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Insights into the regulation of LC-PUFA biosynthesis in wild and aquaculture Atlantic salmon in response to a vegetable oil diet

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Summary

The aim of this master thesis was to use transcriptomics to evaluate if lipid metabolism of wild and aquaculture salmon is different. In recent years, there has been extensive research on lipid metabolism in salmonids related to the changes in lipid content of their commercial diet over the past decade (Bell *et al.* 2003, Bell *et al.* 2004, Naylor *et al.* 2009, Sprague *et al.* 2016, Tacon and Metian 2013). Fish and other vertebrates are not able to produce poly-unsaturated fatty acids (PUFA) *de novo,* so they are required in the diet (Tocher *et al.* 2003). When Linolenic acid (LA) and alpha-Linolenic acid (ALA) are available, these essential fatty acids (EFAs) can then be converted to long-chain poly-unsaturated fatty acids (LC-PUFA), which are required for fish health, but also important for the health benefits of human salmon consumption. Because salmon is a very important source of LC-PUFA in humans, there is much interest in understanding the workings of the salmonid apparatus for endogenous LC-PUFA synthesis. Globally, half of all fish consumed comes from aquaculture and especially in Europe has the production from fisheries steadily declined while that of aquaculture grows (FAO 2016). As aquaculture is supplying an increasing amount of the total fish for human consumption, the industry is working on maintaining or improving the LC-PUFA content of fish products while also switching to more sustainable feeds (Sprague *et al.* 2016). The ability of the fish to

endogenously synthesise LC-PUFA from EFAs is an important part of this objective. One aspect to understanding and perhaps improving LC-PUFA synthesis in aquaculture salmon, is to understand the difference between the diets of aquaculture and wild salmon (Bell *et al.* 1994), and how endogenous LC-PUFA synthesis is affected by these. In this project we set out to look for differences between aquaculture and wild salmon in the activity of their endogenous LC-PUFA synthesis in response to two industrial diets with different LC-PUFA content.

We hypothesised that wild salmon are more efficient than aquaculture salmon at converting fatty acid precursors to LC-PUFAs in response to a plant-based diet which is low in LC-PUFA, most notably the particularly healthy omega-3s docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Gillard *et al.* 2018). This was based on the fact that wild salmon have a more variable supply of food and eat prey low in DHA and EPA in the river life-stage (Gillard *et al.* 2018), while aquaculture salmon are kept in environments with enormous surplus of nutrients. Hence, wild populations are experiencing natural selection favouring individuals with the ability to effectively regulate endogenous LC-PUFA synthesis upon insufficient dietary levels. Such selection pressure for maintaining a likely costly metabolic response has not been present for the past 15 or so generations of domestication in aquaculture salmon.

Genes required for the synthesis of LC-PUFA are found to be more highly expressed in salmon fed VO than those fed FO, regardless of fish strain. Further, aquaculture salmon have higher expression of a set of genes important to LC-PUFA synthesis. It appears that the response to a low LC-PUFA diet is more pronounced in aquaculture salmon than wild, contrary to our hypothesis. However, the evidence from transcriptomics is limited and there is some variation in the results over time.

Introduction

Aquaculture is a big and still growing industry in Norway and elsewhere. 1.2 million tonnes of Atlantic salmon (*Salmo salar*) are reared in Norway every year (Statistisk Sentralbyrå 2018), constituting more than half of the total reared globally (Steinset 2017). Worldwide, aquaculture accounts for most of the growth in fish production the last 20 years or so (FAO report 2016), with the aquaculture sector far outgrowing the wild sector. There are important health benefits to human consumption of salmon as it is a major source of the LC-PUFAs EPA and DHA (Calder 2014). It is therefore of great importance to the industry to have adequate amounts of EPA and DHA in the salmon flesh. Breeding programs are a significant part of the industry,

where the aim is to improve industrially favourable traits of the aquaculture population. Partially for this reason genetic studies of salmon have become a hot research topic in more recent years, helping breeders in their work and illuminating the genetic basis of important traits for aquaculture salmon.

The endogenous LC-PUFA synthesis pathway

Many, but not all, vertebrates are able to convert the essential fatty acids LA and ALA to LC-PUFA (Carmona-Antoñanzas *et al*. 2013), and salmon are particularly efficient at this compared to other freshwater fishes (Carmona-Antoñanzas *et al*. 2013, Gillard *et al.* 2018). The parent n-3 fatty acid ALA can be converted to the long-chained n-3 fatty acids EPA and further to DHA (figure 1). LC-PUFA synthesis is done through a series of desaturation and elongation steps. The efficiency of this conversion is associated with the possession and expression of the genes that code for the desaturation and elongation enzymes (Castro *et al.* 2016). The key enzymes involved in synthesis of LC-PUFA from parent fatty acids are the desaturases Fadsd5 and Fadsd6 (three isoforms a, b and c) and the elongases Elovl2 and Elovl5 (two isoforms a and b). In addition, there is Elovl4, which catalyses the further elongation step of the 24 carbon LC-PUFA into the very-long chained PUFA (VLC-PUFA).

The LC-PUFA synthesis pathway in salmonids

As mentioned above, salmon are among the most efficient LC-PUFA synthesisers among the saltwater fishes. This may be because key genes of the LC-PUFA synthesis pathway exist in more than one copy in salmon. There are three gene copies of *fadsd6* (*fadsd6a*, *fadsd6b* and *fadsd6c*), and two for *elovl5* (*elovl5a*, *elovl5b*). This may be a result of an important evolutionary event (Carmona-Antoñanzas *et al*. 2013), namely a whole genome duplication (WGD) specific to the salmonids. This salmonid-specific fourth vertebrate whole genome duplication (Ss4R) occurred ~80 million years ago and salmon today still retain duplicate genes for approximately half of their genome (Gillard *et al.* 2018, Lien *et al.* 2016). A WGD results in an increase in the genetic material available for evolution, and new characteristics can evolve as a result (Lorgen *et al.* 2015, Macqueen and Johnston 2014). A species will normally revert back to the pre-WGD state over time, but because the Ss4R is relatively recent, some of that potential "boost" in genetic ingenuity still persist in salmon. As a result, salmonids have more copies of certain genes than other teleost species. This gives salmon a genetic advantage in certain metabolic pathways, if they have double the genes for certain important enzyme

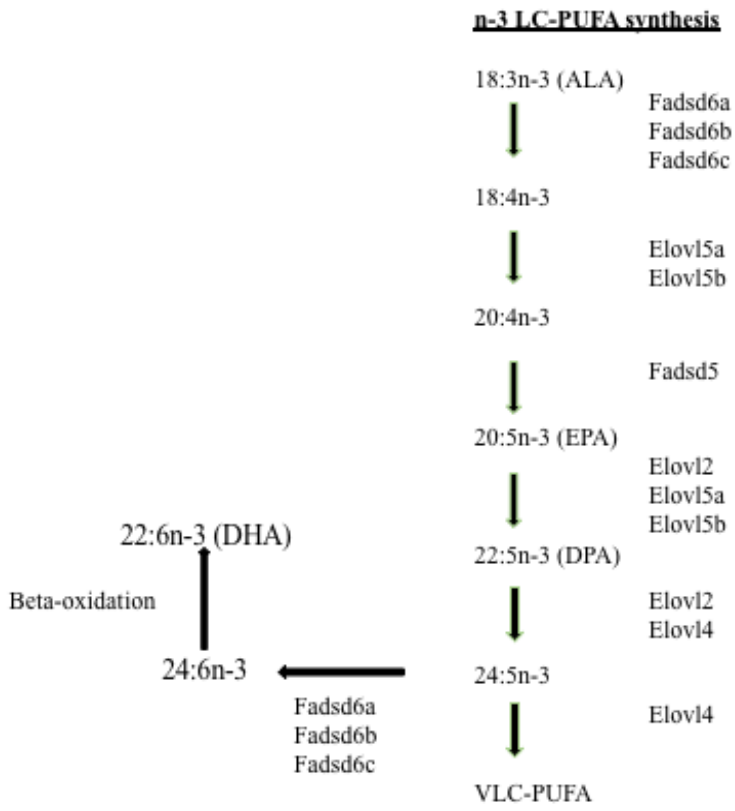

Figure 1. Metabolic pathway of n-3 (omega-3) LC-PUFA and VLC-PUFA from EFAs, showing enzymes involved for each step. Where several enzymes are listed it means one of them is required, not all.

products. While still uncertain, there is evidence to suggest that the existence of gene copies in at least *elovl5* is connected to the Ss4R WGD (Carmona-Antonanzas *et al.* 2016), and this could explain why salmon have higher LC-PUFA synthesis efficiency than other freshwater fishes.

A change towards feeds with less LC-PUFA in aquaculture

Aquaculture salmon have traditionally been fed diets where the lipid component is made of fish oil (FO). Fish oil is high in EPA and DHA and so the deposition of these fatty acids are high in the flesh of aquaculture salmon. As fish oil is an expensive and limited resource, its use as the sole lipid source in fish feed is not sustainable (Shepherd and Jackson 2013). The industry is therefore replacing a significant proportion of the fish oils with plant-based oils (Naylor *et al.* 2009). The exact FA composition of the plant oils vary, depending on the source plants they are made up of, but they generally contain high amounts of the EFAs LA and ALA, the precursors to LC-PUFA omega-6s and omega-3s. There has been extensive research into the effect of dietary vegetable oils on salmon health, growth and tissue fatty acid content (Hixson

et al. 2017, Kjær *et al.* 2016, Stubhaug *et al.* 2005, Thorstensen *et al.* 2005, Tocher *et al.* 2003). Studies show that genes related to the LC-PUFA synthesis pathway are more highly expressed when FO is replaced with VO, and conversely less expressed when fed FO (Bell *et al.* 2001, Bell *et al.* 2002, Kennedy *et al.* 2006, Leaver *et al.* 2008, and Zheng *et al.* 2005). In other words, salmon regulates the LC-PUFA synthesis pathway in response to the LC-PUFA content of the diet. This means that their endogenous LC-PUFA synthesis can to a certain extent compensate for lower LC-PUFA dietary content. Endogenous LC-PUFA synthesis is sufficient to make up for the substitution of some FO with VO (Tocher *et al.* 2001), which is now common practice in aquaculture. The goal is to eventually replace all FO with terrestrial alternatives.

Potential differences in LC-PUFA synthesis efficiency of aquaculture and wild salmon

Although aquaculture salmon are able to adjust their endogenous production of omega-3 in response to diets with a deficit, these salmon are bred in a closed off environment, and an adequate food supply is always available to them. Up until recently, that diet has contained high levels of FO, making it a "perfect feed" for the fish. On the other hand, the wild salmon diet consists of invertebrates (both crustaceans and insects), low in the LC-PUFAs EPA, DHA and AA (arachidonic acid). (Gillard *et al.* 2018). Therefore, there could be selection in the wild populations for efficient endogenous LC-PUFA synthesis, and relatively efficient LC-PUFA synthesis possibly evolved as an adaptation (Leaver *et al.* 2008). Because the aquaculture salmon have access to an unlimited amount of a high LC-PUFA diet, this selection pressure is presumably reduced in the aquaculture populations. Therefore, an interesting question emerged: do we find genetic potential for an improved omega-3 synthesis in wild populations?

In this project we want to assess if wild fish are more responsive in their metabolic response to low levels of LC-PUFA in their feed compared to aquaculture fish. In order to do this, we raised several groups of wild and aquaculture salmon on diets that differed in LC-PUFA, specifically EPA and DHA. We then measured gene the expression response in liver to the different diets as a proxy for the functional differences between wild and domesticated lipid metabolism.

Materials and method

Background on experimental data

Atlantic salmon were reared, and samples prepared by Yang Yin of NTNU. The aquaculture salmon are from an AquaGen strain from Kyrksætersrøra which has been selected for good growth and performance for 11 generations. The wild fish were acquired from hatchery Haukvik Kraft AS. All fish were hatched at the Ervig hatchery in Frøya, Norway and then reared from first feeding on diets containing either V) or fish oil FO. The ingredient and fatty acid composition of the two diets are shown in table 1. The lipid component of the FO diet consisted of various fish oils. The lipid component of the VO diet consisted of rapeseed oil, linseed oil and palm oil. The FO diet contained more EPA and DHA than VO, while the VO diet contained more LA and ALA than FO. Feeds were produced by the Sparos Company, Portugal.

Fish we assigned randomly to two replicate tanks of each combination of fish type and diet, 8 tanks in total. Wild and aquaculture fish had different initial mean weights, 0.18 and 0.22 grams respectively. The fish were reared for 94 days after first feeding, and liver from two fish per replicate tank was sampled (4 fish per treatment) on days 0 (before first feeding), 48, 65, 77, and 94. In order to compare fish of similar developmental stage, fish were initially sampled when they reached approximately 1 gram, so day 48 only contains aquaculture fish and day 77 only contains wild fish fed VO diets (Figure 2). RNA extraction and sequencing was performed by Yang Jin. Mapping and counting of the subsequent sequencing data was performed by Gareth Gillard.

RNA extraction, library prep and sequencing

The RNA extraction and library preparation was completed in Centre for Integrative Genetics (CIGENE), Ås, Norway. Total RNA extraction was done using RNeasy Plus Universal Kits (QIAGEN, Hilden, Germany). Library preparation was performed with the TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA). RNA concentration and quality was checked with the Nanodrop 8000 (Thermo Scientific, Wilmington, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RIN values for all samples were above 8, indicating good quality for sequencing. Samples were sequenced by 100bp single-end high-throughput mRNA sequencing (RNA-seq) on Illumina Hiseq 2500 (Illumina, San Diego, CA, USA) in the Norwegian Sequencing Centre (Oslo, Norway).

Read sequences were quality trimmed, removing any Illumina TruSeq adapter sequence and low-quality bases (Phred score < 20) from read ends and length filtered (minimum length 40 bases) using cutadapt (v1.8.1), before being aligned to the salmon genome (ICSASG_v2) using STAR (v2.5.2a). Raw gene counts per sample were generated from read alignments using HTSeq-count (v0.6.1p1) and the NCBI salmon genome annotation (available for download at http://salmobase.org/Downloads/Salmo_salar-annotation.gff3.gz). The uniquely mapped reads, aligned to exon regions, were counted for each gene in the annotation.

Summary of experiment samples

Preliminary results showed that age is a better proxy for similar development than weight. Equal age, regardless of weight differences due to different growth rates, is preferable in regard to background variation between the two fish types. When comparing strains at equal weights we saw more difference in gene expression than when we compared the strains at equal age. For this reason, comparisons between the two strains at the same age is considered the better option to avoid the most development–related differences.

Also, one treatment group had to be removed. Preliminary analysis found that the samples of aquaculture fish fed VO from day 65 could not be used. For this group, a few very highly expressed genes took up much of the available reads in the library for those samples during RNAseq. Therefore, the read counts were very much skewed towards those few genes.

Data analysis

Data analysis was performed in R Studio (version 1.1.442) (R Core Team 2017). Differential expression analysis was done using the package DESeq2 (Love *et al.* 2014), with all analyses done with a false discovery rate of 0.05. KEGG pathways enrichment analysis was done using the package edgeR (Robinson *et al.* 2010). Other packages used can be viewed in the appendix code.

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Table 1. Percentage content of ingredients (a) and nutritional content (b) of the FO and VO diets. In b) the percentage of nutritional components is per feed basis.

a) Ingredient	**FO**	**VO**		*b) Nutrition*	**FO**	**VO**
	%	**%**			**%**	**%**
Fishmeal 70 LT FF Skagen	10	10		***Crude protein***	65.00	65.00
Fish protein concentrate (CPSP 90)	15	15		***Crude fat***	14.11	14.10
Squid meal	25	25		***Fiber***	0.29	0.29
Shrimp hydrolisate	5	5		***Starch***	4.33	4.33
Fish gelatin	2	2		***Ash***	6.34	6.34
Pea protein concentrate	7.5	7.5		***Gross Energy***	20.43	20.43
Wheat Gluten	12.5	12.5		***LA***	0.41	1.48
Potato starch gelatinised	2.5	2.5		***ALA***	0.20	1.53
Fish oil	7.2	0		***AA***	1.45	5.00
Tuna oil	2.3	0		***EPA***	1.12	0.25
Rapeseed oil	0	2.9		***DHA***	1.26	0.48
Linseed oil	0	2.4		***EPA+DHA***	2.40	0.73
Palm oil	0	4.2		***DHA/EPA***	1.10	1.90
Vit & Min Premix	1.5	1.5		***PUFA***	3.35	3.50
Lutavit C35	0.03	0.03				
Lutavit E50	0.12	0.12				
Brewer's yeast	5	5				
Betaine HCl	1	1				
MAP (Monoammonium phosphate)	3	3				

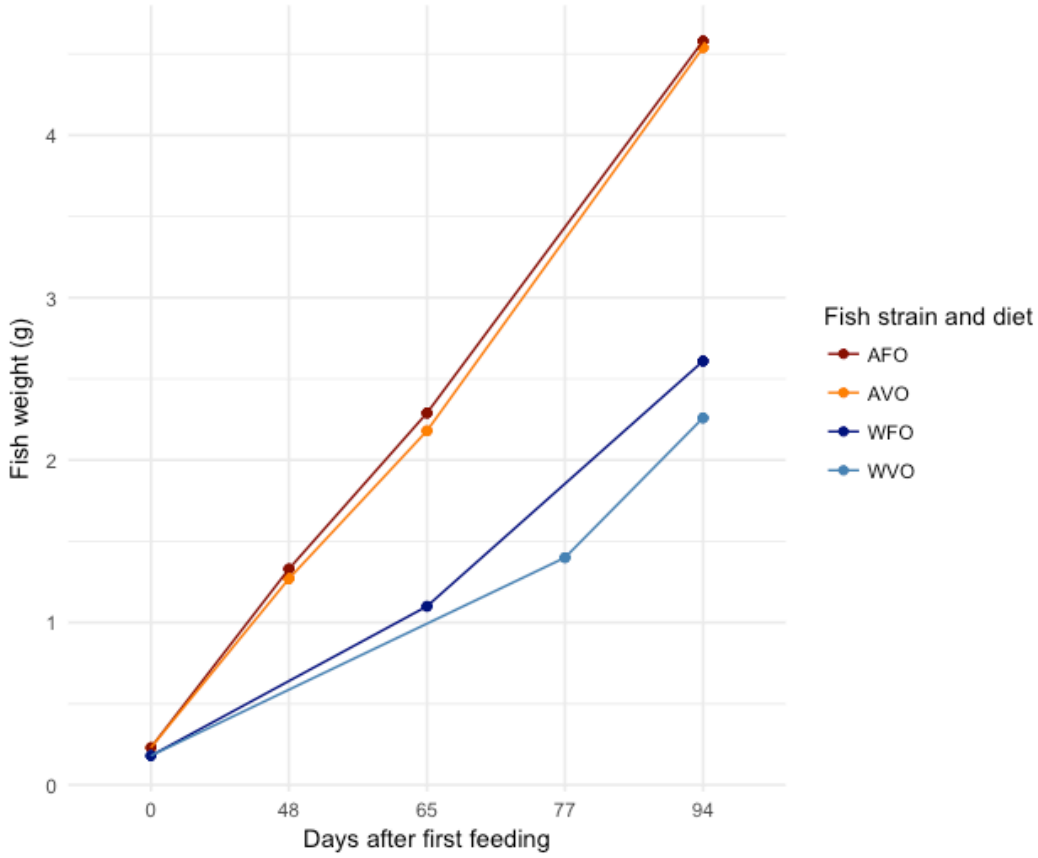

Figure 2. A time and weight summary overview of the samples procured. Each dot represents a point at which a sample was made for that treatment group. Treatment group combination of fish strain and diet is denoted in the legend.

Also, one treatment group had to be removed. Preliminary analysis found that the samples of aquaculture fish fed VO from day 65 could not be used. For this group, a few very highly expressed genes took up much of the available reads in the library for those samples during RNAseq. Therefore, the read counts were very much skewed towards those few genes.

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Data analysis was performed in R Studio (version 1.1.442) (R Core Team 2017). Differential expression analysis was done using the package DESeq2 (Love *et al.* 2014), with all analyses done with a false discovery rate of 0.05. KEGG pathways enrichment analysis was done using the package edgeR (Robinson *et al.* 2010). Other packages used can be viewed in the appendix code.

Comparisons of gene expression are divided into two categories; those comparing the gene expression following different diet treatments for each fish type separately, and those comparing the two fish types for one diet at a time. The first type (contrast class I) illuminates the ways each fish strain responds to the two diets. These contrasts test for differential expression of genes in fish fed the VO diet relative to those fed the FO diet in aquaculture and wild fish separately. The second type of differential gene expression analyses (contrast class II) compares aquaculture and wild fish on the same diets. These contrasts directly test differentially expressed genes between the two fish strains. Comparing results from contrast class I analyses helped evaluate if VO/FO feeds induced gene expression shifts in similar genes and pathways in wild and aquaculture fish, while contrast class II analyses were designed to reveal *expression level* differences between the two fish types.

Results

To get an overview of the similarity of our experimental groups we initially used principle components analysis on the gene expression data (prcomp package in R) for an unsupervised clustering of samples. Gene read counts were transformed by the regularized logarithm transformation (rlog), to account for genes with very high and low counts (Love *et al.* 2014). The principal component analysis (PCA) was performed on the top 5000 variable genes. The first two principal components (PCs) account for 30% of the variance and produced the clearest separation of experimental groups.

Table 2. Summary of variance explained by the first seven PCs in the rlog-transformed read counts.

Importance of components	*PC 1*	*PC 2*	*PC 3*	*PC 4*	*PC 5*	*PC 6*	*PC 7*
Standard deviation	23.371	16.590	15.034	12.002	10.590	9.692	9.094
Proportion of variance	0.201	0.101	0.084	0.053	0.041	0.035	0.031
Cumulative proportion	0.201	0.302	0.386	0.439	0.48	0.515	0.546

The PCA is shown in figure 3. The most distinct separation in the data set is between the two types of salmon, aquaculture and wild. PC1 is separating the fish strains, while PC2 separates the different time points. The four replicates of each treatment combination (fish strain and diet) group satisfactorily. There appears to be some time-dependent structure in the data, but not for diet.

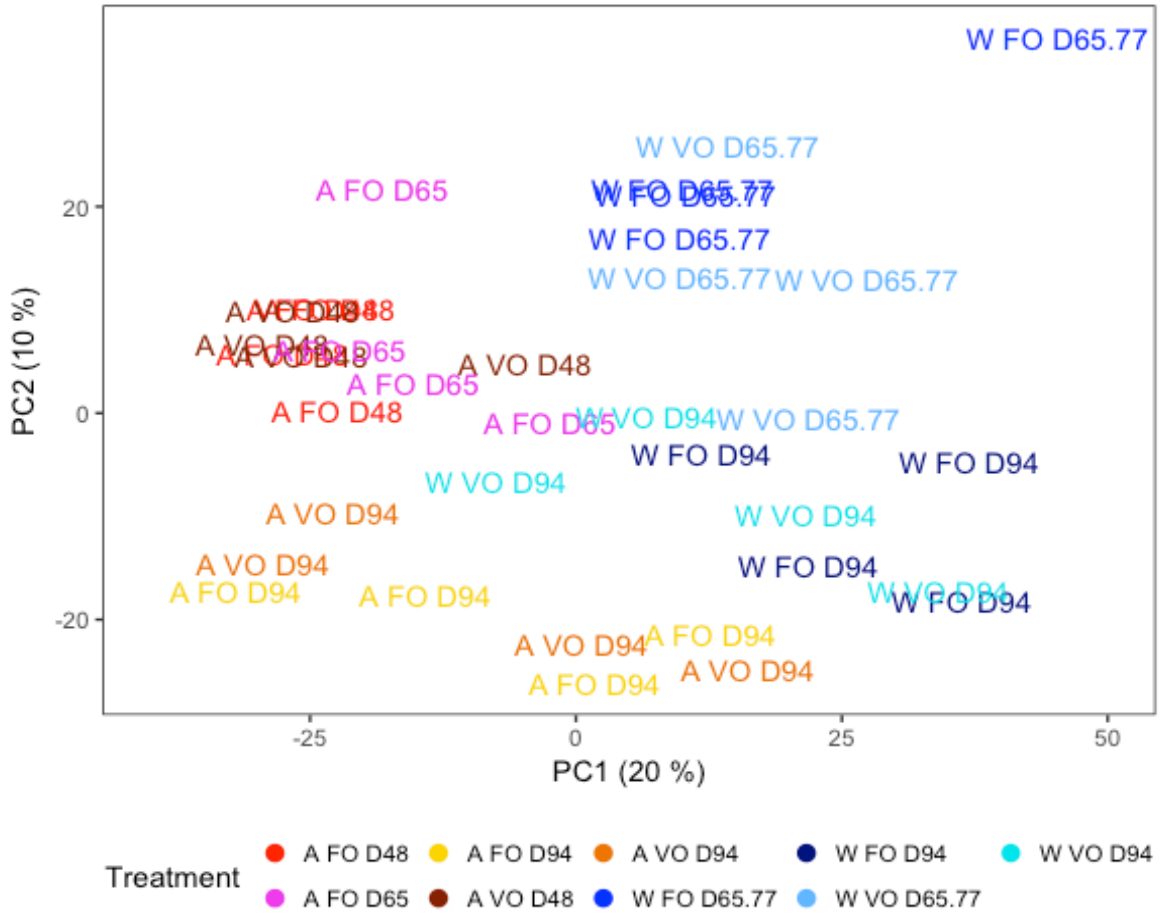

Figure 3. PCA of the treatment groups, showing the first two PCs.

Contrast class I. Diet response in aquaculture and wild salmon

In the introduction we mentioned the three isoforms of Fadsd6: a, b and c. In our data we only observe activity of Fadsd6a. There are also two isoforms of Elovl5: a and b. In our data we only observe activity of Elovl5b. Further, there is the transcription factor SREBP1which will be presented in the results. There are two different isoforms of SREBP1 in mammals, but according to Dong *et al.* (2017) only one has been identified in fish, and so we report this transcription factor as simply SREBP1.

Differential gene expression

Differential expression analysis was performed using DESeq2. Genes with a false discovery rate (FDR) adjusted *p* value (*q*) <0.05 were considered to be differentially expressed genes (DEGs) between the two test conditions. The tables below summarise the number of DEGs between two contrasted groups. They also show the percentage of DEGs that are known lipid genes.

Table 3. DEGs between the two diet treatments for each fish strain separately. Differential expression contrasts were performed on VO relative to FO, i.e. "up-regulation" refers to genes that are up-regulated in the VO group relative to FO group for that contrast.

Strain	***Day***	***VO v FO***	***Number of DEGs***	***% lipid genes***
Aqua	**48**	Up-regulated	19	5.3
		Down-regulated	7	28.6
		Total	26	
	94	Up-regulated	57	33.3
		Down-regulated	19	15.8
		Total	76	
Wild	**65/77**	Up-regulated	441	5.7
		Down-regulated	66	0
		Total	507	
	94	Up-regulated	1	0
		Down-regulated	0	n/a
		Total	1	

It is only at day 94 that *fadsd5* and *fadsd6a* are up-regulated in aquaculture fish fed VO relative to those fed FO, with fold changes of 2.03 and 2.13 respectively. Elongases are not found to be significantly up-regulated for this same contrast. In the wild fish, *fadsd5* and *fadsd6a* are up-regulated in the VO relative to the FO fish at day 65/77, with fold changes of 2.13 and 2.71 respectively.

Visualisation of fold changes from contrasting diet treatments in aquaculture and wild

Figure 4 highlights the differences between aquaculture and wild fish in regulation of LC-PUFA synthesis activity in relation to diet. All genes in the data set are represented as a single dot in the figure, including all not considered significantly up-regulated and subsequently not represented in table 3. This is in order to visualise the pattern of diet response in overall gene expression for the two fish strains. The first thing we can say about this result is that among all genes in the data set, including lipid genes not key to LC-PUFA synthesis, the majority are similarly affected by diet in the aquaculture and wild fish.

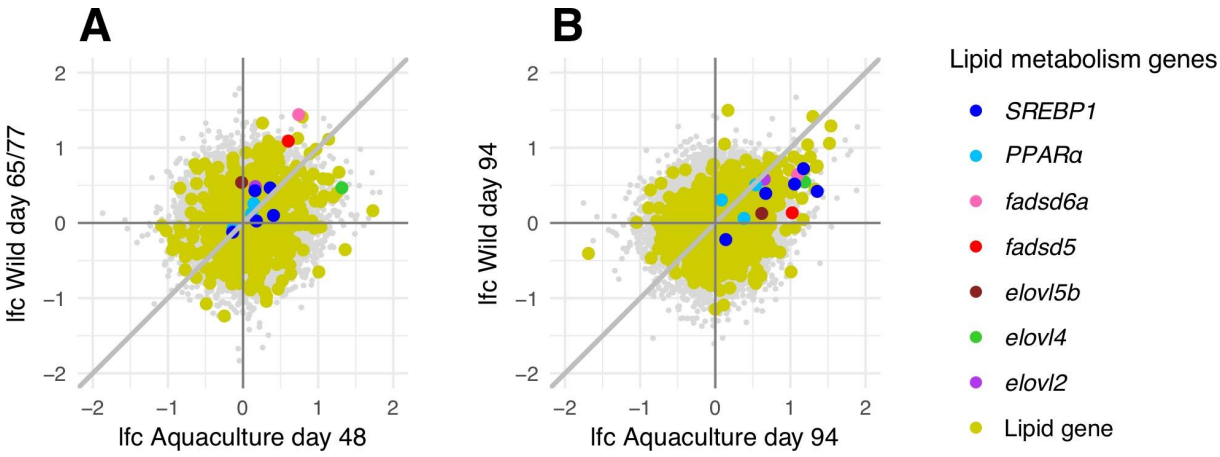

Figure 4. Correlation plots of the log2 fold changes (lfc) of the diet response DE analyses in aquaculture and wild salmon. lfc-values of aquaculture and wild fish are from separate DE analyses contrasting the VO relative to FO diet.

In figure 4, genes positioned directly on the vertical line are not DE between aquaculture fish fed VO relative to the aquaculture fish fed FO. Genes positioned to the right of the vertical line are up-regulated in aquaculture fish fed VO relative to those fed FO, and those to the left are down-regulated. Similarly, genes positioned directly on the horizontal line are not DE between wild fish fed VO relative to wild fish fed FO. Genes positioned above the horizontal line are

up-regulated in wild fish fed VO relative to those fed FO, and those below are down-regulated. From this visualisation, we can see that several key LC-PUFA synthesis genes are in the top right corner of both plot A and B. Genes in this area are up-regulated in both aquaculture and wild fish for the VO v. FO treatment. In other words, we see that several key LC-PUFA genes are up-regulated when salmon of both strains are fed VO rather than FO.

Further, a few other patterns emerge. In the upper right square of figure 4A and B, genes positioned to the left of the diagonal line are more highly up-regulated in wild than aquaculture fish. Those positioned to the right of the diagonal line are more highly up-regulated in aquaculture than wild fish. In figure 4A, we see that the two desaturases are more highly up-regulated in wild than aquaculture fish. Elovl4 is more highly up-regulated in the aquaculture fish. The other key LC-PUFA synthesis genes are similarly affected by diet in aquaculture and wild fish**.** In plot A we see that most key LC-PUFA synthesis genes are more highly up-regulated in aquaculture than wild fish. This is true for all genes, except one copy each of the transcription factors PPARα and SREBP1**.**

Relative expression of diet response genes

Figure 5 shows a heatmap of the relative expression of a set of genes found to be differently expressed between fish of the same strain fed different diets. The list includes those DEGs that are up-regulated by a fold change of 2 or above, or down-regulated by a fold change of 0.5 or below. In other words, these are the genes found to be differently expressed as part of the response to diet in the aquaculture and wild fish. The genes lists were combined, and redundancy removed. Key LC-PUFA synthesis genes that were not represented in the list of diet response genes were added manually, because while they may not have been significantly DE it is still interesting to look at the relative expression of those genes.

Aquaculture fish fed VO cluster away from the three other groups, and there is higher expression of a large majority of these "diet response" genes in that group, including the key LC-PUFA synthesis genes. Further, the similarity in gene expression of these select "diet response" genes is higher within fish strains than for fish fed the same diet, as the two groups of wild fish cluster together and aquaculture FO fish is more similar the aquaculture VO fish than the wild cluster**.**

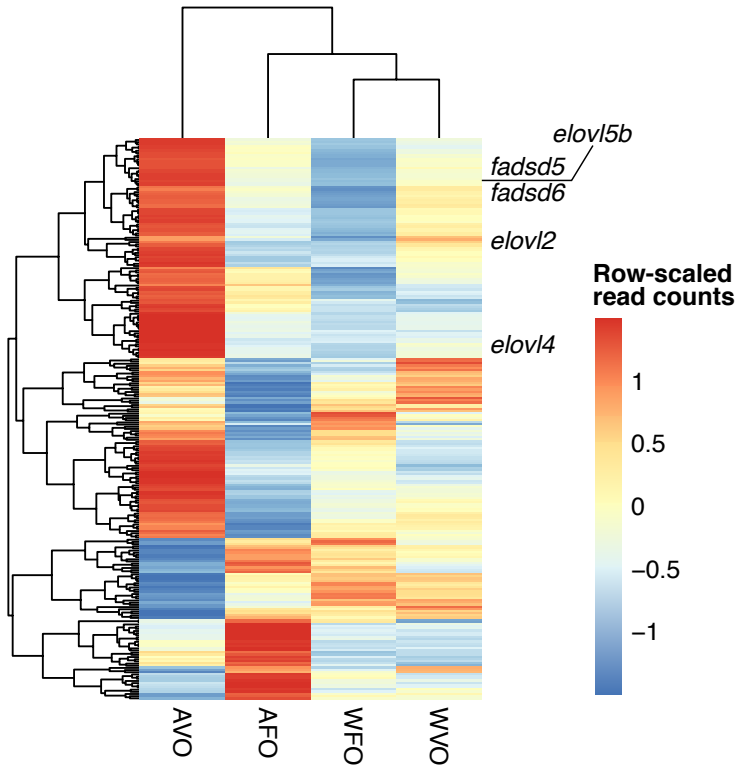

Figure 5. Heatmap of row-scaled rlog-transformed counts of genes found to be differentially expressed between fish fed VO and FO. Red colouring indicates relatively higher expression, blue indicates relatively lower expression and yellow is neutral.

Expression of key LC-PUFA synthesis genes and transcription factors

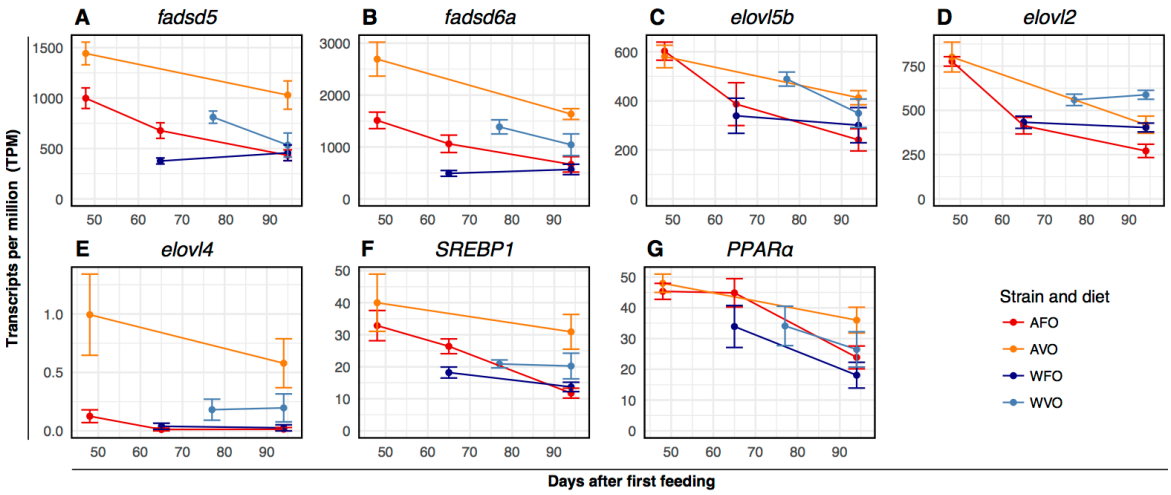

Figure 6. Relative expression over time of the genes of the LC-PUFA synthesis pathway, as well as the transcription factors involved in LC-PUFA synthesis. Expression levels are presented in transcripts per million (TPM) and are shown for each combination of fish type and diet over time.

The expression level of all five LC-PUFA synthesis genes and the transcription factors PPARα and SREBP1 is higher in fish fed VO than FO regardless of fish type. Further, wild fish tend to have less of a clear difference between the VO and FO treatments at day 94 than day 65 and 77. This observation is in line with the fact that there are few DEGs between wild fish of the two diet treatments at day 94 (table 3), and we saw in figure 4B that LC-PUFA synthesis genes are more highly up-regulated in aquaculture than wild fish at day 94. *Elovl4* is the LC-PUFA synthesis gene we have discussed the least, as its activity is specific to the "dead-end" production of VLC-PUFA from LC-PUFA (see figure 1). Here we see that expression levels of *elovl4* are generally low, but aquaculture fish fed VO have higher expression than the other groups. The two most important transcription factors involved in regulation of LC-PUFA synthesis are SREBP1 and PPARα. In figure 6F we see that expression of SREBP1 is highest in aquaculture fish fed VO. PPARα is also more highly expressed in aquaculture than wild fish, and within the aquaculture fish is somewhat more expressed in the VO than FO group.

KEGG pathway enrichment analysis

KEGG pathway enrichment analysis takes a list of up- or down-regulated genes and looks for these genes in metabolic pathways in the KEGG database (Kyoto Encyclopedia of Genes and Genomes). As we saw in table 3, there are fewer down-regulated genes in the diet response of both aquaculture and wild fish. This is reflected in the KEGG analyses, with down-regulated pathways being either completely absent or negligible. Therefore, only *up-regulation* in response to VO relative to FO is shown in figure 7 overleaf.

In the aquaculture fish we see that several pathways involved in LC-PUFA synthesis are more active at both day 48 and day 94. The pathways that are more active at both times are; 'PPAR signaling pathway', 'Fatty acid biosynthesis', 'Fatty acid degradation', 'alpha-Linolenic acid metabolism' and 'Peroxisome'. The LC-PUFA synthesis related pathways that are up regulated at day 48 only are; 'Arachidonic acid metabolism' and 'Linoleic acid metabolism'. The pathways that are up regulated at day 94 only are; "Biosynthesis of unsaturated fatty acids', 'Glycerolipid metabolism' and 'Glycerophospholipid metabolism'.

In the wild fish it is only at day 94 that up-regulated genes in response to VO relative to FO are significantly represented in KEGG pathways. Here too, though, we see that pathways related to LC-PUFA synthesis are more active. These are; 'PPAR signaling pathway', 'Arachidonic acid metabolism', 'Biosynthesis of unsaturated fatty acids' and 'alpha-Linolenic acid metabolism'.

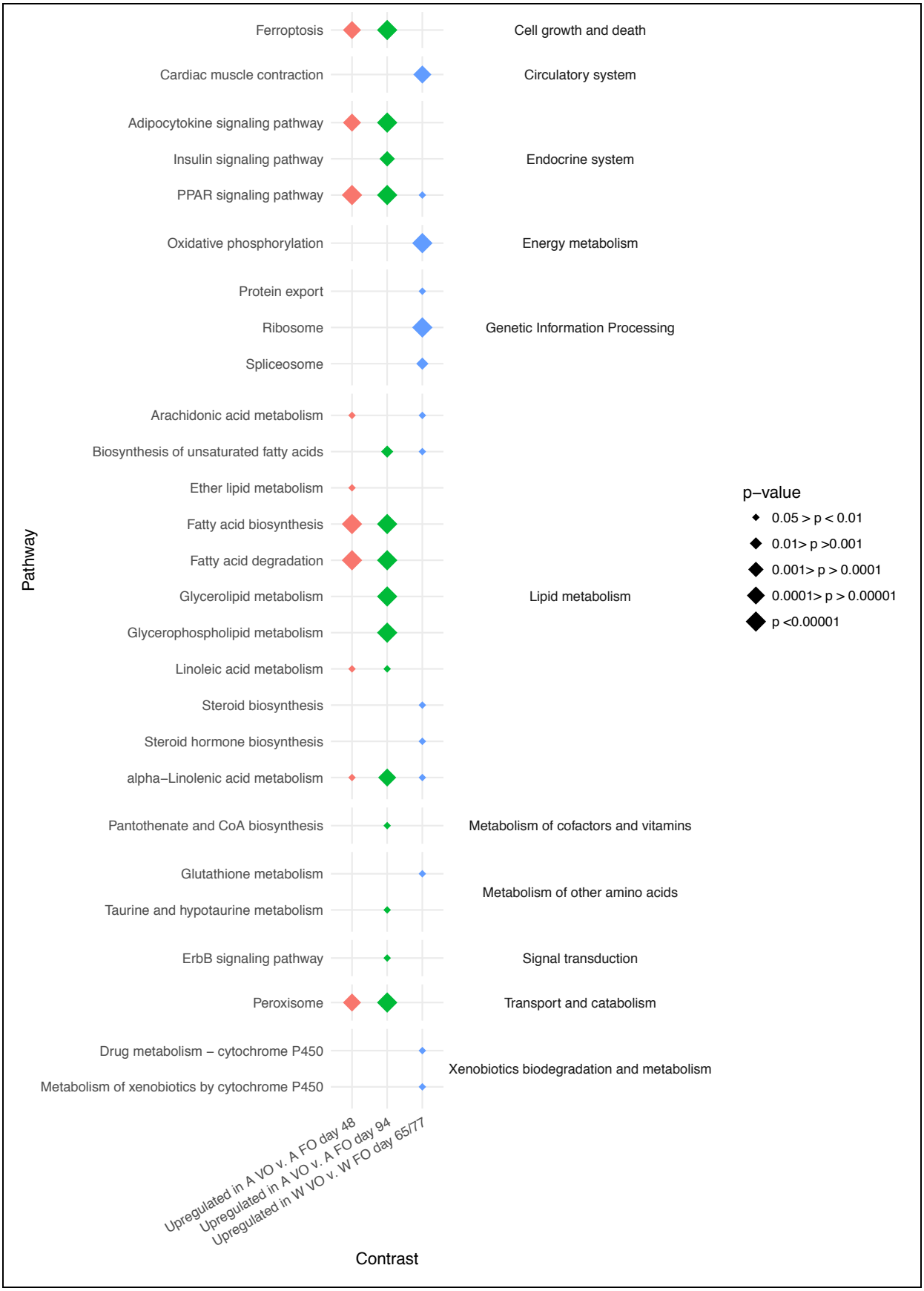

Figure 7. KEGG pathway enrichment results in the aquaculture and wild fish. The results for each contrast are independent of each other, based on lists of DEGs from the contrast of the VO v. FO diet within the aquaculture and wild strains separately.

Contrast class II. Difference between aquaculture and wild salmon in response to diets

Differential gene expression

For differential expression analysis between aquaculture and wild strains all data from the different time points were merged, the results of which are shown in table 4 below. All up-regulated genes reported on are differentially expressed with a fold change of 2 or above, and all down-regulated genes with a fold change of 0.5 or below. We see that there are more DEGs between the two strains for the FO diet than the VO diet, 380 and 242 respectively.

Table 4. DEGs between the aquaculture and wild salmon for each diet separately. Differential expression contrasts were performed on aquaculture relative to wild, e.g. "up-regulation" refers to genes that are up-regulated in aquaculture fish relative to wild fish for that diet contrast.

Diet	***Contrasting aquaculture v wild***	***Number of DEGs***	***% lipid genes***
VO	**Up regulated**	108	19.4
	Down regulated	134	10.4
	Total	242	
	Contrasting aquaculture v wild	***Number of DEGs***	***% lipid genes***
FO	**Up regulated**	163	10.4
	Down regulated	217	7.4
	Total	380	

KEGG pathway enrichment analysis

As with differential expression analysis, data was merged for all time points in the KEGG pathway enrichment analysis between the fish strains. We see that some key pathways related to LC-PUFA synthesis are more active in aquaculture than wild salmon. These are; 'PPAR signaling pathway', 'Biosynthesis of unsaturated fatty acids', 'Fatty acid biosynthesis', 'Fatty acid degradation', 'Fatty acid elongation' and 'Peroxisome'. Apart from 'Fatty acid degradation' and 'Peroxisome', which is only more active in the aquaculture fish for the VO treatment, these pathways are up-regulated in aquaculture fish relative to wild fish regardless of diet.

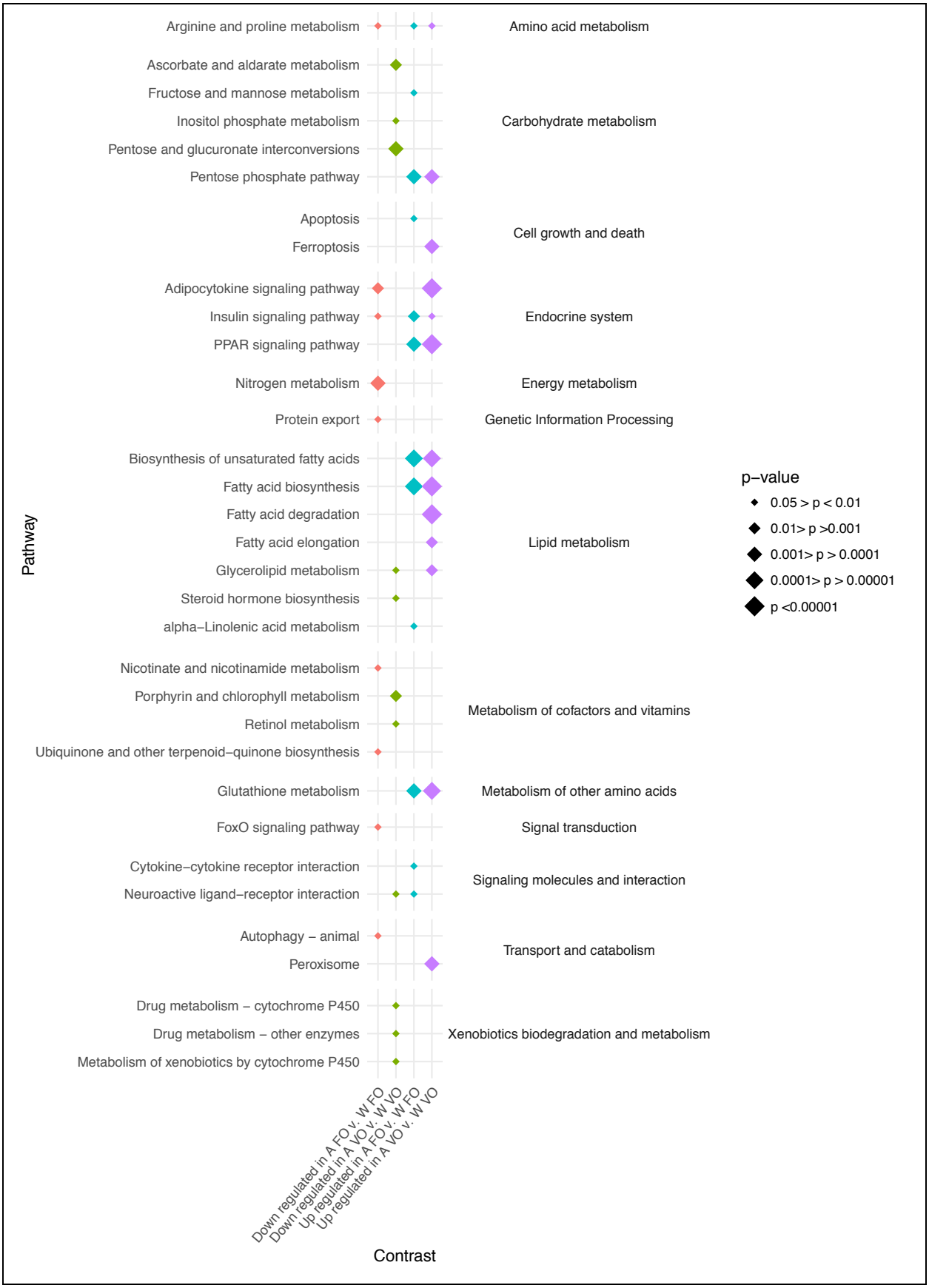

Figure 8. KEGG pathway enrichment results comparing the response of aquaculture and wild salmon to the two diets separately. The results for the VO and FO contrasts between the strains are independent of each other.

Discussion

Vegetable oil diet stimulates LC-PUFA synthesis in salmon

We find that in agreement with the literature, both aquaculture and wild salmon increase LC-PUFA biosynthesis related genes and pathways in response to a VO diet. Both aquaculture and wild fish have overall higher expression of the key LC-PUFA synthesis genes *fadsd5, fadsd6a*, *elovl2* and *elovl5b* for the VO diet relative to the FO diet. The low content of LC-PUFA in VO means the nutritional requirements are not met, and endogenous production of EPA and DHA from precursor fatty acids is induced. This activation of LC-PUFA synthesis is likely the result of either the high availability of the precursor fatty acids, the low concentration of EPA and DHA or some combination of the two (Glencross *et al.* 2015, Hixson *et al.* 2017, Jordal *et al.* 2005 and Tocher *et al.* 2003). Increased activity of LC-PUFA synthesis in salmon fed diets of low LC-PUFA content is well supported (Bell *et al*. 2001, Bell *et al*. 2002, Geay *et al.* 2015, Kennedy *et al*. 2006, Leaver *et al*. 2008, and Zheng *et al*. 2005), with Tocher *et al.* (2003) finding a clear correlation between the amount of VO in the diet and the activity of LC-PUFA synthesis. While the result is obvious in some studies, others report that the observable effect is minimal or not statistically significant (Castro *et al.* 2015, Teoh and Ng 2016).

LC-PUFA may be more efficient in aquaculture than wild salmon

While both strains appear to have up-regulation of LC-PUFA synthesis genes when FO is replaced by VO, the effect is higher in aquaculture fish. Increased activity of LC-PUFA synthesis genes and related pathway may indicate more efficient LC-PUFA biosynthesis in these fish than in the wild ones. However, this needs to be confirmed by other studies and would require measurement of fatty acid compositions of the fish. Assuming that higher expression of related genes indicates a more efficient synthesis pathway, we see that the synthesis efficiency is higher in the aquaculture fish. In figure 6 we see that aquaculture fish have higher expression levels of genes for desaturation enzyme than wild fish. The same is seen for the transcription factors SREBP1 and PPARα. For the elongation enzymes the expression is similar. Figure 4B gives further support to this observation, showing that compared to the wild fish, aquaculture fish have higher up-regulation of key LC-PUFA genes in response to VO. Further, KEGG pathways related to LC-PUFA synthesis are up-regulated under the VO treatment for both aquaculture and wild fish (figure 7) but the effect is much clearer in the aquaculture fish.

Further, KEGG pathway enrichment analysis between the aquaculture and wild fish also shows that compared to the wild fish, aquaculture fish have more active pathways related to LC-PUFA synthesis (figure 8). Interestingly, while this effect is strongest in the comparison of their response to VO, it is also seen for FO. In other words, we see in our results that activity of KEGG pathways related to LC-PUFA synthesis are higher in the aquaculture fish than wild fish for the FO diet too, possibly indicating a higher base level of activity in the synthesis pathway in aquaculture fish.

However, aquaculture fish do not have higher overall levels of the LC-PUFA synthesis genes regardless of diet treatment (see figure 6). In general, wild fish fed VO have higher expression of these genes than aquaculture fish fed FO. So, while KEGG pathways related to LC-PUFA synthesis are more active in aquaculture than wild fish for both diets, this is likely a result of merging the data for all time points. In figure 6 we see that for the aquaculture fish, the expression of LC-PUFA genes is highest at day 48. We do not know what the expression was for wild fish at day 48 as we do not have those samples, and they could very well have been lower than in the aquaculture fish. While we cannot know for sure, we may have been overstating the difference between the strains by merging the data across time points when we compared them directly.

Responsiveness to diet in aquaculture and wild salmon

Expression of LC-PUFA synthesis key genes is higher for the VO diet in both aquaculture and wild fish (figure 6). However, the difference between expression levels in VO and FO are bigger in the aquaculture fish. This greater responsiveness to diet is also reflected in the KEGG pathway analysis (figure 7). While both aquaculture and wild fish have higher activity of LC-PUFA synthesis related pathways, the effect is bigger in the aquaculture fish. Further, up-regulation of these KEGG pathways is seen in the aquaculture fish for both day 48 and day 94, while in the wild fish we see it only for day 65/77. The difference between expression levels of key LC-PUFA synthesis genes is also noticeably small in the wild fish at day 94 (figure 6), and there is only one DEG between the wild fish fed VO and FO at that time. From this, it appears that aquaculture fish have a stronger response to FO being replaced with VO than the wild fish.

In consideration of the strength of diet response, it is worth noting that in the wild fish the FO treatment shows very little change in expression levels of the four most key genes over time (figure 6 A-D). This is in contrast with the aquaculture fish, where the genes are initially at higher expression which then drops over time. However, as we do not have measurements for the wild fish at day 48, the significance of this observation is unknown.

The changes in expression over time of these key genes in the aquaculture fish points to one reason why they may have a more efficient apparatus for LC-PUFA synthesis than wild fish. These genes are expressed more highly early on in the aquaculture fish for both diets and then level off over time. In other words, they have a higher baseline expression level of these key genes.

While it seems like aquaculture fish have a stronger reaction to diet, certain things point in another direction. In terms of DEGs between fish of the same strain fed different diets (table 3), each fish strain has one time point where the key genes *fadsd5* and *fadsd6a* are up-regulated. This is day 94 for the aquaculture fish and day 65/77 for the wild fish. The up-regulation is slightly stronger in the wild fish; for *fadsd5*, wild fish fed VO have a fold change of 2.13 while for the aquaculture fish fed VO it is 2.03. For *fadsd6a*, wild fish fed VO have a fold change of 2.71 while for the aquaculture fish fed VO it is 2.13. However, the difference between wild fish fed VO and FO at day 65/77 is likely high partly because of the age difference between them, and so this difference in *fadsd5* and *fadsd6a* expression might be a result of that.

The responsiveness of wild fish to the two diets is more uncertain than that of the aquaculture fish because of more discrepancies in the results for the wild fish. The number of DEGs in wild fish fed the two different diets in much higher at day 65/77 than day 94 (table 3). In fact, there is only a single gene found to be up-regulated in the wild fish for VO at day 94. This somewhat surprising result adds some uncertainty to our ability to draw conclusions.

These uncertainties aside, it does appear that the aquaculture fish are better able to compensate for a diet low in EPA and DHA, judging by the fact that diet affects the growth rate of wild fish more than aquaculture fish (figure 2). In fact, the growth rate of aquaculture fish fed VO is very near that of aquaculture fish fed FO. This gives further support to the observation that the aquaculture fish are better able than wild fish to up-regulate the activity of the LC-PUFA synthesis pathway in response to VO.

Conclusion

VO induced LC-PUFA synthesis in salmon, both aquaculture and wild

Substitution of FO with VO induces synthesis of LC-PUFA from shorter-chain fatty acid precursors LA and ALA in both wild and farmed salmon. LC-PUFA, especially EPA and DHA, are important for fish health and both strains respond to the low LC-PUFA VO diet by up-regulating endogenous LC-PUFA synthesis to meet the requirements.

Aquaculture salmon has higher up-regulation of LC-PUFA synthesis than wild salmon in response to VO

While VO induced up-regulation of LC-PUFA synthesis in both salmon strains, the activity appears to be higher in the aquaculture fish. This is seen from several lines of evidence; higher expression levels of LC-PUFA synthesis related genes, higher relative expression of diet response genes and more active KEGG pathways related to LC-PUFA synthesis. This last part is somewhat uncertain, because it is based on direct comparison between aquaculture and wild fish. For these class II contrasts data from different time points were merged, potentially overstating the differences between the fish strains.

The physiological response to diet type is bigger in the wild fish

The growth rate of wild fish is affected by the diet, with lower growth rates when fed VO. VO is sub-par to FO in regard to fish health. As we have seen, it appears that the ability of the wild salmon to convert LA and ALA to LC-PUFA is less than that of the aquaculture salmon. It therefore makes sense that the growth rate of the farmed fish is not affected in the same way as for the wild fish.

Concluding remarks

There are limitations to have far we can go in interpreting this gene expression analysis as reflective of the efficiency of the fishes endogenous LC-PUFA synthesis. While we conclude that aquaculture salmon are more efficient at up-regulating the activity of the LC-PUFA synthesis pathway, more studies would be needed to confirm this. It would be especially important to measure the actual fatty acid composition of the fish as they grow to adult sizes, having been reared on diets with various VO and FO content.

Further, there are a few confounding factors in this study to take note of. For one, the loss of samples from aquaculture fish fed VO from day 65 meant we only had one time point where we had samples of both diets for both fish strains – day 94. In order to have more data to work with, we decided to also include the day 48 samples of aquaculture fish. As we did not have samples of wild fish from day 48, this may have overstated differences between the strains.

Lastly, we should consider the possibility that the wild fish may have been under stress from being in the experimental environment, as it is more foreign to them than it is for the aquaculture fish. Wild salmon are not adapted to a life in such a restricted environment as this, nor industrially prepared diets. While it is not necessarily so that the regulation of LC-PUFA synthesis is affected by any potential stress response, it should be kept in mind as a possible confounding factor in direct comparisons of aquaculture and wild salmon.

Statement of ethics

All fish rearing and sampling was done in accordance with Norwegian laws on animal welfare.

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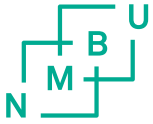

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