New species for EU aquaculture

Technical Leaflets – Atlantic halibut (*Hippoglossus hippoglossus*)

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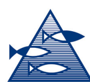
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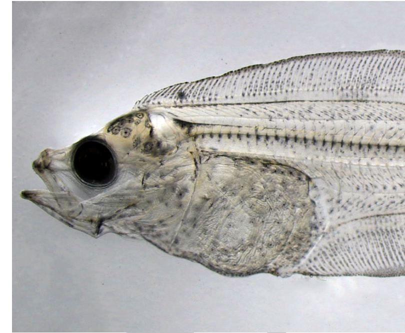

The Atlantic halibut is the world's largest flatfish and can attain a weight of over 300 Kg. It is highly prized at markets worldwide, but availability of wild Atlantic halibut is decreasing. Norwegian stocks are classified as viable, but fisheries are subject to strict regulation. This has led to a higher market demand for Atlantic halibut than cannot be met by fisheries alone. Cultured Atlantic halibut has an excellent reputation. The Atlantic halibut is a semi-fat fish, rich in omega-3 fatty acids, with a characteristic flaky white meat with few bones. In terms of product diversification, Atlantic halibut is traditionally marketed as large fish steaks or cutlets. It can be smoked or marinated in the typical Scandinavian style. These characteristics led to the inclusion of Atlantic halibut in DIVERSIFY, as a great candidate for fish species and product diversification in European aquaculture.

Research and cultivation efforts of Atlantic halibut started in the 1980's, and although the total annual production of cultured Atlantic halibut is increasing, it still only reached ~1600 t in 2017 (Norwegian Directorate of Fisheries). In Europe, Atlantic halibut farms exist in Norway and Scotland. The desired market size is 5-10 kg and production time is currently 4-5 yrs. Despite a significant research effort between 1985 and 2000, the complicated life cycle of Atlantic halibut made aquaculture progress slow, and very little research funding has been allocated thereafter. However, during this time slow but steady progress has been made by the farmers in order to improve production stability, and interest in both cage and land-based culture is growing. The remaining bottlenecks for increased and stable production are related to a steady supply of fry and a need to decrease the production time. The latter may be achieved with the recent establishment of "all female" juvenile production (Hendry et al., 2003; Babiak et al., 2012). This is expected to have a major impact on production time as females grow faster and mature later –80% of slaughtered fish <5 kg are mature males (P22 SWH, production statistics). DIVERSIFY addressed these important bottlenecks with a coordinated research effort in reproduction, larval nutrition and husbandry, and vaccine development.

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Reproduction and Genetics

Reproductive performance of wild-capture vs cultured female Atlantic halibut

Birgitta Norberg, Institute of Marine Research (Norway); Børre Erstad, Sterling White Halibut (Norway)

In order to obtain eggs with high viability, females have to be stripped according to their individual ovulatory rhythms, to prevent over-ripening and deterioration of the eggs (Norberg et al., 1991). While wild-captured females generally adapt well in captivity, displaying high fecundity with egg batches spawned at regular intervals, hatchery-produced F1/F2 females appear to suffer from a reproductive dysfunction, releasing small batches of eggs at irregular intervals. There is, however, a lack of thoroughly documented evidence to support the hypothesis of such a reproductive dysfunction in farmed females. Consequently, reproductive performance of wild-caught halibut and farmed (F1) females was compared.

Based on the information gathered in the first year of the project, one group of wild-captured breeders, held in captivity for at least four years (n=4), and one group of farmed females (F1; n=5) were closely monitored during the spawning season of 2015. All individual females were measured in January, before the start of the breeding season (**Table 1**). Eggs were stripped and fertilized and the following parameters were recorded: Egg batch volume, fertilization rate, batch interval (hours between ovulations), number of batches, total and relative fecundity. Egg yolk content of cortisol and testosterone was analyzed in unfertilized eggs from all collected batches. Based on the results obtained in 2015, egg characteristics (fertilization, diameter, cell division symmetry) and hatching success were compared in selected egg batches from the same wild-caught and farmed females that were used the previous year. Fertilized eggs were photographed at the 8-16 cell stage using a dissecting microscope for measurements of egg diameter and blastomere symmetry. For calculation of hatching percentage, 300 eggs were collected and divided into three beakers containing 500 ml of sterile-filtered seawater (salinity 35‰, temperature 6°C) and incubated in darkness at 6°C for 72 hours. Hatched larvae and dead eggs were counted in a binocular microscope. Larvae were also photographed in a dissecting microscope, to document any possible aberrations from normal development.

Table 1. Biometric and spawning performance data of wild-caught and farmed Atlantic halibut breeders at IMR, Austevoll

	Wild-caught females	**Farmed (F1) females**
n	3 (4[a])	5
length (cm)	150.7 ± 6.2	113.4 ± 3.9
weight (kg)	48 ± 5.7	19.2 ± 2.3*
number of batches · female^{-1}	7.3 ± 0.6	9.4 ± 1.7
spawning interval (hours)	82.2 ± 8.4	72.4 ± 22.9
batch volume (mL)	2300 ± 900	700 ± 300*
total fecundity (mL · female^{-1})	16700 ± 420	6800 ± 130*
relative fecundity (mL · kg^{-1})	347 ± 70	349 ± 84
average fertilization (%)	89 ± 7	61 ± 29

[a] One domesticated female was left undisturbed for most of the season, due to a large skin lesion, and was not included in calculations. *Significant difference (P<0.05; Mann-Whitney U-test)

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Egg diameters were in the range of 2670 to 3700 µm. There were no significant differences in egg diameter between individual fish, but wild-caught females had significantly larger eggs than farmed females and there were significant differences in diameter between viable, dead and unfertilized eggs (**Fig. 1**).

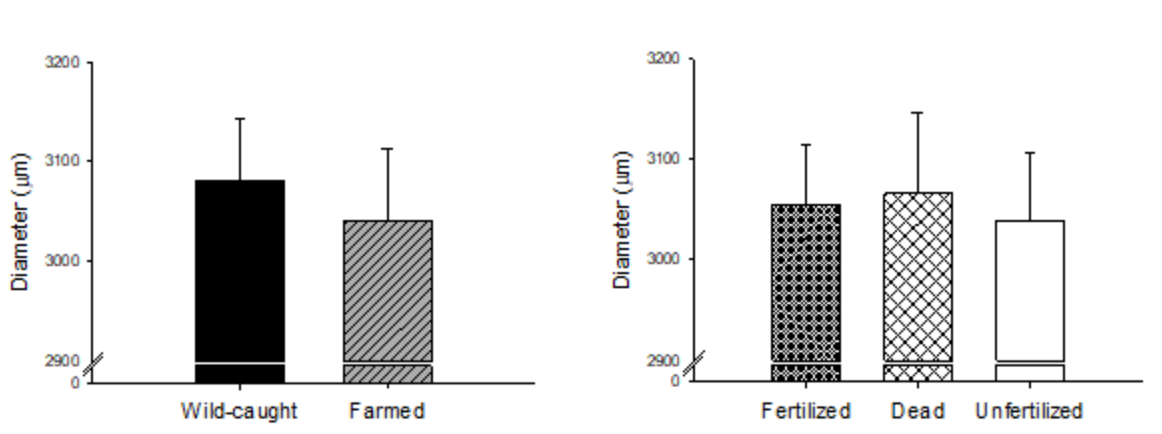

Figure 1. Diameters of) Eggs from wild-caught ("domesticated") and farmed Atlantic halibut females (*Indicates significant difference) (left), and fertilized, dead and unfertilized eggs from wild-caught and farmed Atlantic halibut females (right).

When eggs were characterized according to development, most were either categorized as fertilized/OK or dead. Very few of the fertilized eggs showed signs of asymmetric cell division, and most eggs appeared to be cleared from the ovarian cavity when the batch was spawned (**Fig. 2**).

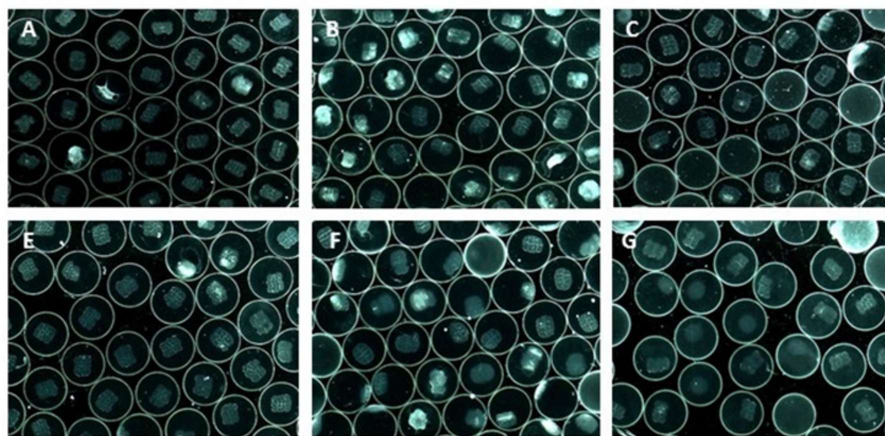

Figure 2. Fertilized eggs at the 8-16 cell stages from wild-caught (A-C) and farmed (E-G) Atlantic halibut.

There was a large variation in the fraction of viable eggs between individuals and between batches from the same individual. Wild-caught fish gave eggs that were more viable than those from farmed fish. Fertilization and hatching success both showed a tendency to be lower in farmed fish, (**Fig 3 left**). Overall, eggs from domesticated females tended to have higher fertilization and had significantly higher hatching success, with less individual variation than farmed females. (**Fig. 3 right**).

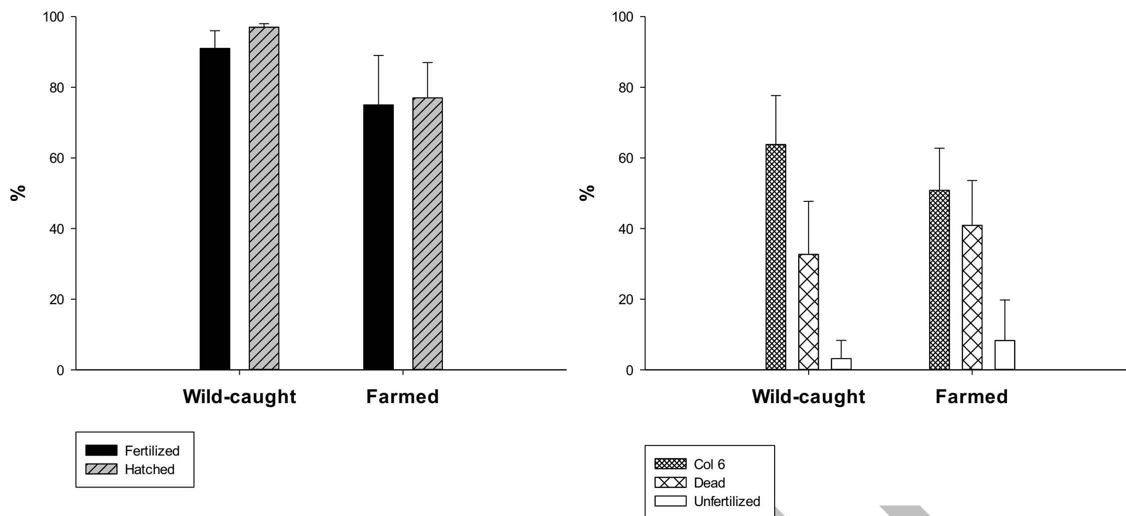

Figure 3. Left graph: Fertilization (black bars) and hatching (grey bars) success in eggs from wild-caught and farmed females. *Indicates significant difference. Right graph: Percent fertilized, viable (left), dead (middle) and unfertilized (right) eggs in wild-caught and farmed females. *Indicates significant difference.

Some differences were observed between eggs and larvae from the different females. Eggs from farmed females tended to be heavier, and would sink to the bottom of the incubator/beaker, while eggs from wild-caught females remained buoyant near the surface. In addition, dead or deformed larvae were observed more frequently when eggs from farmed females hatched (**Fig 4)**. It is not clear what caused the deformities, but one possible cause may be mechanical damage of the heavy eggs, that sank and rested at the bottom of the beaker for two days.

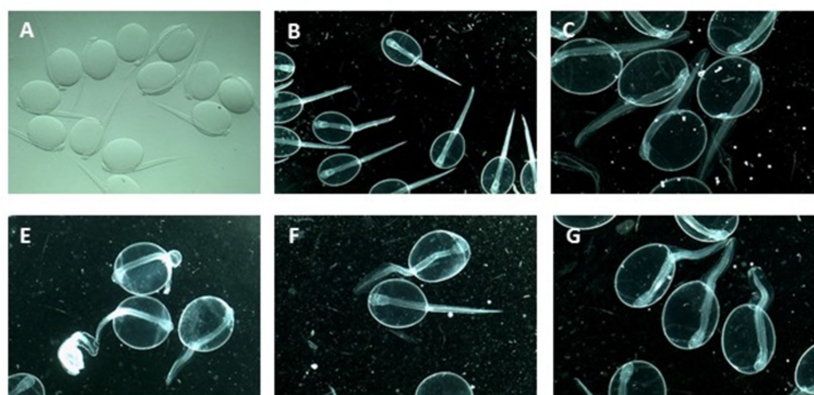

Figure 4. Newly hatched larvae from wild-caught (A-C) and farmed (E-G) Atlantic halibut females. Note spinal deformities in E, F and G.

No significant difference was found in average content of cortisol or testosterone in eggs of wild-caught and farmed female Atlantic halibut. There was a general trend towards decreasing egg content of steroids through spawning.

In conclusion, wild-caught, domesticated females were predictable spawners that consistently gave eggs of very high quality (>85% fertilization). Farmed females also produced eggs of high quality when their ovulatory cycles were identified and stripping carried out close to ovulation. For commercial, as well as breeding purposes, it is not practical to rely on wild-caught females. However, relatively few farmed females consistently produced eggs with fertilization rates >80-85%. As a consequence, it may be necessary to include wild-caught broodstock also in future breeding groups in order to ensure a broad enough genetic material. Identifying potential high-quality breeders and concentrating the strip-spawning effort on those females may be useful in order to reduce the very considerable work load connected with spawning and egg collection of halibut.

GnRHa therapy to improve spawning performance

Birgitta Norberg, Institute of Marine Research (Norway); Constantinos C. Mylonas, Hellenic Center for Marine Research.

Atlantic halibut females are periodic spawners that release their eggs in 5-10 batches during the annual spawning season (Norberg et al., 1991). Wild-captured Atlantic halibut females mature and release eggs of good quality in captivity. However, sometimes females of the F1/F2 generation have been reported to display reproductive dysfunctions, including irregular spawning cycles, low and unstable fertilization, low gamete survival and lower realized fecundity than wild females. One way of mitigating these dysfunctions might be to use gonadotropin releasing hormone agonist (GnRHa) therapy. Thus, we tested GnRHa implantations on F1/F2 halibut females from two populations, one produced and held at IMR, Austevoll Research Station (Norway), and one population that was part of a commercial broodstock at the SWH Reipholmen hatchery (Norway).

All females had a total length between 100 and 115 cm. The fish were divided in three groups (n=4), that received either GnRHa implants for an effective dose of 50 μg kg^{-1} or 100 μg kg^{-1}, or were sham injected (Control group). After treatment, fish were left undisturbed for 3 days, and were then checked daily for signs of ovulation.

In response to treatment with the GnRHa implants, Atlantic halibut started ovulating after 7 days (**Fig. 5**). There were no significant differences between fish receiving the two GnRHa, so the data from the two GnRHa doses were pooled. One Control fish ovulated a day later, while the others ovulated 2 to 3 weeks later or not at all. Control fish also ovulated fewer times and over a more prolonged period.

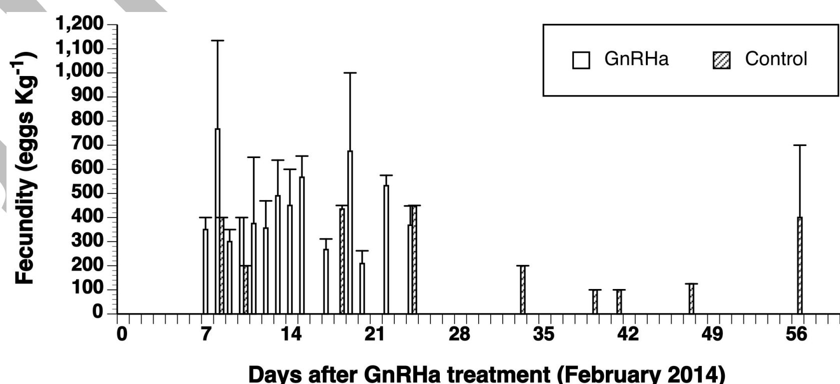

Figure 5. Mean (±SEM) daily egg production of Atlantic halibut treated with GnRHa implants (50 or 100 μg kg^{-1}) or sham-injected as Controls.

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Implanted fish spawned more batches than Control fish and a marked trend towards higher egg production was evident (**Fig. 6**).

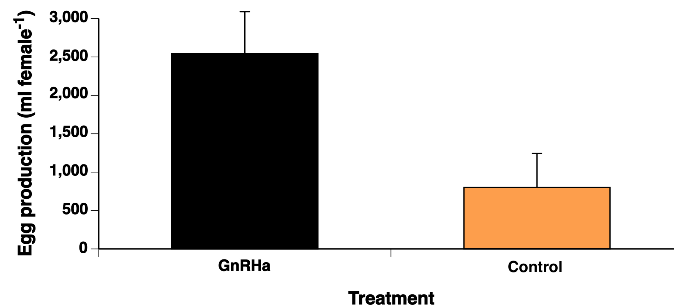

Figure 6. Total mean (±SEM) egg production of Atlantic halibut treated with GnRHa implants or sham-injected as Controls.

The GnRHa treatment appeared to synchronize spawning between individuals, as seen when cumulative egg production in the three groups was compared (**Fig. 7**). All GnRHa-implanted fish completed spawning between March 5 and March 22, within a period of 17 days. Control females spawned between March 5 and April 26, a period of 52 days. The spawning periods lasted for 12.8±2.9 days in the group that received 100 µg GnRHa kg^{-1}, for 11±5 days in the group that received 50 µg GnRHa·kg^{-1} and for 14.7±3.2 days in the Control group.

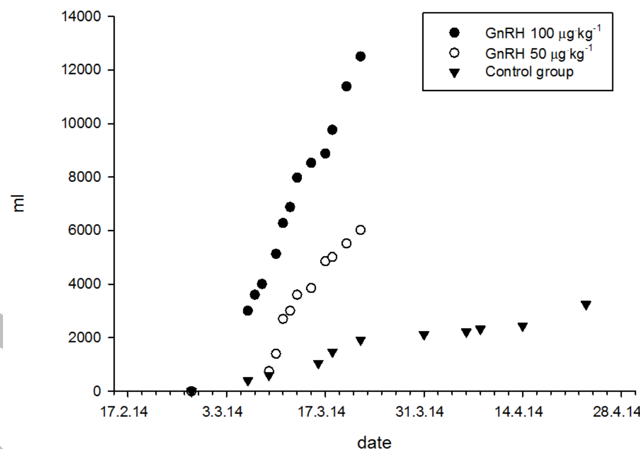

Figure 7. Cumulative egg production in female Atlantic halibut treated with 100 or 50 µg kg^{-1} GnRHa implants or sham-injected as Controls.

Although GnRHa implantation did not advance spawning time significantly in Atlantic halibut females, in two of the trials there was an apparent synchronization in spawning time between individuals, as all treated females had completed spawning 1 month before all Control fish were spent. Spawning in Atlantic halibut normally occurs during a period of 2 to 3 months both in captive broodstock and in natural populations (Norberg et al, 1991; Haug1990). This is most likely an adaptation that will ensure production of viable offspring independent of year-to-year fluctuations in temperature and feed availability for larvae. In a commercial production, however, synchronization between individuals can be an advantage as staff efforts can be concentrated to a relatively short period. Atlantic halibut females ovulate and release their eggs (*i.e.* spawn) in captivity, but fertilization of eggs released in the broodstock tank happens only occasionally. Therefore, Atlantic halibut breeders need to be monitored for ovulation and stripped on a regular basis, and eggs are

fertilized *in vitro*. Therefore, the use of GnRHa implantation offers a logistic advantage to the commercial broodstock management of the species, by reducing the spawning season.

On the other hand, spawning performance in terms of fecundity per female and fertilization success was not significantly affected by GnRHa treatment in Atlantic halibut females. In the first experiment, which was carried out at IMR, females implanted with GnRHa had a marked trend towards a higher fecundity than Control females. In the 2 commercial trials, however, this trend was not observed. So, at this stage the use of GnRHa therapy to increase fecundity and/or fertilization success is not confirmed. Apparently, spontaneously maturing and ovulating females may produce as many eggs as GnRHa treated individuals. However, GnRHa was demonstrated to be highly effective in ensuring that all females matured and ovulated, as all treated females ovulated at least 3 to 4 egg batches, whereas in all trials some of the Control fish did not ovulate and appeared to resorb their ovaries. So, these results indicate that GnRHa implantation may be a useful tool to ensure that all females in a broodstock group reach maturation and ovulation, increasing parentage contribution to the next generation and increasing overall broodstock fecundity, without having deleterious effects on egg viability.

Fecundity regulation

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Some significant differences in spawning performance between wild-captured and farmed Atlantic halibut females exist. Our results showed no differences in fecundity between wild-caught and farmed females, but ovulatory intervals seemed more irregular in the farmed broodstock (Table 1). Fertilization and hatching rates were lower and egg diameter was slightly but significantly lower in farmed females (Figs 1&3).. To investigate possible differences in endocrine regulation of maturation, blood samples were taken at 3-5 week intervals from September 2016 to July 2017. The samples were analysed for the sex steroids estradiol-17ß (E2) and testosterone (T), and the gonadotropins Fsh and Lh.

Plasma concentrations of sex steroids were similar to what has been reported previously in Atlantic halibut, with annual profiles following ovarian growth and maturation. Highest E2 levels were recorded just prior to spawning, in the beginning of February, while both E2 and T remained elevated through the spawning period. No differences in average concentrations were seen between wild-caught and farmed females, although the highest individual E2 concentrations were detected in wild-caught females. The wild-caught females were larger that the farmed ones, had a higher total egg production and hence a larger total ovary weight. This would result in a higher total capacity for steroid production which may explain the higher plasma concentrations in some individuals.

Plasma concentrations of the gonadotropins, Fsh and Lh, were documented for the first time in Atlantic halibut. Mean Fsh concentrations were relatively stable during vitellogenesis, from October to early February. Fsh decreased to low levels during spawning but increased again after spawning was completed. This is consistent with previously reported results in other teleosts, including flatfish. Mean Fsh concentrations were higher in wild-caught females than in farmed fish, but individual variations were high and further studies are needed to confirm if this result is consistent. Lh concentrations showed large individual variations through the reproductive cycle, but peak levels were apparent during spawning, in accordance with results in other teleost fish.

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Nutrition

Kristin Hamre, Torstein Harboe, Øystein Sæle (Institute of Marine Research), Børre Erstad (Sterling White halibut), Ramon Fontanillas (Skretting Aquaculture Research Center)

Early weaning of Atlantic halibut larvae

Slow growth in late larval stages and labor-intensive production of *Artemia* may be overcome by early weaning. Most often, weaning of Atlantic halibut occurs only at 60 days post first-feeding (dpff), but attempts have been made to introduce formulated diets from 20 and 50 dpff, with varying results. The first problem arising is that the larvae refuse to eat formulated feed (Harboe, Hamre and Erstad, unpublished results). It has frequently been observed, however, that they ingest inert particles such as *Artemia* cysts and pollen from pinewood, the main similarity being that both particles have neutral buoyancy and a bright color. Previous experiments have also shown better feed ingestion with floating compared to sinking feed particles. Furthermore, the structure of the visual system of Atlantic halibut larvae indicates that they hunt prey in the horizontal plane (Helvik, pers. com.), favoring feed intake when particles stay in the same position in the water column for some time.

In 2015 we chose three candidate feeds (AgloNorse, Otohime and Gemma micro) based on chemical content and earlier experience, and tested them for weaning of Atlantic halibut larvae 28 dpff in a 5-d experiment. The larvae were fed *Artemia* nauplii from 1 until day 28 dpff and then transferred to 50-l tanks (**Fig. 8**). In the experiment, the larvae were fed either Gemma micro, AgloNorse or Otohime in triplicate tanks for 5 d. Larvae in one tank were fed *Artemia* to have a quality control. Each tank had continuous water supply of 10 l / hour, central aeration and a belt feeder. Clay was added to the tanks three times a day to create turbidity. Larvae were fed formulated feeds continuously, using belt feeders, and were also hand fed two times during a day. In the morning before handfeeding and clay addition and in the evening after handfeeding, 28 larvae were examined for gut content using a strong flashlight. The categories used for gut fullness were full, partly full and empty

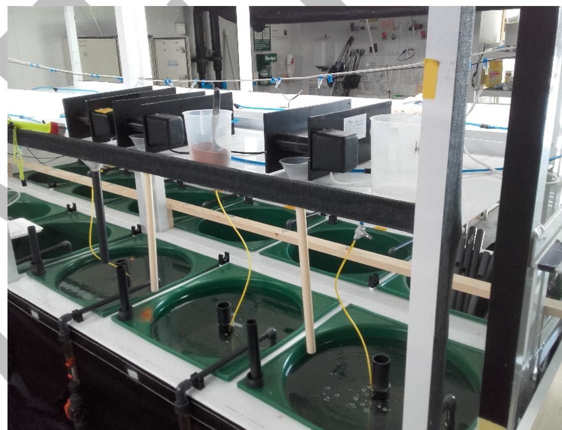

Figure 8. Tanks used for early weaning.

The gut fullness of larvae was lower in the morning than in the evening in both experiments, possibly because the gut fullness was measured before and after handfeeding and clay addition in the morning and evening, respectively. According to the evening measurements in the first experiment **(Fig. 9)**, larvae fed *Artemia* were almost full after one day and stayed full for the rest of the experiment. Larvae fed Otohime showed increasing fullness over the whole period and on day 5, almost 100% of the larvae were full in the evening. The fraction of larvae with food in their gut increased more slowly on Gemma and Aglonorse. On the evening of day 5, 12.0±0.6 and 14.7±1.2 larvae, respectively, out of 28 had filled guts, while 0-0.3 larvae had partly filled guts on these diets.

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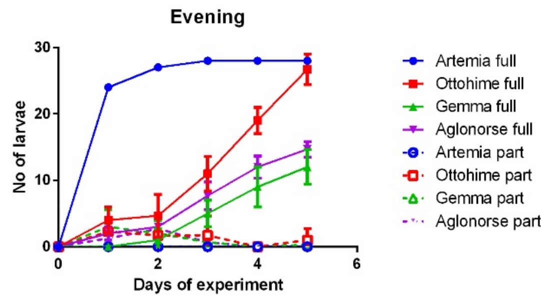

Figure 9. Gut filling in Atlantic halibut larvae fed different diets for 5 days from 28 dpff. The experiment was run in triplicate tanks and 28 larvae were examined for gut filling morning and evening each day. The categories for gut filling were full, partly full and empty.

In 2016, Otohime was given to Atlantic halibut larva at 15, 22 and 28 dpff, again for 5 d, using the same experimental conditions as in 2015. The gut filling of the larvae was observed in the morning and in the evening and the evening data are given in **Fig. 10.** The number of larvae with filled guts was very low when weaning started on 15 dpff, concomitant with almost total mortality during the five days the experiment lasted. When weaning started on 22 dpff, both feed intake and survival were higher than on 15dpff. Weaning on 28 dpff resulted in a mortality of only 3±1 out of 17±2 larvae and all surviving larvae were full on day 3 of the experiment. The low success rate at the two early time points may be connected to the rearing system, which may have been suboptimal for pelagic larvae or to the quality and bioavailability of the feed due to insufficient development of the digestive tract.

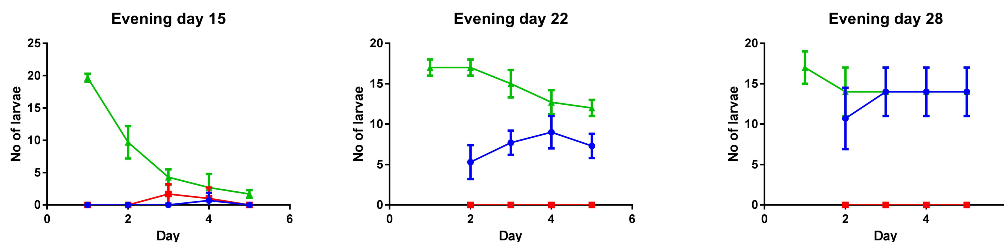

Figure 10. Gut filling and mortality of Atlantic halibut larvae fed the Otohime diet for 5 d, starting from 15, 22 and 28 dppf. The gut filling of the larvae was observed in the morning before handfeeding and in the evening after handfeeding. The data are mean±SD of full, partly full and surviving larvae in three tanks.

Conclusion

Otohime was the best of the three diets for weaning of Atlantic halibut larvae. The diet gives good feed intake during weaning of Atlantic halibut larvae from 28 dpff, while earlier weaning may give high mortalities. The ability of the larvae to digest and grow on the diet must be tested in further experiments. Early weaning should be further tested in rearing systems that are better adjusted to pelagic halibut larvae. Due to their size and swimming abilities we have so far not been able to first feed them in tanks smaller than 100cm in diameter. It seems that the larvae need time to adjust to the prey or feed particle before intake of the particle. Floating characteristics of the particle is also important since *Artemia* cysts are a preferred particle for first feeding halibut larvae.

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On-grown Artemia: Production method and suitability as feed for Atlantic halibut larvae

A possible strategy to increase growth in the later larval stages of Atlantic halibut and improve juvenile quality (**Fig. 11)** is to use on-grown *Artemia*. Olsen et al., (1999) showed that halibut larvae fed on-grown *Artemia* develop into juveniles with better pigmentation and eye migration than Atlantic halibut fed *Artemia* nauplii. This was verified in a feeding experiment performed in 2005 (Harboe and Hamre, unpublished).

Figure 11. Halibut juvenile quality of approximately 0.5 g (Photo: Øystein Sæle)

Production method for on-grown Artemia

In DIVERSIFY, *Artemia* cysts (EG, INVE Aquaculture) were hatched in a separate tank, held for 24 h from hatching, and then transferred to either short-term enrichment or on-growing tanks. Conical 300-l fiberglass tanks were used both for hatching, short-term enrichment and on-grown *Artemia*. All tanks were equipped with temperature (500 W, and Carlo Gavazzi 600+ temperature regulator) and oxygen control systems (Ocea). Hatching and short-term enrichment was performed at stagnant conditions, while on-growing tanks had a flow-through system (**Fig. 12**).

Seawater was pumped from 160 m depth. For hatching and short-term enrichment, the water was treated with chlorine and thereafter thio-sulphuric acid for at least 18 hours. For the on-growing tanks the 160 m depth water was only filtered down to 5 µm before being connected to the tanks. Flowrate was 15 l h^{-1} for the entire period. 100 g of the disinfectant Sanocare ACE (INVE Aquaculture, Belgium) was mixed with 1 l of freshwater using a blender (Hamilton Beach commercial) for 2 min and added to the tanks daily.

OriGreen from Skretting AS (Stavanger, Norway) was used for grow-out of *Artemia* nauplii. In a pilot trial, *Artemia* was fed using a belt feeder, but due to variation in how the feed dispersed in the *Artemia* on-growing tanks, the feed was mixed with 1 l of freshwater using a blender (Hamilton Beach commercial) for 2 min and added to the tanks twice a day. The *Artemia* were fed 20 g of OriGreen in each meal.

Larviva Multigain (Biomar, Denmark) was used for short-term enrichment of both nauplii and on-grown *Artemia,* using the manufacturer's standard procedure for short-term enrichment of *Artemia*. Enrichment period was 12 hours and density of *Artemia* was 200 ind ml^{-1}.

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Figure 12. *Artemia* tank and outlet sieve.

Experiments

An additional experiment was performed at the hatchery of Sterling White Halibut (SWH) using similar methods as the one outlined above, to check that that the results were reproducible and could be transferred to the industry. Growth and survival of Artemia are seen in **Fig. 13**.

Artemia grown for 3-4 days on Oriculture and enriched with Multigain, obtained an improved nutrient profile in many aspects. The protein, free amino acid, and taurine contents increased, lipid and glycogen decreased, while the ratio of phospholipid to total lipid increased. These effects were consistent at the two hatcheries and are assumed to improve the nutritional quality of feed for marine fish larvae. The fatty acid composition improved at IMR, but not at SWH. The micronutrient profiles were similar or improved by culture of *Artemia* on Oriculture.

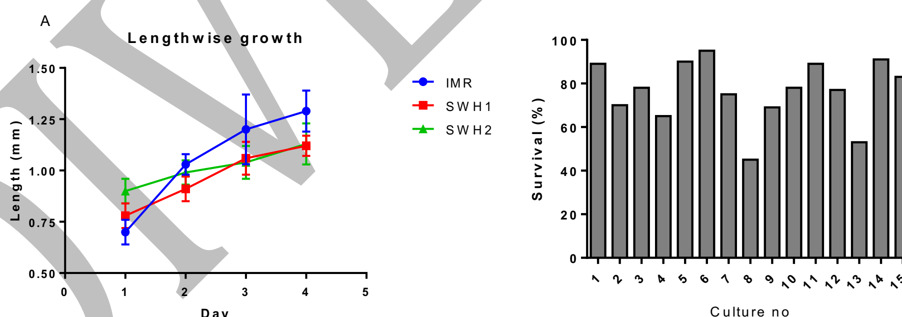

Figure 13. A. Growth of *Artemia* cultured for four days at IMR and SWH (mean±SD). B. Survival in 15 batches of on-grown *Artemia* cultured for three days at IMR. Numbers are based on numbers of *Artemia* pr. ml. at start and end of the 3 days culture period.

Atlantic halibut larvae were fed *Artemia* nauplii from 1 until 14 dpff (days post first-feeding). Then one group of larvae was fed nauplii, and the other group on-grown *Artemia* (2+ out of 3 meals) in triplicate tanks until 28 dpff. There were no significant differences in larval performance. Both groups showed good growth and survival, 100% normal pigmentation and good eye migration (score: more than 2.5/3,).

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Even though we saw positive effects of on-grown Artemia on halibut metamorphosis in the past (Olsen et al., 1999; Harboe and Hamre unpublished), the larvae in both groups in this experiment showed good performance **(Fig. 14).** The reason may be that general production methods, such as tank design and dynamics, have improved since 1999 and 2005, so that the larvae can use more of their nutrients and energy on growth and development instead of having to cope with stress.

Furthermore, use of enrichment diets based heterotrophic algae has improved the nutrient composition of Artemia. Accordingly, our experiment shows that good larval performance can be obtained using Artemia nauplii and that on-grown Artemia have limited benefits compared to the costs of facilities and labor needed to produce them

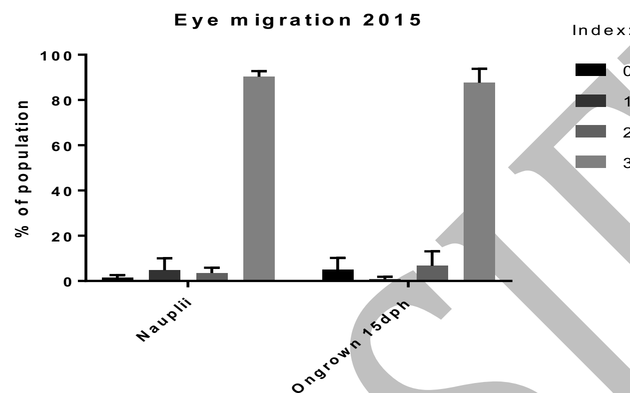

Figure 14. Juvenile quality as represented by eye migration in Atlantic halibut fed Artemia nauplii or on-grown Artemia on the larval stage (0 represents no eye migration, 3 – complete eye migration)

Effect of dietary phospholipids on digestion, absorption and metabolism of lipids in Atlantic halibut juveniles.

The benefit, or even essential need for high inclusion of phospholipids (PLs) in marine larvae is well documented (Coutteau et al., 1997). But adding PLs to the feed of juvenile fish has also shown to be beneficial for a wide spectre of species (Attar et al., 2009, Niu et al., 2008, Sotoudeh et al., 2010).

PLs are vital for lipid transfer from the intestinal tissue to the blood, probably due to limited capacity of de novo PL synthesis in the intestine. Limited PL synthesis will also inhibit membrane metabolism in the larval body, and thereby growth. Lipids are transported from enterocytes to other tissues in chylomicrons. Besides proteins, chylomicrons consist of a core of TAG and cholesterol esters and a monolayer of PL on the surface. Chylomicron production starts with the formation of PL rich particles; thus PL synthesis is a potential bottleneck for lipid transport.

We have shown that juvenile Ballan wrasse increase the growth rate by up to 40% when lipids are added as PL instead of triacylglycerols (TAG, Sæle et al., unpublished), while requirements for PL in Atlantic halibut juveniles are not known.

Experiment

Approximately 5000 Atlantic halibut larvae were transferred from a yolk sac incubator (silo) to a standard 1.5-m diameter 0.8-m depth first feeding tank. The larvae were fed *Artemia* nauplii from 1 until day 28 dpff and then transferred to 15, 50-l tanks. Here, the larvae were fed Otohime until ca 1 g. Each tank had continuous water supply of 10 l / hour, central aeration and a belt feeder. Clay was added to the tanks three times a day to create turbidity. Larvae were fed formulated feeds

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continuously, using belt feeders, and were also hand fed two times a day. In the morning before clay addition and in the evening after handfeeding.

When the juvenile fish reached 0.92 ± 0,42 g with a total length of 46 ± 7 mm, they were fed diets with increasing PL/TAG ratio. The experiment design was a regression with 3 replicates and 5 levels, and the experiment lasted for two months. The larvae were sampled at a fixed time after the first meal in the morning. Measured and snap frozen on liquid nitrogen analysis.

The fish were fed five different diets, mainly based on marine ingredients, but the PL added to the diets at graded levels was from soy, and soy oil was used to balance the feeds with regard to dietary lipid levels (Table 1). The analyses showed increasing levels of PL, but more or less stable levels of neutral lipids. This gave a slight increase in total lipid from diet 1-5 **(Table 2)**.

After two months, the halibut had grown from 0.92 ± 0,42 g to 10.12 ± 3,84 g and from a total length of 4.63 ± 0.71 cm to 9.85 ± 1.30 cm, however there were no differences in growth between the treatment groups **(Fig. 15)**. Therefore, growth in halibut this size, did not benefit from increasing inclusions of PL up to 31 % of total lipid. In Atlantic salmon increased PL/TAG ratio led to better growth in juveniles up to 2.5 g. However, this growth effect disappeared after the fish had reached 2.5 g (Taylor et al., 2006).

Table 2. Lipid class (mg/g ww) analysis (HPTLC) of diets 1 to 5 (TL, total lipids)

Diet	**1**	**2**	**3**	**4**	**5**
LysoPC	4,1	6,3	8,4	10,5	9,7
Sphingomyelin	1,4	1,5	1,7	1,9	2
Phosphatidylcholine	8,1	13,3	19	23,9	29,1
Phosphatidylserine	1,2	3,2	7,6	9,8	11,6
Phosphatidylinositol	0	2,3	5,6	7,1	8,3
Cardiolipin	0,1	0,2	0,4	0,4	0,5
Phosphatidylethanolamine	2	5,2	9,4	11,5	13,1
Diacylglycerol	1	1,3	1,7	1,5	1,3
Cholesterol	6,9	7,9	7,8	7,6	7,9
Free fatty acid	10,5	13	15,2	15,2	16,5
Triacylglycerol	149	161	152	144	139
Cholesteryl ester	nd	nd	nd	nd	nd
Sum Phospholipids	16,9	31,9	52,2	65	74,1
Sum Neutral lipids	167	183	177	169	164
Sum Lipids	184	215	229	234	238
Phospholipids (% of TL)	9	15	23	28	31

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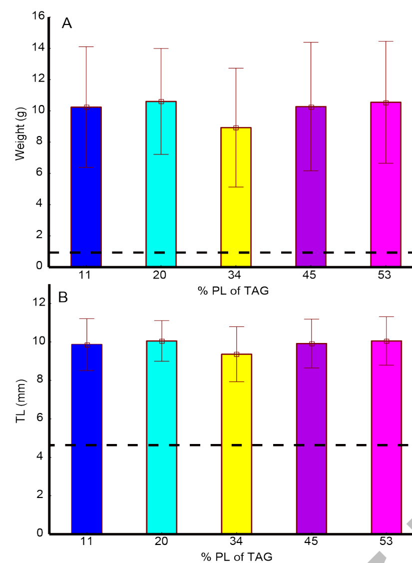

Figure 15. Final weight (A) and length (B) of halibut. Stippled line show start weight and length.

Results and discussion

In the present study, intestinal, muscle or liver lipid composition were not affected by the diets, but time after the meal influenced lipid level and composition in intestinal tissue. The relative concentration of neutral lipids such as TAG, DAG and FFA were high at 1 and 4 hours and decreased until 24 hours postprandial. In the same period, the relative concentration of cholesteryl esters (CE), ceramides (CER) and some PL were lower at 1 and 4 hours and increased until 24 hours postprandial. qPCR showed increased expression at 4 compared to 1 and 24 hours, of some of the genes involved in absorption and remodelling of lipids in the enterocytes.

Conclusion

It appears that Atlantic halibut juveniles regulate their lipid species composition to be independent of the diet when a range of 9 to 31% dietary PL of total lipid is applied. Furthermore, absorption and metabolism of lipids in the enterocytes seems to be too fast for different dietary PL/TAG ratios to be detected by analyses of intestinal tissue.

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Larval husbandry

Recirculation vs flow through larval rearing systems

Torstein Harboe and Audun Nerland, Institute of Marine Research

The commercial production of halibut fry is currently carried out in flow through systems (FT), while there is a growing consensus that a recirculation system, RAS, would offer more stable environmental and chemical water parameters that would lead to improved larval performance. The yolk sac and first feeding stages in halibut are performed in different rearing systems. RAS systems for both these stages are presented here.

The yolk sac stage lasts for 43 days at 6 C° in halibut. Fertilised eggs are transferred to the silos approximately 3 days prior to hatch. At this time, a salinity gradient has been established in the upper part of the silo by use of freshwater. Hatching is synchronized by use of light, which arrests hatching, and thereafter darkness to induce hatching. The salinity gradient is present during hatching and for one or two more days, depending of the buoyancy of the larvae. Recirculation is not used in this period. The silos used for water treatment and for larval rearing, are 5000 litres in volume. Approximately 1 to 2 litres of eggs (40 000-80 000 eggs) are normally incubated in one silo, depending on the size of the egg batch. There is no feeding or any addition of organic material during this period.

Two trials were conducted before the protocol was finalized. In the first trial, water temperature was adjusted between the RAS silo (without larvae) and the silo inoculated with larvae. In the second trial this temperature adjustment was done within the RAS silo, resulting in a more even temperature profile (**Fig. 16**).

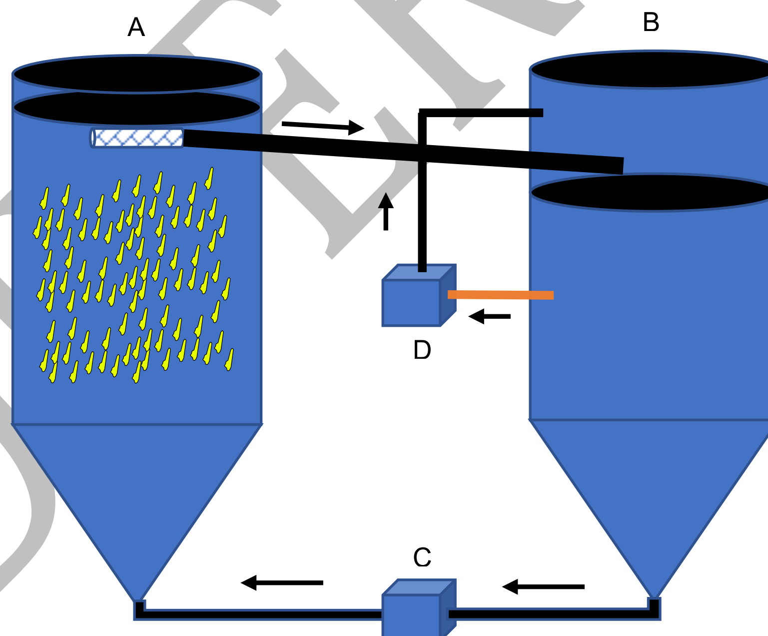

Figure 16. Illustration of the RAS used for yolk sac larvae. A= silo with larvae, B=water treatment, C= water pump including flowmeter. D= water cooler.

Only small differences in survival were observed during yolk sac incubation between FT and RAS both in 2015 and 2017 (**Fig. 17**). Proportion of jaw deformed larvae was 14% in 2015 and 11% in 2017 for the RAS larvae and 9% in 2015 and 17% in 2017 for the FT larvae.

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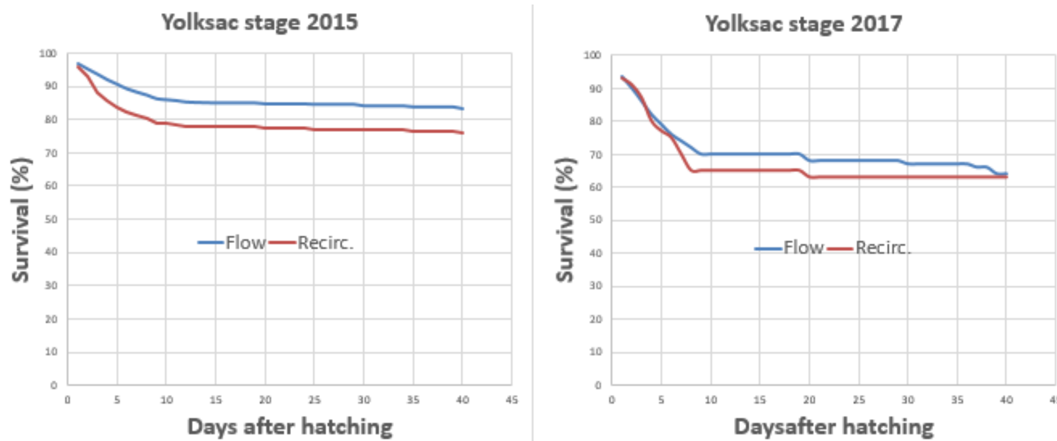

Figure 17. Larval survival (%) in 2015 and 2017.

To avoid use of antibiotics and to decrease mortalities use of a RAS was tested, in order to establish a stable microbial environment. It is not clear whether the intestinal microflora of halibut larvae is determined by the feed or by water quality parameters. Short time enriched *Artemia* is most widely used for first feeding of halibut larvae. The feeding period is normally 45 to 50 days before they are weaned to a dry diet.

A RAS system from Tropical Marine Centre (TMC) has been used by the IMR for research on several cold-water and warm-water marine species. In this set up three first feeding tanks were connected to the system (**Fig. 18).**

Figure 18. First feeding tanks attached the RAS system.

The first-feeding tanks were flat bottomed, with a volume of 1100 l and a water flow of 5 l per minute. Water temperature was 12 ± 0.3°C during the whole period. The tanks had shadow frames to avoid illumination of the walls and fluorescent (daylight) light sources placed 70 cm above the water surface, giving a light intensity of approximately 400 lux at the surface. The tanks had central aeration near the bottom. The water outlet sieves were also in the center of the tanks, reaching from

the bottom to the surface. Water inlets were placed near the tank wall approximately 10 cm below the surface. An automatic cleaning device (car wipers) were mounted in each tank and were run once a day. After one rotation, dead material was removed by a siphon. The water volume that was removed daily by siphoning represented the water exchange in the RAS system. The recirculating volume was calculated to 97%. Water turbidity was created by use of dissolved clay (Sibelco, Vingerling K148, white) to an initial turbidity of 2 NTU. Approximately 10g of clay was dissolved in one liter of freshwater and added to each tank twice a day. Before the water returned to the RAS unit it was filtered to remove *Artemia* and part of the clay. The reminding clay was left in the RAS unit, mostly in the reservoir.

Larval growth was significantly higher in the FT system compared to RAS in 2016. In 2017 the situation was opposite as the larval growth was significantly higher in the RAS group. There were no differences in growth between the FT group in 2016 and the RAS group in 2017 (**Fig. 19**).

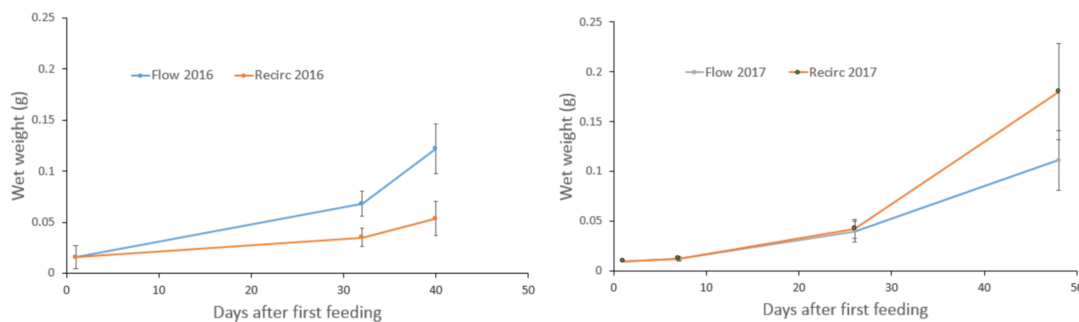

Figure 19. Larval weight development in 2016 (a) and 2017 (b)

In 2016, the RAS system was started only a short time prior to larvae incubation. The concentration of un-ionized ammonia was significantly higher in the RAS tanks compared to the FT tanks. In 2017 the RAS system was started one month before larval incubation and fed daily with 1,7 g of NH_4CL. At the end of the experiments there was clay in the unit, but the concentration of un-ionized ammonia did not increase (**Fig. 20**).

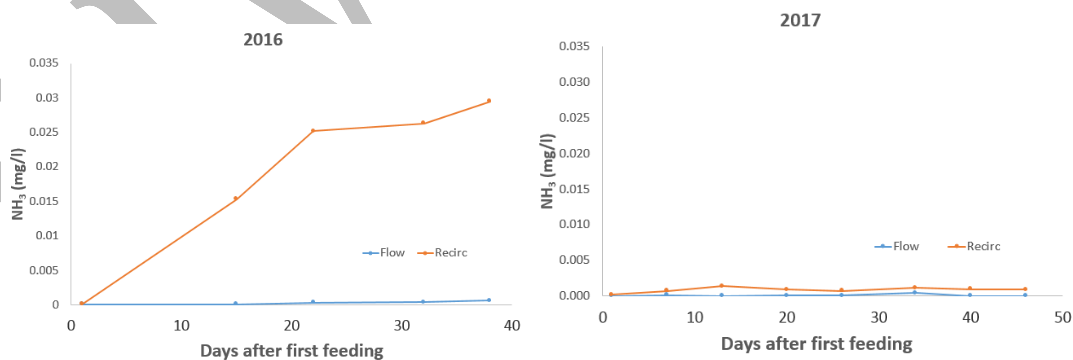

Figure 20. Concentration of unionized ammonia for the FT and RAS systems in 20016 (a) and 2017 (b).

There were no significant differences in survival through first feeding between FT and RAS tanks in 2016. In 2017, high mortality occurred in one of the RAS tanks (**Fig. 21**).

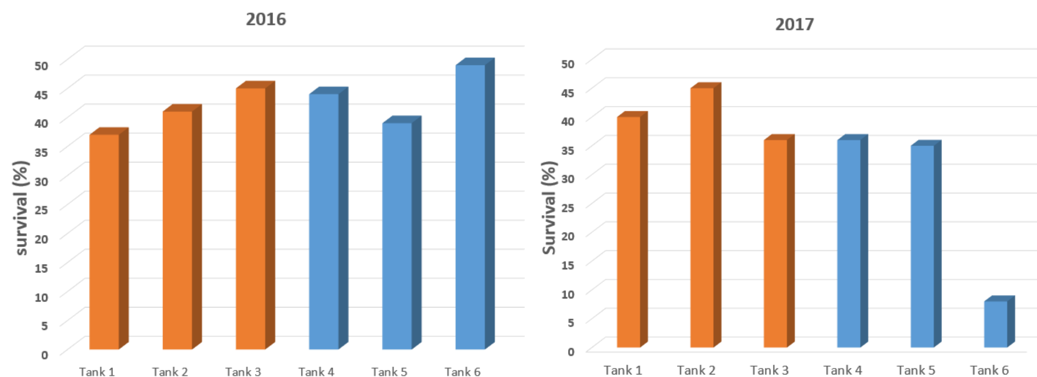

Figure 21. Survival (%) of larvae at end of the experiments. Tank 1, 2 and 3 =RAS. Tank 4, 5 and 6=FT. The survival is based on estimated number of larvae incubated from start and counted larvae at the end of the experiment.

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Atlantic halibut differs from other marine cold-water species by its comparatively long lasting yolk sac stage. This stage has been a bottleneck in the production of fry, mainly due to the changes that appear in buoyancy from egg to larva. Small changes in temperature or salinity strongly influence the positioning of the larvae in the water column resulting in mortality and malformations like jaw deformity. We expected RAS to give lower variation in water density compared to an FT system, which would result in better production stability. Water temperature during this stage was 6°C and no organic material was added. There was therefore no need for a specific biofilter. The yolk sac incubators are large in volume and are normally incubated with 1 to 2 l of eggs, depending of the size of the egg batch. The experiment was repeated since it is not possible to have triplicate tanks. In both runs there were only small differences in survival between the treatments. Appearance of jaw deformities was larger in the RAS larvae then for the FT larvae in the first run. However, in the second run the case was the opposite. Both runs were within what we normally expect both when it comes to survival and proportion of jaw deformed larvae.

The first feeding tanks were smaller than the yolk sac incubators and contained a lower number of larvae per unit. Although larval survival in the RAS system was stable and high for both runs, larval growth was significantly lower in the first run, both compared to the FT control and to RAS larvae in the second run. This was likely due to the high concentration of un-ionized ammonia, caused by insufficient priming of the RAS system in the first run. In the second run, the FT systems appeared less stable than the RAS system, as seen by both growth and mortality: growth was almost twice as high in the RAS system, and larval mortality increased in one FT tank in the second half of the period leading to a loss of >90% of the larvae in that tank. However, RAS larval growth in the second run did not differ from the growth of the FT larvae in the first run. Taken together, the results suggest that with adequate conditioning in the RAS system, a stable system is established where growth and survival of larvae is as good as, or better than in FT systems with optimal conditions.

Based on these two experiments we conclude that RAS was a more stable rearing system for Atlantic halibut larvae compared to the FT system.

Probiotics and larval microbiota in industrial larval rearing protocols
Torstein Harboe and Audun Nerland, Institute of Marine Research

Two trials were conducted before the protocol was finalized. In the first trial, water temperature was adjusted between the RAS silo (without larvae) and the silo inoculated with larvae. In the second trial this temperature adjustment was done within the RAS silo, resulting in a more even temperature profile (**Fig. 22).**

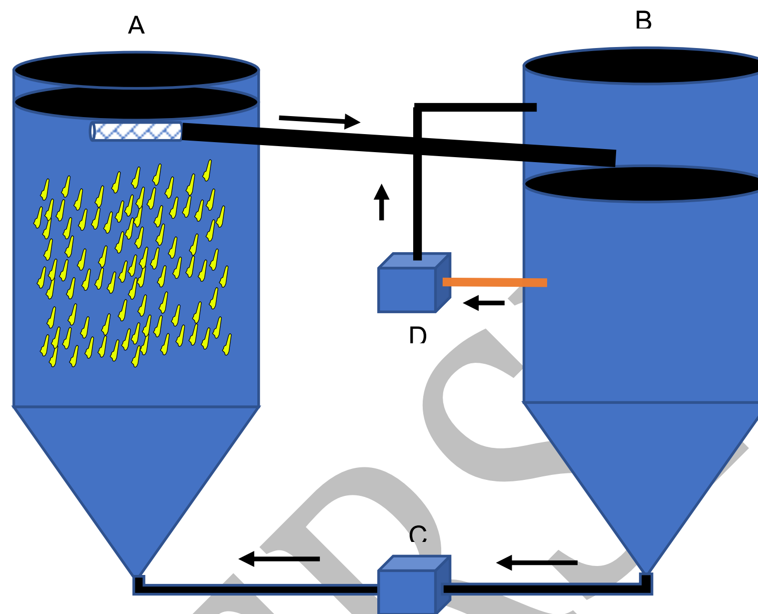

Figure 22. Illustration of the RAS used for yolk sac larvae. A= silo with larvae, B=water treatment, C= water pump including flowmeter. D= water cooler.

Protocol for halibut yolk sac larvae:
Fill the yolk sac incubator and the RAS silo with seawater of full salinity.

Establish a salinity gradient in the larvae incubator approximately two days before egg incubation.

Maintain circulation of new seawater during hatching and until the larvae are distributed evenly in the incubator and the salinity gradient is removed. This can take one to 3 days depending on the buoyancy and distribution of the larvae.

Change the incoming water from FT to RAS.

Increase water flow from one l·min^{-1} at the start to a maximum of 5 l·min^{-1} within five days. Measure water temperature and the distribution of the larvae in the incubator daily. If the larvae are distributed in the bottom half of the incubator create a small turbulence by adding oxygen bobbles (ceramic diffusor) from the bottom of the silo.

Renew the layer of brackish water at the surface of the incubator (above the outlet sieve) once a week.

At approximately 260 day-degrees remove the layer of brackish water by lifting the outlet sieve. Illuminate the surface of the incubator and collect the larvae as they are coming to the surface with a 10-liter bucket and transfer them to start feeding units.

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First feeding stage:

A RAS system from Tropical Marine Centre (TMC) has been used by the IMR for research on several cold-water and warm-water marine species **(Fig. 23**). In this set up three first feeding tanks were connected to the system.

Figure 23. RAS system P5000P MARINE from Tropical Marine Centre. The system consists of a reservoir (650 liter), filter bags, sand filter, re-gassing / trickling biofilter and a protein skimmer.

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Protocol for feeding halibut larvae under RAS condition:

Priming:

Fill the RAS unit with seawater more than 30 days prior to larvae incubation. During this period the recirculation unit without tanks, a total of 650 l, must be conditioned by addition of a daily amount of 1.5 g NH_4Cl.

Preparation:

Measure NH_4 concentration and pH value once a week to see if the biofilter in the unit removes NH_4.

Keep water temperature stable at 12°C.

Fill the first feeding tanks with seawater the day before larvae incubation. Adjust aeration, water flow and turbidity. Connect the tanks to the RAS unit.

Incubate approximately 5000 larvae per tank. Feed short-time enriched *Artemia* according to feeding protocol.

Daily routines:

Check and if necessary adjust water flow in protein skimmer (venturi pump), sand filter and biofilter in the RAS unit.

Exchange and clean bag filters prior to the RAS unit (excess *Artemia* and clay) and the bag filters in the RAS unit.

Refill sea water after the larvae tanks have been tended. Use a water level mark in the reservoir.

Weekly routines:

Measure NH_4 concentration and pH value.

Siphon clay from the bottom of the reservoir.

Other:

Feed and remove dead larvae according to larvae rearing protocol.

Use of on-grown Artemia in first feeding larvae of Atlantic halibut

Torstein Harbo, and Kristin Hamre, Institute of Marine Research

Børre Erstad, Sterling White Halibut

A possible strategy to alleviate the slow growth in the later larval stages of Atlantic halibut and improve juvenile quality is to use on-grown *Artemia*. On-grown *Artemia* are larger, contain more protein and phospholipids and have a different micronutrient status from *Artemia* nauplii. They also have a lower shell to nutrient content.

Atlantic halibut larvae from one single egg batch were hatched and further incubated in two 5m^3 siloes until 260 day-degrees post hatch. They were then transferred to 6 first feeding tanks and stocked at ca. 5000 larvae tank^{-1}. The first feeding tanks were 1,5 m in diameter and 0,8 m in height. The tanks had continuous water supply entering near the surface and an outlet sieve in the middle of the tank. Each tank had a fluorescent light above its center and was equipped with a shadow frame, to reduce light reflections from the tank wall which can attract the larvae. The tanks also had central aeration from near the bottom and an automatic cleaning system. The temperature of the rearing water was 12 ± 0,2°C. Water flow started at 1 l min^{-1} at the start of incubation and increased within the next 4 days to 5 l min $^{-1}$, where it was held for the remainder of the experiment. Dissolved clay (30 g morning and 30 g evening) was added to each tank daily to keep turbidity high during the live feed period. Live feed was added three times a day at 10.00, 15.00 and 21.00. Light was on from 07.00 to 24.00.

Artemia cysts (EG, INVE Aquaculture) were hatched in a separate tank, incubated for 24 hours and then transferred to either short term enrichment or on-growing tanks. Conical 300 l fiberglass tanks were used for hatching, short term enrichment and on-grown *Artemia*. Hatching and short-term enrichment were performed in stagnant conditions, while on-growing tanks had a flow-through system. For hatching and short-term enrichment, the water was treated with chlorine and thereafter thiosulphate for at least 18 hours. For the on-growing tanks water pumped from 160 m was filtered (5 µm) before entering the tanks. Flow rate was 15 l h^{-1} for the entire period. The disinfectant Sanocare ACE was mixed with one l of freshwater for 2 minutes and added to the tanks daily.

ORI-GO from Skretting AS was used for grow-out of *Artemia* nauplii. LARVIVA MULTIGAIN (Biomar, Denmark) was used for short term enrichment of both nauplii and on-grown *Artemia,* using the manufacturer's standard procedure for short term enrichment of *Artemia*. Enrichment period was 12 hours and density of *Artemia* was 200 ind·ml^{-1}.

The larvae in all six tanks were fed *Artemia* nauplii from 1 until 14 dpff (days post first feeding). Then one group of larvae was fed nauplii, and another group on-grown *Artemia* (2 out of 3 meals) in triplicate tanks until 28 dpff. The amount of *Artemia* fed each meal was based on the clearance rate of *Artemia* in each larvae tank. This was done by examining 100 ml of rearing water from each tank for *Artemia* content, which should be zero at least one hour before next meal. Parameters such as the

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remaining number of *Artemia*, water flow, temperature and number of *Artemia* fed the larvae were recorded daily.

Larval growth.

There was no significant difference in growth at any time of the experiment (**Fig. 24**). However, the standard deviations in this parameter at the end of the experiment were large for both experimental groups. Larval growth in this experiment did not differ from earlier growth studies on halibut larvae.

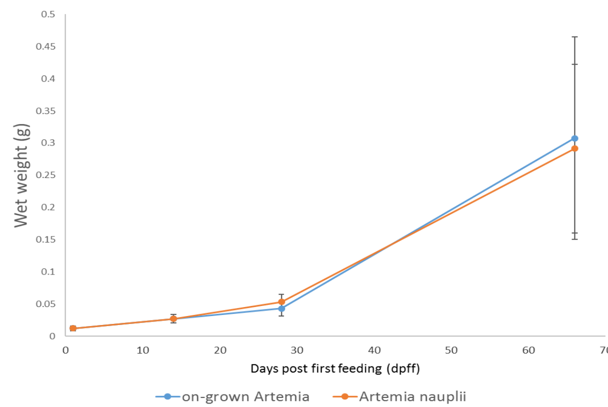

Figure 24. Larval growth (wet weight) from first feeding until fry weaned to a dry diet. Blue arrow indicate period were larvae were fed on-grown *Artemia.*

Larval survival.

Larval survival from first feeding until end of experiment at day 65 dpff was ca. 50% in all six tanks. An exact number is not possible to calculate as the initial number of larvae stocked was estimated. The first feeding tanks were tended daily by use of the cleaning system described above, and number of dead larvae was counted. Highest mortality (more than 90%) was observed from day 4 to 7 dpff , thereafter mortality was low throughout the rest of the experiment.

Eye migration and pigmentation.

More than 12.000 halibut fry were produced in this experiment and only two individuals were mal-pigmented. Degree of eye migration was evaluated on a scale from 0 to 3, where 3 is complete eye migration and 0 is no eye migration. In this experiment both groups scored higher than 2.5.

Conclusions

Use of on- grown *Artemia* during the critical period of metamorphosis in Atlantic halibut larva did not differ from use of *Artemia* nauplii with regard to growth, mortality and fry quality. In addition, the production of on-grown *Artemia* is labour-intensive, and high personnel costs may be prohibitive in implementation of this live feed source in commercial larviculture.

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Fish health

Sonal Patel, Institute of Marine Research (Norway); Audun H. Nerland, Institute of Marine Research (Norway)

One of the diseases, viral encephalopathy and retinopathy (VER) affecting culture of Atlantic halibut is caused by the Viral Neural Necrosis (VNN) virus, which is also known as betanodavirus. Nodavirus infections and disease outbreaks in halibut are seen in larval and early juvenile stages and often leads to up to 100% mortality (**Fig 25**). Prevention measures are required to avoid the loss of larvae and disease outbreaks. Vaccination is one of the important prevention strategies and thus production of nodavirus capsid protein in different expression systems and its potential to give protection to late larval stages was assessed.

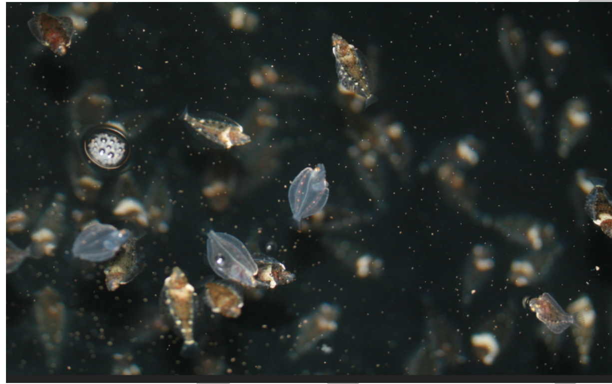

Figure 25. One of the halibut larval stages prone to VER disease outbreaks. Photo: Sonal Patel, IMR

Production of vaccine candidates against nodavirus

Several alternatives were explored to express the protein antigen in systems having different post-translational modifications and test if that could influence the antigenicity of the proteins. Recombinant capsid protein from nodavirus expressed in *E. coli* has been shown to induce protection when formulated in a vaccine. However, bacterial cells do not glycosylate the expressed protein, as do higher eukaryotes. It might be that other expression systems may provide antigens more like the naive viral proteins as produced after viral infection. By expressing the capsid protein of nodavirus recombinantly also in other systems as *Leishmania tarentolae*, and tobacco plants it would be possible to find out if post-translational modifications might influence antigenicity and thereby its ability to induce protection when used as antigen in a vaccine. Recombinant capsid protein has also been expressed in *Pichia*, from which it can be isolated as virus like particles (VLP) for integration in a vaccine.

We managed to express the nodavirus capsid protein in all three systems. Although, it was only in the *E. coli* system that we achieved sufficient and high expression for use of the protein as antigen for vaccination purposes, the expression in other systems did offer enough expression for assessment in a small-scale experiment and were explored to be used in oral delivery and/or by injection.

Vaccine delivery and assessment of protection to nodavirus.

It is important to have knowledge about when the larvae are immune-competent to plan the time-point for vaccination and avoid immune-tolerance (Patel et al 2009, Øvergård et al 2011). Since nodavirus outbreaks occur at larval and early juvenile stages, size of the fish to be stimulated is a hindrance for traditional injection vaccination. An alternative is to bath vaccinate or deliver the vaccine orally. To achieve oral vaccination the antigen has to be presented in a way that the target fish will accept and ingest. If we succeed to get uptake of the antigen by the *Artemia* offered as food

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item, we anticipate that it can act as a vector for oral uptake to the larvae. We hypothesized that vaccination of halibut during the late larval stages would provide protection during the transition period from live- to commercial- feed, and thus protection from VNN outbreak during some of the phases when halibut are prone to get the disease outbreak can be achieved.

The nodavirus capsid proteins expressed in several different systems were given as feed to Artemia that were not enriched beforehand. 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. 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* (**Fig .26**).

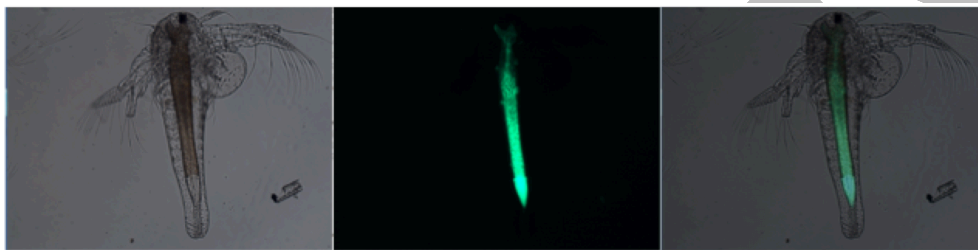

Figure 26. Microscopic pictures of *Artemia* fed with GFP organisms, here exemplified with Artemia fed with *L. tarentolae* expressing GFP. To the left a light microscope image; in the middle a fluorescence microscope image, and to the right an overlay of the left and middle images.

Artemia that were ready for feeding were incubated with purified protein or live organisms expressing recombinant capsid protein (**Fig. 27**). At the end of the incubation period, the *Artemia* were filtered through a plankton mesh, washed once with sea water and fed to the halibut larvae. Treatment was repeated 3 days in a row. On the second day after the experiment start, the treatment groups to be injected intraperitoneally (*i.p.*) received single injections (**Figure 27C**), and the adjuvanted vaccine was visible in the peritoneum of the larvae. The analysis revealed that *Artemia* will digest the recombinant protein, but the degradation depends on the expressing host. 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.

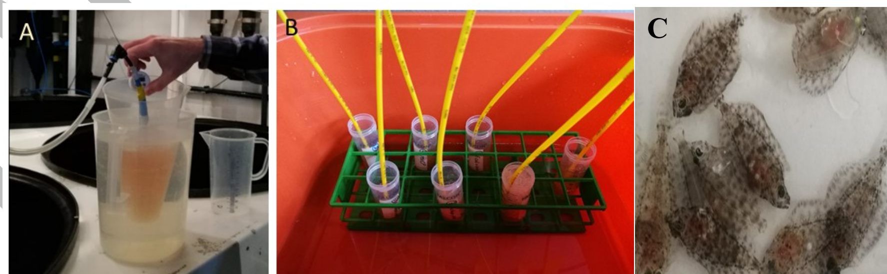

Figure 27. (A) *Artemia* incubated in water bath before being fed different formulations containing recombinant capsid protein. (B) Tubes with *Artemia* with aeration during incubation for uptake of specific protein or live organisms for oral delivery. (C) Halibut larvae/juveniles *i.p.* injected with VNN capsid protein formulated with mineral oil adjuvant. Correct delivery in the form of white traces in the peritoneum can be seen.

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At the end of 10 weeks of vaccination, the juveniles that had survived within each treatment were transferred to the wet lab challenge facility, IMR, Bergen and the number of individuals that survived within each treatment group were challenged with nodavirus by *i.p.* injection of 50 µl at $1x10^{7.5}$ $TCID_{50}$ /ml. At the end of the experiment brain from all fish were collected and analyzed for nodavirus using a RNA2 specific real time rt-PCR assay (Korsnes et al, 2005) to assess the effect of different vaccination treatment. The individuals within each treatment group and between treatment groups were in varying developmental phases and during the experiment, weight and development of the individuals continued to differ and this was reflected in the large individual differences seen at the end of the experimental period. We aimed to test possible protection to nodavirus infection by oral delivery of the nodaviral antigens as this has been shown to induce an immune response in different fish species, and in some cases a higher survival rate was observed (Reviewed in Yong et al 2017). Several different ways for oral and bath delivery have been tried including through the use of live feed *Artemia* for larval stages. A relatively high amount of nodavirus RNA2 was detected in the juveniles that were not- treated and later challenged (NCI) confirming that the challenge model worked. The amount of virus detected in the treatment groups was quite similar to the non-vaccinated challenged control (NCI), apart from a few individuals where the Ct <30 **(Fig. 28)**. The size of the individuals in vaccinated and non-vaccinated groups at the start of the experiment was comparable but with large individual variation, and several larvae had not reached the end phase of metamorphosis.

Earlier studies have shown that at 94 days post hatch just before the transition from live feed to commercial dry pellets, the larvae have a developed immune system where also IgM^{+} (B) cells could be detected using immunohistochemical analysis (Patel et al, 2009; Øvergård et al, 2011). Thus, most larvae were expected to have a fully functional immune system at the time of vaccination. However, apart from the observation that the adjuvanted injected groups showed a slightly lower amount of virus, none of the treatments gave any protection to the juveniles. In general, it seems that the larvae did not respond to the vaccination at all, nor did the individuals develop tolerance (as higher viral amounts in vaccinated groups were not observed). There might be several reasons for these findings. As mentioned earlier, most larvae were underdeveloped for the phase they were in, and very few had reached the expected developmental stage.

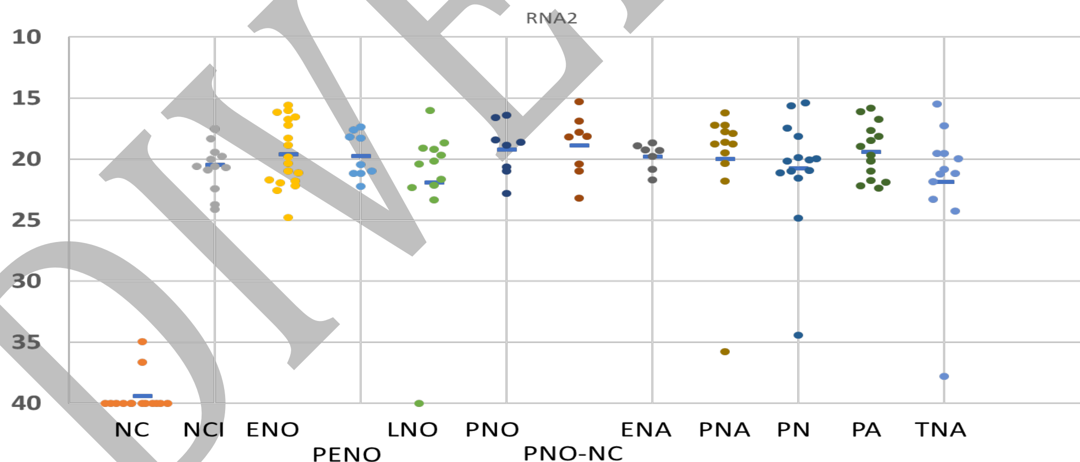

Figure 28. Ct values of RNA2 analysis of all the experimental groups. Each individual has been shown along with the median value (blue horizontal dash). NC – Non vaccinated negative control, NCI – Non vaccinated control challenged, ENO – Live *E. coli* expressing capsid protein orally delivered through *Artemia*, PENO – Purified inclusion bodies containing nodavirus capsid protein expressed by *E. coli* delivered through *Artemia*, LNO - Live *L. tarentolae* expressing capsid protein orally delivered through *Artemia*, PNO – Freeze fried *Pichia* without nodavirus capsid protein orally delivered through *Artemia* as Pichia negative control, PNO-NC – Freeze dried *Pichia* expressing capsid protein orally delivered through *Artemia*, ENA - Purified inclusion bodies containing

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nodavirus capsid protein expressed by *E. coli* formulated with mineral oil and delivered by *i.p.* injection, PNA – VLPs expressed by Pichia formulated with mineral oil and delivered by *i.p.* injection, PN - VLPs expressed by Pichia without mineral oil and delivered by *i.p.* injection, PA – PBS with adjuvant as negative control for *i.p.* delivery, TNA - Purified nodavirus capsid protein expressed in Tobacco leaves formulated with mineral oil and delivered by *i.p.* injection.

The oral treatments were carried out once a day for three days in a row, and we could see that the capsid protein was broken down into smaller proteins in *Artemia*, and thus it is difficult to know if the larvae received enough antigen and if the antigen was in the right conformation to induce a protective immune response. If delivering the antigen for a longer period would have had a better effect is unknown. The antigenic formulation with and without adjuvant that was delivered by *i.p.* injection did not go through the same route via *Artemia* and hence could have given protection. However, it can be speculated that due to the very small peritoneum it was extremely difficult to deliver enough antigen, and most larvae received around 10-20 µl of either pure antigen or adjuvanted antigen, and thus the concentration of antigen was much lower than planned. In an earlier study using one of the formulations, adjuvanted purified capsid protein expressed in *E. coli*, when delivered by *i.p.* injection to halibut weighing approximately 25 g elicited protection in most of the juveniles (Øvergård et al., 2013). The same formulation that was used as a positive control in this study did not give any protection to larvae, leading us to speculate that the amount of antigen that was delivered could have been too low.

Testing these antigens in larvae that are sorted such that all individuals are in the same developmental phase or by delivering the antigens along with dry pellets rather than through *Artemia* would reveal if the antigens can give protection at a stage earlier than at 25 g.

Conclusion:
Although it has been shown that *Artemia* will take up and accumulate the various forms of recombinant nodavirus capsid proteins and act as a vector for oral delivery to larvae of Atlantic halibuts, it can be concluded from the challenge experiments that this strategy of antigen delivery does not induce protection against nodavirus infection, at least under the conditions used in this study.

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