Changes in histidine metabolism through smoltification and effect on cataract development in Atlantic Salmon (*Salmo salar L.*)

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Abstract

Cataract is the collective definition of any light scattering opacities of the eye. Cataractogenesis is caused when there is a noticeable irregular light scattering because there has been a change in the tridimensional structure of the crystalline. This can be caused by two varieties of cataract, one irreversible, and one reversible osmotic form. The last type only happens when salmon move from freshwater to saltwater. Cataract investigations during the 90`s and early 2000`s revealed high prevalence and an increasing severity. The most believed reason for the major increase was the removal of bloodmeal from the feed, the second was the transition from fish oil to plant oil. As of 1995 it turned into one of the most economically important diseases in farmed salmon. This pushed research to find an understanding for the high prevalence of cataract and how to prevent it. The main purpose of this study was to investigate the interaction effect of histidine supplementation and dietary lipid in freshwater. Concentrations of NAH, histidine and histidine derivatives in heart, lens and muscle were decided through HPLC and ninhydrin detection. For three samplings fish were scored for cataract using the scale 0-4 developed by Wall and Bjerkås (1999). Findings in previous research have discovered histidine supplementation to have a preventative effect on cataract development. Histidine and histidine derivatives have important functions in the eye metabolism and osmotic function. The replacement of fish oil with a RAFOA mix (vegetable oil) has not previously shown to have any effect on cataract development. The findings in the present study support previous findings that histidine supplementation prevents cataract, but this study does show that vegetable oil has a negative effect on cataract development in freshwater.

1. Introduction

1.1 Definition of cataract

Cataract is a collective definition of any light scattering opacity of the lens (Michael and Bron, 2011), opacity is the loss of transparency. Cataractogenesis is caused when there is a noticeable irregular light scattering because there has been a change in the tridimensional structure of the crystalline. (Midtlyng *et al.*, 1999). This is caused by changes in the epithelial tissues surrounding the lens fibers or by the composition changing in the structures of the lens fibers (Bjerkås et al., 2006). In most species including fish, cataract can appear as an irreversible damage to the lens fibers and a proliferation of the lens epithelium. In Salmonids a reversible cataract is also known, which is called osmotic cataract (Bjerkås et al., 2006).

Osmotic form of cataract only involves the lens fiber cells, and will appear as opacities around sutures (Hargis Jr, 1991). This form of cataract only happens in salmon moving from freshwater (FW) to saltwater (SW) and is caused by a temporary osmotic imbalance. It is reversible in some developing stages (Hargis Jr, 1991). Osmotic cataract observed by Iwata et al can be reversed if the swelling does not last for a long period or has caused any disruption to the lens fibers. Cataract is scored depending on the severity of opacity, on a scale of 0-4, which is developed by Wall T and Bjerkås (1999).

Cataract development has been documented in almost all life stages, but it looks like salmon is especially exposed during the parr-smolt stage (Bjerkås et al., 2006). Due to the changes in the physiological state, which includes changes in amino acids, imidazole concentrations and osmolyte composition (Breck *et al.*, 2005; Bjerkås et al., 2006)

1.1.1 Lens morphology

Fish lenses are built up by two different types of cells. Outer monolayer epithelial cells and underlying fiber cells, they are nourished by the outer monolayer. Crystallin's, is the main protein of which the fish lens is built up by. When they are bound in their natural structures, they are responsible for the transparency of the lens (Hargis Jr, 1991; Bjerkås et al., 2006). When lenses grow the epithelial cells convert into fiber cells that will cover the previous fiber

cells and create more layers like that of an onion, this process continues its whole life and slows down with age like in mammals (Hargis Jr, 1991). Fiber cells will eventually deteriorate and lose their nuclei and other organelles. (Waagbø *et al.*, 2009).

Salmon eyes compared to other vertebrates are harder and have lower water content, but with higher protein content (Bjerkås *et al.*, 2003). The higher protein content in the eye is believed to make the lens more susceptible to cataract development, as protein aggregation is a cause of light scattering (Wegener *et al.*, 2001). The lens of fish is dependent on nutrient and electrolyte transport to make sure the eye maintains normal function and transparency (Iwata *et al.*, 1987). Growth and metabolism of the lens can be affected by factors in the water and by substances absorbed through the digestive tract which is converted to aqueous humor (Bjerkås *et al.*, 2003). Teleost eyes have similarities to other vertebrates, the differences are adaptations to the aquatic environment.

The adaptations are lack of an eyelid and an immobile pupil, which makes it necessary to make any photomotor response at retinal level (Wall, 1998). Teleost's eye is constantly exposed to the elements since it is missing an eyelid and protrusive. Which causes toxins in the water to easily cross the membrane and cause damage to the eye (Hargis Jr, 1991) Most species of teleost are also not able to control the entry of light, as they cannot vary the iris (Hargis Jr, 1991). Teleost eyes also differ from other vertebrates with that their retinas grow continuously throughout their life's (Fernald, 1988). Salmon and other anadrome fish will during their life have to change osmoregulation when moving from FW to SW (Bjerkås *et al.*, 2003).

1.2 Challenges with cataract and background

First reported cataract was in rainbow trout *(Oncorhynchus mykiss*) (Hess, 1935). Which was an ocular cataract, and the study was done in hatcheries in New York state. They investigated the cataract as they suspected the cause was a contagious disease. Cataract was more observed and a major challenge during the 90`s and early 2000`s. As of 1995 it turned into one of the most economically important diseases in farmed salmon (Menzies *et al.*, 2002). In a study conducted in 1998 along the Norwegian coast, a cataract prevalence in 49 salmon was found to be 82% (Ersdal et al., 2001). The high prevalence of cataract in farmed fish during the mid-

90`s provoked a push towards the understanding of the causes and preventing it within European aquaculture. (Midtlyng *et al.*, 1999). Previous research around cataract has been focused on biotic factors, parasites, pathogen infestation and on the theory of genetic predisposition (Peuhkuri et al., 2009).

A series of investigations in Ireland found a prevalence that ranged from 50- 90 % in Irish farms 1995-96, and clinical findings were similar in Scotland in 1996 (Wall, 1998). A year later prevalence varying from 5-90% was reported from Norway (Wall, 1998; Ersdal et al., 2001). Over 38,000 fish from 46 different farms were screened for cataract, this was done during a 3-year period. Scotland 1995, Ireland 1996 and Norway 1997 (Wall, 1998). The removal of blood meal from the diet was one of the first causations of cataract, the second was the transition from fish oil to plant oil (Midtlyng *et al.*, 1999; Tacon and Metian, 2008).

Salmon is a visual feeder, which means it is dependent on its vision to find food (Bjerkå *et al.*, 1996). Damaged vision caused by cataract is not only a fish welfare problem, but a financial hindrance to the production of salmon. There are previous studies that have suggested that there is a reduction in growth rate in fish with cataract (Bjerkås *et al.*, 1996). In this study they found a correlation between growth and cataract formation, the strongest correlation was between body length and cataract formation. As less feed the salmon gets the less, he grows. It causes severe economic problems, based on a cost estimation model the average annual direct costs of cataract is 27,865,000 Euros (Menzies *et al.*, 2002). This is based on calculating an estimate of weight loss because of cataract.

Cataract is very often observed in salmonoids, this might be an indirect consequence of salmon being the most observed fish in the world. Challenges with cataract are the severe outbreaks that can cause blindness, a secondary infectious disease and in causality high mortality (Waagbø et al., 2003).

1.3 Development of cataract

The knowledge that there are multiple factors, both environmental and nutritional, that causes cataractogenesis is well established (Peuhkuri *et al.*, 2009). The controllable environmental factors during rearing are water temperature and light. Nutritional factors such as histidine, amino acid composition and source of lipid have also been shown to cause higher prevalence and severity of cataract (Bjerkås and Sveier, 2004; Trösße *et al.*, 2013; Remo *et al.*, 2014).

1.3.1 Water temperature

Fluctuation in water temperature has been associated with the formation of cataract (Ersdal et al., 2001). At higher temperature feeding will also cease as oxidative pressure increases (Sambraus *et al.*, 2017).Trials that have been done with pre smolt in FW have shown a connection between rapid growth and the development of cataract (Breck and Sveier, 2001).

In an another study it was shown a high correlation between body length and cataract formation ($p<0.01$) (Bjerkås *et al.*, 1996). A study has also shown an increase in prevalence of cataract in adult Atlantic salmon when the water temperature was increased from 12°C to 18.5 °C (Waagbø *et al.*, 2010).

1.3.2 Nutritional imbalance

Several advances have been made in fish nutrition during the late 90`s early 2000`s (‘Fish Diseases and Disorders, Svazek 3’, 2011). This is caused by the spike of focus on fish welfare and the growth of a sustainable aquaculture industry (Midtlyng *et al.*, 1999; Nasopoulou and Zabetakis, 2012). Advances in fish nutrition had to be made as blood meal was removed as an ingredient in fish feed, because of the risk of transmitting bovine spongiform encephalopathy (BSE). Omission of blood meal in fish feed caused a spike in cataract prevalence. Since blood meal is very rich in dietary histidine, and studies have seen the preventative effect of the histidine in blood meal (Wall, 1998; Breck *et al.*, 2003; Waagbø *et al.*, 2010)

In addition to histidine, other nutritional deficiencies were proposed for causing cataract. Zinc, riboflavin, tryptophan, thiamine and methionine deficiency (Ersdal et al., 2001) Feeding

studies done early 1980`s showed a methionine deficiency to be a cause of cataract (Tacon, 1992). Iron deficiency has also been suggested as a cause for higher severity and prevalence of cataract (Breck *et al.*, 2003). The replacement of fish oil with plant oil is also considered to be a risk factor for both cataract and in general under developing salmon (Torstensen *et al.*, 2008; Trösße *et al.*, 2013).

1.4 Transition from fresh to saltwater, smoltification

Smoltification is a physiological, morphological, and behavioral change in salmon when going from FW to SW. All salmonoids start their life in FW and as an anadromous species they make a transition to SW at a juvenile stage. Smoltification is the biological change salmon goes through to make use of both FW and SW during its transition. The development from parr to smolt involves multiple endocrine systems (Björnsson et al., 2011).

During the stage of parr-smolt transformation salmon changes from hyperosmotic osmoregulation of water and ions to hypoosmotic osmoregulation (Bjerkås *et al.*, 2003). Levels of nutrient in diet has been an auxiliary factor for the accelerated smoltification, healthier smolts and an improved survival rate after release (Higgs *et al.*, 1985; Ogata and Murai, 1994).

1.4.1 Light

Major factors for the process of smoltification is the photoperiod salmon is exposed to (Stefansson *et al.*, 1991). Salmon can be exposed to different light intervals (photoperiod) to manipulate growth and development (McCormick *et al.*, 1998). A photoperiod could be 12:12, which is 12 hours light and 12 hours darkness. That photoperiod is a winter signal to stimulate growth (Stefansson *et al.*, 1991). Lights are used when fish are reared to have control over when the fish is going to go from parr-smolt, and it is possible to have year round production of smolt (Berrill *et al.*, 2002). Under natural condition salmon matures consistently with the light cycle during the year (McCormick *et al.*, 1998).

1.5 Amino Acids

In fish the major organic component in the tissue is proteins, it makes up around 65-75% for the basis of total dry weight (Wilson, 2013). In the diet for salmon, it makes up 35-55% with highest levels at early life stages. Young salmonids need around 45-55%, juveniles 40% and yearlings 35% (Wu, 2009). Fish consume proteins to obtain free amino acids, which are used by various tissues to synthesize new protein. Proteins are used continually by fish (Wilson, 2013). Some fish's protein requirements are affected by temperature, chinook salmon have been examined to require 40% protein at 8 degrees and 55% at 15 (Wu, 2009).

Amino acids consumed through diet is essential for fish as it is used for energy substrate, endogenous protein synthesis and to regulate metabolic pathways (Andersen et al., 2016). Amino acids are divided into either essential amino acids (EAA) or nonessential (NEAA).When animals digest an amino acid imbalanced diet it responds by lowering its feed intake, because it has the ability to perceive the amino acid deficiency in the feed (Averous *et al.*, 2003). This is assumed to be the case for fish also (A. Khan, 2018).

In 2011 the national research council (NRC) published recommendations for AA requirements in fish and shrimp. (Council, 1993; Andersen et al., 2016). There are some drawbacks to the studies done by the NRC. The feed used by the NRC had high levels of fish meal whilst feed today has higher plant protein levels. Some studies have shown a reduction of feed intake, growth and protein utilization when fish have been feed a plant protein based diet. (Kaushik *et al.*, 1994; Ruyter *et al.*, 2015; Andersen et al., 2016). Growth is used to measure requirements, which can overlook the metabolic requirements (Andersen, Waagbø and Espe, 2016). Histidine requirements are also too low levels to prevent damaging oculars and causing cataractogenesis (Remo *et al.*, 2014).

1.6 Histidine and derivatives

Histidine classifies for fish as an EAA (essential amino acid) (Andersen et al., 2016). As it cannot be synthesized de novo (Wu, 2009). The histidine molecule viewed chemically, has a functional positively charged imidazole group which can act as an ampholyte (Andersen et al.,2016). Histidine and histidine derivatives might function as antioxidants and can also mitigate the impact of oxidative stress (Andersen et al., 2016).

In fish the white muscle is known to have large amounts of histidine and its related dipeptides, which are anserine (β-alanyl-N-methylhistidine), carnosine (β-alanyl-L-histidine), or β-alanine (β-alanyl-L-histidine-τ-methyl-L-histidine) (Ogata and Murai, 1994).

Different feeding trials with an increased dietary histidine have shown a reduction in cataract. (Rhodes *et al.*, 2010). Histidine is able to attach to and control the absorption of zinc, copper and iron (Wade and Tucker, 1998). Histidine levels can affect the availability of zinc, and reduced levels of zinc could have an effect on cataract development (Ketola, 1979).

First time they could show that higher histidine levels in the feed had a preventative effect on cataract development it was demonstrated in adult Atlantic salmon. That study showed preventative effect in salmon during their second year in SW (Breck *et al.*, 2003).

The required histidine levels in feed for salmonids are said to be 8 g histidine/kg (Council, 1993; Andersen et al., 2016). For salmon weighing 62g the histidine requirement is 2% of crude protein in the feed (A. Khan, 2018). Breck et al showed that the diet had to be supplemented with histidine levels far above the levels required for growth. 18 g histidine/kg showed to significantly reduce the prevalence of cataract in smolt (Breck *et al.*, 2005). The higher levels of histidine required for adult salmon has been confirmed later by Remø et al, where 13.4 g histidine/kg was needed to minimize cataract Atlantic salmon smolt and at 12.8 g histidine /kg in adult salmon in SW (Waagbø *et al.*, 2010)

N-acetylhistidine (NAH) which is a histidine metabolite and can be found in the lenses of poikilothermic vertebrates (Baslow, 1998). Study done in 2010 showed that NAH is the major osmolyte in the salmon lens (Rhodes *et al.*, 2010). It is a prominent biomolecule in brain,

retina and lens of poikilothermic vertebrates, and NAH has a rapid hydrolysis, and only trace amounts are present in ocular fluid in vivo at any given time (Baslow and Guilfoyle, 2015). Remø et al (2014) showed that NAH in heart tissue increases after sea transfer and reaches a tissue saturation at moderate dietary histidine concentrations (Remo *et al.*, 2014).

NAH is synthesized from L-histidine and the energy-rich acetyl Co-enzyme A (AcCoA). Acetate (Ac) derived from D-glucose (Glc) metabolism (Baslow and Guilfoyle, 2015). The cycling of NAH and HIS appears to be an energy driven pump mechanism operating at the lens/ocular fluid interface (Baslow and Guilfoyle, 2015).

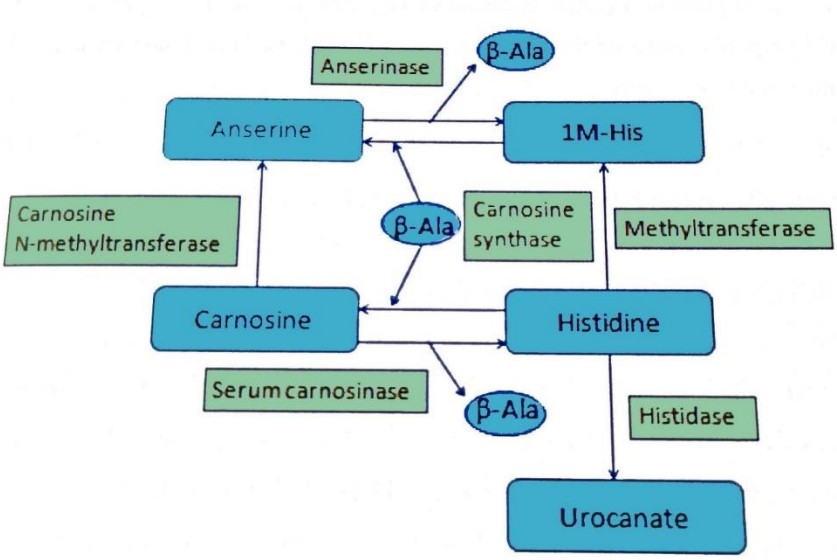

Figure 1 Schematic illustration of the histidine dipeptide metabolism, based on the KEGG pathway.

1.6.1 Derivatives

Of the 4 histidine derivatives anserine and carnosine seems to be the amino acids of most importance for proper bodily functions in salmon (Ogata and Murai, 1994; Ogata et al., 1998; Snyder *et al.*, 2012). The four derivatives as shown in figure 1 can influence each other as they are synthesized from each other.

Anserine is an amino acid that is synthesized from 1-methyl-histidine (1-MHis) and B-alanine by carnosine synthase. Or through the formation of carnosine from histidine, then followed by methylation of carnosine (carnosine-N-methyl-transferase) (figure 1). Anserine has a strong buffering capacity and this is important for anaerobic burst swimming ability (Ogata and Murai, 1994). In rainbow trout it is shown to act as a lactic acid modulator in white muscle (Snyder *et al.*, 2012).

Ogata et al (1994) hypothesized that smolt accumulate anserine in their muscle to heighten their buffering capacity. This accumulation is also shown in adult salmon after transfer to seawater (Tröße *et al.*, 2010). Salmonoids and most other fish, both marine and freshwater seem to selectively produce anserine in muscle rather than histidine and the other dipeptides (Abe, 1983; Ogata and Murai, 1994).

Carnosine is two amino acids (histidine and β-alanine) bonded together with an amide bond (Guiotto *et al.*, 2005). It is methylated to form anserine and it can also be cleaved by carnosinase back into its constituent's histidine and β-alanine. Carnosine is found exclusively in animal tissue, and is therefore of extra interest when studying free amino acids in fish (Snyder *et al.*, 2012).

1.7 Lipids

Lipids are a vital part of the salmon's diet, and account for more than 30% of the total weight. It is a major source of energy as Atlantic salmon has a high capacity of using fat as an energy source (Torstensen et al., 2000). Lipids are defined as soluble compounds in organic solvents which usually contains fatty acids esterified to alcohol groups, if it is a glyceride, and amino groups when it is a sphingolipid. Lipids can be divided into two groups, the first group is polar lipids, which is composed of phospholipids. The second group is neutral lipids, which is composed of triacylglycerols (Tocher, 2003).

Lipids with their constituent fatty acids are together with proteins the major organic constituents in fish (Tocher, 2003). Carbohydrates are quantitatively much less prominent in fish, and the lipid content can exceed the protein content. This can show how an important role the lipids and more specifically their constituent fatty acids have as a source for metabolic energy in fish for growth. Which also includes reproduction and movement.

The main role of fatty acids in all organisms is to generate metabolic energy. Organisms create energy in the form of ATP through mitochondrial B-oxidation. (Sargent et al., 1952). All known organisms including fish, are able to biosynthesize de novo saturated fatty acids 16:0 and 18:0 (Sargent et al., 1952). Another important role of fatty acids (phosphoglycerides) is to make up the cell membrane bilayers (Sargent et al., 1952)

1.7.1 Source of lipid

The accessibility of omega 3 fatty acids is one of the biggest limiting factors for further growth in aquaculture. The demand for omega 3 fatty acids EPA and DHA has increased over the last several years. Normally fishmeal and fish oil has more than enough nutritional value by themselves, but todays raw resource situation makes it hard to make feed solely composed of marine oils (Ruyter et al., 2019). Today's production of fish meal and fish oil is a stable but limited resource. A consequence of reducing the amount of fish meal/oil is the natural lowering of EPA and DHA, since there is not any viable vegetable replacement.

In 2014 the feed was composed of 70% canola oil and 30% fish oil. (Ytrestøyl et al., 2014). A report from 2019 showed that in 2016 the total composition of feed was composed of 20.1% plant oils and 9.4% marine oils (Aas et al., 2019). A conclusion from a study done in 2006 says that Atlantic salmon utilize diets based on plant protein sources without fish meal satisfactory as long as the dietary amino acid composition mimics the amino acid composition of the control fish meal diet (Espe *et al.*, 2006). Vegetable oils do not naturally contain any of the n-3 fatty acids , but are instead rich in saturated n-6 fatty acids (Torstensen et al., 2000).

Several previous studies have investigated the consequences of replacing fish with plant oil, a review from 2009 concluded that 60-75% of fish oil can be replaced with alternative lipid sources (Turchini, Torstensen and Ng, 2009). 2019 rapport on finding new sources for omega 3, they had findings that a modified rapeseed oil was a safe oil alternative in salmon feed (Ruyter *et al.*, 2019)

Between 80 and 90% of all fish oil produced is used for fish feed, given that this is an expensive and limited resource when compared to other raw materials. When looking at it sustainability and financially there is a need to find and use other sources (Nasopoulou and Zabetakis, 2012).

There is limited knowledge if replacing fish oil with plant oil influences cataract development and in cause effects growth. There have been two studies on this and they are described by fat for fish health (Sissener *et al.*, 2016) The risk of cataract development with replacing plant oil with fish oil was investigated in 2014 (Remo, 2014). An earlier study in 2005 showed 4 to 5 times higher prevalence when salmon was fed 75% and 100% VO blend when compared to fish fed FO (RAFOA, 2005).

1.8 Goal for the study

1.8.1 Objectives:

The overall objective of the present study was to investigate cataract development in FW in relation to dietary lipids and histidine supplementation.

This included

- Investigate whether histidine supplementation influenced prevalence or severity of cataracts.
- Investigate whether dietary lipids modulated the susceptibility to cataracts.
- Study interaction effects between dietary lipids and histidine.
- Study changes in histidine and histidine derivatives in lens, heart and muscle during parr-smolt transformation, and whether these could be influenced by dietary lipids or his supplementation.

2. Material and method

2.1 Experimental design

This master thesis is conducted as a part of a project at institute for marine research. Project name sophisticat and project number 15493. The project studies if the interactions between dietary lipids and histidine in the FW diet modulate the risk of cataract development after sea water transfer.

2.1.1 Feeding trial

The feeding trial was conducted at the Institute of Marine Research (Matre Research Station, Norway). The fish were reared by standard production procedures at Matre research station and came from the Aquagen strain. From first feeding, 2 groups of Atlantic salmon fry were given diets containing either 100% FO (fish oil) or VO (plant oil mix, RAFOA), the average dietary lipids are shown in table 2. The experimental feeds were produced by Biomar. When the fish reached ~30 grams the fish were split further into four groups and were fed fish oil (F) with high levels histidine (H), F with low levels of histidine (L), vegetable oil (V) with H and V with L in triplicate tanks. The experimental diets are shown in table 1. Both diets had a His content above the optimum for growth (NRC). The fish were smoltified by giving them a winter period (photoperiod 12:12) followed by a period with continuous light (photoperiod 24:0). The fish were fed daily to satiation by continuous feeders and temperature was 13°C for the duration of the experiment.

Table 1 Feed recipe and analyses of content. Analyses performed by technical staff at IMR

(g/100g)	F His -	F His +	V His -	V His +
Fish oil	16	16		
RAFOA oil			16	16
Soya SPC	13	18	13	18
Pea Protein	5	5	5	5
Wheat Milling quality	12	10	12	10
Wheat Gluten	15	11	15	11
Fish meal	39	40	39	40
Lecithin Soy, Liquid	0,50	0,50	0,50	0,50
Additives and crystaline amino acids	0,48	0,57	0,48	0,57
Vitamins and minerals	0,48	0,48	0,48	0,48
Lucantin Pink	0,03	0,03	0,03	0,03
Mono-sodium Phosphate (MSP)	1,49	1,59	1,49	1,59
Proximate feed composition				
Protein	51	53,5	51	53
Lipid	20	18	19	18
DM	95		95	
Ash				
Histidine (mg/g)	10,9	13,7	10,4	13,7

Table 2 Average dietary lipids. Analyzed by technical staff at IMR

Analysis	**FO**	**SD**	**VO**	**SD**
06:0 (mg/g ww)	<0.01	-	<0.01	-
08:0 (mg/g ww)	<0.01	-	<0.01	-
10:0 (mg/g ww)	<0.01	-	<0.01	-
12:0 (mg/g ww)	0,21	0,02	0,19	0,02
14:0 (mg/g ww)	11,3	1,2	2,2	0,1
14:1n-9 (mg/g ww)	0,2	0,0	<0.01	-
15:0 (mg/g ww)	0,72	0,04	0,18	0,01
16:0 (mg/g ww)	26	1	27	2
16:1n-9 (mg/g ww)	0,5	0,1	<0.01	-
16:1n-7 (mg/g ww)	9,3	0,9	1,6	0,3
17:0 (mg/g ww)	0,64	0,03	0,22	0,01
16:2n-4 (mg/g ww)	1,30	0,24	0,35	0,01
18:0 (mg/g ww)	4,0	0,5	4,8	0,3
16:3n-3 (mg/g ww)	0,7	0,4	<0.01	-
18:1n-11 (mg/g ww)	0,5	0,2	<0.01	-
18:1n-9 (mg/g ww)	17	2	65	4
18:1n-7 (mg/g ww)	3,8	0,7	3,7	0,5
16:4n-3 (mg/g ww)	1,5	0,4	<0.01	-

18:2n-6 (mg/g ww)	5,9	0,4	25,3	1,8
18:3n-6 (mg/g ww)	0,21	0,01	<0.01	-
20:0 (mg/g ww)	0,39	0,05	0,73	0,05
18:3n-3 (mg/g ww)	2,1	0,1	19,3	1,7
20:1n-11 (mg/g ww)	0,99	0,42	0,22	0,04
20:1n-9 (mg/g ww)	9,4	3,7	2,6	0,1
20:1n-7 (mg/g ww)	0,40	0,01	<0.01	-
18:4n-3 (mg/g ww)	4,17	0,59	0,61	0,03
20:2n-6 (mg/g ww)	0,27	0,05	<0.01	-
20:3n-9 (mg/g ww)	<0.01	-	<0.01	-
20:3n-6 (mg/g ww)	<0.01	-	<0.01	-
22:0 (mg/g ww)	0,20	0,01	0,30	0,02
20:3n-3 (mg/g ww)	<0.01	-	<0.01	-
20:4n-6 (ARA) (mg/g ww)	1,18	0,13	0,26	0,02
22:1n-11 (mg/g ww)	16	8	2	1
22:1n-9 (mg/g ww)	1,0	0,1	0,4	0,1
20:4n-3 (mg/g ww)	1,0	0,1	0,2	0,0
20:5n-3 (EPA) (mg/g ww)	17	2	3	1
24:0 (mg/g ww)	<0.01	-	<0.01	-
22:4n-6 (mg/g ww)	<0.01	-	<0.01	-
21:5n-3 (mg/g ww)	0,91	0,03	<0.01	-
24:1n-9 (mg/g ww)	0,95	0,17	0,31	0,04
22:5n-6 (mg/g ww)	0,36	0,02	<0.01	-
22:5n-3 (DPA) (mg/g ww)	2,1	0,2	0,3	0,1
22:6n-3 (DHA) (mg/g ww)	16,1	0,5	3,7	0,3
24:5n-3 (mg/g ww)	0,4	0,2	<0.01	-
24:6n-3 (mg/g ww)	0,2	0,0	<0.01	-
Sum unudentified (mg/g ww)	8	2	1	0
Sum identified (mg/g ww)	159	10	166	9
Sum fatty acids (mg/g ww)	167	7	167	9
Sum saturated (mg/g ww)	43	1	36	2
Sum 16:1 (mg/g ww)	9,8	0,9	1,6	0,3
Sum 18:1 (mg/g ww)	22	2	69	4
Sum 20:1 (mg/g ww)	10,8	4,1	2,7	0,2
Sum 22:1 (mg/g ww)	17,5	7,6	2,8	0,5
Sum mono-unsaturated (mg/g ww)	61	9	76	4
Sum EPA + DHA (mg/g ww)	33	2	6	1
Sum n-3 (mg/g ww)	46	1	27	1
Sum n-6 (mg/g ww)	8	0	26	2
Sum polyunsaturated (mg/g ww)	55	1	52	3
n-3/n-6	5,8	0,3	1,0	0,1
n-6/n-3	0,2	0,0	1,0	0,1

2.1.2 Sampling

At the first sampling, at the mid and final sampling, organ samples were taken from 6 fish per tank. Sampled fish were killed with anesthesia, length and weight was measured. Cataract was also evaluated on fish sampled for whole fish analysis.

Cataract was evaluated using a Heine HSL 150 hand-held slit lamp (HEINE Optotechnik GmbH & Co. KG, Herrsching, Germany) in a dark room (start n=108. Middle n=108. End n=144). The fish was scored, and each eye was scored separately using a scale from 0-4. 0: No sign of cataract in any eye. 0: Normal lens 1: Changes affecting less than 10% when seen straight through the lens 2: Changes affecting 10-50 % of the lens 3: Changes affecting 50-75 % of the lens, and with a clear nucleus. 4: Complete cataract according to Wall and Bjerkås (1999).

The head was then removed. From the head the lenses were removed and placed in vials. Lenses from the first sampling was pool sampled to obtain enough material for analysis and individually sampled for the middle and end sampling (start, n=72. Middle, n=72. End, n=72)

Heart, viscera, and liver was removed from the abdomen and weighed. Heart was pool sampled for the first sampling and individually sampled for the middle and end sampling (start, n=72. Middle, n=72. End, n=72). A part of muscle was cut out and white muscle was sampled individually, and the same sample was pool sampled for all three samplings. All samples were frozen immediately on liquid nitrogen and stored at -80C until analysis.

2.2 Methods

2.2.1 Method used for deciding free NAH (Na-acetyl-1-Histidine) and Histidine in lens and heart tissue.

The principle is to make a homogenous solution and filtrating it and running it through reverse face HPLC.

Heart tissue samples were weighed and transferred to Eppendorf tubes (Sigma-Aldrich) containing 1000µl 80% Et-OH and a 5mm round stainless-steel beads. They were then homogenized using a mixer mill (Retsch) on frequency 30 for 5min. After removing the steel bead carefully with a magnetic rod, the samples were then spun down using an Eppendorf centrifuge (Sigma-Aldrich), for 15min at 8000rpm. 200µl of the supernatant were extracted and transferred to a new Eppendorf tube (Sigma-Aldrich). Samples were then left to evaporate in a heating cupboard set to 40°C. Next day the samples were made into a solution by adding 200µl phosphate buffer and shaken for 1 hour on a vibramixer. The solutions were finally filtered through a 22µm syringe filter (Sigma-Aldrich) into a vial.

Lenses analyzed for NAH and histidine were prepared like heart tissue. Differences were instead of a round steel bead you used a ufo shaped bead, which is for easier homogenizing of a harder lens. And only adding 600µl 80% Et-OH.
All the final filtered samples were run through reverse phase HPLC (waters corporation, milford, MA, USA).

2.2.2 Method for deciding physiological amino acid in muscle, Ninhydrin detection.

All the samples were half frozen at the time of sampling. Samples were only brought out from the freezer two and two to make sure that not much defrosting happened. This is to assure that the samples can be used again. If the samples would have gotten defrosted, they could not be used again. The samples were a pool sample from 6 fish from each tank from each sampling. The muscle samples were homogenized first with a scalpel on a glass surface. The muscle was homogenized by cutting it up, smashing with scalpel and folding it. Samples were weighed directly into a brand eppendorf tube. All samples weighed ≈0.3 g. A round 5mm

stainless steel bead was added to each tube, before 600 µl 10% sulfosalicylic acid was added. All the samples were then homogenized on a retch mill (Retch, Haan, Germany) for 4 min at 30 frequency. They were left at room temperature for one hour. After the steel bead was carefully removed with a magnet rod, if not removed the tubes might crack when spun down using an eppendorf centrifuge (Sigma-Aldrich) for 15 min at 8000 rpm. Transfer to a new 1,5 ml eppendorf tube 300 µl of the supernatant and 300 µl loading buffer. Then add 150 µl internal standard, before mixing well and storing at -20°C until running the test.

Before running, the samples were filtered through 0,22 µm Millex filters into chromasol vials 25V without insert. The test was performed with a Biochrom 20 plus after the protocol from Biochrom (Cambridge, UK).

2.3 Statistical analyses and calculations

Statistical analyses were performed using Statistica, a Tibco software (Palo alto, California, USA). Graphs and analyses were performed using Prism GraphPad software v. 8 (San Diego, California, USA). Two-way analysis was performed to see the effect of different HIS and lipid in the feed. The variables tested were growth, length, condition factor, NAH, Histidine, cataract, and all FAA. Parametric statistical tests are established on meeting a set of underlying assumptions.

When running an ANOVA, three assumptions needed be made. 1: The three sampling points are independent; 2: Homogeneity of variance; and 3: The dependent variables should be normally distributed. All variables were assessed for normality with Levene's test, all of the variables did meet the assumption. All variables were also tested for correlation towards cataract. To test for negative or positive correlation. Variables were also tested for correlation towards each other using the spearman rank-order.
Specific growth rate (SGR percentage day^{-1}) was calculated using SGR= 100(ln Wt – ln Wo) t^{-1}Wt is the final body weight, and Wo is the initial body weight.

3. Results

3.1 Cataract

Table 3 Prevalence and average cataract score ± SD start (n=120) middle (n=108) and end (n=144) sampling and number per score. Only two groups in the start sampling. Feed groups: F-H: Fish oil, high histidine V-H: Plant-oil mix high histidine F-L: Fish oil, high histidine V-H: Plant-oil mix high histidine F-L: Fish oil, low histidine V-L: Plant-oil mix low histidine.

	F-H			V-H			F-L		V-L	
Score Sum Left and right	Start	Middle	End	Start	Middle	End	Middle	End	Middle	End
0	34	7	11	31	2	4	3	5	3	0
1	21	13	18	18	17	17	11	17	11	17
2	5	4	6	11	5	6	8	7	5	12
3	0	3	1	0	2	8	5	7	7	7
4	0	0	0	0	1	1	0	0	1	0
Prevalence	43%	74 %	69 %	48%	92 %	88 %	88 %	86 %	88 %	100 %
Average Score	0,52±0.65	1.11±0.93	0.91±0.76	0,66±0.77	1.37±0.88	1.58±1.05	1.55±0.93	1.44±0.96	1.70±1.10	1.72±0.77

During the whole trial only 100 out of 372 fish did not show any signs of cataract (table 3). The lowest prevalence is for the samplings with the F-H group (43,74%,69%) (table 3). Highest average prevalence's was in the V-L group (48%, 88%, 100%) (table 3). V-L group was the only group with a 100% prevalence at the end sampling. That same sampling also had the highest average cataract score (1.72) F-H group had the lowest average scores each sampling and lowest prevalence.

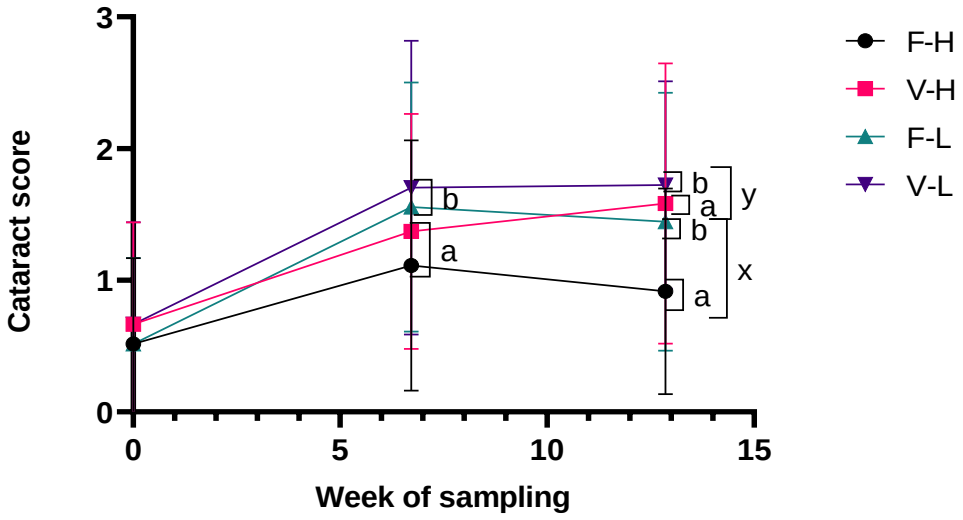

Figure 2 Cataract score over time (n= 3tanks). Significant differences are denoted by lower case letters. Significance is denoted individually per sampling. Middle sampling His (a, b) (p<0.038), end sampling His (a, b) (p<0.028) Lipid (x, y) (0.002) Points are represented by three samplings. Photoperiod LD 12:12 first period and 24:0 last period. Feed groups: F-H: Fish oil, high histidine V-H: Plant-oil mix high histidine F-L: Fish oil, low histidine V-L: Plant-oil mix low histidine. Data is represented as mean values +/- the standard deviation of mean.

At the start sampling there was no significant difference in cataract score in the two dietary lipid groups (fig 2). For the middle and end sampling there was a significant difference in the groups given high levels of histidine (p<0.038)(p<0.002). There was also a significant difference in the dietary lipid at the end sampling (0.002). From the middle to end sampling the two groups given fish oil as lipid source show a small reduction in average cataract score (fig 2). Both groups also show a reduction in prevalence (table 3).

3.2 Growth

*Table 4 Average ± SD weight, length and k-factor for all samplings and feed groups. (S) Start (n=60), (M) middle (n=27), (E) end (n= 36) Also tested for two-way anova. *only two groups in first sampling. Photoperiod LD 12:12 first period and 24:0 last period. Feed groups: F-H: Fish oil, high histidine V-H: Plant-oil mix high histidine F-L: Fish oil, low histidine V-L: Plant-oil mix low histidine. Data is represented as mean values +/- the standard deviation of mean. Weight (g), Length (cm), K-factor (Fulton's k-factor (100*g/cm^3))*

Sampling	F-H	V-H	F-L	V-L	Statistics (Anova)
Weight S*	31 ± 7	30 ± 6			n.s
Weight M	61 ± 11	63 ± 9	59 ± 8	58 ± 9	n.s
Weight E	115 ± 23	113 ± 24	111 ± 29	112 ± 24	n.s
Length S*	13 ± 1	13 ± 0.7			n.s
Length M	17 ± 0.90	17 ± 0.7	17 ± 0.6	17 ± 0.8	n.s
Length E	21 ± 1	21 ± 1	21 ± 1	21 ± 1	n.s
K-factor S*	1.2 ± 0.07	1.2 ± 0.07			n.s
K-factor M	1.2 ± 0.05	1.3 ± 0.08	1.2 ± 0.06	1.3 ± 0.07	n.s
K-factor E	1.2 ± 0.08	1.2 ± 0.10	1.2 ± 0.09	1.2 ± 0.06	n.s

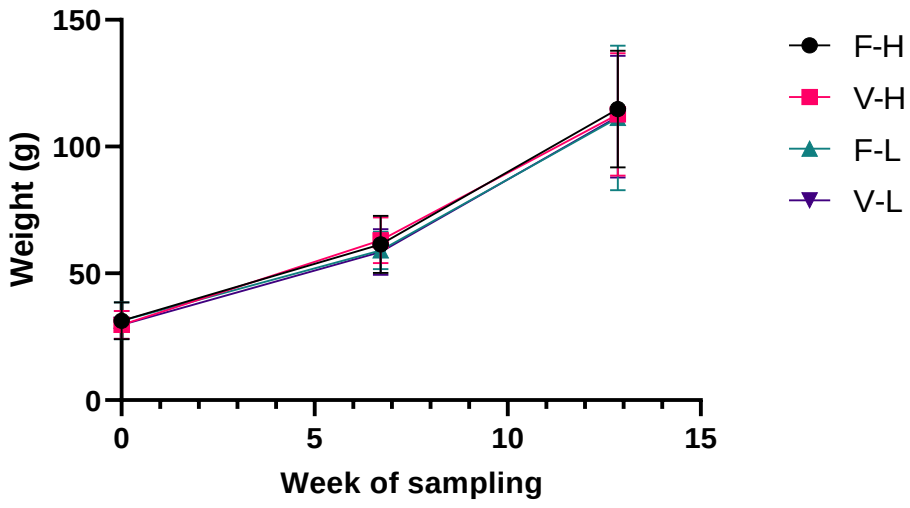

Figure 3 Average weight over time (n=3 tanks) Points are represented by three samplings. Photoperiod LD 12:12 first period and 24:0 last period. Feed groups: F-H: Fish oil, high histidine V-H: Plant-oil mix high histidine F-L: Fish oil, low histidine V-L: Plant-oil mix low histidine. Data is represented as mean values +/- the standard deviation of mean.

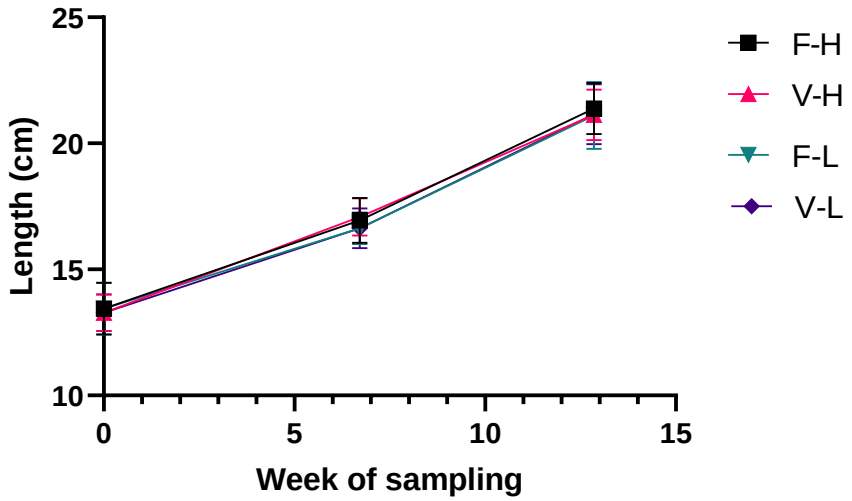

Figure 4 Average length (cm) over time (n= 3tanks). Points are represented by three samplings. Photoperiod LD 12:12 first period and 24:0 last period Feed groups: F-H: Fish oil, high histidine V-H: Plant-oil mix high histidine F-L: Fish oil, low histidine V-L: Plant-oil mix low histidine. Data is represented as mean values +/- the standard deviation of mean.

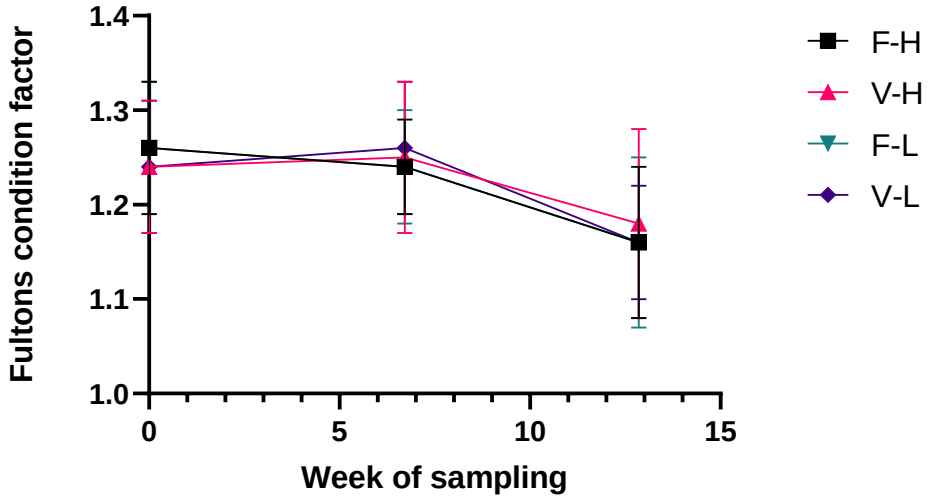

*Figure 5 Average condition factor (n= 3tanks). Points are represented by three samplings. Photoperiod LD 12:12 first period and 24:0 last period. Feed groups: F-H: Fish oil, high histidine V-H: Plant-oil mix high histidine F-L: Fish oil, low histidine V-L: Plant-oil mix low histidine. Data is represented as mean values +/- the standard deviation of mean. K-factor (Fulton's k-factor (100*g/cm^3))*

Table 5 The specific growth rate (SGR) for the four feed groups. Photoperiod LD 12:12 first period and 24:0 last period. Feed groups: F-H: Fish oil, high histidine V-H: Plant-oil mix high histidine F-L: Fish oil, low histidine V-L: Plant-oil mix low histidine.

Feed group	Specific growth rate
F-H	1.42
V-H	1.39
F-L	1.46
V-L	1.45

At the start of the trial, the mean weight of the sampled fish fed F diet was 31±7 and was like the fish fed V diet (30±6). Dietary His supplementation or lipid source did not influence weight, weight gain, length, condition factor and SGR during the trial.

There were no significant differences in weight, length, or condition factor amongst any of the given diets ($p>0.05$)(table 4)(fig 3, 4 and 5). Weight and length had a steady increase through the whole trial. The condition factor was lower at the last sampling compared to the two previous samplings. The specific growth rate had no significant differences and did not differ much between groups (table 5) ($p>0.05$).

3.3 Lens Histidine and NAH

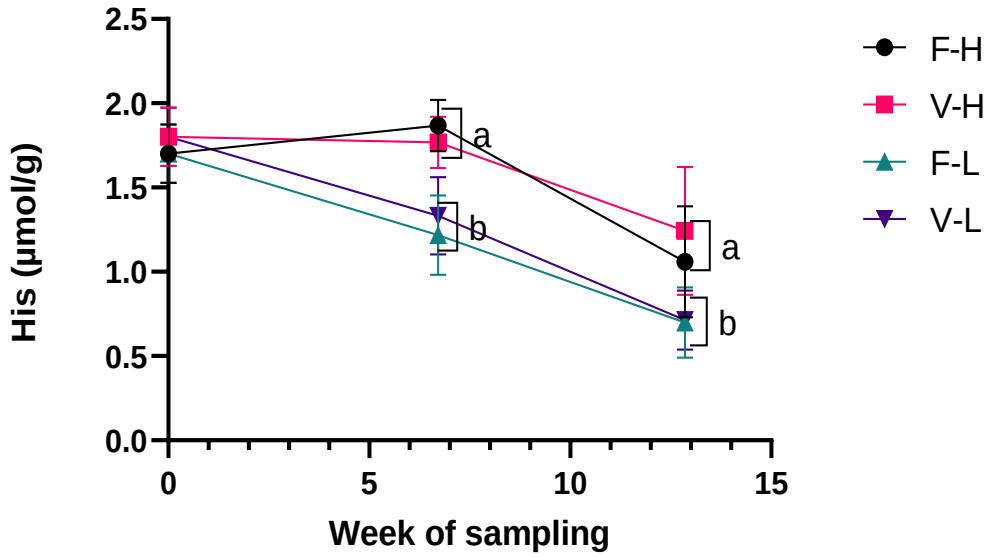

Figure 6 HIS concentration in the lens over time. Significant differences are denoted with lower case letters. Significance is denoted individually per sampling. Middle sampling (p<0.0014), end sampling (p<0.000) (n= 3 tanks) Photoperiod LD 12:12 first period and 24:0 last period. Feed groups: F-H: Fish oil, high histidine V-H: Plant-oil mix high histidine F-L: Fish oil, low histidine V-L: Plant-oil mix low histidine. Data is represented as mean values +/- the standard deviation of mean.

Lens His concentration at the start of the experiment was 1.7 µmol/g.

There was a significant difference in His concentrations in the fish that were fed either high or low histidine levels in the feed, middle sampling ($p<0.0014$), end sampling ($p<0.000$). The lipid composition had no significant effect on the levels of histidine.

His concentrations in the lens had a steady decline from start sampling to end sampling in the two groups fed low histidine (fig 6). The F-H group had a small increase from start to middle sampling and the V-H had a small decrease from start to middle.

The two groups fed high histidine show the same tendencies as the concentrations in the heart, with a slight increase.

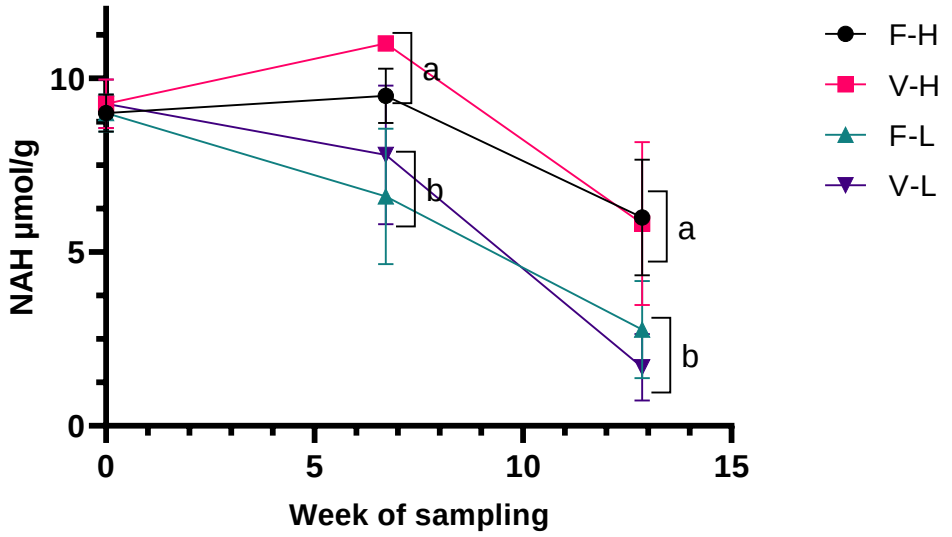

Figure 7 NAH concentrations in the lens over time. Significant differences are marked with lower case letters. Significance is denoted independent per sampling. Middle sampling ($p<0.00656$), End sampling ($p<0.0000$) ($n= 3$ tanks) Points are represented by three samplings. Photoperiod LD 12:12 first period and 24:0 last period Feed groups: F-H: Fish oil, high histidine V-H: Plant-oil mix high histidine F-L: Fish oil, low histidine V-L: Plant-oil mix low histidine. Data is represented as mean values +/- the standard deviation of mean.

Lens NAH concentrations were similar in the beginning of the experiment (Fig 7). NAH concentrations at the start of the experiment were at 9 µmol/g. The concentration showed significant differences in the two groups with high His in the feed, both for the middle ($p<0.00656$) and end sampling ($p<0.0000$). Dietary lipids did not influence lens NAH concentrations at any time. The two groups with high histidine had a slight increase in concentration up towards the middle sampling, before decreasing from middle to end sampling. Low histidine groups showed a constant decrease from start to end sampling.

3.4 NAH/Histidine in the heart

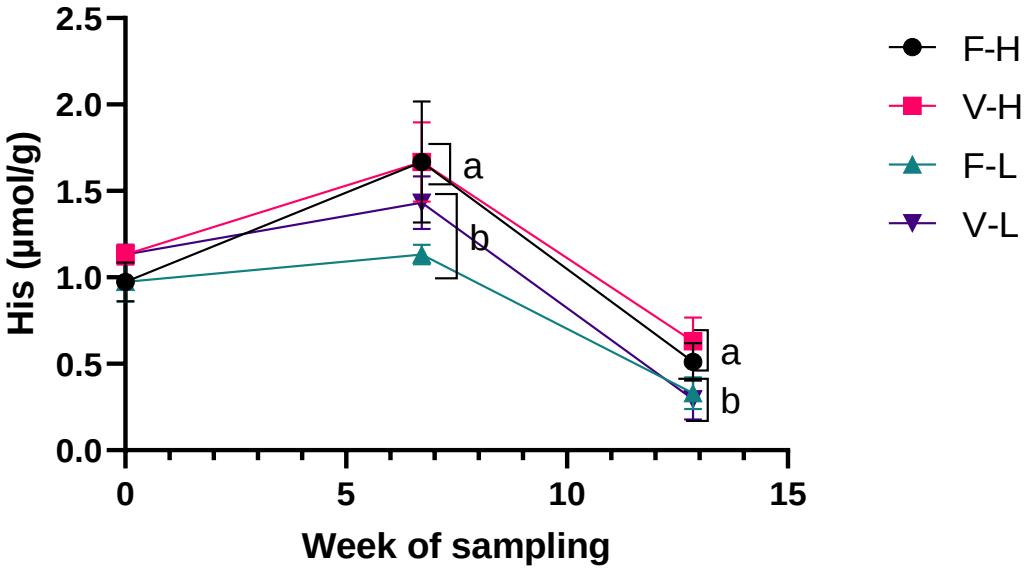

Figure 8 Histidine concentrations in the heart over time. Significant differences are denoted by lower case letters. Significance is denoted individually per sampling. Middle sampling ($p<0.01$) and end sampling ($p<0.00$). (n= 3 tanks) Points are represented by three samplings. Photoperiod LD 12:12 first period and 24:0 last period Feed groups: F-H: Fish oil, high histidine V-H: Plant-oil mix high histidine F-L: Fish oil, low histidine V-L: Plant-oil mix low histidine. Data is represented as mean values +/- the standard deviation of mean.

At the start of the experiment, the mean His concentration in the heart was 0.97 µmol/g no differences were seen in the heart His concentration between fish previously fed the F or V diets (fig 8). The heart His concentration was significantly higher in the two groups with high histidine in the feed both at the middle ($p<0.01$) and end sampling ($p<0.00$). Irrespective of dietary lipid. There was a small increase in all groups between start and middle sampling, and a decrease in all groups from middle sampling to end sampling.

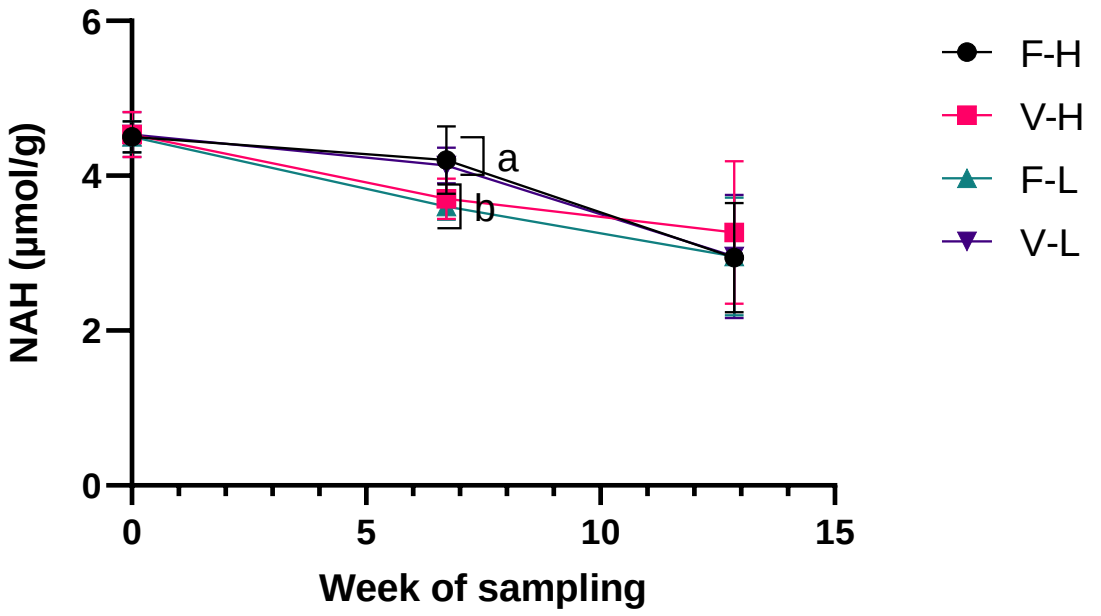

*Figure 9 NAH concentrations in the heart over time (n= 3 tanks) Points are represented by three samplings. Significant differences are denoted with lower case letters. Interaction effect His*lipid middle sampling (p<0.01571). Photoperiod LD 12:12 first period and 24:0 last period. Feed groups: F-H: Fish oil, high histidine V-H: Plant-oil mix high histidine F-L: Fish oil, low histidine V-L: Plant-oil mix low histidine. Data is represented as mean values +/- the standard deviation of mean.*

At the start of the experiment, heart NAH concentration was 4.5µmol/g.
NAH concentration in the heart showed significance in the interaction effect between His and lipid for the middle sampling ($p<0.01571$). But had no significant values when looking at either the lipid or the histidine concentration as a single independent variable. The experimental diets did not influence the heart NAH concentration as there were no significant differences in the start of the experiment or at the end. NAH concentration in the heart showed a steady decline from the start sampling until the end sampling (fig 9). And the concentrations showed minimal differences between the groups (fig 9)

3.5 FAA in muscle

All free amino acids in the muscle were analyzed. The results were focused on the amino acids involved in histidine metabolism. Which is histidine, anserine, β-alanine, carnosine, and 1-Methylhistidine.

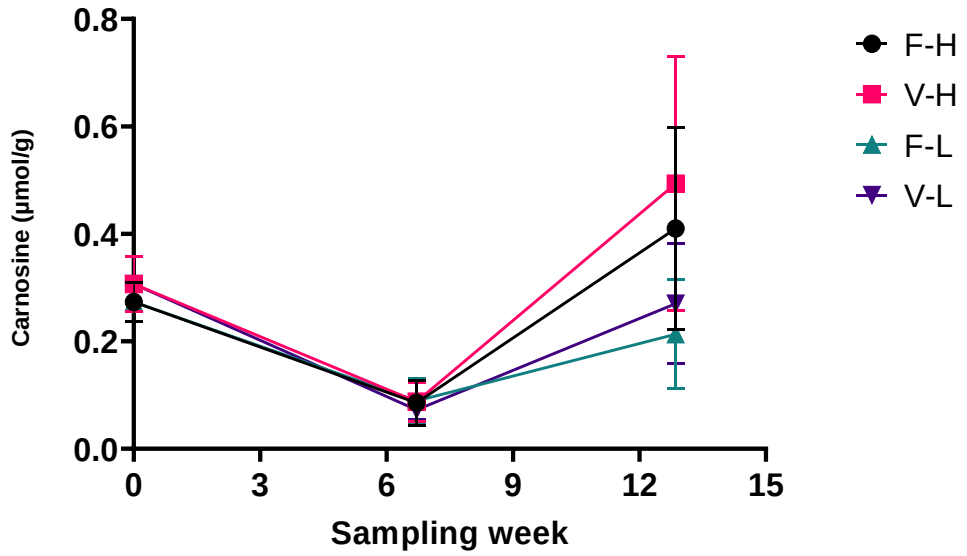

Figure 10 Carnosine concentration in muscle over time. Points are represented by three samplings. Photoperiod LD 12:12 first period and 24:0 last period. Feed groups: F-H: Fish oil, high histidine V-H: Plant-oil mix high histidine F-L: Fish oil, low histidine V-L: Plant-oil mix low histidine. Data is represented as mean values +/- the standard deviation of mean.

At the start of the experiment the mean concentration of carnosine was 0.28 µmol/g.
There was no significance in the free muscle concentration of carnosine (Figure 10). The concentration showed a decrease towards middle sampling and a small increase in all groups, somewhat higher in the two groups with high histidine.

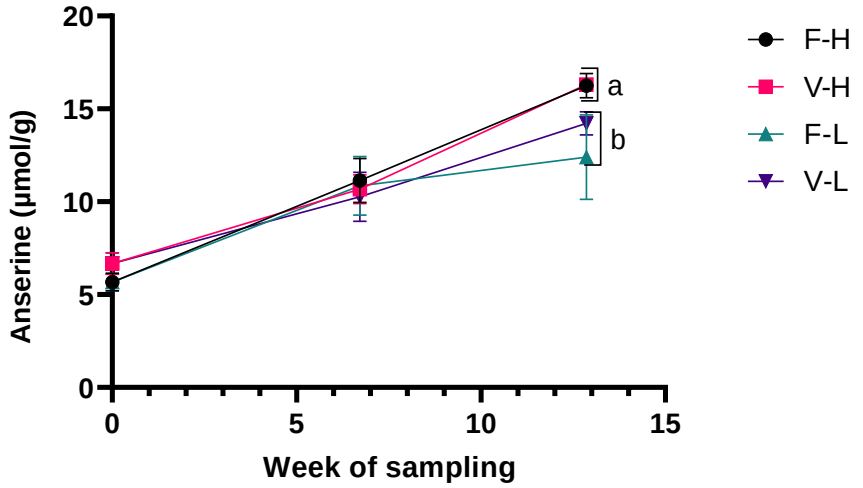

Figure 11 Anserine concentration in muscle over time. Significant differences are denoted by lower case letters. End sampling showed significant values for histidine (p<0.0031). Points are represented by three samplings. Photoperiod LD 12:12 first period and 24:0 last period. Feed groups: F-H: Fish oil, high histidine V-H: Plant-oil mix high histidine F-L: Fish oil, low histidine V-L: Plant-oil mix low histidine. Data is represented as mean values +/- the standard deviation of mean.

At the start of the experiment the mean anserine score was 6.1 µmol/g.

Anserine concentrations increased steadily throughout the experiment in all four groups, with higher concentrations on the groups given high histidine. There was a significant difference at the end sampling (p<0.0031) in the groups given high histidine. Dietary lipid had no effect on anserine concentrations.

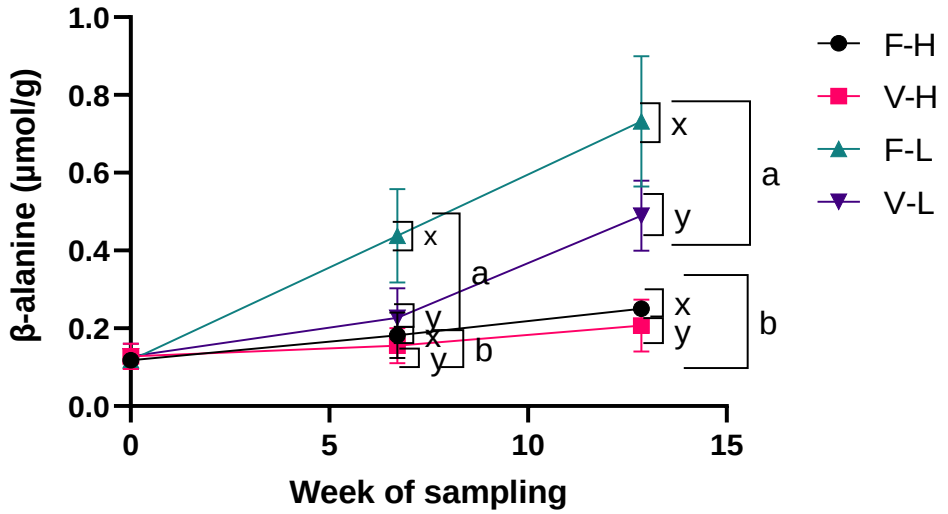

*Figure 12 β- Alanine concentration in muscle over time. Significant differences are denoted by lower case letters. Middle sampling histidine (a,b)(p<0.000), lipid (x,y) (p<0.000) and interaction effect His*lipid (p<0.002). End sampling show significant values (p<0.0001) for histidine (a,b) and (p<0.038) for lipid (x,y). Significance is denoted independent per sampling Points are represented by three samplings. Photoperiod LD 12:12 first period and 24:0 last period. Feed groups:*

F-H: Fish oil, high histidine V-H: Plant-oil mix high histidine F-L: Fish oil, low histidine V-L: Plant-oil mix low histidine. Data is represented as mean values +/- the standard deviation of mean.

Starting mean concentrations of β – Alanine was 0.12 µmol/g.
β - Alanine levels increased through all four groups during the period. Less increase in the two groups with high histidine Significantly higher increase in the two groups with low histidine. Statistically it showed ($p<0.038$) when looking at lipid as the main effect and ($p<0.0001$) for histidine.

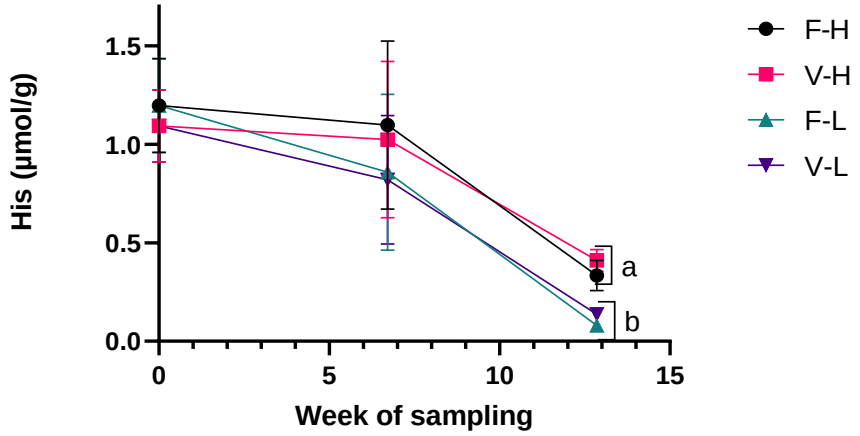

Figure 13 Histidine concentration in muscle over time. Significant differences are denoted by lower case letters. End sampling showed significant values for histidine ($p<0.0000$). Points are represented by three samplings. Photoperiod LD 12:12 first period and 24:0 last period. Feed groups: F-H: Fish oil, high histidine V-H: Plant-oil mix high histidine F-L: Fish oil, low histidine V-L: Plant-oil mix low histidine. Data is represented as mean values +/- the standard deviation of mean.

At the start of the experiment the mean concentration of His was 1.13 µmol/g.
At the start and middle sampling there were no significant differences in the histidine concentrations. At the end sampling there was a significance in given histidine levels in the feed ($p<0.0000$). The free histidine follows the same trend as His in heart and lens.

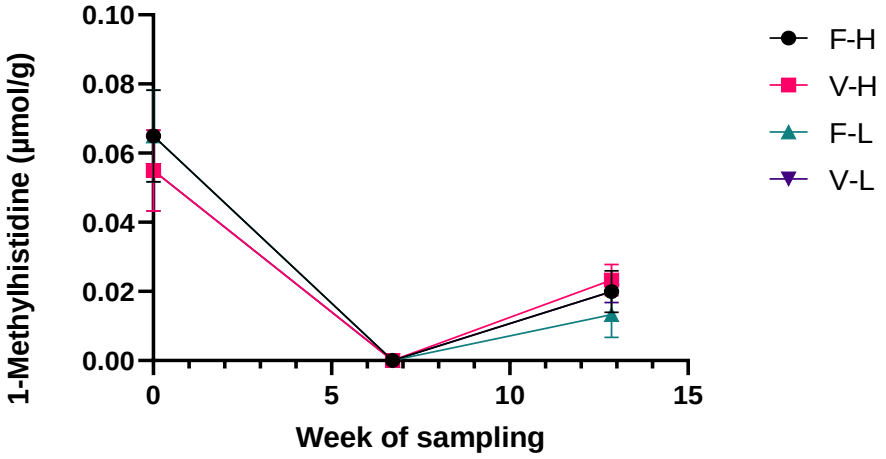

Figure 14 1-Methylhistidine concentration in muscle over time. Points are represented by three samplings. Photoperiod LD 12:12 first period and 24:0 last period. Feed groups: F-H: Fish oil, high histidine V-H: Plant-oil mix high histidine F-L: Fish oil, low histidine V-L: Plant-oil mix low histidine. Data is represented as mean values +/- the standard deviation of mean.

At the start of the experiment the mean concentration of 1-Methylhistidine was 0.05 µmol/g. There were no significant values for the concentration of 1-1-Methylhistidine. During the middle sampling, the concentration was not sufficient to measure.

4. Discussion

The present study was done to investigate cataract development in FW, and whether this could be influenced by a change in dietary lipid source and histidine supplementation, and the interaction effect between them. The study builds on previous knowledge on how histidine supplementation can influence cataract development and that plant lipids was is suspected to have a negative effect.

4.1 Cataract development

The results from the present study show that both dietary His supplementation and lipid source can influence cataract development during the parr-smolt transformation phase in FW. During the first 6 weeks of the experiment, fish given His supplementation had a lower cataract score compared to fish given the un-supplemented feed, while dietary lipid source did not influence cataract development. At the end of the FW phase, both dietary His and dietary lipids influenced cataract development.

4.1.1 Histidine prevents cataract development in freshwater.

In the present study the highest average cataract scores were in the groups with lowest histidine. There was also a 100% prevalence at the end sampling in one group with low histidine. Our findings also showed statistically significant differences between the two different histidine groups during both the middle ($p<0.038$) and end sampling ($p<0.028$). Which is a significant finding as it also was high prevalence in the groups with high histidine.

Although several studies have shown that His supplementation can minimize cataract development after SW transfer, fewer studies have investigated this in FW. Two studies to highlight and compare with the present study is Breck et al., (2005) and Sambraus et al., (2017). Sambraus et al., (2017) used comparable His levels to this study, where the cataract preventive effect was evident at high temperature 16C, but not at 10C in FW (Sambraus *et al.*, 2017). Whereas Breck et al (2005) tested different strains of salmon that were fed diets with low or high histidine concentrations. They had a 9-week period in FW before transfer to SW,

and samplings at 1 week and 6 weeks. This study was to see the effect of dietary histidine on cataract development.

Contrary to the present study they could not see any differences on cataract development between the groups in FW. However, fish who had been fed diets that were supplemented with His showed a significant reduction in cataract severity in SW. The dietary His concentrations in the study by Breck et al (2005) was higher than what was used in the present study with 11.7 g His/kg (low histidine) and a group 18 g his/kg (high histidine, which may explain the differences in findings. The rearing temperatures and smoltification regime also differed between the studies.

According to NRC 2011, salmon needs 8 g his/kg for growth (NRC- National Research Council, 2011). They did mention in the rapport that it is not high enough dietary histidine to prevent cataract. A study from 2010 concluded that also adult salmon in SW had higher requirements for dietary histidine than recommended by the NRC (Waagbø *et al.*, 2010). Work continuing on this by Remø et al 2014 concluded that the dietary levels of histidine to reduce the risk of cataract has to be 14.4 g His/kg and lowest severity of cataract can be achieved with 13.4 g His/kg in salmon smolt after SW transfer (Remo *et al.*, 2014).

We can draw general conclusion from previous studies and the present study done on salmon in both FW and SW that histidine has a preventative effect.

4.1.2 Plant oil replacement for fish oil

The use of vegetable oil in replacement of fish oil has long been considered a risk factor for the development of cataract. The present study included VO from start feeding to test previous hypothesis that there is an increased risk of cataract when VO is given from start to slaughter.

At the end of the experiment, the fish given the VO feed had significantly higher cataract scores compared to the fish given FO. This difference was only seen after smoltification. There have been studies that have studied the severity and prevalence when fish oil has been completely or partially replaced with vegetable oil (RAFOA, 2005;Waagboø *et al.*, 2013;

Remo *et al.*, 2014). In a study where salmon were fed VO (same RAFOA mix as used in the present study), a higher severity of cataracts was seen at the time of harvest (Waagbø et al., 2004, RAFOA). However, later studies investigating whether including different plant oils in the SW phase has not seen an increased risk of using plant oils compared to fish oils in salmon (Sissener *et al.*, 2016)

The effects of replacing fish oil completely or partially with plant oil in the diet of salmon has been tested by replacing fish oil 75% and 100% with a RAFOA mix (Sissener *et al.*, 2016). Where they found higher prevalence and severity of cataract in fish given the RAFOA mix compared to fish given only fish oil. Turchini 2009 concluded that a significant part of the FO in the diet can be replaced with an alternative lipid source without significantly affecting the fish (Turchini et al., 2009).

The statistical difference at the end sampling might have been influenced by the fact that the F-H group has so much lower scores then the three remaining groups. The F-L has lower scores and prevalence then the rest but not significantly since mentioned before the histidine groups also differed significantly. However, with the present study and previous studies it is possible to make an observation that the lipid source does influence cataract development in FW. His supplementation in the diet reduced the cataract score in the fish given FO, but not the fish given VO at the end of the experiment.

4.1.3 Temperature and growth

Cataract was observed already at the start of the trial, and a contributor to this fast development might have been the high rearing temperature used in the present study. The prevalence and severity of cataract in the present study also appear to be higher than what has been reported in previous studies investigating cataract development in FW.

Low temperatures with low growth rate has been shown to have lower incidence of cataract then fish reared in higher temperatures in FW (Bjerkås *et al.*, 2001). It was also concluded in the same study that fish which was exposed to fluctuating temperatures developed more cataract, and fish that were exposed to higher temperatures grew faster and developed more cataract then fish kept at a constant low temperature (Bjerkås *et al.*, 2001).

In the study by Sambraus et al., (2017), fish reared at 16°C developed a significantly higher mean score for diploid (1.5±0.3) and triploid (4.3±0.1) than fish reared at 10°C. The 10°C reared fish even with low histidine in the diet showed lower scores diploid (0.3±0.1) and triploid (0.8±0.1). The average scores for the fish reared at 10°C were much lower than all the groups in the present study. The diploid groups reared at 16°C were similar to the groups in the present study with the highest average scores, the triploid group had much higher scores.

When comparing to other studies the high prevalence, although low severity observed in this study may be due to the high rearing temperature (13°C) used in the present study. Cataract development has been linked to both rearing temperatures and growth rate. The calculated SGR throughout the trial indicated a high growth rate, but this was similar between all groups. Even though high growth rate there was not found any significant values in the present study when looking at length, weight, or k-factor. Which indicates that this is not a factor that influenced cataract score.

Our findings do not correlate to two previous studies that have shown a correlation between growth and cataract development. In the first study, the highest correlation was found between

body length and cataract ($p<0.01$) (Torstensen et al., 2008). Experiment done in 1998 measured weight and calculated SGR with three different diets, the following numbers are from their trial in FW. Their calculated SGR was between 0.57-0.66. With an initial weight averaging 45g and final weight 126g (Breck et al., 2003).

4.2 Changes in metabolism during smoltification

4.2.1 Changes during parr-smolt transformation

The three sampling points during the experiment were chosen to get a good representation of the salmon developing from parr to smolt. It was important to separate the samplings sufficiently to make sure that the fish had developed and that they differed from the last sampling. The parr-smolt transformation was induced using a light regime with a winter signal (12:12) The photoperiods which is used in the experiment also matched up with the sampling points, when a 6 week period is the previously tested time for photoperiods (Stefansson *et al.*, 1991; McCormick *et al.*, 1998). In the present study the fish was reared with a photoperiod 12:12 which is a winter signal, for 7 weeks before it was switched to continuous light 24:0 for 5 weeks. This was to stimulate growth and an important step to initiate the smoltification (Stefansson *et al.*, 1991; McCormick *et al.*, 1998).

The favoring of length over weight which happened slightly at in the present study, is hypothesized to be a smoltification adaption when going from FW to SW (McCormick *et al.*, 1998). There are hypotheses on why the length is favored. One hypothesis is that salmon has a high energy expenditure when they prepare to enter seawater, and they utilize the stored energy to modify and adapt the functions in the body. Salmon then become lengthier and does not have sufficient energy to increase its weight. A loss of total lipid during smolting also lowers the condition factor. The reason for this can be an adaptive change for swimming performance or it is because of the energetic demands of smolt transformation, but this is still unclear (McCormick *et al.*, 1998).

The changes that occurred in the metabolism does not correlate with the development of cataract. Average cataract scores stay stable from the middle sampling to the end sampling in all groups, whereas all measurements of histidine and histidine derivatives changes. The two

groups with high histidine do not differ significantly from each other in any measurements. From that observation it is possible to draw a conclusion that the changes that occur towards smoltification does not affect cataract development in such a way it is significant.

4.2.2 Histidine metabolism changes during smoltification

Changes in the muscle and eye histidine metabolism are hypothesized by several studies to be a part of the adaptions salmon go through before and during transfer to SW (Munakata *et al.*, 2000; Breck *et al.*, 2005; Waagbø *et al.*, 2010). This can also be seen in the present study, however, the concentration of histidine and anserine in lens and muscle changed in different direction. In the present experiment, the lens His and NAH concentrations were higher at the start than at the end of the experiment, while the muscle anserine concentrations increased.

Lens and heart His concentration was influenced by the dietary His supplementation, and during the middle and end sampling there was significant differences between the low and high groups. However, for NAH only the lens was influenced by the His supplementation. The lens also had more of a decline in concentration compared to the heart which kept more stable concentrations. The heart seems to be the tissue least effected by the feed, which coincides with what Remø et al., (2014) found.

Histidine levels were almost depleted in groups fed low histidine during the present study. The depletion could be seen in all three places measured histidine, heart, muscle, and lens. All three analyses showed a steady decline (result figure 6, 8 and 13) This correlates to the increase in cataract score and decrease in NAH. The slopes on the graphs for histidine in the heart and lens show a similarity to the graphs showing NAH concentration.

In the present study it was not possible to find any strong correlation between lens NAH and severity of cataract. The correlation was believed to be there based on a previous study showing a negative correlation between the lens NAH status and the severity of cataract, with lower NAH levels giving higher cataract scores (Waagbø *et al.*, 2010). The present study also showed low NAH levels in the groups given low histidine concentrations, but there was not observed any direct correlation between the two values.

In SW there seems to be a link between cataract development and the concentration of the histidine derivative NAH (Breck *et al.*, 2005). NAH is shown to be an important osmolyte in the salmon lens, and the rise of osmolality in the lens coincides with the increase of NAH when salmon is moved from FW to SW (Rhodes *et al.*, 2010). Previous data suggests that there is an increase in NAH synthesis connected to the parr- smolt transformation process after seawater transfer (Rhodes *et al.*, 2010). This is a very important role in anadromous fish as a deficiency in NAH will cause a malfunction in the normal osmoregulatory processes and it is assumed that this will cause cataract formation in fish that have low concentrations of histidine (Rhodes *et al.*, 2010).

A calculation done on NAH in the lens with the correlation between dietary histidine and reducing cataract prevalence and severity, summarized that the µmol NAH/g needed is 10.8 and 8.8 µmol NAH/g (Remo *et al.*, 2014). This study was done in the SW phase, with the first sampling on underyearling smolt with initial mean body weight 71.4. In the present study, the start sampling had NAH concentrations 9 and 9.26 µmol NAH/g. For the middle sampling, the NAH concentrations differed between the groups with high or low histidine, high (9.5-11) and low (6.6-7.8).

Experiment done in 2005 on how the dietary histidine affected lens protein turnover and N-acetyl histidine showed similar concentrations during the middle sampling as in this study. They measured NAH concentrations after 37 days (Breck *et al.*, 2005). Another study from 2005 (Breck *et al.*, 2005) that had samplings week 1 and week 6 in freshwater showed similar histidine concentrations in the lens. The NAH concentrations were much lower in their study compared to the present study, even in the groups they had with high histidine in the feed.

In the present study, anserine concentration in the muscle had a steady incline throughout the whole project period. Similar results were seen in a study by Ogata and Murai (1994) when done on Masu salmon. The anserine levels in the smolt were measured in 3 different feed groups, and in all three groups the concentrations were always higher in smolt than in parr (Ogata and Murai, 1994).

Histidine and its compound anserine were examined in rainbow trout for its buffering capacity in white muscle, and it showed that they only have a supporting role (Abe and Okuma, 1991).

The data they presented makes it clear that higher concentrations of histidine related compounds in muscle increases muscle buffering capacity, which increases muscle anaerobic capability (Abe and Okuma, 1991; Okuma and Abe, 1992). This is clearly shown in highly active and fast swimmers like tuna and mackerel.

After transfer to SW it is observed that salmon build up on tissue specific histidine compounds, anserine in muscle and NAH in the lens. This effectively traps histidine intracellularly and making it unavailable for protein syntheses (Trößе *et al.*, 2010).
In this study it cannot be seen with NAH in the lens, but anserine increases in the muscle whilst the other FAA like histidine are almost depleted.

There is an increase in B-alanine, but the concentration is low. In humans B-alanine is the limiting factor for the synthesis of carnosine and in causation can lower buffer activity (Artioli *et al.*, 2010). In fish it is shown in this study and in previous studies that B-alanine gets piled up when the histidine concentrations are low. In the present study it shown by significant differences between the groups given low or high histidine at the end sampling, and it is shown in the study by Ogata and Murai (1994). There was also a strong negative correlation (-0.8) between histidine levels and β-alanine at the end sampling. Which means higher β-Alanine concentrations mean lower histidine. Which just strengthens the statement that β-alanine gets piled up when histidine levels get low.

Carnosine have been suggested to have important biological roles in rainbow trout, similar to anserine as a buffer and as an antioxidant (Guiotto *et al.*, 2005; Snyder *et al.*, 2012). An increase of carnosine in feed resulted in a significant increase in anserine concentration. In this study there was no significant values of carnosine, it was almost depleted during the middle sampling. As there was no significant difference in carnosine levels or anserine, there could be made assumptions that all carnosine is used to make anserine. And that pathway is not influenced by histidine levels.

Previous studies that have examined FAA in salmon have done so right after sea transfer and a period in seawater. The sampling right after sea transfer show similar levels as the end sampling in our project. Concentrations were similar for muscle free histidine and anserine (Trößе *et al.*, 2010; Remo *et al.*, 2014).

5. Conclusion

- Dietary His supplementation reduced the severity of cataract.
- Fish given the VO feed had more severe cataract scores at the end of the trial,
- There was not shown any interaction effect between histidine and dietary lipid.
- There were changes in histidine and histidine derivatives in lens, heart, and muscle during the parr-smolt transformation and they were influenced by both dietary lipids and his supplementation.

6. Future perspectives

For a future study there are several adjustable factors that could make an impact on future results if the study is repeated.

In the present study the only adjusted factors in the feed were either a 100% replacement of plant oil for fish oil and low or high histidine. Different other adjustments in the feed have been previously studied to see the effect on preventing cataract. And are any of them a viable? A review from 2009 stated that if the industry is to grow it needs to find and utilize other sources of lipid then fish oil (Turchini et al., 2009)

Bjerkås et al 2014 concluded with that having an optimal level of histidine is important for preventing the development of cataract, but only correcting that factor will not prevent cataract all together. Adding dietary NaCl prevented later formation of cataract when added before and after smoltification (Bjerkås and Sveier, 2004). They also tested increased taurine, which had no preventive effect. Low level of dietary fat or high concentrations of carbohydrates showed an increase in cataract.

Study done on rainbow trout showed insufficient zinc levels caused higher prevalence of cataract (Ketola, 1979). The adding of zinc and iron to the feed was done in a trial to experiment on the effect of removing blood meal from feed and looking at their effect on reducing cataract. They could only see an affect when adding HIS and Fe, since the concentration of dietary zinc was the same in the blood meal diet as in the control diet (Breck *et al.*, 2003). They concluded with that HIS and/or Fe are the nutritional components that have an influence on cataract development.

For future studies adding higher concentrations of histidine from start feeding might show a preventative effect as there was a difference in both the middle and end sampling. The addition of Fe in the given feed during the present study might have an effect.

In the present study, the fish were kept at a constant 13°C temperature throughout the experiment. Which is the optimum temperature for smolt (Handeland, Imsland and Stefansson, 2008). And was chosen to get a good growth throughout the experiment.

Fish have an optimum temperature where growth and survival are increased (Gadomski and Caddell, 1991; Handelandt et al., 2008). This optimum temperature usually changes with age and size. For cod it is shown that larger cod prefer temperatures a couple degrees lower than smaller cod (50-1000g) (Pedersen and Jobling, 1989). There have been different studies done on salmon, and Handeland et al (2008) describes experiments where optimum temperature for growth and development, around 13°C for smolt (40-60g) for parr (4-12g) the temperature is assumed to be higher. Low temperatures with low growth rate has been shown to have lower incidence of cataract then fish reared in higher temperatures in FW (Bjerkås *et al.*, 2001).

In Breck et al experiment the cataract score ranged from 0.53-1.07 for the whole trial, with a frequency of 40-52%. Which is a significant lower frequency then in this study.
In the experiment there were several factors that could have influenced the lower cataract score. The temperature was much lower in the 1998 experiment compared to the project. Their temperature was around 10°C for their FW period, which is a less optimum temperature for growth in smaller salmon (Handeland et al., 2008). The growth rate is later influenced by the photoperiod. Breck et al had natural light period, and the trial was conducted at summertime.

Repeating this study with lower temperatures, could give a better indication on the effect of histidine. When not influenced by the high growth rate caused by the optimum temperature for salmon growth.

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Appendix

Buffers and solutions

80% Et-OH

- Measure out 83,3 ml of 96% Et-OH in a 100ml measuring cylinder
- Dilute up to the mark with purified water

Eluent 1 (0,1 M natriumphosphate buffer pH, 2,0):

- Weigh in 17,8 g Na_2PO_4* $2H_2O$
- Dilute to the mark in a 1000ml measuring flask
- Pipette out 13,5ml H_3PO_4 and dilute to 2000ml with purified water
- Take barely out 350ml of the Na_2PO_4 solution
- Transfer to a 2000ml flask
- Add the H_3PO_4 solution until the natriumphosphate reaches a pH=2,0

0,5mM NAH – 0,5mM HIS standard

- Weigh in 0,0538g NAH (Cat. No 85,754-8, Aldrich) and weigh 0,0388g HIS (Cat. No H-8776, Sigma Aldrich) in separate weighing boats.
- Transfer to a 100ml measuring flask.
- Add some natrium phosphate buffer and dissolve, then dilute to the mark with the buffer.
- Transfer 5ml to a 25ml measuring flask and dilute to the mark with buffer.

0,25mM NAH – 0,25mM HIS standard

- Mix 0,5mM NAH and 0,5mM HIS standard med natriumphosphate 1:1, mix well.

Eluent II (Methanol :Water):

- Mix methanol and purified water 1:1

10% sulfosalicylic acid

- 10 g Sulfosalicylic (Cat. No 33619, Riedel-deHaen)
- Transfer to 100ml measuring flask and dilute to the mark with water.

Hydrochloric acid, 6 M

- 500 ml 37% hydrochloric acid to water in a 1000 ml measuring flask.
- Fill to the mark when the mixture has cooled down.

Internal standard I (2.5 mM Nor)

- Weigh in approximately 0,3280 g Norleucin (Cat. No. 1398, Sigma).
- Transfer to a 1000ml measuring flask and dissolve 17 ml 6 M hydrochloric acid.
- Dilute to the mark with water.

2.5 mM glutamin

- Weigh in approximately 0,0365 g Glutamin (Cat. No. G-3126, Sigma).
- Transfer to 100 ml measuring flask and dilute to the mark with water.

Working standard, 0,625 mM

- Pipette out 500 µl standard A/N (Cat. no. A-6407, Sigma), 500 µl standard B (Cat No. A-6282, Sigma), 500 µl internal standard I and 500 µl loading buffer (cat no. 80-2038-10, Biochrom) to a 4 ml sample glass
- Mix well on the whirl mix
- Store at -20 °C up to a year

External standard

- Pipette accurately 200 µl of the working standard
- Add 50 µl glutamin
- Mix well on the whirl mixer

Ninhydrinreagent

- Set ninhydrin solution (Cat. No. 80-2110-76, Biochrom) in an ultrasound bath for 10min
- Transfer Ultrasolve (Cat. No. 80-2110-75, Biochrom) to a 2 L light filtered blue corked flask (leave some ml to rinse the ninhydrin solution)
- Set to stirring added nitrogen for 10min
- Transfer the sonicated ninhydrin solution to ultrasolve (rinse the flask with the rest of the ultrasolve)
- Continue the stirring with nitrogen for max 10min.

0.1 % DEPC

- 1.8 ml DEPC
- 1800 ml ddH_20
- Let the solution stand for 1 hour in 37 °C. Autoclave in 121 °C for 15min.

1M NaOH

5M NaOH

75 % EtOH m DEPC

- 75 ml absolute ethanol

- 25 ml 0.1 % DEPC H_2O
- Store at -20 °C

RNA 6000 ladder (Cat. NO. 7152, Ambion)

- Heat treat for 3min, 70 °C for the first-time use

70 % EtOH (washing ethanol)

- 25 µl 100 mM dATP
- 25 µl 100 mM dTTP
- 25 µl 100 mM dGTP
- 25 µl 100 mM dCTP
- 900 µl dd H_2O
- Mix the solution in a 1.5 mL RNase free tube.

Info about the fish that were used for statistical analyses

Table 1: Relevant information about the individuals of Atlantic salmon that were sampled at the first sampling

Fish	Length	Weight	K-factor	Lipid	Right Eye	Left Eye
1	14,3	37	1,2653003	F	0	0
2	15,1	44,7	1,29830486	F	0	0
3	13,4	28,3	1,17617526	F	0	0
4	14,5	32,4	1,0627742	F	0	0
5	13,3	30,4	1,29216704	F	1	0
6	13,4	30,2	1,25514109	F	0	0
7	13,5	30,7	1,24777727	F	1	1
8	13,8	34,5	1,31274942	F	0	0
9	12	20,4	1,18055556	F	0	1
10	11,8	18,7	1,13813973	F	0	0
11	13,9	30,5	1,13567859	F	0	0
12	14,2	35	1,22237092	F	0	0
13	14,2	37,3	1,30269816	F	0	0
14	14	35,4	1,29008746	F	0	0
15	13	27,4	1,24715521	F	0	0
16	13,2	29,5	1,28262654	F	0	0
17	13,1	27,6	1,22770831	F	1	0
18	13,8	31,6	1,20240237	F	0	1
19	9,2	8,9	1,14294814	F	0	0
20	14,2	35	1,22237092	F	0	1
21	13,7	33,6	1,30670507	V	1	1
22	12,5	22,3	1,14176	V	0	0
23	13	28,1	1,27901684	V	1	0
24	13,5	31,1	1,26403495	V	1	1
25	12,6	25,9	1,29475659	V	0	0

26	12,3	21,7	1,1661231	V	0	0
27	12,1	19,8	1,11765838	V	0	0
28	12,6	26,2	1,30975377	V	0	0
29	11,4	18	1,21494873	V	0	0
30	12,7	24,3	1,18630159	V	1	0
31	13,1	27,7	1,23215653	V	1	1
32	14,6	43	1,38168769	V	0	1
33	13,6	32,3	1,28406142	V	1	0
34	14,5	41,7	1,36782976	V	0	0
35	12	21,7	1,25578704	V	0	0
36	13,2	28,6	1,24349556	V	1	1
37	13,6	26	1,03360981	V	0	0
38	11,6	18,4	1,17881012	V	1	0
39	12,4	21,3	1,11715787	V	0	1
40	13,8	34,4	1,30894435	V	1	1
41	13,7	31	1,20559099	F	1	0
42	13,8	35,7	1,35841027	F	0	0
43	11,6	20	1,28131535	F	0	0
44	15	43,7	1,29481481	F	1	0
45	13	34,5	1,57032317	F	0	0
46	14	33,2	1,20991254	F	0	0
47	13,2	30,9	1,34349695	F	0	1
48	13,6	32,5	1,29201226	F	0	1
49	12,8	25,8	1,23023987	F	1	1
50	13,5	31,8	1,2924859	F	1	0
51	12,9	26,6	1,23911754	F	0	0
52	13	30,1	1,37005007	F	0	0
53	13,3	30,5	1,29641759	F	0	0
54	13,3	29,5	1,2539121	F	0	0
55	13,5	31,6	1,28435706	F	1	1
56	13,2	30	1,30436597	F	0	1
57	12,9	24,9	1,15992582	F	0	1
58	13,6	31,2	1,24033177	F	1	1
59	13	25,6	1,16522531	F	1	0
60	11,5	17,9	1,17695406	F	0	0
61	13,8	33,6	1,27850379	V	0	0
62	13,8	33	1,25567336	V	1	0
63	13	28,4	1,29267183	V	0	1
64	13,5	32	1,30061474	V	1	1
65	13	28	1,27446518	V	0	0
66	13,3	32,4	1,37717803	V	0	1
67	14	33,9	1,23542274	V	0	0
68	12,8	24,1	1,14917755	V	0	0
69	12,6	24,6	1,2297688	V	0	0
70	12,5	25,3	1,29536	V	0	1
71	13,3	29,8	1,26666375	V	0	0

72	13,2	28,3	1,2304519	V	0	0
73	13,7	32,7	1,27170404	V	0	0
74	14	34,1	1,24271137	V	1	0
75	13,8	33,6	1,27850379	V	0	0
76	13,9	33,7	1,25483175	V	1	0
77	13,1	28,5	1,26774228	V	0	0
78	13,2	26,4	1,14784206	V	1	1
79	13,2	28,3	1,2304519	V	0	0
80	13,5	23,6	0,95920337	V	0	0
81	14,2	37,3	1,30269816	F	0	0
82	14,5	38,4	1,25958424	F	0	1
83	13,6	33	1,31188938	F	0	0
84	13,6	31,4	1,24828262	F	0	0
85	14,4	36,3	1,21567966	F	1	0
86	12,8	26,4	1,2588501	F	1	0
87	11,7	20,9	1,30493446	F	0	1
88	15	45,9	1,36	F	1	1
89	13,5	28,7	1,16648885	F	1	0
90	13,3	28,5	1,2114066	F	0	0
91	12,1	21,9	1,23619791	F	0	0
92	14,4	37,1	1,24247149	F	0	0
93	15,2	49,2	1,40098775	F	1	0
94	15,4	50,4	1,37996596	F	0	0
95	14,3	40,2	1,37473168	F	0	0
96	12,9	27,2	1,26706756	F	1	0
97	13	27,5	1,25170687	F	0	0
98	12,6	24	1,19977444	F	0	Missing
99	13,8	35,2	1,33938492	F	1	0
100	14,2	35	1,22237092	F	0	0
101	13,3	30,4	1,29216704	V	0	0
102	13,4	31,6	1,31332644	V	0	0
103	13,6	31,4	1,24828262	V	0	1
104	13	28,6	1,30177515	V	1	0
105	14,1	33,9	1,20932313	V	1	0
106	13,3	28,5	1,2114066	V	1	0
107	12,5	23,5	1,2032	V	0	0
108	12,6	25	1,24976504	V	1	1
109	14,2	36,4	1,27126576	V	1	1
110	12,5	23,9	1,22368	V	0	0
111	13,3	29,9	1,2709143	V	0	0
112	14,7	40,6	1,27812706	V	0	0
113	13,3	29,7	1,2624132	V	0	0
114	14,2	36,7	1,28174323	V	1	0
115	13,1	30,2	1,34336199	V	0	0
116	14	35,4	1,29008746	V	0	0
117	14,6	41,3	1,32706283	V	1	1

118	14	36,5	1,33017493	V	0	0
119	14	34,4	1,25364431	V	1	1
120	13,5	29,9	1,2152619	V	1	0

Table 2: Relevant information about the individuals of Atlantic salmon that were sampled at the middle sampling

Fish	length	weight	K factor	Lipid	HIS	Left Eye	Right Eye	Sum
1	17,00	62	1,261958	F	High	0	1	1
2	15,10	44	1,277973	F	High	0	0	0
3	17,30	67	1,294007	F	High	0	0	0
4	15,90	48	1,194125	F	High	0	1	1
5	17,30	62	1,197439	F	High	1	0	1
6	16,70	54	1,15943	F	High	0	0	0
7	15,60	48	1,26435	V	High	1	1	2
8	17,20	64	1,257751	V	High	1	0	1
9	16,50	55	1,224365	V	High	0	1	1
10	17,00	67	1,363729	V	High	1	0	1
11	17,70	73	1,316446	V	High	0	1	1
12	17,30	64	1,236066	V	High	0	1	1
13	17,30	66	1,274693	F	Low	0	1	1
14	17,60	70	1,283985	F	Low	0	1	1
15	16,30	55	1,269989	F	Low	1	0	1
16	17,90	70	1,220503	F	Low	1	1	2
17	16,50	61	1,357932	F	Low	0	0	0
18	17,00	60	1,22125	F	Low	0	1	1
19	15,90	49	1,219003	V	Low	0	1	1
20	16,40	56	1,26957	V	Low	0	1	1
21	15,80	50	1,267648	V	Low	0	0	0
22	15,70	49	1,266185	V	Low	2	1	3
23	15,30	47	1,312271	V	Low	0	1	1
24	17,10	61	1,219949	V	Low	1	1	2
25	17,20	61	1,198794	F	High	1	1	2
26	18,10	80	1,349131	F	High	1	0	1
27	17,50	69	1,287464	F	High	1	0	1
28	16,50	56	1,246626	F	High	1	2	3
29	16,80	54	1,138848	F	High	1	1	2
30	17,30	66	1,274693	F	High	1	0	1
31	18,40	79	1,268159	V	High	2	2	4
32	18,90	85	1,259023	V	High	1	0	1
33	17,30	68	1,31332	V	High	1	1	2
34	17,60	70	1,283985	V	High	1	0	1
35	17,40	53	1,00607	V	High	0	1	1
36	16,40	59	1,337582	V	High	1	0	1
37	16,30	55	1,269989	F	Low	1	1	2
38	16,30	51	1,177626	F	Low	1	2	3

39	16,50	52	1,157581	F	Low	1	0	1
40	17,30	64	1,236066	F	Low	0	1	1
41	17,00	59	1,200896	F	Low	0	1	1
42	16,00	49	1,196289	F	Low	1	1	2
43	15,50	48	1,28898	V	Low	1	0	1
44	15,90	61	1,517534	V	Low	0	0	0
45	16,00	51	1,245117	V	Low	1	0	1
46	17,20	62	1,218446	V	Low	2	2	4
47	17,00	63	1,282312	V	Low	1	1	2
48	15,90	51	1,268758	V	Low	1	2	3
49	17,60	70	1,283985	F	High	1	0	1
50	16,60	55	1,202371	F	High	1	1	2
51	15,90	48	1,194125	F	High	0	0	0
52	17,90	74	1,290246	F	High	1	0	1
53	15,80	51	1,293001	F	High	1	2	3
54	16,00	49	1,196289	F	High	1	1	2
55	17,80	71	1,258921	V	High	0	0	0
56	17,40	63	1,195894	V	High	2	1	3
57	16,20	52	1,22309	V	High	2	1	3
58	16,60	56	1,224232	V	High	1	1	2
59	16,00	50	1,220703	V	High	1	0	1
60	16,60	59	1,289816	V	High	1	1	2
61	17,90	72	1,255375	F	Low	1	1	2
62	16,60	51	1,114926	F	Low	0	0	0
63	17,30	72	1,390574	F	Low	0	1	1
64	17,00	58	1,180541	F	Low	2	1	3
65	17,00	66	1,343375	F	Low	1	2	3
66	16,70	55	1,1809	F	Low	0	0	0
67	17,40	67	1,271824	V	Low	1	0	1
68	17,00	60	1,22125	V	Low	1	2	3
69	16,70	59	1,266784	V	Low	1	0	1
70	16,50	54	1,202104	V	Low	1	1	2
71	16,40	53	1,201557	V	Low	1	2	3
72	17,00	62	1,261958	V	Low	1	1	2
73	18,80	83	1,249121	F	High	0	0	0
74	18,10	74	1,247947	F	High	0	1	1
75	16,70	63	1,352668	F	High	1	0	1
76	16,50	67	1,491499	V	High	1	0	1
77	17,60	65	1,192272	V	High	0	1	1
78	17,00	62	1,261958	V	High	0	1	1
79	15,50	47	1,262126	F	Low	1	1	2
80	17,20	62	1,218446	F	Low	1	0	1
81	16,00	50	1,220703	F	Low	1	1	2
82	17,00	61	1,241604	V	Low	0	0	0
83	16,50	50	1,113059	V	Low	0	1	1
84	16,70	56	1,202371	V	Low	0	1	1

85	17,00	60	1,22125	F	High	0	0	0
86	16,80	61	1,286477	F	High	1	0	1
87	15,80	49	1,242295	F	High	0	1	1
88	16,10	49	1,174136	V	High	0	0	0
89	16,20	54	1,270132	V	High	0	1	1
90	17,50	65	1,212828	V	High	0	1	1
91	15,90	50	1,24388	F	Low	1	1	2
92	16,90	60	1,243057	F	Low	0	1	1
93	16,90	57	1,180904	F	Low	1	1	2
94	17,00	61	1,241604	V	Low	1	0	1
95	17,70	73	1,316446	V	Low	1	0	1
96	17,60	71	1,302328	V	Low	2	1	3
97	18,70	87	1,330437	F	High	1	2	3
98	16,00	47	1,147461	F	High	0	1	1
99	17,30	64	1,236066	F	High	0	0	0
100	17,30	63	1,216752	V	High	0	1	1
101	17,70	75	1,352513	V	High	1	1	2
102	17,30	67	1,294007	V	High	1	0	1
103	17,30	66	1,274693	F	Low	1	0	1
104	16,20	55	1,293653	F	Low	2	1	3
105	17,50	68	1,268805	F	Low	1	2	3
106	18,40	85	1,364475	V	Low	1	1	2
107	15,60	47	1,23801	V	Low	1	2	3
108	17,90	70	1,220503	V	Low	2	1	3

Table 3: Relevant information about the individuals of Atlantic salmon that were sampled at the end sampling

Fish	Length	Weight	K-factor	Lipid	HIS	Left Eye	Right Eye	Sum
1	23	161	1,32	F	High	0	1	1
2	18,9	78	1,16	F	High	1	0	1
3	20,5	103	1,20	F	High	0	0	0
4	22,1	125	1,16	F	High	1	0	1
5	23	166	1,36	F	High	1	0	1
6	20,6	100	1,14	F	High	1	1	2
7	22,5	133	1,17	V	High	1	1	2
8	21	104	1,12	V	High	2	1	3
9	21,6	116	1,15	V	High	1	0	1
10	21	106	1,14	V	High	2	1	3
11	22,6	159	1,38	V	High	1	0	1
12	19,3	79	1,10	V	High	0	1	1
13	19,8	91	1,17	F	Low	0	1	1
14	21,4	107	1,09	F	Low	1	0	1
15	20,3	86	1,03	F	Low	0	0	0
16	22,3	130	1,17	F	Low	1	0	1
17	20	99	1,24	F	Low	0	0	0

18	19,2	82	1,16	F	Low	0	0	0
19	20,5	100	1,16	V	Low	1	1	2
20	21,3	118	1,22	V	Low	1	0	1
21	21,4	113	1,15	V	Low	1	1	2
22	21,5	107	1,08	V	Low	1	1	2
23	19	82	1,20	V	Low	0	1	1
24	20,1	91	1,12	V	Low	0	1	1
25	20,7	98	1,10	F	High	1	1	2
26	22	123	1,16	F	High	0	0	0
27	21,5	110	1,11	F	High	1	0	1
28	20,3	105	1,26	F	High	1	1	2
29	20,4	99	1,17	F	High	1	1	2
30	23,8	169	1,25	F	High	1	0	1
31	21,7	131	1,28	V	High	1	0	1
32	20,2	104	1,26	V	High	2	2	4
33	21	96	1,04	V	High	2	1	3
34	20	87	1,09	V	High	2	1	3
35	20,5	100	1,16	V	High	0	1	1
36	20,2	93	1,13	V	High	1	0	1
37	22,3	149	1,34	F	Low	0	1	1
38	21	116	1,25	F	Low	0	0	0
39	19	75	1,09	F	Low	1	0	1
40	20,5	102	1,18	F	Low	1	0	1
41	21,7	120	1,17	F	Low	1	2	3
42	21,4	110	1,12	F	Low	1	1	2
43	19,2	75	1,06	V	Low	1	1	2
44	23,5	163	1,26	V	Low	0	1	1
45	21,3	107	1,11	V	Low	1	1	2
46	21,4	113	1,15	V	Low	0	1	1
47	21	113	1,22	V	Low	1	1	2
48	19,7	91	1,19	V	Low	0	1	1
49	22,4	130	1,16	F	High	0	1	1
50	21,7	123	1,20	F	High	0	1	1
51	21,3	118	1,22	F	High	0	0	0
52	20,7	90	1,01	F	High	1	1	2
53	22,4	131	1,17	F	High	1	0	1
54	22,1	120	1,11	F	High	1	1	2
55	21,8	114	1,10	V	High	0	1	1
56	20,5	87	1,01	V	High	0	1	1
57	22,3	141	1,27	V	High	1	0	1
58	20,7	107	1,21	V	High	1	1	2
59	22,7	121	1,03	V	High	0	1	1
60	20,2	97	1,18	V	High	0	1	1
61	20,2	108	1,31	F	Low	0	1	1
62	22,6	141	1,22	F	Low	1	0	1
63	22,6	131	1,13	F	Low	0	1	1

64	22,4	119	1,06	F	Low	0	1	1
65	21,7	108	1,06	F	Low	1	2	3
66	20,3	101	1,21	F	Low	0	1	1
67	20,7	103	1,16	V	Low	0	1	1
68	22,7	139	1,19	V	Low	0	1	1
69	20,7	100	1,13	V	Low	1	0	1
70	23	147	1,21	V	Low	1	2	3
71	19,4	80	1,10	V	Low	1	2	3
72	21,1	110	1,17	V	Low	0	1	1
73	21,2	113	1,19	F	High	0	0	0
74	21,3	106	1,10	F	High	0	0	0
75	22	124	1,16	F	High	0	0	0
76	21,4	117	1,19	F	High	0	1	1
77	21	109	1,18	F	High	0	0	0
78	21,4	109	1,11	F	High	1	0	1
79	22,3	141	1,27	V	High	0	1	1
80	20,3	100	1,20	V	High	0	0	0
81	21	110	1,19	V	High	1	0	1
82	23,2	177	1,42	V	High	1	2	3
83	20,9	100	1,10	V	High	1	2	3
84	21,3	113	1,17	V	High	1	1	2
85	18,6	69	1,07	F	Low	1	0	1
86	20,9	91	1,00	F	Low	1	1	2
87	20,7	96	1,08	F	Low	0	0	0
88	20	105	1,31	F	Low	1	0	1
89	20,2	94	1,14	F	Low	0	1	1
90	19,3	80	1,11	F	Low	2	0	2
91	24,2	195	1,38	V	Low	2	1	3
92	20,3	101	1,21	V	Low	1	0	1
93	21	99	1,07	V	Low	1	1	2
94	20,3	98	1,17	V	Low	1	1	2
95	22	126	1,18	V	Low	0	1	1
96	21,3	102	1,06	V	Low	0	1	1
97	21,5	115	1,16	F	High	0	0	0
98	20,4	96	1,13	F	High	1	0	1
99	22,8	173	1,46	F	High	0	1	1
100	19,7	77	1,01	F	High	1	0	1
101	20,9	102	1,12	F	High	1	0	1
102	21,8	113	1,09	F	High	0	1	1
103	20,4	102	1,20	V	High	0	0	0
104	22,3	143	1,29	V	High	1	0	1
105	21,5	120	1,21	V	High	2	1	3
106	20,1	93	1,15	V	High	1	1	2
107	21,8	128	1,24	V	High	0	0	0
108	19,2	80	1,13	V	High	1	1	2
109	23,1	150	1,22	F	Low	1	1	2

110	22	118	1,11	F	Low	1	2	3
111	20,1	84	1,03	F	Low	1	0	1
112	20,8	102	1,13	F	Low	0	1	1
113	23,2	172	1,38	F	Low	0	1	1
114	21,5	108	1,09	F	Low	1	2	3
115	21,3	113	1,17	V	Low	0	1	1
116	22,3	129	1,16	V	Low	1	0	1
117	20	92	1,15	V	Low	1	1	2
118	21,5	125	1,26	V	Low	1	0	1
119	20,7	98	1,10	V	Low	1	1	2
120	22	123	1,16	V	Low	1	2	3
121	21,4	105	1,07	F	High	1	2	3
122	22,7	132	1,13	F	High	0	0	0
123	21	108	1,17	F	High	0	1	1
124	20,2	91	1,10	F	High	0	1	1
125	21,1	98	1,04	F	High	0	0	0
126	20,5	98	1,14	F	High	0	0	0
127	20,3	92	1,10	V	High	1	1	2
128	21,6	117	1,16	V	High	1	0	1
129	20,6	101	1,16	V	High	0	1	1
130	22,8	173	1,46	V	High	0	1	1
131	21,1	100	1,06	V	High	0	0	0
132	20,3	93	1,11	V	High	1	2	3
133	22	124	1,16	F	Low	1	2	3
134	20	90	1,13	F	Low	1	2	3
135	21,5	110	1,11	F	Low	1	1	2
136	21,4	110	1,12	F	Low	1	1	2
137	24,9	218	1,41	F	Low	1	2	3
138	20,7	109	1,23	F	Low	1	1	2
139	20,1	94	1,16	V	Low	1	1	2
140	22,7	148	1,27	V	Low	1	0	1
141	23	130	1,07	V	Low	1	2	3
142	21	106	1,14	V	Low	1	1	2
143	20,6	97	1,11	V	Low	1	2	3
144	20,3	97	1,16	V	Low	1	2	3

One-way analyses of variance for the start sampling:

Table 4 : One-way Analysis of Variance (ANOVA) test on the weight (g) of the Atlantic salmon. P>0.05 indicates that there was not a general significant difference in weight

	Univariate Tests of Significance for Weight. Sigma-restricted parameterization Effective hypothesis decomposition				
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	111337,4	1	111337,4	2570,577	0,000000
Lipid	81,3	1	81,3	1,878	0,173150
Error	5110,8	118	43,3		

Table 5 : Levene's Test for Homogeneity of Variances

	Effect: Lipid Degrees of freedom for all F's: 1, 118			
	MS Effect	MS Error	F	p
Weight	20,67253	18,74831	1,102634	0,295835

Table 6: One-way Analysis of Variance (ANOVA) test on the length (cm) of the Atlantic salmon. P>0.05 indicates that there was not a general significant difference in length.

Effect	Univariate Tests of Significance for Length. Sigma-restricted parameterization. Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	21440,13	1	21440,13	27000,77	0,000000
Lipid	0,77	1	0,77	0,97	0,327395
Error	93,70	118	0,79		

Table 7: Levene's Test for Homogeneity of Variances

	Effect: Lipid Degrees of freedom for all F's: 1, 118			
	MS Effect	MS Error	F	p
Length	0,855704	0,358318	2,388112	0,124939

Table 8: One-way Analysis of Variance (ANOVA) test on the K-factor of the Atlantic salmon. P>0.05 indicates that there was not a general significant difference in K-factor.

Effect	Univariate Tests of Significance for K-factor Sigma-restricted parameterization. Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	188,8258	1	188,8258	31900,94	0,000000
Lipid	0,0044	1	0,0044	0,75	0,389231
Error	0,6985	118	0,0059		

Table 7: Levene's Test for Homogeneity of Variances

	Effect: Lipid Degrees of freedom for all F's: 1, 118			
	MS Effect	MS Error	F	p
K-factor	0,000737	0,002775	0,265386	0,607407

Table 9: One-way Analysis of Variance (ANOVA) test on cataract score of the Atlantic salmon. P>0.05 indicates that there was not a general significant difference in score

	Univariate Tests of Significance for Sum. Sigma-restricted parameterization Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	42,00833	1	42,00833	82,18265	0,000000
Lipid	0,67500	1	0,67500	1,32053	0,252821
Error	60,31667	118	0,51116		

Table 10: Levene's Test for Homogeneity of Variances

	Effect: Lipid Degrees of freedom for all F's: 1, 118			
	MS Effect	MS Error	F	p
Sum	0,320333	0,095509	3,353956	0,069566

Table 11: One-way Analysis of Variance (ANOVA) test on His heart of the Atlantic salmon. P>0.05 indicates that there was not a general significant difference in score.

Effect	Univariate Tests of Significance for Start HIS Heart Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	6,657067	1	6,657067	818,4918	0,000009
Lipid	0,038400	1	0,038400	4,7213	0,095504
Error	0,032533	4	0,008133		

Table 12: One-way Analysis of Variance (ANOVA) test on NAH heart of the Atlantic salmon. P>0.05 indicates that there was not a general significant difference in score.

Effect	Univariate Tests of Significance for Start NAH Heart Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	122,4017	1	122,4017	1984,892	0,000002
Lipid	0,0017	1	0,0017	0,027	0,877390
Error	0,2467	4	0,0617		

Table 13: One-way Analysis of Variance (ANOVA) test on His lens of the Atlantic salmon. P>0.05 indicates that there was not a general significant difference in score.

Effect	Univariate Tests of Significance for Start His lens Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p

Intercept	18,37500	1	18,37500	612,5000	0,000016
Lipid	0,01500	1	0,01500	0,5000	0,518519
Error	0,12000	4	0,03000		

Table 13: One-way Analysis of Variance (ANOVA) test on NAH lens of the Atlantic salmon. P>0.05 indicates that there was not a general significant difference in score.

Effect	Univariate Tests of Significance for Start NAH lens Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	500,5067	1	500,5067	1294,414	0,000004
Lipid	0,1067	1	0,1067	0,276	0,627193
Error	1,5467	4	0,3867		

Two-way anova analyses for middle sampling:

Table 14: Two-way Analysis of Variance (ANOVA) test on weight of the Atlantic salmon. P>0.05 indicates that there was not a general significant difference in weight.

Effect	Univariate Tests of Significance for weight Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	395912,2	1	395912,2	4388,467	0,000000
Lipid	4,1	1	4,1	0,045	0,831940
HIS	310,1	1	310,1	3,437	0,066581
Lipid*HIS	44,1	1	44,1	0,489	0,486095
Error	9382,5	104	90,2		

Table 15: Levene's Test for Homogeneity of Variances

	Effect: Lipid*HIS Degrees of freedom for all F's: 3, 104			
	MS Effect	MS Error	F	p
weight	35,32014	31,04034	1,137879	0,337360

Table 16: Two-way Analysis of Variance (ANOVA) test on length of the Atlantic salmon. P>0.05 indicates that there was not a general significant difference in length.

	Univariate Tests of Significance for length. Sigma-restricted parameterization Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	30731,07	1	30731,07	50489,63	0,000000
Lipid	0,02	1	0,02	0,03	0,863243
HIS	2,31	1	2,31	3,80	0,054019
Lipid*HIS	0,62	1	0,62	1,02	0,314182
Error	63,30	104	0,61		

Table 17: Levene's Test for Homogeneity of Variances.

	Effect: Lipid*HIS Degrees of freedom for all F's: 3, 104			
	MS Effect	MS Error	F	p
length	0,209737	0,196383	1,067999	0,366047

Table 18: Two-way Analysis of Variance (ANOVA) test on K factor of the Atlantic salmon. P>0.05 indicates that there was not a general significant difference in K factor.

Effect	Univariate Tests of Significance for K factor. Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	169,3611	1	169,3611	35785,70	0,000000
Lipid	0,0068	1	0,0068	1,44	0,232679
HIS	0,0001	1	0,0001	0,01	0,907337
Lipid*HIS	0,0002	1	0,0002	0,04	0,836927
Error	0,4922	104	0,0047		

Table 19: Levene's Test for Homogeneity of Variances.

	Levene's Test for Homogeneity of Variances Effect: Lipid*HIS Degrees of freedom for all F's: 3, 104			
	MS Effect	MS Error	F	p
K factor	0,000457	0,002295	0,198984	0,896869

Table 20: Two-way Analysis of Variance (ANOVA) test on cataract score of the Atlantic salmon. P<0.05 indicates that there was a general significant difference in cataract score.

Effect	Univariate Tests of Significance for Sum. Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	222,4537	1	222,4537	237,8713	0,000000
Lipid	1,1204	1	1,1204	1,1980	0,276246
HIS	4,0833	1	4,0833	4,3663	0,039095
Lipid*HIS	0,0833	1	0,0833	0,0891	0,765909

Error	97,2593	104	0,9352		

Table 21: Levene's Test for Homogeneity of Variances.

	Levene's Test for Homogeneity of Variances Effect: Lipid*HIS Degrees of freedom for all F's: 3, 104			
	MS Effect	MS Error	F	p
Sum	0,463124	0,296940	1,559654	0,203662

Table 22: Two-way Analysis of Variance (ANOVA) test on HIS Heart of the Atlantic salmon. P<0.05 indicates that there was a general significant difference in cataract score.

	Univariate Tests of Significance for Middle HIS Heart Sigma-restricted parameterization Effective hypothesis decomposition				
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	26,10750	1	26,10750	513,5902	0,000000
Lipid	0,06750	1	0,06750	1,3279	0,282454
HIS	0,44083	1	0,44083	8,6721	0,018571
Lipid* HIS	0,06750	1	0,06750	1,3279	0,282454
Error	0,40667	8	0,05083		

Table 23: Two-way Analysis of Variance (ANOVA) test on NAH Heart of the Atlantic salmon. P<0.05 indicates that there was a general significant difference in cataract score.

	Univariate Tests of Significance for Middle NAH Heart Sigma-restricted parameterization Effective hypothesis decomposition				
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	183,3008	1	183,3008	2135,544	0,000000
Lipid	0,0008	1	0,0008	0,010	0,923933
HIS	0,0208	1	0,0208	0,243	0,635485
Lipid* HIS	0,8008	1	0,8008	9,330	0,015713
Error	0,6867	8	0,0858		

Table 24: Two-way Analysis of Variance (ANOVA) test on HIS lens of the Atlantic salmon. P<0.05 indicates that there was a general significant difference in cataract score.

	Univariate Tests of Significance for Middle HIS left lens Sigma-restricted parameterization Effective hypothesis decomposition				
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	28,67521	1	28,67521	736,0481	0,000000

Lipid	0,00021	1	0,00021	0,0053	0,943500
HIS	0,88021	1	0,88021	22,5936	0,001439
Lipid* HIS	0,03521	1	0,03521	0,9037	0,369613
Error	0,31167	8	0,03896		

Table 25: Two-way Analysis of Variance (ANOVA) test on NAH lens of the Atlantic salmon. P<0.05 indicates that there was a general significant difference in cataract score.

	Univariate Tests of Significance for Middle NAH Left lens Sigma-restricted parameterization Effective hypothesis decomposition				
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	913,5075	1	913,5075	434,4863	0,000000
Lipid	5,4675	1	5,4675	2,6005	0,145496
HIS	27,9075	1	27,9075	13,2735	0,006558
Lipid* HIS	0,0675	1	0,0675	0,0321	0,862253
Error	16,8200	8	2,1025		

Two-way anova analyses for end sampling

Table 26: Two-way Analysis of Variance (ANOVA) test on weight of the Atlantic salmon. P>0.05 indicates that there was not a general significant difference in weight.

	Univariate Tests of Significance for weight Sigma-restricted parameterization Effective hypothesis decomposition				
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	1827679	1	1827679	2906,286	0,000000
Lipid	23	1	23	0,036	0,850042
HIS	185	1	185	0,293	0,588915
Lipid*HIS	65	1	65	0,104	0,747678
Error	88042	140	629		

Table 27: Levene's Test for Homogeneity of Variances

	Effect: Lipid*HIS Degrees of freedom for all F's: 3, 140			
	MS Effect	MS Error	F	p
Weight	50,56301	286,6846	0,176372	0,912253

Table 28: Two-way Analysis of Variance (ANOVA) test on length of the Atlantic salmon. P>0.05 indicates that there was not a general significant difference in weight.

Effect	Univariate Tests of Significance for length. Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	64693,92	1	64693,92	49086,84	0,000000
Lipid	0,28	1	0,28	0,22	0,642962
HIS	0,54	1	0,54	0,41	0,524009
Lipid*HIS	0,90	1	0,90	0,68	0,409355
Error	184,51	140	1,32		

Table 29: Levene's Test for Homogeneity of Variances.

	Effect: Lipid*HIS Degrees of freedom for all F's: 3, 140			
	MS Effect	MS Error	F	p
length	0,568421	0,468262	1,213895	0,307035

Table 30: Two-way Analysis of Variance (ANOVA) test on the K factor of the Atlantic salmon. P>0.05 indicates that there was not a general significant difference in K factor.

Effect	Univariate Tests of Significance for K factor. Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	195,7781	1	195,7781	24168,70	0,000000
Lipid	0,0025	1	0,0025	0,31	0,581536
HIS	0,0019	1	0,0019	0,24	0,625440
Lipid*HIS	0,0028	1	0,0028	0,35	0,555523
Error	1,1341	140	0,0081		

Table 31: Levene's Test for Homogeneity of Variances

	Effect: Lipid*HIS Degrees of freedom for all F's: 3, 140			
	MS Effect	MS Error	F	p
K factor	0,007263	0,003589	2,023941	0,113329

Table 32: Two-way Analysis of Variance (ANOVA) test on cataract score of the Atlantic salmon. P<0.05 indicates that there was a general significant difference in cataract score.

Effect	Univariate Tests of Significance for sum. Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	289,0000	1	289,0000	356,1271	0,000000
Lipid	8,0278	1	8,0278	9,8924	0,002027
HIS	4,0000	1	4,0000	4,9291	0,028016
Lipid*HIS	1,3611	1	1,3611	1,6773	0,197420
Error	113,6111	140	0,8115		

Table 33: Levene's Test for Homogeneity of Variances

	Levene's Test for Homogeneity of Variances Effect: Lipid*HIS Degrees of freedom for all F's: 3, 140			
	MS Effect	MS Error	F	p
Sum	0,824789	0,228284	3,612999	0,014919

Table 34: Two-way Analysis of Variance (ANOVA) test on HIS Heart of the Atlantic salmon. P<0.05 indicates that there was a general significant difference in cataract score.

Effect	Univariate Tests of Significance for End HIS Heart Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	7,040178	1	7,040178	529,5026	0,000000
Lipid	0,016044	1	0,016044	1,2067	0,280178
HIS	0,603211	1	0,603211	45,3684	0,000000
Lipid*HIS	0,052900	1	0,052900	3,9787	0,054656
Error	0,425467	32	0,013296		

Table 35: Two-way Analysis of Variance (ANOVA) test on NAH Heart of the Atlantic salmon. P<0.05 indicates that there was a general significant difference in cataract score.

Effect	Univariate Tests of Significance for End NAH heart Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	330,6336	1	330,6336	516,9516	0,000000
Lipid	0,2336	1	0,2336	0,3653	0,549863
HIS	0,2025	1	0,2025	0,3166	0,577572
Lipid*HIS	0,2336	1	0,2336	0,3653	0,549863
Error	20,4667	32	0,6396		

Table 35: Two-way Analysis of Variance (ANOVA) test on HIS Lens of the Atlantic salmon. P<0.05 indicates that there was a general significant difference in cataract score.

Effect	Univariate Tests of Significance for End HIS lens Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	29,20864	1	29,20864	357,9799	0,000000
Lipid	0,08144	1	0,08144	0,9982	0,325744
HIS	1,68158	1	1,68158	20,6094	0,000085
Lipid*HIS	0,06040	1	0,06040	0,7403	0,396392
Error	2,44779	30	0,08159		

Table 36: Two-way Analysis of Variance (ANOVA) test on NAH Lens of the Atlantic salmon. P<0.05 indicates that there was a general significant difference in cataract score.

Effect	Univariate Tests of Significance for End NAH Lens Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	561,2865	1	561,2865	203,6468	0,000000
Lipid	3,3243	1	3,3243	1,2061	0,280838
HIS	115,0110	1	115,0110	41,7285	0,000000
Lipid*HIS	1,7264	1	1,7264	0,6264	0,434897
Error	82,6853	30	2,7562		