Deliverable Report

Deliverable No:	D11.3	**Delivery Month:**	36
Deliverable Title	Report on the nutrient retention and digestive physiology in Atlantic halibut larvae fed *Artemia* nauplii and on-grown *Artemia*		
WP No:	11	**WP Lead beneficiary:**	P17. NIFES
WP Title:	Nutrition – Atlantic halibut		
Task No:	11.3	**Task Lead beneficiary:**	P17.NIFES
Task Title:	Nutrient retention and digestive physiology of Atlantic halibut juveniles fed *Artemia* nauplii or on-grown *Artemia*		
Other beneficiaries:	P17. IMR	P15. ULL	
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Objective: The objective of this Deliverable was to improve growth in late larval stages, and juvenile quality, through feeding with on-grown *Artemia*.

Introduction

A possible strategy to alleviate the slow growth in the later larval stages of Atlantic halibut and improve juvenile quality (**Figure 1**) is to use on-grown *Artemia*. Ongrown *Artemia* are larger, contain more protein and phospholipids and have a different micronutrient status from *Artemia* nauplii (Hamre and Harboe, unpublished; DIVERSIFY Task 11.2). They also have a lower shell to nutrient content. Olsen et al. (1999) showed that Atlantic 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 This was verified in a feeding experiment performed in 2005 (K. Hamre and T. Harboe, unpublished). The industry is considering implementing this knowledge in their production line, but will need further documentation.

Figure 1. Atlantic halibut juveniles of approximately 0.5 g, showing different characteristics related to their quality. (Photo: Øystein Sæle)

A holistic understanding of feeding and digestive functions is important for designing diets for fish larvae and the adaptation of rearing conditions to meet requirements for the best presentation of prey and microdiets, and their optimal ingestion, digestion and absorption (Rønnestad *et al.*, 2013). In this sense, it is obvious that a better knowledge of larval digestive ontogeny and its physiology when using different scientific approaches and techniques such as that of feeding Atlantic halibut larvae with *Artemia* nauplii or on-grown *Artemia*, will contribute to the optimization of diets and rearing conditions. The analysis of the main digestive enzymes under these two different rearing sceneries may help to this understanding of functions and limitations in processing capacity of the digestive system of a species such as Atlantic halibut, that is, the plasticity of their digestive processes to deliver nutrients to the rapidly growing larval tissues under changeable feeding and environmental conditions.

Materials and methods

Atlantic halibut larvae were fed *Artemia* nauplii from 1 until 14 days post first-feeding (dpff). Then one group of larvae was fed *Artemia* 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, See ***Deliverable D17.4 Comparison of feeding on-grown Artemia versus Artemia nauplii on Atlantic halibut larval performance*** for details on larval rearing and performance). Samples were taken for nutrient analyses (P17. NIFES) and analyses of digestive capacity (P15. ULL), after the end of feeding with ongrown *Artemia*. The nutrients were analyzed by ISO accredited methods at NIFES (**Table 1**).

Samples of larvae were collected for comparisons of the main digestive enzyme activities. Eleven larvae at day 15 (start) and 6-9 larvae at day 28 from each stock (*Artemia* nauplii or on-grown *Artemia* fed larvae) were pooled after previous dissection and discard of heads and tails. The samples were completely homogenized (Ultra-Turrax T8, IKA©-Werke, Germany), in 5 volumes (v/w) of ice-cold Milli-Q water, centrifuged at 3300 x g for 3 min at 4ºC, the supernatant removed for enzyme quantification and kept at -80ºC until further analysis. Enzymatic determinations for total amylase, lipase, alkaline protease and pepsin activities were based on methods performed and described by Gisbert and co-authors (see Gisbert et al. 2009). In brief, total alkaline proteases were measured using azocasein (0.5%) as substrate in Tris-HCl 50 nmol.l^{-1}, and pH 9. Alkaline protease activity is measured in nmoles azo dye per minute and per ml of tissue homogenate at 366 nm. Alpha-amylase (E.C. 3.2.1.1) activity was determined according to Métais and Bieth (1968) using 0.3% soluble starch. Amylase activity was defined as the amount of starch (mg) hydrolyzed during 30 min per ml of tissue homogenate at 37ºC at 580 nm. Bile salt-activated lipase (BALT, E.C. 3.1.1) activity was assayed for 30 min at 30ºC using p-nitrophenyl myristate as substrate. The reaction was stopped with a mixture of acetone: n-heptane (5:2), the extract centrifuged for 2 min at 6080 x g and 4ºC and the increase in absorbance of the supernatant read at 405 nm. Bile salt-activated lipase activity was defined as the amount (nmol) of substrate hydrolyzed per min per ml of enzyme extract (Iijima et al., 1998). Finally pepsin activity was defined as the nmol of tyrosine liberated per min at 37ºC per ml of tissue homogenate at 280 nm (Worthington, 1993).

All enzymatic activities were expressed as specific activity defined as units per milligram of larvae or protein. Soluble protein of crude enzyme extracts was quantified by means of the Bradford's method (Bradford, 1976) using bovine serum albumin as standard. All the assays were made in triplicate from each pool of larvae and absorbance read using a spectrophotometer (Beckman Coulter DU800, Fullerton, CA).

Statistical analysis

Nutrient concentrations are given (mean±SD) on wet weight or as % of total fatty acids. Differences between larvae fed *Artemia* nauplii and on-grown *Artemia* were identified using a t-test after checking for homogenous variances by Levenes test, software Statistica (ver 12, Statsoft Inc, Tulsa, OK).

Enzyme activities are expressed as mean ± SD. Normal distribution was checked for all data with the one-sample Kolmogorov–Smirnoff test and homogeneity of the variances with the Levene test. The group data were statistically tested using one-way ANOVA followed by the Tukey test. When variances were not

homogeneous, a non-parametric Kruskal-Wallis test was applied. The significance level for all the analysis was set at 5%. All the data were statistically treated using a SPSS Statistical Software System 15.0 (SPSS, www.spss.com).

Table 1. Analytical methods: Principles and references

Analyte	Principle	Reference
Protein	N x 6.25 Leco N Analyzer	(Hamre and Mangor-Jensen, 2006)
Free amino acids	HPLC and post column derivatization	(Srivastava *et al.*, 2006)
Fatty acids	Transmethylation extraction and GC/FID	(Lie and Lambertsen, 1991)
Glycogen	Hydrolysis and spectrometric detection	(Hemre *et al.*, 1989)
Thiamine	HPLC	(CEN, 2003b)
Vitamin C	HPLC	(Mæland and Waagbø, 1998)
Vitamin A	HPLC	(Moren *et al.*, 2002)
Vitamin D	HPLC	(CEN, 1999)
Vitamin E	HPLC	(Hamre *et al.*, 2010)
Sum vitamin K[3]	HPLC	(CEN, 2003a)
Iodine	ICPMS	(Julshamn *et al.*, 2001)

Results and discussion

In the industry, the routine method is to feed *Artemia* nauplii and it is quite common to produce large fractions of Atlantic halibut larvae with abnormal pigmentation and lack of eye migration, although the Atlantic halibut juvenile quality has improved in recent years. In this study, larvae fed the *Artemia* nauplii had perfect pigmentation and eye migration, so the juvenile quality could not be improved further by feeding on-grown *Artemia*. The nutrient concentrations of Atlantic halibut larvae fed *Artemia* nauplii and on-grown *Artemia* from 15 until 28 dpff were similar, except that the on-grown group had a slightly lower level of EPA than larvae fed *Artemia* nauplii, a difference that is probably biologically insignificant (**Table 2**). The similarity in nutrient composition of the larvae is another possible explanation of the lack of differences in growth and larval performance between the two treatments. It was very labor-intensive to produce the on-grown *Artemia* needed for the experiment, so on some occasions the on-grown group had to be fed *Artemia* nauplii to get enough feed. As the fish grow, more feed is needed and due to capacity problems, the feeding period had to be shortened to last until 28 dpff instead of 45 dpff as was planned. These are all possible reasons that no differences in performance were detected between the groups.

The average enzymatic activities measured per mg larvae are shown in **Table 2**. The activity of amylase, alkaline protease and pepsin, but not lipase, were higher in larvae fed *Artemia* nauplii and sampled at 28 dpff than in larvae sampled at 15 dpff. The activity of amylase and alkaline protease was lower in larvae fed the on-grown *Artemia* than in those fed *Artemia* nauplii and a similar, non-significant tendency was seen for pepsin activity. Lipase activity did not change from 15 to 28 dpff and was similar in the two larval groups at the end of the experiment.

Taking into consideration expression data from the study performed by Murray et al. (2006), and enzyme activity data from Gawlicka et al. (2000), it seems that Atlantic halibut have the capacity to digest both proteins and lipids from as early as 45 dph, approximately at first feeding. However, their potential for gastric protein digestion is limited until stomach development and gastric gland formation is completed at 80

dph (35 dpff). Therefore, the increment in the pepsin activity displayed by the older larvae is probably insufficient for efficient gastric digestion of proteins. Activities of amylase and protein digestive enzymes were generally higher in larvae fed *Artemia* nauplii compared to those fed on-grown *Artemia*. The reason for this is not known, but a possible explanation may be that *Artemia* nauplii are less digestible and that a higher digestive capacity is needed in these larvae.

Table 2. Nutrient concentrations in Atlantic halibut larvae fed *Artemia* nauplii and on-grown *Artemia* from 15 until 28 dpff. Significant differences are marked with red (t-test, $p<0.05$).

On wet wt		Start	Nauplii	Ongrown
Protein	%	-	11.4±0.4	11.5±0.6
Sum FAA	mg/g	3.5	4.4±1.1	4.8±0.3
Taurine	mg/g	1.4	1.8±0.2	1.8±0.1
Glycogen	mg/g	0.95	1.6±0.3	1.4±0.3
20:4n-6 %	%TFA	5.2	6.2±0.1	6.2±0.2
20:5n-3 EPA %	%TFA	8.4	7.8±0.3	6.9±0.2
22:6n-3 DHA %	%TFA	14	9.6±0.9	8.4±0.7
Total FA	mg/g	16	15±3	19±2
Thiamin	mg/kg	2.6	2.1±0.2	2.2±0.2
Vitamin C	mg/kg	158	155±31	136±12
Vitamin-D3	mg/kg	0.02	0.01±0.00	0.01±0.00
Vitamin E	mg/kg	37	25±2	23±1
Vitamin A1	mg/kg	0.7	1.2±0.1	1.1±0.1
Iodine	mg/kg	0.26	0.28±0.02	0.26±0.01

Table 2. Activities of digestive enzymes in Atlantic halibut larvae (U/mg larvae) fed *Artemia* nauplii or on-grown *Artemia* from 15 (Start) until 28 dpff. Values are expressed as mean ± SD (n=3).

	Start	Artemia naupli	Ongrown Artemia
Amylase	11.2 ± 0.4^{a}	20 ± 1^{c}	14.2 ± 0.5^{b}
Alkaline Protease	132 ± 33^{a}	293 ± 24^{b}	120 ± 53^{a}
Lipase	57 ±12	36 ± 8	48 ± 4
Pepsin	28 ± 24^{a}	100 ± 28^{b}	51 ± 11^{ab}

Comparisons among groups were performed by one way ANOVA followed by Tukey´s test. Different letters within a row indicate significant differences ($P < 0.05$). Amylase (U) = mg starch hydrolyzed during 30 min at 37oC (580 nm); Alkaline protease (U) = nmol azodye per min (366 nm); Lipase (U) = nmol myristate per min (405 nm); Pepsin (U) = nmol tyrosine per min at 37oC (280 nm).

Conclusion

In the present experiment, there was no effect on growth or juvenile quality of Atlantic halibut with respect to eye migration and pigmentation, or digestive capacity when feeding on-grown *Artemia* instead of *Artemia* nauplii to Atlantic halibut larvae. The reason may be that the control group already showed good results and that the labor required for producing on-grown *Artemia* made it difficult to produce enough big *Artemia*. As a consequence, sometimes the group fed the on-grown *Artemia* was given *Artemia* nauplii and the experiment had to be finalized earlier than planned. Even though the on-grown *Artemia* and the *Artemia* nauplii had different nutrient composition, the nutrient composition and the growth of the fish were similar. On-grown *Artemia* had a positive effect on growth and juvenile quality of larvae in previous experiments (Olsen et al., 1999; K. Hamre and T. Harboe, unpublished), but then the control groups had high incidence of abnormal pigmentation and eye migration. In the industry, such problems are still encountered in some batches of juveniles. It is possible that improvement of the rearing protocols for Atlantic halibut larvae will prevent malformation of juveniles and therefore reduce the need for using on-grown *Artemia*.

Deviations: There were no deviations from the approved DOW.

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