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Evaluation of the impact of camelina oil-containing diets on the expression of genes involved in the innate anti-viral immune response in Atlantic cod (*Gadus morhua*)

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ABSTRACT

To improve sustainability of aquaculture, especially for carnivorous species like Atlantic cod, replacement of fish oil-based diets with vegetable oil-based diets has been studied. The use of vegetable oil in fish feeds can significantly change the fatty acid composition of fish tissues, and given the importance of fatty acids in inflammation and immunity, this change could potentially impact the immune response and health of the fish. The oilseed *Camelina sativa* is a promising source for this vegetable oil, because of the high oil content of its seeds (40%), a higher n-3 fatty acid content than most other oilseeds, and a high amount of γ-tocopherol. This study aims to investigate the effect of the replacement of dietary fish oil with oil from *Camelina sativa* on the immune response of Atlantic cod, as measured by the gene expression in spleen.

Juvenile cod were fed on a fish oil-based diet (FO) or one of two diets in which camelina oil replaced 40% or 80% of fish oil (40CO and 80CO respectively) for 67 days, after which they were injected with either the viral mimic polyriboinosinic polyribocytidylic acid (pIC), or phosphate-buffered saline (PBS) as a control. Microarray analysis was used to determine the effect of the diet on the basal spleen transcriptome (pre-injection), and on the response to pIC (24 h post-injection). No marked differences in the spleen transcriptome were found between the three diets, either before or after injection with pIC. All fish, regardless of diet, showed a strong anti-viral response 24 h after pIC injection, with more than 500 genes having a significant difference of expression of 2-fold or higher compared to the PBS-injected fish for the FO, 40CO and 80CO diets. Gene Ontology annotation analysis of the three pIC-responsive gene lists indicated they were highly similar, and that the term 'immune system process' was significantly enriched in the pIC-responsive gene lists for all three diets. QPCR analysis for 5 genes with a known function in the anti-viral innate immune response (LGP2, STAT1, IRF1, ISG15 and viperin) showed modestly (smaller than 2-fold) up-regulated basal expression of LGP2, IRF1 and STAT1 in fish fed 40CO compared to the other diets. After pIC injection, all 5 genes were significantly and strongly up-regulated in pIC-injected fish compared to PBS-injected fish, but no significant differences were found between any of the diets.

In conclusion, replacement of up to 80% of fish oil with camelina oil in Atlantic cod diets does not have a strong effect on basal spleen gene expression. Atlantic cod fed on camelina oil-containing diets are capable of mounting a strong anti-viral immune response, which is comparable to that in cod fed with a fish oil diet.

1. Introduction

As seafood production from commercial capture fisheries has leveled off, the role of the aquaculture industry has increased, and aquaculture is currently supplying almost half of the world's food fish for human consumption [1]. The Atlantic cod (*Gadus morhua*) commercial fishery has put pressure on wild stocks to the point that several of those stocks collapsed in the early 1990's, which led to a moratorium on cod fishery in Canadian waters [2]. Although cod populations in some areas seem to have recovered, stocks in the northwest Atlantic are recovering only very slowly [3]. Atlantic cod is an important food fish, and since history has shown that wild cod

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stocks are very sensitive to overfishing, and harvests of wild cod are unpredictable and variable [4,5], Atlantic cod aquaculture will be important to meet the demand in a sustainable manner [6].

The major source of lipids for aquaculture fish feed, especially for carnivorous species like Atlantic cod, is fish oil, which is mostly harvested from wild stocks of oily fish such as capelin or herring. The world fish oil production has slowly but steadily declined since the late 1990's, while the use of fish oil in aquaculture is predicted to increase slowly, especially for marine species [7,8]. To improve sustainability of the aquaculture industry, research is being done into replacing fish oil with alternatives, such as vegetable oil.

Like all vertebrates, fish need a dietary supply of essential fatty acids linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3). These are the precursors of the biologically active long-chain polyunsaturated fatty acids (LC-PUFA) arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). The n-3 LC-PUFA EPA and DHA are beneficial for the growth and health of the fish, and also for the health of the human consumer. Most freshwater fish have the capability of converting LA and ALA to LC-PUFA, but Atlantic cod and other marine fish have mostly lost this capability [9]; therefore it is important that the LC-PUFA are supplied in the diet [10,11].

Fish oil is rich in n-3 LC-PUFA EPA and DHA, and low in n-6 PUFA. Terrestrial vegetable oils do not contain EPA, DHA or ARA; instead they contain mainly shorter chain C18 fatty acids. Most vegetable oils are rich in n-6 fatty acids (LA, e.g. soy, sunflower) or n-9 fatty acids (oleic acid, e.g. canola), and low in n-3 fatty acids. Many studies have shown that the tissue fatty acid composition of fish fed with vegetable oil-based diets reflects the fatty acid composition of the diets, with low levels of n-3 fatty acids, particularly EPA and DHA; however despite the absence of EPA and DHA in vegetable oil, the replacement of fish oil with vegetable oil generally does not significantly affect fish growth (reviewed in [11]). For Atlantic cod, this has been shown for various vegetable oils such as soybean oil [12,13], echium oil [14], combinations of palm, rapeseed and linseed oils [15], and camelina oil [16,17].

Camelina sativa, or false flax, is an oilseed of the Brassicaceae family with several positive agronomical characteristics. It can grow in soil with low fertility, does not require as many nutrients as other oilseed crops, and it is tolerant to frost, insects and weeds [18]. As a potential ingredient for fish diets it compares favorably to other plants such as canola and soy, because of the high oil content of its seeds (40%), the unusually high amount of ALA, which gives it a higher n-3 fatty acid content than most other oilseeds, and the high amount of the antioxidant γ-tocopherol [18–20].

Hixson et al. have shown that replacing up to 80% of fish oil with camelina oil does not have an effect on Atlantic cod growth performance [16]. Morais et al. [17] showed the same for Atlantic cod that were fed diets replacing up to 100% fish oil with camelina oil. However, these studies showed that the fatty acid profiles of liver, muscle and intestine of cod fed the camelina oil diets had changed to reflect the fatty acid profile of the diets. Some of the most noticeable differences between the cod fed fish oil diets and camelina oil diets were the higher levels of terrestrial-type fatty acids ALA and LA, the lower levels of marine-type LC-PUFA EPA and DHA, and the lower level of ARA in the tissues of cod fed the camelina oil diets. The overall n-3:n-6 fatty acid ratio in cod fed the camelina oil diets was lower than in cod fed the fish oil diets [16,17].

Fatty acids play an important role in inflammation and immunity (reviewed in [21,22]). They act on both the innate and adaptive immune response and modulate diverse processes such as expression of adhesion molecules, production of inflammatory cytokines, phagocytosis, chemotaxis, eicosanoid production, antigen presentation and T-cell activity. Saturated fatty acids are generally pro-inflammatory and up-regulate immune processes [23,24]. LC-PUFAs are substrates for the synthesis of eicosanoids [25,26]. ARA-derived eicosanoids are potent and pro-inflammatory. EPA and DHA give rise to weaker eicosanoids, as well as protectins and resolvins, which are anti-inflammatory and suppress immune processes. Since many of these compounds compete for the same enzymes and receptors, the balance between EPA/DHA and ARA and their products determines whether the inflammatory response is promoted or suppressed. EPA and DHA also exert their anti-inflammatory effect through other mechanisms [21,22]. In the form of membrane phospholipids, they can disrupt lipid rafts; as free fatty acids, they can activate the anti-inflammatory transcription factor peroxisome proliferator-activated receptor gamma (PPARγ), or signal through G-protein coupled receptor 120. Many of those mechanisms are also linked to the inactivation of pro-inflammatory transcription factor nuclear factor kappa B (NFκB). It has been shown that the consumption of EPA and DHA by humans led to changes in gene expression in peripheral blood mononuclear cells [27] or whole blood [28], with down-regulation of genes involved in immunity and inflammation pathways, such as eicosanoid synthesis and NFκB signaling.

Although Hixson et al. [16] and Morais et al. [17] have shown that Atlantic cod growth was not affected by the replacement of fish oil with camelina oil, it was hypothesized that given the importance of fatty acids in inflammation and immunity, the resulting changes in tissue fatty acids might have an impact on the immune response. The current study used the same fish population and fish diets as in Hixson et al. [16], with the aim to investigate the effect of camelina oil diets on the immune function of Atlantic cod, as measured by gene expression in spleen. We studied both the effect of the diet alone on basal spleen gene expression levels, as well as the effect of the diet after fish were injected with the synthetic double-stranded RNA polyriboinosinic polyribocytidylic acid (pIC), which mimics a viral immune stimulus and has been shown to elicit potent anti-viral spleen transcriptome responses in Atlantic cod [29,30].

2. Materials and methods

2.1. Feeding trial, immune challenge and fish sampling

The Atlantic cod used for the immune challenge experiment were part of a feeding trial [16]. In this feeding trial, juvenile cod (average initial weight 19.4 g) were kept in 9 experimental 620 L tanks, each containing 110 fish, at the Dr. Joe Brown Aquatic Research Building (Ocean Sciences Centre, St. John's, Newfoundland, Canada). Fish were kept in flow-through seawater at a temperature of 10 °C, oxygen saturation >90% and a photoperiod of 12 h light/12 h dark. Triplicate tanks were fed for 67 days with one of three practical diets: a control diet using herring oil as a lipid source (FO diet), and two experimental diets using vegetable oil from *Camelina sativa* to replace 40% or 80% of herring oil (40CO and 80CO diets). All three diets were formulated to meet the nutritional requirement of gadoids based on previously published formulations [31,32] and to be isonitrogenous and isolipidic. Diet formulations, proximate composition and fatty acid composition were published previously in Hixson et al. [16] and are summarized in Table 1 for easy reference. Fish were fed 1% of body weight of feed per tank twice daily in order to ensure equal consumption of feed among treatments.

At the end of the feeding trial we performed the immune challenge experiment for each of the three diets. The triplicate design was maintained by dividing 48 fish from each of the triplicate tanks over two separate 620 L tanks of 24 fish each, one for PBS and one for pIC injection (see Supplementary Figure S1A for a schematic overview of the experimental setup). Feeding of the FO,