**Megan Mitchell: East Bio Summer placement report (3rd June 2024 – 26th July 2024)**

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**How Omega-3 sources in salmon feeds modulate immune gene expression and resolve inflammation**

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**Introduction**

Atlantic Salmon (*Salmo salar*) rely on long-chain omega-3 fatty acids such as Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA), for their health and development (Sundell et al., 2022). These fatty acids are crucial components of the cell membrane contributing to the maintenance of its structure and function by forming the lipid bilayer (Zhang et al., 2023). Additionally, poly-unsaturated fatty acids (PUFAs) like EPA and DHA also have key roles in the immune function (Zhang et al., 2023) and act as precursors for both pro-inflammatory and anti-inflammatory eicosanoids (Caballero-Solares et al., 2024). The balance of eicosanoids is essential for a healthy inflammatory response, a critical aspect of the immune system’s defence against pathogens.

Salmon are a key dietary source of omega-3 for humans. However, over the years, the levels of EPA and DHA have decreased in farmed salmon due to the increased use of terrestrial fatty acids, such as vegetable and seed oils, these terrestrial oils have replaced the fish / marine oils that were traditionally used in fish feed (Sprague et al., 2016). This change in feed composition is driven by the aquaculture industry’s reliance on more sustainable and cost-effective plant-based oils.

In an effort to address this nutritional shift, transgenic oilseed crops like *Camelina sativa*, which are engineered to produce high levels of EPA, have been developed as alternative feed sources. Oils from these genetically modified crops, supplied by Prof Jonathan Napier from the Rothamsted Institute, provide a unique opportunity to assess the effects of plant-based omega-3 sources on salmon health.

My East Bio placement worked along side a funded BBSRC project “Novel Omega-3 Sources in Feeds and Impacts on Salmon Health” (BBSRC grant BB/S005919/1), my work compares the expression of immune-related genes in salmon fed with oils derived from transgenic *Camelina sativa* against those fed with traditional fish oil and sunflower oil as controls (Broughton et al., 2022).

The specific immune genes investigated in this study include pro-inflammatory markers such as **IL1β** and **IL8 B** that are proinflammatory cytokines and **ALOX5AP, ALOX5, COX2** that encode genes involved in ecisonoid production  (Buchmann et al., 2022; Katikaneni et al., 2020). Additionally, the gene encoding the cytokine  **IL11**  was examined due to its debated role in inflammation, with current literature suggesting it may have both pro-inflammatory and regulatory functions (Buchmann et al., 2022). By understanding how different omega-3 sources modulate immune gene expression in response to stimulant, this research aims to provide insights into optimizing salmon feed formulations to enhance health and disease resistance.

**Methods**

**Diets and Feeding Trial**

Diets which were isonitrogenous and isoenergetic but constituted different omega-3/omega-6 polyunsaturated fatty acid (PUFA) ratios were produced at BioMar Tech-Centre (Brande, Denmark, Table 1). A total of 180 Atlantic salmon parr of weight ~30g from Buckieburn were divided into 3 tanks and fed on these experimental diets from September to December 2021 until weighing approximately 75g.

Table 1: Diet with source of oil, the % EPA and DHA, the total omega-3 PUFA % and omega-6 PUFA % and the omega-3:omega-6 ratio

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Diet** | **Oil source** | **EPA (%)** | **DHA (%)** | **Total n-3**  **PUFA (%)** | **Total n-6**  **PUFA (%)** | **n:3/n:6** |
| C | ECO GM Camelina | 9.36 | 0.66 | 16.4 | 36.8 | 0.45 |
| G | Southern fish oil | 18.8 | 10.4 | 37.2 | 3.44 | 10.8 |
| H | Sunflower oil | 0 | 0 | 0.09 | 61.3 | 0 |

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*Figure 1: Experimental design outline. Fish were fed experimental diet (ECO GM Camelina) or control diet (Southern Fish Oil or Sunflower oil. 6 fish per diet were selected and stimulated with either PBS (a control) or Y. rukeri flagellin (Bacterial stimulent). Samples of head kidney tissue were taken and RNA was extracted, cDNA was synthesised from the RNA and qPCR assay was performed.*

***In vivo* stimulation**

Fish fed experimental diets for four months were selected and stimulated with a bacterial mimic. After the fish were lightly anaesthetised with MS-222 (PHARMAQ, Norway), six fish from each diet group were intraperitoneally injected with 0.1mL of Phosphate Buffer saline (PBS). Another six for each diet were injected with 0.1 mL of*Yersinia ruckeri* recombinant flagellin protein (FLAG) prepared in 0.02 M PBS to a working solution of 75 ngµl-1. The fish were returned to their respective tanks to allow for recovery post-injection. 24 hours post-recovery, fish were euthanised and weights and lengths were recorded. Head kidney tissue for each fish was sampled and stored in RNAlaterTM (Ambion Inc., USA) according to the manufacturer’s protocol.

**RNA extraction and cDNA synthesis**

From each head kidney sample, approximately 50 mg was taken for RNA extraction using standard TRIzol® reagent (Ambion by Life Technologies, Carlsbad, CA, United States) extraction protocol as described (Król et al., 2020). Spectrophotometry (NanoDrop Technologies, Santa Clara, CA, United States) and electrophoresis (Agilent Technologies, Santa Clara, CA, United States) were used to determine RNA quantity and integrity respectively.

The Quantitect cDNA Synthesis kit (Qiagen) was used for the synthesis of cDNA from 1000 ng of total RNA in a volume of 12μl with water. 2μl of gDNA wipeout was added and this was incubated at 42°C for 2 min to remove any genomic DNA contamination. A mastermix created from 4μl buffer, 1 μl RT primer mix and 1 μl Quantiscript Reverse Transcriptase per sample was added to the treated RNA and incubated at 42°C for 25 min followed by a final 95°C incubation for 5 min. Each sample was diluted to a final concentration of 5 ng μl-1.

cDNA was tested by standard PCR with a mastermix consisting of 15.5μl water, 0.5μl Mytaq enzyme (Qiagen), 1μl forward primer (Elongation Facter 1, 10 μM), 1μl reverse primer (Elongation factor 1, 10 μM) and 5μl of buffer per sample. 23μl of the mastermix was added to 2μl of cDNA (total of 10ng cDNA) to give a total of 25μl. PCR cycling conditions were 2min at 95°C to denature the cDNA, followed by 30 cycles of 95°C for 15 sec, 55°C for 15sec and 72° for 30sec for annealing. The PCR finished with an extension of 72° for 3min and was held a 4°C. To visualise the results of PCR amplification, 8μl of the PCR product was loaded on a 1.8% agarose gel stained with Midori green.

**Gene expression of immune genes by qPCR**

Real time PCR was used to assay the mRNA expression of 6 different genes (IL1β2, ALOX5ap1, ALOX5a1, COX2a2, IL8 and IL11a1) for which sequence analysis confirmed the specification of primers. A mastermix of 1μl water, 7.5μl 2 × GoTaq® SYBR-green qPCR master mix (Promega), 0.75μl forward primer and 0.75μl reverse primer per sample for each gene run was made up and 10μl was aliquoted into each well. 5μl of cDNA at 5ng μl-1 was added to the well. The PCR cycling consisted of 95°C for 3min then 40 cycles of 20s at 95°C and 20sec at 64°C with a final cycle of 1min at 95°C, 30sec at 55°C and 30sec at 95°C to produce a melting curve.

Arbitrary expression values were calculated using Bio-Rad CFX Manager Software (version 3.0) and expression levels were normalised against the reference gene elongation factor 1α (Elf-1α).

**Statistical Analysis**

To determine any significant changes in mRNA expression for each gene in response to stimulation and / or diet, a 2-way ANOVA was carried out with stimulant and diet as factors. Diagnostic plots of the residuals allowed for testing of the models in R and post-hoc testing, Tukey’s multiple comparison test, was carried out when significance was recorded. p values < 0.05 were considered to be significant.

**Results**

**IL1β**

Interleukin-1β2 (IL1β2) mRNA expression measured by qPCR was significantly in creased in expression in thefish stimulated with a bacterial mimic than in control fish (p = 0.028), but no significant effect of diet was identified.



**Figure 2a.** Mean relative abundance of the expression of mRNA of the IL1B gene for each diet (C, G or H) and stimulant (PBS and FLAG) with Standard error of the mean bars included **Figure 2b**. Mean fold change of mRNA expression of the IL1B gene against the PBS control for each diet (C, G and H) with Standard error of the mean bars included.

Table 2: Each factor of the 2-way ANOVA test with degrees of freedom and the F- and P-values for Mean Relative Abundance (MRA) and Mean Fold Change (MFC) for the IL1B gene

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **MRA F-value** | **MRA P-value** | **MFC F-value** | **MFC P-value** |
| **Diet** | 2.715 | 0.0824 | 1.624 | 0.214 |
| **Stimulent** | 0.153 | 0.6982 | 6.332 | 0.028 |
| **Diet:Stimulent** | 0.023 | 0.9774 | 1.624 | 0.214 |

**ALOX5ap1**

Arachidonate 5-Lipoxygenase Activating Protein (ALOXap1) mRNA expression measured by qPCR was significantly higher in relative abundance and fold change of fish stimulated with a bacterial mimic than in control fish (p = 0.0282 and 0.0352), but no significant effect of diet was identified.



**Figure 3a**: Mean relative abundance of the expression of mRNA of the ALOX5ap1 gene for each diet (C, G or H) and stimulant (PBS and FLAG) with Standard error of the mean bars included; **Figure 3b**: Mean fold change of mRNA expression of the ALOX5ap1 gene against the PBS control for each diet (C, G and H) with Standard error of the mean bars included.

Table 3: Each factor of the 2-way ANOVA test with degrees of freedom and the F- and P-values for Mean Relative Abundance (MRA) and Mean Fold Change (MFC) for the ALOX5ap1 gene

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **MRA F-value** | **MRA P-value** | **MFC F-value** | **MFC P-value** |
| **Diet** | 0.186 | 0.8313 | 1.160 | 0.3272 |
| **Stimulent** | 5.315 | 0.0282 | 4.865 | 0.0352 |
| **Diet:Stimulent** | 0.904 | 0.4155 | 1.160 | 0.3272 |

**ALOX5a1**

Arachidonate 5-Lipoxygenase (ALOX5a1) mRNA expression measured by qPCR was significantly higher in relative abundance in fish stimulated with a bacterial mimic than in control fish (p = 0.0237), but no significant effect of diet was identified.

 

**Figure 4a:** Mean relative abundance of the expression of mRNA of the ALOX5a1 gene for each diet (C, G or H) and stimulant (PBS and FLAG) with Standard error of the mean bars included; **Figure 4b**: Mean fold change of mRNA expression of the ALOX5a1 gene against the PBS control for each diet (C, G and H) with Standard error of the mean bars included.

Table 4: Each factor of the 2-way ANOVA test with degrees of freedom and the F- and P-values for Mean Relative Abundance (MRA) and Mean Fold Change (MFC) for the ALOX5a1 gene

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **MRA F-value** | **MRA P-value** | **MFC F-value** | **MFC P-value** |
| **Diet** | 0.968 | 0.3916 | 1.900 | 0.167 |
| **Stimulent** | 5.681 | 0.0237 | 2.098 | 0.158 |
| **Diet:Stimulent** | 2.976 | 0.0662 | 1.900 | 0.167 |

**COX2a2**

Cytochrome c oxidase polypeptide II (COX2a2) mRNA expression measured by qPCR was significantly higher in relative abundance of fish stimulated with a bacterial mimic than in control fish (p < 0.001). A significant effect was detected on fold change of mRNA expression measured by qPCR when diet H was compared to diet C (p = 0.008) and G (0.007).

 

**Figure 5a**: Mean relative abundance of the expression of mRNA of the COX2a2 gene for each diet (C,G or H) and stimulant (PBS and FLAG) with Standard error of the mean bars included; **Figure 5b**: Bar graph for the mean fold change of mRNA expression of the COX2a2 gene against the PBS control for each diet (C, G and H) with Standard error of the mean bars included.

Table 5: Each factor of the 2-way ANOVA test with degrees of freedom and the F- and P-values for Mean Relative Abundance (MRA) and Mean Fold Change (MFC) for the COX2a2 gene.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **MRA F-value** | **MRA P-value** | **MFC F-value** | **MFC P-value** |
| **Diet** | 1.380 | 0.267203 | 7.119 | p < 0.001 |
| **Stimulent** | 19.941 | P < 0.001 | 18.723 | p < 0.001 |
| **Diet:Stimulent** | 2.294 | 0.118284 | 7.119 | P < 0.001 |

Table 6: Results of the Post-Hoc Tukey comparison test and the significant comparisons with their relevant P-values for both Mean Relative Abundance (MRA) and Mean Fold Change (MFC) for the COX2a2 gene.

|  |  |  |
| --- | --- | --- |
| **COMPARISON** | **MRA P-Value** | **MFC P-Value** |
| **H-C** | N/A | 0.0078228 |
| **H-G** | N/A | 0.0070835 |
| **H:FLAG-C:FLAG** | 0.0395221 | p < 0.001 |
| **H:FLAG-G:FLAG** | N/A | p < 0.001 |
| **C:PBS-H:FLAG** | p < 0.001 | p < 0.001 |
| **G:PBS-H:FLAG** | p < 0.001 | p < 0.001 |
| **H:PBS-H:FLAG** | p < 0.001 | p < 0.001 |

**IL8**

Interleukin-8 (IL8) mRNA expression measured by qPCR was significantly higher in relative abundance and fold change of fish stimulated with a bacterial mimic than in control fish (p = 0.004 and 0.004), but no significant effect of diet was identified.

 

**Figure 6a**: Mean relative abundance of the expression of mRNA of the IL8 gene for each diet (C, G or H) and stimulant (PBS and FLAG) with Standard error of the mean bars included; **Figure 6b**: Bar graph for the mean fold change of mRNA expression of the IL8 gene against the PBS control for each diet (C, G and H) with Standard error of the mean bars included.

Table 7: Each factor of the 2-way ANOVA test with degrees of freedom and the F- and P-values for Mean Relative Abundance (MRA) and Mean Fold Change (MFC) for the IL8 gene

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **MRA F-value** | **MRA P-value** | **MFC F-value** | **MFC P-value** |
| **Diet** | 1.357 | 0.27270 | 2.302 | 0.11744 |
| **Stimulent** | 9.888 | 0.00373 | 9.640 | 0.00413 |
| **Diet:Stimulent** | 2.368 | 0.11091 | 2.302 | 0.11744 |

**IL11a1**

Interleukin 11a (IL11a1) mRNA expression measured by qPCR was significantly higher in relative abundance and fold change of fish stimulated with a bacterial mimic than in control fish (p < 0.001). A significant effect was detected on relative abundance and fold change of mRNA expression measured by qPCR when diet H was compared to diet C (p = 0.014) and G (p = 0.038), when bacterial mimic stimulated fish were compared to control fish (p < 0.001).

 

**Figure 7a**: Mean relative abundance of the expression of mRNA of the IL11a1 gene for each diet (C, G or H) and stimulant (PBS and FLAG) with Standard error of the mean bars included; **Figure 7b**: Bar graph for the mean fold change of mRNA expression of the IL11a1 gene against the PBS control for each diet (C, G and H) with Standard error of the mean bars included.

Table 8: Each factor of the 2-way ANOVA test with degrees of freedom and the F- and P-values for Mean Relative Abundance (MRA) and Mean Fold Change (MFC) for the IL11a1 gene

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **MRA F-value** | **MRA P-value** | **MFC F-value** | **MFC P-value** |
| **Diet** | 5.342 | 0.01059 | 13.46 | p < 0.001 |
| **Stimulent** | 48.006 | p < 0.001 | 35.11 | p < 0.001 |
| **Diet:Stimulent** | 6.126 | 0.00604 | 13.26 | p < 0.001 |

Table 9: Results of the Post-Hoc Tukey comparison test and the significant comparisons with their relevant P-values for both Mean Relative Abundance (MRA) and Mean Fold Change (MFC) for the IL11a1 gene

|  |  |  |
| --- | --- | --- |
| **Comparison** | **MRA P-value** | **MFC P-value** |
| **H-C** | 0.0143812 | p < 0.001 |
| **H-G** | 0.0389670 | 0.0041421 |
| **H:FLAG-C:FLAG** | 0.0011946 | p < 0.001 |
| **H:FLAG-G:FLAG** | 0.0287306 | p < 0.001 |
| **G:PBS-G:FLAG** | 0.0373224 | N/A |
| **H:PBS-G:FLAG** | 0.0359500 | N/A |
| **C:PBS-H:FLAG** | p < 0.001 | p < 0.001 |
| **G:PBS-H:FLAG** | p < 0.001 | p < 0.001 |
| **H:PBS-H:FLAG** | p < 0.001 | p < 0.001 |

**Discussion**

During this EastBio placement I examined the expression of several pro-inflammatory genes in Atlantic salmon (*Salmo salar*) fed different diets and exposed to either a bacterial mimic (FLAG) or a phosphate buffer solution to stimulate an immune response. The genes studied included **IL1β, IL8, ALOX5ap1, ALOX5a1, COX2a2,** and **IL11a1**. Our findings showed that stimulation with the bacterial mimic caused a significant increase in the expression of all six genes, this indicates an active immune response. The expression of the pro-inflammatory genes **IL1β, ALOX5ap1, ALOX5a1**and **IL8** did not show a significant difference in response to diet. This suggests that, under the conditions studied, the omega-3 sources in the ECO camelina feed did not modulate the expression of these genes. However, the expression of the **COX2a2** and **IL11a1** genes did show a significant difference in response to a change in diet.

The mRNA expression of bacterial mimic (FLAG) stimulated fish showed a significant increase (P < 0.05) in comparison to the control (PBS) across all genes studied. This indicates a strong inflammatory response in all fish studied. Each gene, **IL1β, ALOX5ap1, ALOX5a1, COX2a2** and **IL8**, have important roles in mediating inflammation and so, these results are consistent with our expectations. **IL1β, ALOX5ap1,** and **ALOX5a1** are each involved in the early inflammation response and leukotriene synthesis (Buchmann et al., 2022; Katikaneni et al., 2020).

In summary we are able to show that manipulating the diet by feeding the fish with higher levels of DHA can alter how the fish respond to bacterial stimulants. This information will help design future diets that may improve fish health.

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