Deliverable Report

Deliverable No:	D11.5	**Delivery Month:**		58
Deliverable Title	Report on the effect of dietary phospholipids on Atlantic halibut juveniles			
WP No:	11	**WP Lead beneficiary:**		P17. NIFES
WP Title:	Nutrition - Atlantic halibut			
Task No:	11.1	**Task Lead beneficiary:**		P7. IMR
Task Title:	Effect of dietary PL on digestion, absorption and metabolism of lipids in Atlantic halibut juveniles			
Other beneficiaries:	P.20 SARC			
Status:	Delivered		**Expected month:**	48
……..				

Lead Scientist preparing the Deliverable: Sæle, Øystein (IMR/NIFES)*

Other Scientists participating: Harboe, Torstein (IMR), Hamre, Kristin (IMR/NIFES)*, Fontanillas, Ramon (SARC)

*NIFES and IMR merged in January 2018.

Objective: The objective of this Deliverable was to investigate the effect of increased inclusion of dietary PL on Atlantic halibut (*Hippoglossus hippoglossus*) juveniles.

Introduction

Atlantic halibut larvae are approximately 12 mm in standard length (SL) at first-feeding, and because of their relatively large larval size they are first-fed on *Artemia* nauplii. The main constrains for Atlantic halibut hatcheries are (1) slow growth during the late larval stages and (2) high mortalities caused by opportunistic bacteria and 3) slow growth after weaning. The slow growth post weaning is persistent, also in juvenile fish of 1 g and more **(Fig. 1)**.

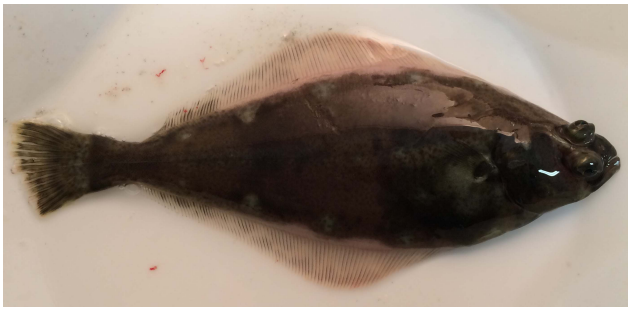

Figure 1. Atlantic halibut juvenile

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 been also shown to be beneficial for a wide spectre of species (Atar et al., 2009, Niu et al., 2008, Sotoudeh et al., 2010). Phospholipids 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 triacylglycerols (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 their growth rate by up to 40% when lipids are added as PL instead of TAG (Sæle et al., unpublished), while requirements for PL in Atlantic halibut juveniles are not known.

The main objective of this study was to investigate lipid composition in intestinal tissue as a function of dietary ratio of PL/TAG and postprandial time. We know that high dietary PL gives better growth in other marine fish juveniles, and we have also shown that dietary PL can affect intestinal transport of TAG. The hypothesis is therefor that the low PL/TAG diet will give fish with more total lipid and in particular TG in intestinal tissue. We are also curious as to how the PL/TAG ratio might affect liver and muscle lipid composition and are therefore including some samples from these tissues taken 24 h post feeding.

Materials and methods

Fish

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 days post first feeding (dpff) and then transferred to 15, 50-l tanks. Here, the larvae were fed Otohime until approximately 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 continuously using belt feeders, and were also hand fed two times a day: in the morning before clay addition and in the evening after the end of belt-feeding.

When 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. Sampled larvae were euthanized with an overdose metacaine (MS-222TM; Norsk medisinaldepot AS, Bergen Norway). Total length (TL) and weight were registered before intestine, liver and muscle tissues were snap frozen on liquid nitrogen for lipidomics analysis and RNA isolation for qPCR analysis.

Diets

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**).

Table 1: Diet compositions (%)

Diet	**1**	**2**	**3**	**4**	**5**
Water	3.36	3.36	3.36	3.36	3.36
Soy oil	6.00	4.50	3.00	1.50	0.00
Soy lecithin	0	1.50	3.00	4.50	6.00
Krill meal	2.50	2.50	2.50	2.50	2.50
Wheat	15.37	15.37	15.37	15.37	15.37
Fish Meal North-Atlantic	74.63	74.63	74.63	74.63	74.63
Fish meal hydrolysate	2.50	2.50	2.50	2.50	2.50
Fish oil North-Atlantic	2.06	2.06	2.06	2.06	2.06
Vitamin premix	0.10	0.10	0.10	0.10	0.10
Yttrium premix	0.10	0.10	0.10	0.10	0.10
Mineral premix	0.10	0.10	0.10	0.10	0.10
Calculated proximate composition:					
Volume	100.00	100.00	100.00	100.00	100.00
Dry matter	93.00	93.00	93.00	93.00	93.00
Protein	57.00	57.00	57.00	57.00	57.00
Lipid	18.00	18.00	18.00	18.00	18.00
Ash	10.56	10.56	10.56	10.56	10.56
Rest	8.76	8.76	8.76	8.76	8.76

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

Lipid class analyses

Lipid class composition of the diets was analyzed using high-performance TLC, as described by Bell et al. (1993) and Jordal et al. (2007). The plates (20 x 10 cm) were developed at 5 cm in methyl acetate – isopropanol – chloroform – methanol–0·25% (w/v) aqueous KCl (25:25:25:10:9, by vol.) to separate PL classes from neutral lipids running at the solvent front (Vitiello and Zanetta, 1978). After drying, the plates were developed fully in isohexane – diethyl ether – acetic acid (80:20:1·5, by vol.) to separate neutral lipids and cholesterol. Lipid classes were visualized by charring at 160˚C for 15min after spraying with 3% copper acetate (w/v) in 8 % (v/v) phosphoric acid and identified by comparison with commercially available standards. Lipid classes were quantified by scanning densitometry using a CAMAG TLC Scanner 3 and calculated using an integrator (WinCATS- Planar Chromatography, version 1.2.0).

Lipidomics

Lipids were extracted from tissue samples using dichloromethane and methanol in a modified Bligh-Dyer extraction in the presence of internal standards with the lower, organic, phase being used for analysis. The extracts were concentrated under nitrogen and reconstituted in 0.25mL of dichloromethane:methanol (50:50) containing 10mM ammonium acetate. The extracts were placed in vials for infusion-MS analyses, performed on a SelexION equipped Sciex 5500 QTRAP using both positive and negative mode electrospray. Each sample was subjected to 2 analyses, with IMS-MS conditions optimized for lipid classes monitored in each analysis. The 5500 QTRAP was operated in MRM mode to monitor the transitions for over 1,100 lipids from up to 14 lipid classes. Individual lipid species were quantified based on the ratio of signal intensity for target compounds to the signal intensity for an assigned internal standard of known concentration. Lipid class concentrations were calculated from the sum of all molecular species within a class, and fatty acid compositions were determined by calculating the proportion of individual fatty acids within each class.

qPCR

The effect of dietary lipid composition on expression of genes in several pathways involved in lipid metabolism was measured by qRT-PCR. Tissue pieces were homogenized on Quiazol (Qiagen) using a Precellys 24 (Bertin Technologies). Total RNA was extracted from the whole fish on a Bio Robot EZ1 using the EZ1 RNA Universal Tissue Kit with the RNase-free DNase Set (Qiagen), according to the manufacturer's instructions. The quantification and purity of RNA was assessed with the NanoDrop ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies). For all total RNA samples, the optical density ratio at 260/280 nm ranged between 1.70 and 1.98. RNA integrity numbers (Imbeaud 2005) were between 8.2 and 9.7.

RT reactions and qPCR were run according to published methods (Sæle et al., 2009). Genes coding for proteins involved in assembly and transport of chylomicrons, ER-stress, cytosolic lipid droplet metabolism and mitochondrial ß-oxidation were analysed (**Table 3**). Results were calculated as the arithmetic mean using ubiquitin and beta-actin as reference genes based on the study of (Sæle et al., 2009). Normalization was performed using the geNorm Visual Basic for Applications applet for Microsoft as previously described by (Vandesompele et al., 2002). Primer sequences were deduced from halibut genome sequenced in-house in IMR.

Table 3. Primers used in the qPCR assay.

Gene	Primer sequence F	Primer sequence R
Tubb2	CTACAATGAGGCTTCAGGTGG	TCCCTCTGTGTAGTGACCCTTG
ActB	GAGAAGATGACTCAGATCATGTTCG	CCAGCCAGGTCCAGACGG
SLC27A4	AGTAGAGTCTGTCCGGCACT	GCCATCGCTGATCCGTCTAA
CD36 F	GGAAAAACGCACAGCACCAT	TCGATCCAGGCTCAATGACG
ApoAIV	GCAGAGATCAGTGACGACGA	GTCATGCTGGACAAAGACGC
MTTP	GCTACTGAGGATGACGTCCG	GAGTCAGAGGTGGGAGCCTA
CHPT1	GCGCAGAGCTGCTATTTGTC	TCCGTGTTGGGCCTTCTTAC
LPCAT1	CGGGGATGGCATCGAAGTAG	CTGGGACGCTCTGAGTTTGT
LPCAT2	TATAGTGCCATCGCCGTTCC	GGGAGGACGCATCACTATCG
LPCAT5	TCCTCTCTGGACTCGGCTAC	CCACGCGTTTGTGTTGATGT
GPAT4	CGGCATGGTGAACTACCTGT	TTACCTTTCCGCGCTTCAGT

Results and Discussion

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. 2**).

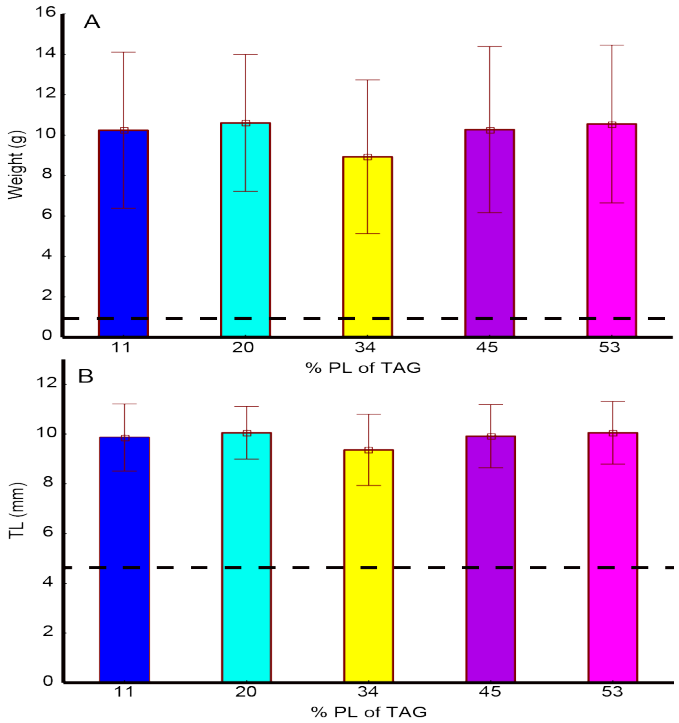

Figure 2. Final weight (A) and length (B) of halibut. Stippled line shows start weight and length.

Growth in halibut this size, did not benefit from higher inclusions of PL. 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., 2015).

To analyse the difference in lipid transport from the intestinal epithelium to peripheral tissues, the concentration of lipid classes in intestinal epithelium at 1, 4 and 24 hours post prandial in fish fed the different diets were compared. There was no effect of diet type on the concentration of lipid classes, but time post prandial had an effect on the relative concentrations of cholesterol (CE), ceramide (CER), diacylglycerol (DAG), dihydroceramides (DCER), phosphatidylinositol (PI), sphingolipid (SM) and triacylglycerol (TAG) (**Fig. 3**).

The three diets included in this analysis varied in PC and TAG, it is therefore interesting to observe that PC was not significantly different in intestinal tissue. The general picture is that neutral lipids are transported out of the epithelium and some of the polar lipids then increase in concentration.

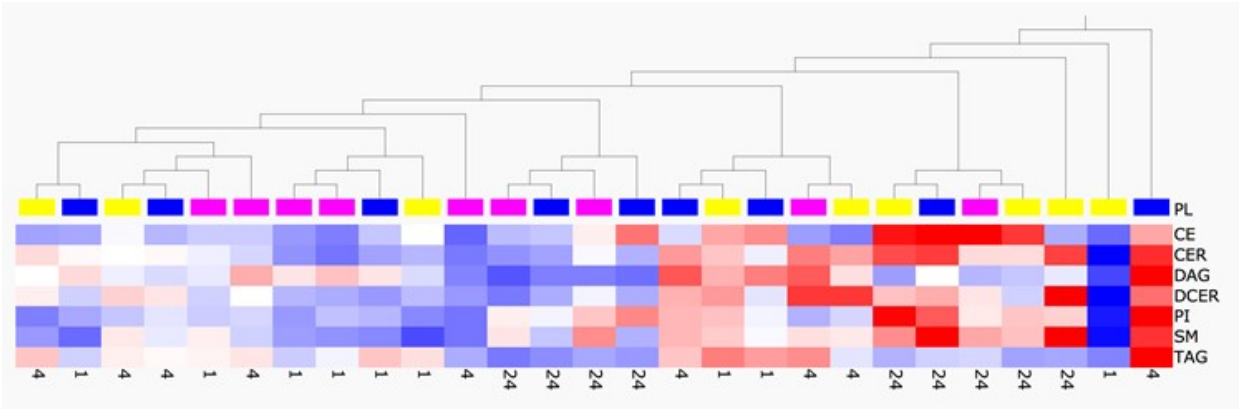

Figure 3. Lipid classes in intestinal tissue: Heat map showing the effect of diet and postprandial time on the relative concentrations of lipid classes. The concentrations range of lipid classes is from 2(red) to -2 (blue) fold of mean and time is given as 1, 4 or 24 hours post prandial. The colours in the first row represent the diets (Blue, low; Yellow, medium; Pink, high PL)

When looking at all the different fatty acid combinations within the lipid classes, referred to as lipid species, 160 out of 1061 species were significantly regulated by postprandial time, but not by diet (**Fig. 4a,b**). The clustering of samples based on post prandial time show a very clear image, where neutral lipids, such as TAG, DAG and FFA, are transported out of the intestinal tissue after the meal and concentrations of CE, CER and some of the phospholipids increase. One sample from the 4 h post prandial low PC group and one from the 1 h post prandial medium PC group appear as outliers (far right **Fig. 4b**) by having very low levels of lipid. Even though only fish that had feed in the stomach (1 h) or intestine (4 h) were included in the analysis, it seems like these two specimen did not digest and or absorb lipids in the same rate as the remaining fish in these groups. Otherwise, the 4 and 1 h groups cluster together based on higher concentrations of neutral lipid species.

Soy lecithin, used to regulate PL in the diets is particularly rich in linoleic acid (18:2). The only PC species that was differentially regulated postprandially was PC (18:2/18:2).

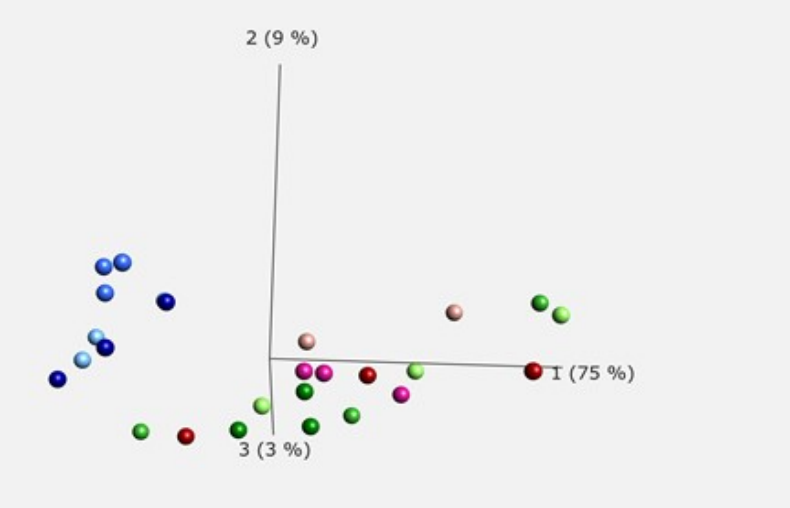

Figure 4a. PCA plot based on all lipid species in intestinal tissue**.** The 24h sampling clusters away from 1 and 4h, but there is no separation between 1 and 4h. Nor does PL level in the diets have any effect. The same data are represented in a heatmap below.

Also phosphatidylinositol (PI) and phosphatidylethanolamine (PE) species with linoleic acid follow the same pattern of depletion in intestinal tissue over time after a meal. As expected, intestinal tissue with the highest linoleic acid species, neutral or polar, were found in the 1 and 4 h post prandial group, but in the feed groups given the diets with low or medium levels of PC.

Since linoleic acid may be considered a fingerprint of the soya lecithin and oil used in the experimental diets (Howell and Collins, 1957), we analysed the fatty acid concentrations within the different lipid classes **(Fig. 5)**. As seen in the above-mentioned analysis of lipid species, the 24 h postprandial samples grouped, but the diets did not show any relationship based on FA concentrations. Surprisingly, no fatty acid was differently regulated in the PC class and only lauric acid (12:0) was significantly regulated in the treatment groups in the TAG class.

Liver and muscle samples taken at 24 h postprandial did not vary in lipid composition in response to the different diets (data not shown).

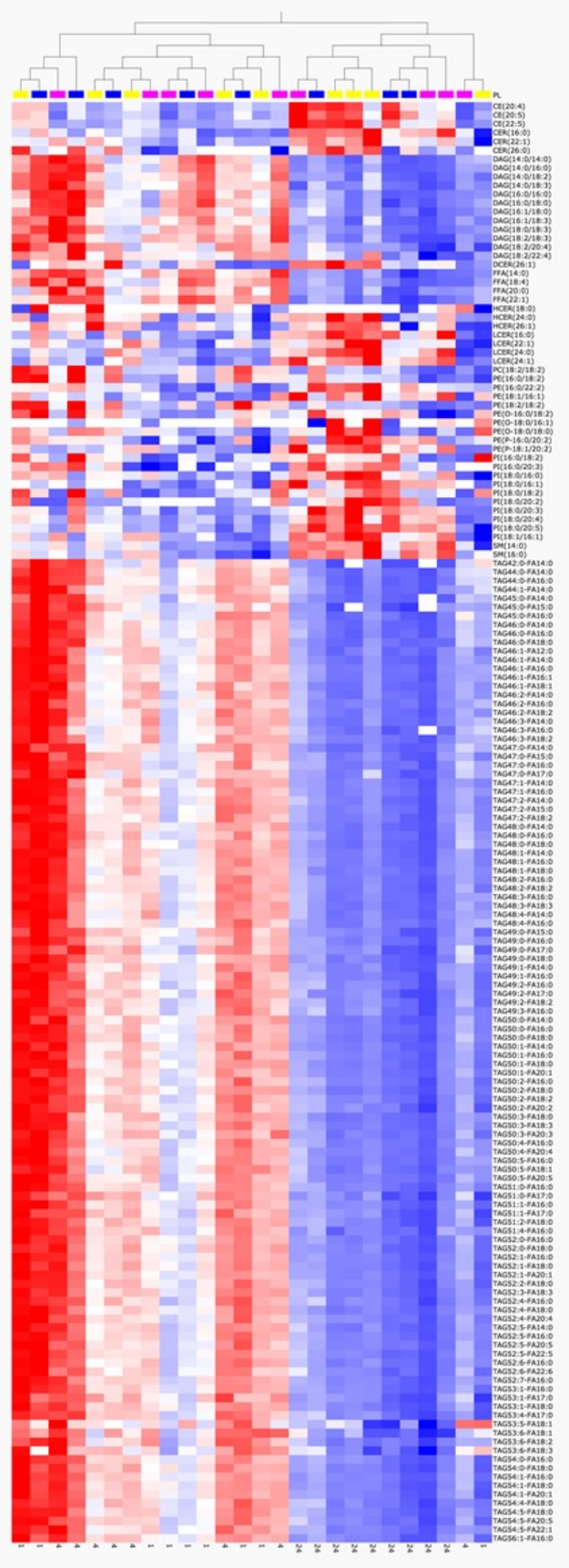

Figure 4b. Lipid species in intestinal tissue**.** Heatmap of 79 differently regulated lipid species out of 1061, analyzed in Atlantic halibut fed diets with 9, 23 or 31% PL in total lipid. The colors of the first row represent the diets, while the numbers at the bottom of the figure represent time post-prandial. (Blue, low PC; yellow, medium PC; pink, high PC).

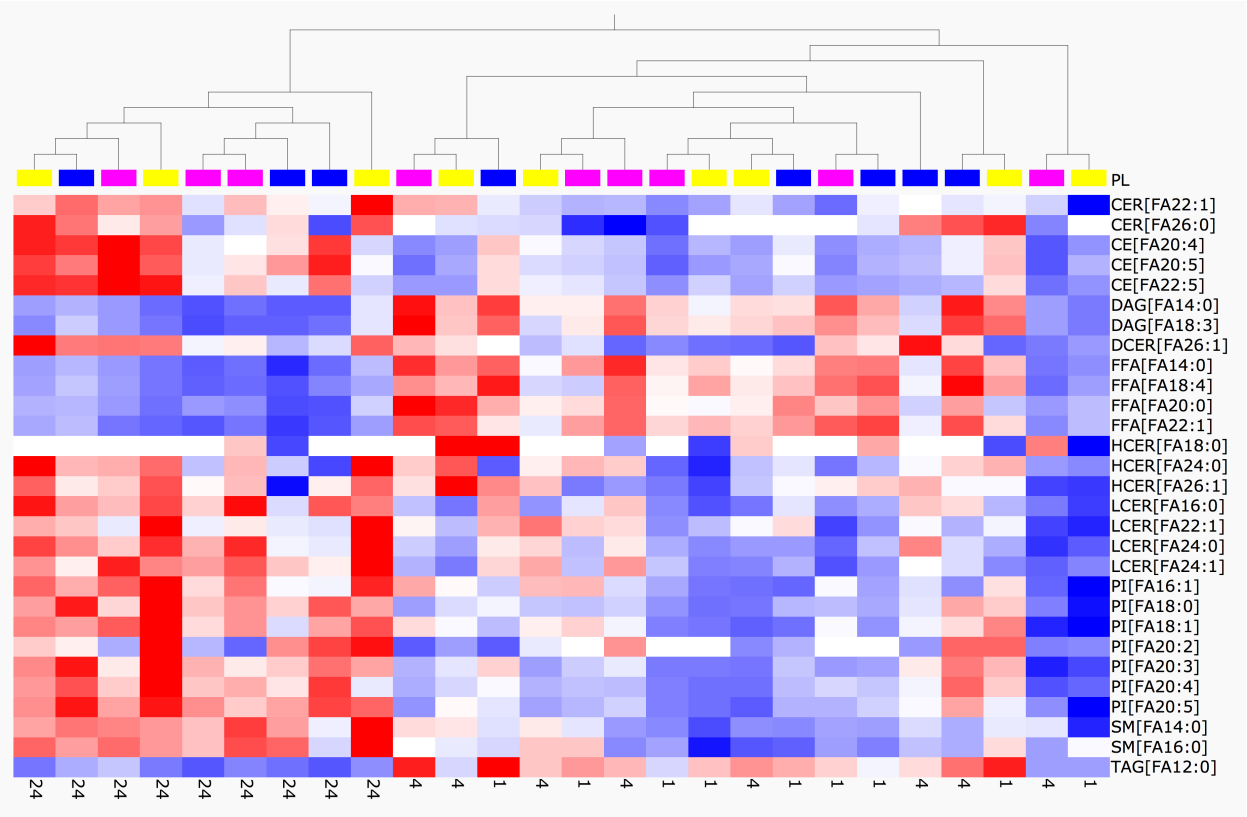

Figure 5: Fatty acids in intestinal tissue**.** Heat map showing clustering of feed groups and post prandial time based on fatty acids grouped per lipid class that were regulated according to time ($p<0.05$). The dominating fatty acid of soy: linoleic acid (18:2) is not differently regulated in any lipid class.

An array of genes involved in lipid absorption and metabolism in enterocytes (**Table 3**) was analysed in intestinal tissue from the diet groups given high, medium and low levels of PC at 1, 4 and 24 h post prandial. No effect of diet type was found, but time postprandial had an effect on CD36 molecule (CD36), Lysophosphatidylcholine acyltransferase 1 (LPCAT1), Choline phosphotransferase 1 (CHPT1) and Glycerol-3-phosphate acyltransferase 4 (GPAT4) (**Fig. 6**). CD36 is one of the factors transporting digested fatty acids into the enterocyte. We suspect the lipid absorption to be at the highest 4 h post prandial, and an up-regulation of CD36 would naturally correlate with that. However, the amount of lipids in the intestinal tissue does not show a significant increase between 1 and 4 hours. GPAT4 and CHPT1 are both involved in new synthesis of lipids and LPCAT1 remodels lyso-PL into PL. All these genes are expected to be up-regulated when a lipid diet is being absorbed and metabolized in the enterocyte. However, genes associated with lipid transport from the enterocyte to periphery tissues, such as ApoA4 were not regulated as expected.

We hypothesise that lipid absorption after a meal is at its highest 4 h post prandially, but the transport out of the intestinal tissue is too rapid to be detected by measuring the actual lipid load in the tissue. However, supporting the hypothesis is the regulation of the broad spectre of genes associated with absorption and re-synthesis of dietary lipids.

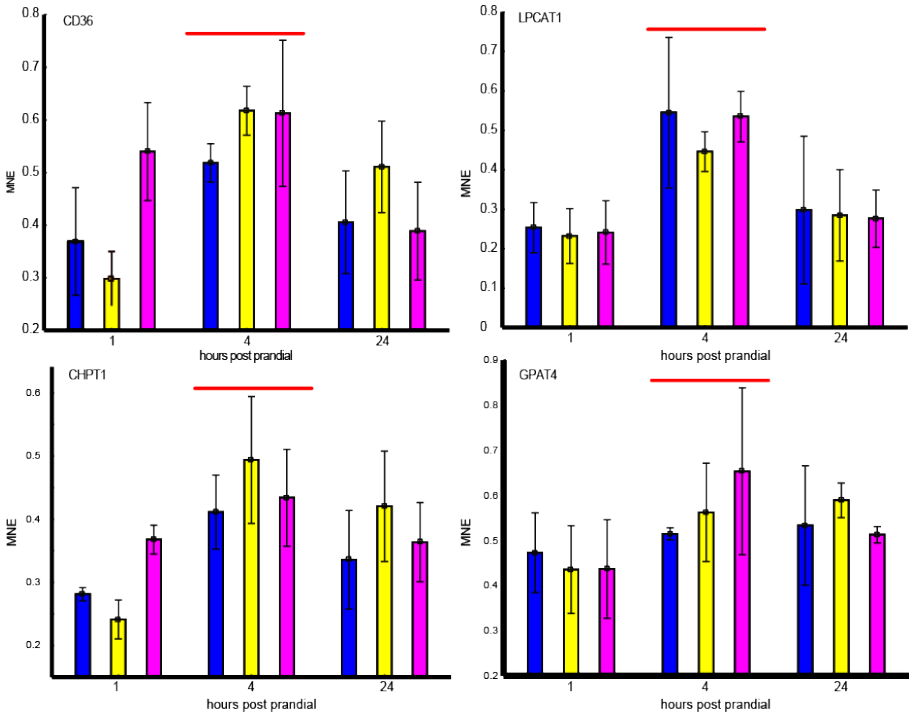

Figure 6. Gene-expression of CD36 (FA absorption), LPCAT1 (PL remodeling), CHPT1 (phosphatidylcholine de novo synthesis) and GPAT4 (de novo glycerolipid synthesis) 1, 4 and 24 hours post prandial. Blue bars indicate low PC diets, yellow indicate medium PL diets and pink the high PL diet. Diet type did not affect any of the genes analyzed, but time had an effect with higher expression after 4 h.

Summary and conclusions

Dietary PL varying from 9 to 31 % of dietary lipid did not affect growth of Atlantic halibut juveniles. Intestinal, muscle or liver lipid composition was 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 CE, 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.

It appears that Atlantic halibut juveniles regulate their lipid species composition to be independent of the diet when a range of PL/TAG as in the present study 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.

Deviations: This task was reported 10 months later than planned and included lipidomics analyses that were financed by internal NIFES funds. Otherwise, there were no deviations from the approved DOW.

References

Atar, H.H., Bekcan, S., Olmez, M., 2009. The Effects of Dietary of Soybean Lecithin on the Growth Performance Feed Conversion and Body Composition of Tilapia (*Oreochromis niloticus* L.) Fry. Journal of Animal and Veterinary Advances 8, 1678–1684.

Bell, J.G., Dick, J.R., McVicar, A.H., Sargent, J.R., Thompson, K.D., 1993. Dietary sunflower, linseed and fish oils affect phospholipid fatty acid composition, development of cardiac lesions, phospholipase activity and eicosanoid production in Atlantic salmon (*Salmo salar*). Prostaglandins, Leukotrienes and Essential Fatty Acids 49, 665–673. doi:10.1016/0952-3278(93)90075-8

Coutteau, P., Geurden, I., Camara, M.R., Bergot, P., Sorgeloos, P., 1997. Review on the dietary effects of phospholipids in fish and crustacean larviculture. Aquaculture 155, 149–164. doi:10.1016/S0044-8486(97)00125-7

Howell, R.W., Collins, F., 1957. Factors Affecting Linolenic and Linoleic Acid Content of Soybean Oil 1. Agronomy Journal 49, 593–597. doi:10.2134/agronj1957.00021962004900110007x

Imbeaud, S., 2005. Towards standardization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces. Nucleic Acids Research 33, e56–e56. doi:10.1093/nar/gni054

Jordal, A.E.O., Lie, Ø., Torstensen, B.E., 2007. Complete replacement of dietary fish oil with a vegetable oil blend affect liver lipid and plasma lipoprotein levels in Atlantic salmon (*Salmo salar* L.). Aquaculture Nutrition 13, 114–130. doi:10.1111/j.1365-2095.2007.00455.x

Niu, J., Liu, Y.J., Tian, L.X., Mai, K.S., Yang, H.J., Ye, C.X., Zhu, Y., 2008. Effects of dietary phospholipid level in cobia (*Rachycentron canadum*) larvae: growth, survival, plasma lipids and enzymes of lipid metabolism. Fish Physiol. Biochem. 34, 9–17. doi:10.1007/s10695-007-9140-y.

Sotoudeh, E., Kenari, A.A., Rezaei, M.H., 2010. Growth response, body composition and fatty acid profile of Caspian brown trout (*Salmo trutta* Caspius) juvenile fed diets containing different levels of soybean phosphatidylcholine. Aquacult. Int. 19, 611–623. doi:10.1007/s10499-010-9376-x

Sæle, Ø., Nordgreen, A., Hamre, K., Olsvik, P.A., 2009. Evaluation of candidate reference genes in Q-PCR studies of Atlantic cod (*Gadus morhua*) ontogeny, with emphasis on the gastrointestinal tract. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 152, 94–101. doi:10.1016/j.cbpb.2008.10.002

Taylor, J.F., Martinez-Rubio, L., del Pozo, J., Walton, J.M., Tinch, A.E., Migaud, H., Tocher, D.R., 2015. Influence of dietary phospholipid on early development and performance of Atlantic salmon (*Salmo salar*). Aquaculture 448, 262–272. doi:10.1016/j.aquaculture.2015.06.012

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3, research0034. doi:10.1186/gb-2002-3-7-research0034

Vitiello, F., Zanetta J.P. (1978) Thin-layer chromatography of phospholipids. J. Chromatogr. 166, 637-640.

Co-funded by the Seventh Framework Programme of the European Union