RNAi-mediated treatment of two vertically transmitted rhabdovirus infecting the salmon louse (*Lepeophtheirus salmonis*)

Aina-Cathrine Øvergård, Lars Are Hamre, Heidi Kongshaug & Frank Nilsen

Rhabdoviruses are a family of enveloped negative-sense single-stranded RNA viruses infecting a variety of hosts. Recently, two vertically transmitted salmon louse (*Lepeophtheirus salmonis*) rhabdoviruses (LsRV) have been identified. The prevalence of these viruses was measured along the Norwegian coast and found to be close to 100%, and with the present lack of suitable cell lines to propagate these viruses, it is challenging to obtain material to study their host impact and infection routes. Thus, virus free lice strains were established from virus infected lice carrying one or both LsRVs by treating them with N protein dsRNA twice during development. The viral replication of the N protein was specifically down-regulated following introduction of virus-specific dsRNA, and virus-free lice strains were maintained for several generations. A preliminary study on infection routes suggested that the LsRV-No9 is maternally transmitted, and that the virus transmits from males to females horizontally. The ability to produce virus free strains allows for further studies on transmission modes and how these viruses influences on the *L. salmonis* interaction with its salmonid host. Moreover, this study provides a general fundament for future studies on how vertically transmitted rhabdoviruses influence the biology of their arthropod hosts.
pre-adult stages before the final molt to adult. The louse feeds on mucus, skin, and blood\textsuperscript{23,24}; hence, lice infestation can increase the susceptibility to other pathogens as it disturbs the osmotic balance and stress the fish\textsuperscript{25–27}. Thereby, RNAi has been used to study the function of various salmon louse genes by soaking of nauplius I larva or by injections of the larger pre-adult and adult stages\textsuperscript{28–32}, in order to identify potential targets for new treatments and countermeasures.

Recently, two vertically transmitted salmon louse rhabdoviruses, LsRV-No9 and No127, have been identified\textsuperscript{33}. Rhabdoviruses are a family of enveloped viruses with a non-segmented negative-sense single-stranded RNA genome, infecting a variety of host such as mammals, fish, birds, reptiles, insects, crustaceans and plants\textsuperscript{34}. The LsRVs have been identified in salmon louse gland tissue\textsuperscript{33}, and is potentially secreted onto the salmonid host skin where the expression of genes generally believed to be virus induced often are found to be moderately increased following lice infestation\textsuperscript{35–37}. Studies on transmission routes and how the LsRVs affect louse biology and the ability to immune modulate the host is therefore of importance. There are at present no cell lines or infection models available for propagating the LsRVs\textsuperscript{33}. In order to study LsRV transmission routes and their effect on louse biology and the host-parasitic interaction, we therefore aimed to produce virus infected (LsV) and virus free (LsVF) salmon louse strains from a common virus infected origin by using RNA interference. Prior to this, the presence of the LsRVs in lice from or near Norwegian salmon farms was analysed, as to be able to make strains reflecting the wild population of salmon lice.

**Results and Discussion**

**Prevalence of the LsRV strains No9 and No127.** The presence of LsRV-No9 and No127 in salmon louse sampled from farmed fish or fish nearby farms at 13 locations along the Norwegian coast was examined. Samples were obtained from: Veranger, Tana, Alta, Senja, Harstad, Bodø, the coast of Helgeland, Bjugg, Agdenes, Romsdalen, Gulen, Hardanger and Dirdal (gray circles, location names listed from North to South). The graph shows the frequency of lice with both viral strains (No9 and No127), with No9 only, with No127 only, and with none of the two viral strains. The map, ”Kommuner med hav”, was downloaded from the Norwegian Mapping Authority (https://kartkatalog.geonorge.no/metadata/kartverket/norge-illustrasjonskart/a374867-60c0-4524-9eda-b15ab4d12858), and incorporated into the figure after changing it to grayscale and excluding text. License of use: https://creativecommons.org/licenses/by/4.0/deed.no.

![Prevalence of the two LsRV strains No9 and No127 in 75 lice sampled from farmed fish or fish nearby farms at 13 locations along the Norwegian coast.](https://kartkatalog.geonorge.no/metadata/kartverket/norge-illustrasjonskart/a374867-60c0-4524-9eda-b15ab4d12858)

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**Figure 1.** Prevalence of the two LsRV strains No9 and No127 in 75 lice sampled from farmed fish or fish nearby farms at 13 locations along the Norwegian cost. Samples were obtained from: Veranger, Tana, Alta, Senja, Harstad, Bodø, the coast of Helgeland, Bjugg, Agdenes, Romsdalen, Gulen, Hardanger and Dirdal (gray circles, location names listed from North to South). The graph shows the frequency of lice with both viral strains (No9 and No127), with No9 only, with No127 only, and with none of the two viral strains. The map, ”Kommuner med hav”, was downloaded from the Norwegian Mapping Authority (https://kartkatalog.geonorge.no/metadata/kartverket/norge-illustrasjonskart/a374867-60c0-4524-9eda-b15ab4d12858), and incorporated into the figure after changing it to grayscale and excluding text. License of use: https://creativecommons.org/licenses/by/4.0/deed.no.
louse were further analyzed for the presence of the two LsRVs, however, only the LsRV-No9 was detected in the LsGulen and LsAlta strains, while the LsOslo strain was negative for both viruses.

**Specificity of knockdown.** RNA interference represents an important sequence-specific innate immune response towards viruses in invertebrates, however, sequence-independent responses have been reported in crustaceans like shrimps. Therefore, the specificity of viral knockdown was analyzed in the present study. The viral N protein RNA level decreased in free-living copepods soaked in LsRV N protein dsRNA and not in lice soaked in control dsRNA or N protein dsRNA targeting the other rhabdovirus strain (Fig. 2). Hence, the induced anti-viral response was considered sequence-specific. An increase in the viral N protein RNA level were seen in control animals at 8 dpi, indicating that the LsRV-No9 overcomes the salmon louse RNAi defense mechanism likely as the viral dsRNA formed during viral replication and transcription are concealed from the RNAi system. In other rhabdoviruses, encapsulation of the viral genome during replication and the use of the ribonucleoprotein complex as template for primary transcription rather than the naked RNA have been demonstrated.

**Viral knockdown and production of a virus free lice strain.** After confirming the specificity of viral knockdown, attempts to produce virus free lice strains using the LsRV-No9 infected LsGulen strain were made. Samples obtained from the LsVF1 F0 generation of lice at the chalimus I, pre-adult and adult stages displayed a gradual increase in viral N protein RNA level after the first pre-adult stage (LsVF1a, Table 1, Fig. 3), suggesting that only one RNAi treatment is not sufficient to produce virus free egg bearing adults. Also in previous RNAi studies targeting salmon louse genes, a decrease in knockdown during development has been demonstrated. Therefore, a group of lice were given a second dsRNA treatment at the pre-adult stage, and an average knockdown of 97 and 87% was achieved in adult males and females, respectively (LsVF1b, Table 1). Viral N protein RNA were not detected in six out of ten adult female lice sampled or in the following LsVF 1b generations. These results were confirmed in experiment 2, using a lower density of knockdown lice on the host fish. Here, an average knockdown of 99.9% was seen and six out of eight female lice from the dsRNA treated group were LsRV negative (Table 1). N protein RNA was not detected in the offspring from these eight lice, including the offspring from two slightly positive females. Probably, a certain level of LsRV-No9 virions needs to be present in the parenting female lice to ensure vertical transfer of the virus. Moreover, no viral N protein RNA was detected in subsequent generations. A second round of dsRNA treatment at the pre-adult stage thus increases the percentage of negative lice substantially, and is therefore required to establish a virus free strain of salmon lice. It was not tested whether an injection of the dsRNA treatment in the previous generation, and this may explain the high treatment efficiency. This suggests that only one RNAi treatment is not sufficient to produce virus-free offspring, however; at this point in development the ovaries have started to develop, increasing the risk of transferring the LsRVs during reproduction.

To test if it was possible to knockdown both LsRVs simultaneously, lice from the Hardanger fjord (LsHardanger) were taken into the lab and confirmed to be positive for both LsRVs. Eggs were incubated and lice were subjected to dsRNA treatment as in previous experiments. However, in this third experiment the LsV3 and LsVF3 F0 generation originated from the third egg string pair of adult F0 females, displaying a modest average knockdown of 66 and 83% of LsRV-No9 and No127 strain, respectively (Table 1). Thereby, only one out of five females produced offspring negative for both viruses (Fig. 4). RNAi efficiency has been shown to decline when attempting to knockdown two salmon louse genes simultaneously. However, as demonstrated in experiment 1, viral RNA level rise over time post dsRNA treatment, and when using the first egg string pairs in experiment 4a, three out of five F0 females produced negative offspring (LsVF1a). This knockdown success is not altogether very different from that in experiment 1 and 2. This demonstrates the importance of collecting early egg strings to establish the virus free F1 generation, before the viral level increases above the threshold for vertical transmission. This also demonstrates that it is possible to simultaneously knockdown both viruses sufficiently to produce virus free strains from a double infected origin. Interestingly, in experiment 4b, dsRNA treatment of the nauplius cohort originating from LsVF3 F0 females apparently cured all treated individuals, and the LsVF4b strain remained negative for three generations (Table 1). This nauplius cohort had lower virus levels than normal due to the dsRNA treatment in the previous generation, and this may explain the high treatment efficiency. This suggests...
that increasing the dose of administered dsRNA may potentially remove both viruses from a lice cohort over one generation. However, since it is not possible to analyze the viral status of lice without sacrificing them, it is recommended to use the F1 generation to obtain material for experimental studies rather than curing the F0 generation.

**Horizontal transmission of LsRV-No9.** The degree of horizontal transmission of the LsRV-No9 virus was studied due to its wide distribution along the Norwegian coast. Virus free adult males (LsVF2) were cohabitated with virus infected pre-adult females (LsV2) on naïve fish and later sampled as adults. After 35 days of cohabitation, three out of seven males had become marginally positive for the LsRV-No9 and eight out of 14 lice slightly positive at 56 days of cohabitation (Fig. 5a). As the LsRVs have been localized to lice glandular tissue and on salmon skin at the chalimi attachment site33, it is likely that virus particles are secreted onto the salmon skin and potentially consumed by the lice. The uptake of virus particles through the gut seems, however, to be limited as the level of viral RNA was low, still after 56 days of cohabitation. This could suggest that virus particles were only present in the gut content of the positive males or adhered to the louse mucoid coat. Thus starving LsVF male cohabitants in continuous flow-through incubators prior to analysis could answer whether males obtained the infection horizontally.

To study transmission of LsRV-No9 from males to females, pre-adult II LsVF2 females were placed on naïve fish together with LsV2 adult males. After 35 days of cohabitation the females had molted into adults, become fertilized by LsRV-No9 positive males by the attachment of a sperm sac, and produced their first external fertilized offspring. The degree of horizontal transmission was estimated by performing F1 offspring analyses. As no virus was detected in the material sampled, it is likely that the virus did not spread to the F1 generation by horizontal transmission.

**Table 1.** RNAi mediated down regulation of the viral N protein genes in treated _L. salmonis_. Data from experiment 1–4 are listed, showing the lice origin, virus strain, percent down regulation at various days post infection (DPI) and the number of virus negative lice. Percent down-regulation ± SD were calculated from pooled samples of planktonic copepodids (cop free) and chalimus I (chal I), and from single animals for the pre-adult (pad) and adult stages. LsV = virus infected strain, LsVF = virus free strain All the subsequent LsVF generations maintained post treatment were tested (N generations) for presence of virus and found negative.
Figure 4. N protein RNA Ct values in parenting females LsV3 and LsVF4 (75 days post infestation (dpi)) and LsV4, LsVF4a and LsVF4b (56 dpi) plotted against the Ct values of their respective offspring at the copepodid stage. (A) LsRV-No9 and (B) No127. The reference gene was highly stable among the samples. A Ct value of 40 indicates undetected levels of viral RNA.

Figure 5. Level of LsRV-No9 N protein RNA given as average Ct values for each biological replicate in the transmission experiment. (A) LsVF₂ females (black circle) cohabitated with LsV₂ males (gray triangle). (B) LsV₂ females (gray circle) cohabitated with LsVF₂ males (black triangle). (C) Ct values from LsV₂ and LsVF₂ females sampled at 56 days post infestation (dpi) plotted against the Ct values of their respective offspring at the copepodid stage. A Ct value of 40 indicates undetected levels of viral RNA. The reference gene was highly stable among the samples.
egg strings. At this stage none of the females were LsRV positive (Fig. 5b), hence if viral particles were transferred with the spermatophore, the viral RNA level must have been below the detection limit. After 56 days of cohabitation, seven out of 13 LsVF2 females were positive, and six of these had equally high viral N protein RNA level as the LsVF2 lice. Surprisingly, four of these seven positive LsVF2 females produced negative offspring, while the other three females produced only marginally positive offspring (Fig. 5c). In contrast, the LsV2 females, with similar N protein RNA level, all produce virus infected offspring displaying significantly higher N protein RNA levels. Presumably, the virus was vertically transmitted from females horizontally infected by males, and not directly from male sperm. The females were infected by virus particles entering either through the gut, or via the seminal fluid delivered during mating. Considering that LsVF2 males showed only marginally elevated levels of viral RNA after cohabitation with LsV2 females, the latter is more likely. Similarly, sigma viruses that infect Mediterranean fruit flies (Ceratitis capitata), Drosophila immigrans, and speckled wood butterflies (Pararge aegeria) have been shown to be transmitted maternally, with only lower rates of paternal transmission seen. The presence of highly positive females producing offspring that were either negative or slightly positive for the LsRVs indicates that the infection needs to develop beyond a threshold for vertical transfer in females before entering the eggs at some point during oocyte development. At any point in time, an adult female carry at least four batches of eggs in different phases of development. One batch are developing embryos within external egg strings, one batch of eggs undergoing vitellogenesis in the genital segment, one batch developing in the oviduct (this phase include the development of oogonium cell walls) and at least one batch of future oocytes developing within the large tubular syncytium that make up the ovary. At 10 °C, each batch require around 9–10 days before moving to the next stage of development. Thus, the oocytes giving rise to the egg strings collected at 56 days were formed in the ovary roughly 20–30 days earlier, at a point in time where females tested negative for the virus. Previously, high levels of both LsRV transcripts have been detected in the salmon louse ovaries. Altogether, this suggests that the LsRV-No9 virus transmits to eggs in an early phase of oocyte development.

Conclusion. The present study shows that it is possible to simultaneously remove two vertically transmitted rhabdoviruses from a strain of *L. salmonis* by subjecting the lice to two dsRNA treatments during development. As the degree of horizontal transmission of LsRV-No9 through the salmon louse gut seems to be limited, the main mode of transmission is most likely vertically as previously suggested. A low horizontal transmission rate may have been vital for the present success of RNAi mediated viral treatment in salmon louse since variability in the degree of knock-down and virus proliferation could potentially have produced a significant horizontal infection pressure increasing the virus level above the threshold for vertical transfer. The present study indicates that LsRV-No9 is, although to a lesser extent, horizontally transmitted with male sperm. This emphasizes the need to sample the first egg string before the females become re-infected by virus positive males. The fact that multiple infections appear to be common advocates the need to study transmission mechanisms in lice strains carrying several viruses. Moreover, since the LsRVs seems to be omnipresent at sites with extensive farming of salmon, further research on how these rhabdoviruses influence louse biology and the interaction between the lice and its salmonid host are of importance. As no cell lines for LsRVs cultivation are presently known, the method reported herein provide a vital fundament for further research on vertically transmitted RNA viruses in sea lice. In principle, this study also demonstrates in more general terms that it is possible to remove vertically transmitted virus from strains of arthropods by means of dsRNA treatment. This allows for experimental designs comparing virus infected and virus free strains from a common origin, reducing the genetic variability within the system observed.

Method

Source and culture of salmon lice. Laboratory strains of salmon lice were maintained on farmed Atlantic salmon (*Salmo salar*) according to Hamre et al. Three laboratory strains were analyzed for LsRVs, originating from Gulen (LsGulen), Alta (LsAlta) and the fjord of Oslo (LsOslo). Moreover, two to ten lice taken from or near salmon farms in Varanger, Tana, Alta, Senja (Laksfjord), Bodø (Skjerstadfjorden), the coast of Helgeland, Bjugn, Agdenes, Romsdalen, Gulen, Hardanger, and Dirdal (Hogsfjorden) were analyzed for the presence of the two LsRVs. All experimental procedures were performed in accordance with national legislation for animal welfare, and approved by the governmental Norwegian Animal Research Authority (NARA, [http://www.fdu.no/ldu/](http://www.fdu.no/ldu/)).

RNA purification and cDNA synthesis. Lice for RNA purification were stored in RNAlater (Qiagen). Total RNA from nauplius, copepods and chalimus stages was isolated with a combined Tri reagent (Sigma Aldrich) and RNeasy (Qiagen) method, as previously described. Pre-adult and adult lice were purified with Tri reagent (Sigma Aldrich) according to the Trizol reagent protocol described by Invitrogen. Samples were either frozen at –80 °C until use, or cDNA synthesis was done directly. For real time RT-PCR, cDNA synthesis was carried out using the AffinityScript qPCR cDNA Synthesis Kit (Stratagene) according to the supplier recommendations, adding 200 ng total RNA. The cDNA samples were diluted 3 times and stored at –20 °C until use. For PCR, the qScript cDNA SuperMix (Quanta Bioscience) was used, applying 1 µg total RNA.

Virus detection. For the detection of viral N protein RNA, real time RT-PCR was performed with 1x SYBR Select Master mix (Life Technologies), 500 nM gene specific primers (Table 2) and 2 µl cDNA in 10 µl reactions. Samples were run in duplicate on an Applied Biosystems 7500 Real-Time PCR System under standard conditions (50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 15 seconds and 60 °C for 1 min, followed by a melt curve analysis at 60–95 °C). The efficiency of each assay was tested with a five-point standard curve of 4-fold dilutions,
Establishment of virus free sea louse strains using RNA interference. The general procedure applied for establishing a virus free strain of salmon louse involved RNAi knock-down of the LsRV N protein to inhibit virus replication in a F0 generation of adult females in order to inhibit vertical transmission and produce a virus free F1 generation. To produce a virus free and virus infected strain, naïve fish kept in single tanks were divided into five groups. One group were not added any dsRNA (untreated) while the rest of the groups were added control dsRNA, N protein dsRNA from the No9 strain, N protein dsRNA from the No127 strain, or N protein dsRNA from both No9 and No127 strain. Samples were taken at 0, 4 and 8 days after soaking to analyze the viral N protein RNA level.

Table 2. Primers used for virus detection and production of dsRNA.

| Name                  | Primer 5′→3′                          | Application         |
|-----------------------|---------------------------------------|---------------------|
| Fw1 LsRV-No9          | TCCAGTTTAAAGACGGGTGATCGGACG           | Virus detection     |
| Rev1 LsRV-No9         | CACCATGCTACAGCTTCGCCGGAATGTC          | Virus detection     |
| Fw1 LsRV-No127        | CACCAAGCGGTTCCTCTGCCGTTCAATGG         | Virus detection     |
| Rev1 LsRV-No127       | CGAACGAGTTGTCGAGTATTACGGGACA          | Virus detection     |
| Fw2 LsRV-No9          | TTCCTCCGGAACCCGATAGGA                 | RNAi                |
| Rev2 LsRV-No9         | AGGGAATTGGCGGTGACTG                   | RNAi                |
| Fw2 LsRV-No127        | GGACTCGCGGAGGTATGACC                  | RNAi                |
| Rev2 LsRV-No127       | AAGGGGCCGTTGCTCAATCTTA                | RNAi                |

given by the equation $E\% = (\frac{10^{1/\text{dose}} - 1} {100})$. The salmon louse elongation factor 1 alpha (eEF1α) was used to normalize the data[52], and always included in the run. When the relative differences in threshold cycle between the viral genes and the reference gene ($\Delta\Delta$CT) and expression relative to controls ($\Delta\Delta$CT) were calculated, they were transformed by the equation $2^{-\Delta\Delta\text{CT}}$. If only CT values were displayed, the reference gene was always run and evaluated to be stably expressed. For detection of viral RNA in pre-adult and adult animals, one animal per sample was purified, while 10–50 animals were pooled in one sample for the copepodid and chalimus stages.

RNA interference. RNAi was performed as previously described[29,30], with primers listed in Table 2. In short, the MEGAscript RNAi Kit (Ambion) was used to produce double stranded RNA according to supplier’s instructions. The dsRNA was made targeting the N protein of the two viruses (Accession no.: KJ958535 and KJ958536).

Specificity. To analyze the specificity of the knock-down, nauplius I from three females positive for both viruses were each divided into five groups. One group were not added any dsRNA (untreated) while the rest of the groups were added control dsRNA, N protein dsRNA from the No9 strain, N protein dsRNA from the No127 strain, or N protein dsRNA from both No9 and No127 strain. Samples were taken at 0, 4 and 8 days after soaking for analyzing the viral N protein RNA level.

For soaking, a batch of newly hatched nauplii was incubated overnight in 1.5 µg of each dsRNA. After molting into the nauplius II stage, the nauplii were returned to flow through incubators. RNAi in pre-adults was performed by injecting around 250 ng of each dsRNA into the cephalothorax.

For analyzing the viral N protein RNA level, the groups were added control dsRNA, N protein dsRNA from the No9 strain, N protein dsRNA from the No127 strain, or N protein dsRNA from both No9 and No127 strain. Samples were taken at 0, 4 and 8 days after soaking to analyze the viral N protein RNA level.

Primers used for virus detection and production of dsRNA.

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| Rev1 LsRV-No9         | CACCATGCTACAGCTTCGCCGGAATGTC          | Virus detection     |
| Fw1 LsRV-No127        | CACCAAGCGGTTCCTCTGCCGTTCAATGG         | Virus detection     |
| Rev1 LsRV-No127       | CGAACGAGTTGTCGAGTATTACGGGACA          | Virus detection     |
| Fw2 LsRV-No9          | TTCCTCCGGAACCCGATAGGA                 | RNAi                |
| Rev2 LsRV-No9         | AGGGAATTGGCGGTGACTG                   | RNAi                |
| Fw2 LsRV-No127        | GGACTCGCGGAGGTATGACC                  | RNAi                |
| Rev2 LsRV-No127       | AAGGGGCCGTTGCTCAATCTTA                | RNAi                |
treatment at the pre-adult stage. Further, the first egg string produced by the females was sampled at 55 dpi, and the LsVF4b strain was followed for three generations.

**Transmission experiment.** To study the degree of horizontal transmission of LsRV-No9, pre-adult II females and adult males from the F2 generation of LsV2 and LsVF2 were used to infest two groups of naïve fish. Ten LsV2 female lice were put together with ten LsVF2 males on one fish in a single fish tank, while ten LsVF2 female lice were put on a second fish together with ten LsV2 males. After 35 days, when the females had become egg bearing adults (63 dpi), the lice were sampled and tested for virus.

A second transmission experiment was carried out using the LsV2 and LsVF2 F3 generation, with an equal experimental design, only this time adding eight females and eight males to each of three fish for each combination. Adult lice were sampled after 56 days (81 dpi), and the level of viral RNA was analyzed. Egg strings were collected and incubated in flow-trough incubators, and the copepodids were sampled and tested for virus by real time RT-PCR.

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

We carefully reviewed the ethical standards of the journal and we hereby certify that the procedures used with the investigated species comply fully with those standards.

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Author Contributions
A.C.Ø. designed and carried out the experiments and data analysis, and main authored the manuscript. L.A.H. designed and participated in experiments, discussed and helped writing the manuscript. H.K. participated in experiments and performed data analysis. F.N. conceived the project, discussed and helped writing the manuscript. All authors reviewed and approved of the manuscript before submission.

Additional Information
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