Deliverable Report

Deliverable No:	D26.2	**Delivery Month:**	48
Deliverable Title	Determine immune response and effectiveness of orally delivered VNN capsid protein on protection of Atlantic halibut larvae		
WP No:	26	**WP Lead beneficiary:**	P7. IMR
WP Title:	Fish Health – Atlantic halibut		
Task No:	26.2	**Task Lead beneficiary:**	P7. IMR
Task Title:	Monitor and assess immune response and protection		
Other beneficiaries:			
Status:	Delivered	**Expected month:**	48
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Lead Scientist preparing the Deliverable: Patel, S. (IMR)

Other Scientists participating: Nerland, A.H. (IMR)

Objective: The objective of this deliverable was to determine uptake of recombinant capsid protein expressed either in *E. coli*, tobacco plants or by the two eukaryotic expression systems into *Artemia* and further to late larval stages of Atlantic halibut.

Background: Atlantic halibut are affected by nodavirus especially at larval and early juvenile stages. Therefore, classical vaccination regimes by injection will not be a viable route of administration due to the size of the fish. Thus, it is important to assess if it is possible to deliver the vaccine orally through feed during the larval stages. Recombinant capsid protein from nodavirus expressed in *E. coli* has been shown to induce protection when formulated in a vaccine. By expressing the capsid protein of nodavirus recombinantly in different systems, and feeding these to *Artemia*, it would be possible to check if *Artemia* take up the protein, and also if larvae after feeding on these *Artemia* can respond to the recombinant protein as an antigen, thereby achieving protection against the virus.

Description:
The aim was to use the recombinant protein antigen expressed in different host systems. The host system might impact on the immune response for two reasons in this situation. The first is that the host cells might protect the recombinant antigen differently, initially in the digestive system of the *Artemia*, then in the digestive system of the larvae, both of which might influence where and how the antigen will stimulate the immune system. The other reason could be differences in post-translational modifications and its impact on the immunogenicity of the antigen. For example the eukaryote *Leishmania tarentolae* modifies proteins post-translationally quite similarly to higher eukaryotes (such as fish), whereas the prokaryote *E. coli* has little or no post-translational modifications.

Materials and methods:
The nodavirus capsid proteins expressed in several different systems (Deliverable 26.1) were given as feed to *Artemia* that were not enriched beforehand. As a result of collaboration with Targetfish project, we received nodavirus capsid protein expressed in *Pichia* as crude freeze-dried extract and also nodavirus VLPs expressed in *Pichia* and purified, and these were also included in the testing for uptake in *Artemia*. Enriched *Artemia* were produced according to the standard protocol used at IMR and used for the purpose. The antigen expressed by *E. coli* was purified and fed to *Artemia* and then further fed to halibut larvae. The larvae were fed for three days in a row and later fed with normal *Artemia* feed. The halibut larvae used in this study were 100 days post-hatch.

Testing of uptake of microbes to *Artemia*: To test if *E. coli* and *L. tarentolae* were taken up when fed to *Artemia*, genes encoding GFP (green flourescent protein) were introduced to the bacterium and the protozoan.

<u>Preparation of *E. coli GFP*:</u> The plasmid pTZ19R (Pharmacia) was fused to a plasmid isolated from *Aeromonas salmonicida* by digesting both plasmids with *Bam*HI, followed by ligation and selection on ampicillin containing agar plates. The resulting plasmid, which contained two *Eco*RI sites, was digested by *Eco*RI and self-ligated, resulting in deletion of most of the sequences coming from the *Aeromonas*. Into this plasmid a *Sal*I, *Xba*I fragment containing the GFP from pEGFP-N1 was inserted. The resulting construct designated pAsEGFP, gave very bright green fluorescence when transformed into *E. coli* and illuminated with UV-light around 480 nm. Before feeding *Artemia*, *E. coli* GFP was cultivated overnight in LB (Luria broth) containing 100ug/ml ampicillin, centrifuged at 3,000 g for 10 min and resuspended in an equal volume LB without antibiotic.

<u>Preparation of *L. tarentolae* GFP:</u> The plasmid pLEXSY-egfp-sat2 was obtained from Jena Bioscience, Germany, and digested with *Swa*I. The fragment containing the gene encoding GFP was isolated and transformed into *L. tarentolae* using the BTX-electroporator ECM 830 (Harvard Bioscience, USA) and following the protocol of Li *et al.* (2007), and the transformants were selected by using 100 µg/ml NTC (nourseothricin). The GFP-gene in this vector is flanked by sequences from the Leishmania 18S gene, causing the GFP-gene to be inserted into the chromosome of the protozoan. Before feeding to *Artemia*, the *L. tarentolae* was cultivated in BHI medium (Jena Bioscience) supplemented with 5 µg/ml hemin, 50 µg/ml streptomycin and 50 µg/ml penicillin G at 30°C for 3 days, centrifuged at 3,000g for10 min and resuspended in BHI without supplements.

Artemia were enriched as described below and kept in seawater at 8°C until use. Before feeding, 20 ml *Artemia* (1,000 organisms/ml) were transferred to a 50 ml tube and kept at 20°C for 1 h with aeration (Fig.1) then added either to 1 ml *E. coli* GFP or 1 ml *L. tarentolae* GFP and kept for another hour under the same conditions. Samples were taken, filtered through a plankton mesh, washed in seawater, spotted on an object glass, overlaid by a cover slip and inspected live in a florescence microscope (Leica DMRE).

<u>**Uptake of antigen by *Artemia*:**</u>
Early morning each day, the enriched *Artemia* were concentrated to 1,000 *Artemia* per ml, washed and incubated at 20°C for ~1 h so that the *Artemia* open their jaws and are ready for feeding (**Figure 1A**). At the end of incubation, the *Artemia* were mixed well and 35 ml per 50 ml tube was distributed. Either purified protein or live organisms expressing recombinant capsid protein were

added to the respective treatment tubes, and the tubes incubated in a water bath with aeration to maintain 20°C during the feeding period (**Figure 1B**).

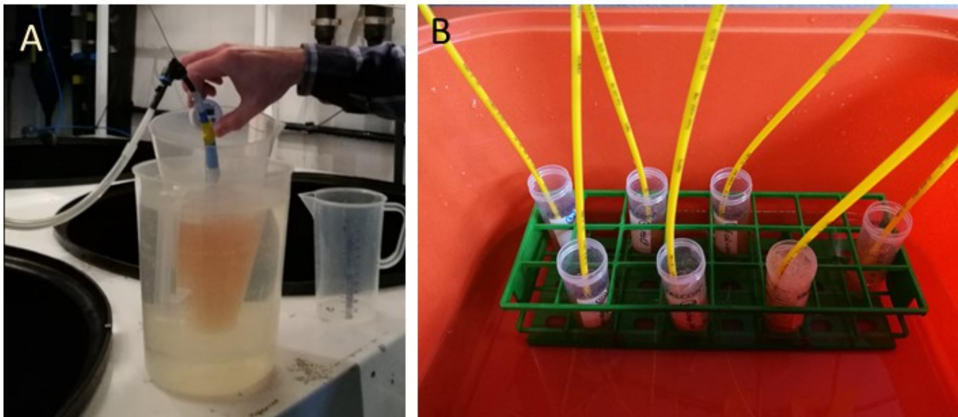

Figure 1 (A) *Artemia* incubated in water bath before being fed different formulations containing recombinant capsid protein. (B) Tubes with *Artemia* with aeration during incubation for uptake of specific protein or live organisms for oral delivery.

At the end of the incubation period, the *Artemia* were filtered through a plankton mesh, washed once with sea water and samples were collected from each of the groups for analysis. The samples were maintained at 4°C for a maximum of 3 h, and upon reaching the laboratory were frozen and stored at -20°C until analysed by SDS-PAGE and immunoblotting (Western blot).

Delivery of antigens to larvae:

For the different treatment groups 50 halibut larvae were kept in tanks with 15 L seawater at 9°C (**Figure 2).** The larvae were starved for the first delivery of feed in the morning. Before feeding with *Artemia*, the water circulation in the tanks was turned off, and the tanks were aerated during the feeding time of 2 h.

The seven different groups of larvae were orally fed as follows:

1. *Pichia* expressing recombinant capsid protein
2. *Pichia* with empty vector (no capsid protein)
3. *Artemia* fed live *L. tarentolae* expressing recombinant capsid protein
4. *Artemia* fed live *E. coli* expressing recombinant capsid protein
5. *Artemia* fed purified recombinant capsid protein (inclusion bodies) from *E. coli*
6. *Artemia* fed purified recombinant capsid protein (inclusion bodies) from *E. coli* for sampling of larvae for analysis of uptake of antigen.
7. Negative control

At the end of the feeding time, the water flow was restarted to remove the *Artemia* that had not been taken up as feed by the larvae. The larvae/juveniles were given one feed portion of routine enriched *Artemia* late in the evening and starved in the morning to repeat the recombinant capsid fed *Artemia* in the afternoon. The process was repeated for 2 days in a row.

The larvae from group 6 were sampled at days 1, 3, 5, 7, 9 and 11 (6 larvae/juveniles per sampling day) to analyse for uptake of the protein. At the end of the feeding time, the water flow through was restarted to remove the *Artemia* that were not taken up as feed by the larvae as before.

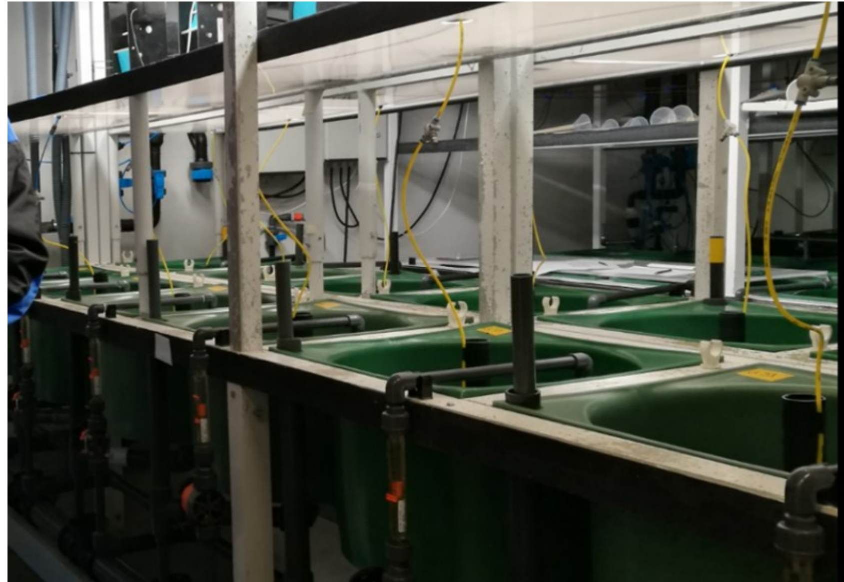

Figure 2 *Artemia* that had been fed with recombinant capsid protein expressed in different systems were fed to halibut larvae/juveniles and the water in the tanks was aerated during the feeding period.

SDS-PAGE and immunoblot:

For analyzing the uptake of antigen, samples of around 200 *Artemia* were added to NuPAGE™ LDS sample buffer (Invitrogen) according to the manufacturer´s recommendations, grinded with a Pellet Pestle (Kontes) and heated at 80°C for 10 min. Likewise, the intestines from the sampled larvae were dissected, added to sample buffer and handled as described for the larvae.

Samples were applied to a 4-12% NuPage Bis-Tris gel (Invitrogen), followed by blotting to a nitrocellulose filter. The filter was handled according to the protocol from the WesternBreeze chromogenic anti-rabbit kit (Invitrogen), where the rabbit-anti recombinant nodavirus capsid protein K233 (Sommerset et al 2005) was used as the primary antigen.

Results:

Visualizing the uptake of *E. coli* and *Leishmania* by GFP:

Figures 3 and 4 show the pictures taken of *Artemia* fed *E. coli* expressing GFP and *L. tarentolae* expressing GFP, respectively. Both microorganisms were taken up very efficiently by the *Artemia*. As the *Artemia* (as well as the microbes) were inspected live, it was observed that the *Artemia* filtered and accumulated both type of microbes.

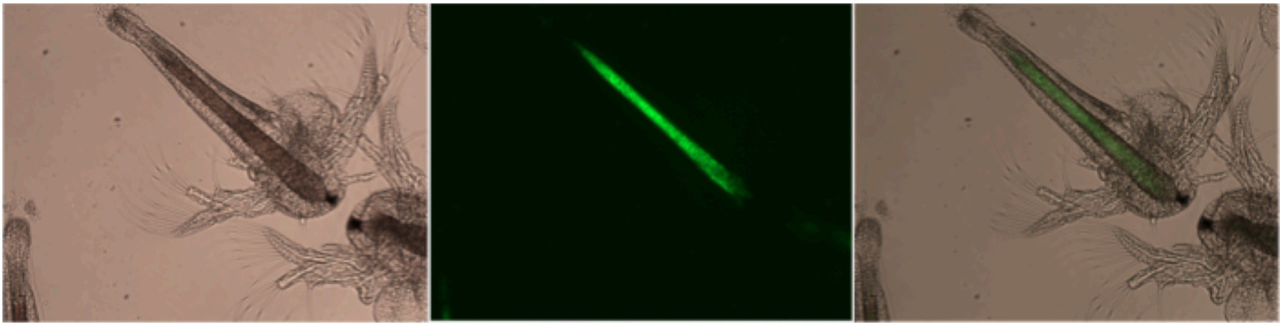

Figure 3. Microscopic pictures of *Artemia* fed with *E. coli* expressing GFP. To the left a light microscope image; in the middle a fluorescence microscope image, and to the right an overlay of the left and middle images.

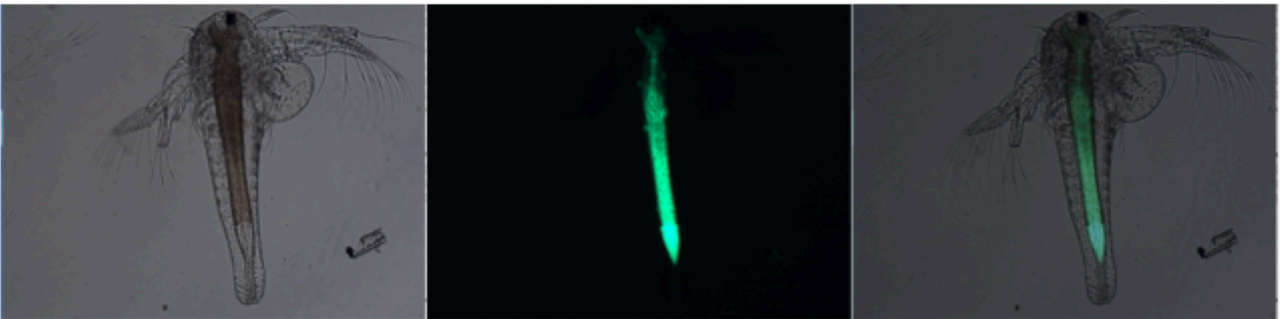

Figure 4. Microscopic pictures of *Artemia* fed with *L. tarentolae* expressing GFP. To the left a light microscope image; in the middle a fluorescence microscope image, and to the right an overlay of the left and middle images.

Uptake of recombinant capid protein by *Artemia*

To examine the presence of the recombinant antigen in the *Artemia* after being fed by the different formulations, and later the presence of recombinant antigens in the larvae after having been fed by the *Artemia*, samples were applied on a SDS-PAGE and analysed be immunoblotting.

Figures 5 and 6 reveal that the recombinant capsid proteins were present but were degraded by the *Artemia*. The capsid antigen fed to the *Artemia* as inclusion bodies was degraded from the original 43 kD protein to one of around 17 kD (**Figures 5 and 6**, lane 1) which also appears when applying the recombinant protein on the gel (**Figures 5 and 6**, lane 10). For *Artemia* fed with *E. coli* expressing recombinant antigen there were positive signals on the blot, but the size indicates a partial degradation of the protein. For the recombinant protein expressed in *Pichia* the signals indicate proteins larger than 43 kD in **Figure 5**, but in **Figure 6** there is a signal around 43 kD, which might be due to overloading of the total amount of protein on the gel shown in **Figure** 5. For *Artemia* fed with *L. tarentolae* expressing the recombinant protein it was difficult to see any signal at all. However, these results are qualitative, and not quantitative. *Leishmania* gives less expression of recombinant protein than the other host systems, and it might be that the amount of protein applied to the gel is lower that the detection limit. For days 1 and 2 the pictures were quite similar, while for day 3 the signals were weaker, but still with the same tendency.

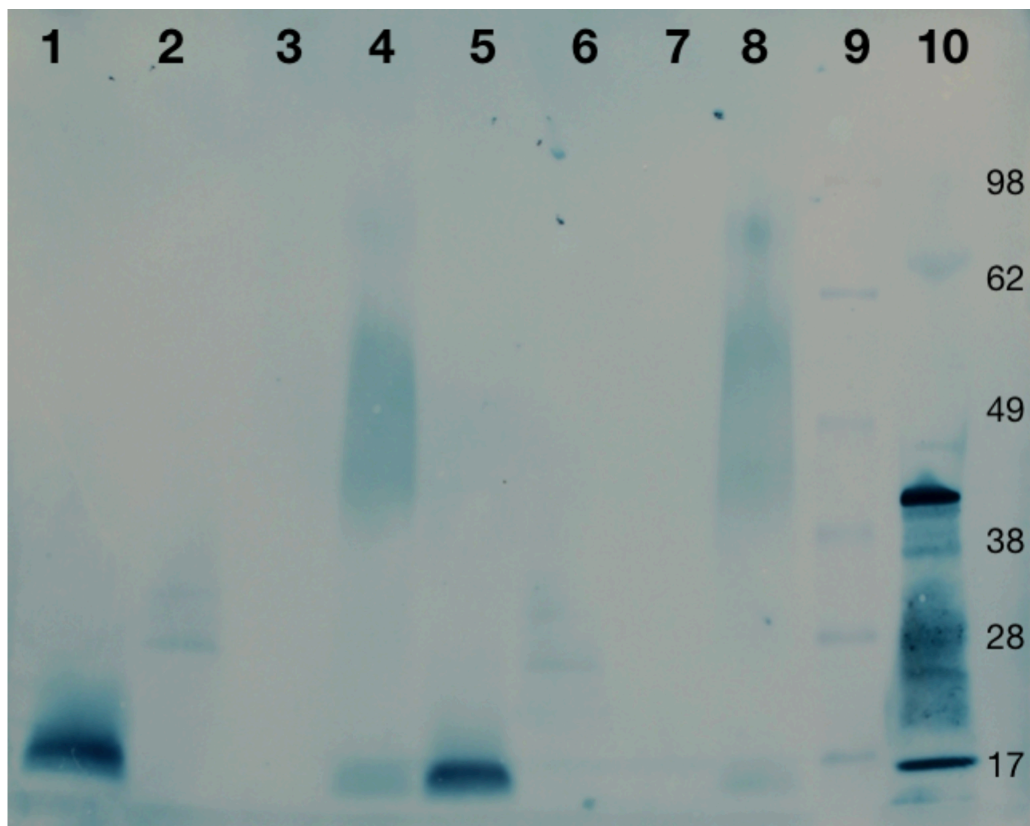

Figure 5. Immunoblot using anti-recombinant capsid protein as primary antibody, with the following samples: In lanes 1- 4 *Artemia* fed the antigens in the different forms at day 1 of the feeding experiment (1. *Artemia* fed purified recombinant protein expressed in *E. coli*, 2. *Artemia* fed *E. coli* expressing capsid protein, 3. *Artemia* fed *L. tarentolae* expressing capsid protein, 4. *Artemia* fed *Pischia* expressing capsid protein). Lanes 5-8 similar order of samples as 1-4 taken at day 2. Lane 9: marker protein (mw indicated to the right), Lane 10: positive control (purified recombinant capsid protein).

For the larvae fed with the *Artemia* containing isolated recombinant capsid protein (expressed in *E. coli*) there was a signal from one of the larvae from day 1 after the last feeding, giving a sharp band corresponding to 20-25 kD. For the other 5 larvae from day 1, and the larvae sampled at days 3, 5, 7, 9 and 11 post feeding, no detectable protein was observed. The gels with no detectable proteins for the rest of the sampling days (containing only the signal for the positive control) have not been shown.

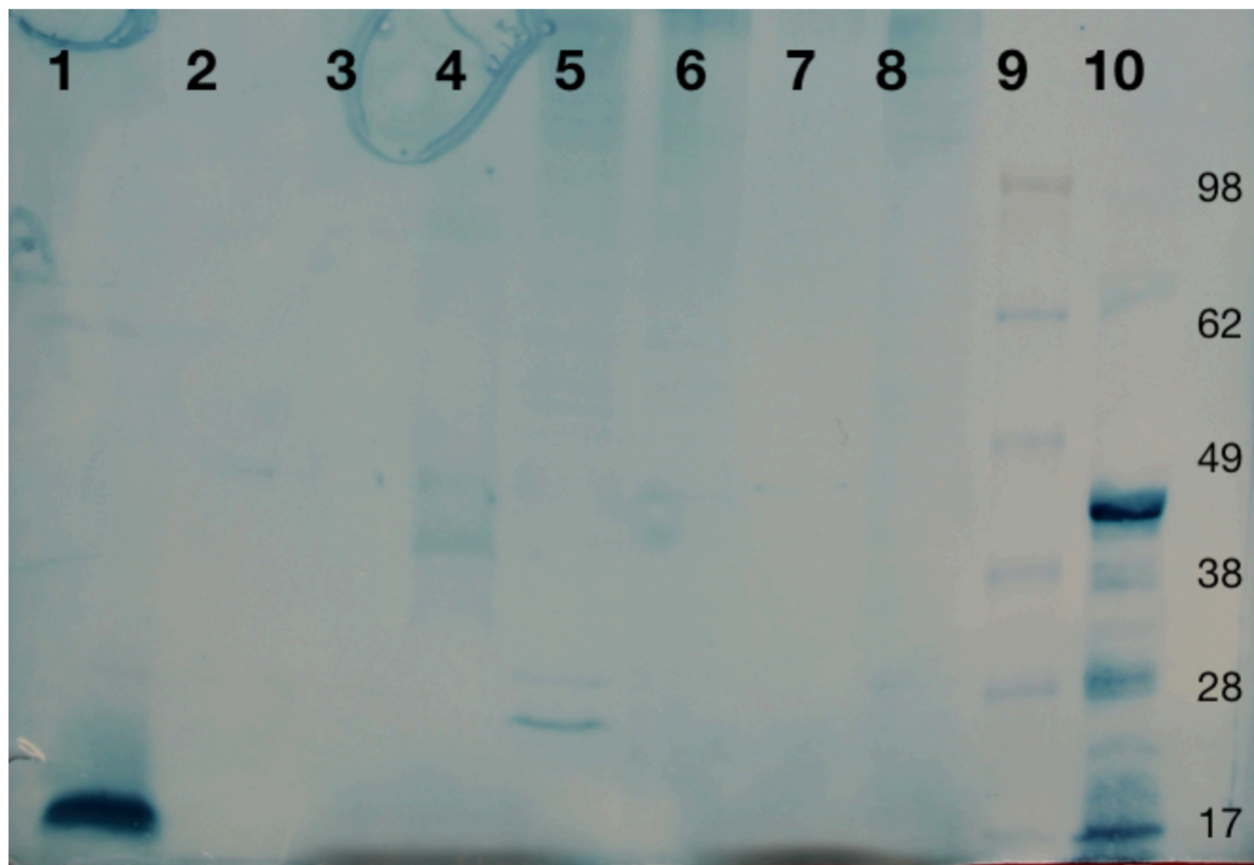

Figure 6. Immunoblot using anti-recombinant capsid protein as primary antibody. In lanes 1- 4 *Artemia* fed the antigens in the different forms at day 3 of the feeding experiment. 1. *Artemia* fed purified recombinant protein expressed in *E. coli*, 2. *Artemia* fed *E. coli* expressing capsid protein, 3. *Artemia* fed *L. tarentolae* expressing capsid protein, 4. *Artemia* fed *Pichia* expressing capsid protein. Lanes 5-8: Intestine samples of 4 larvae sampled at day 1 after the larvae had been fed for 2 days, Lane 9: marker protein (mw indicated to the right), Lane 10: positive control (purified recombinant capsid protein).

Conclusion:

By using GFP expressing microbes it was shown that both *E. coli* and *L. tarentolae* are readily filtered from the surroundings and taken up by *Artemia* (Figures 3 and 4). It is difficult to conclude from this experiment, however, the extent to which the microbe or its harboring proteins are degraded in the intestine of the *Artemia*. Presumably this will also be dependent on the different types of proteins. For example, GFP might be more stable than the recombinant capsid protein.

SDS-PAGE and immune-blotting reveal that *Artemia* will digest the recombinant protein, but the degradation depends on the expressing host (Figures 5 and 6). The recombinant antigen could be detected in the intestine of the larvae only at day 1, post end of the feeding and in just one out of six larvae examined.

References:

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Sommerset, I., Skern, R., Biering, E., Bleie, H., Fiksdal, I.U., Grove, S., and Nerland, A.H. (2005). Protection against Atlantic halibut nodavirus in turbot is induced by recombinant capsid protein vaccination but not following DNA vaccination. Fish Shellfish Immunol. *18*, 13–29.

Li, Y., et al. (2007). "Identification of new kinetoplast DNA replication proteins in trypanosomatids based on predicted S-Phase expression and mitochondrial targeting." Eukaryotic Cell 6(12): 2303-2310.

Deviations: None