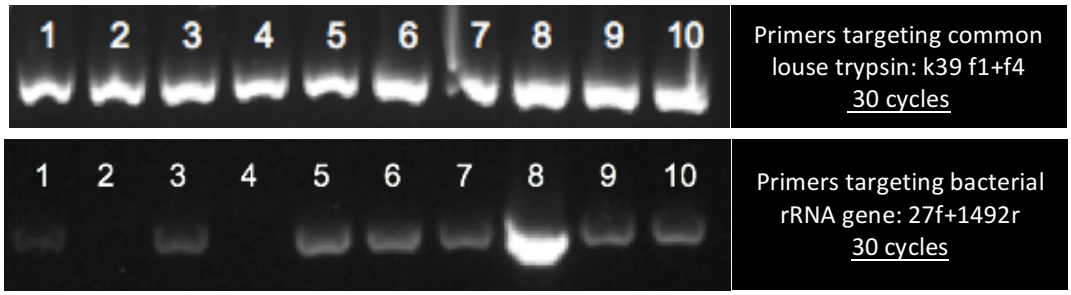


*Figure 6.4 1% agarose gel of PCR products generated by primer pair K39 f1+f4 (top image) and PCR products generated by primer pair 27f+1492r (bottom image). Lanes 1-5 correspond to the 5 animals in the untreated group. Lanes 6, 7 and 8 correspond to type A injection animals. Lanes 9 and 10 correspond to type B injection animals.*

### 6.1.3 Cephalothorax injections: the double gene knockdown experiment

Tables 6.10 and 6.11 in conjunction with Figure 5.1 illustrate and condense the information regarding the results of the two successive injection procedures of the double gene knockdown experiment. Cumulative mortality for the experiment was 37% (43% in group X and 33% in group Y).

*Table 6.10 Data from the first injection procedure in the double gene knockdown experiment. CT stands for Cephalothorax injection*

| Targeted gene - dsRNA | Type of injection | Number of injected animals (t=0) | Surviving animals at t=312h | Mortality at t=312h |
|---|---|---|---|---|
| LsSID | CT | 69 | 44 | 36% |
| CPY | CT | 64 | 45 | 29% |

*Table 6.11 Data from the second injection procedure in the double gene knockdown experiment. Cephalothorax injection was once again the chosen dsRNA delivery method*

| Gene targeted in the first injection - dsRNA | Gene targeted in the second injection - dsRNA | Number of injected animals at t=312h | Surviving animals at t=480h | Mortality between t=312h and t=480h | Group mortality between t=312h and t=480h (SID vs CPY groups) |
|---|---|---|---|---|---|
| LsSID | LsYAP | 13 | 13 | 0% | |
| LsSID | LsSub | 13 | 12 | 8% | **8%** |
| LsSID | LsTryp1 | 13 | 11 | 15% | |
| | | | | | |
| CPY | LsYAP | 15 | 14 | 7% | |
| CPY | LsSub | 15 | 14 | 7% | **4%** |
| CPY | LsTryp1 | 15 | 15 | 0% | |

## 6.2 Evaluation of gene knockdown in the double gene knockdown experiment

### 6.2.1 LsSID knockdown evaluation

#### 6.2.1.1 LsSID gene knockdown evaluation at t=312h

A SYBR green assay was set up using the primer pairs b434+b435 (EL1α), b3821+b3822 (LsSID1a), b3823+b3824 (LsSID1b) b3823+b3824 (LsSID1b) and b3630+b1640 (LsSID1b). EL1α was used as a reference gene and fold variations were calculated using the ΔΔCt method (Livak and Schmittgen, 2001). The results, taking into account the 95% confidence intervals, show a moderate downregulation of LsSID1a in the animals terminated at t=312h, immediately before the second round of dsRNA injections (Figure 6.5). Depending on the primer pair chosen for the LsSID1b SYBR green assay, a close to non-existent downregulation is seen (primer pair b3823+b3824; Figure 6.6) or a low level of gene downregulation is present (primer pair b3630+b1640; Figure 6.7).

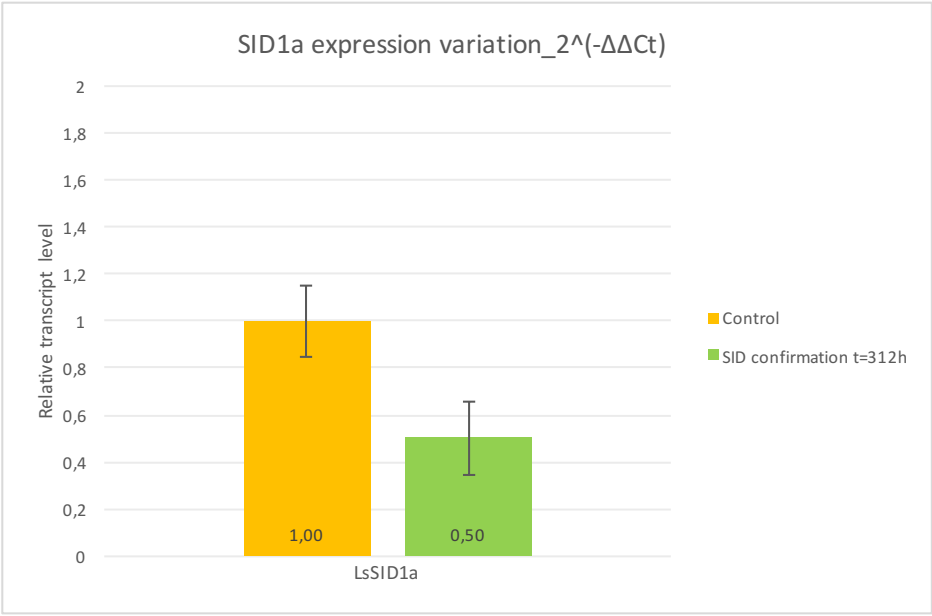


*Figure 6.5 Relative SID1a gene expression normalized to EL1α (elongation factor 1 alpha). Control group is composed of 5 adult female lice injected with CPY. SID confirmation group is composed of 5 adult female lice injected with a equimolecular mixture of SID1a and SID1b dsRNAat t=0 (cephalothorax injection) and were terminated at t=312h (before the second round of dsRNA injections) (see figure 4.1). The error bars indicate 95% confidence intervals.*

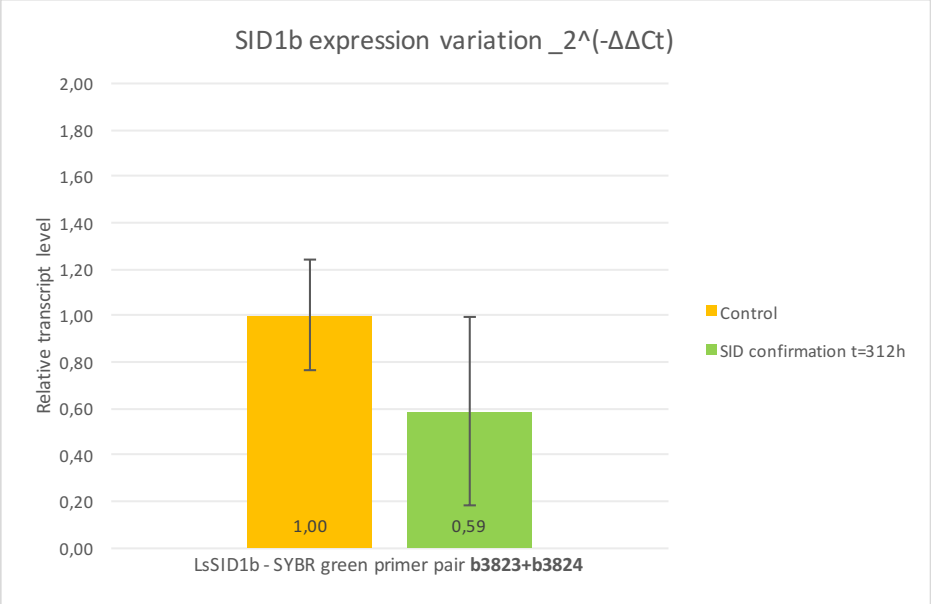


*Figure 6.6 Relative SID1b gene expression normalized to EL1α (elongation factor 1 alpha). Control group is composed of 5 adult female lice injected with CPY. SID confirmation group is composed of 5 adult female lice injected with a equimolecular mixture of SID1a and SID1b dsRNA at t=0 (cephalothorax injection) which were terminated at t=312h (before the second round of dsRNA injections) (see figure 4.1). Primer pair used in the SYBR green assay:* ***b3823+b3824****. The error bars indicate 95% confidence intervals.*

As previously mentioned in the material and methods section, the use of two sets of primers to evaluate LsSID1b downregulation in SYBR green assays is due to the the overlap between the portion of the sid1b gene that was used in the production of the dsRNA and the portion of the gene that is amplified during the SYBR green assay (primer pair b3823+b3824; Figure 6.6). The second pair of primers used (b3630+b1640; Figure 6.7) amplifies a section of the gene which is different from the one that was used in the production of the LsSID1b dsRNA. The results obtained from the two LsSID1b SYBR green assays show that in fact, the presence of high amounts of dsRNA that overlap with the region of the gene that is being amplified in those assays affects (slightly in this case) the correct measurement of relative gene expression.

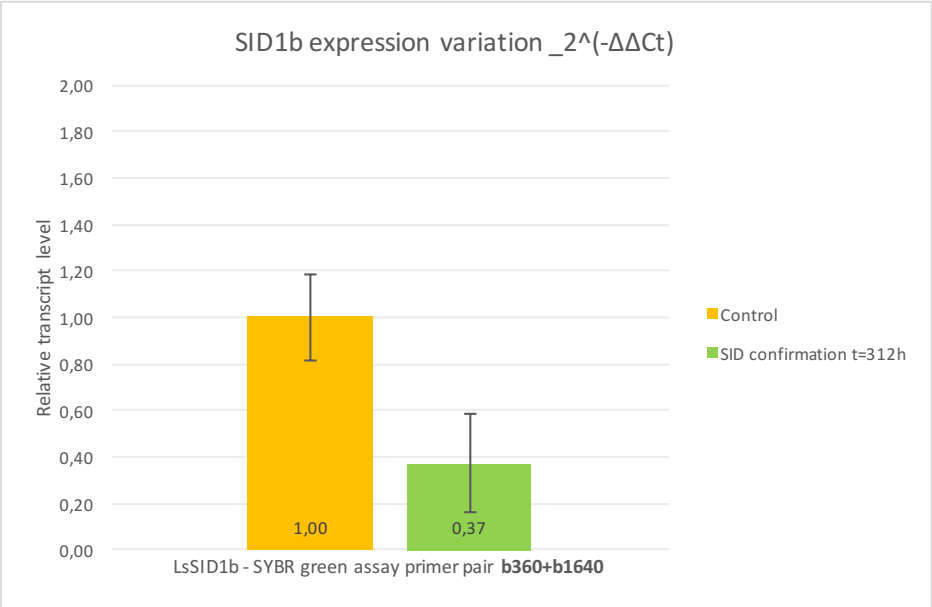


*Figure 6.7 Relative SID1b gene expression normalized to EL1α (elongation factor 1 alpha). Control group is composed of 5 adult female lice injected with CPY. SID confirmation group is composed of 5 adult female lice injected with a equimolecular mixture of SID1a and SID1b dsRNA at t=0 (cephalothorax injection) which were terminated at t=312h (before the second round of dsRNA injections) (see figure 4.1). Primer pair used in the SYBR green assay:* ***b3630+b1640****. The error bars indicate 95% confidence intervals.*

### 6.2.1.2 LsSID gene knockdown evaluation at t=480h

In order to evaluate the LsSID gene knockdown effect at the termination of the double knockdown experiment (t=480h), a SYBR green assay was set up using the primer pairs b434+b435 (EL1α), b3821+b3822 (LsSID1a), b3823+b3824 (LsSID1b) and b3630+b1640 (LsSID1b). Although there seems to exist some degree of downregulation in the SID-Tryp1 double knockdown group of animals, when taking into account the 95% confidence intervals, the effect is rather small. There is also no visible downregulation in the animals in the other two groups: SID-YAP double knockdown group and SID-Sub double knockdown group. (Figures 6.8, 6.9 and 6.10)

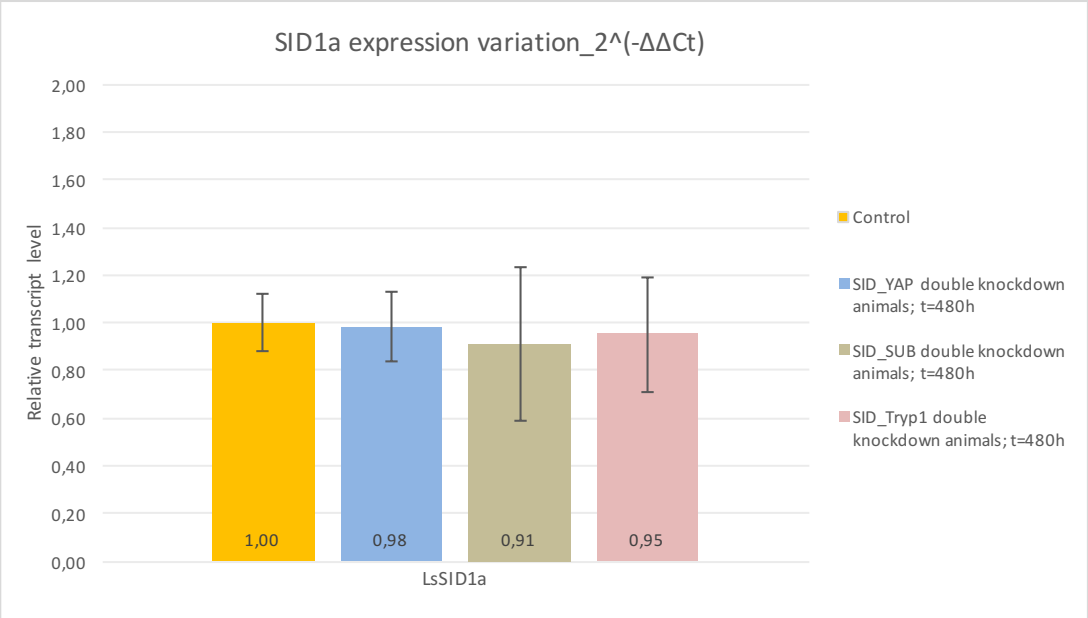


*Figure 6.8 Relative SID1a gene expression normalized to EL1α (elongation factor 1 alpha). Control group is composed of 2 adult female lice injected with CPY. Remaining groups are composed of 3 animals that underwent a LsSID dsRNA cephalothorax injection at t=0 and a second dsRNA injection at t=312h (target genes: LsYAP, LsSub or LsTryp1). Animals were terminated at t=480h. The error bars indicate 95% confidence intervals.*

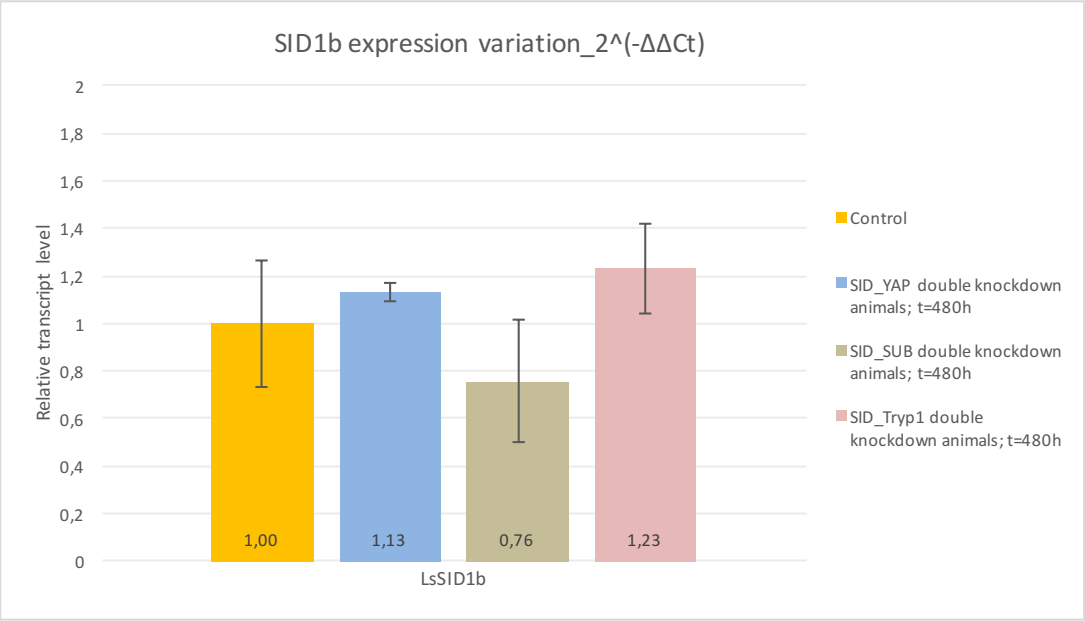


*Figure 6.9 Relative SID1b gene expression normalized to EL1α (elongation factor 1 alpha). Control group is composed of 2 adult female lice injected with CPY. Remaining groups are composed of 3 animals that underwent a LsSID dsRNA cephalothorax injection at t=0 and a second dsRNA injection at t=312h (target genes: LsYAP, LsSub or LsTryp1). Animals were terminated at t=480h. LsSID1b primer pair used for SYBRgrenn assay:* ***b3823+b3824****. The error bars indicate 95% confidence intervals.*

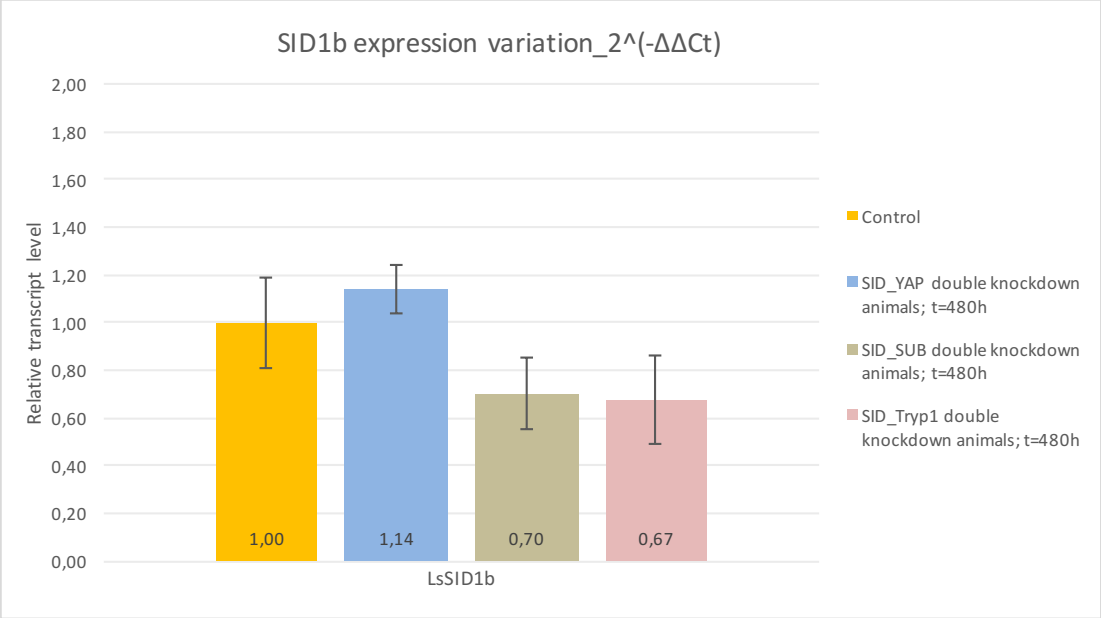


*Figure 6.10 Relative SID1b gene expression normalized to EL1α (elongation factor 1 alpha). Control group is composed of 2 adult female lice injected with CPY. Remaining groups are composed of 3 animals that underwent a LsSID dsRNA cephalothorax injection at t=0 and a second dsRNA injection at t=312h (target genes: LsYAP, LsSub or LsTryp1). Animals were terminated at t=480h. LsSID1b primer pair used for SYBRgrenn assay:* ***b3630+b1640****. The error bars indicate 95% confidence intervals.*

### 6.2.2 Confirmation of LsSID SYBRgreen assay optimization by the evaluation of newly designed primer pairs effect on amplification efficiency

Due to the need to use a second pair of primers to assess LsSID1b relative gene expression in the SYBR green assays, a primer efficiency test was performed in order to assess comparability of the obtained results by the ΔΔCt method (Livak and Schmittgen, 2001) as well as primer pair effect on the amplification efficiency. Primer pairs b434+b435 (EL1α), b3821+b3822 (LsSID1a), b3823+b3824 (LsSID1b) and b3630+b1640 (LsSID1b) were tested as previously described. All sets of primers are within the efficiency limits (95-105%) previously defined as necessary for an optimized Q-PCR assay. Furthermore, the efficiency of the two sets of LsSID1b primers is equal (100,1%) which makes the assays involving this two sets of primers comparable.

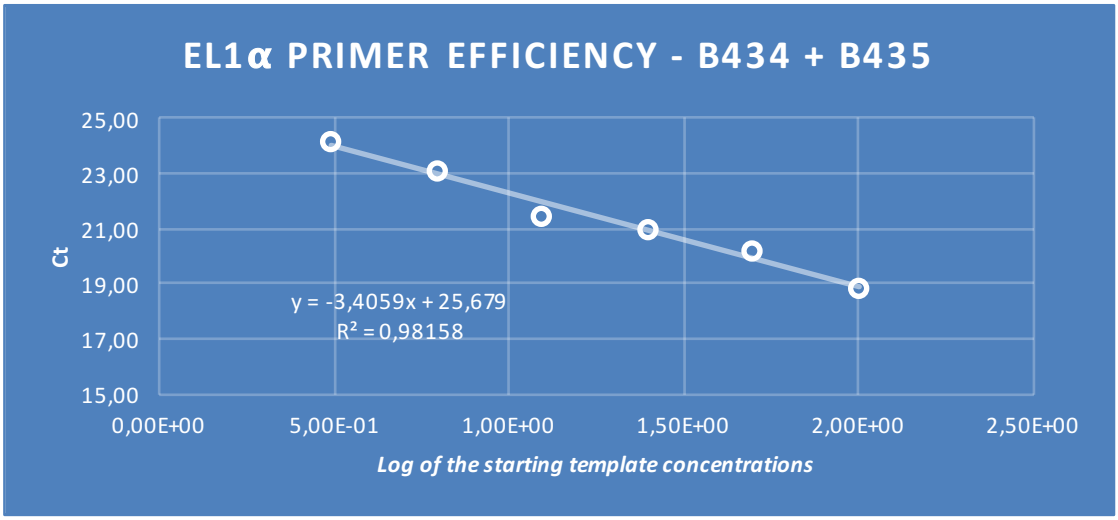


*Figure 6.11 Standard curve with the Ct plotted against the log of the starting concentration of template for each dilution. The equation for the regression line and the $R^2$ value are shown below the graph. The calculated amplification efficiency was 96,6%.*

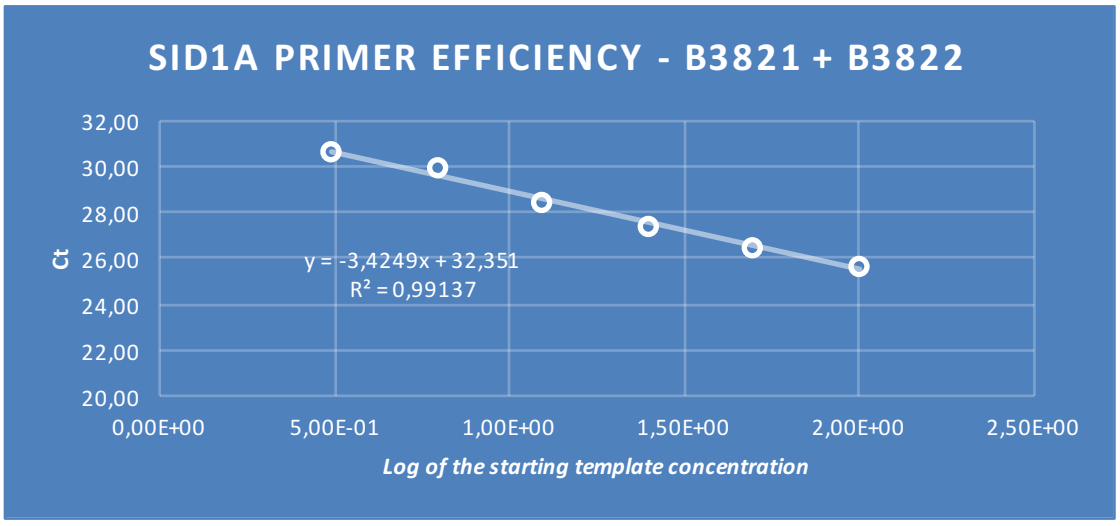


*Figure 6.12 Standard curve with the Ct plotted against the log of the starting concentration of template for each dilution. The equation for the regression line and the $R^2$ value are shown below the graph. The calculated amplification efficiency was 95,9%.*

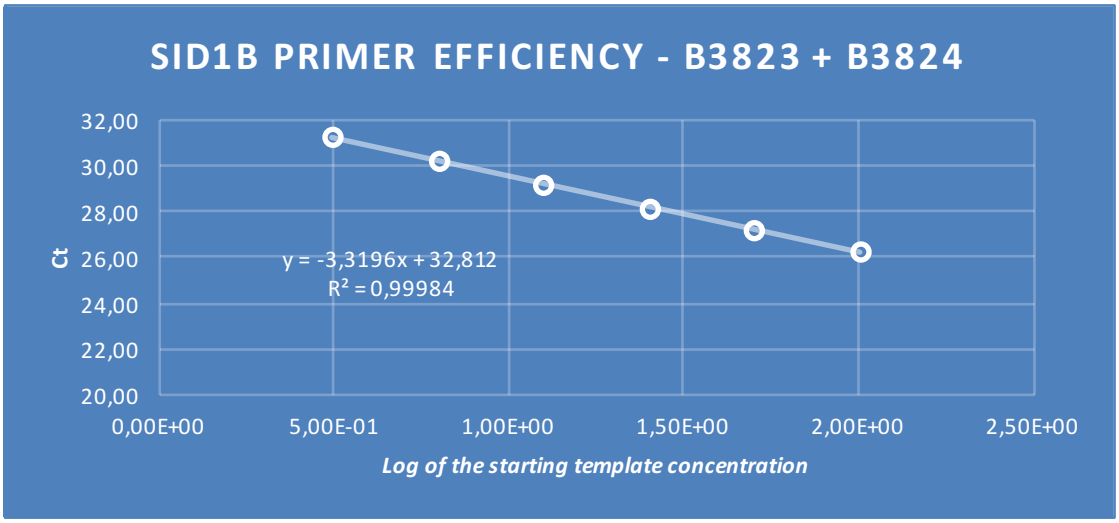


*Figure 6.13 Standard curve with the Ct plotted against the log of the starting concentration of template for each dilution. The equation for the regression line and the $R^2$ value are shown below the graph. The calculated amplification efficiency was 100,1%.*

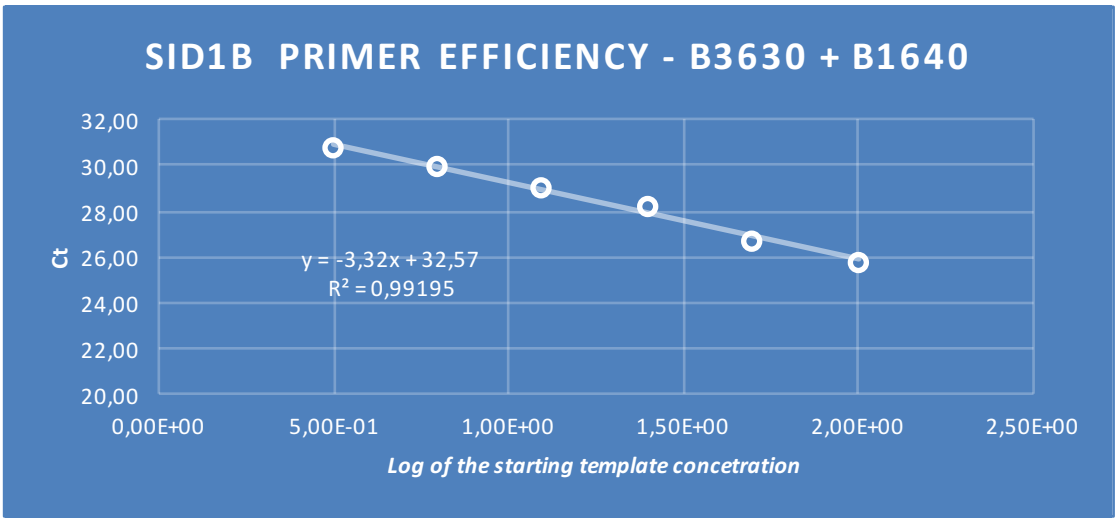


*Figure 6.14 Standard curve with the Ct plotted against the log of the starting concentration of template for each dilution. The equation for the regression line and the $R^2$ value are shown below the graph. The calculated amplification efficiency was 100,1%.*

### 6.2.3 Gene knockdown evaluation of the other genes of interest (LsYAP, LsSub and LsTryp1) in group X and group Y.

Assessment of gene knockdown of the six (6) groups formed from the initial groups X (SID-YAP, SID-Sub and SID-Tryp1 double knockdowns) and Y (CPY-YAP, CPY-Sub and CPY-Tryp1 single knockdowns) was performed via SYBR green assays. Three (3) assays were prepared according to the target of the second dsRNA injection. The first essay evaluated LsYAP gene knockdown using the sets primers b434+b435 (EL1α) and b1137+b1138 (LsYAP) (Figure 6.15). The second assay assessed LsSub gene knockdown using the sets of primers b434+b435 (EL1α) and b1454+b1455 (LsSub) (Figure 6.16). The third essay measured LsTryp1 gene knockdown using the sets of primers b434+b435 (EL1α) and b1262+1262 (LsTryp1) (Figure 6.17). EL1α was used as a reference gene and fold variations were calculated using the ΔΔCt method (Livak and Schmittgen, 2001).

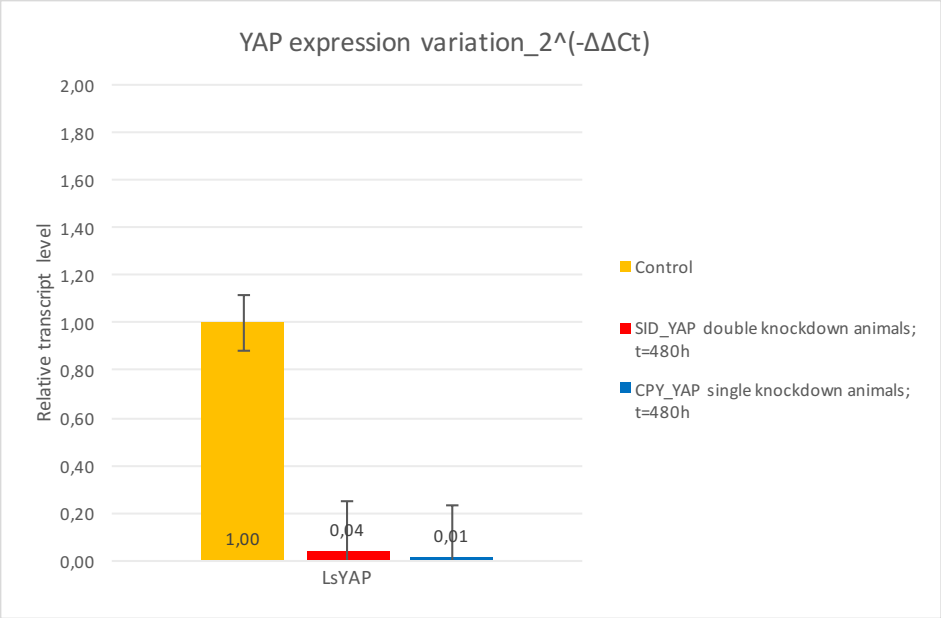


*Figure 6.15 Relative YAP gene expression normalized to EL1α (elongation factor 1 alpha). Control group is composed of 5 adult female lice injected with CPY. Remaining groups are composed of 5 animals that undergone either a LsSID dsRNA (bright red bar) or a CPY dsRNA (blue bar) cephalothorax injection at t=0 and a second LsYAP dsRNA injection at t=312h. Animals were terminated at t=480h. The error bars indicate 95% confidence intervals.*


*Figure 6.16 Relative Sub gene expression normalized to EL1α (elongation factor 1 alpha). Control group is composed of 5 adult female lice injected with CPY. Remaining groups are composed of 5 animals that undergone either a LsSID dsRNA (purple bar) or a CPY dsRNA (grey bar) cephalothorax injection at t=0 and a second LsSub dsRNA injection at t=312h. Animals were terminated at t=480h. The error bars indicate 95% confidence intervals.*

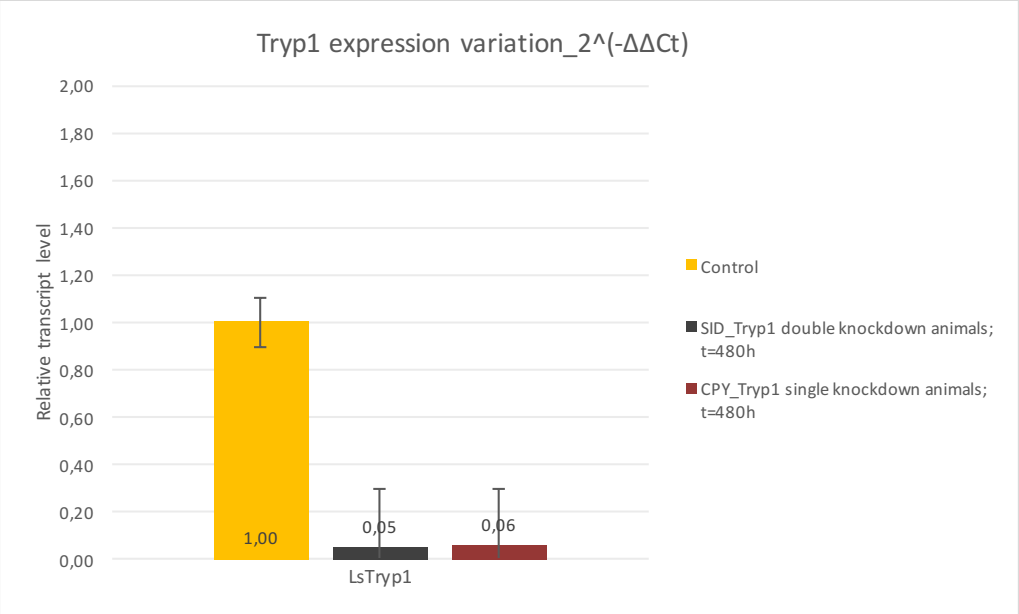


*Figure 6.17 Relative Tryp1 gene expression normalized to EL1α (elongation factor 1 alpha). Control group is composed of 5 adult female lice injected with CPY. Remaining groups are composed of 5 animals that undergone either a LsSID dsRNA (black bar) or a CPY dsRNA (dark red bar) cephalothorax injection at t=0 and a second LsTryp1 dsRNA injection at t=312h. Animals were terminated at t=480h. The error bars indicate 95% confidence intervals.*

Results from these three (3) SYBR green assays show a potent downregulation of LsYAP and LsTryp1 with no significant differences between animals originating from groups X and Y. Regarding the relative expression of LsSub, animals from group X show a slight upregulation. Animals from group Y show an even less pronounced up-regulation of gene expression. Taking into account the 95% confidence intervals, these effects are so slight that a de facto upregulation of LsSub seems unlikely.

## 6.3 Blood and dsRNA feeding to pre-adult males II experiment

### 6.3.1 Observations during the experimental procedure

Due to the 34h starvation period that the lice went through, all animals had a blood free gut before the beginning of the experiment. Upon placing the lice on top of the drop of blood+dsRNA it was fairly obvious that they struggled to find balance for a few seconds but rapidly started ingesting the Atlantic salmon blood and dsRNA mixture. The drop volume was approximately 15 μL in volume and the amount ingested by the lice was variable, with some very actively ingesting it and others showing a more static posture possibly due to their weakened state following the starvation period. Blood was visible in the gut of the majority of the animals, although colour intensity was variable, indicating ingestion of different volumes.

### 6.3.2 ΔCt comparison of control animals versus animals terminated following blood+dsRNA ingestion (t=0)

Taking advantage of the two (2) different sets of LsSID1b primer pairs available, a comparison was made between the ΔCt of a group of untreated animals of the same batch and animals terminated post-procedure (t=0) via SYBR green assay. As previously mentioned, primer pair b3823+b3824 anneals to a region of the LsSID1b cDNA that overlaps with the region in the LsSID1b cDNA that was used to produce the dsRNA. The opposite happens with primer pair b3630+b1640, which anneals with a completely different region of the LsSID1b cDNA. Previous results demonstrate equal primer pair efficiency (Figures 6.13 and 6.14) and support the hypothesis that the injected dsRNA interferes with the SYBR assay results when primer pair b3823+b3824 is used (Figures 6.6 and 6.7).

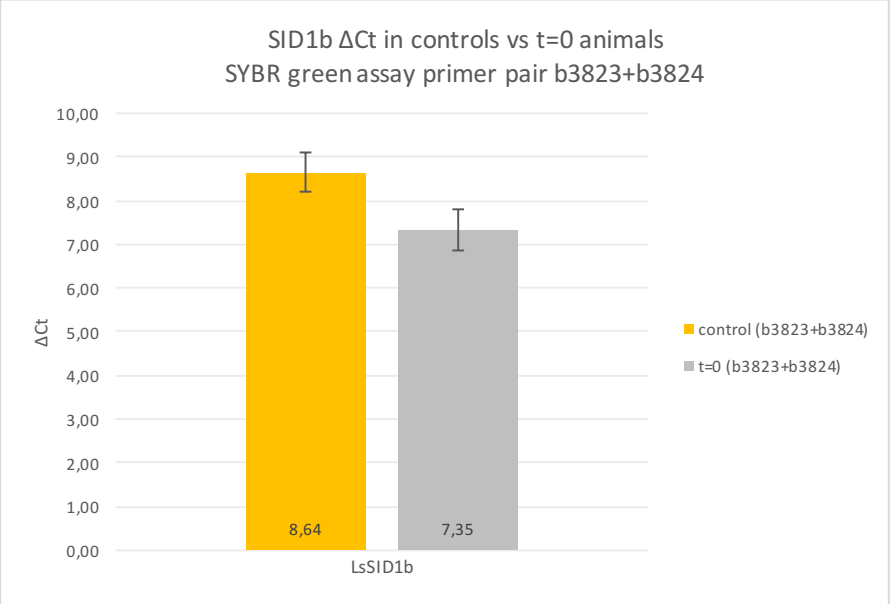


*Figure 6.18 ΔCt of SID1b using the housekeeping gene EL1α (elongation factor 1 alpha) as reference gene. Control group is composed of 5 untreated pre-adult II male lice while t=0 group is composed of 5 five pre-adult II male lice that were terminated following the two (2) minutes exposure to the blood+dsRNA mixture. Primer pair used in the SYBR green assay:* ***b3823+b3824****. The error bars indicate 95% confidence intervals.*

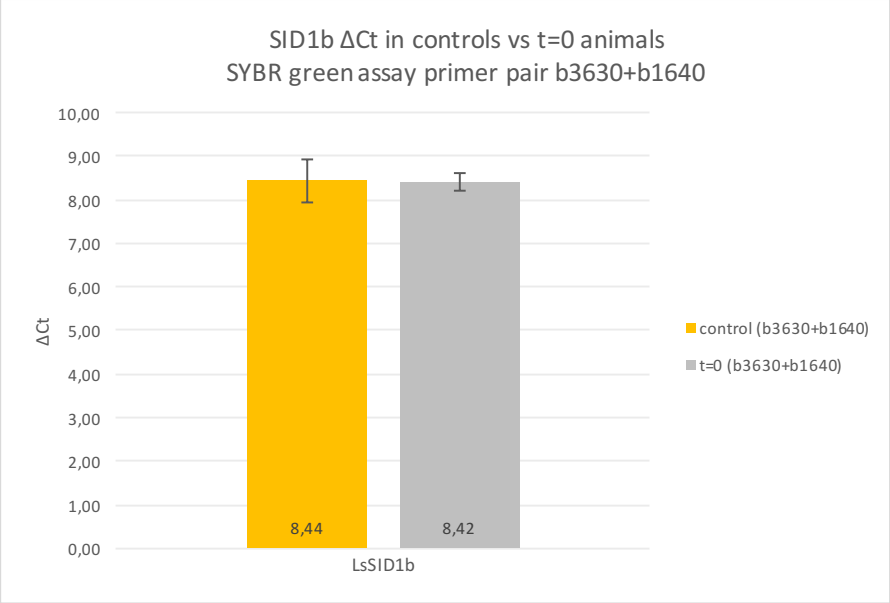


*Figure 6.19 ΔCt of SID1b using the housekeeping gene EL1α (elongation factor 1 alpha) as reference gene. Control group is composed of 5 untreated pre-adult II male lice while t=0 group is composed of 5 five pre-adult II male lice that were terminated following the two (2) minutes exposure to the blood+dsRNA mixture. Primer pair used in the SYBR green assay:* ***b3630+b1640****. The error bars indicate 95% confidence intervals.*

In this context, a lower ΔCt value indicates a lower Ct value for LsSID1b. This indicates that the target reached the threshold at an earlier cycle during the SYBR green assay. Therefore, the comparison between Figure 6.18 and Figure 6.19 suggests that a higher level of LsSID1b was detected using the primer pair b3823+b3824. Taking into account the previously described characteristics of the two different pairs of primers, this is yet another confirmation that the animals that were placed on top of the blood+dsRNA mixture have in fact ingested it and that the dsRNA is inside their gut at termination.

### 6.3.3 ΔCt timeline for LsSID genes using the housekeeping gene EL1α (elongation factor 1 alpha) as reference gene

Following the experimental procedure, the salmon lice were maintained in individual cylinders inside flow-through incubator boxes as previously described. One (1) animal died at the 240h time point in what can arguably be attributed to starvation. Five (5) animals were collected and terminated at t=0, t=24h, t=48h, t=96h, t=120h, t=144h, t=168h and t=192h. One (1) animal was collected and terminated at t=240h. Figures 6.20, 6.21 and 6.22 illustrate the ΔCt timeline using the housekeeping gene EL1α for LsSID1a, LsSID1b (using SYBR green primer pair b3823+b3824) and LsSID1b (using SYBR green primer pair b3630+b1640) respectively. Three (3) animals were tested per time point with the exception of t=240h as only one animal was available for testing.

Results suggest a lack of LsSID1a downregulation in the analysed time points. Regarding LsSID1b there is a marked decrease of ΔCt which strongly suggests gene silencing happening at 192h and extending until 240h. This result is nonetheless close to end of the experiment and more time points would be of use to confirm this scenario. It also visible that the discussed trend between LsSID1b primer pairs b3823+b3824 (overlap with dsRNA fragment) and b3630+b1640 (no overlap with dsRNA fragment) is maintained, with the majority of measured ΔCt values being larger for the former in comparison to the latter. This is yet another result suggesting that the injected dsRNA influences the LsSID1b SYBR green assay results when using primer pair b3823+b3824.

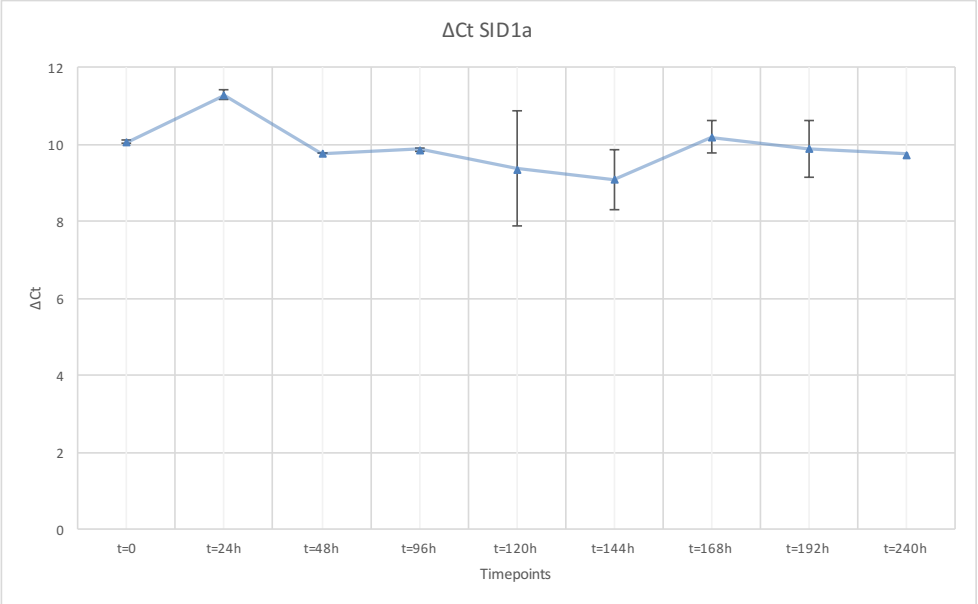


*Figure 6.20 ΔCt of SID1a using the housekeeping gene EL1α (elongation factor 1 alpha) as reference gene. Each time point is generated from groups of 3 pre-adult II males with the exception of t=240h where only 1 animal was available. The error bars indicate 95% confidence intervals.*

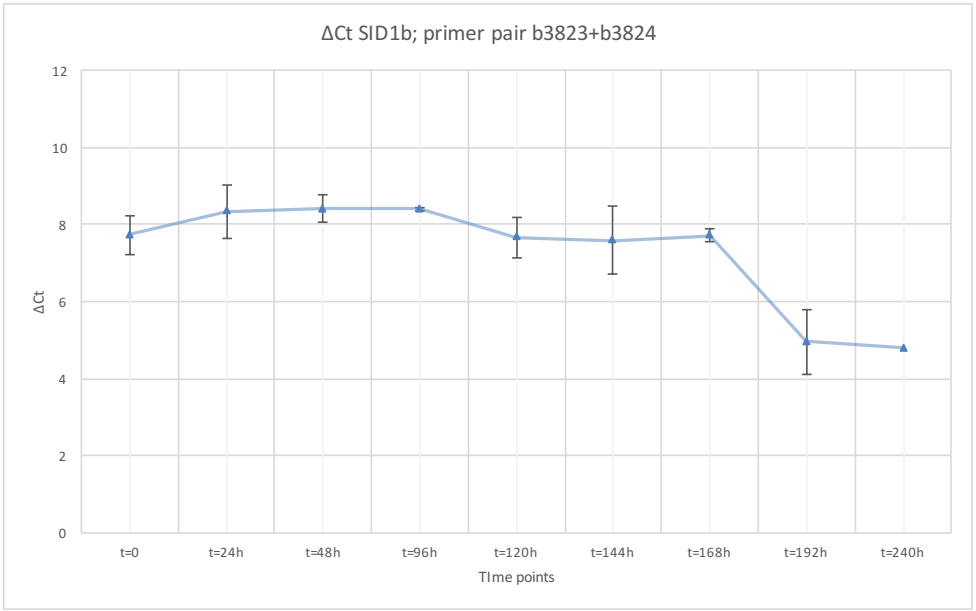


*Figure 6.21 ΔCt of SID1b using the housekeeping gene EL1α (elongation factor 1 alpha) as reference gene. SYBR green primer pair used:* ***b3823+b3824****. Each time point is generated from groups of 3 pre-adult II males with the exception of t=240h where only 1 animal was available. The error bars indicate 95% confidence intervals.*

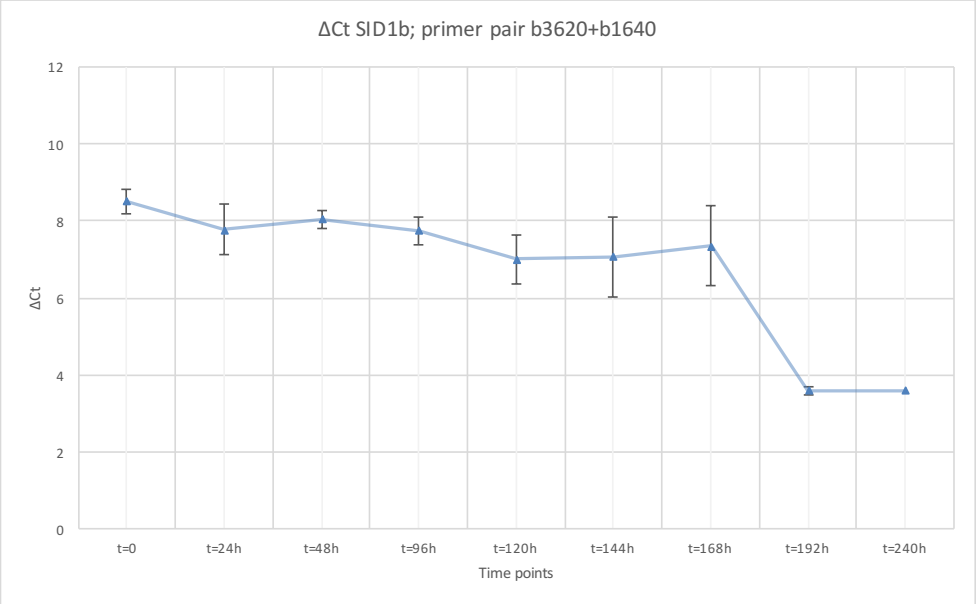


*Figure 6.22 ΔCt of SID1b using the housekeeping gene EL1α (elongation factor 1 alpha) as reference gene. SYBR green primer pair used:* ***b3630+b1640****. Each time point is generated from groups of 3 pre-adult II males with the exception of t=240h where only 1 animal was available. The error bars indicate 95% confidence intervals.*

## 7. Discussion

This study addressed the three (3) aims that were defined in the planning stage and an additional three (3) aims that were added during the course of the experimental work. Building on the work developed at the Sea Lice Research Centre by Dalvin et al. (2009; 2011) and Eichner et al. (2014; 2015a; 2015b; 2015c) where the RNAi technique was applied, this study aimed at exploring the importance of the previously identified *L. salmonis* genes encoding putative SID-1 like proteins (LsSID1a and LsSID1b), in the RNAi process in the salmon louse.

The previously cited works have confirmed that a systemic downregulation of gene transcription can be achieved by soaking (immersion) salmon louse nauplii in a dsRNA+seawater solution and by injecting dsRNA in the haemocoel of cephalothorax of pre-adult and adult lice. They have, however, not been able to clarify the genes and corresponding transcripts that are essential to the occurrence of systemic RNAi. This study proposed to assess the importance of *L. salmonis* genes LsSID1a and LsSID1b in this process. In order to achieve that goal, a dsRNA micro injection into the louse's intestine was attempted in order to create a model by which it would be possible to infer the function of the putative SID-1 like proteins through selective transcriptional knock-down of the genes encoding those proteins. It would at the same time give some insight as to whether the injected dsRNA would be able to survive the louse's enzyme packed intestine and, additionally, if the SID-1 like proteins have a role similar to the SID-1 protein in *C. elegans*, where it has been classified as a selective importer of extracellular dsRNA into the cytosol (Jose, 2015; Winston et al., 2002). The working hypothesis was that upon knock-down of LsSID1a and LsSID1b, in the first round of dsRNA intestine injections, lack of systemic RNAi upon a second of dsRNA intestine injections targeting three (3) other genes (LsYAP, LsSub and LsTryp1) which have previously been successfully knocked-down by RNAi, would indicate that the SID-1 like proteins had a relevant role in the RNAi mechanism in the salmon louse.

The results obtained from the louse's intestine injections forced the preparation of a new approach to the problem. At that point, three additional aims were added to this study in order to circumvent the intestine injection but, nonetheless, be able to evaluate the role of the purported *L. salmonis* SID-1 like proteins.

The several louse intestine injection trials were the most time consuming part of the experimental work performed. As previously mentioned, the technique had not been tested in the salmon louse before and involved a great degree of training and preparation even before the injection trials began. The work developed shows that the louse is not able to survive that kind

of injection (mortalities ranged from 76% to 93% in intestine injected lice). As the results reflect, care was taken to confirm that lice handling was not a cause of the results obtained (see injection trial 5). A hypothesis was formed arguing that the intestine injection resulted in a major intestinal tissue damage to the lice, with the consequent leakage of intestinal content to the haemocoel, resulting in the release of bacteria which had previously been limited to the intestine. In order to get more information regarding this hypothesis, total RNA of two (2) sections of the cephalothorax (see figure 6.3) of the surviving adult female lice from injection trial 6 (n=5) was extracted and, following the previously described protocol, converted to cDNA. The same procedure was followed for five (5) untreated adult female lice. This procedure was executed avoiding the louse's intestine in order not to include bacteria which remained contained in that organ. The concentration of those ten samples was normalized (10 ng/μL) and used for two (2) PCR's that were run in parallel. The first PCR used a set of primers that target a common trypsin in the louse (k39f1+f4) while the second one used a set of primers that target bacterial 16S rRNA (27f+1492r). Resulting PCR products were run in 1% agarose gel. Figure 6.4 (top) shows rather similar bands for all the tested lice (treated and untreated) in the first PCR. This confirms that cDNA content in both groups of samples (treated and untreated) is similar and the results are comparable. The second PCR (Figure 6.4, bottom) shows a marked difference in band number and band intensity. This results suggest that, in fact, the intestine injected lice have a considerable higher amount of bacterial content in the haemocoel of the cephalothorax, which indicates that it is highly likely that indeed some of their intestinal bacterial content leaked to the haemocoel and contributed to the high mortality in the intestine injection trials.

As previously mentioned, the intestine of the adult salmon louse is a rather simple tube, divided into a short cuticle covered foregut and hindgut, and a long undifferentiated midgut (Nylund et al., 1992). The peristaltic movement of the intestine is continuous and the gut content is pumped back and forward unremittingly (Nylund et al., 1992), which in the case of the intestinal injections was an advantage because it provided the ability to almost instantly identify successful injections by the appearance of a blue line following the length of the intestine. The drawback to this morphologic characteristic it that any rupture in the intestinal tissue will lead to some kind of leakage, as the intestinal content is under constant muscular pressure to maintain movement. The adult female sea louse used in the intestine injections frequently had a blood filled gut that gave it a distinct red colour, facilitating the injection. In some of the injected lice, upon the removal of the injection mechanism, a drop of blood

appeared in the point of penetration. Nonetheless, after being placed in sea water, there was no visible leakage through that same point and furthermore, the blue colour that was visible post injection was maintained for several hours.

Salmon lice are blood sucking ectoparasites but there is scarce information at molecular level regarding how the salmon louse copes with the response mounted by the fish's immune system. Present knowledge suggests that infected hosts have increased reactive oxygen species (ROS) (Sutherland et al., 2014) and up-regulate some inflammatory genes (Fast, 2014). This response is normally not sufficient to expel the parasite, which suggests that the louse is capable of lessening the effect of such response. Given that most of the injected salmon lice still had Atlantic Salmon blood in the intestine at the time of the injection, it is not possible to exclude the possibility that some of these molecules present in the salmon's blood, escaped the closed louse's intestine, wreaking havoc in the haemocoel and other internal structures and contributed to the high mortality of the intestine injection trials.

It is also important to refer that the intestine injection procedure itself is, from the operator's point of view, more stressful for the louse than the simpler and quicker cephalothorax injection (Dalvin et al., 2009; Eichner et al., 2015a, 2015c; Sandlund et al., 2015; Tröße et al., 2014). The latter is done quicker, reducing the exposure of the louse to room temperature, reduced humidity and microscope illumination. In addition, no rupture of the exoskeleton is required as the needle is easily slid under the cephalothorax exoskeleton plates. In fact, intestine injection trials 1 to 4 tried to take advantage of the easy penetrable cephalothorax plates with no success.

As defined in the aims of this project, a successful intestine injection in the salmon louse could allow the exploration of the systemic RNAi mechanism in the salmon louse, specifically by knocking down the known sid1 like genes present in the louse and, potentially, reveal their dsRNA selective importer ability by attempting a second dsRNA injection in the intestine upon confirmation of LsSID1a and LsSID1b knock-down. Insight into the role of LsSID in the transport of dsRNA from the intestinal lumen and its relevance for the systemic RNAi that is known to take place in the salmon louse in different developmental stages upon introduction of dsRNA (Campbell et al., 2009, Dalvin et al., 2009, Eichner et al., 2015a, 2015c; Sandlund et al., 2015; Tröße et al., 2014) could have been gathered.

After a period of approximately four (4) months pursuing a successful salmon louse intestine injection, exhaustion of the possibilities was reached and a new experiment was designed using the previously tested cephalothorax injection. The initial results (sections 6.2.1.1) were less than satisfactory as there was a quite small downregulation of LsSID1a (Figure 6.5) and a low downregulation of LsSID1b at 312h post-injection (Figure 6.7). That trend was slightly

different at 480h post-injection (section 6.2.1.2) with no LsSID1a downregulation in all groups (Figure 6.8) and a similar result for LsSID1b with the exception of very slight downregulation of LsSID1b in the SID-Sub and SID-Tryp1 groups (Figure 6.10). It is however curious to verify that the presence of dsRNA, which is known to be present in injected animals more than thirty (30) days post-injection (Heidi Kongshaug, senior engineer, 2016, personal communication) does in fact interfere with the SYBR green assay (view Figures 6.6 and 6.7), when the primers used in that assay produce a fragment that overlaps with the injected dsRNA. That effect is also visible in the SID-Tryp1 group (Figures 6.9 and 6.10). These observations are reinforced by the Q-PCR efficiency test that was performed which concluded that primer pairs b3823+3824 (Figure 6.13) and b3620+b1640 (Figure 6.14) have equal efficiencies (100,1%) and therefore the results obtained can be directly compared. Figures 6.7 and 6.10 should consequently be considered the most reliable analysis of LsSID1b relative gene expression, as they exclude the dsRNA interference in the SYBR green assay's results.

The conclusion that can be drawn from the results in this section (6.2.1) is that the LsSID knock-down was unsuccessful. Although it is impossible to find references in the literature to *L. salmonis* genes that are not knocked down by introduction of dsRNA targeting that same gene (possibly because those studies are never published) it is known within the research group where this work was performed, that this is a common and frequent occurrence. Personal communication of these failed experiences is rather common and no exact cause has been pinned down. Nevertheless, there are some other possibilities that were not explored due to lack of time. Different dsRNA fragments could have been designed for each gene (using newly designed sets of T7 primers) and directly injected or, in alternative, mixed with the existing fragments and injected into the lice. That would increase of the probabilities of a successful knock-down as more diverse double-stranded short interfering RNAs (ds-siRNA) would be formed and, consequently, more diverse single-stranded short interfering RNAs (ss-siRNAs) would be available inside the cell and would provide RDE-1 with an increased number of guide RNAs to find mRNAs with complementary sequences. There is however no guarantee that the outcome would be positive. And it is, nonetheless, an exercise of cost versus benefit, as ordering primers, producing dsRNA and repeatedly performing the sequence of methods described in the material and methods section is rather costly and time consuming.

One other possibility for the lack of success of the LsSID knock-down is related to the described response of the sid-1 gene in the Pacific white leg shrimp (*Litopenaeus vannamei*). Lv-SID-1 was predicted based on *L. vannamei* expressed sequence tag (EST) sequence

homologous to Sid-1. Labreuche et al. (2010) concluded that after the injection of dsRNA with sizes ranging from 50bp to 200bp, a strong upregulation of Lv-Sid-1 was detected.

Nevertheless, obvious improvements in lice survival were achieved using the cephalothorax injection technique, with a cumulative mortality of 37% (43% in group X and 33% in group Y; see Section 6.1.3 and Figure 5.1 for more details) in the double knock-down experiment, which is within the standard mortality for injection experiments in the research group. It is also important to mention that this cumulative mortality results from two dsRNA injections per group, which implies a doubling of the previously referred stressors to which the lice are exposed.

Analysis of the lice that went through the second round of injections showed a potent downregulation of LsYAP and LsTryp1, two of the genes of interest. There are however, no differences between the group injected with dsRNA targeting the LsSID genes (group X) and the group which was injected with a dsRNA produced from an unrelated cod trypsin (CPY, group Y) during the first round of injection (see Figure 5.1). These results are not surprising given that the LsSID1a and LsSID1b gene knock-down was not successful and therefore, LsSID genes expression should be similar in both groups. Regarding the SID-Sub and CPY-Sub groups, no downregulation of LsSub was detected. Previous work in the research group (Birkeland, 2010) achieved a potent LsSub downregulation by injection of dsRNA. The strategy used to produce the dsRNA was nonetheless different. While this study opted to produce dsRNA via a single PCR with T7 promoters appended to both primers (see section 5.3.1), Birkeland (2010) used a different strategy, opting for two separate PCRs with a single T7 promoter-containing primer in each. The latter strategy requires four (4) PCR primers and two (2) PCRs while the former only requires two (2) PCR primers and one (1) PCR. All the dsRNA in this study was produced via a single PCR with T7 promoters appended to both primers and very good results were achieved for LsYAP and LsTryp1 (Figures 6.15 and 6.16). Nonetheless, future studies involving RNA interference targeting LsSub should take this result into consideration and contemplate using the strategy put in practice by Birkeland (2010), when producing dsRNA.

The final experiment of this master's thesis (section 6.3) was devised after the unsuccessful intestine trials and was designed in order to avoid the necessity of injecting dsRNA into the lice's intestine. Lice were fed Atlantic salmon blood mixed with dsRNA targeting the LsSID genes. Due to unavailability of adult female lice, pre-adult II males were used in this experiment. It is fair to mention that this experiment was initiated the day following the start of the double knock-down experiment and the author had no knowledge of the later confirmed

unsuccessful LsSID knock-down or, for that matter, the success of the LsYAP and LsTryp1 knock-down. Had that been the case, the dsRNA that was mixed in the Atlantic Salmon's blood would have been different. This was once again a first of its kind experiment in the salmon louse, and was conducted after a pre-trial where it was confirmed that the lice in fact "suck up" blood when they are starved for a period of more than 24h and have a blood empty gut. That same pre-trial concluded that the feeding was most efficient when the lice were placed on top of a drop of blood. The two (2) minute timeframe was considered correct and was chosen in order to allow the animals to ingest the blood but at the same time avoid an extended exposure to environmental stressors and reduce the amount of time the lice would be taken out of water.

The blood feeding experiment has some parallels in other species. The silencing of genes by ingestion of dsRNA matching sequences of those same genes was first discovered in *C. elegans* (Timmons and Fire, 1998) and named environmental RNAi or feeding RNAi. It allowed several genome-wide RNAi screens in *C. elegans* (Kamath et al., 2003) where several genes involved in different biological processes were identified.

Direct observation during the experiment confirmed the appearance of a red line in the lice's gut confirming the ingestion of the blood+dRNA mixture. Figure 6.18 provides data that reinforces that observation as LsSID1b is detected at an earlier cycle during the SYBR green assay using the set of primers b3823+b3824. It is safe to conclude that the salmon louse is able to ingest dsRNA orally using Atlantic salmon blood as solute. Had this experiment been started at a later date, dsRNA matching the sequence of either LsYAP or LsTryp1 would have been used to test the ability to induce gene silencing by feeding the salmon louse with dsRNA mixed with blood. There is nonetheless a great potential in this non-invasive and experimentally less demanding technique.

Regarding the ΔCt timeline for the LsSID genes, some promising trends were observed. While it is quite obvious that LsSID1a downregulation is not achieved (Figure 6.20), Figures 6.21 and 6.22 show a significant drop in ΔCt when reaching the 192h time point. This suggests that LsSID1b downregulation was achieved at 192h and maintained up to 240h. However, there was only one (1) surviving animal at 240h, which compromises the confidence of that timeline point. Contrasting with the double knockdown experiment, where LsSID1a and LsSID1b transcription downregulation was not detected at t=312h nor at t=480h, this result was quite surprising. It is nonetheless credible, as RNAi is not a fully understood phenomena in *L.salmonis,* neither regarding mechanism nor timeframe. As previously mentioned, the fact that some genes' transcription is not affected by the introduction of dsRNA in the louse while others

are, is not fully understood. Neither is the time necessary post dsRNA injection to achieve said transcription downregulation. In fact, some genes' transcription downregulation is detected within 24h of dsRNA introduction, while others take quite a longer period of time (Heidi Kongshaug, senior engineer, 2016, personal communication). The obtained result justifies further investigation, as confirmation of LsSID1b transcription knockdown post feeding of dsRNA, would mean that the louse is able to internalize dsRNA from the intestine, as the phenomena takes place inside the cytosol. In addition, it would suggest that at least part of the dsRNA is capable of surviving degradation in the enzyme packed louse's intestine.

This result was nonetheless obtained at a very late phase of this study, due to an instrument breakdown that occurred in the Q-PCR machines at the SLRC following a routine calibration (Applied Biosystems 7500 Real-Time) that forced the repetition of all the SYBR assays with a different machine (BIORAD CFX96 Touch™ Real-Time PCR Detection System). There was no time available to repeat the experiment in order to confirm this rather noteworthy result.

## 8. Conclusions and Future Studies

Taking into consideration both the results and the discussion sections, there are some key conclusion that can be drawn from this study, as well as a few points that need additional clarification.

It is safe to conclude that the intestine (micro) injection is a poor option for dsRNA delivery in the salmon louse. High mortalities, associated with increased bacterial content in the injected lice indicate inability to deal with this procedure and the stressors associated with it. The cephalothorax injection technique used by Dalvin et al. (2009) is considerably less aggressive and invasive, and that is reflected in the lower registered mortalities.

The salmon louse is capable of ingesting dsRNA mixed with Atlantic salmon blood by simply placing it on top of a drop of this mixture following a period of starvation of more than 24h. It is also possible to detect the ingested dsRNA by comparing the results of a SYBR green assay using two different sets of primers: one set that amplifies a section of the gene that overlaps with the cDNA sequence used to produce dsRNA and a second pair of primers that amplifies a sequence of the gene which does not overlap with said sequence. Several examples were shown in this study's experimental work that strongly support that conclusion.

Blood+dsRNA feeding is less invasive than the other explored dsRNA delivery techniques and that is reflected in the 0% registered mortality in the procedure.

Given the results obtained in the double knock-down experiment using the cephalothorax injection technique introduced by Dalvin et al. (2009), LsTryp1 and LsYAP are good targets to assess transcription downregulation using the blood+dsRNA feeding procedure. It is the author's opinion that this would be an interesting future study in order to complement the information gathered in this study. In fact, the ΔCt timeline (presented in section 6.3.3) of blood+dsRNA fed animals targeting LsSID1b suggest that transcription downregulation is present at 192h and up to 240h. However, this result is derived from a total of four (4) animals (3 animals collected at t=192h a 1 animal collected at t=240h) and, given its importance, should be the focus of additional studies. Studies in *C.elegans* using fed dsRNA were very important in gene function identification (Kamath et al., 2003) and could have a similar role in *L.salmonis* if it is capable of internalizing ingested dsRNA.

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## Appendix I

LsSID1a cDNA

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### LsSID1a putative protein sequence
### EMLSAT00000009906

>EMLSAT00000009906 peptide: EMLSAP00000009906 pep:protein_coding
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LsSID1b cDNA

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## LsSID1b putative protein sequence
## EMLSAT00000006871

>EMLSAT00000006871 peptide: EMLSAP00000006871 pep:protein_coding
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