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FHF-prosjekt 900958 - Sluttrapport

Et fullskalaforsøk med lavt innhold av EPA og DHA i fôret – betydning for struktur av tarm og tarminflammasjon

A full scale feeding experiment with low dietary EPA and DHA – potential effects on intestinal structure and inflammation status

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Sammendrag

Hovedmålet med dette prosjektet var å dokumentere effekter av fôr med reduserte innhold av langkjedete n-3-fettsyrer fra 7.5% EPA og DHA til 5.4% av fettet og lavt innhold av fiskemel (Marin Protein Indeks MPI<10) under en fullskala produksjonssyklus i sjø på laksens tarmhelse. Konklusjoner fra forsøket er at lavt nivå av EPA og DHA i fôret (5.4% av lipid) ikke påvirker lipid akkumulering i tarmvevet hos slakteklar laks, sammenlignet med fisk gitt en moderne plantebasert diett med 7.5% EPA og DHA. Fremtidige eksperimenter som undersøker lave innhold av EPA og DHA i fôret med ulike fettkilder bør vurdere oljedråpe dannelse og genuttrykk av perilipin i midttarm som markører for ubalansert fettsyreopptak og transport.

A full scale feeding experiment with low dietary EPA and DHA – potential effects on intestinal structure and inflammation status

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Abstract

The main objective of this project was to document the effects on intestinal health of Atlantic salmon of further reduction in concentration of long chain n-3 fatty acids from 7.5% EPA+DHA of fatty acids to around 5% in salmon feeds low in fish meal (MPI≤ 10) during a full scale production cycle in sea. The overall conclusion from the present study is that the levels of EPA and DHA in the low diet (5.4% of lipid) did not affect lipid accumulation in the intestinal tissue of harvest size Atlantic salmon, when compared to fish given a modern commercial plant based diet with 7.5% EPA+DHA. Future experiments investigating low dietary EPA and DHA levels with varying replacement lipid sources should consider lipid droplet formation and gene expression of perilipin in mid intestine as markers of imbalanced fatty acid uptake and transport.

Background

Due to the current lack of alternative EPA and DHA sources, the availability of fish oil is the single factor in the short term that can limit further growth in salmon production if the level of EPA and DHA should be maintained at the current dietary levels (ca 7.5 % EPA + DHA of the dietary fat). Thus, to be able to further increase fish feed production volumes, the inclusion of fish oil and hence the dietary EPA and DHA has to be reduced. However, the minimum safe level of omega- 3 is determined by the actual nutritional needs for omega-3 fatty acids in Atlantic salmon, and these are not yet known. The official tables (NRC 2011) indicates 0.5-1.0 % of the feed, corresponding to 1.5 to 3.0 % of the fat in the diet. The report "Fett for fiskehelse" (Torstensen *et al.* 2013) concludes, "it is not shown clearly whether fat accumulation in the intestinal cells affects inflammation in the bowel which may affect their health and welfare". It is also conceivable that lipid accumulation is not chronic, but diminishes after a meal and thus does not have adverse health effects on fish.

In an extension to the current full-scale CAC experiment where Atlantic salmon were fed with two different levels of EPA + DHA (low and standard) we have here extended the analysis of gut health - structure and fat accumulation in the intestine in relation to a possible chronic inflammation of the intestinal tissue. Histological examination of the intestine is included in the health surveillance program of the CAC site and will be reported in a later publication.

High dietary n-6/n-3 ratio has been associated with lipid accumulation in intestinal tissue in fish (Olsen *et al.* 2003). To investigate if low levels of EPA and DHA in the diet may influence intestinal lipid accumulation, we looked at lipid droplet accumulation. A number of genes that are involved in lipid droplet formation, assembly of chylomicrons and VLDLs and transport of these (Fig. 1), were also analyzed in intestinal tissue samples.

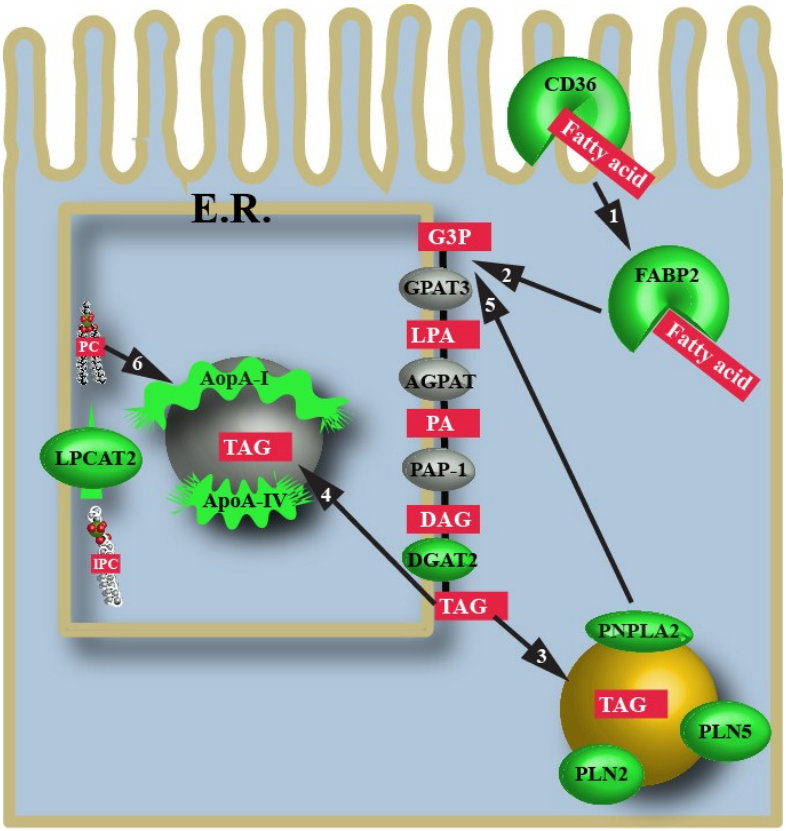

Figure 1. *Simplified model of lipid metabolism in the enterocyte. Proteins of which genes were analyzed in this study are highlighted in green. Long chained fatty acids are actively absorbed by receptors like CD36, FAs are then transported via FABP2 to the ER where they are incorporated into TAG molecules. TAG molecules were incorporated into lipid droplets for storage assisted by PLN2 and PLN5 or into chylomicrons for transport out of the cell.*
Chylomicrons have a core of neutral lipids and a surface of lipoproteins like ApoA-I and IV and phospholipids (PL). In enterocytes, digested PLs are absorbed as lysoPLs and remodeled back to intact PLs in the ER by LPCATs.

Methods

Fish material

Adult Atlantic salmon (approx. 5 kg) from a full cycle and industrial scale experiment at the site of the Centre of Aquaculture Competence (CAC 2012) in Hjelmeland (Rogaland) had been fed two experimental diets, containing low (5.4% of the lipid) and regular (7.5% of the lipid) concentrations of EPA and DHA, from smolt (summer 2012) to harvest (Nov 2013 and Feb 2014). Except for the differences in the long chain n-3 fatty acids (made by replacing fish oil with rapeseed and palm oil), the diets were of similar composition (protein and lipid

sources, and vitamin- and mineral mixes). The diets contained high proportions of plant proteins, with a marine protein index (% marine protein) of approx. 10 %.

The large scale feeding experiment includes health surveys (lice counting, inspections for cataract, bone deformities, vaccine side effects etc), as well as regular examinations of the intestinal tissue by the Veterinary Institute, Bergen (Waagbø *et al* 2013). In the present study we expanded the survey with additional analyses of gut health, i.e. structure and fat accumulation in the intestine. The outcome will be discussed into the context of the general health situation, potential histopathological changes in the intestinal tissue, growth, feed utilization and other health outcomes when these results are available from CAC. The CAC 2012G project will be reported to the Directorate of Fisheries in 2014.

Samples and analyses

All fish had intestinal content indicating an active feeding status. Intestinal tissue samples were taken of the mid intestine ~1 cm behind the pyloric caeca from six fish in each sea cage. Neighboring tissue samples were used for histology and RNA extractions.

Samples for histology were rinsed in PBS, and fixed on 4 % paraformaldehyde in PBS, pH 7.2 over night before they were transferred to 30 % sucrose through a gradient of baths. Tissue samples were then embedded in Tissue-Tek® O.C.T.™ (Sakura, Netherlands) and frozen in a dry ice and methanol slurry.

Fifteen micron thick sections were cut on a cryostat (Leica) dried and stained with oil red O (producer) for 10 min. No counterstain were employed for better quantification of lipid stores. The amount of lipid stored in the epithelium were scored from 0 (no visual lipid droplets) to 10 (epithelium saturated with fat).

Samples for gene expressions were snap frozen on liquid nitrogen. RNA was isolated from tissue, cDNA synthesized and gene expression measured with qPCR according to Sæle *et al* (2009). The primer sequences used are shown in Table 1.

The qPCR data were tested with a nested design ANOVA (StatSoft, Inc., USA). Six fish from each cage were analyzed.

Table 1. *The primer sequences used in the intestinal tissue samples*

Gene*	***Forward***	***Reverse***
PLIN2	AAGAGGGCATACCACAAGGC	CACTCAACCAGGGAGCTCAG
PLIN5	ACAATGGCAGACAGCGAGAA	AGCTCACCAGAGGGATGCTA
FABP2	CCTGGGCGTACAGTTTGACT	ACTAGCTCTCCTCCCACCAG
APO A4	GCTGAAGAAGCTGGACCCAT	TCAGGAGCACTGATGCTTGG
APO A1	ACCCACCAGACCACCATCAT	CAGCTGAGAGGGAGCATCAG
CD36	GGTAGCGGAGTGCTGAAAGT	TGAGCCTTCTTACTGGCACG
LPCAT2	GGTGAAATTGGCCAAGGCTG	GCTCCTTTGAGAGGGTGCAT
DGAT2α	AGCGGAGTTGAACCCAAACA	AGGTCCATAAAGCCACAGGC
PNPLA2	TCAACCGGATGGAGGTGTTG	CATCCTTCCAAGGAGTGCGT
IL-1	TGAAGTCCATCAGCCAGCAG	GGATGGTGAAGGTGGTGAGG

* PLIN2, PLIN5 (perilipin 2, 5); FABP2 (fatty acid binding protein 2);
APO A4, APO A1 (apolipoprotein A4, A1); CD36 (receptor/sensor of diacylglycerol);
LPCAT2 (lysophosphatidylcholine acyltransferase 2); DGAT2α (diacylglycerol acyltransferase-2α);
PNPLA2 (patatin-like phospholipase domain containing 2); IL-1 (interleukin-1)

Results and discussion

There was no effect of the diet on the quantity of lipid droplets in the intestinal epithelium. However, the individual variation was rather large (Fig. 2). Areas of the intestine could be so loaded with lipid that it leaked out of the tissue when sections were mounted on the slides. This was observed as stained droplets on the lumen side of the enterocytes (see star in Fig. 3). Examples of intracellular lipid droplets are indicated with arrows in Fig. 3.

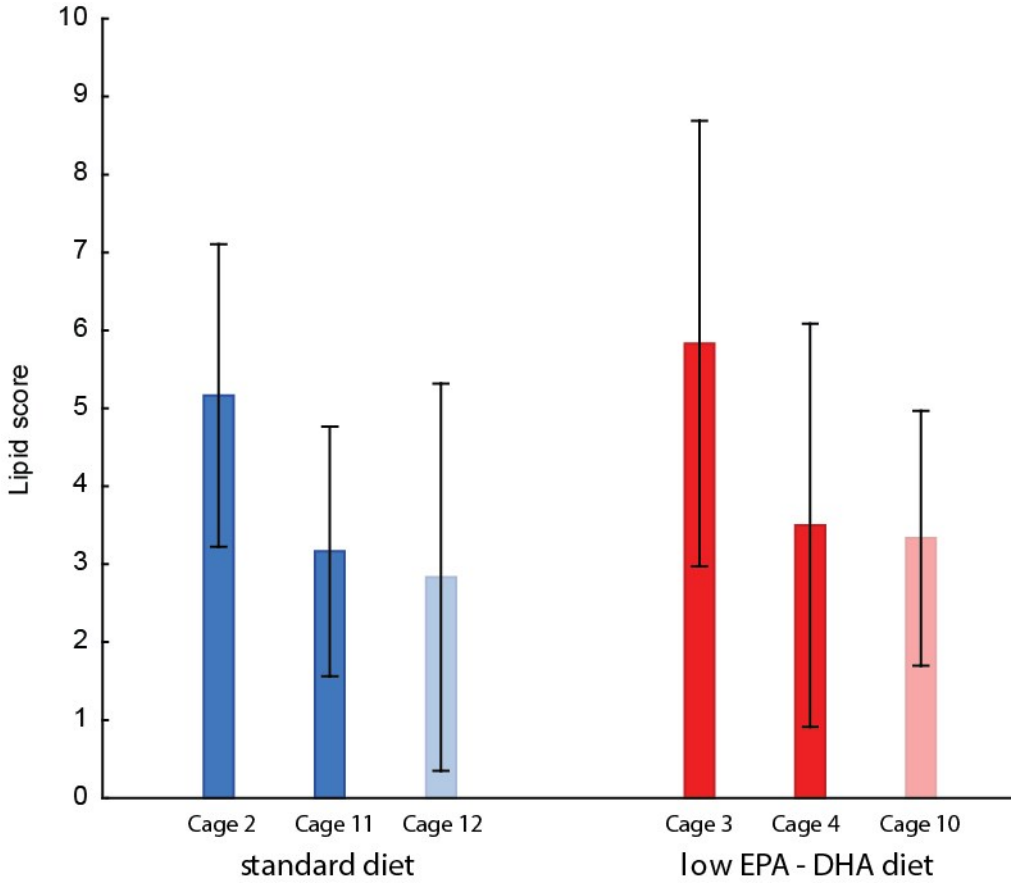

Figure 2. *Amount of lipid stored in the epithelium. Score from 0, no visual lipid droplets to 10, intense red, most of epithelium contained lipid droplets.*

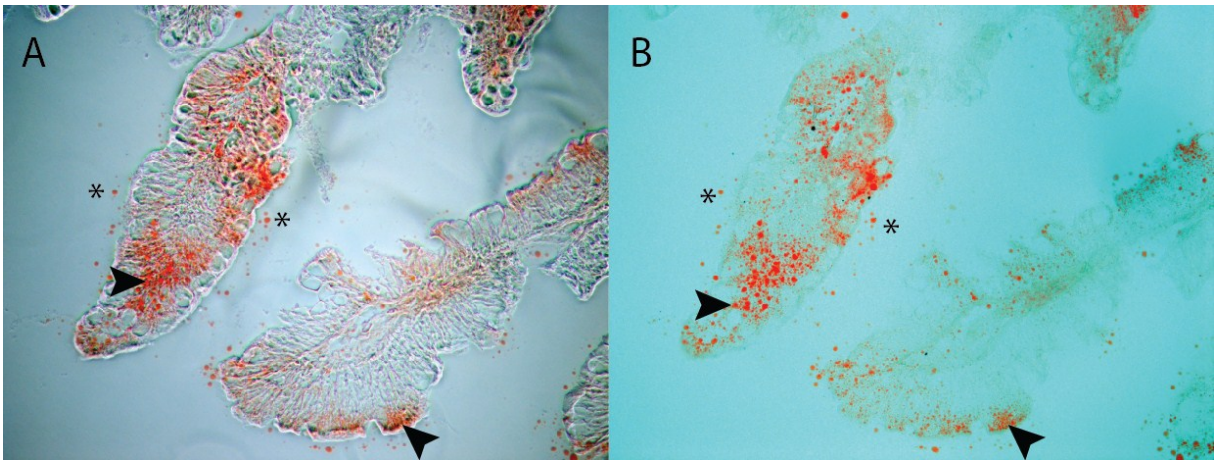

Figure 3. *Photographs (x200) of intestinal tissue with lipid droplets in red. A and B is of the same area, but A has been taken with a DIC filter (brings out contrasts of the tissue) and B with bright field.*

The diets did not influence the gene expression of proteins involved in uptake (CD36), transport (FABP2), and re-synthesis of lipids (DGAT2 and LPCAT2). Neither were genes of lipoproteins (ApoA-I and –IV) vital for chylomicron assembly influenced by the diet. Nor was PNPLA2 which is a lipase that hydrolyses stored lipids in cytosolic lipid droplets. However, one of the perilipines was upregulated in fish given the low EPA-DHA diet (Fig. 4). Perilipines coat cytosolic lipid droplets and are upregulated when droplets are formed. The difference was only registered in PLN5 and not in PLN2. The upregulation in the low diet groups compared to the standard diet groups was quite subtle and this upregulation did not reflect the actual amount of lipid droplets.

The present results on intestine agrees with the preliminary conclusions from the overall feeding experiment, showing that the salmon fed lower levels like 5 % EPA+DHA of the dietary lipid was not negatively affected with respect to growth performance, quality characteristics or health parameters (Rosenlund *et al* 2014). Lipid accumulation have been observed previously in rainbow trout fed similar and higher dietary EPA and DHA levels but with severity depending type of vegetable oil replacing fish oil (Caballero *et al* 2002). Thus, current results indicate that replacing fish oil with the blend used in CAC provide a balanced

fatty acid composition which sustain lipid uptake and transport at a similar rate as fish oil fed Atlantic salmon.

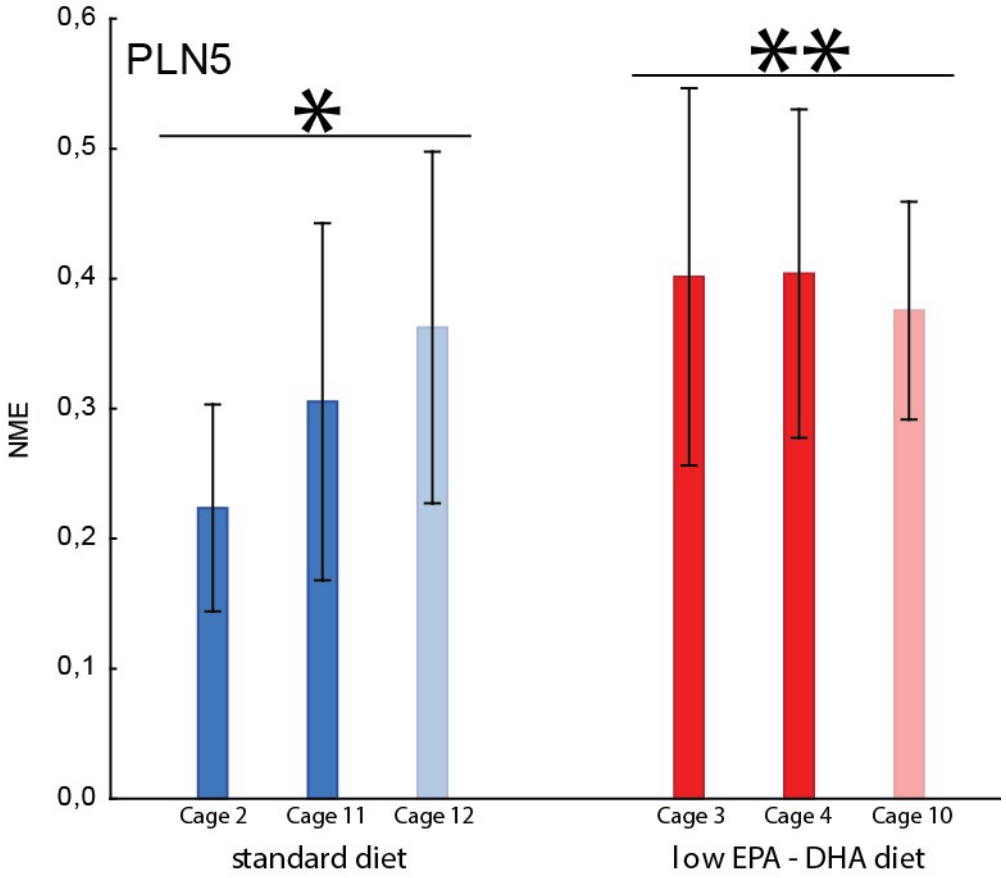

Figure 4. *Gene expression of perilipin 5 (PLN5) in intestinal tissue of fish fed standard diet and low EPA-DHA diet.*

Conclusion

The overall conclusion from the present study is that the levels of EPA and DHA in the low EPA+DHA diet (5.4% of lipid) did not affect lipid accumulation in the intestinal tissue of harvest size Atlantic salmon, when compared to fish given a modern commercial plant based diet with 7.5% EPA+DHA and a marine protein index of 10%.

Future experiments investigating low dietary EPA and DHA levels with varying alternative oil sources, should consider lipid droplet formation and expression of perilipin in mid intestine as a possible indicator of imbalanced fatty acid uptake and transport.

The present data will be considered and included in a scientific publication on intestinal health from the main project (CAC 2012G) covering a commercial scale production of Atlantic salmon under the given dietary regime.

References

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