In vitro bacterial and viral response in head kidney leukocytes of Atlantic salmon (*Salmo salar*) fed dietary insects meal.

Thesis for the degree

Master of Science in Aquamedicine

Oda Kvalsvik Stenberg

Department of Biology

University of Bergen, Norway

June 2018

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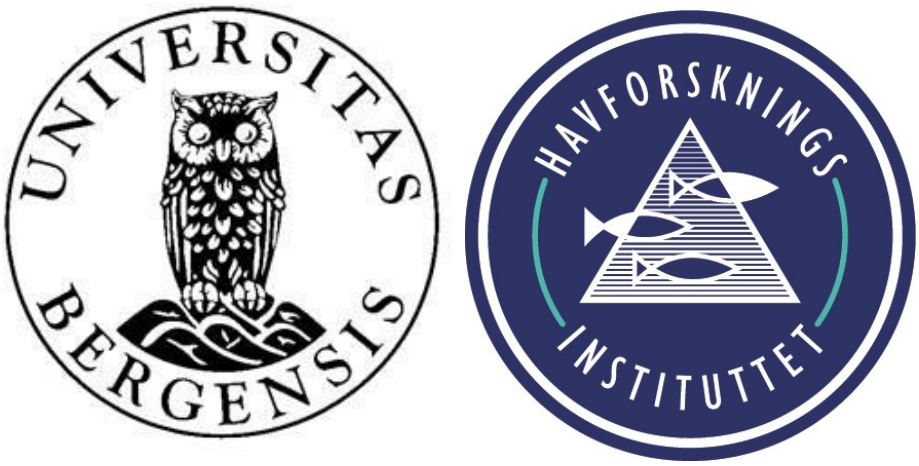

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Abstract

With the fast growth of today's aquaculture industry, the demand for aqua-feeds is expanding dramatically. Insects, which are part of the natural diet of salmonids, could represent a sustainable ingredient for aquaculture feed. The aim of the current study was to test how a partial or total replacement of dietary fishmeal with insect meal affect gene responses involved in inflammation and signaling pathways in Atlantic salmon (*Salmo salar*) head kidney leukocytes after exposure to bacterial or viral mimics.

Insect meal (IM) was produced from black solider fly (BSF, *Hermetia illucens*) larvae. Seawater Atlantic salmon were fed three different diets; a control diet (IM-0, protein from fishmeal and plant based ingredients (25:75) and lipid from fish oil and vegetable oil (33:66); IM-66 and IM-100 diets, where 66 and 100% of fishmeal protein was replaced with IM, respectively. Leukocytes were isolated from the head kidney of fish from the three dietary groups (2 fish per cage, 3 cages per treatment, and 18 fish in total). Isolated leukocytes were seeded into culture wells and added bacterial mimic lipopolysaccharide (LPS) or viral mimic polyinosinic acid: polycytidylic acid (poly I: C) to induce an inflammatory response. Controls (Control) without LPS and poly I: C were included.

The transcription of interleukins *IL-1β*, *IL-8*, *IL-10* and *TNF-1β* was elevated in LPS treated leukocytes isolated from the three dietary groups, which confirmed the suitability of the *in vitro* model in this experiment. The inflammatory related gene expression was not affected by the experimental diets. The gene expression of the fish specific toll-like receptor (*TLR22*) and transcription factor T CCAAT/enhancer-binding protein *β (C/EBP-β)* was down regulated by poly I: C in all diets and by LPS treatment in cells isolated from salmon fed insect-based diets. Similarly, the leukocytes challenged with LPS and isolated from fish fed with IM-66 and IM-100 had lower gene expression of all selected antioxidant enzymes (*SOD* and *GPx1*) and heat shock proteins (*HSP27* and *HSP 70*) compared to the control diet (IM-0). The diets did not affect Prostaglandin D and E synthase (*PTGDS, PTGES*) transcription, but insect-based diet did lower the gene expression of arachidonate 5-lipoxygenase (*5-LOX*). These results suggest that replacement of fishmeal with IM in the diets of Atlantic salmon had no effect on the transcription of pro-inflammatory genes in the head kidney cells. In addition, fish fed with IM had down regulated transcription of antioxidant and stress related genes.

Contents

Abbreviations

AA amino acids

ANOVA Analysis of variance

Ara Polyunsaturated fatty acids

BSF Black solider fly

DAMP Damage associated molecular patterns

CDNA Complementary DNA

DW weight

EPA Eicosapentaenoic acid

FA fatty acids

FM fish meal

FO fish oil

FW final weight

IM Insect meal

IM-0 Control diet, 0% of fishmeal protein replaced with IM

IM-66 Insect meal where 66% of fishmeal protein replaced with IM

IM-100 Insect meal where 100% of fishmeal protein replaced with IM

IW initial weight

LA lauric acid

MCT medium chain-FA

mRNA Messenger RNA

PAMP Pathogen-associated molecular patterns

PG Prostaglandin

PGH2 2-series prostaglandin H2

PGH3 3-series prostanglandin H3

ROS Reactive oxygen species

PRR Pattern recognition receptors

VP vegetable protein

1. INTRODUCTION

1.1 Aquaculture development and feed formulation: trends and prospects

Capture fisheries and aquaculture production supplied the world with 167, 2 million tons of fish in 2014 (FAO 2016). Aquaculture is the fastest growing animal food producing sector and covered 44,1 percent of the total fish supply, by providing 73,8 (49,8 finfish) million tons in 2014 (FAO 2016). The leading production of Atlantic salmon is located among the Norwegian coastline, where 1 207 800 tons of Atlantic salmon (*Salmo salar)* were produced in 2017 alone (Hjeltnes et al. 2018). The Norwegian salmon industry consumed in total 182 579 tons of fish oil (FO) and 317 241 tons of fish meal (FM) in 2012 (Ytrestøyl et al. 2014). More details about the ingredients used for salmon aquaculture production in Norway in 2012 and 2013, and specific resource utilization are described in NOFIMA report (Ytrestøyl et al. 2014).

The salmon farming aquaculture industry and its dependence on FM and FO raises concerns about the sustainability of salmon production, and raise questions about how it impacts wild fish stocks (Tacon and Metian 2008; Naylor et al. 2009). Marine ingredients are limited resources with a production of FM and FO that has been relatively constant for the last 20 years. The World-wide annual production did in 2014 reduce 15.8 million tons capture fish to fishmeal (FM) and fish oil (FO) (FAO 2016). However, the need for fish-feed for the salmon industry increased drastically with the fast production growth. Terrestrial plant-based ingredients are prominent substitution prospects for marine ingredients due to their availability and competitive price (Gatlin et al. 2007). A significant decline in the use marine ingredients in aqua-feed was seen, e.g. in the Norwegian salmon industry; from 90% of marine-based diet in 1980s to 37% in 2013 (Figure 1.1) (Ytrestøyl et al. 2014) . The three big fish feed companies in Norway; Ewos (Cargill today), Skretting and Biomar, used 598 861 and 303 592 tons of plant-based protein sources (mainly soy protein concentrate) and marine fish meal, respectively in the diets of Atlantic salmon in 2012 (Ytrestøyl et al. 2014).

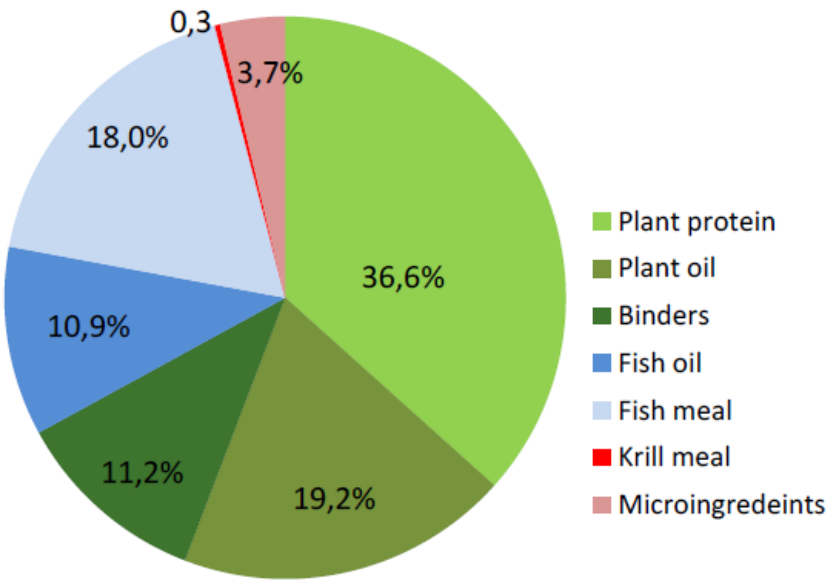


Figure 1.1: Composition of the Norwegian salmon feed production in 2013. Values represent the percentage (%) of total ingredients used. From (Ytrestøyl et al. 2014).

However, plant-based diet used for salmonids and other carnivorous fish species rises concerns regarding the nutritional aspects, such as lower protein content and less balanced amino acid profile compared to FM, high content of carbohydrates and the presence of anti-nutritional factors. Also concerns about environmental issues have arisen regarding the use of plant-based diet for animal production. For example, soybean production for animal use competes for the same cultivated-area, water end energy that could be used directly for human food production. Therefore, it is important to find new alternatives for fish- and plant-based ingredients in aqua-feed that can meet the requirement of salmon, but at the same time maintaining the environmental sustainability. Insects may represent an innovative, and sustainable feed ingredients for aqua-feed today.

1.2 Insects as feed ingredients for fish species

Many fish species prey on insects in the wild, including fresh water Atlantic salmon (Gabler and Amundsen 1999). Introduction of insect in fish feeds are therefore a natural concept, and might from a nutritional perspective be a valuable feed ingredient in aqua-feed. Insect larvae contain high level of lipids and proteins (Makkar et al. 2014). Compared to plant protein, dietary insects contain low levels of carbohydrates and anti-nutritional factors (Gilles et al. 2015). Dietary inclusion of farmed-insects is also interesting in an environmental perspective. Indeed, they have a short life cycle, grow and reproduce easily. Insects are able to grow on a wide range of low quality materials that mainly content carbohydrates, and efficiently convert it into high quality energy, rich in fats and proteins (Čičková et al. 2015). Other environmental advantages of farmed insect are; (1) Minimal land and water is required; (2) Greenhouse gas emissions are lower; (3) Insects have high feed conversion efficiencies (van Huis and Oonincx 2017). The insect farming industry are today not big enough to meet the huge commercial feed production, but it shows a great potential as sustainable ingredients for fish feed in the future.

Insect meal (IM) has been used as feed ingredient for a variety of fish species, e.g. Juvenile turbot (*Psetta maxima)* (Kroeckel et al. 2012), European seabass (*Dicentrarchus labrax)* (Magalhães et al. 2017; Gasco et al. 2016), Rainbow trout (*Oncorhynchus mykiss)* (St-Hilaire et al. 2007; Renna et al. 2017) and Atlantic salmon (*Salmo salar)* (Belghit et al. 2018; Lock et al. 2016). The existing insect diet experiments shown some various results, depending on the investigated fish species and the experimental diet formula. Recent studies have shown no effects on growth performances when using black soldier fly (BSF) larvae meal as a replacement to fish meal in the diet for salmonids (Renna et al. 2017; Belghit et al. 2018; St-Hilaire et al. 2007; Lock et al. 2016).

It's interesting to compare the nutrient content in various dietary insects against the nutrient composition in FM and plant-based ingredients to understand how a replacement of these ingredients can impact the growth and the fish health of fish species. The nutritional composition of dietary insects depends on the species, developmental stage and the feeding medium. For example, the BSF larvae contain high amount of lipid (≈40% of dry weight, dw) and protein (>30% dw) with amino acid (AA) profile similar to fish meal (Liland et al. 2017; Henry et al. 2015; Makkar et al. 2014) The composition of AA has to be balanced in the fish

diet, and the AA content in insect meal varies among different species. It has recently been demonstrated that BSF protein meal contain AA with high bioavailability for Atlantic Salmon, and shown the possibility of including insect meal in combination with insect oil in the diets of in fresh-water salmon without negative effect on the growth performances (Belghit et al. 2018). However, compared to fish meal, the insect meals have lower levels of the amino acids histidine, lysine and threonine, but higher levels of lysine, methionine and tyrosine than soy meal (Barroso et al. 2014). BSF larvae also contain minerals such as, iron, zinc, potassium, phosphorus, manganese, magnesium and a variety of vitamins (Henry et al. 2015; Makkar et al. 2014). Overall, the studies concluded that the nutritional composition of some selected insect species is rich and comparable to plant or fish meal based diets and point out insect meal as a promising replacement for these feed ingredients in aqua-feed (Makkar et al. 2014; Barroso et al. 2014).

In terms of the nutritional value and the farming conditions in the western world, the insect species which are considered as good candidates for fish feed so far are: the larvae or pupae of Diptera black solider fly (*Hermetia illucens*) and Diptera house fly (*Musca Domestica*). In addition, larvae from mealworm (*Tenebrio molitor*) and silkworm pupae are widely studied, and adult *Orthoptera locusts*, grasshoppers and crickets have been investigated.

1.3 Black solider fly

The name of the fly coms from the adult's attitude associated to military troops while the fly rests on surfaces. BSF come from the Diptera of Stratiomyidae family, and lives in the nature worldwide in tropical and sub-tropical areas (Dortmans et al. 2017). The life cycle starts with eggs laid close to decomposing organic matter to ensure the first food apply for the larvae after hatching. The eggs hatch after around four days, depending on the environmental conditions, and the small larvae (a few mm) start feeding on the organic matter. The larvae can fully grow in two weeks under optimal conditions, and this is the only stage where the BSF is feeding. Later, the larvae go through pupation, emerge as flies that lives about one week searching for a partner and the female lays 400-800 eggs before dying (Dortmans et al. 2017).

Figure 1.2: To the left: Newly hatched BSF larvae on substrate. To the right: Adult BSF used for breeding BSF larvae. Photo taken at the facilities at NIBIO in Ullensvang, by Oda Kvalsvik Stenberg

1.4 Fish immune system and immune stimulants

All organisms have a kind of immune system that protects against pathogens. Teleost's have as in higher vertebrates both innate (non-specific) and adaptive (specific) immune responses, as seen in Figure 1.3 (Magnadottir 2006). These systems cooperate, and it is shown that the innate immune system is important for activating and determining the nature of the adaptive immune response (Fearon and Locksley 1996). Compared to mammals, fish species generally lack the lymphatic nodules and the bone marrow. However, unique for teleost fish is that they possess a major lymphoid organ named head kidney (cranial placement). The head kidney, has many function including, cytokine producing lymphoid cells, endocrine cells secreting cortisol and thyroid hormones (Kum and Sekkin 2011). The innate immune system of fish is more developed then the adaptive response, and is primarily important in the fish's defence against pathogens. Fish innate immune system is reflected in the reviews (Magnadottir 2006; Ellis 2001)

Innate immunity is initiated by a recognition of non-self-structures from e.g. a bacteria or virus. The immune cells in the fish and humoral innate component have germline-encoded host receptors, knows as pathogen recognition receptors (PRR), which detect molecular patterns that are typical for microorganisms, referred as pathogen-associated molecular patterns (PAMPs). PAMPs consist of structures which are not normal to find on/in the cells of multicellular organisms e.g.; lipopolysaccharide (LPS), polysaccharides, peptidoglycans, bacterial DNA and double and single stranded viral RNA. When the PRRs recognise a non-self-structure, the innate immune responses react quickly. The fish have also the possibility to discover damage in its own tissues through damage-associated molecular patterns (DAMPs) that in the same way as PAMPs, activate downstream inflammatory response. (Medzhitov and Janeway 2002).

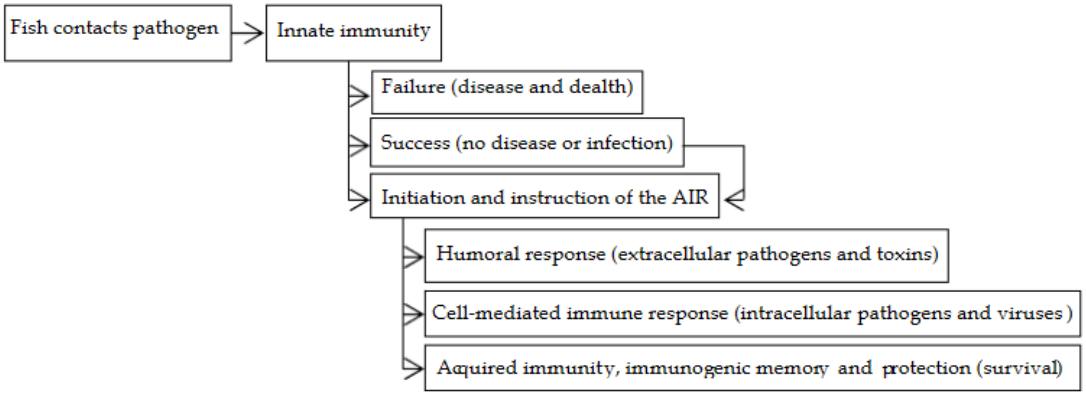


Figure 1.3: Presentation of the immune response of a fish following contact with a pathogen. AIR: Acquired/adapted immune response. From (Kum and Sekkin 2011).

It is common to divide the non-specific immune components into three categories. First, the physical parameters where fish scales, epidermis and mucus act as a barrier against pathogens. The mucus act as a biochemical barrier and contains anti-bacterial peptides and proteins (Kum and Sekkin 2011). Second and third, if the pathogen pass the barrier, cells of the innate immune system and the humoral immune components is activated (Magnadottir 2006). The important cells of the innate immune system are summarized; the phagocytic cells (granulocytes (neutrophils) and monocytes/macrophages) and the non-specific cytotoxic cells, and Epithelial and dendritic cells also participate in the innate defence in fish (Magnadottir 2006). The humoral immune defence consist of soluble components in body fluids or cell associated receptors (PRRs). Toll like receptors TLRs are one example of a group PRRs which bind various PAMPs, e.g. LPS and lipoteichoic acid (Li et al. 2017).

Cytokines are small signalling proteins secreted by cells of both the innate and the adaptive immune systems. They are often produced in a cascade, as one cytokine causes a target cell to make additional cytokines. The function of fish cytokines has been recently reviewed (Jun and Christopher 2016). A cytokine made by one leukocyte and acting on other leukocytes is called Interleukin. Interleukin-1 β IL-1β is an important interleukin in fish, produced in cells mediated by PRRs that have been in contact with PAMPs or DAMPs, and act as a central pro inflammatory cytokine. (Jun and Christopher 2016).

Further, another important classical pro-inflammatory cytokines in fish are IL-6 and Tumor necrosis factor (TNF-a) that have overlapping functions with IL-1β (Jun and Christopher 2016). IL-8 is a pro-inflammatory chemokine (cytokines with chemotactic activities), and it is

suggested that interleukin-8-derived peptide in salmonids have an additional antibacterial activity (Santana et al. 2018). An immune response out of control is potentially harmful to the fish, and therefore the anti-inflammatory cytokines are produced to suppress the immune response. IL-10 has the function of inhibiting inflammation in fish, and is associated with the adaptive immune response in the late phase of an inflammation (Jun and Christopher 2016).

Other signaling proteins with an important activity in the regulation of genes involved in the immune and inflammatory responses is the transcription factor C/EBPβ (Wedel and Lömsziegler-Heitbrock 1995).

The progress of the inflammation is also affected by lipid derived prostaglandins and leukotrienes, which are a signaling molecules derived from phospholipase-released arachidonic acid (Ara) and eicosapentaenoic acid (EPA) (Funk 2001; Wada et al. 2007). The prostaglandins and the leukotrienes are generated by enzymes cyclooxygenase isozymes COX and 5-lipoxygenase LOX5, respectively. These signaling molecules play key roles in the development of the immune response and are considered to act at, or near the producer-cell (Gómez-Abellán and Sepulcre 2016). The prostaglandin biosynthesis pathway is described in Figure 1.4.

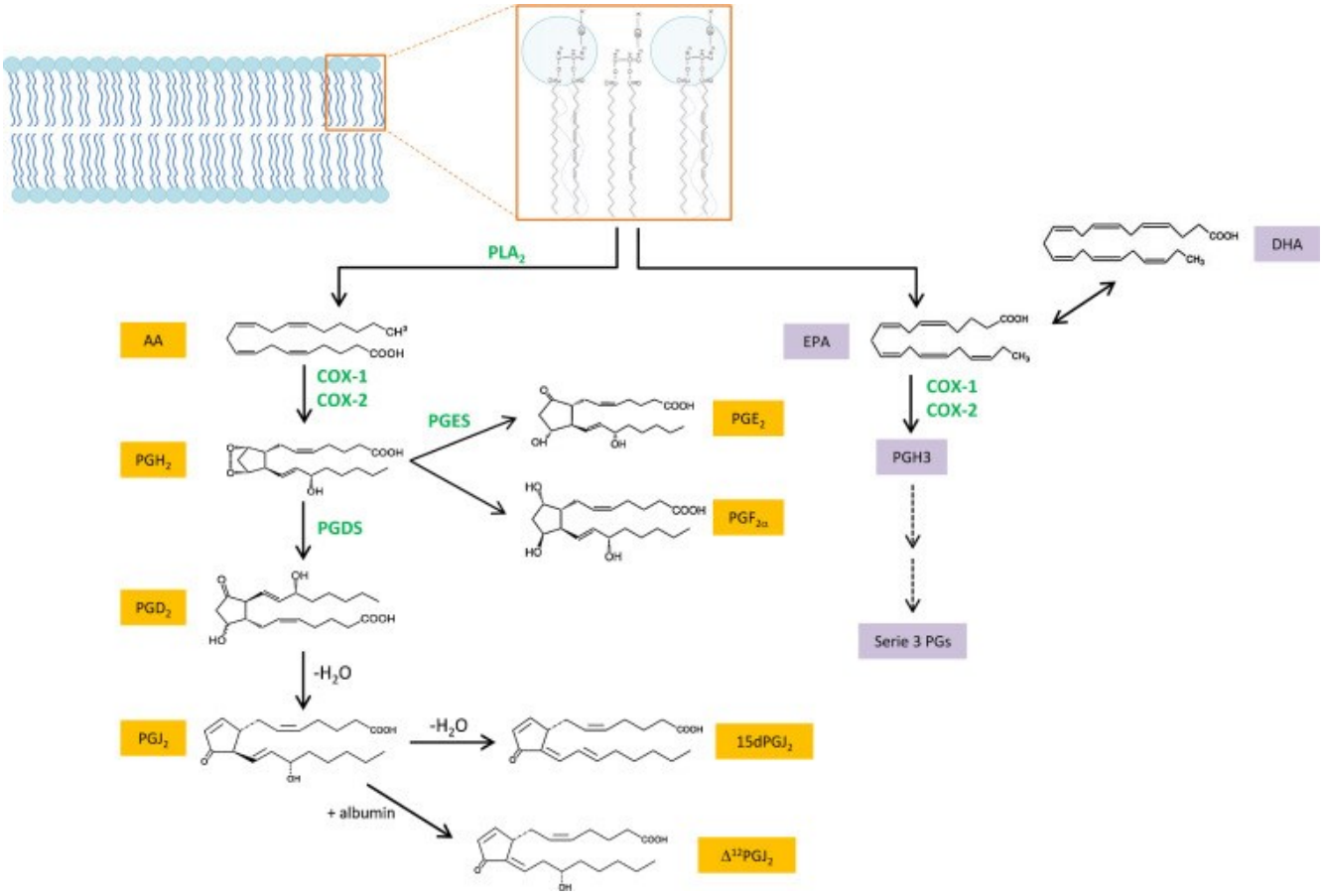


Figure 1.4: **Prostaglandin biosynthetic pathway.** Polyunsaturated fatty acids Ara or eicosapentaenoic EPA are released from the cell membrane by the action of phospholipase enzymes. Further, cyclooxygenase (COX-1 and COX-2) enzymes catalyze the biosynthesis of Ara-derived 2-series prostaglandin H_2 (PGH_2, left in the figure) or EPA-derived 3-series prostanglandin H_3 (PGH_3, to the right in the figures). Then by the activity of specific PG synthases PGDS and PGES, converts PGH_2 and PGH_3 to one of several structurally related prostaglandins of the series 2 and series 3 respectively, including PGE_2 or PGE_3, PGD_2, $PGF_{2\alpha}$. PGD_2 is further converted to cyclopentenone derivates. Figure from (Gómez-Abellán and Sepulcre 2016).

1.5 Oxidative stress response

An increased production of reactive oxygen species (ROS) or reduced biological function in the antioxidant defense against ROS can cause oxidative stress, and lead to damage of vital macromolecules and cells. The book "Oxidative stress in aquatic ecosystems" mention a variety of processes that generate ROS e.g.; Environmental stress factors (temperature change, oxygen levels, ozone levels and ultraviolet radiation) and contaminant induced stress (metal-ions, pesticides and oil spill pollutants) leads to production of ROS (Abele et al. 2011). Activity of the immune defense also induces ROS production. Indeed, activated phagocytic immune cells produce ROS and reactive nitrogen species (RNS) in an oxidative burst response to defend the organism against intruders (Abele et al. 2011). To avoid oxidative stress, a rapid detoxification by enzymatic and non-enzymatic antioxidants is required.

Together with the no-enzymatic antioxidants (carotenoids, flavonoids, vitamins C, A and E), are superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) important enzymatic antioxidants (Abele et al. 2011). The function of SOD is to catalyze the dismutation of superoxide anion (O_2^-) into O_2 and hydrogen peroxide (H_2O_2). There are different types of superoxide dismutase, Cu/Zn-SOD is localized in the cytosol and nucleus, while Mn-SOD is located within the mitochondrial matrix (Olsvik et al. 2005). Following, H_2O_2 is reduced to water by the enzyme catalase (CAT), or enzyme from the family glutathione peroxidase (GSH-Px) included GPx1. The activity of these enzymes are induced by the oxidative stress factors and the expressed levels of SOD, CAT and GSH-Px antioxidant genes have been used as biomarkers for the oxidative stress response in fish species (Olsvik et al. 2005).

Another stress defense marker in fish species is the heat shock response, which include an increased transcription of genes encoding heat shock proteins (HSPs), also referred to as "stress proteins". A variety of stressors promote the production of HSPs (e.g. inflammation, abiotic stressors and environmental contaminants) to enhance survival by protecting the critical cellular functions (Iwama et al. 1998). They are also consistently present in cells to maintain some primarily important cellular processes linked to protein folding and translocation (Iwama et al. 1998).

1.6 Impacts of dietary insects on the immune system

Generally in their natural environment, insect larvae live in a harsh environment, such as compost, animal waste (carrion), rotten plant material or manure, which are often infested with many types of microorganisms. The defence strategies of the insects living in these substrates result in the presence of many native bioactive peptides with diverse pharmacological properties, such as anti-microbial, anti-fungal, anti-viral and anti-tumoral functions (Sergey et al. 2002; Bulet et al. 1999; Elhag et al. 2017; Park et al. 2015). Furthermore, some insect species have been shown to contain components with potentially immunostimulating response in mammalian e.g.; A novel water-soluble polysaccharide named dipteriose was identified in the pupae of the melon fly (*Bactrocera cucurbitae*) as a molecule that activates the innate immune response in mouse macrophages (Ohta et al. 2014).

Only a few studies have investigated the effect of dietary insects on the immune system in fish species. Indeed, a recent study showed that feeding yellow catfish (*Pelteobagrus fulvidraco)* for 5 weeks with dietary inclusion level of between 18-75% of yellow worm meal (*Tenebrio molitor)* improved the immune response and bacterial resistance 24h post bacterial challenge, without negative growth effects (Su et al. 2017). Another study showed an anti-inflammatory effect on European sea bass (*Dicentrarchus labrax)*, with enhanced lysozyme antibacterial activity and serum trypsin inhibition, when *Tenebrio molitor* meal was given for 6 weeks (Henry et al. 2018). Similarly, in African catfish (*Clarias gariepinus*), replacement of fish meal by cricket meal (CM) for 40 days enhanced the innate immune system and disease resistance (Mohd Taufek et al. 2018). These studies demonstrated that insect meal might have a potential to impact the immune response in fish species.

One of the characteristics of the insect species is the polysaccharide chitin, derived from the exoskeletons. The amount of chitin varies among insect species and development stage. Some other chitin-containing organisms have also been described; crustaceans (crabs and shrimps, etc.), bacteria, fungi and parasitic nematodes (Lee et al. 2008). Mammalian, birds and fish do not synthetize chitin, so this non-self-molecule is a potential PAMP as target for PRRs. Immunological effect of chitin have been addressed in different fish species such as; (1) Gilthead seabream head kidney leucocytes were able to phagocytose chitin particles smaller than 10 µm, and activate the innate immunity as a consequence of prior incubation with chitin particles (Cuesta et al. 2003); (2) Feeding rainbow trout (*Oncorhynchus mykiss*) with dietary

BSF larvae meal increased incidence of bacteria of the *Carnobacterium* genus, a well-known probiotic that improves disease resistance in salmonids, and which is probably present in the chitin-exoskeletons of BSF (Bruni et al. 2018). However, at high levels in the fish diets, a negative effect on growth performances were seen due to an often-observed reduction in digestibility in diets high in chitin (Gilles et al. 2015). The high presence of chitin might cause low palatability of insect-based diet and has attributed to a reduced protein intake, and affected growth performance in juvenile turbot fed with dietary BSF meal (inclusion >33%) (Kroeckel et al. 2012).

A great diversity of anti-microbial peptides (AMPs) are found in insects species, where more than 150 insect proteins with antimicrobial activity have been identified (Gasco et al. 2018). AMPs have great microbial potential due to the problems related to antibiotic resistance. Some studies investigated the antimicrobial activity of BSF specifically, such as; (1) One novel AMP has been purified, characterized and showed to have potent activity against gram-positive bacteria, including Methicillin-resistant Staphylococcus aureus MRSA (Park et al. 2015); (2) Seven new gene fragments of three types antimicrobial peptides were established from BSF, named cecropinZ1, sarcotoxin1, sarcotoxin (2a), sarcotoxin (2b), sarcotoxin3, stomoxynZH1, and stomoxynZH1(a) (Elhag et al. 2017); (3) A different antimicrobial peptide (AMPs) and immune related genes expression, depending on the feeding media (higher level of AMPs expression when the larvae were fed with diets supplemented with protein or sunflower oil)(Vogel et al. 2018). However, further investigations are needed to assess the effect of dietary BSF larvae meal on the immune response.

In addition, depending on the substrate, some insects contain a high level of lauric acid (LA). Specifically the BSF prepupae contains up to 60% LA of total fatty acids (Spranghers et al. 2017). LA is a medium-chain fatty acid with 6-12 carbon chains, the principal constituents of medium chain-FA (MCT). These MCT have been widely investigated due to their rapid oxidation and absorption, their antimicrobial (activity against gram positive bacteria) and antiviral properties in gut microbiota (Skrivanova et al. 2005; Dierick et al. 2002). Indeed, *in vitro* and *in vivo* experimental trials with different inclusions levels of BSF larvae, containing a high level of LA, have been conducted in piglet. In this study supplementation of the culture media with LA suppresses growth of lactobacilli, with antibacterial effects against D-streptococci infections in piglet (Spranghers et al. 2018). Dietary fats are also known to affect

the human immune system, depending on quantity and which specific fatty acids are consumed (Fritsche 2015). Little is known about LA and other MCTs effects on fish immune system, and the antimicrobial properties of LA in fish species have to be investigated.

1.7 Objective of the thesis

This thesis is part of the large project AquaFly. The project's objective is to exploit the great potential of insects as highly efficient converters of under-used marine organic material to high value nutrient sources, tailor made for sustainable production of robust Atlantic salmon. As of July 1st 2017 the use of insect meal from; Black Soldier Fly (BSF), Common Housefly (CH), Yellow Mealworm (YM), Lesser Mealworm (LM), House Cricket (HC), Banded Cricket (BC) and Field Cricket (FC) in aqua-feed is allowed, and a tremendous increase in interest and investments in this sector is now seen.

The main goal of the master thesis is to investigate how a partial or total replacement of dietary fishmeal by BSF insect meal affects gene expression involved in inflammation, stress and signalling pathways in head kidney leucocytes, isolated from Atlantic salmon fed a control diet (control) or diets containing insect meal (IM-66 and IM-100). The experimental feeding period lasted for 54 days. Isolated leukocytes were seeded into culture wells and added bacterial mimic lipopolysaccharide (LPS) or viral mimic polyinosinic acid: polycytidylic acid (poly I: C) to induce inflammatory response. Controls without LPS and poly I: C were also included. Gene expression was measured for genes involved in the pro/anti-inflammatory response, eicosanoid pathway related genes and oxidative stress related genes, using quantitative real time RT- qPCR (qPCR).

2. MATERIALS AND METHODS

The experiment was part of a large project financed by: the Norwegian Research Council project AquaFly, grant number 238997 and RAFFPINN, grant number 220634.

2.1 Diets

Insect Meal (IM) used in this study was produced from black soldier fly (BSF) larvae by Protix Biosystems BV (Dongen, The Netherlands). The larvae were grown on media containing grounded seaweed (*Ascophyllum nodosum*) mixed with organic waste (50:50). More details on the rearing and the chemical composition of the BSF larvae are presented in (Liland et al., 2017). At the end of an eight-day growth period, the larvae were mechanically separated from the feeding media, washed and processed immediately to separate IM (Protix Biosystems BV). The experimental diets (Table 2.1) were formulated and produced by Cargill (Dirdal, Norway), and supplemented with 1 % yttrium oxide as an inert digestibility marker. Directly after production, the diets were shipped to the experimental facility and stored at -20 °C until they were fed to the fish. The control diet (IM-0) represent a modern sea-water salmon diet, with proteins from fish meal (FM) and plant-based proteins (25:75) and lipids from fish oil (FO) and vegetable oil (VO) (33:66). Two experimental diets were formulated, wherein 66 and 100% of FM protein source was replaced with IM. The diets were balanced to contain sufficient essential AA (methionine and lysine were added) and some additional FO was included in the diets without FM to provide sufficient LC-PUFAs (Table A.2).

Table 2.1: Formulation and proximate composition of the three experimental diets fed to sea-water Atlantic salmon (*Salmo salar*).

	IM-0	IM-66%	IM-100%
Ingredients (%)			
Fishmeal LT94	10	3.33	0
BSF meal	0	9.84	14.75
SPC	25	25	25
Corn gluten meal	7.5	7.5	7.5
Wheat gluten meal	3.35	5.7	6.88
PPC 55	8.8	4.8	2.84
Fish oil	10.18	13.23	14.76
Veg oil	20.95	16.79	14.73
Binder	12.32	11.72	11.24
Addetives	1.89	2.1	2.29
Yttrium	1	1	1
Proximate analysis			
Dry matter (%)	93	94	95
Crude Protein (%)	38	39	39
Crude Lipid (%)	29	29	29
Ash (%)	4.6	4.5	4.5
Carbohydrates (%)	11.6	11.5	11.4
Gross energy (MJ/kg DM)	24.6	24.8	25.0
TBARS (nmol g^{-1})	3.0	4.2	4.9

BSF=black soldier fly; SPC= soy protein concentrate; PPC= pea protein concentrate; TBARS: Thiobarbituric acid-reactive substances.

2.2 Experimental design and facilities

The feeding trial was run at Gildesål Research Station (GIFAS) in Langholmen, Inndyr, Norway. The Atlantic salmon used in the trial come from the same generation (16G) Aqua Gen strain. Sea-water Atlantic salmon were randomly distributed into 12 cages 125 m^3 (5x5x5m) with 90 fish in each cage with a water temperature of 6º C. The fish were fed one of the three diets (*Table 2.1*)**.** Each diet was distributed by hand until visual satiation, two times per day with minimum 4 hours between the meals. Uneaten feed was collected and deduced from the total daily feed calculation.

Figure 2.1: The facilities at Gildesål Research Station (GIFAS). Private photo.

2.3 Sampling

Fish were collected at the start (7th August, 2017) (≈1200 g), at the middle (3rd October, after 54 days) (≈ 2500 g) and at the end of the trial (4th December, 2017, after 113 days) (≈4200 g). In the current thesis, all the samples were collected during the middle sampling. A total of eighteen fish, two per cage (randomly selected) were killed using a percussive blow to the head, individually weighed and body length measured.

2.4 Culture medium and reagent

L-15 medium was supplemented with 10% fetal bovine serum (FBS) (BioWhittaker, cat#14-801F), 1% glutamax (Gibco, cat#35056), 1% Antibiotic Antimycotic (Penicillin-Streptomycin 50U/mL, Bio- Whittaker, cat#17-602E), and was designated as a complete medium, cL-15. Lipopolysaccharide, specific for the outer membrane of Gram-negative bacteria, was derived from *Pseudomonas aeruginosa* (LPS, cat# L-7018) used as *in vitro* bacterial mimic. Polyinosinic: polycytidylic acid (poly I: C, cat# P9582) with structurally similarity to double-stranded RNA was used *in vitro* to simulate viral infection.

LPS and poly I: C have been used in aquaculture to reduce mortality and improve fish performance (Sakai 1999). These two immunostimulants are widely used in mammals and in different fish species, as *in vitro* models of inflammatory responses, specifically the expression of regulatory genes of the innate immune response (Chettri et al. 2011; Holen et al. 2018).

2.5 Isolation of leukocytes, head kidney

From each Atlantic salmon, head kidney was removed with a sterile spoon and added to a sterile isolation buffer (Table A 3), and then kept in a petri dish on ice to keep the tissue cold. After cutting the head kidney with a scissors, the cells were aspirated with a 2ml syringe to digest and squeeze the tissue through a 100 µm cell strainer. The cells were after this step transferred to 50 ml tubes with a new isolation buffer and washed by centrifugation in a Hettich Zentrifugen (600 g, 5 min, 4 ºC). After the centrifugation, cell pellets were re-suspended in a new isolation buffer.

After the first washing step, the cell suspension contained both erythrocytes and leukocytes, which are both produced by the head kidney in fish. A percoll-gradient (density=1.08 g/ml) (Table A 3) was used to separate the erythrocytes from the leukocytes. Diluted cell suspension was layered carefully in equal amounts on top of six 50 ml tubes, containing each 10 ml of Percoll-gradient solution (Figure 2.2). The gradients tubes were centrifuged (600 g, 5 min) in order to separate the erythrocytes (on the bottom) from the leucocytes (on the top) via the density differences between the types of cell and the percoll-gradient. The leucocytes at the top were collected with a Pasteur pipette, and an additional washing step with isolation buffer was performed before dilution the cells in 50 ml tubes with cL-15 medium and transported to the laboratory on cold-ice.

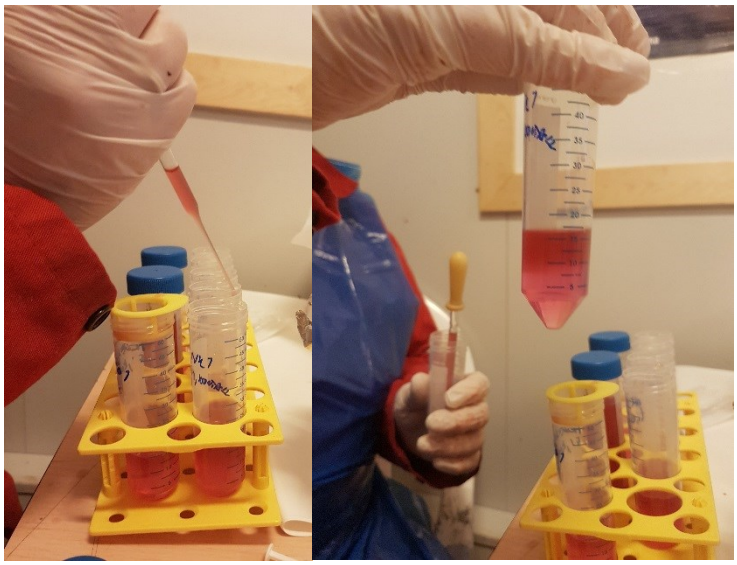


Figure 2.2: Cell suspension carefully layered in equal amounts on top of 50 ml tubes filled with 10 ml Percoll-gradient solution.

2.6 Cell culture calculation and viability

After transportation, another centrifugation was performed in order to re-suspend the cells with new cL-15 medium. The cells were counted using Bürcher chamber, with 20µl of cells in suspension + 0.4% trypan blue (BioWhittaker, cat#17-942E). The viability of the head kidney cells was assessed and cell preparations with viability less than 85% were not processed further. The counting was also used to calculate the amount of cell-suspension needed for each well, in order to assure the same number of cells in all the wells. $1x10^7$ of isolated leucocytes from the same fish were added to 6 well Costae cell-culture plates (CLS-3506, Sigma-Aldrich) and cL-15 medium was added to a final volume of 2 ml.

The cell culture was incubated for 2 days in the dark in a normal atmosphere incubator (Sanyo Incubator) at 9 ºC. The second day, after 24 h of culturing, selected wells (A2 and B2, Figure 2.3) received 100 mg/mL LPS and other selected wells (A3 and B3) received 100 mg/ml Poly I: C . Untreated cultures were included as the controls (A1 and A2). Treated and untreated cells were incubated for an additional 24 h in the incubator device before harvesting for further analysis.

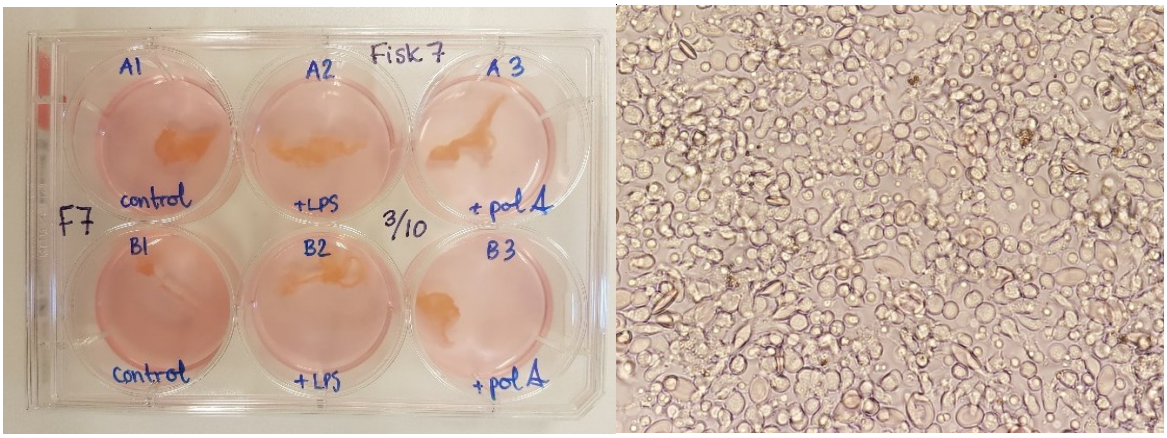


Figure 2.3: Left: 1x107 of isolated cells from fish number 7 added to 6 well culture plate. All wells were added cells and cL-15 medium to final volume of 2 ml. Duplicates with controls (A1, A2), 100 mg/ml LPS after 24 h of culturing (A2, B2) and 100 mg/ml poly I: C treatment after 24h (A3, B3). Right: microscopic photograph of isolated leukocytes cultured after 24h of incubation.

2.7 Cell culture harvesting

Both the stimulated and the untreated head kidney cells were harvested 2 days after seeding into wells. For eicosanoid measurements, the cell suspensions (A1-A3, Figure 2.3) were centrifuged and supernatants were collected and stored at –80 ºC. The pelleted cells left after this step were collected for RNA extraction. The cell pellet was homogenized in 600µl RTL-Plus buffer (RNeasy Plus kit Qiagen) using a syringe and needle for homogenizing 3-4 times and then frozen at -80 ºC. Identical cell-cultures in well B1-B3 were sampled for protein detection. For this method, cells were washed in PBS (table x) and pelleted by centrifugation before dissolving the pellet in 300µl CelLytic reagent (Sigma C2978). The lysates were centrifuged (15000 g, 15 min) to pellet the cellular debris, and then frozen at -80 ºC for further processing using Western Blot method.

2.8 RNA extraction

This procedure was performed under sterile conditions to avoid contamination of the samples. Working table was treated with RNase Zap® (Sigma- Aldrich, Missouri, USA) and plastic gloves (Sempermed, Wien, Germany) and a clean lab coat was used to avoid contamination from ribonuclease (RNase), an RNA degrading enzyme.

Total RNA was extracted using RNeasy Plus Mini kit according to the manufacturer`s protocol; RNeasy Plus mini Handbook. The lysate was slowly melted on ice before centrifugation (max speed, 3 min). Ethanol was added to the lysates, which promote a selective binding of RNA to

the RNeasy membrane, and transferred to a gDNA spin column placed in a 2ml collection tube. This step cleans the sample for DNA by centrifugation (10 000 rpm, 30s) to get the RNA to flow through the column. 600 µl of ethanol (70%) was added to the flow-through and then transferred to RNeasy Mini spin column that binds the RNA. The ethanol flows through the column, and the RNA binds to the membrane. 500 µl of the buffer RPE (RNeasy Plus kit Qiagen) was used x2 to wash the RNA from ethanol before collecting the RNA in a new 1.5 ml collection tube using RNase-free water. All steps were performed by centrifugation in a Hettich Zentrifugen.

2.9 RNA quality

The concentration of RNA after the extraction was measured with Nanodrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The samples used in this experiment had 260/280 nm absorbance ratios of 2.0 ± 0.1 and 260/230 nm ratios of 2.4 ± 0.2. The quality of 12 random selected RNA samples was analysed by RNA6000 Nano LabChip kit, run on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA Integrity Number (RIN) for the RNA range from 1-10 and samples with a RIN value of >8.0 is considered adequate for use in RT-PCR reactions (Imbeaud et al. 2005). A sample with a poor RIN value will indicate degradation of the total RNA in the sample. In addition to the RIN value, the Agilent 2100 BioAnalyzer gives a gel photo and an electropherogram (graph). RIN values of 10 in all the 12 selected RNA samples indicate that the RNA from the head kidney (HK) samples were suitable for RT-qPCR.

2.10 Reverse transcription polymerase chain reaction (RT-PCR)

The enzyme "reverse transcriptase" was used to convert RNA template into the more stable cDNA (complementary DNA) that is used for quantitative qPCR. A pool of all the 50 samples was used to make standard curve with six serial dilutions from 31 to 1000 ng and run in triplicates into a 96 well PCR plate (VWR, AB-06000). The rest of the samples were diluted individually with sterile water free from RNase into a concentration of 50 ng/µl and set up in duplicates in two 96-well PCR plates (10 µl /well) as seen in Figure A 1 . A RT-reaction mix (Table A4) was prepared with the kit TaqMan reverse transcription reagents containing Multiscribe Reverse Transcriptase (50 U/mL) (Applied Biosystems, Foster City, CA, USA) and

added 40 µl/well RT-reaction mix to the diluted RNA samples in the two 96-well PCR plates to a total volume of 50 µl/well. Two negative controls were also run, one non amplification control (nac) without enzymes and one non-template control (ntc) with RNase free water instead of RNA. The 96-well PCR plate setup are showed in *Figure A 1*. The RT reaction was performed with a GeneAmp PCR System 9700 machine at following program; An incubation step for 10 min at 25 °C, continuing with RT reaction at 4°C for 60 min by using oligo dTprimers (2.5 mM) in 50 mL total volume, and finally the inactivation in 5 min at 95 °C. The controls were used to ensure that the kits and water were free for contaminants. PCR plate was stored at –20°C until running qPCR.

2.11 Quantitative real time RT- qPCR (qPCR)

The cDNA plates from the RT-reaction functioned as a template for the qPCR. The following method was used to give a relative quantification of the target gene by measuring cDNA amplification and fluorescence. The gene expression was quantified with a Light Cycler 480 instrument (Roche Applied Sciences, Basel Switzerland). The 50 µl volume in the cDNA plate was diluted with milliQ water into a final volume of 100 µl per well. The PCR plate was centrifuged (1000 x g, 1 minute) and then vortexed (1300 rpm, 5 min) to ensure homogenized samples.

A reaction mix was made for the RT-qPCR (Table A 5) and a pipetting robot was used to transfer 2 µl /well from the cDNA-plate and 8 µl /well reaction mix into a 384 wells Real Time plate. The finished Real Time plate was then covered with an optical adhesive cover without touching the film, and centrifuged (1500 x g, 2 min) before running the qPCR on following program; 5 min activation and denaturizing step at 95 °C followed by 45 cycles of 10s denaturizing step at 95°C, 10s annealing step at 60°C and a 10s synthesis step at 72°C, followed by a melt curve analysis and cooling at 40°C. RT-qPCR program is described in *Table A 6.*

2.12 Statistics

The stability of reference genes was calculated by the program geNorm version 3,5 as the M value. The two reference genes Elongation factor 1α and β-actin got a satisfactory M value of 0.517 and 1.017 in this trial. The reference genes were used to determine the normalization factors. Ct values of each target genes from qPCR were used to calculate the normalized gene expression with its respective normalization value. From this, a gene expression normalization factor was made for each sample. The expression values were re-calculated by dividing each value by its respective normalization factor, giving the MNE- value (mean normalized expression).

Microsoft Excel was used to treat raw data from the feeding trial. Ct-values from the qPCR were imported into Excel where interplate normalization, MNE- value, relative quantities and standard deviations were calculated.

All statistical analyses were performed using the free software "environment R" (R Development Core Team, 2011). The experiment was designed to use a 2x2-way factorial ANOVA design with diets (IM-0, IM-66 and IM-100%) and treatment (Control, LPS and poly I: C) as varying factors. Differences due to dietary treatments were detected by two-way ANOVA and Tukey's post hoc test using the packages *nlme (Pinheiro et al. 2010)* and *multcomp* (Hothorn et al. 2008). All data were tested for homogeneity of variance by Levene's test. Data, which were identified as non-homogeneous, were subjected to a non-parametric analysis (Kruskal Wallis test) (Giraudoux 2011). Data are presented as mean with standard deviation (SD) and a significance level of 95% was used ($P < 0.05$). Figures were made using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA).

3. RESULTS

3.1 Growth

At the start of the trial, the fish had a mean weight of ~1200g (Figure 3.1). After 54 days, the fish grew to similar final weights (~2500g) (Figure 3.1) and there were no significant differences in growth observed between the three dietary groups.

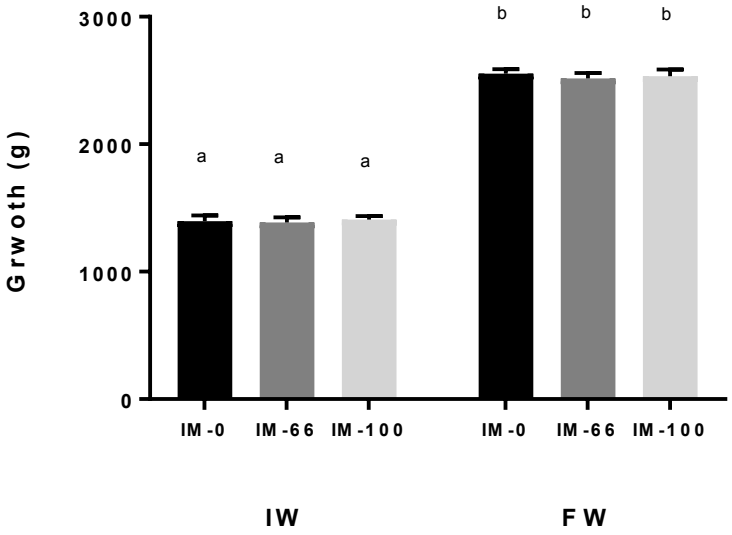


Figure 3.1 **Growth of Atlantic salmon fed control diets (IM-0) or diets containing insect meal IM (IM-66 or IM-100)**. Different letters (a,b)= statistically significant differences between dietary groups. IM-0 = diets without IM inclusion: protein from fish meal (FM) and vegetable protein (VP); IM-66= 66% of FM is replaced with IM; IM-100= complete replacement of FM with IM. IW= initial weight; FW= final weight, after 54 days.

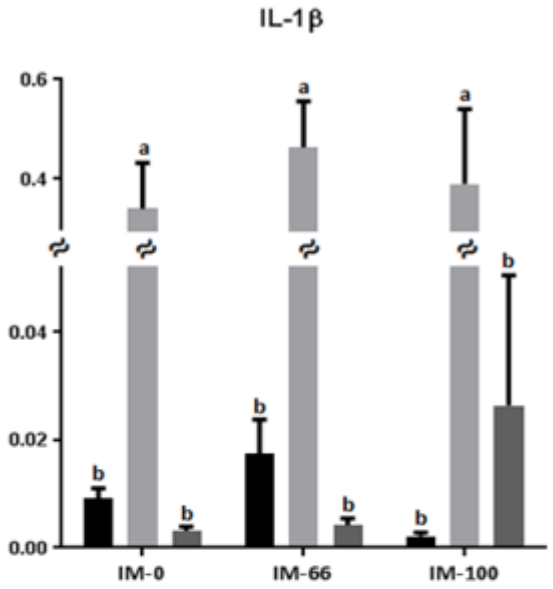
3.2 Inflammatory related gene expression (IL-1β, IL-8, IL-10, TNF-α)

The transcription of inflammatory related genes interleukin IL-1β, IL-8, IL-10 and TNF-α was significantly affected by the treatment (two way ANOVA, $P<0.0001$, $P<0.0001$, $P<0.001$, $P<0.001$ respectively), but not by the diets and no significant interactions were shown.

Treatment effect: The inflammatory related genes interleukin IL-1β, IL-8, IL-10 and TNF-α transcriptions were all significantly up-regulated in LPS treated leukocytes isolated from salmon fed the three dietary groups (IM0, IM66 and IM100), when compared to the non-treated cells (Control) ($P=0.000000003$, $P=0.0000000006$, $P=0.0006$, $P=0.00001$, respectively) and compared to poly I: C treated cells ($P=0.0000001$, $P=0.00001$, $P=0.001$, $P=0.00001$, respectively).

LPS>control	poly I: C=Control	poly I: C<LPS

Cell treatments	<0.0001
Dietary treatments	NS
Interaction	NS



Cell treatments	<0.0001
Dietary treatments	NS
Interaction	NS

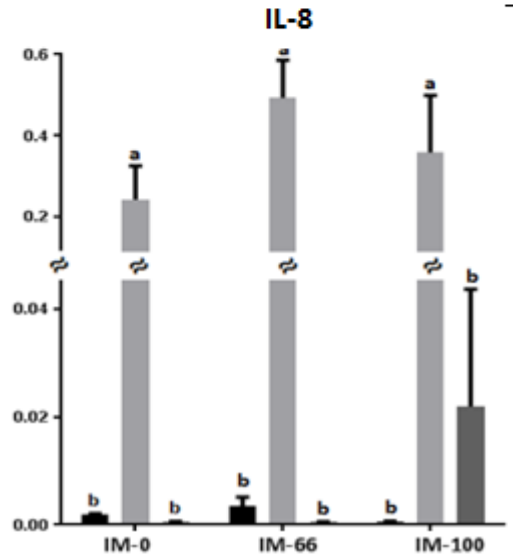
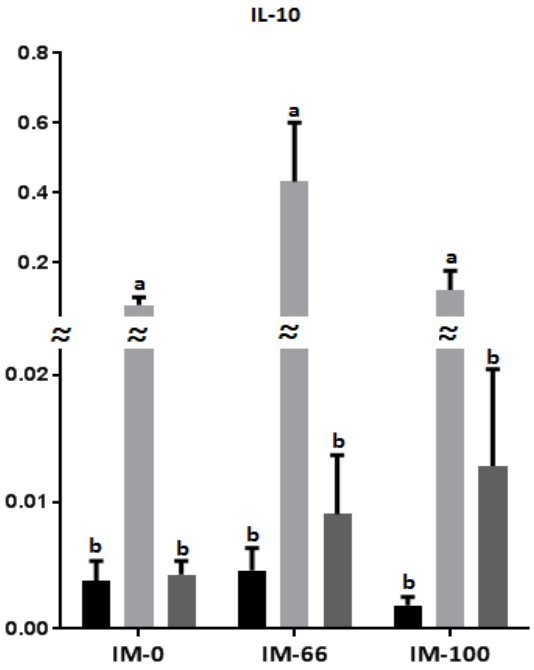


Figure 3.2: **Inflammatory related genes interleukin 1b (IL-1β,) and IL-8 gene expression in leukocytes isolated from head kidney of Atlantic salmon fed control or insect based diets.** Significant up-regulated transcription in LPS treated leukocytes compared to control and poly I: C treated cells. No dietary effects. Different letters (a,b) = statistically significant differences between cell treatments, P<0.05. Cell treatments = control, LPS and poly I: C; diets = IM-0 (without insect meal inclusion), IM-66 and IM-100; interaction between cell treatments and experimental diets.

Cell treatments	<0.001
Dietary treatments	NS
Interaction	NS



Cell treatments	<0.001
Dietary treatments	NS
Interaction	NS

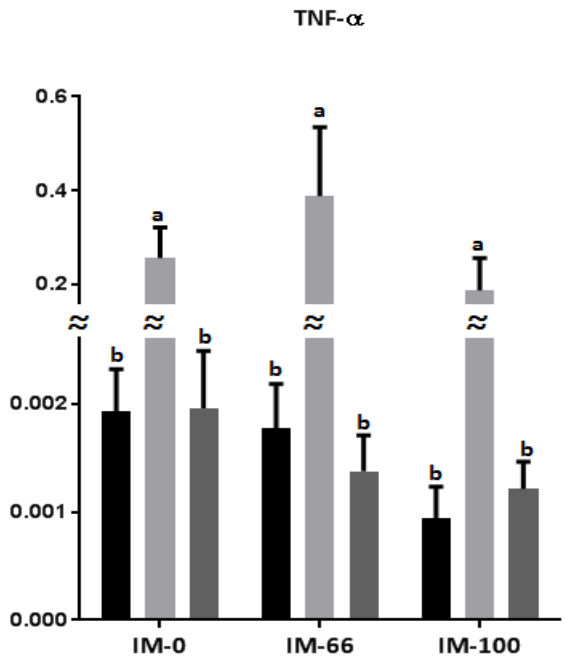


Figure 3.3: **Inflammatory related genes TNF-α and IL-10 gene expression in leukocytes isolated from head kidney of Atlantic salmon fed control or insect based diets.** Significant up-regulated transcription in LPS treated leukocytes compared to control and poly I: C treated cells. No dietary effects. Different letters (a,b) = statistically significant differences between treatments, P<0.05. Cell treatments = control, LPS and poly I:C; dietary diets = IM-0 (without insect meal inclusion), IM-66 and IM-100; interaction between cell treatments and experimental diets.

3.3 Toll-like receptor 22 (TLR22) and transcription factor involved in metabolism and inflammation (CEBpb)

The transcription of TLR 22 was significantly affected by both the treatments and the diets (two way ANOVA, P<0.001 and P=0.001, respectively). No interaction was shown between treatments and diets.

- **Treatment effect TLR 22:** The transcription of this gene was significantly lower in poly I: C treated cells isolated from salmon fed with the three diets (IM-0, IM-66 and IM-100) than the non-treated cells (Control) (P=0.02). Cells treated with LPS were almost, but not significantly lower expressed than the control treatments group (Control) (P=0.058).

LPS=control	poly I: C< Control	poly I: C=LPS

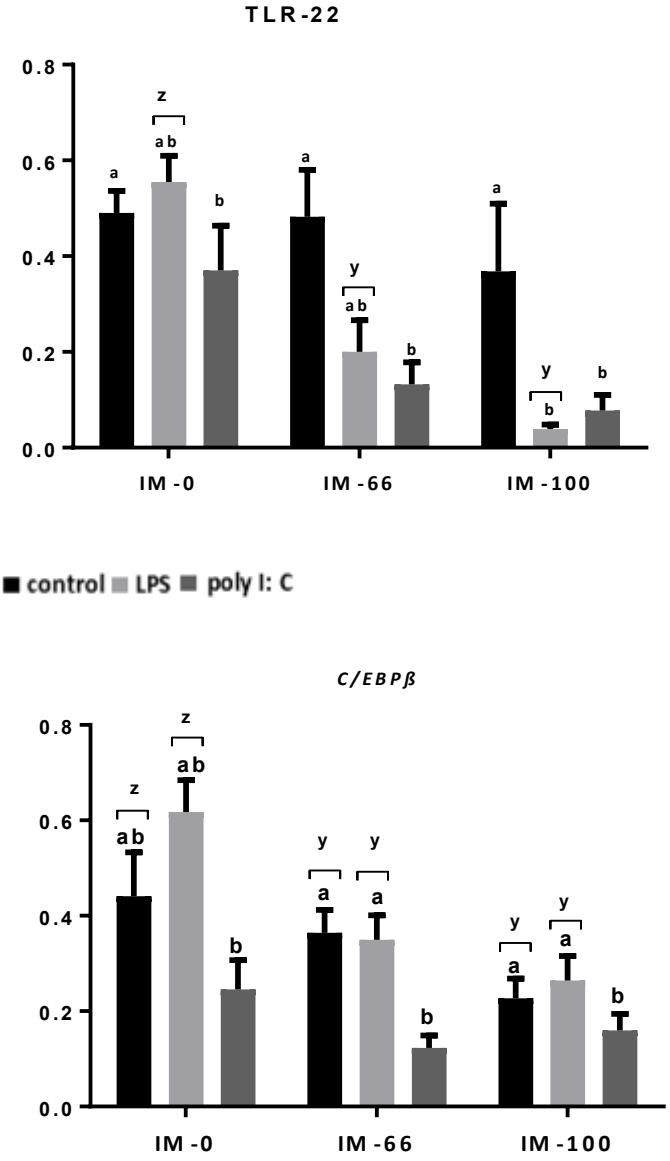
- **Diet effect TLR22:** Head kidney leukocytes isolated from fish fed with IM-66 and IM-100 had lower gene expression of TLR22 compared to the control diet (IM-0) in LPS treated cells (P<0.001).

Similarly, the transcription of the C/EBPβ gene was significantly affected by both the treatments and the diets (two way ANOVA, P<0.001 and P=0.001, respectively). No interaction was shown between treatments and diets.

- **Treatment effect C/EBPβ:** The transcription of this gene was significantly down-regulated in leukocytes treated with poly I: C (P=0.026) compared to the non-treated cells (control), but not significantly different compared to LPS treated cells.

LPS=control	Poly I: C< Control	poly I: C=LPS

- **Diet effect C/EBPβ:** Head kidney leukocytes isolated from fish fed with IM-66 and IM-100 had lower gene expression of C/EBPβ compared the control diet (IM-0) in LPS treated cells (P<0.0001) and in the non-treated cells (Control) (P=0.03).



Cell treatments	<0.001
Dietary treatments	0.001
Interaction	NS

Cell treatments	<0.001
Dietary treatments	<0.001
Interaction	NS

Figure 3.4**: Toll-like receptor 22 (TLR22) and transcription factor (C/EBPβ) gene expression in leukocytes isolated from head kidney of Atlantic salmon fed control or insect based diets**. TLR22 and C/EBPβ transcriptions were significantly lower in poly I: C treated compared to Control and poly I: C treated cells. In addition, head kidney leukocytes isolated from fish fed with IM-66 and IM-100 had lower gene expression of TLR22 compared to the control diet (IM-0) in LPS treated cells. Different letters a,b and y,z= statistically significant differences between treatments and dietary effects, respectively, P<0.05. Cell treatments = control, LPS and poly I: C; dietary treatments = IM-0 (without insect meal inclusion), IM-66 and IM-100; interaction between cell treatments and dietary treatments.

3.4 Gene expression related to eicosanoid pathway (5-LOX, PTGDS, PTGES)

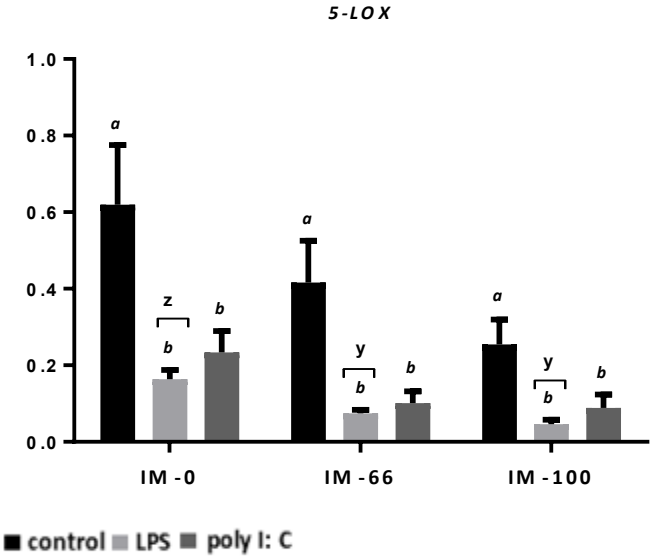
The transcription of 5-lipoxygenase enzyme (5-LOX) was significantly affected by both the treatments and the diets (two way ANOVA, $P<0.001$ and $P<0.001$, respectively).

- **Treatment effect 5-LOX:** The transcription of this gene was significantly down-regulated in LPS and poly I: C treated cells isolated from salmon fed with the three diets (IM-0, IM-66 and IM-100) compared to the non-treated cells (Control) ($P<0.001$).

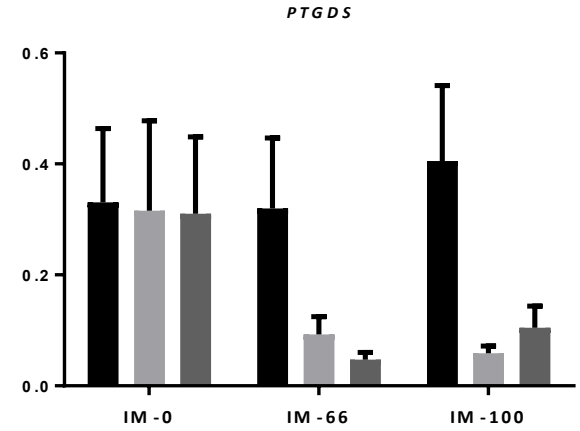
LPS<control	poly I: C< Control	poly I: C=LPS

- **Diet effect 5-LOX:** Head kidney leukocytes isolated from fish fed with IM-66 and IM-100 had lower gene expression of 5-LOX compared the control diet (IM-0) in LPS treated cells ($P=0.01753$).
- No interaction was shown between treatments and diets.

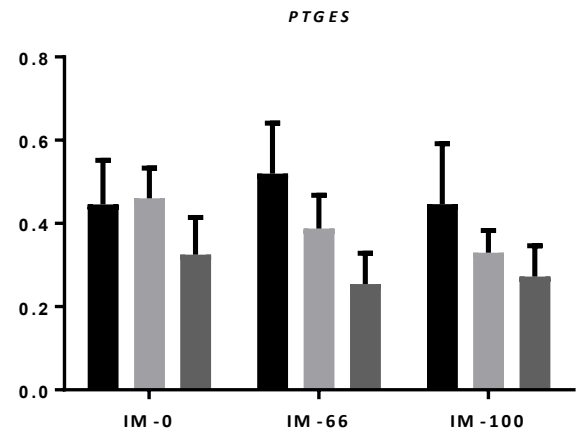
The transcriptions of prostaglandin synthase PTGDS and PTGES were not affected by the treatments or the diets (two way ANOVA, $P>0.005$).



Cell treatments	<0.001
Dietary treatments	<0.001
Interaction	NS



Cell treatments	NS
Dietary treatments	NS
Interaction	NS



Cell treatments	NS
Dietary treatments	NS
Interaction	NS

Figure 3.5: **Eicosanoid related 5-lox, PTGDS and PTGES gene expressions in leukocytes isolated from head kidney of Atlantic salmon fed control or insect based diets.** 5-LOX transcription was significantly down-regulated in LPS and poly I: C treated compared to the non-treated cells (Control), and the cells isolated from salmon fed with IM-66 and IM-100 had a lower gene expression of 5-LOX compared to the control diet (IM-0) in LPS treated cells. The transcriptions

of PTGDS and PTGES were not affected by the treatments or the diets. Different letters a,b and y,z= statistically significant differences between treatments and dietary effects, respectively, $P<0.05$. Cell treatments = control, LPS and poly I: C; dietary treatments = IM-0 (without insect meal inclusion), IM-66 and IM-100; interaction between cell treatments and dietary treatments.

3.5 Stress related gene expression (SOD, GPx1, HSP 27, HSP 70)

The transcription of superoxide dismutase SOD was significantly affected by both the treatments and the diets (two way ANOVA, P<0.001 and P=0.01, respectively). No interaction was shown between treatments and diets.

- **Treatment effect SOD:** The transcription of this gene was significantly down-regulated in poly I: C treated cells isolated from salmon fed with the three diets (IM-0, IM-66 and IM-100) compared to the non-treated cells (Control) (P<0.001) and to LPS-treated cells (=0.04)

LPS=control	poly I: C< Control	poly I: C<LPS

- **Diet effect SOD:** Head kidney leukocytes isolated from fish fed with IM-66 and IM-100 had down-regulated gene expression of SOD compared to the control diet (IM-0) in LPS treated cells (P=0.001).

The transcription of Glutathione peroxidase enzyme GPx1 was significantly affected by both the treatments and the diets (two way ANOVA, P<0.001). No interaction was shown between treatments and diets.

- **Treatment effect GPx1:** The transcription of this gene was significantly down-regulated in poly I: C treated cells isolated from salmon fed with the three diets (IM-0, IM-66 and IM-100) compared to the non-treated cells (Control) (P=0.005) and to LPS-treated cells (P<0.001)

LPS=control	poly I: C< Control	poly I: C<LPS

- **Diet effect GPx1:** Head kidney leukocytes isolated from fish fed with IM-66 and IM-100 had down-regulated gene expression of GPx1 compared to the control diet (IM-0) in LPS treated cells (P<0.001) and in non-treated cells (Control) (P=0.026).

The transcription of heat shock proteins HSP27 was significantly affected by the treatments, but not by the diets (two way ANOVA, P=0.03). No interaction was shown between treatments and diets.

- **Treatment effect HSP27:** The transcription of this gene was significantly down-regulated in poly I: C treated cells isolated from salmon fed with the three diets (IM-0, IM-66 and IM-100) compared to the non-treated cells (Control) (P=0.0282).

LPS=control	poly I: C< Control	poly I: C=LPS

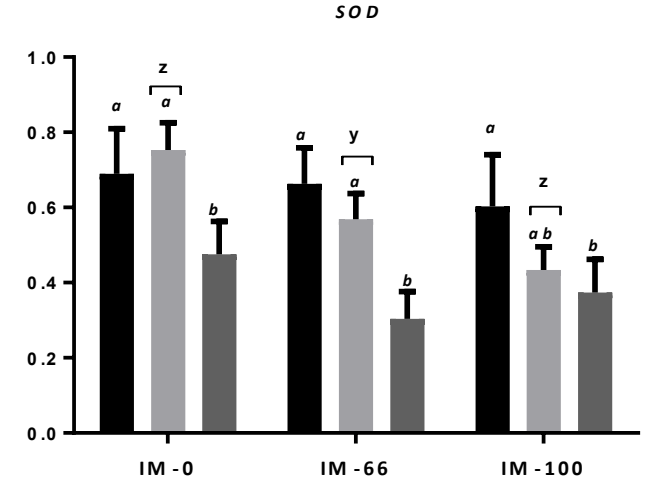
- **Diet effect HSP27:** No diet effects were shown.

The transcription of heat shock proteins HSP70 was significantly affected by both the treatments and the diets (two way ANOVA, P=0.002 and P=0.05, respectively). No interaction was shown between treatments and diets.

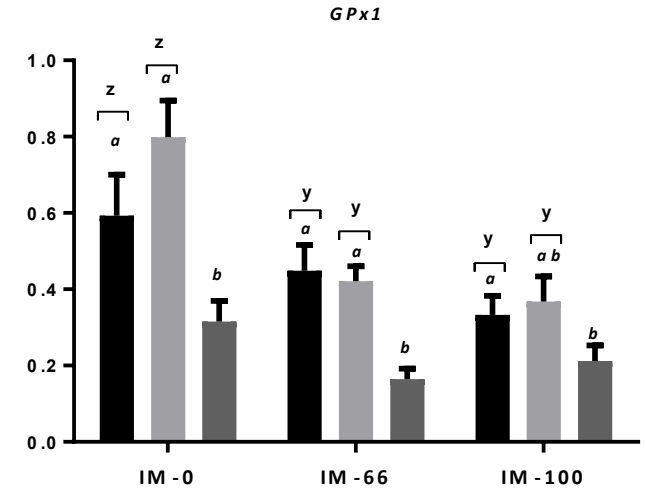
- **Treatment effect HSP70:** The transcription of this gene was significantly down-regulated in poly I: C treated cells isolated from salmon fed with the three diets (IM-0, IM-66 and IM-100) compared to the non-treated cells (Control) (P=0.0068).

LPS=control	poly I: C< Control	poly I: C=LPS

- **Diet effect HSP70:** Head kidney leukocytes isolated from fish fed with IM-66 and IM-100 had down-regulated gene expression of HSP70 compared to the control diet (IM-0) in LPS treated cells (P=0.0085)

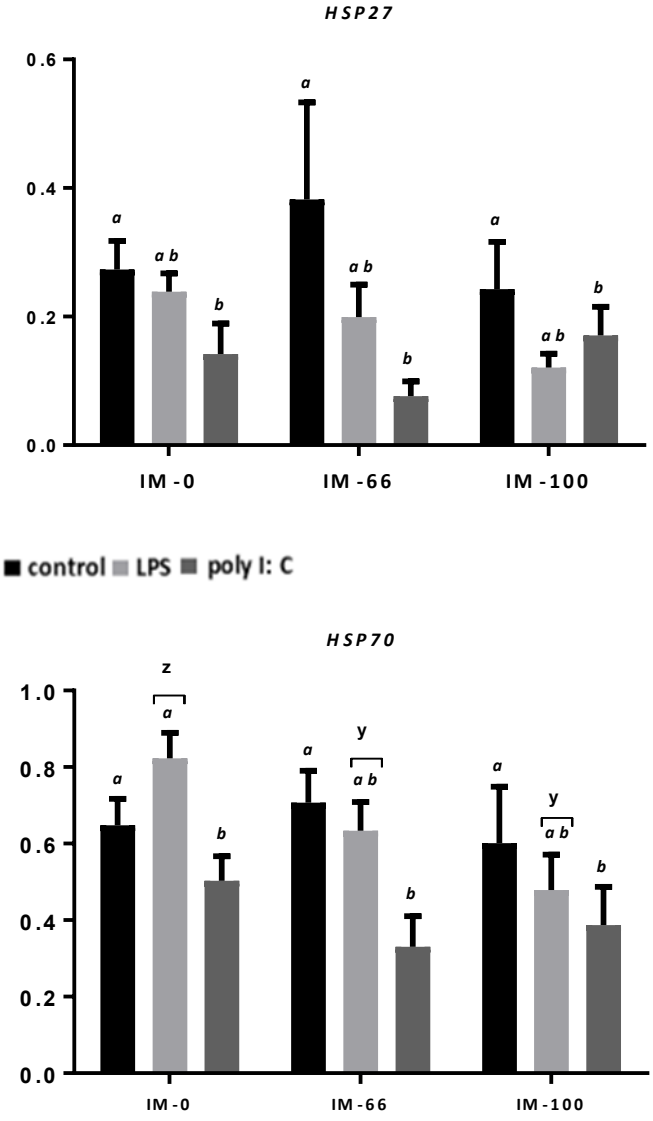


Cell treatments	<0.001
Dietary treatments	0.01
Interaction	NS



Cell treatments	<0.001
Dietary treatments	<0.001
Interaction	NS

Figure 3.6: **Stress related genes Cu/Zn SOD and GPx1 gene expressions in leukocytes isolated from head kidney of Atlantic salmon fed control or insect based diets**. SOD and GPx1 transcriptions were significantly down-regulated in poly I: C treated cells compared to the non-treated cells (Control). The expressions of these two genes were down-regulated in leukocytes of fish fed with IM-66 and IM-100 compared to control diet (IM-0). Different letters a,b and y,z = statistically significant differences between treatments and dietary effects, respectively, $P<0.05$. Cell treatments = control, LPS and poly I:C; dietary treatment = IM-0 (without insect meal inclusion), IM-66 and IM-100; interaction between cell treatments and dietary treatments.



Cell treatments	0.03
Dietary treatments	NS
Interaction	NS

Cell treatments	0.002
Dietary treatments	0.05
Interaction	NS

Figure 3.7: **Stress related genes, heat shock proteins HSP 27 and HSP 70 gene expressions in leukocytes isolated from head kidney of Atlantic salmon fed control or insect based diets.** HSP27 and HSP70 transcriptions were significantly down-regulated in poly I: C treated cells compared to the non-treated cells (Control). Fish fed with IM-66 and IM-100 had down-regulated gene expression of HSP70 compared to the control diet (IM-0) in LPS treated cells. Different letters (a,b)= statistically significant differences between treatments (z,y) = statistically significant differences between the diets, $P<0.05$. Cell treatments = control, LPS and poly I:C; dietary treatment = IM-0 (without insect meal inclusion), IM-66 and IM-100; interaction between cell treatments and dietary treatments.

4. Discussion

The current study evaluated the effects of diets containing BSF larvae meal in partial or total replacement of fish meal on the immune responses in head kidney leukocytes of sea-water Atlantic salmon. After the experimental feeding period of 54 days, the fish had grown to approximatively two-fold of the initial body weight, and no differences between the insect-based diet and the control group were shown. These results are in consistence with previous trial, using BSF protein meal or whole insect meal as replacement for FM in diets for different fish species (Belghit et al. 2018; St-Hilaire et al. 2007; Renna et al. 2017), and demonstrated that the larvae meal of BSF might be a promising alternative to fish meal in aqua-feed.

After ensuring that all the dietary groups had grown similarly and had adapted to the experimental diets (8 weeks), the leukocytes were isolated from the head kidney of the salmon. The responses to viral and bacterial pathogens represented by poly I: C and LPS, respectively, in head kidney leukocytes have been demonstrated for different fish species, e.g Atlantic cod and Atlantic Salmon (Holen et al. 2012; Holen et al. 2018). In the following sections, we highlight the main effects of partial and total replacement of FM by IM in the diets on gene expressions involved in inflammatory, eicosanoids pathways and stress responses.

4.1 Effects of LPS and poly I: C treatment on inflammatory gene transcriptions

In this study, exposure to the bacterial mimic (LPS) produced upregulation of the pro-inflammatory IL-1β, IL-8, TNF-α, and the anti-inflammatory IL-10. These results confirmed the suitability of the *in vitro* model used in the current experiment, as these cytokines act to mediate resistance to infections. However, compared to the bacterial mimic, the responses of the selected inflammatory related genes were slightly induced after exposure to the viral poly I: C. These results are consistent with previous studies in head kidney cells isolated from Atlantic cod, where it has been shown that the immune response in fish to either bacteria or virus mimic act through different pathways, and the expression of these genes are different in Poly I: C than LPS treated cells (Holen et al. 2012). Many of the common diseases in salmonid aquaculture per today are viral diseases, and this is an important topic for further investigation.

Conversely, Fierro-Castro et al., showed that the genes related to the innate immune system were upregulated in the head kidney macrophages isolated for rainbow trout after exposure to Poly I: C (Fierro-Castro et al. 2013). In this study, the authors exposed the cells to the immunostimulants (LPS and poly I: C) during 4 and 24 h in 18 °C. The highest immune-related gene expression was observed after 4h, rather than the 24h of exposure to 100 µg/ml Poly I: C (Fierro-Castro et al. 2013). In our study, Poly I: C was also added at 100 µg/ml in the head kidney cells, however exposed for 24h to Poly I: C and in a different temperature of 9 °C. Therefore, the differences observed between the cells isolated from rainbow trout and the head kidney cells of the current trial, could be due to the time of exposure to poly I: C, different species and the temperature.

In the present study, the pro-inflammatory gene IL-6 was not expressed in the head kidney leucocytes isolated from Atlantic salmon, even after LPS exposure (24h after culturing). Chettri et al. showed in their study that IL-6 expression increased in leukocytes isolated from rainbow trout after 4h of LPS exposure, but the expression decreased drastically following 12h of exposure (cultured in 15 °C) (Chettri et al. 2011). The authors associated the changes in expression of IL-6 gene at the later phase of exposure (12h) with the expression of the putative regulatory cytokine, IL-10. They showed a supressing effect on the pro-inflammatory IL-6 after 4h when IL-10 gene increased significantly, and it is today known that fish have a similar cytokine feedback mechanism as described in mammals (Jun and Christopher 2016). Our study

showed as mentioned an activity in IL-10 gene after 24h exposure to LPS. The time we looked at gene expression (after 24h) can be a reason why IL-6 was not expressed neither in the control nor treated cell cultures in our study, truly related to IL-10 supressing effect. However, Chettri at al. studied another salmonid species and the cells were cultured in other temperature conditions.

Furthermore, the viral exposure (poly I: C) is capable of activating the antiviral responses via the recognition of different PRRs in specific combinations. Numbers of PRRs that are involved in the immune response have been described in teleost fish (Boltaña et al. 2011), and TLRs is an important group of PRRs. The only PRR expressed in our study was the fish specific toll like receptor TLR22. Previous studies have shown that the TLR22 gene expression might be induced by various PAMPS in multiple fish species, included Atlantic salmon (Li et al. 2017). Li et al. have shown up-regulated expression levels of CcTLR22 mRNA after intraperitoneal injections with poly (I: C) in a variety of tissues, included the head kidney in the fresh water common carp. The authors suggested that CcTLR22 plays a significant role in the defense after viral and bacterial infection in this species (Li et al. 2017). Our results showed down-regulated TLR 22 expression after poly I: C challenging for 24h in Atlantic salmon head kidney cells.

Transcription factors control the transcription of DNA to mRNA by binding to specific DNA sequences. The transcription factor C/EBP-β is known for having target genes including those for cytokine genes in monocytes/macrophages in mammals (Wedel and Lömsziegler-Heitbrock 1995). Indeed, C/EBP-β has been showed to be expressed in immunologically important organs in salmonids, suggesting that it is likely to be involved in the immune response just as in mammals (Fujiki et al. 2003). In our study, the transcription factor C/EBP-β was expressed in all treatments and was down-regulated in Poly I: C-treated cells compared to non-treated cells.

4.2 Effects of partial and total replacement of FM by IM in the diets on inflammatory gene transcriptions

Recently, some studies investigated the impacts of insect meal on the immune response in fish species; in yellow catfish and European sea bass fed with dietary yellow worm meal (Henry et al. 2018; Su et al. 2017), and in African catfish fed with dietary cricket meal (CM) (Mohd Taufek et al. 2018). In our work, we were not able to show statistically significant differences between the experimental diets (IM-66 and IM-100) compared to the control diet (IM-0) in cytokine inflammatory related gene expression IL-1b, IL-8, IL-10, TNF-α. This might indicate that the BSF as a protein source did not affect the cytokine transcription, and neither bad nor good effects in the innate immune response in the head kidney leucocytes isolated from salmon. To our knowledge, no study has investigated *in vitro* gene expressions in fish fed insect-based diets, and more studies are required. However, it might be possible that the experimental insect based diets affect the gene expression of cytokine and other immune response genes not investigated in this experimental setup.

The immunological effects of chitin have been addressed in many fish species (Cuesta et al. 2003; Bruni et al. 2018). Indeed, feeding rainbow trout with a dietary BSF larvae meal, increased the presence of bacteria of the Carnobacterium genus, a well-known probiotic that improves disease resistance in salmonids, and which is probably caused by the dietary chitin present in the exoskeletons of BSF (Bruni et al. 2018). However, in the current trial, the insect ingredients did not affect the inflammatory responses in the leukocytes stimulated with bacterial and viral mimics. In order to reduce the concentration of chitin, the mantle of the larvae has been separated from the rest of the insect body before being processed to protein meal. With a low amount of chitin (estimated between 3-5% in our diets), there may not be enough to activate the immune response in the head kidney of salmon.

4.3 Effects of partial and total replacement of FM by IM in the diets on eicosanoid pathway related gene expressions (5-LOX, PTGDS, PTGES)

Prostaglandin D and E synthase (PGDS, PGES) are important enzymes in the biosynthetic pathway of prostaglandins made from arachidonic acid (Ara) or eicosapentaenoic acid (EPA) (Gómez-Abellán and Sepulcre 2016). Previous studies suggested that the n-6/n-3 ratio might impact the eicosanoid production in the head kidney isolated from salmon, and showed that PGDS and PGES were significantly upregulated in leukocytes isolated from salmon fed with soybean oil (n-6/n-3 ratio 2.4) diet compared to palm oil (n-6/n-3 ratio 0.7), rapeseed oil (n-6/n-3 ratio 0.9) and high soybean oil (n-6/n-3 ratio 4.1) diets (Holen et al. 2018). Our experimental diets did not have a big differences in n-6/n-3 ratios and range from 0.7-0.9 for all dietary groups (Table A.2)**.** The transcriptions of eicosanoid related genes, prostaglandin D and E synthase, were not significantly affected by the insect based diets in our study. These results also correspond to the cytokine gene expressions that were not affected by the diets.

5-LOX is a key enzyme in the biosynthesis of leukotrienes (Funk 2001). In the current study, exposure to both bacterial (LPS) and viral (poly I: C) mimic down-regulated the transcription of this enzymes in all dietary groups. Similarly, Holen et al 2018. observed the same effect in salmon head kidney cells stimulated with LPS (Holen et al. 2018). In addition, the expression of 5-LOX was downregulated in the cells isolated from fish fed with dietary IM-66 and IM-100 compared to the respective control diet in cells treated with LPS. Our results indicate that the activity of lipooxygenase pathway might be affected by the experimental diets during a bacterial infection. The experimental diets in our trial had balanced amino acids profile, added minerals and vitamins to the same levels and the same amounts of omega3 FA. However, the only difference in FA composition was the lauric acid (12:0) content, which was detected only in the insect based ingredients (IM-66 and IM-100) and was below the quantification limit (0.01 mg kg^{-1} sample) in the control diet (IM-0). Difference in dietary FAs can make changes in the fatty acid composition in the cell membranes of cells involved in the inflammatory response, and then affect the production of eicosanoids (Calder 2010). The FA profile in our experimental diets may have caused alterations in the composition of the cell membranes, and further may have led to changes in eicosanoid production.

4.4 Effects of partial and total replacement of FM by IM in the diets on stress related gene expressions (SOD, GPx1, HSP 27, HSP 70)

Oxidative stress within cells or tissue have profound effects on fish health, and the anti-oxidant substances that reduce oxidative stress therefore play a significant role as health-benefiting factors. The antioxidant genes are easily induced by ROS (oxidative stress), and the levels of antioxidant genes can be used to quantify oxidative stress in the cell (Olsvik et al. 2005). Surprisingly, our results showed that the leukocytes isolated from salmon fed with IM diet (IM-66 and IM-100) in combination with LPS treatment had a lower gene expression of antioxidant enzymes (SOD and GPx1) and heat shock protein (HSP 70). The lowered expression of genes involved in the oxidative stress might indicate a reduced ROS production in the cells when the salmon were fed with IM, compared to fish fed with a commercial diet containing FM and VP. The changes in oxidative stress in the fish might be due to a number of processes e.g.; Environmental stressors, changes in the metabolism, activity of the immune defense and different levels of contaminants in the diets (Abele et al. 2011). In contrast, a down-regulation of the antioxidant genes could also play an aggravation role of the oxidative stress response, if the antioxidant defense system against ROS is reduced. Our results indicated that the difference in stress related genes were observed between the dietary treatments only when the cells were stimulated with LPS. Overall, our results indicated that the experimental diets most likely did not influence the salmon during viral or bacterial diseases, but had an impact on the salmon cellular stress response. More studies are required to understand the function of insects in the diet.

5. CONCLUSION

The present results indicate that the *in vitro* model used in this experiment is suitable for investigating the impact of insect meal inclusion on the immune response related gene expression. Growth differences were not observed between insect based diets and the fish meal based control diet groups, but the experimental diets altered at a great number of the genes studied in the current trial.

The study showed a normal transcription of the pro-inflammatory genes in the head kidney leukocytes isolated from salmon fed insect-based diets; Inflammatory related genes IL-1β, IL-8, IL-10 and TNF-α had elevated gene expressions in LPS treated cells. The experimental diets, IM-66 and IM-100, did not affect IL-1β, IL-8, IL-10 and TNF-α gene expression.

66 and 100% replacement of fish meal by insect meal in the diets lowered the gene expression of Toll-like receptor 22 and transcription factor T CCAAT/enhancer-binding protein β (C/EBP-β) when the cells were treated with LPS.

66 and 100% replacement of fish meal by insect meal in the diets induced lower levels of arachidonate 5-lipoxygenase (5-LOX) expression, but did not affect the expression of Prostaglandin D and E synthase (PTGDS, PTGES) transcriptions.

Interestingly, the leukocytes isolated from salmon fed with IM diets in combination with LPS treatment had down-regulated gene expression of the antioxidant enzymes (SOD and GPx1) and the heat shock protein (HSP 70) compared to control diet.

In conclusion, in this study the Atlantic salmon fed dietary insect meal did not show differences in cytokine inflammatory transcription during viral or bacterial diseases, but the diets may have had an impact on the cellular stress response in salmon during a bacterial infection.

6. FURTHER STUDIES

Insects are promising feedstuffs for fish as they contain not only valuable nutrients, but also particular compounds that seem to be able to optimize fish health. So far, there has been little work on the effects of those insect derived compounds in fish and animal feeding trials, however this initial investigation showed promising results. The experiment has to be repeated to support the results and analyze more genes related to the immune response. It would also be interesting to explore the effect of insect ingredients *in vivo* model on the mortality and disease resistance, by challenging the Atlantic salmon fed with dietary insect meal with certain pathogens. The valuable nutrients of insect species are well documented, however, the effects of chitin, lauric acid, and anti-microbial peptides present in the insects are not well known and need further investigation.

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APENDIX

Table A 1. Fatty acid composition (g/100 g total fatty acids) of the three experimental diets

Fatty acid	IM-0	IM-66%	IM-100%
12:0	n.d	1.2	2.3
14:0	2.2	3.2	3.6
16:0	8.5	9	9.1
16:1n-7	2.0	2.6	2.8
18:0	2.9	2.9	2.9
18:1n-9	40	33	30
18:1n-7	2.5	2.2	2.0
18:2n-6	14	12	11
20:0	0.4	0.4	0.4
18:3n-3	6.5	5.6	5.1
20:1n-9	4.5	5.6	5.9
18:4n-3	1.4	1.9	2.0
20:4n-6 ARA	0.2	0.2	0.3
22:1n-11	5.2	6.8	7.4
20:5n-3 EPA	3.0	4.0	4.4
22:5n-3 DPA	0.3	0.4	0.4
22:6n-3 DHA	2.9	4.0	4.1
Saturated FA	15	17	19
Sum 16:1	2.0	2.8	3.0
Sum 18:1	42	35	32
Sum 20:1	4.7	5.9	6.2
Sum 22:1	5.8	7.5	8.0
Sum MUFA	55	52	50
Sum EPA+DHA	5.9	8.0	8.5
Sum n-3	14.8	16.5	17
Sum n-6	13.8	12.3	11.6
Sum PUFA	29	29	29
n-3/n-6	1.1	1.3	1.4
n-6/n-3	0.9	0.7	0.7

Table A.2. Total amino acid composition (g kg-1 wet weight) of the four experimental diets

	IM-0	IM-66%	IM-100%
ALA	19.3	19.2	19.7
ARG	22.5	20.0	20.6
ASP	36.0	34.6	34.6
GLU	73.0	74.8	79.0
GLY	16.6	15.2	15.6
HIS	8.4	8.2	8.8
HYP	1.0	0.5	0.3
ILE	14.2	13.8	14.7
LEU	33.5	32.7	33.7
LYS	20.6	19.5	20.2
MET	10.0	9.9	10.0
PHE	20.3	19.2	20.0
PRO	23.4	25.0	27.5
SER	20.0	19.5	20.3
TAU	0.5	0.2	0.0
THR	14.4	13.8	14.3
TYR	13.7	14.5	16.2
VAL	16.5	16.5	18.0

Table A 3 : Solutions used for cell isolation

Solution	**contents**
1.5M NaCl	87.6 g NaCl MQ-water until 1 L
Stock Isotonic Percoll (SIP)	1 part 1.5M NaCl 9 part Percoll
Percoll-gradient solution, density 1.08 g/ml Abouth 51% percoll	50 ml: 28.5 ml SIP 21.4 ml L-15 media
Complete L-15 medium for cell culture 10% Foetal bovine serum (FBS) 1% Glutamax 1% Antibiotic Antimycotic	500 ml: 440 ml L-15 50 ml FBS 5 ml Glutamax (10X) 5 ml Antibiotic Antimycotic (10X)
Isolation buffer	9 gram NaCl, 7 g EDTA, 1l dH_20, pH 7.2

Table A 4: **reaction mix for the RT reaction**

	Reagents	**Volume (µl)**	**Concentration**
	ddH2O	8,9	
	10x RT buffer	5	1X
Non enzymatic reagents	10x MgCl2	11	5,5 mM
Enzymes	10 mM dNTP mix 50µM oligo d(T)16 RNase Inhibitor (20 U/µl)	10 2,5 1	500µM per dNTP 2,5 µM 0,4 U/µl
	Multiscribe Reverse Transcriptase (50U/µl)	1,67	1,67 U/µl

Table A 5: SYBRGreen RT-qPCR reaction mix

Reagents	**Volume pr sample (ul)**
ddH2O	2.8
Primer forwards	0.1
Primer Rewers	0.1
TaqMan universal PCR Master Mix (SYBRGreen)	5

Table A 6: RT-qPCR reaction program

reaction program Steps	**Temperature (°C)**	**Time (min: sec)**	**Comment**
Pre- incubation	95	05:00	Denaturation and activation of FastStart Taq DNA polymerase
Amplification			45 cycles, 3 steps
Denaturation	95	00:10	Separating DNA strands
Annealing	60	00:10	Primer binds to DNA strand
Elongation	72	00:10	Synthesis of double stranded DNA
Melting point analysis			1 cycle, 3 steps
Denaturation	95	00:10	
Annealing	65	01:00	
Melting	97		
Cooling	40	00:10	

Table A 7: Primers and function used for RT-qPCR, gene expression analyses

Gene	Forward sequence Reverse sequence	name	Function
IL-1β	*GTATCCCATCACCCCATCAC* *GCAAGAAGTTGAGCAGGC*	Interleukin 1 beta	Cytokine pathaway
IL-6	TGA AGA AGG AGT ACC CCG ACA AT GGT GCC TCA TCT TTT CCT CAA TG	Interleukin 6	Cytokine pathway
IL-8	GAGCGGTCAGGAGATTTGTC TTGGCCAGCATCTTCTCAAT	Interleukin 8	Cytokine pathway
IL-10	GGCTTCCCTGTTGGACGAAG TCAGTGTTTGCGCCTCTTAG	Interleukin 10	Cytokine pathway
TNF-α	GGC GAG CAT ACC ACT CCT CT TCG GAC TCA GCA TCA CCG TA	Tumor necrosis factor alpha	Cytokine pathway
TGF-β	GTGCAAACGTACATACCGCC TCAAAAAGGATCAGCCCCCA	Transforming growth factor beta	
TLR3	TGGTTAATGAGTGCAATAGTGG GTTTCATGCTCAATTACAGTAGG	Toll-like receptor 3	Toll like reseptor
TLR (ref)	ATAGTGGCCGCCAAAGATCC ACATGAACAGCTGCCGTGTA	Toll-like receptor	Toll like reseptor
TLR9	CTCAGCCTCTGTCCTTTCACTT CAGAGAATGCATCCTTCCCTAC	Toll-like receptor 9	Toll like reseptor
TLR22	AAAGGATGAGGACCCGATG GCCAACCTCTCCCTGCTAC	Toll-like receptor 22	Toll like reseptor
Cox 2	GGAGGCCTACTCCAACCTATT CGAACATGAGATTGGAACC	Cyclooxygenase 2	Eicosanoid pathway
5-LOX	ACTAAGTTTGCTGCTTCGG CTGACTCCAGACCTCGTG	Arachidonate 5-lipoxygenase	Eicosanoid pathway
PTGDS	ATC CCA GGC CGC TTC AC ACA CGC ATG TCA TTT TCA TTG TT	PGD synthase (PGDS)	Eicosanoid pathway
PTGES	TCC AGC CAA TGT CTT AGT AAG CAC GGT ATA ACT GAAC	PGE synthase (PGES)	Eicosanoid pathway
CD83	CAAACTGGTCCAGACAGGGT CAGCGTGATAGACTCGTTC	Antigen (Activated B Lymphocytes, Immunoglobulin Superfamily)	
CD36	GGA TGA ACT CCC TGC ATG TGA TGA GGC CAA AGT ACT CGT CGA	Antigen (Collagen Type I Receptor, Thrombospondin Receptor)	
C/EBPβ	CGC GTG GAG CAG CTG TCA AGA TGG GCA CTC CGG TGT GGC TA	Ccaat-enhancer-binding protein β	Transcription factor involved in metabolism and inflammation
Mn-SOD	GTTTCTCTCCAGCCTGCTCTAAG CCGCTCTCCTTGTCGAAGC	Mn superoxide dismutase	Stress response
Cu/ZnSOD	CCACGTCCATGCCTTTGG TCAGCTGCTGCAGTCACGTT	CuZn superoxide dismutase	Stress response

catalase	CCAGATGTGGGCCGCTAACAA TCTGGCGCTCCTCCTCATTC	Catalase	Stress response
GPx1	TCTCCTGCCATAACGCTTGA GTGATGAGCCCATGGCCTTA	Glutathione peroxidase 1	Stress response
HSP27	CCA	Heat shock protein 27	Stress response
HSP 70	CCCCTGTCCCTGGGTATTG CACCAGGCTGGTTGTCTGAGT	Heat shock protein 70	Stress response
P38	GGC ACA CAG ACG ATG AGA TG ACA GCG TTC TGC CAG TGAG	P38 mitogen-activated protein kinases	Stress response
EF1A	TGCCCCTCCAGGATGTCTAC	CAGCGTGATAGACTCGTTC	Reference gene
ACTB	CCA AAG CCA ACA GGG AGAA ACG AGC TAG AAG CGG TTT GC	Beta-actin (refgen)	Reference gene

				HK								Plate 1
A	Delution curve 1000	Delution curve 1000	Delution curve 1000	Delution curve 500 ng	Delution curve 500	Delution curve 500	Delution curve 250 ng	Delution curve 250 ng	Delution curve 250 ng	Delution curve 125 ng	Delution curve 125 ng	Delution curve 125 ng
B	Delution curve 62.5 ng	Delution curve 62.5 ng	Delution curve 62.5 ng	Delution curve 31.25 ng	Delution curve 31.25 ng	Delution curve 31.25 ng	2017-1399/1 500 ng	2017-1399/1 500 ng	2017-1399/3 500 ng	2017-1399/3 500 ng	2017-1399/5 500 ng	2017-1399/5 500 ng
C	2017-1399/7 500 ng	2017-1399/7 500 ng	2017-1399/11 500 ng	2017-1399/11 500 ng	2017-1399/13 500 ng	2017-1399/13 500 ng	2017-1399/15 500 ng	2017-1399/15 500 ng	2017-1399/17 500 ng	2017-1399/17 500 ng	2017-1399/19 500 ng	2017-1399/19 500 ng
D	2017-1399/21 500 ng	2017-1399/21 500 ng	2017-1399/23 500 ng	2017-1399/23 500 ng	2017-1399/27 500 ng	2017-1399/27 500 ng	2017-1399/31 500 ng	2017-1399/31 500 ng	2017-1399/33 500 ng	2017-1399/33 500 ng	2017-1399/35 500 ng	2017-1399/35 500 ng
E	2017-1400/1 500 ng	2017-1400/1 500 ng	2017-1400/3 500 ng	2017-1400/3 500 ng	2017-1400/5 500 ng	2017-1400/5 500 ng	2017-1400/7 500 ng	2017-1400/7 500 ng	2017-1400/9 500 ng	2017-1400/09 500 ng	2017-1400/11 500 ng	2017-1400/11 500 ng
F	2017-1400/09 500 ng	2017-1400/11 500 ng	2017-1400/13 500 ng	2017-1400/13 500 ng	2017-1400/15 500 ng	2017-1400/15 500 ng	2017-1400/17 500 ng	2017-1400/17 500 ng	2017-1400/09 500 ng	2017-1400/11 500 ng	ntc	nac
G												
H												

Figure A 1: 96-well PCR plate number 1. Standard curve/dilution curve in triplicate from 1000-31 ng. 10 µl /well samples in wells with 40 µl/well RT-reaction mix in duplicate and two negative controls. Ntc: Nac: no reverse transcriptase