

**Stress Response and Recovery in Atlantic Salmon
(*Salmo salar* L.) Fed Diets Low in the Marine n-3
Fatty Acids EPA and DHA**

***Master's thesis in Nutrition of Aquatic Organisms in
Aquaculture***

Chan Cai

Department of Biology
University of Bergen

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N I F E S
NATIONAL INSTITUTE
OF NUTRITION AND
SEAFOOD RESEARCH

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Abbreviations

ACTH	Adenocorticotropic hormone
CAT	Catalase
CPO	Crude palm oil
DHA	Docosahexaenoic acid
EFAs	Essential fatty acids
EPA	Eicosapentaenoic acid
GPx	Glutathione peroxidase
FA	Fatty acid
FO	Fish oil
HMW	High molecular weight
HPI	Hypothalamic pituitary interregnal
HSPs	Heat shock proteins
HUFA	Highly unsaturated fatty acids
IAA	Indispensable amino acids
LMW	Low molecular weight
LO	Linseed oil
MNE	Mean normalized expression
PUFA	Polyunsaturated fatty acid
RNase	Ribonuclease
RO	Rapeseeds oil
ROS	Reactive oxygen species
RT reaction	Reverse transcription reaction
SGR	Specific growth rate
SOD1	CuZn-superoxide dismutase
VOs	Vegetable oils

1. Abstract

With the fast growth of today's aquaculture industry, the demand for aquafeeds is expanding dramatically. Finding sustainable alternative sources to fishmeal and fish oil (FO) is becoming an urgent issue; vegetable oil (VO) and plant meal are potential candidates. Replacing the fishmeal and FO with plant materials means fish eat low levels of polyunsaturated fatty acids including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA); this may affect the fish growth and health such as stress response. The objective of this study was to investigate the effect of low level of dietary EPA and DHA on stress response in Atlantic salmon (*Salmo salar* L.).

Atlantic salmon were fed four diets with increasing levels of EPA and DHA, ranging from 1.3% to 7.4% of total dietary fatty acids (FAs) for five months. All diets contained 10% fishmeal of the total diets, and the remainder of protein was provided by plant sources. To examine the effects of the different diet treatments on stress response, concentrations of plasma cortisol and glucose were analyzed before stress, and 1h, 2h and 23h after stress. The gene expression of stress protein HSP27 and the antioxidant enzymes (SOD1, CAT, GPx1), and SOD1 enzyme activity in liver were tested at the same time points.

Replacing dietary FO with vegetable oils significantly affect fish growth, the final weight of fish fed two lower levels of EPA and DHA (1.3% and 2.7%, respectively) was significantly lower than control group (7.4% EPA and DHA). Significantly higher SGR% and weight gain rate% were observed in fish fed 4.4% and 7.4% EPA and DHA compared to fish fed 1.3% and 2.7% EPA and DHA. A significantly lower concentration of plasma cortisol in diet C (4.4% EPA and DHA) compared to the control group was seen. No effect of the four diets was found on plasma glucose, transcription of HSP27, SOD1 CAT and GPx1 in liver, and SOD1 enzyme activity in liver.

2. Introduction

2.1 General introduction

The aquaculture industry has become the fastest growing food production sector, with an average annual growth rate of 8.8% in the last three decades (1980-2010) (FAO, 2012). Globally, farmed fish accounts for 50% of human fish consumption and this proportion will continue to grow with the decreasing amount from capture fisheries and the increasing demand for seafood due to a growing world population (Turchini et al. 2009). The fast growing aquaculture industry leads to an expanding demand for aquafeeds, 40% fish oil (FO) are used in commercial salmon feeds (Turchini et al. 2009). The demand for FO from aquaculture may soon exceed supply. Fishmeal and FO are mainly produced from lower value fishes like small pelagic species, especially anchoveta, which are strongly influenced by climatic factors such as El Nino.

Fishmeal production decreased drastically in recent years from 30.2 million tons in 1994 to 15.0 million tons in 2010, mainly due to the reduction of anchoveta catch (FAO, 2012). Additionally, some human induced events like pollution, environmental degradation and over exploitation of resources will all increase this problem. For these reasons, finding alternatives to FO in farmed fish feeds is becoming an increasingly urgent issue.

Increasing amounts of plant ingredients are added in diets for farmed fish, a consequence of this is a decrease in the dietary level of the n-3 highly unsaturated fatty acids (HUFA), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). A well balanced composition of the dietary FAs is vital to fish growth and welfare, thus it is possible that partial replacement of FO by vegetable oils (VOs) in fish feeds will influence the balance. It is important to investigate how such diets

may affect fish health and robustness, including in stressful situations where the fish may struggle to maintain homeostasis. A previous study has shown a tendency for increased plasma cortisol levels in Atlantic salmon fed low levels of EPA and DHA (Jutfelt et al., 2007), and some previous results have also shown differences in how quickly the fish recover from stress based on what VO was used in the feed (Bell et al. 1991; Ganga et al. 2011). These observations warrant a more in depth study regarding stress response and recovery from stress in Atlantic salmon fed diets with high levels of VO and low levels of EPA and DHA.

2.2 EPA and DHA in fish feed

Some farmed fish, especially marine fish, require inclusion of long chain unsaturated n-3 essential fatty acids (EFAs) such as EPA (22:5n-3) and DHA (22:6n-3). EFAs means a series of FAs that are necessary in fish growth and health but cannot be produced *in vivo* by fish and needs to be obtained from dietary sources. EPA and DHA are necessary for neural development, cellular activity and for maintaining cell membrane structure in fish (Corraze, 1999; Tocher, 2003). The requirements of EFAs in fish vary with both species and different developmental stages (Tocher, 2010). Freshwater fish can synthesize EPA and DHA in sufficient amounts from linolenic acid (18:3n-3). While some fish species, especially marine fish, have low activities of the $\Delta 6$ -desaturase and $\Delta 5$ -desaturase enzymes which are necessary for the formation of EPA and DHA from their precursor: 18:3n-3 (Tocher, 2010). Atlantic salmon, an anadromous fish species, which spends part of its lifecycle in seawater, can synthesize some of EPA and DHA but not enough (Tocher, 2010). Studies have shown improved growth in Atlantic salmon when EPA and DHA were provided directly from the diet, compared to when only 18:3n-3 was provided (Ruyter et al. 2000). It has been reported that feeding Atlantic salmon a diet consisting of 100% VO caused several diseases such as heart lesions, muscle necrosis and even enhanced the mortality of fish in a stress situation (Bell et al. 1991; Seierstad et al. 2005). In order to fulfill the EFAs requirements in farmed fish, it seems that EPA and DHA have to be added in feeds of salmonids. Traditionally, the dietary source of EPA and DHA is mainly from wild catch FO like anchoveta (*Engraulis ringens*), sardines (*Sardina*

pilchardus), and capelin (*Mallotus villosus*) etc. Replacement of FO by VOs in aquafeeds has increased considerably in recent thirty years. The overview of different VOs sources and the effect of VOs feed on fish is in next section of this paper.

2.3 Vegetable oils (VOs) utilized in fish feed

The VOs are generally considered as substitutable sources of oil for use in aquafeeds, due to its large productivity. The largest production among VOs in the world is crude palm oil (CPO) (Turchini et al. 2009). Palm oil contains a high concentration of the saturated FA palmitic acid (16:0) and the mono-unsaturated FA oleic acid (18:1n-9). Partial replacement of FO with CPO in Atlantic salmon feed has shown no significant effect on fish growth, survival and fillet quality (Rosenlund et al. 2001; Bell et al. 2002). It has been reported that approximately 10% (wt/wt) CPO in feeds of Atlantic salmon did not affect FA digestibility and growth of fish (Ng et al. 2004). 100% palm oil replaced with FO in Atlantic salmon resulted in poor FA digestion (Torstensen et al. 2000).

The second largest VO production next to CPO is rapeseeds oil (RO) (Turchini et al. 2009), the dominant FA in RO used in fish feeds is oleic acid. It has been observed as the acceptable VO in Atlantic salmon and rainbow trout when compared with linseeds oil and sunflower oil, since a significantly higher feed intake of fish in RO group has been found (Geurden et al. 2005, 2007). No effect of RO feed in Atlantic salmon on fish growth and health has been reported in several studies, but the fish fillet FAs compositions changed, especially EPA and DHA contents were significantly reduced when VO replaced FO (Bell et al. 2003, 2001; Tocher et al. 2000; Torstensen et al. 2004).

Linseed oil (LO) is also a potential candidate for FO replacement since it contains large amounts of linolenic acid (18:3n-3), which is the precursor for synthesis of EPA and DHA. In addition, LO is rich in linoleic acid (18:2 n-6), therefore it has an 18:3(n-3)/18:2(n-6) ratio of 3-4:1 (Bell et al. 2003). Growth was not affected by LO dietary treated fish (Rosenlund et al. 2001; Bell et al. 2004, 2003), while FA

compositions in fish fillet were positively correlated with FA in diet (Bell et al. 2004).

VOs are different from FO in long chain unsaturated FAs, especially for lacking of EPA and DHA. Partial replacement of FO with VOs has shown no significant effect on fish growth (Rosenlund et al. 2001; Bell et al. 2002; Tocher et al. 2000; Torstensen et al. 2004), but it alters the FAs composition in fish tissue that may lead to change the lipid metabolism, immune system and even stress susceptibility in fish (Bell et al. 1991).

2.4 Replacement of fishmeal with plant meal in fish feed

Like VOs, plant meal is also an alternative to fish feeds. However, change the dietary composition means changed nutrients in the feed. For example, the FAs as mentioned above and the less indispensable amino acids (IAA) provided by plant protein than by fishmeal. When using the plant meal in aquafeeds, some problems like complex carbohydrates levels and some antinutritional factors may be introduced into fish feeds, which may affect digestion and nutrient metabolism in fish (Francis et al. 2001; Krogh et al. 2005). 100% fishmeal replaced by plant meal reduced final weight of Atlantic salmon, mainly due to the imbalance of AA and lacking IAA (Espe et al. 1991; 1993). Some of the problems can be solved such as by adding crystalline IAA to balance the AA requirements, and by reprocessing the plant meal to remove antinutritional factors (Espe et al. 2006). It has been reported that 90% fishmeal replaced by plant meal with balanced AA did not affect fish growth; detailed influence of plant protein on growth of Atlantic salmon has been discussed by Espe et al. (2006). Nearly total replacement of both the FO and fishmeal with plant materials (contains really low EPA and DHA) resulted in decreased final weight and elevated plasma glucose concentration in Atlantic salmon (Torstensen et al. 2008; Sissener et al. 2013). The diets used in the present study all had the same amount of fishmeal and plant proteins.

2.5 Stress in fish

Fish are exposed to stressors not only from aquaculture practices like handling, sorting, grading, transport and poor water quality but also from various methods of fish capture, physical trauma and environmental contaminants (Barton, 1997). Stress response of fish involves all levels of organization from cells and organisms of individual fish to the fish population.

2.5.1 Physiological stress response

There are three physiological response stages when fish undergo a stressful situation. The primary response includes the release of stress hormones like catecholamines and cortisol into the circulation. When the fish are under stress condition two axes are stimulated in the fish brain: the simpatico-chromaffin cell axis innervates chromaffin cells to release catecholamines, and the hypothalamic pituitary interregnal (HPI) axis induces adenocorticotrophic hormone (ACTH) to stimulate adrenaline to release cortisol (Figure 2-1) (Montero and Izquierdo, 2010). Plasma cortisol is used as a general stress indicator from the 1960s since many studies demonstrated the increase in corticosteroids in fish elicited by handling and confining stressor (Barton, 1997). The secondary response represents the biochemical and physiological effects in blood chemistry and haematology, activated by the stress hormones mentioned previously. For example the elevated adrenaline and cortisol is shown to increase plasma glucose concentration to cope with the energy metabolism in fish, and the plasma glucose has also been used as an indicator of stressed fish. The tertiary response involves the whole fish performance and population level change such as reproduction, growth and even altered community species and diversity (Iwama, 1998).

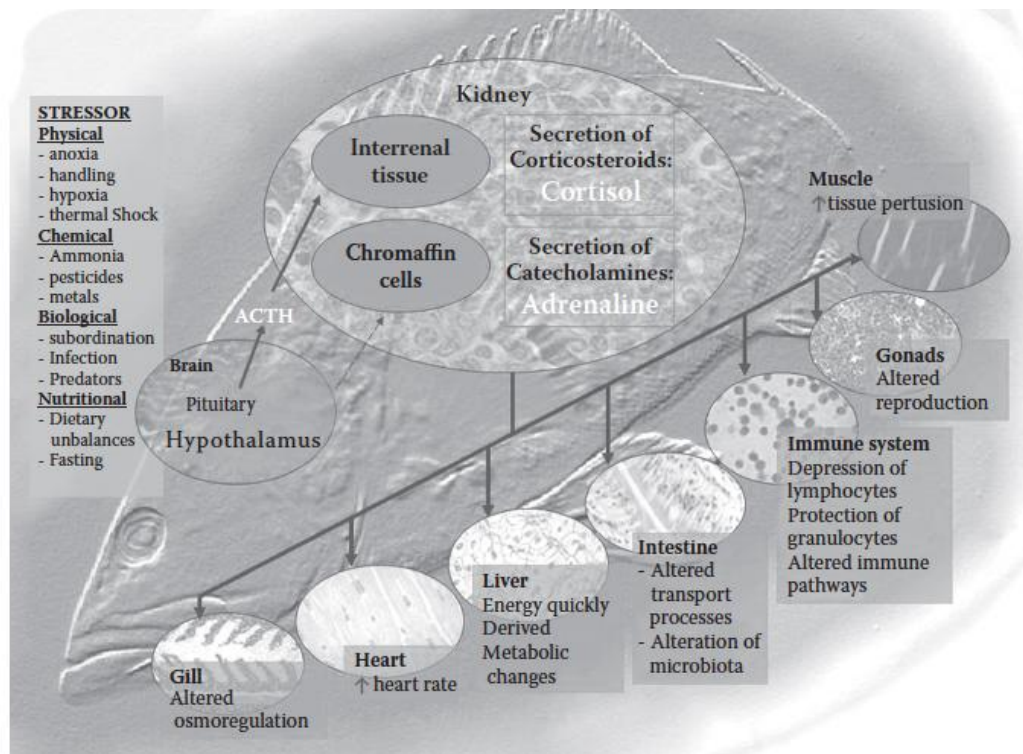


Figure 2-1 General procedure of stress response in fish.

Once the fish are under a stressed situation, the sympathetic chromaffin cell axis (dotted line) and the hypothalamus pituitary interrenal (HPI) are stimulated in the fish brain, which result in releasing two stress hormones (catecholamines and cortisol) into the blood circulation. The consequence of stress response involves changing different organs function. Source Montero, et al. (2010).

2.5.2 Stress protein- heat shock proteins (HSPs)

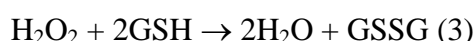
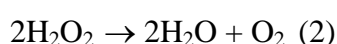
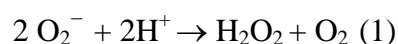
On a cellular level, heat shock proteins (HSPs) have been used as indicators of stressful situations in fish, since this group of protein is present in nearly all organisms and expressed in response to biotic and abiotic stressors (Iwama et al. 1998; Iwama et al. 2004). The HSPs play a constitutive role in cells and are expressed to protect cellular processes involving protein folding, fidelity and translocation in the unstressed condition (Welch, 1993; Hightower, 1994). The HSPs can be divided into two sections based on the molecular mass, the high molecular weight (HMW) section: HSP100, HSP90, HSP70, HSP60, and the low molecular weight (LMW) from 16 to 30kDa (Morimoto et al., 1994). Most of HMW HSPs have been documented that they increased under stressful condition, as they have the constitutive function and repair the misfolded and denatured proteins. While the LMW HSPs, such as HSP27, are more species specific (Iwama et al. 1998) and play the role as molecular

companions, preventing irreversible protein aggregation (Derham and Harding, 1999).

The study of HSPs in fish is still in early stages when comparing with mammals, bacteria and yeast (Iwama et al. 1998). Seldom research has been focused on the effect of FAs in fish diets on HSPs, lower HSP27 level was found in head kidney leukocytes of Atlantic salmon fed soybean oil when compared with fish fed FO (Holen et al., 2011), while HSP27 in intestine was elevated in rainbow trout fed 35% soybean meal (Sealey et al., 2013).

2.5.3 Oxidative stress and antioxidants (CAT, SOD, GPx1)

Oxidative stress is caused by the imbalance between the generation of reactive oxygen species (ROS) and the ability of the biological system to neutralize and eliminate them. Generation of ROS occurs naturally during aerobic metabolism in mitochondria of aerobic animals. ROS include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($\bullet OH$), etc. (Evans and Halliwell, 2001). In addition, environmental factors such as increase or decrease in water temperature, elevated metal ions, and chemical pollutants can also produce ROS. Elevated ROS may damage DNA, protein, and FAs (Halliwell and Gutteridge, 1999). Endogenous antioxidants like CuZn-superoxide dismutase (SOD1), catalase (CAT), and glutathione peroxidase (GPx) are easily oxidized and ensure the protection against oxidative stress in cell (Michiels et al. 1994; Hermes-Lima 2004). SOD is considered as a vital antioxidant in most of the organisms exposed to oxygen. The basic reaction of SOD is performed in equation (1); it dismutates O_2^- to H_2O_2 , which can be removed by CAT and GPx as indicated in equation (2) and (3):



CAT is usually located in peroxisomes to catalyze hydrogen peroxide, a by-product of FA oxidation, to water and oxygen. In addition to reduce H_2O_2 to water, GPx can

catalyze organic peroxides (ROOH) to the corresponding stable alcohols (ROH) (Tocher, 2003).

VOs in the fish feeds change the FAs composition in fish tissues, which potentially may affect the oxidative system in salmonids. Dietary imbalances might increase ROS generation and thus increase oxidative stress in fish. Few studies have addressed the replacement of FO and possible effects on antioxidants activity and transcription. Down regulation of SOD and CAT transcription was seen in liver of Atlantic salmon fed soybean oil compared with fish feed FO (Olsvik et al. 2011), indicating that VO supplement diet may affect antioxidative defense system in salmon.

2.6 Effect of dietary EPA and DHA on fish stress response

Dietary FAs can affect stress resistance in fish under different types of stressor (Kanazawa, 1997; Montero et al. 2004; 1998). Stress tolerance was enhanced with the increasing DHA content in VO fed fish (Kanazawa 1997; Kraul et al. 1993). Bell et al. (1991) reported a transportation-induced shock syndrome that caused 30% mortality in Atlantic salmon fed for 16 weeks on a fishmeal-based diet where lipid was provided by sunflower oil. In juvenile sea bream, lower EPA and DHA FA diet induced a long-term elevated plasma cortisol levels as well as under stressful condition (Montero et al. 1998).

Few studies focused on the stress response of fish fed VOs as FO substitutes. Some previous studies show that plasma cortisol level in gilthead sea bream was not affected by dietary VOs (Montero et al. 2003; Diaz-Lopez et al., 2009). Fish fed 60% linseed oil diets showed a significantly higher level of plasma cortisol compared with FO fed fish after stress, while neither rapeseeds oil or soybean oil had a significant effect (Montero et al. 2003). Atlantic salmon fed soybean oil and elevated plasma cortisol was found during parr-smolt transformation. (Jutfelt et al. 2007).

2.7 Aim of the study

To investigate the effect of low dietary levels of EPA and DHA from 1.3%-7.4% (of the FAs) on stress responses at three levels of biological organization; the physiological level, the cellular level and the molecular level. This was achieved by investigating plasma cortisol and glucose, RNA transcripts of heat shock protein27 (HSP27) in liver and gene transcript levels of antioxidant enzymes (SOD1, CAT and GPx1) and SOD1 enzyme activity in liver.

3. MATERIALS AND METHODS

The experiment was part of a large project financed by the Norwegian Research Council, project number 225086, “Tailoring salmon feeds of the future to maximize utilization of EPA and DHA”.

3.1 Fish experiment

3.1.1 Experimental design

The feeding trial was run by Skretting ARC, the research department of a leading manufacturer of feed for farmed fish in Norway, through most of the seawater production cycle for Atlantic salmon, from April 2012 to February 2013. In the beginning, 23rd of April 2012, 1200 fish of about 600g were put in a common 8m tank and were fed the same diet with 3% EPA+DHA, to “wash out” and reduce tissue levels of these FAs, reared at 8°C water temperature. Then, 16th of August 2012, the fish were transferred to five 3m tanks for acclimation, and the temperature was gradually increased from 8 to 12°C, and the fish were still fed with 3% EPA+DHA. On the 29th of September 2012, the fish were about 1450g and were distributed in eight 3m tanks with 70 fish per tank, and were fed the 4 experimental diets with 1.3, 2.7, 4.4 and 7.4% EPA+DHA of total FAs for about 5 months before sampling of tissues.

All diets contained 10% fishmeal of the total diet, while the remainder of the protein was provided by plant sources. In diet A, all added lipid was a VO mix (palm oil 30%, rapeseed oil 55% and linseed oil 15%), meaning that all EPA+DHA was from the

fishmeal at a level of about 1.3% of total FAs and 0.4% of the diet. Diet B to D were added some FO to give an EPA+DHA level of about 2.7% to 7.4% in the different diets (0.9%-2.2% of the diet). Table 3-1 shows the formulation and proximate composition of the diets and Table 3-2 shows the FA composition of the diets.

Table 3-1 Formulation and proximate composition (g kg^{-1}) of the experimental diets (8 mm)

Diet	A	B	C	D
Ingredients:				
Fish meal ¹	100	100	100	100
Wheat gluten ²	101.9	101.9	101.9	101.9
Sunflower meal ³	40	40	40	40
Faba beans, dehulled ⁴	60	60	60	60
Soya concentrate ⁵	275	275	275	275
Wheat ⁴	50	50	50	50
Fish oil ⁶	0	31.0	66.5	110.9
Rapeseed oil ⁴	175.0	156.8	137.2	112.8
Palm oil ⁷	94.0	85.5	74.9	61.5
Linseed oil ⁸	47.0	42.8	37.4	30.8
DL-Methionine ⁹	0.39	0.39	0.39	0.39
L-Lysine 99% ⁹	6.28	6.28	6.28	6.28
Premixes ¹⁰	50.43	50.43	50.43	50.43
<i>Proximate composition:</i>				
Dry matter	926	933	932	931
Protein	382	383	386	382
Fat	337	343	347	331
Ash	66	67	65	57
Astaxanthin, mg kg^{-1}	39.4	39.1	39.4	39.7

1. Scandinavian fish meal, Skretting, Stavanger, Norway
2. Cargill Cerestar, Hautbourdin, France
3. Linas Agro AS, Aalborg, Denmark
4. Skretting, Stavanger, Norway
5. Selecta, Goiânia-GO, Brazil
6. South American and Northern hemisphere fish oil (50:50), Skretting, Stavanger. Norway
7. Fritex 24, Aarhus Karlshamns, Karlshamn, Sweden
8. Elbe Fetthandel GmbH, Geesthacht, Germany
9. Trouw Nutrition, Boxmeer, The Netherlands
10. Include vitamins and minerals; Trouw Nutrition, Boxmeer, the Netherlands, proprietary composition Skretting ARC, vitamin and mineral supplementation as estimated to cover requirements according NRC (2011)

Table 3-2 Fatty acid composition (area % and mg/g) of the experimental 8 mm diets

Diet	A		B		C		D	
	Area%	mg g ⁻¹	Area%	mg g ⁻¹	Area%	mg g ⁻¹	Area%	mg g ⁻¹
<i>Fatty acid</i>								
C14:0	0.70	2,4	1,1	4,2	1,7	6,2	3,2	10,6
C16:0	15,1	53,2	14,9	54,6	15,0	53,7	14,5	47,4
C16:1n-7	0.60	2,0	1,1	4,0	1,8	6,3	3,0	9,7
C16:2n-6	<0,01	<0,1	0,1	0,3	0,1	0,5	0,3	1,0
C18:0	2.5	8,7	2,3	7,6	2,6	9,1	2,6	8,2
C18:1n-9	40.4	139,0	38,6	137,6	35,5	124,3	29,5	93,7
C18:1n-7	2.30	8,0	2,1	8,2	2,3	7,9	2,3	7,3
C18:2n-6	16.50	56,5	15,6	55,1	14,3	49,7	12,6	39,8
C18:3n-3	11.70	39,8	11,10	39,0	10,1	34,9	9,0	28,1
C18:4n-3	0.10	0,5	0,3	1,0	0,5	1,8	1,0	3,3
C20:1 sum isomers	1.5	5,2	1,8	6,4	2,3	8,0	3,1	9,7
C20:4n-6	<0,02	1,0	0,1	1,2	0,2	8,2	0,3	0,8
C20:4n-3	<0,02	<0,2	0,1	0,2	0,1	0,4	0,3	0,9
C20:5n-3 EPA	0.60	2,0	1,4	4,9	2,4	7,9	4,1	12,5
C22:1 sum isomers	1,1	3,8	1,4	5,0	2,2	7,4	3,7	11,3
C22:5n-3	0.10	0,4	0,2	0,7	0,3	1,1	0,5	1,7
C22:6n-3 DHA	0.70	2,4	1,3	4,4	2,0	6,7	3,3	9,8
C24:1n-9	0.2	0,5	0,2	0,6	0,2	0,7	0,3	1,1
Sat. FA not listed	0.8	2,3	0,8	2,4	0,9	2,5	1,1	2,5
Monoenes not listed	0.3	1,0	0,3	0,1	0,2	0,7	0,3	0,8
n-6 FA not listed	0.10	0,2	0,1	0,4	0,2	0,5	0,3	1,0
n-3 FA not listed	<0,02	<0,2	0,1	0,1	0,1	0,3	0,3	0,5
Others	0.1	0,2	0,1	0,7	0,1	1,2	0,2	1,8
Sum Sat FA	19,1	66,6	19,1	69,4	20,2	71,5	21,4	68,7
Sum monoenes	46.4	159,5	45,5	162,2	44,5	155,3	42,2	133,6
Sum n-6 FA	16.60	57,8	15,9	57,0	14,8	58,9	13,5	42,6
Sum n-3 FA	13,20	45,5	14,5	50,3	15,5	53,1	18,4	56,8
Unsat/Saturated	4.00	3,95	3,97	3,88	3,7	3,74	3,46	3,39
n-6/n-3	1.3	1,27	1,1	1,13	0,95	1,11	0,73	0,75
EPA+DHA	1.30	4,4	2,7	9,3	4,4	14,6	7,4	22,3
Sum FA		329,6		339,6		340,0		303,5

This results in a regression design (see Figure3-1.), and each diet was fed to duplicate tanks (n=2)

Fish tanks (8 in total):

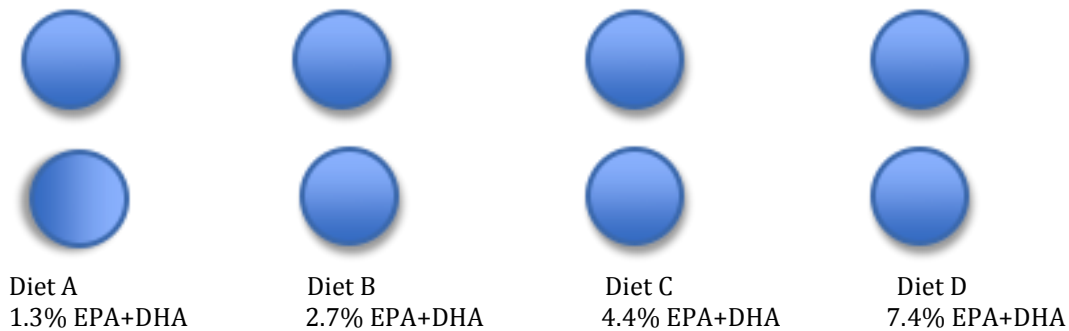


Figure 3-1 Design of the regression trial with 4 diets containing 1.3%-7.4% EPA+DHA and each diet was fed to duplicate tanks

3.1.2 Sampling

The sampling took place at Lerang (Skretting ARC's research station) from 20th to 22nd of February 2013. Samples were collected before stress, and 1hr, 2hr and 23 hours after stress. Stress was induced by reduction of the water level in the tank for 30 min before it was filled up again (Fig 3-2). The sampled fish were anesthetized, weighed and length was measured. Blood samples were taken from the caudal vessel using vacutainers. These were centrifuged for 7 min at 3000 g, to obtain the plasma fraction which was flash frozen and stored at -80 °C until analyses of glucose and cortisol. Livers were weighed and comments were written down for livers deviating from the rest in color, appearance etc. Two small pieces (500-800mg per piece), one for gene expression analysis and one for enzyme assay, at the same place from each liver were cut and placed into eppendorf tubes and then frozen in liquid nitrogen (Fig 3-3, 3-4, 3-5, 3-6). Five fishes from each tank at each time point were taken as parallels.



Figure 3-2 Protocol for stress test: water reduced in tank for 30 minutes, and then filled up again



Figure 3-3 Liver sampling first cut



Figure 3-4 Liver sampling for stress test



Figure 3-5 Sampled liver placed into eppendorf tube

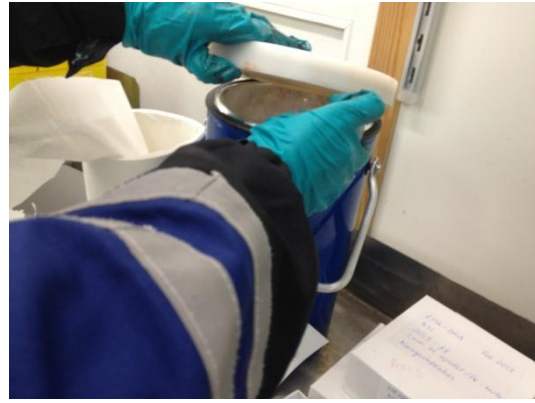


Figure 3-6 Sample frozen in liquid nitrogen

3.2 Analytical methods

3.2.1 Determination of cortisol in plasma

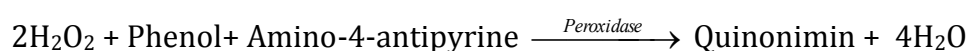
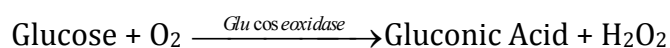
Pooled plasma samples from 8 fish, n=2 per diet group were analyzed before stress, and individual samples from 5 fish per tank for the time points after stress (except three plasma samples that were missing).

Cortisol in plasma was determined with a GammaCoat [¹²⁵I] Cortisol Radioimmunoassay Kit (DiaSorin, Stillwater, Minnesota, USA). The kit procedure is principally based on a radioimmunoassay, which counts the number of the binding antibody by use of the specific antigens. The plasma sample and reagents were thawed in room temperature and then mixed thoroughly by vortex. Five calibrate points ranging from 1-60 µg/dL and a blank were prepared for making a standard curve. 10µL calibrator or plasma sample was added at the same place on the bottom of the antibody-coated GammaCoat tube where antibody is fixed. All the tubes were added 1ml cortisol tracer-buffer and mixed gently by vortex for 1 min. Then tubes were incubated in a 37°C water bath for 45 minutes. After the incubation the tubes were drained in an inverted position for 30 minutes and tap the tubes on absorbent paper to remove the water. The tubes were counted in a gamma counter and the concentration of cortisol in sample was calculated from a calibrated standard curve. In order to get a good average, all the calibrator and the samples were tested in

duplicate.

3.2.2 Determination of glucose in plasma

Glucose concentration in plasma was determined with a colorimetric enzymatic method by using a commercial kit (GLUCOSE PAP, Maxmat S.A., Montpellier, France) on Maxmat instrument with PL Multipurpose diagnostic analyzer system. Plasma samples were thawed on ice before putting into the instrument. The method was calibrated with the calibrator Maxcal (ref. RM MXCA0018V, Maxmat S.A., Montpellier, France) and controlled with Maxtrol N(ref. MNCO0030V, Maxmat S.A., Montpellier, France) and Maxtrol P (ref. RM MPCO0030V, Maxmat S.A., Montpellier, France). The principle is that glucose is oxidized by glucose oxidase resulting in gluconic acid and hydrogen peroxide. Hydrogen peroxide is subsequently used for the generation of a Quinonimin dye with the presence of other reagents (peroxidase, phenol and Amino-4-antipyrine). The intensity of the color is proportional to the concentration of glucose in the sample, and is measured at 500nm. Glucose concentration in the sample is calculated from an external standard curve. Enzymatic determination of glucose was done according to the following reactions:



3.2.3 Gene expression analyses for SOD1, CAT, GPx1 and HSP27

Four samples were selected randomly from each diet group (2 per tank) from each of the 4 time points to analyze the gene expression.

1) RNA isolation

To avoid the liver samples being contaminated by ribonuclease (RNase), an RNA degrading enzyme, RNase Zap (Sigma- Aldrich, Missouri, USA) was used to remove the RNA from fume hood table, pipette and all the sample preparation tools during the procedure. About 25mg of each sample was cut, and added into a homogenizer tube (tube for Precellys) together with 750 μ L Qiazol lysis reagent (Qiagen, Austin, TX, USA) and 5 zirconium beads. Since RNA is easily degraded at room temperature, all the samples were treated on the dry ice before homogenization. The tissue was then homogenized by Precellys 24 (AH Diagnostics, Aarhus, Denmark) at 6000rpm 10 sec for 3 times. After standing for 5 min, 150 μ L chloroform was added into the tube to separate RNA from proteins and DNA. Then the tube was shaken by hand for 15-30 sec and kept for 3 minutes on ice before centrifuging (Eppendorf centrifuge 5415R) at 1200 rpm for 15 minutes at 4°C. The supernatant was collected into the sample tube (tube for Biorobot EZ1) and added 10 μ L DNase for purifying RNA using the Biorobot EZI (Qiagen, Austin, TX, USA). The samples were then frozen at -80°C for later analysis.

2) Check RNA quality:

NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to get the concentration and purity of RNA. Samples were diluted to 50ng/ μ L with milliQ water (RNA-free water). Twelve samples were selected randomly to determine the RNA integrity on Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) with RNA 6000 Nano Assay Kit. Since all downstream applications depend on a high-quality RNA, we accepted a 260/230 ratio (nucleic acid purity) of 1.75-2.3 from NanoDrop 1000 Spectrophotometer and RIN (integrity of RNA)>7.5 from Agilent 2100 Bioanalyzer. The RIN of all selected samples were > 8.3 in this study.

3) Reverse transcription reaction (RT reaction)-from RNA to cDNA

A standard curve with 6 dilution points from 31 ng to 1000 ng, each run in triplicate on the cDNA plate, was made from a pooled sample using 2 μ L from each of 48 RNA samples. Two 96-well cDNA plates (Roche Applied Sciences, Basel, Switzerland) were used to set up 48 samples, which all after stress, in triplicate with the standard

curve and two negative controls; a non-amplification control without enzymes (nac) and a non-template control without RNA (ntc). For economical reason, the 16 before stress samples were set up in the cDNA plate of other study (Effect on lipid metabolism in Atlantic salmon (*Salmo salar* L.) fed diet low in the marine n-3 FAs EPA and DHA) under same project. 10 μ L RNA sample and 40 μ L master mix (TaqMan[®] Reverse Transcription Reagents) were added in each well. The RT reaction took place on a PCR machine (GeneAmp PCR 9700) with a thermal program (Table 3-3). The cDNA plates were frozen with a plastic film cover at -20°C for later Real-time PCR analysis.

Table 3-3 Thermal program for Reverse transcription reaction

Step	Temperature (°C)	Time (min)
Incubation	25	10
Reverse transcriptase activation	48	60
Reverse transcriptase inactivation	95	5
End	4	∞

4) OneStep PCR

The specificity of the new primer SOD1 was tested by QIAGEN OneStep RT-PCR Kit. The primer was firstly diluted to a concentration of 0.05nmol/ μ L with TE buffer and the master mix reagent was made according to the QIAGEN kit. The RT reaction took place on PCR machine (GeneAmp PCR 9700) with a setup program (Table 3-4)

Table 3-4 Thermal program for OneStep RT PCR reaction

Step	Temperature (°C)	Time
Reverse transcriptase	50	30 min
PCR activation	95	15 min
Denaturation	94	45 sec
Annealing	60	45 sec
Exstension	72	1 min
Final extension	72	10 min

The PCR product was tested on an agarose gel with Gel Red Nucleic Acid Stain as a dye by charged voltage as 80V. After one hour, the gel was photographed by a UV

light to check the result. A single band of the expected size was found in the gel (Figure 3-7).



Figure 3-7 Agarose gel

*represents the target gene SOD1 in liver. Others are CPT-1, ICDH, G6PDH and ME (for other study in the same project) from the left to right respectively.

5) Real-time quantitative PCR (qPCR)

The cDNA plates were thawed on ice before being vortexed on MixMate for 5 minutes at 1500rpm. A reaction mix, which contained 570 μ L Sybrgreen, 319 μ L MiliQ water, 11.4 μ L forward primer and 11.4 μ L reverse prime, was prepared. A pipetting robot (Biomek® 3000) was used to transfer 8 μ L reaction mix and 2 μ L cDNA to each well of a 384-well qPCR plate with setup program. Before putting into Roche Lightcycler 480 System, the plate was centrifuged at 1500rpm for 2 minutes with optical plastic film cover. The qPCR reaction performed according to a thermal cycling program (Table 3-5).

Table 3-5 Thermal cycling program for qPCR reaction

Step	Temperature (°C)	Time
Incubation	95	5 min
45 cycles of Amplification with 3 steps: denaturation, annealing and elongation	95	10 sec
	60	20 sec
	72	30 sec
Melting curve analysis	95	5 sec
	65	1 min
	97	

The software Light Cycler® 480 (version 1.5.039) was used to calculate the reaction efficiencies and the standard curve.

3.2.4 Determination of CuZn superoxide dismutase (SOD1) enzyme activity in liver

In order to compare gene expression and enzyme activity the liver samples from the same fish which were analyzed for gene expression and the SOD enzyme assay. 60-90mg of individual liver samples were prepared by adding cold 20 mM HEPES buffer (pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose) (Sanden et al. 2013). Samples were kept on ice the whole time during preparation. The tissue was then homogenized by Precellys 24 homogenizer with 3*10 seconds at 6000 rpm and centrifuged at 10000 g for 15 min at 4 °C. The supernatant was collected and was frozen at -80°C for later analysis. SOD enzyme activity was determined using the SOD Assay Kit (Item No. 706002, Cayman Chemical Company, Ann Arbor, MI, USA). The procedure of the Kit is based on the principle which use tetrazolium salt to detect the superoxide radicals created by xanthine oxidase and hypoxanthine (Figure 3-8).

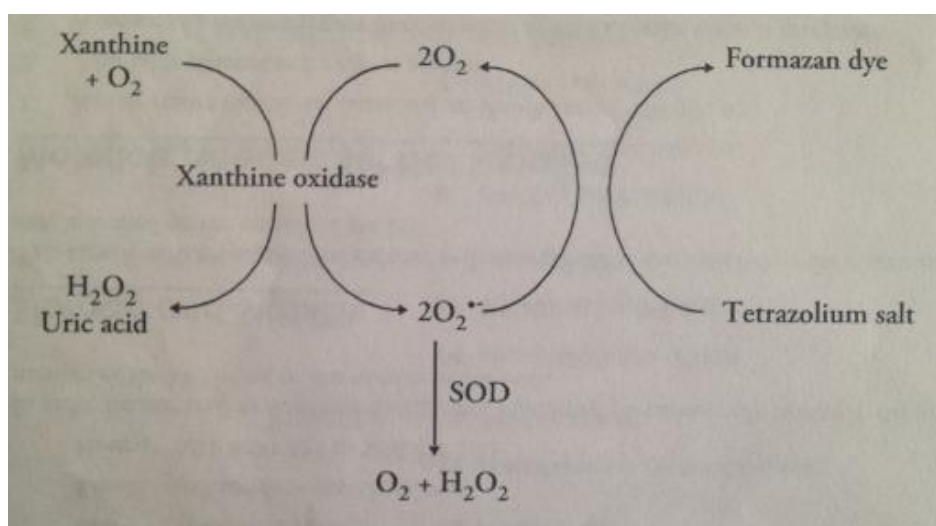


Figure 3-8 Formula of the Superoxide dismutase (SOD) assay.

Source: Instruction of Superoxide Dismutase Assay Kit Item NO. 706002

One unit of SOD is defined as the amount of enzyme needed to cause 50% dismutation of the superoxide radical. Two 96-well plate were used to set up 64 samples in duplicate parallel. Seven SOD1 standards activity from 0 U/ml to 0.25 U/ml were prepared for standard curve. The absorbance was read by a plate reader at 450 nm. The Pierce® 660nm Protein Assay (Thermo scientific, Pierce Biotechnology, Rockford, IL, USA) was used to determine the protein concentration. The sample supernatant was diluted five times within the assay's working range. The SOD activity (U/ml) was calculated from the equation:

$$\text{SOD (U/ml)} = \left[\left(\frac{\text{sample LR-Y intercept}}{\text{slope}} \right) \times \frac{0.23 \text{ ml}}{0.01 \text{ ml}} \right] \times \text{sample dilution}$$

The final results will be given as SOD(U)/mg protein.

3.3 Statistics

Microsoft Excel (2010) was used to treat the experimental data such as calculating mean values, weight gain rate, sepcific growth rate (SGR) and standard deviation (SD) ect. The stability of reference genes was caluculted by the program geNorm (version 3.5), as the M value. The two reference genes Elongation factor 1 α and UBA52 got a satisfactory M value of 0.463 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.

Statistica software (STATISTICA 10., Statsoft Inc, Tulsa, USA) was used for analyzing all the data. Regression analysis was performed to evaluate dietary effects on glucose, cortisol, gene expression (HSP27, CAT, GPx1 and SOD1) and SOD1 enzyme activity within the four different time points seperately. Nested ANOVA was used to check the diet and tank effect (with diet as fixed factor and tank as random factor to account for the fact that individual fish from the same tank are pseudoreplicates) on initial weight, final weight, weight gain rate and SGR, using individual fish as the statistical n. Weight gain rate was calculated as: 100%[End weight (g) – Start weight (g)]/Start weight (g); and SGR as: 100%[ln(End weight) – ln (Start weight)]/ Experimental days (145 days). Two-way ANOVA was used to

investigate both diet and time point factors effect on glucose, cortisol, gene expression (HSP27, CAT, GPx1 and SOD1) and SOD1 enzyme activity, using tanks (mean values) as the statistical n. Nested ANOVA was also used here to check tank and diet factor effect within the four time points (ANOVA run separately for each time point) and with tank nested in diets using individual fish as the statistical n. P-values < 0.05 were considered as statistically significant and Tukey HSD post hoc test was performed when the ANOVA gave p-values <0.05 . Levene's test was used to check the homogeneity of variances before ANOVA analysis. Correlation matrices analysis was used to check the correlation between cortisol and glucose in plasma and gene expression of SOD1 and SOD1 enzyme activity.

4. Results

4.1 Growth

The fish appeared in good health with an average weight gain of 1939 ± 192 g, and no mortality occurred during the 5 months of feeding. Fish fed diet A and B showed the lowest mean final weight (3252 ± 689 g and 3255 ± 630 g, respectively) compared to fish fed diet C and diet D (3420 ± 625 g and 3597 ± 703 g, respectively), and fish fed diet D had significantly higher final weight than fish fed diet A and diet B (Tukey HSD test $p=0.0002$ and $p=0.0003$, respectively; Figure 4-1). A significantly higher SGR% was observed in fish fed two higher levels of EPA and DHA groups with 0.60% and 0.62%, respectively (Table 4-1), when compared with the two lower levels of EPA and DHA groups with 0.55% (Tukey HSD test $p<0.024$; Figure 4-2). A similar situation was seen for the weight gain rate %; a lower weight gain rate % (126%) was seen for fish fed diet A and B compared to fish fed diet C and D (141% and 151%, respectively) (Tukey HSD test $p<0.013$; Figure 4-3). No significant tank effects were found.

Table 4-1 Mean initial weight (g) (n=120), mean final weight (g) (n=120), weight gain rate (%) and specific growth rate SGR (%) for Atlantic salmon fed the four different diets: A (1.3% EPA and DHA), B (2.7% EPA and DHA), C (4.7% EPA and DHA) and D (7.4% EPA and DHA). The initial weight and final weight are given as mean \pm SD

Diet	Mean weight initial \pm SD(g)	Mean weight final \pm SD(g)	Weight gain rate (%)	SGR (%)
A	1446 \pm 202	3252 \pm 689	125.5	0.55
B	1449 \pm 211	3255 \pm 630	125.9	0.55
C	1426 \pm 209	3420 \pm 625	141.2	0.60
D	1443 \pm 219	3597 \pm 703	150.8	0.62

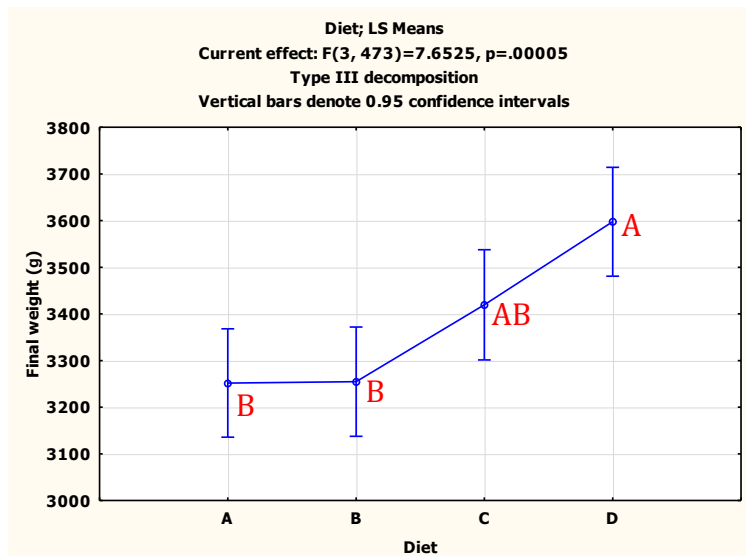


Figure 4-1 Final weight of Atlantic salmon fed the four different diets: A (1.3% EPA and DHA), B (2.7% EPA and DHA), C (4.4% EPA and DHA) and D (7.4% EPA and DHA) all time points combined. Results are presented as mean \pm sd (n=120). Data were analyzed using nested ANOVA. Significantly different diet groups are indicated by different letters

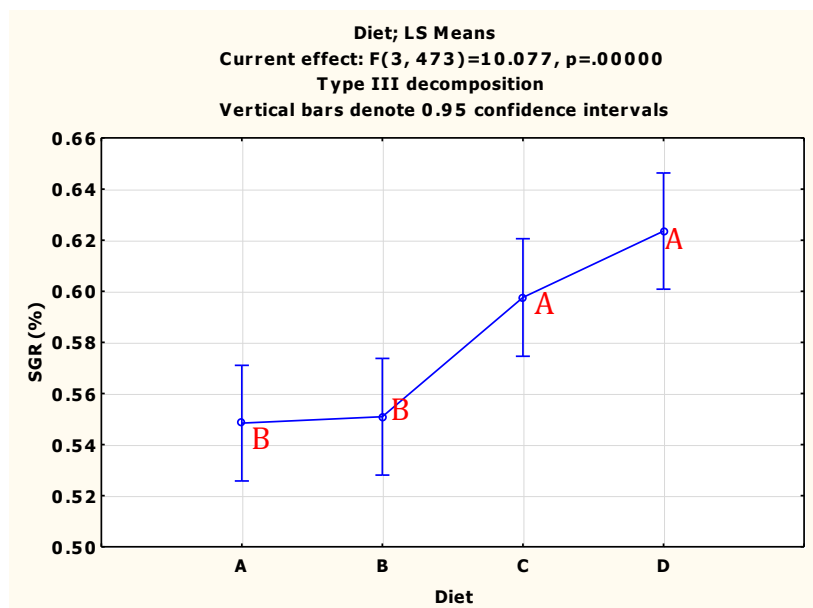


Figure 4-2 Specific growth rate (SGR%) of Atlantic salmon fed the four different diets: A (1.3% EPA and DHA), B (2.7% EPA and DHA), C (4.4% EPA and DHA) and D (7.4% EPA and DHA) all time points combined. Results are presented as mean \pm sd (n=120). Data were analyzed using nested ANOVA. Significantly different diet groups are indicated by different letters.

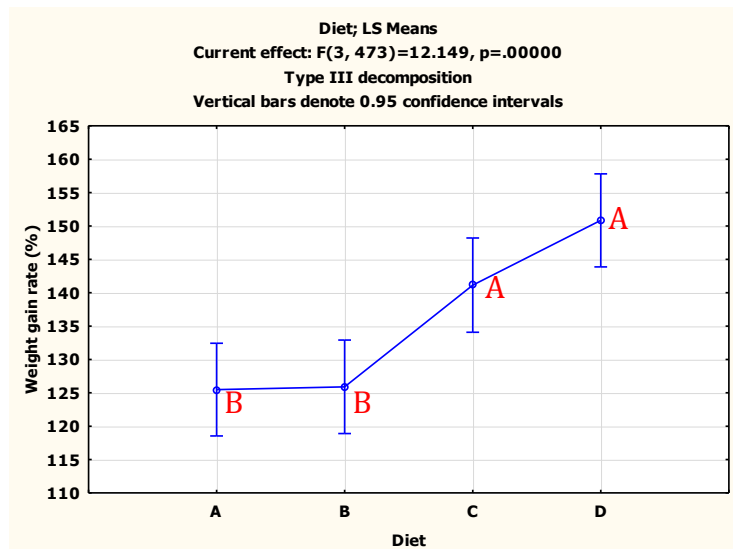


Figure 4-3 Weight gain rate (%) of Atlantic salmon fed the four different diets: A (1.3% EPA and DHA), B (2.7% EPA and DHA), C (4.4% EPA and DHA) and D (7.4% EPA and DHA) all time points combined. Results are presented as mean \pm sd (n=120). Data were analyzed using nested ANOVA. Significantly different diet groups are indicated by different letters

4.2 Cortisol and glucose in plasma

4.2.1 Cortisol in plasma

Table 4-2 shows the mean cortisol concentration in plasma of Atlantic salmon fed the four different diets at four time points. The cortisol concentration increased sharply from 5.0 ± 3.3 ug/dl to 14.7 ± 3.8 ug/dl 1h after stress and continued to increase to 19.8 ± 6.4 ug/dl 2h after stress, then decreased to a lower value at 3.96 ± 1.7 ug/dl 23h after stress (Figure 4-5). The plasma cortisol values 1h after stress were significantly higher than before stress and 23h after stress (Tukey HSD test $p=0.0002$). The highest mean value of plasma cortisol happened 2h after stress, which was significantly higher than 1h after stress (Tukey HSD test $p=0.01$, Figure 4-3). Significant differences were found between the four diets independent of time (Figure 4-4, two-way ANOVA). Fish fed diet C (10.43 ± 6.2 ug/dl) showed significantly lower plasma cortisol levels compared to fish fed Diet D (13.65 ± 8.1 ug/dl) (Tukey HSD test, $p=0.013$). A linear regression model was tested and revealed no linear relationship between plasma cortisol and EPA and DHA content in the diet. From

nested ANOVA results we know that there is no significant tank effect on plasma cortisol of individual fish within four time points separately.

Table 4-2 Cortisol concentration in plasma of Atlantic salmon fed the four different diets: A (1.3% EPA and DHA), B (2.7% EPA and DHA), C (4.4% EPA and DHA) and D (7.4% EPA and DHA) at four different time points: before stress, 1h after stress, 2h after stress and 23h after stress. The data are given as mean \pm SD before stress (n=2), 1h after stress (n=10), 2h after stress (n=10) and 23h after stress (n=10, except for Diet A n=8, Diet B n=9).

Cortisol (μ g/dl)	Diet A	Diet B	Diet C	Diet D
Before stress	6.7 \pm 5.0	3.8 \pm 2.8	1.8 \pm 1.0	7.6 \pm 0.8
1h after stress	14.9 \pm 5.1	15.3 \pm 3.6	13.2 \pm 1.6	15.4 \pm 4.4
2h after stress	20.4 \pm 4.8	21.0 \pm 9.0	16.3 \pm 3.4	21.7 \pm 6.4
23h after stress	3.5 \pm 1.7	3.5 \pm 1.2	3.6 \pm 1.8	5.1 \pm 1.7

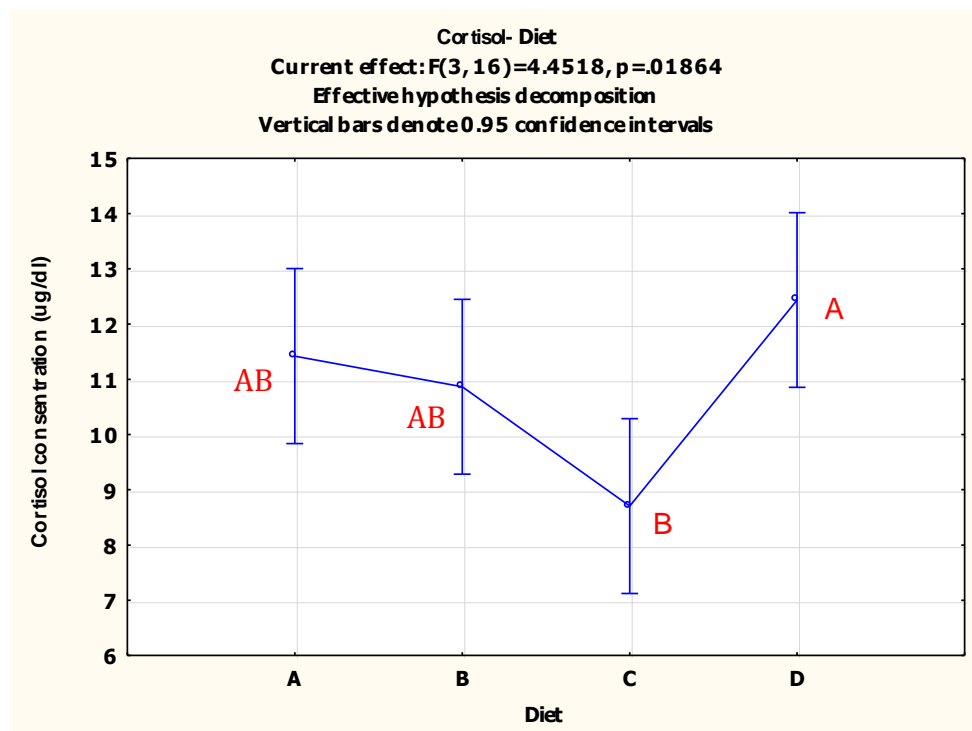


Figure 4-4 Cortisol concentration in plasma of Atlantic salmon fed the four different diets: A (1.3% EPA and DHA), B (2.7% EPA and DHA), C (4.4% EPA and DHA) and D (7.4% EPA and DHA) all time points combined. Results are presented as tank mean \pm sd (n=2). Data were analyzed using two-way ANOVA within the factor DIET. Significantly different diet groups are indicated by different letters.

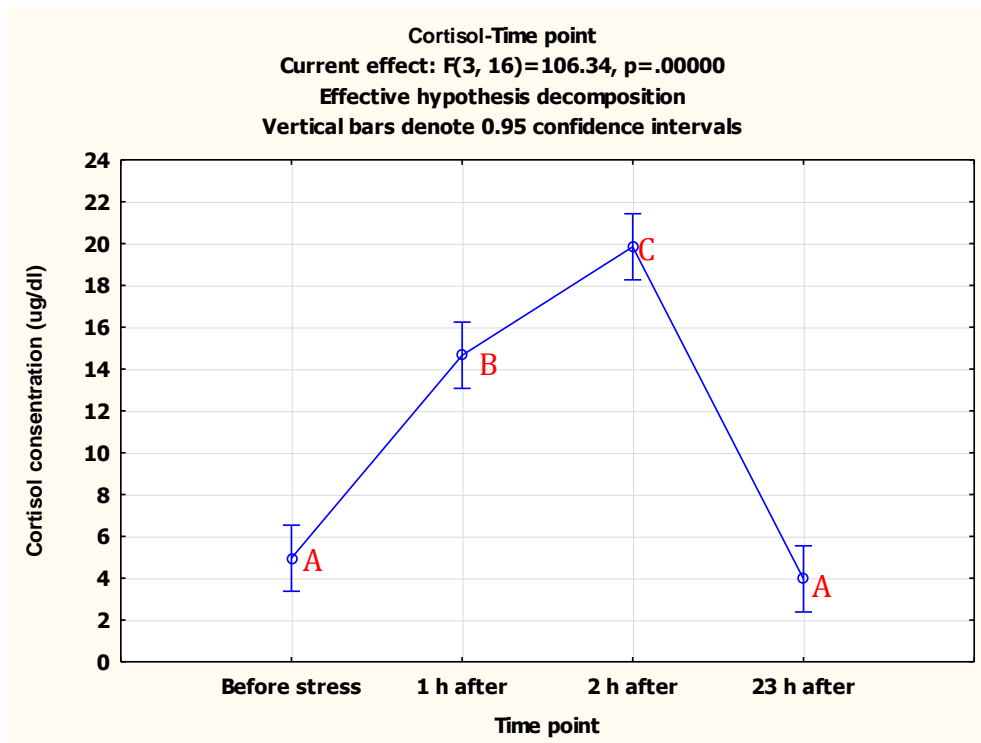


Figure 4-5 Cortisol concentration in plasma of Atlantic salmon before stress, 1 h, 2h and 23 h after stress, all diet groups combined. Results are presented as tank mean \pm sd (n=2). Data were analyzed using two-way ANOVA within the factor TIME. Significantly different diet groups are indicated by different letters.

4.2.2 Glucose in plasma

Table 4-3 shows the mean glucose concentration in plasma for Atlantic salmon fed the four different diets at the four time points. Glucose concentration increased sharply from 5.9 ± 0.9 mmol/L to 10.3 ± 1.6 mmol/L 1h after stress and continued to increase to 11.8 ± 2.6 mmol/L 2h after stress. The glucose concentration decreased to a lower value (6.3 ± 1.0 mmol/L) 23 hours later, which was close to the glucose values before stress (Figure 4-6). Same like plasma cortisol, the plasma glucose values 1h after stress were significantly higher than before stress and 23h after stress (Tukey HSD test $p=0.0002$). The highest mean value of plasma glucose happened 2h after stress, which was also significantly higher than 1h after stress (Tukey HSD test $p=0.026$). There was no significant difference between the four diets on plasma glucose (two-way ANOVA). A linear regression model was tested, but no linear relationship was observed at any time point between plasma glucose and EPA and DHA content in the diet. There was no significant tank effect.

Table 4-3 Glucose concentration in plasma from Atlantic salmon fed the four different diets: A (1.3% EPA and DHA), B (2.7% EPA and DHA), C (4.4% EPA and DHA) and D (7.4% EPA and DHA) at four different time points: before stress, 1h after stress, 2h after stress and 23h after stress. The data are given as mean±SD before stress (n=2), 1h after stress (n=10), 2h after stress (n=10) and 23h after stress (n=10, except for Diet A n=8, Diet B n=9).

Glucose (mmol/L)	Diet A	Diet B	Diet C	Diet D
Before stress	5.6±1.1	6.2±0.3	4.9±0.1	6.7±1.0
1h after stress	9.8±1.3	11.0±1.8	10.5±1.3	10.1±1.7
2h after stress	12.6±2.0	10.5±2.3	11.8±3.1	12.5±2.6
23h after stress	6.5±0.9	6.5±1.4	5.9±1.2	6.2±0.6

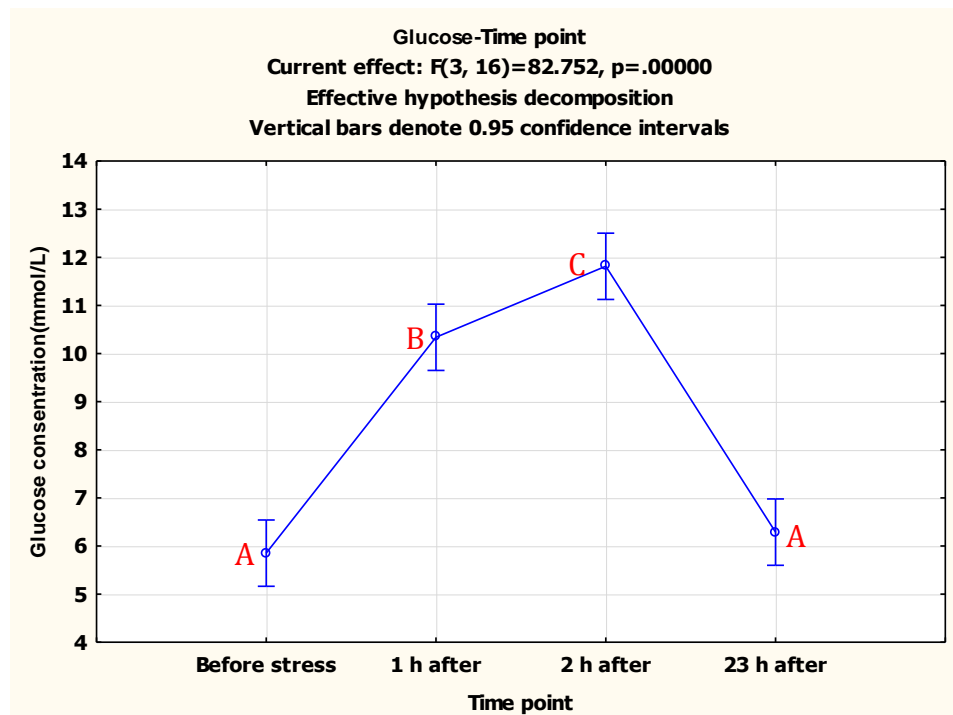


Figure 4-6 Glucose concentration in plasma of Atlantic salmon before stress, 1 h, 2h and 23 h after stress, all diet groups combined. Results are presented as tank mean ±sd (n=2). Data were analyzed using two-way ANOVA within the factor TIME. Significantly different diet groups are indicated by different letters.

4.2.3 Correlation of cortisol and glucose

Figure 4-7 shows a high correlation between cortisol and glucose in plasma ($r=0.704$), when cortisol levels were low in plasma the glucose level was also low for individual fish.

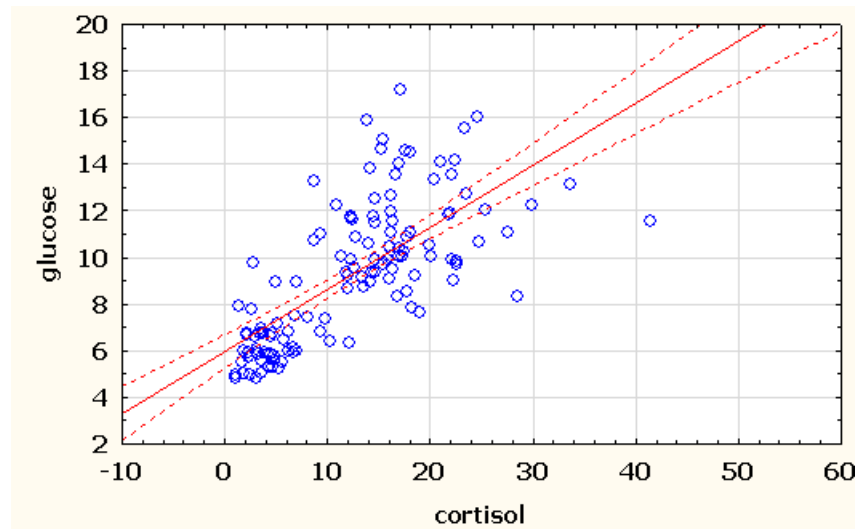


Figure 4-7 Correlation matrices analyze with cortisol and glucose in plasma of Atlantic salmon before stress, 1 h, 2h and 23 h after stress fed four different diets, the points represent the individual fish sample. X axis represents cortisol concentration, Y axis represents glucose concentration.
Glucose = $5.9764 + 0.26721 * \text{cortisol}$, Correlation: $r = 0.70474$

4.3 Gene expression analyses for HSP27, CAT, SOD1 and GPx1

The four genes (HSP27, CAT, SOD1, GPx1) showed decreased expression after stress and expression was quite stable between the four diet groups (Figure 4-8).

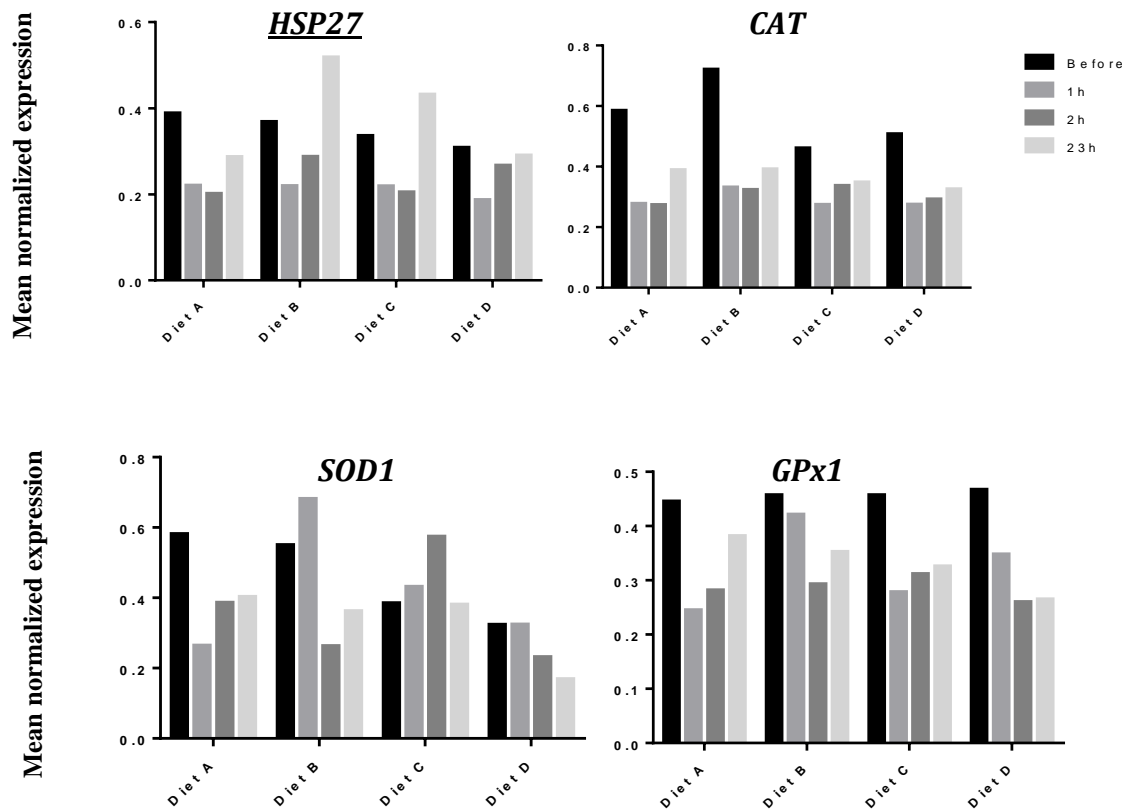


Figure 4-8 Mean normalized expression (MNE) of Heat shock protein 27(HSP27), Catalase (CAT), CuZn Superoxide dismutase (SOD1) and Glutathione peroxidase (GPx1) in liver tissue from Atlantic salmon fed the four different diets: A (1.3% EPA and DHA), B (2.7% EPA and DHA), C (4.4% EPA and DHA) and D (7.4% EPA and DHA) at four time points: before stress, 1h after stress, 2h after stress and 23h after stress. Results are presented as tank mean \pm sd (n=2).

Mean normalized expression (MNE) of HSP27 was 0.35 ± 0.13 before stress and decreased to 0.21 ± 0.07 and 0.24 ± 0.09 1 h and 2 h after stress, respectively and then increased to 0.38 ± 0.18 23h after stress (Figure 4-9). MNE for HSP27 before stress was significantly higher than 1h after stress (Tukey HSD test, $p=0.038$) and 23h after stress was significantly higher than 1h after (Tukey HSD test, $p=0.01$) and 2h after stress (Tukey HSD test, $p=0.034$).

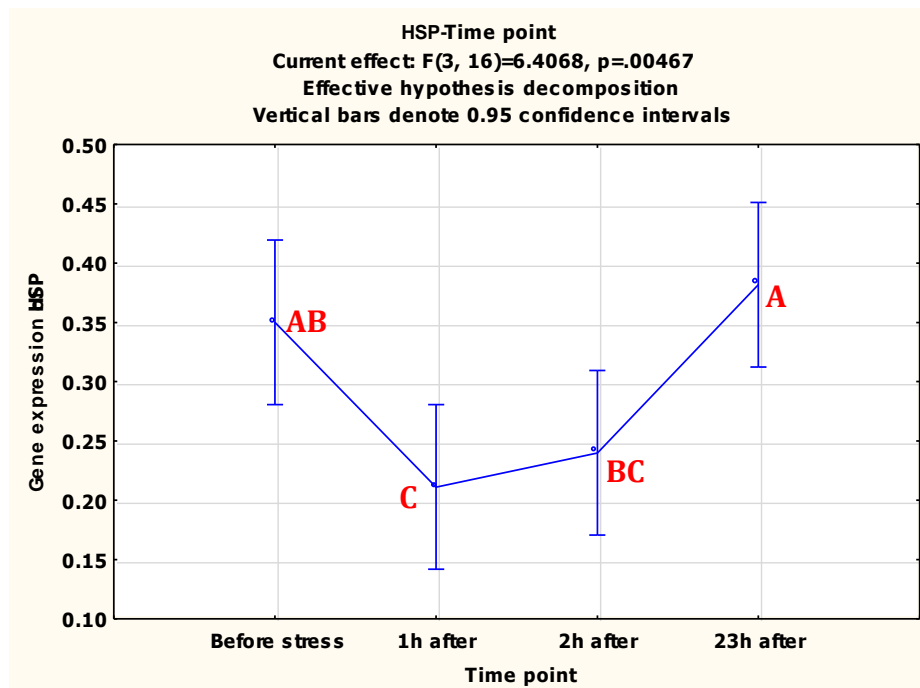


Figure 4-9 Mean normalized expression of Heat shock proteins (HSP) in liver of Atlantic salmon before stress, 1 h, 2h and 23 h after stress, all diet groups combined. Results are presented as tank mean \pm SD ($n=2$). Data were analyzed using two-way ANOVA within the factor TIME. Significantly different diet groups are indicated by different letters.

MNE for CAT decreased sharply from 0.57 ± 0.20 before stress to 0.29 ± 0.07 1h after stress and then showed a low expression level 2h to 23h after stress (0.31 ± 0.13 and 0.36 ± 0.09 , respectively) (Figure 4-10). Two-way ANOVA followed by tukey HSD test of MNE for CAT showed that the expression level was significantly higher before stress compared to the other time points (Tukey HSD test, $p<0.003$).

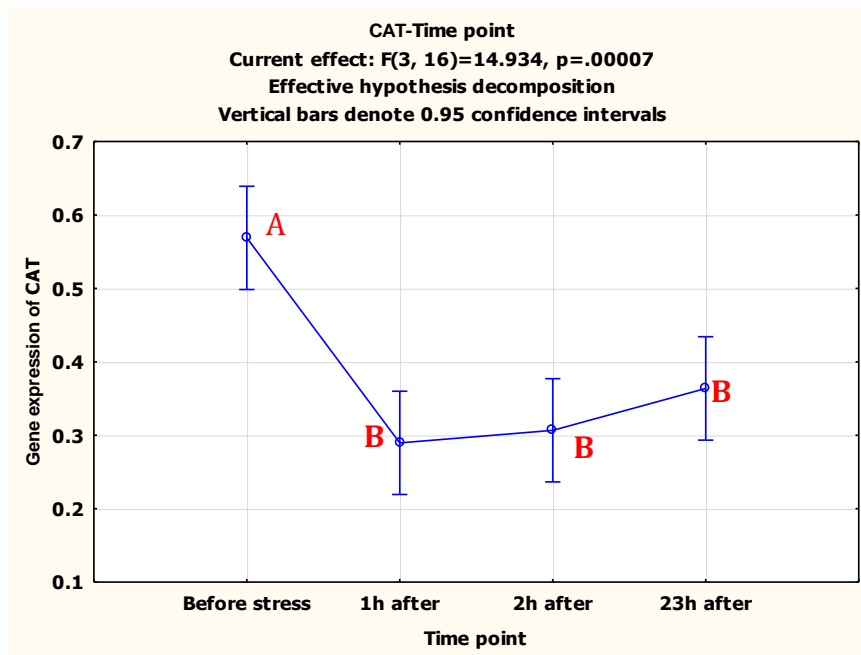


Figure 4-10 Mean normalized expression of Catalase (CAT) in liver of Atlantic salmon before stress, 1 h, 2h and 23 h after stress, all diet groups combined. Results are presented as tank mean \pm SD(n=2). Data were analyzed using two-way ANOVA within the factor TIME. Significantly different diet groups are indicated by different letters.

MNE for SOD1 was decreased much stable when compared to HSP and CAT from 0.46 ± 0.16 before stress to 0.32 ± 0.11 1h after, then continued to decrease to 0.29 ± 0.08 2h after and increased to 0.33 ± 0.10 (Figure 4-11). MNE for SOD1 before stress was significantly higher than 2h after stress (Tukey HSD test, $p=0.04$).

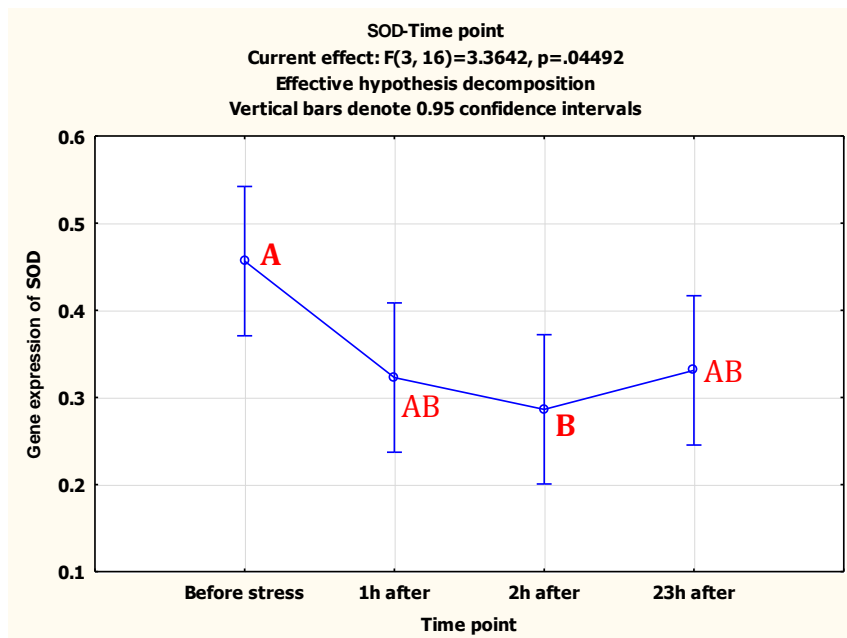


Figure 4-11 Mean normalized expression of CuZn superoxide dismutases (SOD1) in liver of Atlantic salmon before stress, 1 h, 2h and 23 h after stress, all diet groups combined. Results are presented as tank mean \pm SD (n=2). Data were analyzed using two-way ANOVA within the factor TIME. Significantly different diet groups are indicated by different letters.

MNE for GPx1 decreased quite stable from before stress to 23h after stress (0.46 ± 0.16 , 0.32 ± 0.18 , respectively) compared to CAT and SOD1, and the differences were not significantly between four time points. No significant dietary effects were seen for the four genes, neither by ANOVA nor by linear regression at the different time points.

4.4 CuZn superoxide dismutase (SOD1) enzyme activity in liver

4.4.1 SOD1 enzyme activity

Similar to the gene transcript levels (mRNA levels) for SOD1 the SOD1 enzyme activity (protein levels) decreased after stress from 0.57 ± 0.04 U/mg protein before stress to 0.53 ± 0.09 U/mg protein 1h after stress. There was no significant diet effect on the SOD1 enzyme activity (two-way ANOVA, $p > 0.05$, Figure 4-12).

A significant difference was found between 1h after stress (0.53 ± 0.09 U/mg) and 23h after stress (0.69 ± 0.08 U/mg) (Figure 4-13, Tukey HSD test, $p = 0.0175$).

Linear regression was tested and showed no relationship between four diets, and no difference was found with nested ANOVA.

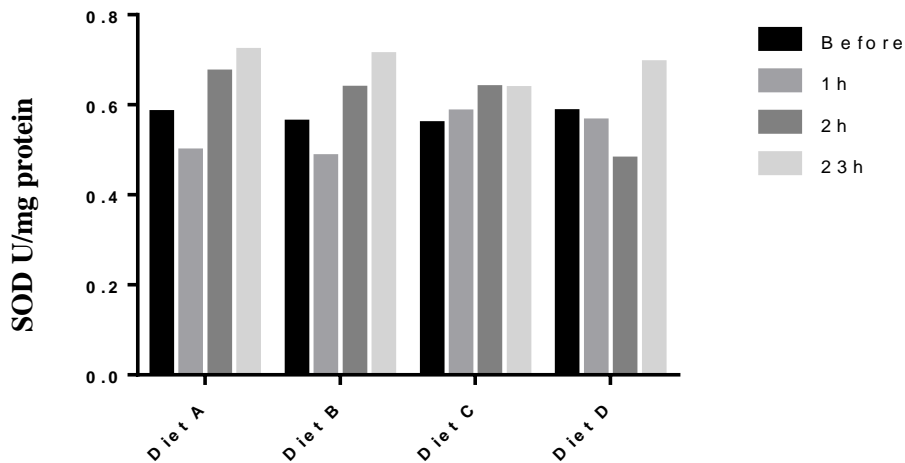


Figure 4-12 CuZn superoxide dismutase (SOD1) enzyme activity in liver of Atlantic salmon fed the four different diets: A (1.3% EPA and DHA), B (2.7% EPA and DHA), C (4.4% EPA and DHA) and D (7.4% EPA and DHA) at four time points: before stress, 1h after stress, 2h after stress and 23h after stress. Results are presented as tank mean \pm sd (n=2).

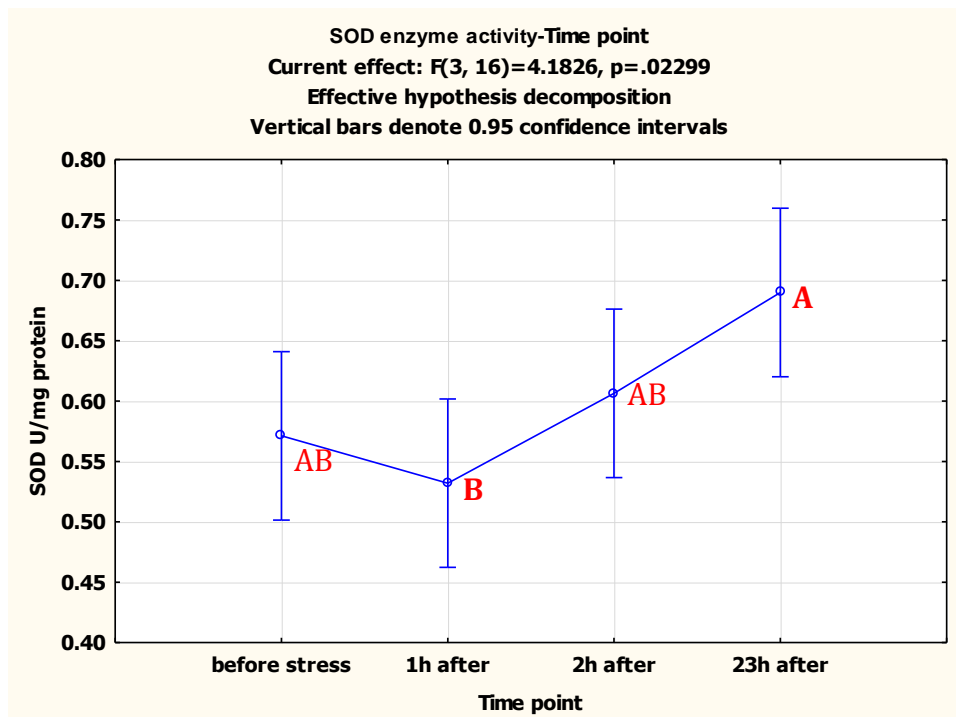


Figure 4-13 CuZn superoxide dismutase (SOD1) enzyme activity in liver of Atlantic salmon before stress, 1 h, 2h and 23 h after stress, all diet groups combined. Results are presented as tank mean \pm SD(n=2). Data were analyzed using two-way ANOVA within the factor TIME. Significantly different diet groups are indicated by different letters.

4.4.2 Correlation of mean normalized expression (MNE) for SOD1 and SOD1 enzyme activity

Figure 4-14 shows the correlation between the transcript levels of SOD1 (MNE values) and protein levels of SOD1 (enzyme activity) in liver of Atlantic salmon, and there was a low correlation between SOD1 gene transcript levels and SOD1 protein activity (correlation $r=0.076$).

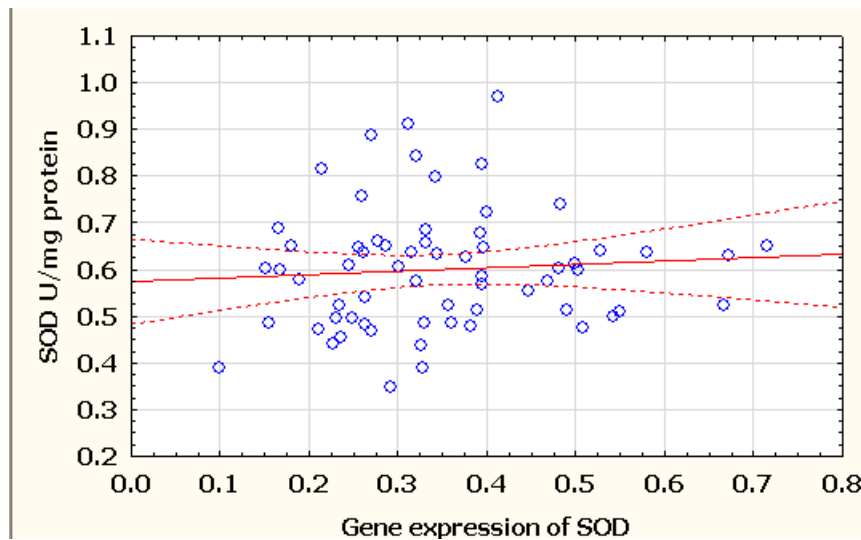


Figure 4-14 Correlation matrices analyze with mean normalized expression (MNE) for SOD1 and SOD1 enzyme activity in liver of Atlantic salmon before stress, 1 h, 2h and 23 h after stress fed four different diets, the points represent the individual fish sample. X axis represents MNE for SOD, Y axis represents SOD enzyme activity. $SOD\ U/mg\ protein = 0.57419 + 0.07363 * MNE\ for\ SOD$, Correlation: $r = 0.07621$

5. Discussion

5.1 Discussion of materials and methods

5.1.1 Experimental design

The trial was set up as a regression design with 4 diets containing 1.3%, 2.7%, 4.4% and 7.4% EPA and DHA of total FAs (0.4%-2.2% EPA and DHA of total diets), and each diet fed to duplicate tanks. Regression is one of the most used experimental designs for dietary nutrient requirement studies for fish (Shearer, 2000). The high point (7.4% EPA and DHA) in this experiment is approximately the amount used in commercial salmon diets at the time the trial was run. Ideally, if resources were unlimited more replicates at many levels would have given better accuracy of the model. However, Mead (1988) have certified that replication is not necessary when using regression, in fact, when the total observation (levels \times replicates) is over 20, more observation will give little improvement. In the present study there were 4 levels (1.3%, 2.7%, 4.4% and 7.4%) and 2 replicates for each level, which gives 8 observations. Eight points in a graph will give clear trends if there are linear relationships.

5.1.2 Feed and sampling

All nutrient supplementations (FAs, protein, vitamin and mineral) except EPA and DHA met the requirements to according NRC (2011). In order to investigate the difference between salmon groups fed different diets, the diet groups were treated as

similarly as possible. However, there are still some uncertainties that can affect the results such as different feed intake of fish within the same tank, different sex, appetite and diseases. In addition, the water exchange system, temperature, light, and gas pressure can lead to variance between tanks. However, in the present study the temperature, light and gas conditions were similar for all tanks throughout the whole study.

5.1.3 Statistics

In order to get representative and homogenous samples it is suggested to analyze large enough samples, but this is not always possible mainly due to economic constraints. In this study the mean value of the fish from one tank counted as one independent sample when using ANOVA, since single fish from one tank cannot be regarded as independent. There were only two tanks per diet in this experiment, and because we had three factors (time, tank and time), it was impossible to use nested ANOVA when including both of the factors, since there would be too many factors in the analysis relative to the degrees of freedom. The solution was to separate different time points like we did in the regression analysis and only include two factors (diet and tank) in the nested ANOVA design.

5.2 Discussion of results

5.2.1 Effect of low EPA and DHA on growth of Atlantic salmon

Low dietary levels of EPA and DHA (1.3%-7.4%) influenced the final weight, specific growth rate (SGR) and weight gain rate in this trial. Some previous studies on FO replaced by VOs have shown similar results (Turchini et al., 2009; Bransden et al., 2003; Torstensen et al., 2005). For example, studies with Atlantic salmon fed

diets with 100% replacement of FO by soybean oil have shown significantly lower growth compared with fish fed with 100% FO (Grisdale-Helland et al., 2002). Stubhaug et al. (2007) found a significant lower SGR in Atlantic salmon fed VO compare with fish fed FO. In a feeding trial by Ruyter et al. (2000) it was shown that growth rates of salmon fry increased fast with increasing levels of n-3 FAs up to 1% in diet, and salmon fed the diet including EPA and DHA grew faster than fish fed only 18:3n-3. On the other hand, many previous studies on replacement of FO with VOs have shown that VO diets do not significantly affect fish growth (Rosenlund et al. 2001; Bell et al. 2001, 2002, 2003, 2004; Ng et al. 2004; Tocher et al. 2000; Torstensen et al. 2004; Rosenlund et al.). However, most of these studies used fishmeal based diets, which contain some EPA and DHA. Turchini et al. (2009) indicated that a minimum of 1.6% EPA and DHA of dietary FAs is important in partial replacement of FO to guarantee a good fish growth of salmonids. While Diet B (2.7% EPA and DHA of total FAs) in this experiment still significantly affected Atlantic salmon growth, this may because different fish species and life stages of fish require different minimum of EPA and DHA in dietary FAs.

5.2.2 Effect of low of EPA and DHA on cortisol in plasma of Atlantic salmon after stress

Plasma cortisol of the control group (Diet D, before stress) was 75.7 ± 8 ng/ml, which is in accordance with the results obtained by Wiik et al. (1989) that reported 76 ng/ml under non-stressed condition for Atlantic salmon post-smolts. Recent research has shown that the plasma cortisol vary between different age of salmonids. For example, Einarsdottir et al. (1996) reported that plasma cortisol of immature seawater adapted Atlantic salmon was 23 ng/ml before stress. 8 ng/ml and 28-83 ng/ml have been reported by Mazur et al. (1993) in juvenile Atlantic salmon and Waring et al. (1992) in cannulated fish, respectively.

In this experiment plasma cortisol increased rapidly 2h after stress in all groups, then returned to a lower level 23h after stress which was close to the pre-stress levels. Our results are in agreement with the trial by Ganga et al. (2011) on sea bream fed diets

containing linseed and/or soybean oil, and also similar with the results on stress response in Atlantic salmon obtained by Waring et al. (1992). While different stressors or experimental methods may lead to different cortisol response of Atlantic salmon, a feeding trial by Einarsdottir et al. (1996) reported plasmas cortisol peak levels at 20min which then returned to resting level 24h after introduction of the stressor. A maximum cortisol level at 1h after handling stress (15s out of water) was seen in a study by Fast et al. (2008) and returned to prestress level 6h after stress.

There were significant differences between the four diet groups on cortisol responses of Atlantic salmon subjected to acute stress, diet C (4.4% EPA and DHA) was significantly lower than control group diet D (7.4% EPA and DHA). However, the fact that no linear regression was seen, and no effect was seen on the two diet groups fed even less EPA and DHA (1.3% and 2.7%), may indicate that this was just a random difference, not caused by the diets. Several previous studies indicate that basal plasma cortisol increased when fish were fed VOs (Ganga et al. 2011, Jutfelt et al. 2007). The inclusion of sunflower oil in a diet with 5% EPA and DHA fed to Atlantic salmon caused elevated plasma cortisol during the parr-smolt transformation (Jutfelt et al., 2007), and sea bream fed 100% FO substituted by linseed oil by Ganga et al. (2011) also found the same situation. While no effect was found for rapeseed or soybean oil fed fish (Montero et al. 2003). The authors indicated that linseed oil based diets produced higher *in vitro* adrenocorticotrophic hormone (ACTH), which induced the release of cortisol, so different ratio of VO replacing FO may affect cortisol response in fish farming. Few studies have focused on specific FAs and their effects on cortisol response in fish farming, more studies are required to clarify the VO effect on cortisol response and cortisol related hormone in fish (Montero et al. 2010).

5.2.3 Effect of low EPA and DHA on glucose in plasma of Atlantic salmon after stress

Plasma glucose increased and reached high levels 1h to 2h after acute stress in this study. This is in accordance with the results by Fevolden et al. (1991) and Fast et al. (2008) in Atlantic salmon when stress was induced by confining and handling,

respectively. Thomas et al. (1991) stressed red drum by 2-min in air transfer stressor, and the plasma glucose was maintained at a higher level from 30min to 2h after stress. It has been indicated that glucose response differ between fish species (Davis et al. 2009). There was a high correlation between plasma glucose and plasma cortisol in this experiment. It is known that the rise of plasma glucose levels has been highly related to high level of plasma cortisol and energy requirements under stress condition (Gamperl et al., 1994. McCormick et al., 1998).

No significant difference in plasma glucose response of Atlantic salmon between the four diet groups were found in this experiment. Similar results were found by Diaz-Lopez et al. (2009), who tested gilthead seabream juveniles fed 50:50 FO :Echium oil. Lee et al. (2003) fed juvenile starry flounder low n-3 HUFA levels (0.0%-2.7%) with VOs indicated that plasma glucose level were not affected by dietary VO in fish. From all these studies we can not directly conclude that low dietary EPA and DHA does not affect plasma glucose response in fish under acute stress condition, since some previous studies may give us other point of view involving FAs effects on fish stress response. However, it is possible that the effects of VOs in other studies were caused by other FAs than EPA and DHA, for instance high levels of n-6 FAs. A feeding trial by Menoyo et al. (2006) in Atlantic salmon partially fed sunflower oil (rich in n-6) and found that plasma glucose levels tend to decrease with increasing sunflower oil levels in diet, this could be explained by the high n-6 diet which may delay cortisol release (Ganga et al. 2011) and changed n-3/n-6 ratio in fish diet may affect stress response (Bell et al. 1991). It has been reported that total replaced FO and fishmeal with plant materials resulted in elevated plasma glucose when compared to FO and fishmeal diet fish (Sissener et al. 2013).

5.2.4 Effect of low EPA and DHA on gene expression of HSP27 in liver of Atlantic salmon after stress

In this experiment the mean normalized expression (MNE) of heat shock protein 27(HSP27) in liver decreased significantly 2h after stress and returned to the pre-stress level after 23h. The same response of HSP27 in liver of Atlantic salmon fed soybean meal elicited by the stress of reduced water level was reported by Sissener et

al. (2009). Many studies have shown that HSPs are up-regulated in response to a wide range of stressors, as they have a role in repair and degradation of misfolded or denatured proteins (Iwama et al. 1998). While as mentioned in the introduction, the low molecular weight (LMW) HSP, such as HSP27, are much more species specific compared with high molecular weight HSPs. The LMW HSP normally play a role as molecular chaperones, preventing irreversible protein aggregation (Derham and Harding, 1999).

No difference in HSP27 expression in liver under stress situation was found between the four diets in this study. No previous studies on the effect of FAs effect on transcription of HSP27 in liver seem to have been conducted. Comparing to results from other tissues, Atlantic salmon fed soybean oil showed significantly lower HSP27 in head kidney leukocytes compare to fish fed FO (Holen et al., 2011) and HSP27 in intestine was elevated in rainbow trout fed 35% soybean meal (Sealey et al., 2013). The HSP response can vary with different tissue (Smith et al., 1999), and stressors (Iwama et al., 1998) and the HSP expression may also vary with the fish species (Nakano and Iwama, 2002), age (Martin et al., 2001), and season (Fader et al., 1999).

Research is still in an early stage on a cellular level of fish stress (Iwama et al. 1998). Some studies on the relationship between stress hormones (cortisol and adrenaline) and HSP have just emerged recently. Handling stressor increase cortisol levels but reduce the gill tissue levels of HSP30 in Atlantic salmon, which suggests that cortisol may down regulate HSP30 synthesis (Ackerman et al. 2000). This maybe the same reason that can explain the decreased expression of HSP27 in liver after stress in the present study. Since the detailed principles of these relationships are not clear yet, future research needs to focus on HSP gene in fish stress response at molecular level (Kayhan and Duman, 2010; Iwama et al. 2004).

5.2.5 Effect of low EPA and DHA on gene expression of CAT, SOD1 and GPx1 in liver of Atlantic salmon after stress

The mean normalized expression (MNE) of the three antioxidant genes SOD1, CAT

and GPx1 all decreased after stress. This is in agreement to the results of Olsvik et al. (2013) in Atlantic salmon responding to high temperature stress. The authors suggested that a high temperature reduced ROS production in mitochondria and then reduced overall metabolism in fish. From previous studies we can find that different stressors may affect MNE of antioxidant genes; MNE of SOD1 and GPx1 increased and CAT decreased in brown trout exposed in metal related stress (Hansen et al., 2007). Since no previous study has been conducted related to the effect of physical stress on MNE of antioxidant genes, this area is not clear yet.

There was no significant difference between the four diet groups for MNE of SOD1, CAT and GPx1 in this study. This is in line with Olsvik et al. (2007) on 100% replacement of FO by VO in Atlantic salmon on MNE of CAT. But some different points were found by other studies such as the MNE of SOD1 decreased in Atlantic salmon fed VOs (Olsvik et al., 2011). The author suggested that the VOs contains lower polyunsaturated fatty acids (PUFA) compared to FO, which may lower the superoxide production in fish liver. Saera-Vila et al. (2009) investigated that dietary FO replaced with VOs decreased the expression of GPx1 in liver of gilthead sea bream. While Bowyer et al. (2012) in yellowtail kingfish fed canola oil had opposite results in expression of GPx1. The authors indicated that different PUFA:SFA ratio in fish diet may vary the expression of GPx1.

5.2.6 Effect of low EPA and DHA on SOD1 enzyme activity in liver of Atlantic salmon after stress

The SOD1 enzyme activity decreased significantly 1h after stress. The same result has been found by Heink et al. (2013) in rainbow trout exposed to ozone, while SOD1 activity in liver increased in other two species in the same experiment. The author suggested that SOD1 enzyme activity vary with different species by specific stressor and in certain tissues.

No significant difference of SOD1 activity was seen between the four diet groups in this experiment. This is similar with the results by Mercan et al. (2013) in rainbow

trout fed soybean oil when compared with FO group. A significantly higher total SOD (CuZn-SOD+Mn-SOD) enzyme activity in liver of Atlantic salmon fed FO and fishmeal diet compared with plant oil and plant meal group was found by Olsvik et al. (2011). The authors indicated that some antioxidants like α -tocopherol and astaxanthin concentrations were much higher in the marine material diet group, while a high γ -tocopherol concentration was only present in plant material group. Maybe the total antioxidants or the antioxidant status in fish are equal in both of the diet groups.

Low correlation between SOD1 enzyme activity and MNE of SOD1 in liver was seen in this study, this result can also be found in some previous studies (Anderson et al., 1997, Olsvik et al., 2007, Sagstad et al., 2007). While Mercan et al. (2013) investigated that rainbow trout fed goose fat (rich in saturated FAs and monounsaturated FAs) showed highest level of SOD1 enzyme whereas the minimum expression level was also observed in this group, the author explained that maybe goose fat suppress the expression of SOD1 gene.

5.3 Conclusions

Overall, low levels of EPA and DHA (1.3%-7.4%) do not seem to affect stress response and recovery in Atlantic salmon (*Salmo salar* L.).

A significantly lower plasma cortisol concentration in diet C (4.4% EPA and DHA) compared to control group (7.4% EPA and DHA), possibly due to the random effect (type 1 error), as no similar trend was seen for diet group A and B. No dietary effect on plasma glucose was found in this study, but there was a high correlation ($r=0.705$) between plasma cortisol and glucose.

On the cellular level, no significant difference between four diet groups has been found on transcription of HSP27 in stress situation.

The transcriptions of antioxidant enzymes (SOD1, CAT, GPx1) and the SOD1 enzyme activity were not influenced by low levels of EPA and DHA in Atlantic salmon in this study.

Both plasma glucose and cortisol, as well as transcription of stress related genes and enzyme activity was affected by stress (significantly different between the time points). This shows that we successfully managed to stress the fish, and thus should have been able to detect differences between the diet groups if EPA and DHA at the current levels had an impact on stress response and recovery.

6. Future perspectives

In this study we looked for the effect of low dietary levels of EPA and DHA on stress response in Atlantic salmon, with diets high in plant based materials. To obtain an overview of stress response in salmon, it would be interesting to include more diet groups such as use other VOs, since different n-3/n-6 may influence the release of stress hormones, or include different levels of fishmeal replacement to see if plant meal affect stress response in fish.

The effect of dietary fatty acids on gene expression of HSPs in liver of Atlantic salmon is still at an early stage. The gene expression of HSPs vary with different organ in fish under stress situation, but the principal of this is not clear. More study needs to be done on this area in the future, especially on the interaction between stress hormones and HSPs.

Physiological stress decrease the gene expression of antioxidants and SOD1 enzyme activity in this study, since no previous study has been conducted related to the effect of physical stress on antioxidant enzymes; it would be interesting to find the principal of this, especially on relationship between plasmas cortisol concentration and antioxidant enzymes activity. The effect of low dietary HUFA on antioxidant enzyme activity is still not clear, more study needs to be done on this area as well.

7. References

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