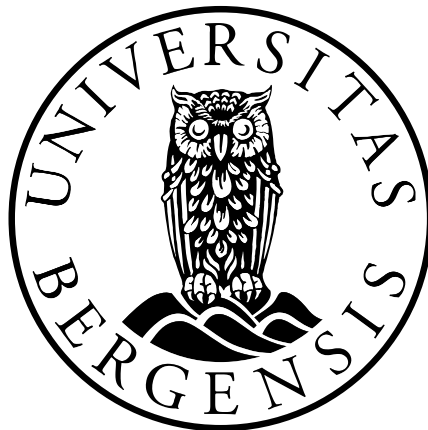
Mechanisms of cataract development in adult Atlantic salmon growers relative to dietary histidine and plant feed ingredients

Christiane Tröße



Dissertation for the degree of philosophiae doctor (PhD)
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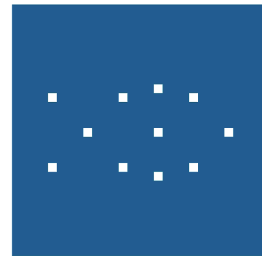

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Paper I **Christiane Tröße, Rune Waagbø, Olav Breck and Pål A. Olsvik.** Expression of stress-responsive genes in lenses of Atlantic salmon (*Salmo salar* L.) smolt during sampling and anaesthesia. Fish Physiology and Biochemistry (submitted).

Paper II **Rune Waagbø, Christiane Tröße, Wolfgang Koppe, Ramon Fontanillas and Olav Breck.** Dietary histidine supplementation prevents cataract development in adult Atlantic salmon, *Salmo salar* L in seawater. British Journal of Nutrition (submitted).

Paper III **Christiane Tröße, Rune Waagbø, Olav Breck, Anne-Kristin Stavrum, Kjell Petersen and Pål A. Olsvik.** (2009) Genome-wide transcription analysis of histidine-related cataract in Atlantic salmon (*Salmo salar* L). Molecular Vision, 15: 1332-1350

Paper IV **Christiane Tröße, Jeremy D. Rhodes, Julie Sanderson, Olav Breck and Rune Waagbø.** (2009) Effect of plant based feed ingredients on osmoregulation in the Atlantic salmon lens. Comparative Biochemistry and Physiology, Part B (accepted). DOI: 10.1016/j.cbpb.2009.12.002

These four papers are referred to in the following text by their roman numerals. In the case where a paper has not yet been published, the style of the manuscript is that required by the respective journal it has been submitted to.

Abbreviations

AGE	advanced glycation end product
ANOVA	analysis of variance
BA-DH	betaine aldehyde dehydrogenase
CA	correspondence analysis
CHOL	cholesterol
EST	expressed sequence tag
FAA	free amino acid
FDR	false discovery rate
FFA	free fatty acid
FM	fish meal
FO	fish oil
GO	gene ontology
GPX	glutathione peroxidase
GPX4	glutathione peroxidase 4
GR	glutathione reductase
GSEA	gene set enrichment analysis
GSH	glutathione
GST	glutathione S-transferase
His	α-L-histidine
HPLC	high performance liquid chromatography
HSP	heat shock protein
HSP70	heat shock protein 70
IMR	Institute of Marine Research
MNE	mean normalised expression
MT-A	metallothionein A
MT-B	metallothionein B

NAH	N-acetylhistidine
NIFES	National Institute of Nutrition and Seafood Research
NL	neutral lipid
PC	phosphatidyl choline
PCR	polymerase chain reaction
PE	phosphatidyl ethanolamine
PI	phosphatidyl inositol
PL	polar lipid
PP	plant protein
PS	phosphatidyl serine
qRT-PCR	quantitative real-time reverse transcription PCR
RIN	RNA integrity number
ROS	reactive oxygen species
RVD	regulatory volume decrease
RVI	regulatory volume increase
SAM	significance analysis of microarrays
SE	standard error
SOD	superoxide dismutase
SPARC	secreted protein acidic and rich in cysteine
UEA	University of East Anglia
VO	vegetable oil

Abstract

Cataracts are defined as opacities of the eye lens and can be caused by a large number of risk factors. In aquaculture, cataracts in farmed Atlantic salmon (*Salmo salar* L.) represent an ethical problem and can cause economical losses. A series of studies have shown the cataract mitigating effect of dietary histidine (His) levels above the currently recommended minimum requirement in Atlantic salmon smolt and that dietary His levels are reflected in the concentrations of the His-derivative N-acetylhistidine (NAH) in the lens. However, the mechanism of lens protection by dietary His is not clear and no studies with adult Atlantic salmon growers have previously been carried out.

Gene expression analysis in the lens is a powerful tool to investigate the molecular mechanisms of cataract formation in Atlantic salmon. In an initial study, the possible effects of different sampling procedures and tissue preservation methods on lens gene expression and lens RNA quality were investigated. Although there was no difference in RNA quality, tissue preservation in liquid nitrogen instead of RNA*later* was recommended due to practical conditions in RNA extraction. Sampling procedures lasting up to two hours and procedures not employing anaesthetics provoked expression changes of selected antioxidant and stress-responsive genes. Thus, a quick sampling protocol not exceeding 30 minutes and the use of anaesthetics were recommended for future studies.

A feeding trial was carried out to investigate the effects of different dietary His concentrations and His feeding regimes on lens and muscle imidazole concentrations and on cataract formation in adult Atlantic salmon growers. Fish in their second year in sea were fed one of three diets only differing in the His content [low His (L): 9 g/kg diet, medium His (M): 13 g/kg diet and high His (H): 17 g/kg diet] for four months (June to October). Dietary His concentrations were reflected in lens NAH and

to a lesser degree in muscle anserine concentrations. Between July and September, fish developed cataracts with different severity in response to dietary histidine levels fed between June and September. Feeding at least 13 g His/kg diet from June to July mitigated later cataract formation.

To identify molecular mechanisms involved in cataract formation, microarray analysis was applied to compare lens gene expression in groups of fish with cataracts of different severity caused by high or low dietary His levels. The differences in gene expression between the dietary groups were more pronounced in lenses sampled in October at a late stage of cataract formation than at an earlier state in September. At the late stage, 514 transcripts were significantly differentially expressed, while the same only applied to eight transcripts at the earlier state. Among the differentially expressed transcripts at the late stage were metallothionein A and B, as well as transcripts involved in lipid metabolism, carbohydrate metabolism, regulation of ion homeostasis and protein degradation. The differentially expressed transcripts could be categorized as “early” or “late” responsive, according to their expression pattern relative to progression in cataract formation. “Early” responsive transcripts might be used as molecular markers for early cataract formation in future studies; and one of the most promising candidates was SPARC (secreted protein acidic and rich in cysteine).

In a second feeding trial, Atlantic salmon growers were fed one of four diets with varying inclusion levels of plant proteins and plant lipids (100% fish meal and 100% fish oil, 80% plant protein and 35% vegetable oil blend, 40% plant protein and 70% vegetable oil blend and 80% plant protein and 70% vegetable oil blend) for 12 months. The His concentrations differed in the diets dependent on the level of plant protein inclusion and were reflected in lens NAH concentrations. Fish fed the highest inclusion level of plant lipids had decreased growth rates and higher lens water contents than fish fed the marine control diet or the lower inclusion levels of plant lipids. The lenses of all groups fed plant feed ingredients were smaller than lenses of the control fish. Inclusion of dietary plant lipids led to slightly different lens fatty acid

and lipid class composition. No severe cataracts were observed during the feeding study and there was no difference in cataract prevalence between the dietary groups.

Lenses of fish from the plant feeding trial were examined for their ability to withstand osmotic disturbances *ex vivo*. Lens whole-organ culture in hypoosmotic and hyperosmotic media led to increase and decrease of lens volume, respectively. Lenses of plant diet-fed fish were less resistant to swelling and shrinking, released less NAH into the culture medium and accumulated His and NAH at higher rates than control lenses. Culture in hypoosmotic medium resulted in higher cataract scores than in hyperosmotic and control medium. mRNA expression levels of the selected genes glutathione peroxidase 4 (GPX4) and SPARC were affected by the diet and betaine aldehyde dehydrogenase by osmotic treatment of the lenses. The role of NAH as a lens osmolyte was confirmed. However, the ability to osmoregulate under hypoosmotic conditions was related to dietary plant lipid inclusion levels rather than lens NAH concentrations.

1. Introduction

"Cataract" is a collective name for any kind of light-scattering opacity of the eye lens. Cataracts can affect the vision on a range from not noticeable, over hazy vision, glare, contrast sensitivity reduction, diplopia and up to complete blindness (Brown and Bron 1996). Cataracts are a major health problem for humans and have been a subject of biochemical research for about a hundred years (Hockwin et al. 2002). Cataracts have also been observed in many terrestrial animals and in a number of fish species, among them Atlantic salmon (*Salmo salar* L.), which is a popular food fish and a major aquaculture species in northern Europe. Cataracts were frequently observed in populations of farmed Atlantic salmon in the early beginnings of fish farming, and in the nineties and early 2000. Today, although less often, cataracts still occur in farmed Atlantic salmon. Atlantic salmon are visual predators and fish with severe cataracts may experience decreased growth rates and be more susceptible to secondary diseases due to decreased feed intake (Menzies et al. 2002). Cataracts represent an ethical problem for the aquaculture industry, being subject to steadily increasing public attention on fish welfare. In addition, severe cataract outbreaks may lead to economical losses for the aquaculture industry.

In a recent series of studies, the amino acid histidine (His) has been identified as a key preventative factor in the development of cataracts in Atlantic salmon parr and smolt. Elevated levels of dietary His - reached by supplementation of the diet with mammalian blood meal, crystalline His, or by the use of His-rich fish meals - have been shown to suppress, or, to a lesser degree, to prevent cataract formation in Atlantic salmon (Breck et al. 2003; Breck et al. 2005a). The levels of His in the diet were reflected in the lens concentrations of His and especially the His-derivative N-acetylhistidine (NAH). NAH has thus been proposed as a marker for the His status in the Atlantic salmon lens (Breck et al. 2005a; Breck et al. 2005b). However, the effect of elevated dietary His on cataract development in adult Atlantic salmon growers

during the second summer in sea water has not been studied. Also, the exact mechanism of the protective function of elevated dietary His and thus elevated lens NAH concentrations is not clear.

Due to the versatile chemistry of the His molecule, NAH might have many possible functions in the lens, including osmoregulation (Baslow 1998), anti-oxidation (Babizhayev 1989; Babizhayev et al. 2004), anti-inflammation (Wade and Tucker 1998), anti-glycation (Hobart et al. 2004) and increased buffering capacity (Abe et al. 1985). A role of NAH in the hypoosmotic regulation in the salmon lens *ex vivo* has been proposed (Breck 2004). Since several mechanisms might be involved in cataract formation in Atlantic salmon, the study of gene expression might be a valuable tool to explore the mechanisms of cataractogenesis in the Atlantic salmon lens at the molecular level. Microarray analysis is an effective "hypothesis generator" and a well-suited method to screen for differentially expressed genes in the transcriptomes of cataractous and clear lenses. However, gene expression analysis methods depend on good quality RNA preparations and elimination of confounding factors such as sampling stress.

Aquaculture is a worldwide rapidly growing industry, while the amounts of available marine raw materials are limited (FAO 2009). To secure a sustainable production of Atlantic salmon, increasing amounts of alternative feed ingredients, including plant raw materials, are used (Torstensen et al. 2008; Turchini et al. 2009). In a recent study, severe outbreaks of cataract have been observed in adult Atlantic salmon fed high inclusion levels of vegetable oils (Waagbø et al. 2004). Thus, the effect of plant-derived feed ingredients on lens function and cataractogenesis in Atlantic salmon needs further investigation.

2. Aims of the study

The overall objectives of the present thesis were to investigate the cataract mitigating effect of dietary His in relation to commercial-like diets based on marine ingredients and plant-based diets in adult Atlantic salmon growers, and to find the mechanisms by which dietary His interferes with cataract formation, applying biochemical and gene expression analyses to the lens. The specific aims were:

1. To investigate the possible impact of tissue preservation methods on the quality of RNA extracted from lenses of Atlantic salmon and to explore if sampling stress caused by different sampling procedures - varying in duration and use of anaesthetics - affect the expression levels of specific cataract-related target genes in Atlantic salmon lenses (Paper I).

2. To investigate the effects of different dietary His concentrations (Paper II, III and IV) and His feeding regimes (Paper II) on the concentrations of major His-derivatives in lens and muscle tissue, and on cataract formation in adult Atlantic salmon growers.

3. To apply microarray analysis to screen the lens transcriptome for differentially expressed genes in Atlantic salmon with His-related cataracts of different severity in order to understand the molecular mechanisms of cataract formation and to find possible expression markers for early cataract diagnosis (Paper III).

4. To investigate the possible effects of high inclusion levels of plant-derived dietary proteins and lipids on lens lipid and fatty acid class composition, lens NAH and His concentration and cataract formation in adult Atlantic salmon growers (Paper IV).

5. To apply an *ex vivo* whole organ lens culture model to investigate the osmoregulatory capacity of lenses dissected from Atlantic salmon fed plant-based diets or a marine control diet and exposed to moderate hypoosmotic or hyperosmotic conditions, and to analyse mRNA expression levels of selected genes in the cultured lenses (Paper IV).

3. Background

3.1 The fish eye and lens

The basic construction of the eye is similar in all fish species, including Atlantic salmon (Hargis 1991) (Figure 1). The fish lens is spherical and is surrounded by the eye chamber fluids, the aqueous humour anteriorly and the more jelly-like vitreous humour posteriorly. The lens is avascular, and nutrients are taken up from - and metabolites released into - the eye chamber fluids by the lens epithelial cells. The fish lens protrudes through the pupillary opening, bringing the anterior part of the lens close to the cornea. The cornea regulates the fluid movement into the eye chamber and thereby contributes to keeping the osmolality of the eye chamber fluids constant. Through the close placement to the cornea, fish lenses are more susceptible to osmotically induced anterior opacifications than mammalian lenses (Doughty et al. 1997; Midtlyng et al. 1999).

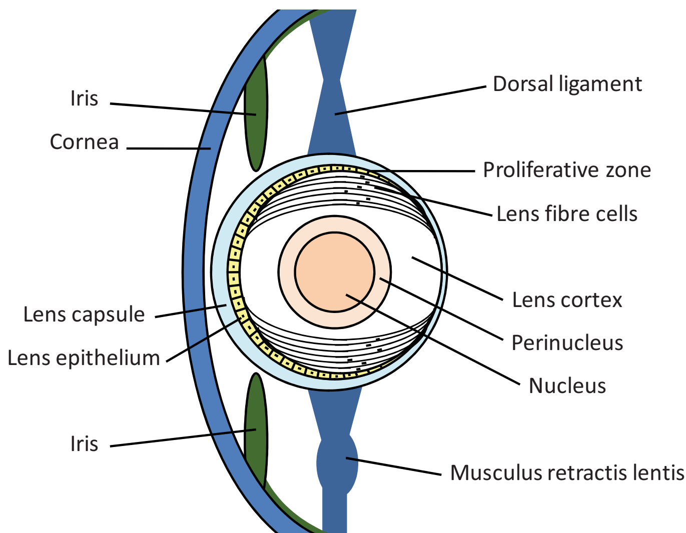

Figure 1. Schematic section of the teleost eye. Adapted from Midtlyng et al. (1999) and Bjerkås (1998).

The basic structure of the lens is similar in all organisms, although the shape can vary from globe-like, as in teleost fish, to the biconvex ellipsoid shape of human lenses. A general overview over the lens structure is given by Brown and Bron (1996). The lens consists of the nucleus, the perinucleus and the cortex - all three made up of fibre cells - as well as the lens epithelium and the lens capsule (Figure 1). The capsule, a thick basal lamina, serves as a physical protection of the lens and is fully permeable for water and small organic molecules. The monolayer of epithelial cells executes the main metabolic and osmoregulatory activity in the lens (Bhat 2001). In the proliferative zone, the epithelial cells differentiate into fibre cells. The resulting new layer of fibre cells spreads upon underlying older layers of fibre cells. The older fibre cells, eventually coming closer to the lens nucleus, lose their nuclei and other organelles, thus losing metabolic activity and the ability to transcribe DNA.

The fish eye and lens continue to grow throughout the entire lifetime (Fernald 1985; Fernald 1990). Unlike in most other tissues, lens cells are never replaced and there is no protein turnover in the central region of the lens. The fibre cells contain high concentrations of the major lens proteins, the crystallins. These proteins are highly ordered and tightly packed in the cells, which enables light to pass through the lens. Any disruption of this order through protein modifications and aggregation, if not removed by lens repair mechanisms, will lead to light scattering and thus cataract formation (Brown and Bron 1996).

3.2 General principles of cataract

Cataracts are defined as opacities of the eye lens (Brown and Bron 1996). Studies of the human lens and cataracts have a long history and are still a highly important field of interest in medical research due to the frequent occurrence of cataracts. Cataracts are one of the main causes of blindness worldwide. About 20% of the population in the western world are estimated to have cataracts at the present time-point and this number is expected to increase dramatically in the coming years, mainly due to the increase in the number of elderly in the population (Congdon et al. 2004).

Cataracts represent a multifactorial disease, and any insults causing changes in the microstructure of the crystalline lens can cause opacities if not repaired instantly and properly. Such insults are for instance oxidative stress, osmotic stress and the formation of chemical adducts (Bunce et al. 1990). Human cataracts have been intensively studied and a high number of risk factors have been identified. Radiation, including UV light (Bochow et al. 1989) and X-ray (Ham Jr 1953) has been shown to be a risk factor for cataract formation. Nutritional factors affecting cataract formation in humans, such as antioxidant vitamins and amino acid deficiencies, are discussed in detail by Bunce et al. (1990). Also smoking of cigarettes (Christen et al. 1992) and beedies (Ramakrishnan et al. 1995) has been shown to increase the risk for cataract formation. However, the main risk factors for cataract development in the Western world are advancing age and diabetes. The main mechanisms involved in both age-related and diabetic cataract formation are oxidative stress and chemical modification (Spector 1995; Truscott 2005; Vinson 2006; Williams 2006; Chan et al. 2008).

Oxidative stress is caused by free radicals, so called reactive oxygen species (ROS), which are highly reactive and can oxidise a broad range of biomolecules, including DNA, lipids and proteins. Especially thiol groups are prone to oxidation. The human lens contains high concentrations of the antioxidant glutathione (GSH) (Reddy 1990; Giblin 2000) and a system of interacting antioxidant enzymes to keep the redoxregulation in the lens in balance (Lou 2003). Among the antioxidant enzymes present in the lens are superoxide dismutase (SOD) (Reddy et al. 2004), glutathione peroxidase (GPX) and glutathione reductase (GR) (Lou 2003), glutathione S-transferase (GST) (Raghavachari et al. 1999), and metallothionein A (MT-A) and B (MT-B) (Oppermann et al. 2001; Hawse et al. 2006). Chemical modifications of proteins, like glycation and glycoxidation, lead to highly complex cross-linked molecules called AGEs (advanced glycation end products). AGE formation impairs the structure of the lens crystallins and thus causes lens opacification (Ahmed 2005). Although the principle mechanisms of oxidative stress and chemical modification are valid in all organisms, it is currently not known to what extent they are involved in cataract formation in Atlantic salmon.

3.3 Cataracts in Atlantic salmon and other teleost fish

Cataracts have been observed in both wild living and farmed teleost fish. The majority of the reported cataracts have been found in salmon and trout species, at least partly as a direct consequence of farmed salmonids being more thoroughly inspected than wild living species. In 1998, a cataract prevalence as high as 82% was found in 49 Atlantic salmon sea farms along the Norwegian coast (Ersdal et al. 2001).

Two types of cataracts can be observed in fish. The first type, transient cataracts caused by osmotic imbalance, has been described in both wild and farmed fish species (Loewenstein and Bettelheim 1979; Iwata et al. 1987; Bruno and Raynard 1994; Bjerkås et al. 1998; Bjerkås et al. 2000; Breck and Sveier 2001). The second type, irreversible cataracts, can be caused by a large number of different factors. Different environmental conditions have been identified as risk factors for cataract development in salmonids: fluctuating water salinity causing severe or repeated osmotic stress (Bjerkås et al. 2003; Bjerkås and Sveier 2004), temperature changes (Bruno and Raynard 1994; Bjerkås and Bjørnestad 1999; Bjerkås et al. 2001), water gas supersaturation (Krise and Smith 1993), water hyperoxygenation (Waagbø et al. 2008) and UV radiation (Hargis 1991; Cullen et al. 1994; Doughty et al. 1997). Also several toxins have been shown to cause cataracts in salmonids: polycyclic aromatic hydrocarbons (PAH) (Laycock et al. 2000), organophosphates (Fraser et al. 1990) and thioacetamide (von Sallmann et al. 1966). Furthermore, intraocular infections or inflammations can lead to secondary changes causing cataract formation in fish (Ashton et al. 1969; Shariff et al. 1980; Karlsbakk et al. 2002). Genetically linked predisposition to cataract formation in salmonids has been described (Steucke et al. 1968; Kincaid 1989; Wall and Richards 1992; Breck et al. 2005a).

In addition to the above listed risk factors for irreversible cataracts, numerous nutritional linked cataracts have been described in farmed salmonids in the past, caused by deficiencies in: thiamine (Hughes 1985), riboflavin (Poston et al. 1977; Hughes et al. 1981), methionine (Poston et al. 1977; Barash et al. 1982; Hughes 1985; Cowey et al. 1992), tryptophan (Poston and Rumsey 1983; Akiyama et al.

1986), manganese (Yamamoto et al. 1983) or zinc. Zinc deficiency is caused either directly by low dietary concentrations or by reduced availability due to formation of chelates in the intestine or competitive uptake of other minerals (Ketola 1979; Barash et al. 1982; Richardson et al. 1985; Shearer et al. 1992; Maage and Julshamn 1993). Also feeding of energy-dense diets, leading to rapid growth, has been related to cataract development (Waagbø et al. 1998; Breck and Sveier 2001). It has also been shown that high dietary levels of iron, manganese and lipids increased, and dietary antioxidants decreased the frequency of cataract formation, respectively (Waagbø et al. 2003). With the development of more balanced and optimised feeds, the nutrition-related cataracts listed above have mostly disappeared from modern aquaculture.

However, a new interest was sparked in the study of nutrition related cataracts when the omission of mammalian blood meal as a protein source in salmonid feeds in the early 1990s led to several severe outbreaks of cataract incidences in European salmonid fish farms, especially in Ireland (Crockford 1998; Wall 1998). Since blood meal is known to contain high amounts of His and iron, a NRC-funded research project (No.134965/120 "Importance of dietary histidine, iron and zinc concentrations on cataract development in two strains of Atlantic salmon") was run from 2000 to 2003 to elucidate the role of these dietary components. Elevated levels of dietary His were found to possess a cataract suppressing effect in Atlantic salmon smolt (Breck et al. 2003; Breck 2004; Breck et al. 2005a; Breck et al. 2005b). These studies led to a further optimisation of feed for Atlantic salmon smolt and laid the fundament on which the work of the present thesis is built.

3.4 His and His-related compounds: chemistry and possible functions in the lens

In contrast to most mammalians, His is an essential amino acid for fish (Espe et al. 2001), meaning that fish cannot synthesise His *de novo*. Thus, His has to be supplied by diet intake. A minimum concentration of 7 g His per kg diet is currently recommended to cover the requirement of Atlantic salmon (NRC 1993). However,

several studies have shown a cataract preventative or cataract mitigating effect of dietary His above the suggested minimum requirement in Atlantic salmon smolt (Breck et al. 2003; Bjerkås and Sveier 2004; Breck 2004; Breck et al. 2005a; Breck et al. 2005b). The His-derivative NAH is a major component of the free amino acid pool of the Atlantic salmon lens. The lens concentrations of NAH have been shown to reflect dietary His levels, and NAH has thus been established as a lens-specific marker for the His status of Atlantic salmon (Breck et al. 2005a; Breck et al. 2005b). A role of NAH as an osmolyte in the Atlantic salmon lens has been proposed (Breck 2004; Breck et al. 2005b).

His is one of the 20 amino acids which function as the monomers that build up peptides and proteins in all living organisms (Nelson and Cox 2000). Amino acids have also a metabolic function and are the main energy source in the liver of carnivorous fish, including Atlantic salmon (Espe et al. 2001). Hence, His may occur in a cell as part of a protein or oligopeptide, or as a free amino acid. In either form it may be modified, e.g. by methylation or acetylation. Modification of free His makes it unavailable for protein synthesis and thus metabolically relatively inert.

Like all other amino acids, the His molecule contains an amino-group, a carboxyl-group and a characteristic side chain attached to the α-C atom. All amino acids except glycine exist in the L or D-form, depending on the position of the side chain on the α-C atom. Only the L-stereoisomers are used in protein synthesis. In the present thesis, including the Papers I-IV, α-L-histidine is commonly referred to as histidine for simplifying reasons. The specific His side chain features a nitrogen containing ring structure, the imidazole ring. The special chemistry of the imidazole ring makes the His molecule and its derivatives unique and versatile compounds, suited to fulfil a number of possible functions in the lens cells. An overview over these possible functions is given the following paragraphs. The molecular structures of His and three His-derivatives found in Atlantic salmon lens and muscle tissue are shown in Figure 2.

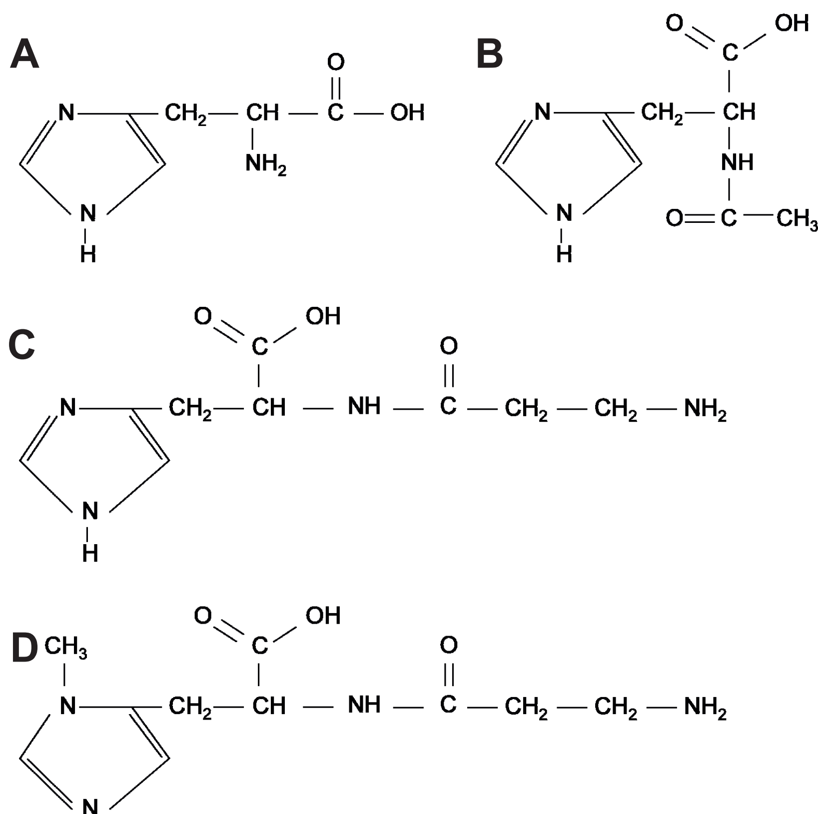

Figure 2. Structural formulas of His (**A**) and the His derived compounds NAH (**B**), Carnosine (**C**) and Anserine (**D**). NAH is lens-specific, while carnosine and anserine are muscle-specific. His is present in both tissues.

His is the only amino acid with an ionisable side chain with a pK_a near the neutral pH (pH 7). Because of the ability to serve as a proton-donor/acceptor at neutral pH, His residues are often found in the reactive centres of enzymes (Nelson and Cox 2000). The imidazole ring is one of the strongest bases existing at pH 7. Since the intra- and intercellular fluids of most animal tissues have a neutral pH, His constitutes an effective physiological buffer. His-related compounds are the major buffering compounds in the skeletal muscle of fish (Abe et al. 1985) and vertebrates (Abe 2000). His and the His-derivative NAH (Figure 2 B) are present in the free amino acid pool of the Atlantic salmon lens. The buffering capacities of lenses

containing different concentrations of His and NAH have recently been tested. In lenses with a higher NAH concentration, the buffering capacity was found to be slightly higher than in lenses with a lower NAH concentration, although the difference was not significant (Breck et al. 2005a).

His-derived dipeptides like carnosine (Figure 2 C), homocarnosine and anserine (Figure 2 D), that occur at high concentrations in muscle and brain of many vertebrates, have been reported to have antioxidant activity (Boldyrev et al. 1988; Kohen et al. 1988; Aruoma et al. 1989). The potential of carnosine-related β-dipeptides to bind prooxidative metal ions, to quench a number of reactive free radicals and to bind to hydroperoxides or aldehydes, including dialdehyde lipid peroxidation products, has been confirmed by Babizhayev (1989; 2006). The antioxidant capacity of N-acetylcarnosine, especially protecting lens membrane lipids from lipid peroxidation and lens proteins from non-enzymatic cross-linking glycation reactions, is utilised in eye drops aimed at non-surgical cataract prevention and treatment in humans (Babizhayev et al. 2004).

Carbonyl groups derive from direct oxidation of amino acid side chains by reactive oxygen species (ROS), the reaction of lipid peroxidation products with basic side chains, or glycation phenomena, all of them being age-related processes and known to be involved in cataract formation. The binding of carnosine to carbonylated proteins prevented cross-linking reactions with primarily unaffected proteins, which had an anti-ageing effect on cultured cells (Hipkiss 2000; Hipkiss et al. 2001). The anti-crosslinking activity of His was even higher than carnosine, while NAH showed no anti-crosslinking activity (Hobart et al. 2004). Other possible functions of His and His-related compounds in the lens include osmoregulation (Baslow 1998) and anti-inflammation (Wade and Tucker 1998).

3.5 Plant feed ingredients and cataracts in farmed Atlantic salmon

Aquaculture is a worldwide growing industry and is actually growing more rapidly than any other animal food-producing sector (FAO 2009). This leads to a steady increase in the amounts of feed raw materials that are needed to produce adequate diets to feed the farmed fish. Atlantic salmon is a carnivorous fish species, and traditionally farmed Atlantic salmon are fed diets based on ingredients of marine animal origin, like fish meal and fish oil (Tacon and Metian 2008). Currently, close to 85% of the entire fish oil production is consumed by the aquaculture industry and more than half of this is used for salmonid feeds alone (FAO 2009). Due to stagnating capture fisheries production the resources for marine ingredients are limited and will not be sufficient to cover the increased demand in the future. Fish meal and fish oil prices have increased in 2008 and are expected to remain high (FAO 2009). Plant ingredients are attractive alternatives to fish meal and fish oil, since they are readily available and the costs are relatively low (Torstensen et al. 2008; Turchini et al. 2009).

Several feeding studies have addressed the effects of plant feed ingredients on growth performance, tissue composition and fish welfare. The dietary lipid composition has been shown to affect the lipid class and fatty acid composition in various salmon tissues (Waagbø et al. 1991; Torstensen et al. 2000; Bell et al. 2001; Torstensen et al. 2001; Bell et al. 2002). The effect of dietary vegetable oils on cataractogenesis in Atlantic salmon was investigated in two long-term feeding studies in Norway and Scotland, as parts of the RAFOA (Researching Alternatives to Fish Oils in Aquaculture, Q5RS-2000-30058, http://www.rafoa.stir.ac.uk/) project (Waagbø et al. 2004). In these studies, Atlantic salmon were fed fish meal based diets with 75-100% fish oil replacement by a common vegetable oil blend (30% palm oil, 15% linseed oil and 55% rapeseed oil) from start feeding to slaughter. Cataract screenings revealed high cataract prevalence in both groups, whereas the severity was considerably and significantly higher in fish fed the vegetable oil diet. Further investigations of the

effects of plant feed ingredients on lens composition, lens function and cataractogenesis as measures of fish welfare are needed to ensure the safe use of plant feed ingredients in aquaculture.

3.6 Principles of osmoregulation in Atlantic salmon

Osmosis is the diffusion of water through a semi-permeable membrane from an area with low solute concentration to an area with high solute concentration. The cell membrane is relatively permeable to water, but not to larger biomolecules and ions. Thus, cells in a hyperosmotic environment will release water and shrink, and cells in a hypoosmotic environment will take up water and swell. To prevent cell damage caused by shrinking or swelling, cells actively regulate their volume back to the initial state. Upon hypoosmotic stress, a regulated volume decrease (RVD) occurs. In RVD, there is an efflux of both inorganic ions and osmolytes to the extracellular environment, which leads to the concomitant loss of water and return to the normal cell volume. Regulated volume increase (RVI) occurs upon hyperosmotic stress and involves the fast uptake of ions that are subsequently slowly replaced by compatible osmolytes (Kwon and Handler 1995; McManus et al. 1995).

Many organisms use osmolytes to control the cell volume. Osmolytes are small soluble organic molecules whose concentrations are up-regulated or down-regulated to adjust the osmolality in the cell. They are often called “compatible”, because they do not interact with cytosolic macromolecules in detrimental ways, as would inorganic ions at high concentrations. Several osmolytes might have additional cytoprotective functions, e.g. as antioxidants, in redox balance, in sulphide/sulphate detoxification, or in protein stabilisation (Yancey 2005; Burg and Ferraris 2008). There are five major categories of osmolytes: small carbohydrates (e.g. trehalose), polyols (glycerol, inositol, sorbitol, etc.) and their derivatives, amino acids (glycine, proline, taurine, etc.) and their derivatives (e.g. NAH), methylamines (e.g. N-trimethylamine oxide (TMAO) and glycine betaine) and methylsulphonium solutes including urea (Yancey et al. 1982; Yancey 2005).

Euryhaline species like Atlantic salmon can adapt to a life under different osmotic conditions. Atlantic salmon have an anadromous lifestyle, migrating from freshwater streams to the sea after the early life stages and returning to freshwater after one or several years in the sea to spawn. Before the migration from freshwater to seawater, Atlantic salmon parr pre-adapt to the increased salinity in seawater and become smolt. They undergo complex physiological, morphological and behavioural changes, called parr-smolt transformation or smoltification (McCormick et al. 1998; McCormick 2001).

Atlantic salmon are osmoregulators, i.e. they aim to keep their body fluids osmolalities constant, in contrast to osmoconformers, which maintain about the same osmotic pressure as their environment (Yancey 2005). The main organs to control the osmotic balance in Atlantic salmon are the gut, skin and gills, as these are the major contact sites to the aquatic environment. In freshwater, specialised cells of the gut and the gills function to take up and retain ions, while in saltwater the opposite is the case and cells actively secrete ions to keep the osmotic balance in the organism. The plasma osmolality in Atlantic salmon is slightly higher in seawater-adapted compared to freshwater-adapted salmon (Iwata et al. 1987; Hansen 1998). The osmolyte glycine betaine (in the present thesis called betaine for simplifying reasons) can be added to the diet during smoltification to increase seawater-tolerance in Atlantic salmon smolts (Virtanen et al. 1989). The impact of osmotic stress on cataract development in Atlantic salmon is detailed in section 3.3. The His-derivative NAH has been suggested to function as an osmolyte in the lens of goldfish (*Carassius auratus*) (Baslow 1998) and Atlantic salmon (Breck 2004; Breck et al. 2005b).

3.7 The stress response in fish

Intensive fish farming implies a number of possible stressors to the fish, which may predispose them to compromised growth and health, and may promote disease. Therefore, a considerable amount of work has been done to investigate the potential stressors and the stress responses in fish (Iwama et al. 1997). The results of this

research are highly important - not only in aquaculture, but also for the experimental biologist working with fish - and help to distinguish if experimental observations are mainly caused by the primary objective of the experiment or by responses to accompanying stress. This is crucial to ensure reproducible and reliable research, as well as ethical handling of the fish as research animals.

The stress response in fish can be divided into primary, secondary and tertiary responses (Mazeaud et al. 1977; Wedemeyer et al. 1990; Barton 2002). The primary response consists of relatively fast endocrine changes, including temporary increases in plasma catecholamines (adrenaline and noradrenaline) through the adrenergic response and in plasma cortisol through the hypothalamo-pituitary-interrenal (HPI) response. To the secondary responses belong metabolic changes (like hyperglycaemia and changes in plasma free fatty acids), haematological changes (like changes in blood haemoglobin, red and white blood cell numbers and hematocrit) and changes in plasma ion and acid-base-balance. Primary and secondary responses are tissue-specific, while tertiary responses, such as changes in the growth performance, reproduction rate and disease resistance affect the whole body (Iwama et al. 1997). In addition to this stress response affecting the whole organism, a stress response exists at the cellular level. The cellular stress response includes the increased expression of stress proteins, such as certain members of the family of heat shock proteins (HSPs) (Iwama et al. 1998; Iwama et al. 1999). HSPs are expressed in virtually all studied organisms and have highly conserved sequences. These unique characteristics have made HSPs popular and widely used molecular markers for responses to different stressors in various tissues and organisms (Iwama et al. 1998).

4. Methodological considerations

The analytical methods and experimental procedures applied in the present thesis are described in the respective papers (Paper I-IV). Challenges, cautions, adaptations and validity of selected methods and models are discussed in more detail in the following sections.

4.1 Fish trials

The lenses analysed for Paper I were sampled after a short-time (2 hours) experiment where different sampling procedures were applied to groups of six Atlantic salmon to investigate possible effects on lens gene expression. All sampled fish came from the same batch of smolts obtained from the Institute of Marine Research's (IMR) research station at Matre (Hordaland County, Norway) in April 2006. The fish had been reared in a 1500 litre outdoor tank at the IMR in Bergen, Norway, until sampling at the end of June 2006. The actual experiment was carried out in smaller (80 litre) tanks without replicates. The use of replicate tanks and nested ANOVA statistics is usually recommended for aquaculture studies, to control any additional variation caused by tank-specific environmental factors or by hierarchical feeding that may appear in fish feeding trials (Ruohonen 1998; Ling and Cotter 2003). In the present study, however, feeding was not an experimental factor and due to the short duration of the experiment, tank-specific variations were considered to have much less effect than the experimental treatment.

Applying the findings of Paper I, fish sampled for Paper II, III and IV were anaesthetised before killing and the time from anaesthesia to dissection of lenses and other tissues was kept as short as possible, not exceeding 30 minutes. The lens samples were flash-frozen in liquid nitrogen or by placing them on dry ice.

A feeding trial to demonstrate the cataract preventative effect of dietary His regimes in adult Atlantic salmon growers was conducted in sea net pens at Skretting ARC's research station at Lerang (Rogaland County, Norway) from June to October 2006. Lerang research station was chosen due to previous cataract observations at this sea site. Adult Atlantic salmon growers in their second year in sea water were fed commercial-like diets with or without His supplementation [low-His diet (L), medium-His diet (M) and high-His diet (H)] in duplicates. Since low dietary His concentrations alone might not have been sufficient to provoke cataract formation, the experimental period was laid to the warmer summer months, as high water temperatures and increased growth have been proposed as risk factors for cataract formation (Breck and Sveier 2001). The cross-over design of the feeding trial (Paper II) allowed the investigation of time-dependent effects of varying dietary His levels relative to possible cataract formation. Lens and muscle tissue samples for the evaluation of His nutrition (Paper II) and the investigation of the molecular aspects of His-related cataracts (Paper III) were obtained in July, September and October.

Another feeding trial from which lenses for Paper IV were obtained was carried out as a part of the IP-EU project "Aquamax" (016249-2; http://www.aquamaxip.eu) at IMR's research station at Matre (Hordaland County, Norway) from June 2006 until June 2007. Atlantic salmon growers were reared in triplicate tanks and received one of four diets varying in the content of marine and vegetable lipid and protein sources (FMFO: 100% fish meal and 100% fish oil, 80PP35VO: 80% plant protein and 35% vegetable oil blend, 40PP70VO: 40% plant protein and 70% vegetable oil blend, and 80PP70VO: 80% plant protein and 70% vegetable oil blend). For simplifying reasons, only the marine diet (FMFO) and the most extremely substituted diet (80PP70VO) are presented and discussed in Paper IV, as diet M and P, respectively. However, the same analyses as in Paper IV have also been carried out for the intermediate dietary groups. These groups are briefly referred to in the discussion of Paper IV and will be dealt with in more detail in the general discussion, to support the results of Paper IV and to try to clarify whether impacts of the diets originated from the protein or lipid ingredients.

For paper I and IV, lenses were dissected from fish also sampled for other purposes. This “sharing” of sampled fish between different projects is an important measure to reduce the number of animals treated - and killed - for research purposes. Reduction is one of the “three Rs”, a main principle in modern laboratory animal science introduced by Russell and Burch (1959). The three Rs stand for **R**eplacement, **R**eduction and **R**efinement. Russell and Burch (1959) prompted the researchers to find alternatives for their animal research, by replacing the use of laboratory animals by other techniques that give the same or similar answers (e.g. applying cell culture methods), by reducing the number of research animals used for an experiment (e.g. by choosing appropriate experimental designs and statistical tests) and by refining the experimental procedures and protocols to reduce stress and suffering for the research animals.

4.2 Assessment of the cataract status in lenses

Many classification systems exist for application in clinical practice and in etiological and intervention studies (Brown and Bron 1996). In the present thesis, cataract status of the lenses was diagnosed by slit lamp biomicroscope inspection and a simple scoring system as described by Wall and Bjerkås (1999) was applied. The cataract score per individual lens was assessed on a scale from zero (clear lens) to four (completely opaque lens), summing up to a possible maximum score of eight per fish (Figure 3). This scoring system does not give morphological information or information about the localisation of the observed opacifications, and such additional information had thus to be noted separately. Since the scoring system is relatively easy to apply and only requires darkened conditions and a portable slit lamp biomicroscope, it has proven to be the method of choice for eye and lens screenings of fish in aquaculture research. Scheimpflug photography has been suggested in addition to slit lamp biomicroscope inspection in farmed fish to reveal causative factors for the observed cataracts (Wegener et al. 2001). In the experiments presented in this thesis, however, this method was not applied since the additional gain of

information was considered not sufficient to legitimate the complication of the experiments by the sophisticated instrumentation.

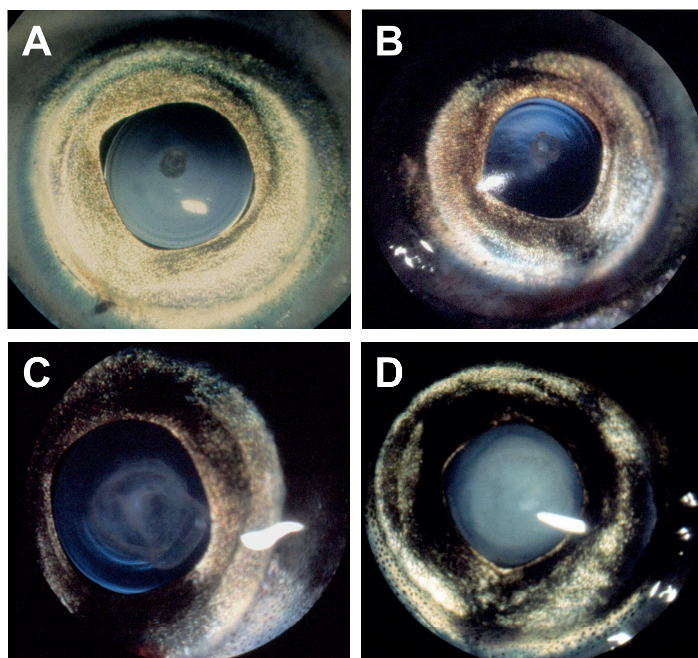

Figure 3. Atlantic salmon lenses with cataracts of different severity, graded after the score system of Wall et al. (1999). (**A**) Score 1. (**B**) Score 2. (**C**) Score 3. (**D**) Score 4. Photographs from Bjerkås et al. (2006), with permission.

4.3 Considerations on the quality of isolated RNA

High quality RNA preparations are of major importance to ensure reliable results of gene expression analysis methods (Bustin and Nolan 2004; Imbeaud et al. 2005; Perez-Novo et al. 2005; Fleige and Pfaffl 2006). The quality of an RNA preparation has traditionally been determined by the ratio between the 28S rRNA band and the 18S rRNA band after gel electrophoresis. Today, microcapillary electrophoresis has become a material-saving alternative and new methods to assess RNA quality have emerged (Imbeaud et al. 2005; Copois et al. 2007). In the present thesis, the quality of RNA samples was judged by the RNA integrity number (RIN) calculated by the

Agilent 2100 Bioanalyzer software (Schroeder et al. 2006). The RIN of a sample can range from 1 (completely degraded RNA) to 10 (high quality RNA). The quality of RNA isolated from different Atlantic salmon tissues can be affected by the method of tissue storage (Olsvik et al. 2007).

The possible effects of lens tissue preservation in liquid nitrogen or RNA*later* on the quality of RNA isolated from Atlantic salmon lens were investigated in Paper I. No significant difference between the methods was found. However, the preservation of lenses in liquid nitrogen instead of RNA*later* was recommended, since the frozen lenses were easier to handle during the RNA purification procedure. High RNA quality (RIN between 8.0 and 9.4) was obtained applying an adapted version of the purification method commonly used at NIFES at that time-point. The results obtained in Paper I were applied in the following gene expression analysis experiments in Paper III and IV.

In the His-feeding trial on which the microarray experiment in Paper III is based, a massive cataract outbreak was observed between July and September. It was originally planned to compare fish that had received high or medium-His feed at two time points, the July-sampling before the cataract outbreak and the September-sampling as an acute stage of cataractogenesis. However, due to problems during the RNA purification (probably overheating of the samples during the homogenisation step) these samples did not meet the quality demands of a RIN ≥ 8 and could thus not be used for microarray analysis.

Figure 4 shows a typical electropherogramme of a high-quality RNA sample (Figure 4A), one of the impaired samples (Figure 4B), as well as a heavily degraded RNA sample obtained from another experiment (Figure 4C). Basically, the impaired sample in Figure 4B lacks the 28S peak, while the baseline remains similarly low as in the high-quality sample in Figure 4A. A raised baseline preceding the 18S peak, as in the heavily degraded sample in Figure 4C, indicates low molecular degradation products. Due to the lacking 28S peak in the impaired sample in Figure 4B, the RIN could not be calculated; however, the impaired samples did not display the typical

pattern of random degradation observed in Figure 4C. Salt contaminations may lead to the observation of lacking 28S peaks in total RNA samples as in Figure 4B. In the present case, however, the samples have been cleaned by an extra column-based step in the purification protocol (Paper III), which virtually excludes salt contamination as the cause of our observations. Since the impaired samples did not meet the quality demands for a microarray experiment, we had to select alternative samples from the cross-over feeding groups sampled in September and from the October-sampling. However, from a preliminary experiment there is evidence that the rejected samples are suitable for qRT-PCR analysis (data not shown).

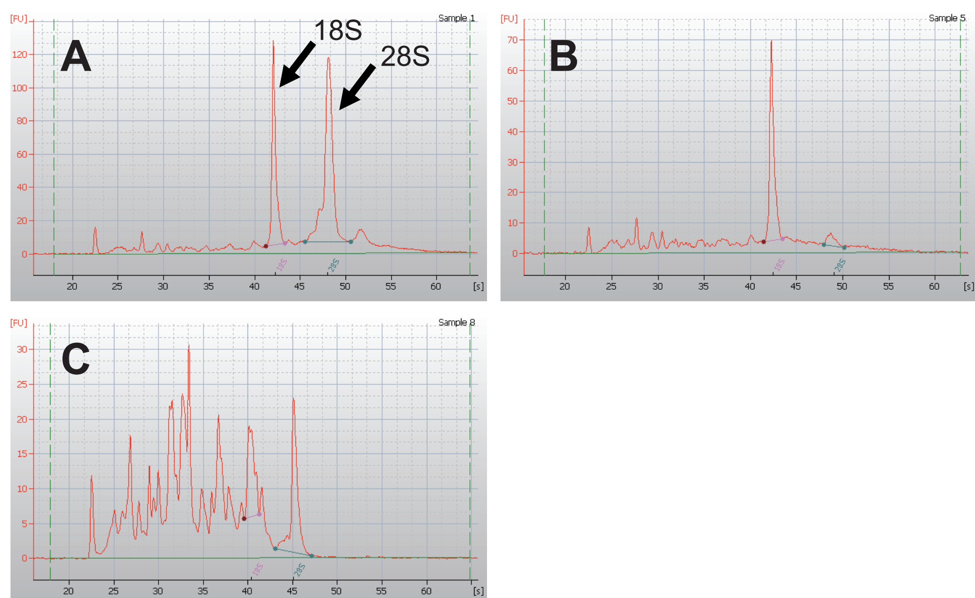

Figure 4. Electropherogrammes of Atlantic salmon total RNA samples produced on an Agilent 2100 Bioanalyzer. (**A**) High-quality lens total RNA sample used in the microarray study, displaying an integrity number (RIN) of 9.0. The 18S and 28S peaks are marked with arrows. (**B**) Lens total RNA sample not used in the microarray study. The RIN was not applicable due to lack of the 28S peak. (**C**) Heavily degraded total RNA sample from another experiment, with a RIN of 5.4.

4.4 Gene expression analysis

Two methods were used to analyse gene expression in the present thesis: qRT-PCR (Paper I, Paper III and Paper IV) and cDNA microarray (Paper III), which are discussed in separate subsections below. Both methods share, however, some potential pitfalls that will be dealt with firstly.

The results obtained by single qRT-PCR and microarray experiments are like snapshots of the transcriptome in the assayed cells or tissues. This is valuable information; however, no answers about mRNA transcription rates, mRNA turnover, protein synthesis, or post-translational modifications and thus protein activity are given. The regulation of gene expression induced by experimental treatment is in most cases time-dependent and different genes may be regulated in different ways. Time course experiments are often the best way to find peak expression levels of a regulated gene, but were beyond the scope of the present thesis.

The term "nutrigenomics" has been used to describe the study of the effects of nutrition on gene expression by high-throughput analytical methods (Müller and Kersten 2003). Ideally, nutrigenomics studies, as any other gene expression studies, should be complemented by studies on the expression levels of proteins ("proteomics") and metabolites ("metabolomics"), to assess the whole spectra of responses to nutrients in cells, tissues, or whole organisms (Müller and Kersten 2003). In the present thesis, gene expression data were combined with results of biochemical analyses (Paper III and IV) and with physiological responses to experimental treatment (Paper IV) to mutually increase the reliability of the results and explore the response mechanisms. Small fold changes are frequently reported in nutrigenomics studies (Jordal 2006; Garosi et al. 2007) and thus a high number of biological replicates were analysed in the experiments presented in this thesis to secure sufficient statistical power to draw reliable conclusions.

In all gene expression analyses presented in this thesis, RNA was isolated from whole lenses. Studies on human cataracts often focus on the lens epithelium alone

(Kantorow et al. 1998b; Zhang et al. 2002; Hawse et al. 2003; Ruotolo et al. 2003). Lens epithelial cells and lens fibre cells have partly different functions in the lens. Thus, clearer results could possibly have been obtained if only epithelial cells or only fibre cells had been used for gene expression analysis. However, to separate the two types of lens cells would have increased the need of biological material since samples from many lenses had to be pooled to ensure high enough yields of purified RNA to run the desired analyses. Besides the resulting need for more experimental animals, sample pooling might have masked differentially expressed genes. In addition, sample pooling would have made it impossible to correlate individual gene expression data to other analysed parameters, such as individual cataract scores, as applied in Paper III. The dissection of lens epithelial cells and fibre cells had also made the experiments more time-consuming and cost-intensive.

4.4.1 Microarray analysis

Microarrays are powerful tools to screen for expression levels of a large number of genes - up to hundreds of thousands - at the same time, thus producing great amounts of individual gene expression data. Microarray analysis is a complex, multistep technique with many sources for variation, both systematic (technical) and non-systematic (biological). To gain meaningful results, experiments have to be carefully planned, standardised protocols used, and correct data normalisation and analysis methods applied (Quackenbush 2001; Imbeaud and Auffray 2005; Jaluria et al. 2007). The experimental design, data normalisation and data analysis for Paper III were thus carried out in collaboration with specialists from the Computational Biology Unit (CBU), Bergen Center for Computational Science (BCCS), at the University of Bergen. Sample labelling and microarray hybridisations were done at the Norwegian Microarray Consortium (NMC) national technology platform in Trondheim, supported by the Functional Genomics Program (FUGE) in the Research Council of Norway (NRC). The Minimum Information About a Microarray Experiment (MIAME) guidelines (Brazma et al. 2001) were followed.

In the present study, the GRASP 16K salmonid microarray was used. This salmonid cDNA microarray was designed to represent a substantial part of the whole transcriptome (von Schalburg et al. 2005; http://web.uvic.ca/grasp). The expressed sequence tags (ESTs) printed on the array originated from a variety of tissues at different developmental stages from Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*), both belonging to the family of Salmonidae and thus displaying high DNA sequence similarity. Samples from several teleost species distantly related to salmonids have been shown to hybridise with the array (von Schalburg et al. 2005). However, since no lens-specific EST library was among the libraries used for its production, the array is very likely not representative for the entire lens transcriptome. Thus, specific genes or isoforms of genes exclusively expressed in the lens - with a potential role in His-related cataract formation - may not be represented among the sequences printed on the array. As a result, the expression patterns of these missing lens-specific genes could not be detected in the microarray study presented in Paper III. One of these potentially interesting genes was aquaporin 0, the major lens aquaporin, which is expressed in the fibre cells and has water transport and structural functions. Mutations in aquaporin 0 have been shown to cause congenital cataracts in human and mice (Verkman et al. 2008).

It is known that technical variation caused by sample processing by different persons or at different time points, as well as the order of sample processing, can influence the results of microarray experiments significantly. In the present study, the various steps of the sample processing were thus carried out by the same person and with randomised sample order. For each processing step, all samples were processed at the same time; and when this was not possible, the samples were divided into batches containing equal numbers of samples of each dietary group in randomised order.

Several of the purchased array slides used in this study bore pronounced fingerprint-like marks at arrival (Figure 5). These marks appeared as flaws about 2-4 pixels in size in the image analysis and are presumably caused by salts of a buffer solution that

evaporated on the slides. The affected spots have been “flagged out” and thus removed from the analysis, to ascertain that the results of the study were not affected.

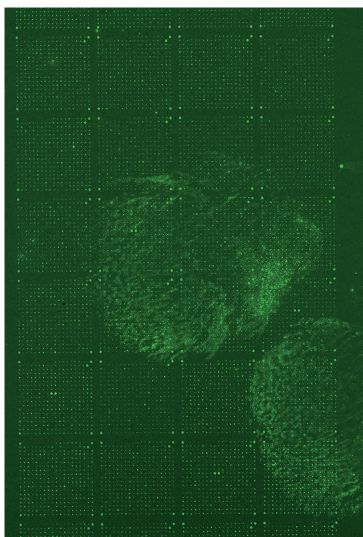

Figure 5. “Fingerprints” on one of the microarray slides scanned before hybridisation.

The differences in gene expression levels between two experimental groups are often presented as fold changes (i.e. the ratios between the two groups). In the past, it has been a common practice in microarray analysis to set arbitrary fold change cut-off values, often at two-fold to four-fold, below which the effect of differential expression was considered not significant (Quackenbush 2002). If such a practice had been applied in the present study, the list of differentially expressed transcripts would have been shortened from several hundreds to less than ten. Many potentially interesting transcripts would have been lost from the analysis. The fold change alone should not be used as a measure of biological significance for differentially expressed genes.

A considerable number of the ESTs on the 16 K salmonid microarray are redundant, i.e. coding for the same transcript, but representing different parts of the sequence of this transcript. These ESTs will thus be annotated with the same gene name. In microarray data analysis, one can choose to treat these ESTs as separate transcripts, or combine the expression data from several ESTs annotated with the same gene name to a new average value for this gene ("collapsed dataset"). This has implications on the results of cluster analyses and enrichment analyses, where artificially low false discovery rates (FDR) will be detected in uncollapsed datasets. However, due to genome duplication, many genes in Atlantic salmon exist in several isoforms and it is often impossible to determine whether small sequence differences between ESTs are due to experimental variance (like sequencing errors), differences between individual fish, or actually represent different isoforms of a gene. In order not to lose potentially important information on different isoforms, as discussed by (Olsvik et al. 2008), we decided to analyse uncollapsed data in Paper III.

Gene ontology (GO; http://www.geneontology.org) terms can be applied to describe the molecular function, biological process and cellular component of a gene product in a standardised way (Ashburner et al. 2000; Harris et al. 2004). In the present study, the transcripts on the array were functionally annotated with GO terms. This functional annotation is deposited in a so called mapping file. Gene set enrichment analysis (GSEA) is a powerful tool to detect functional clusters of genes that are differentially expressed, but where the individual members of the cluster may not be recognised significantly differentially expressed due to variations in expression levels and low fold changes (Subramanian et al. 2005). In the present study, GSEA was applied to the dataset using both the GO-mapping file provided by the array producer and a GO-mapping file created using the Blast2GO platform (Conesa et al. 2005; http://www.blast2go.de). However, both approaches produced results of small biological relevance and have thus not been included in Paper III.

The limitations in data analysis caused by incomplete annotation of the EST clones on the array are discussed in Paper III. Briefly, about 25% of the transcripts in the

dataset were unknown sequences. In addition, about one third of the known transcripts lacked a functional annotation. Thus the functional enrichment analysis results could not give a complete view of the functional categories enriched in the dataset. New sequence information is continuously created and this progress in sequence identification and annotation will eventually fill the gaps that currently exist in the dataset.

Finally, it should be underlined that the experiment presented in Paper III was a screening study. Microarray analysis was used as a "hypothesis generator" and further investigation is needed to fully explore the findings of this study.

4.4.2 qRT-PCR analysis

Two types of quantification are currently common in qRT-PCR analysis, nonspecific detection using DNA-intercalating dyes, e.g. SYBR Green, and specific detection by template-specific fluorescent probes (Bustin and Nolan 2004). Fluorescent probes ensure higher specificity than the unspecific DNA-binding dyes, but increase the costs for an experiment. In the present thesis, SYBR Green was applied for qRT-PCR analysis (Paper I, III and IV). The expected sizes of the respective PCR products were confirmed by DNA melt curve analysis, as recommended by Bustin and Nolan (2004). A further improvement, however beyond the scope of the present thesis, would have been to sequence the obtained PCR products, to confirm that the expected sequences had been amplified.

Differences in the amplification efficiency may lead to different results when samples are assayed for the same gene in separate qRT-PCR runs. Thus, for direct comparison of samples analysed on different plates, the expression values obtained from both plates have to be "calibrated" via one or more common samples. This process is called interplate calibration (Kubista and Sindelka 2007). Interplate calibration was not needed in the experiments described in Paper I, III and IV, where all samples could be assayed for a certain gene on one plate. However, for the present thesis, the samples of the two additional dietary groups not included in Paper IV have been

analysed on a separate plate. Interplate calibration was thus necessary to be able to compare these additional results with the two main dietary groups. The same standard curve of pooled samples was run on both plates and interplate calibration was done as described by Kubista and Sindelka (2007).

Proper normalisation of qRT-PCR data is crucial to obtain reliable results (Vandesompele et al. 2002; Bustin and Nolan 2004). In all qRT-PCR experiments presented in this thesis, the best reference genes have been selected from a pool of tested genes applying the *geNorm* VBA applet for Microsoft Excel version 3.4 (Vandesompele et al. 2002; http://medgen.ugent.be/~jvdesomp/genorm). Two to three reference genes have been used to normalise the data in the various experiments.

Recently, an expert group of leading qRT-PCR specialists has proposed guidelines for the minimum information that should be enclosed when qRT-PCR experiments are published. These guidelines, called “The Minimum Information for Publication of Quantitative Real-Time PCR Experiments” (MIQE), will help the reader to evaluate the quality of the results and to reproduce the results (Bustin et al. 2009). To enable a straightforward and transparent data exchange between qRT-PCR instruments, different data analysis software, collaborating colleagues and any other interested researchers, the XML-based “Real-Time PCR Data Markup Language” (RDML) has been introduced by the RDML consortium (Lefever et al. 2009; http://www.rdml.org). Most of the essential points on the MIQE checklist are described in the traditional way in the materials and methods section in Paper I, III and IV, and RDML was not used. However, the MIQE guidelines and the use of RDML will presumably be integrated into the standard procedures at NIFES in the near future.

4.5 *Ex vivo* whole-organ culture of lenses

Organ culture of lenses is a useful tool in cataract research, since it permits the study of lens responses to various defined treatments, such as H_2O_2-induced oxidative stress (Spector 1995), lipid peroxidation products (Zigler et al. 1983), hypolipidemic drugs (De Vries and Cohen 1993), UV-A radiation (Dovrat and Weinreb 1999) and Ca^{2+}-overload induced by a Ca^{2+} ionophore (Sanderson et al. 2000). An *ex vivo* whole-organ culture model of Atlantic salmon lenses has been established in co-operation with the University of East Anglia (UEA) (Breck 2004). The lenses were cultured in a standard serum-free cell culture medium, Eagle's Minimum Essential Medium (EMEM). Lenses cultured for over four weeks maintained their transparency, and the lens membrane voltage and protein synthesis rate remained relatively constant over two weeks, proving the functioning of the culture model (Breck 2004).

The same *ex vivo* culture model was applied on Atlantic salmon lenses from fish fed a plant-based or a marine control diet in the study presented in Paper IV. In this study, the lenses were subjected to hypoosmotic or hyperosmotic culture media for four successive days. The osmolalities for the hypoosmotic (237 mOsm/kg) and hyperosmotic (399 mOsm/kg) medium were chosen in a range that might be expected to occur upon osmotic challenge *in vivo*, whereas in previous studies, a wider range of osmolalities including more extreme values was applied (Breck 2004). The osmolality of the control medium (321 mOsm/kg) used in Paper IV was chosen based on previous measurements of aqueous humour osmolality in seawater-acclimated Atlantic salmon smolt that ranged from 320 to 324 mOsm/kg (Breck et al. 2005a). To ensure an adequate osmolality of the control medium in future experiments applying *ex vivo* lens whole-organ culture, the measurement of aqueous humour osmolality in fish that are to be used as lens donors could be included as a routine.

The cultured lenses were imaged approximately every 24 hours, prior to medium exchange. In contrast to the previous studies carried out at UEA (Breck 2004), the apparatus used at NIFES to image the cultured lenses was improvised (Figure 6 A). A LED ringlight placed under the object table was used as the light source and the

pictures were taken under darkened conditions with a digital camera with constant focus and aperture. Lens sizes were calculated from three measurements of the lens diameters relative to the opening for the light source in the object table (known constant diameter of 13 mm) on the digital photos (Figure 6 B). The digital images of the lenses did not allow for quantification of opacities using appropriate software, which would have added an additional parameter to the analysis. However, opacities of the cultured lenses were assessed on the last day of culture using a slit lamp biomicroscope and applying the scoring system described in paragraph 4.2. This approach made it possible to directly compare the obtained values to *in vivo* field measurements.

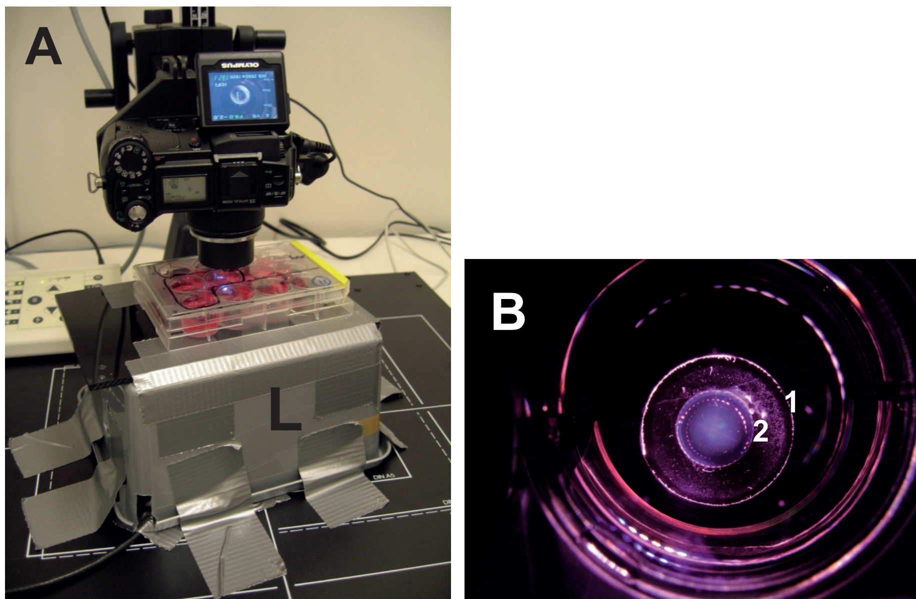

Figure 6. (**A**) Experimental setup for lens imaging applied in Paper IV. The light source is located under the covering marked (L) on the image. Photo: Rune Waagbø. (**B**) Example for one of the images of the cultured lenses taken daily for Paper IV. Several rings can be seen on the image. While the outer rings are reflections from the walls and the lid of the cell culture plate, the inner ring marked (1) on the image is the opening for the light source in the object table. The cultured lens is marked (2) on the image. The bright spots on the lens are reflections of the LED ring light.

4.6 Statistics

The statistics connected to microarray analysis are described in a separate section in Paper III. The statistical tests applied to other measured parameters are stated in the statistics section in the respective papers. Parametric methods were applied whenever possible. If the underlying assumptions for parametric testing were not fulfilled, i.e. the measured values in a group were not normally distributed and/or the variances were significantly different between the groups, non-parametric equivalent tests were applied. Non-parametric methods were used to test cataract scores and gene expression levels in lenses of different experimental groups. Non-parametric methods have usually less power than their parametric equivalents, but are less restrictive about the format of the data (Zar 2009).

5. General discussion

The general discussion will focus on the main findings presented in Paper I-IV, and discuss them in relation to the overall aims of this thesis. The effects of dietary His on tissue composition and cataract formation in adult Atlantic salmon growers are discussed first, followed by the discussion of the alteration of lens gene expression by sampling stress and by His-related cataract formation. Furthermore, the impact of plant feed ingredients on lens osmoregulation and cataract formation are discussed. Finally, speculative considerations about the mechanisms behind His-related cataracts in Atlantic salmon are made.

5.1 Effects of dietary His on tissue composition and cataract formation

In previous studies with Atlantic salmon smolt, it has been shown that elevated levels of dietary His were reflected in the concentrations of the major His-derivative in the lens, NAH, and that this prevented or slowed the progression of cataract development (Breck et al. 2003; Breck 2004; Breck et al. 2005a; Breck et al. 2005b). The work in the present thesis aimed to investigate this relationship in adult Atlantic salmon growers. The effects of dietary His levels and His feeding regimes on lens biochemical composition and cataract formation in adult Atlantic salmon growers are presented and discussed in Paper II and partly in Paper III.

Briefly, the results of Paper II confirm the above stated findings to be valid in adult Atlantic salmon growers in their second year in the sea. A major outbreak of cataracts was observed among the fish in the second period of the feeding trial from July until September. The cataract severity in the feeding groups was directly related to the dietary histidine levels fed during the first period from June until July. Feeding the medium- and high-His diets (containing 12.8 and 17.2 g His/kg diet, respectively)

prevented severe cataract formation, while feeding the low-His diet (containing 9.3 g His/kg diet) resulted in significantly higher cataract scores. It should be noted that the His content of the low-His diet is in the range of current standard commercial diets for adult Atlantic salmon growers.

The cross-over design of the feeding experiment allowed us to determine the cataract outcome relative to the period of feeding the different diets. Supplementation of His during the first period from June until July and then switching to a low-His diet was found to be equally efficient in cataract mitigation as His supplementation throughout the whole experimental period. However, switching from the low-His diet during the first period to the medium or high-His diet during the second period still lowered the mean cataract scores in these dietary groups compared to fish fed the low-His diet during the first and second period. Dietary His levels during the third period had only a minor effect on the cataract scores, which stayed nearly constant from September to October in the respective dietary groups. We would thus recommend a diet containing at least 12.8 g His/kg diet before and during phases of expected increased risk to prevent severe cataract formation in adult Atlantic salmon growers in their second year in the sea. Also, once cataract formation is observed in a population of fish due to low dietary His levels, increasing the His level in the diets up to 17.2 g His/kg can mitigate the severity of the developing cataracts.

The levels of dietary His were reflected by lens imidazole concentrations in adult Atlantic salmon growers before visible cataract formation (Paper II and III), as has previously been shown for Atlantic salmon smolt (Breck et al. 2005a; Breck et al. 2005b). Lens NAH concentrations were also negatively correlated to cataract scores in the respective lenses in September and October (Paper II and III). These findings suggest that the NAH concentrations in cataract-free lenses can be used as a marker for the potential of cataract formation in adult Atlantic salmon growers.

Anserine is the major imidazole compound in the white muscle of Atlantic salmon and its concentration in the muscle depends also on the dietary His intake (Breck et al. 2005a). In Paper II it was found that the muscle anserine concentrations in adult

Atlantic salmon growers were less affected by dietary His levels and feeding regimes than lens NAH concentrations. This indicates that His is more easily accessible from the plasma for white muscle tissue than for the lens, or that the imidazole supply to white muscle tissue is prioritised compared to the lens (Paper II).

Cataracts in Atlantic salmon are most likely initiated by an intricate interplay between several nutritional and/or environmental factors (Breck 2004; Bjerkås et al. 2006). Although low His/NAH concentration in the lens has been identified as a risk factor for cataract formation in Atlantic salmon, fish with low His/NAH status do not necessarily develop cataracts, as seen in Paper IV. Also, although mitigating cataract formation, high His/NAH concentrations in the lens can in most cases not totally prevent cataract development (Paper II and III). This indicates the presence of one or more other factors influencing cataract development in Atlantic salmon that are not entirely controllable by lens imidazoles. Water temperature changes are a known risk factor for cataract formation in Atlantic salmon (Wall 1998; Bjerkås et al. 2001). In the His-feeding trial described in Paper II, the rise in water temperature between June and July was probably one of the contributing factors leading to the development of severe cataracts in the fish fed the low-His diet.

As discussed by Breck (2004) for Atlantic salmon smolt, the currently recommended minimum requirement level of 7 g His/kg diet (NRC 1993) is not sufficient to impede cataract formation in adult Atlantic salmon growers in their second year in the sea (Paper II). In the present study, fish fed the low-His diet containing 9.3 g His/kg diet developed severe cataracts. The dietary His levels did not affect the growth rate in adult Atlantic salmon growers, but influenced the susceptibility for cataract formation (Paper II). It is thus clear that growth performance alone is not sufficient to decide if His requirements are met in Atlantic salmon. The term "beyond deficiency requirement" has been introduced to describe the beneficial role of vitamins beyond the prevention of deficiency diseases (Sauberlich and Machlin 1992). Our findings suggest a "beyond deficiency" role of His in cataract prevention in Atlantic salmon, where the practical requirement in phases of increased risk is higher than the dietary

level required for optimal growth. The outcome of the present histidine feeding study may thus be used as a nutritional guideline for cataract prevention in adult Atlantic salmon growers in their second year in sea water in periods of increased risk.

5.2 Effects of sampling stress on lens gene expression

A general question often posed in studies with fish is to what extend experimental results are affected by handling stress, since the organisms stress response might interfere with the response to experimental treatment. There is no entirely stress-free sampling procedure, and thus researchers working with fish have to be aware of possible effects of the cellular stress response on the measured analytical parameters. The effects of different sampling procedures on the expression levels of selected genes in the lens were investigated to sort out confounding effects of sampling regimes on potential cataract-induced changes (Paper I).

The expression level of HSP70 was found to be significantly elevated in lenses from fish sampled after 30 minutes without anaesthesia, compared to control fish immediately killed by a blow to the head; probably as a result of handling stress (Paper I). In a study on the effects of different water oxygenation levels on cataract formation, mRNA expression levels of HSP70 were found to be significantly decreased in lenses of fish exposed to 125% O_2 compared to the normoxic control group (Waagbø et al. 2008). HSP70 is a stress-responsive protein with chaperone function, and increased expression of HSP70 is often used as a biological marker for different stresses (Iwama et al. 1998). However, the understanding of the HSP response in fish is still imperfect (Iwama et al. 2004) and its use as an indicator for hatchery stress has been questioned (Zarate and Bradley 2003).

Antioxidant genes might be applied as markers for cataract formation. The expression levels of the antioxidant genes glutathione reductase (GR) and manganese superoxide dismutase (Mn SOD) were decreased in lenses of the fish that had been treated for two hours with or without anaesthesia (Paper I). qRT-PCR analysis of the same genes

in gills tissue of the same fish revealed similar expression patterns and the gene expression values in lenses and gills of the same fish were found to be significantly correlated (data not shown). This was a surprising finding, since the avascular lens is considered a relatively inert organ due to its isolated location embedded in eye chamber fluids, while the fish gills are known to have a fast cellular response to external stressors and are also involved in the stress response in fish (Wendelaar Bonga 1997). Our findings might thus indicate that the lens is more sensitive to external stresses than assumed, and that the antioxidant response in lenses and gills tissue functions similarly fast. To avoid possible effects of handling stress on lens gene expression, short sampling procedures applying anaesthetics were used when material for Paper II-IV was collected.

5.3 Lens gene expression screening – suggestion of early markers of cataract formation

In Paper III it was shown for the first time that lens gene expression in two groups of Atlantic salmon growers was affected by feeding different dietary His levels, resulting in cataracts of different severity. Among the differentially expressed genes were metallothionein A and B, and transcripts involved in lipid metabolism (lipocalin precursor, fatty acid-binding protein 2 (FABP2), apolipoprotein Eb and clusterin precursor), carbohydrate metabolism (fructose-bisphosphate aldolase B, glyceraldehyde-3-phosphate dehydrogenase, hexokinase-2 and triosephosphate isomerase) and in the regulation of ion homeostasis (Na,K-ATPase α1C) and protein degradation (the regulatory and catalytic subunits of calpain, and cathepsin L and B). The potential role of these transcripts in cataract formation in adult Atlantic salmon growers was discussed in Paper III. Metallothioneins, Na,K-ATPase, calpains and cathepsins have previously been shown to be involved in cataract formation in mammalians (Paper III), suggesting mechanistic parallels in Atlantic salmon and mammalians.

The two groups of Atlantic salmon compared in Paper III had been fed diets containing 9 and 13 g His/kg diet, respectively. A marked increase in cataracts occurred between July and September, leading to significantly higher mean cataract scores in the low-His group compared to the medium-His group. Samples from both groups were taken at the end of the feeding trial in October. Some of the observed differences in gene expression levels might thus have reflected secondary reactions of the lens cells to cataractous changes over a longer period of time, like compensatory or repair mechanisms. However, lens samples taken in September, closer to the acute stage in cataract formation, were also subjected to microarray analysis in the same way as described for the October-sampling in Paper III. Because of the less pronounced differences between the dietary groups sampled in September, and for simplifying reasons, the findings from the September-sampling presented below have not been included in Paper III.

It was originally planned to use fish that had been fed the medium-His or low-His diet for the entire experimental period from start until sampling, but due to problems during the RNA purification (see section 4.3) alternative samples had to be used. These alternative fish had received the high-His or low-His diet from June to July and the medium-His diet from July to September (HM and LM, respectively). Both lens NAH concentrations and cataract status of the two alternative groups were significantly different. However, receiving the medium-His diet during the period of serious cataract formation may have blurred differences in gene expression and might thus partly explain the less pronounced differences found between the dietary groups in the September-sampling compared to the October-sampling.

Applying correspondence analysis (CA) to the September samples (Figure 7 A), the separation of the two dietary groups was not as clear as for the October-sampling (Figure 7 B). Significance analysis of microarrays (SAM) revealed that fewer genes were significantly differentially expressed than in the October-sampling. Only the first eight transcripts in the SAM list had a q-value of 0%. The ninth gene already had a q-value above the 5% significance threshold. For the October sampling, as much as

514 transcripts were found to be significantly differentially expressed (Paper III). The maximum fold changes were 1.6 and -1.6 for the September-sampling, compared to 2.1 and -2.5 for the October-sampling.

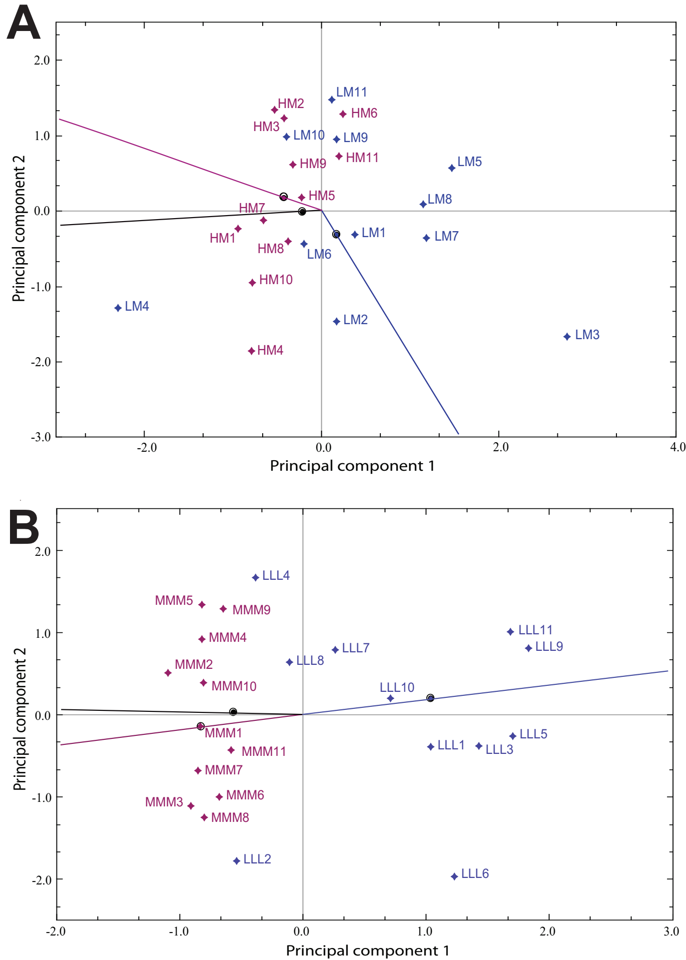

Figure 7. Correspondence analysis (CA) plot of the selected His feeding groups for the September-sampling (**A**) and the October-sampling (**B**). The two principal components explaining the highest variance in the dataset are shown on the x- and y-axis of the plot, respectively. The samples and group medians are coloured according to the dietary groups: low-His: blue, high-His: dark red. The orientation of the lines plotted from the point of origin through the respective group medians indicates the separation of the samples between the two groups.

Appendix 1 shows all 97 transcripts in the SAM list with a q-value of 31% or less. When functional enrichment analysis was applied to the September samples as described in Paper III, fewer functional classes were enriched in the SAM top list than for the October-sampling (data not shown).

Several transcripts showed the same expression patterns in the compared groups from the September-sampling and the October-sampling. Among these transcripts were FABP2, plasma retinol-binding protein II, lipocalin precursor, transaldolase, SPARC (secreted protein acidic and rich in cysteine) and the catalytic subunit of Calpain-2. FABP2, plasma retinol-binding protein II and lipocalin precursor are all lipid-binding proteins. Whether they are involved in lens metabolic processes or have a pure lipid-transporting role related to changes in lens lipid composition due to temperature changes is not clear (Paper III). Calpains have previously been found to be activated in various types of cataracts in rodents and are thought to cleave aggregated lens proteins (Paper III). Transaldolase was down-regulated in low-His lenses both in September and October. Transaldolase is the rate-limiting enzyme in the nonoxidative phase of the pentose phosphate pathway (Heinrich et al. 1976). The pentose phosphate pathway provides ribose-5-phosphate for nucleic acid synthesis and NADPH for lipid biosynthesis and the regeneration of the antioxidant GSH from its oxidised form GSSG. GSH is an important antioxidant in the lens and GSH levels are lowered in cataractous lenses compared to clear lenses (Reddy 1990; Spector 1995; Giblin 2000; Lou 2003). The pentose phosphate pathway has been shown to be stimulated in the rabbit lens in response to oxidative stress (Giblin et al. 1981). Decreased expression of transaldolase has been found to increase the activity of glucose-6-phosphate dehydrogenase, which led to increased GSH levels through stimulating the pentose phosphate pathway and thus protection against oxidative stress (Banki et al. 1996; Banki et al. 1998).

Based on their expression patterns in lenses with different progression in cataract development, selected transcripts were suggested as possible biological markers for early cataract diagnosis in Atlantic salmon (Paper III, Figure 8). As for the October-

sampling, about 10% of the significantly differentially regulated transcripts of the September-sampling were found to be "early" regulated when the same method as in Paper III was applied. The multifunctional extracellular matrix protein SPARC was among the "early" up-regulated transcripts found in the October-sampling that have been proposed as potential molecular marker for early cataract formation in Atlantic salmon (Paper III). The involvement of SPARC in cataract formation has been discussed in Paper III. Briefly, increased levels of SPARC mRNA and protein have been found in human cataractous lenses compared to clear lenses (Kantorow et al. 1998a; Kantorow et al. 2000; Hawse et al. 2003) and the deletion of the SPARC gene in mice leads to cataract development (Gilmour et al. 1998; Norose et al. 1998).

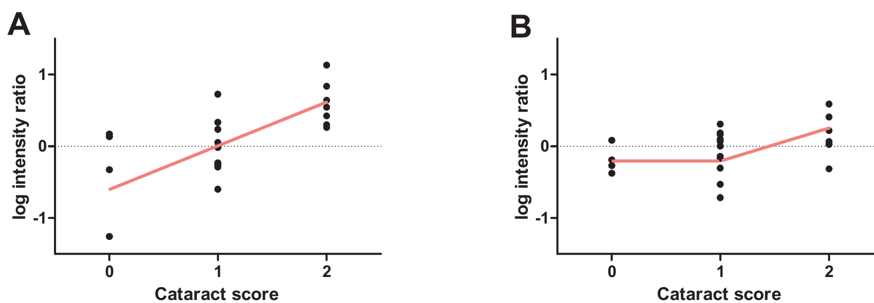

Figure 8. Transcripts with different expression patterns in lenses sampled in September related to cataract scores in the same lenses. The log intensity ratios are plotted against the cataract score of the respective sample, not taking into account which dietary group the samples belong to. The pink line was added to the graphs to help visualise the expression pattern. (**A**) SPARC precursor (SPARC; accession nr. CB488287): "Early" up-regulated. (**B**) Metallothionein A (MT-A; accession nr. CB505763): "Late" up-regulated.

Of the "early" regulated transcripts found in the October-sampling (Paper III), only SPARC was among the most differentially expressed transcripts in the September-sampling. Also here, SPARC was found to be "early" regulated (Appendix 1, Figure 8). This coherence considerably strengthens the potential of SPARC as a molecular marker for early cataract formation in Atlantic salmon. In the lens culture experiment presented in Paper IV and discussed in section 5.5, SPARC expression was found to

be significantly affected by the diet and near to significantly by the osmotic challenge. This additional proof of the regulation of SPARC gene expression by cataract-provoking factors further confirms that SPARC is a strong candidate for early cataract diagnosis by lens gene expression analysis. Table 1 lists “early” regulated transcripts found in the October-sampling (Paper III) and the September-sampling (Appendix 1) that are proposed as potential candidates for molecular markers for early cataract diagnosis in Atlantic salmon.

Table 1. Potential molecular markers for early cataract diagnosis from the datasets obtained from the lenses sampled in September and October. The transcripts were identified by their expression patterns relative to the cataract scores in the respective lenses (method described in Paper III). For the September-sampling, transcripts with a q-value up to 30% were included, while for the October-sampling, only the most significantly differentially expressed transcripts with q=0 were included in this table. A q-value up to 5% was considered significant (Paper III).

Time	Rank	Accession nr.	Transcript name	Fold Change	q-value
September	45	CB488287	SPARC precursor	1.46	18
	58	CB510751	UNKNOWN	1.19	20
	59	CB493904	Endothelin-converting enzyme 1	-1.30	20
	64	CA062809	Polyadenylate-binding protein 4	1.24	21
	66	CA041001	UNKNOWN	-1.17	21
	68	CN442545	Cytochrome c oxidase subunit 3	1.26	21
October	3	CA051877	UNKNOWN	-1.86	0
	4	CB507722	Metallothionein B	1.58	0
	5	CB493454	Lipocalin precursor	-1.67	0
	12	CB509992	Lipocalin precursor	-1.53	0
	15	CA063208	UNKNOWN	-1.95	0
	27	CA059685	UNKNOWN	1.71	0
	29	CA058895	Apolipoprotein Eb precursor	2.11	0
	37	CA041385	Proactivator polypeptide precursor	1.40	0
	38	CA042089	Ependymin precursor	-1.68	0
	49	CB494396	Glycylpeptide N-tetradecanoyltransferase 1	-1.72	0
	64	CB512365	UNKNOWN	-1.45	0
	86	CA057824	Apolipoprotein Eb precursor	1.63	0
	91	CB512134	Myosin light polypeptide 6	1.42	0
	106	CB511219	Gamma crystallin M2	-1.50	0
	108	CA056981	UNKNOWN	-1.37	0
	121	CB492597	Ependymin precursor	-1.41	0
	134	CA037913	UNKNOWN	1.49	0
	141	CA043660	Nuclear receptor 0B2	1.38	0

To prove the functionality of these transcripts as molecular expression markers, they have to be tested in lenses that will develop cataract, but before visible cataract formation is observed. Such experimental material was obtained at the first sampling of the His feeding trial in July. Fish fed the low-His diet until July have been shown to develop more severe cataracts between July and September than the medium and high-His groups (Paper II and III). The lenses sampled from the different dietary groups in July might thus be used to verify the differential expression of possible molecular expression markers in cataractous versus clear lenses by qRT-PCR.

5.4 Interactive effects of plant feed ingredients on lens osmoregulation and cataract formation

Several studies have addressed the possible negative effects of the use of plant feed raw materials in Atlantic salmon (Waagbø et al. 1991; Torstensen et al. 2000; Bell et al. 2001; Torstensen et al. 2001; Bell et al. 2002), but there is only one previous report on the effect of dietary vegetable oils on cataract formation in Atlantic salmon growers (Waagbø et al. 2004). In Paper IV, the effects of plant feed ingredients on lens composition and cataract formation in adult Atlantic salmon growers were investigated.

As a consequence of the different amino acid composition of plant proteins and marine proteins, the His concentration in the diets differed according to the inclusion level of plant proteins. The use of 80%, 40% and 0% plant protein lead to dietary His concentrations of 6.4 to 7.4 g/kg, 9.0 g/kg and 14.3 g/kg, respectively (Torstensen et al. 2008). This in turn led to respective differences in His and NAH concentrations in the lenses of fish of the different dietary groups (Figure 9). Interestingly, the relation between dietary His concentration and lens His and NAH concentrations was not linear. Inclusion of 40% plant protein resulted in lens His and NAH concentrations similar to the marine control group, compared to equally low concentrations in both 80% plant protein groups (Figure 9). This might indicate that a saturation of the lens His and NAH stores was reached by feeding 14.3 g His/kg diet and that feeding 9 g

His/kg diet was nearly sufficient to reach this saturation level in the one year feeding study with constant experimental conditions and relatively low temperature.

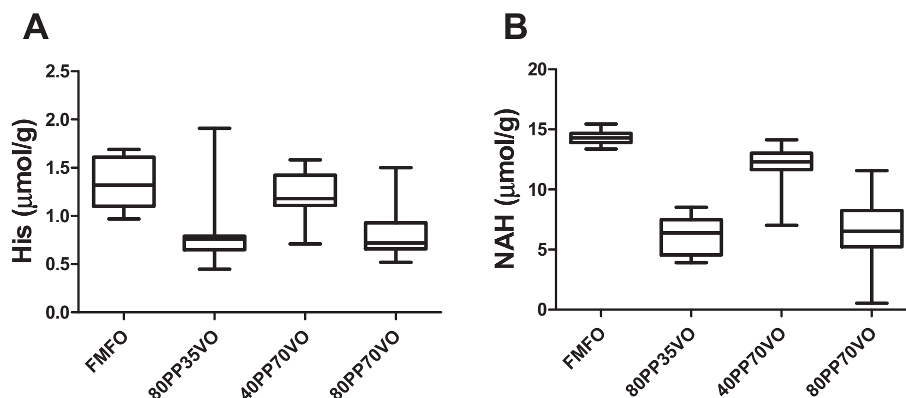

Figure 9. Molar concentrations of (**A**) histidine (His) and (**B**) N-acetylhistidine (NAH) in lenses of Atlantic salmon fed diets containing 100% fish meal (FM) and fish oil (FO), or the indicated percentages of plant protein (PP) and vegetable oil (VO) instead of FM and FO. The fish had been reared for 12 months in sea water. The concentrations of His and NAH are given in μmol/g lens wet weight. The boxes show the group median and 25% and 75% percentiles, and the whiskers show the minimum and maximum values. N=12-15 per dietary group.

The cataracts observed at the end of the trial were mild and there was no significant negative effect of plant feed ingredients on cataract formation during the experimental period, although the relatively low concentrations of His and NAH in lenses of the plant diet fed-fish would suggest an increased risk for cataract development for these fish (Paper IV). The low incidence of cataract in the present long term feeding study might, at least in part, be due to the stable experimental conditions, especially the constant and relatively low water temperature (Paper IV).

The highest dietary inclusion levels of plant proteins led to decreased growth in adult Atlantic salmon growers due to decreased feed intake (Torstensen et al. 2008). The inclusion of plant feed ingredients resulted in decreased lens sizes. Lenses of fish fed the 80PP35VO, 40PP70VO and 80PP70VO diet were all similar in size, while lenses

of fish fed the marine control diet (FMVO) were larger. Lens sizes were thus not directly related to the size of the fish, as would be expected in normally growing fish without any nutrient deficiencies (Pankhurst and Montgomery 1994). The same effect of plant feed ingredient inclusion on lens sizes in Atlantic salmon was seen in a previous feeding trial (Waagbø et al. 2004). Also the lens water content was slightly, but significantly, different in the dietary groups in the present study. Lenses of fish fed diets with 70% vegetable oil inclusion contained more water than lenses of fish fed the marine control diet and the diet with 35% vegetable oil inclusion. The increased water content might be an indication of impaired osmoregulatory ability. The possible implications of these findings for cataract susceptibility in the different dietary groups are not known at the present. The osmoregulatory capacities of lenses of the different groups were further investigated in an *ex vivo* lens culture experiment (Paper IV).

NAH has been shown to be involved in lens water homeostasis in goldfish (*Carassius auratus*) (Baslow 1998) and in Atlantic salmon (Breck 2004). NAH efflux into the culture medium of Atlantic salmon lenses cultured under hypoosmotic conditions *ex vivo* (Paper IV) clearly confirmed a role of NAH as a lens osmolyte in Atlantic salmon. With a larger pool of lens NAH due to higher dietary His levels (Figure 9 B), lenses of fish fed the diets with the highest marine protein content (FMFO and 40PP70VO) were able to release more NAH into the culture medium (Figure 10 E). Since the Atlantic salmon lens contains free His at much lower concentrations than NAH and the medium His concentrations were similar in all dietary groups under both hypoosmotic (Figure 10 H) and hyperosmotic (Figure 10 I) conditions despite diet-dependent differences in lens His concentrations between the groups (Figure 9 A), it can be concluded that the role of free His as an osmolyte was not significant.

Even though lenses of fish fed the 80PP35VO diet had about the same NAH concentrations as lenses of fish fed the 80PP70VO diet (Figure 9 B), it seems clear from Figure 10 B that high dietary plant lipid inclusion is the factor impairing the ability of lenses to withstand swelling on the first day of hypoosmotic treatment. The

situation is not as clear for the hyperosmotic treatment (Figure 10 C). However, high intra-group variation due to the low number of observations prevented reliable conclusions from the intermediate groups (80PP35VO and 40PP70VO).

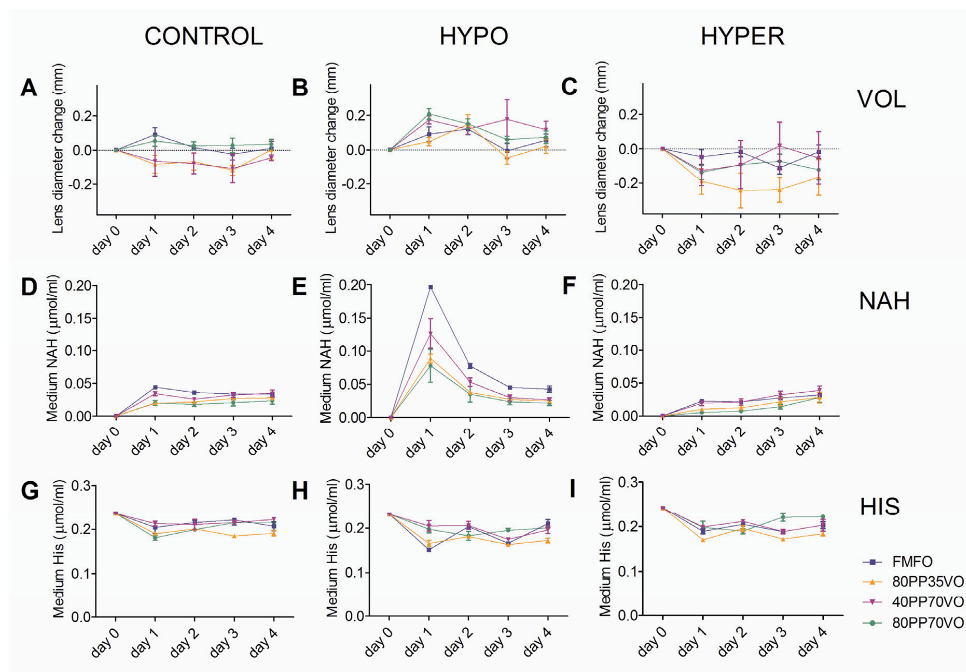

Figure 10. Responses of cultured Atlantic salmon lenses to osmotic challenge *ex vivo*. The graphs show the status before start of the experiment at day 0 and on the four successive treatment days. The volume changes of the lenses cultured in control (**A**), hypoosmotic (**B**) and hyperosmotic (**C**) medium were calculated from lens diameters measured on digital photographs of the lenses. The molar concentrations of NAH in control (**D**), hypoosmotic (**E**) and hyperosmotic (**F**) medium, and likewise His concentrations in control (**G**), hypoosmotic (**H**) and hyperosmotic (**I**) medium were measured by HPLC. Error bars show the standard error of the mean (SE). N=3-4 for lens diameter measurements in the 80PP35VO and 40PP70VO groups, N=10-12 for lens diameter measurements in the FMFO and 80PP70VO groups, and N=3-4 for medium NAH and His measurements in all groups.

Appendix 2 and 3 show the relative lipid class and fatty acid compositions, respectively, of lenses of Atlantic salmon fed the four diets FMFO, 80PP35VO,

40PP70VO and 80PP70VO. The relative amounts of the lipid classes in the lenses of the different dietary groups (as a percentage of the total lens lipid concentration) were similar except for phosphatidyl inositol (PI). Lens PI concentrations seemed to depend on combined inclusion levels of plant protein and lipid sources, rather than plant lipid sources alone. A significant difference in relative lens phosphatidyl serine (PS) concentration between the marine control (FMVO) group and the plant diet (80PP70VO) group was found in Paper IV. When all dietary groups were analysed, this effect was no longer significant (Appendix 2). Lens PS concentrations were obviously related to dietary plant lipid inclusion. The fatty acid profile in the lenses of the four dietary groups was similar despite the marked differences in the composition of marine and plant lipid sources (Appendix 3). The only significant differences were found for linoleic acid (18:2n-6; making out 0, 0.2, 0.3 and 0.4% of total lens fatty acids in the FMFO, 80PP35VO, 40PP70VO and 80PP70VO groups, respectively) and the n-3/n-6 ratio (being 20, 18, 13 and 15 in the FMFO, 80PP35VO, 40PP70VO and 80PP70VO groups, respectively). These differences were obviously caused by the high concentration of linoleic acid and other n-6 fatty acids in plant lipids. These results indicate a tight regulation of the lipid class and fatty acid composition in the Atlantic salmon lens (Paper IV).

It is known that the composition of a membrane can affect the membrane water permeability (Ehringer et al. 1991; Robertson and Hazel 1999) and the activity of integral membrane transport proteins involved in osmoregulation, e.g. Na,K-ATPase (Mizuno et al. 1981; Yeagle et al. 1988; Vemuri and Philipson 1989), which could explain the observed differences in the ability to regulate lens volume between the dietary groups in the present study. It should be noted, however, that the changes described in the above mentioned studies are an order of magnitude higher than the differences in fatty acid and lipid class composition observed in the present thesis and Paper IV. Also, Na,K-ATPase activity was most likely not changed in the present study, since Na,K-ATPase α1C mRNA expression levels were not affected by the different diets. Lens lipid composition may have an influence on cataract formation, as it has been shown to differ between cataractous and clear lenses both in rats

(Mizuno et al. 1981), humans (Borchman et al. 1993; Huang et al. 2005) and Atlantic salmon (Toivonen et al. 2004). However, the lens lipid composition differs markedly between different animal species (Deeley et al.), which makes it difficult to relate data obtained from rat and human lenses to our findings. In the study with Atlantic salmon, the lens lipid compositions were influenced by the different diet compositions in the feeding groups and it is thus not clear if lens lipid composition was the factor causing cataract formation in this study (Toivonen et al. 2004).

In humans, high intakes of dietary linoleic acid (18:2n-6) and linolenic acid (18:3n-3) increased the risk to develop age-related nuclear opacities for women (Lu et al. 2005). It is known that the fatty acid composition affects the rigidity of phospholipid membranes, with highly unsaturated n-3 fatty acids decreasing the rigidity of eye membranes (Nguyen et al. 2007). It may be concluded that lens lipid and fatty acid class composition may play a role in cataract formation and should be further investigated. However, in the present thesis we could not show any significant effect of the vegetable oil mix used in the diets on cataract formation in adult Atlantic salmon growers, although the ability of the lenses to osmoregulate *ex vivo* seemed to be affected. An osmotic challenge test under practical conditions could have verified this relevance.

Gene expression levels of selected genes in lenses of fish fed the marine control diet (FMFO) or a plant-based diet (80PP70VO) were studied in Paper IV. When all four dietary groups were analysed together, glutathione peroxidase 4 (GPX4, Figure 11A), SPARC (Figure 11B), FABP2 (Figure 11C) and betaine aldehyde dehydrogenase (BA-DH) (Figure 11D) were found to be significantly differentially expressed (Kruskal-Wallis ANOVA, $p<0.05$). From Figure 9 and Figure 11 it becomes evident that the expression of GPX4 was most likely increased by the inclusion of plant feed ingredients, seemingly independently of the inclusion level. The expression levels of SPARC and FABP2 were decreased by the inclusion of plant feed ingredients and seemed to correlate with dietary His concentrations or other factors connected to

dietary marine protein levels. The expression pattern of BA-DH seemed only partly to be related to dietary factors.

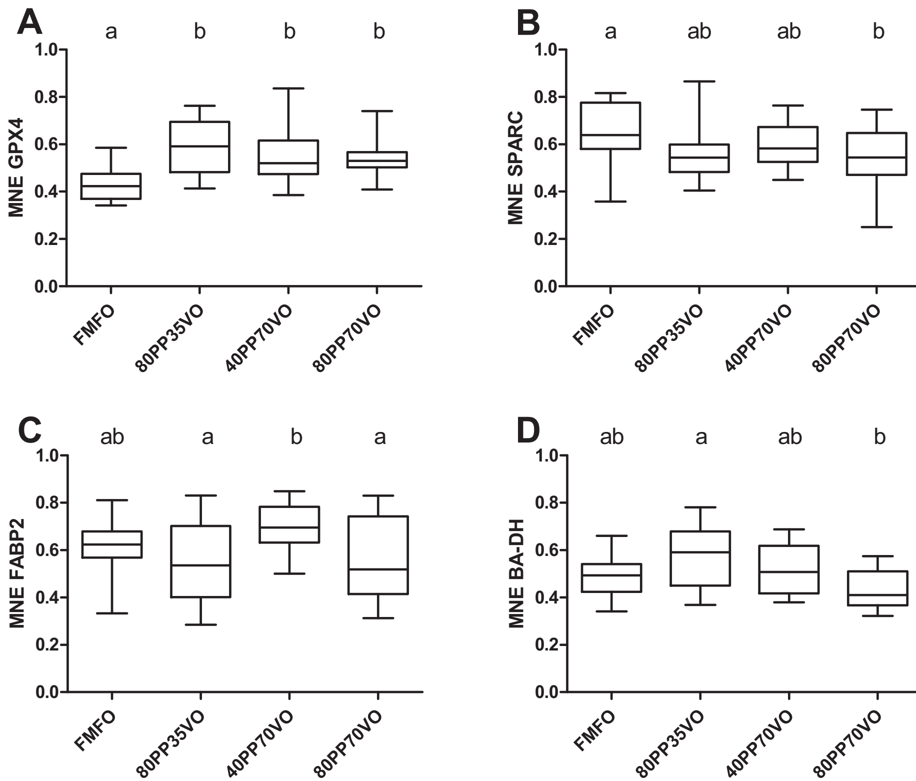

Figure 11. Mean normalised expression (MNE) levels of the selected genes GPX4 (**A**), SPARC (**B**), FABP2 (**C**) and BA-DH (**D**), in lenses of Atlantic salmon fed diets containing 100% fish meal (FM) and fish oil (FO), or the indicated percentages of plant protein (PP) and vegetable oil (VO) instead of FM and FO, after *ex vivo* culture as described in Paper IV. MNE values of lenses cultured in control, hypoosmotic and hyperosmotic media were combined in the graphs. The boxes show the group median and 25% and 75% percentiles, and the whiskers show the minimum and maximum values. Different letters above the boxes show a significant difference between the respective dietary groups (Kruskal-Wallis ANOVA, $p<0.05$). N=14-18 per dietary group.

mRNA expression levels of BA-DH were also affected by osmotic challenge in the two extreme groups (Paper IV). When all four dietary groups were analysed together,

BA-DH (Figure 12 A) and GPX4 (Figure 12 B) were significantly differentially expressed (Kruskal-Wallis ANOVA, $p<0.05$). The trends for ATPα1C and SPARC were close to significant. These findings indicate that the expression levels of a wide range of genes with different functions were affected by osmotic challenge of Atlantic salmon lenses. BA-DH, GPX4, ATPα1C and SPARC were also significantly differentially expressed between cataractous and clear lenses (Paper III).

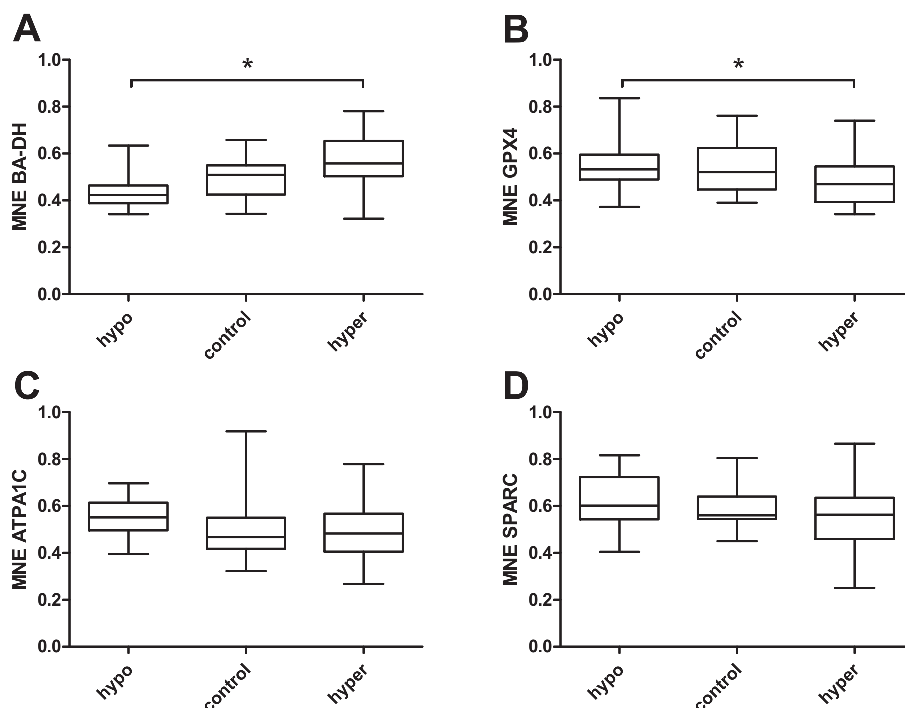

Figure 12. Mean normalised expression (MNE) levels of the selected genes BA-DH (**A**), GPX4 (**B**), ATPα1C (**C**) and SPARC (**D**), in lenses of Atlantic salmon after *ex vivo* culture in hypoosmotic (hypo), control or hyperosmotic medium. MNE values of lenses from fish of al four dietary groups were combined in the graphs. The boxes show the group median and 25% and 75% percentiles, and the whiskers show the minimum and maximum values. An asterisk (*) indicates a significant difference between the respective dietary groups (Kruskal-Wallis ANOVA, $p<0.05$). N=14-18 per osmotic challenge group.

5.5 Speculations about the mechanisms behind His-related cataracts in Atlantic salmon

NAH is the main lens imidazole in Atlantic salmon and high concentrations of lens NAH reached by adequately high levels of dietary His are known to have a cataract preventative effect (Breck et al. 2003; Breck 2004; Breck et al. 2005a; Breck et al. 2005b; Paper II; Paper III). Furthermore, lens NAH has been shown to be involved in lens osmoregulation (Breck et al. 2005b; Paper IV) and lens antioxidative response (Remø 2009) in Atlantic salmon. The up-regulation of calpains and cathepsins in cataractous Atlantic salmon lenses might indicate increased aggregation of lens proteins that have to be cleaved proteolytically in an attempt to maintain lens transparency (Paper III). Kinoshita (1974) was the first to describe the osmotic stress model of diabetic cataract in humans, stating that osmotic stress caused by accumulation of the sugar alcohol sorbitol leads to cataract development through lens fibre cell swelling, ionic imbalance and membrane disruption. Chan and co-workers (2008) recently refined this model, specifying that in acute diabetic cataract models where lens opacification occurs within days or weeks, the accumulation of sorbitol and the associated increased osmotic stress are the main contributing factor, whereas in human diabetic cataracts that develop more slowly, chronic oxidative stress and overexertion of the osmoregulating enzymes lead to premature failure of the lens osmoregulatory mechanism.

There might be mechanistic parallels between diabetic cataracts in mammalians and humans and the His-related cataracts found in Atlantic salmon. Both changes in water salinity and in plasma osmolality can cause changes in aqueous humour osmolality, which in turn leads to osmotic stress for the lens. It has indeed been shown that changed water salinity can cause reversible osmotic cataracts in Atlantic salmon and repeated and/or severe osmotic stress may eventually lead to the formation of irreversible cataracts (Bjerkås et al. 2003; Bjerkås and Sveier 2004). One of the reactions of fish to stressors, such as handling stress, is release of catecholamines and corticosteroids, which in turn lead to elevated plasma glucose levels (Wendelaar

Bonga 1997). There is also evidence for carnivorous fish like Atlantic salmon not being able to control blood glucose concentration as tightly as mammalians (Cowey et al. 1977; Hemre et al. 2002). Hence, farmed Atlantic salmon may experience slightly "diabetic" conditions throughout their life, increasing the incidence of oxidative stress and protein glycation in the lens.

Chronic oxidative stress, non-enzymatic glycation, the exertion of the osmoregulatory system in the lens due to changing environmental conditions - such as water salinity and temperature - and poor regulation of blood glucose levels may eventually lead to impairment of the lens osmoregulatory system in Atlantic salmon. In consequence, once a certain threshold is exceeded, the lens will no more be able to handle osmotic stress, even at levels that were manageable before, and lens fibre disruption and impaired ion balance will lead to cataract development. The above mentioned osmoregulatory dysfunction model for slowly developing human diabetic cataracts proposed by Chan et al. (2008) might thus apply to the cataracts observed in farmed Atlantic salmon.

6. Conclusions

Two main conclusions can be drawn from the present thesis: i. Elevated levels of dietary His mitigate cataract formation in adult Atlantic salmon growers in their second year in sea water (Paper II and III). ii. NAH acts as an osmolyte in the Atlantic salmon lens (Paper IV), which might at least in part explain the cataract mitigating function of high dietary His levels. In more detail, it can be concluded that:

1. The quality of RNA isolated from Atlantic salmon lenses was not affected by the tissue preservation techniques tested. A quick sampling protocol including the use of anaesthetics and not exceeding 30 minutes duration is recommended to avoid changes in lens gene expression caused by sampling stress (Paper I).

2. Dietary His concentrations were reflected in lens NAH (Paper II, III) and, to a lesser extent, in muscle anserine (Paper II) concentrations in adult Atlantic salmon growers in their second year in sea water. Feeding a diet containing more than 12.8 g His/kg diet prior to a period of increased risk due to high water temperature prevented severe cataract formation. Increasing the dietary His level during a period of cataract formation mitigated cataract severeness (Paper II).

3. Cataracts in adult Atlantic salmon growers caused by a low level of dietary His led to significant changes in lens gene expression. The number of significantly differentially regulated genes was higher in lenses sampled at a late stage of cataract formation in October than at a more acute stage in September (Paper III). Based on the expression patterns in lenses with different progression in cataract development, selected transcripts - the most promising candidate

being SPARC - were suggested as possible biological markers for early cataract diagnosis in Atlantic salmon (Paper III, Appendix 1).

4. The inclusion of plant feed ingredients affected lens sizes in a long term feeding study with adult Atlantic salmon growers at stable water temperature. Lens water contents and lipid and fatty acid class compositions were altered by dietary plant lipid inclusion. Lens His and NAH concentrations were related to dietary plant protein inclusion. Mild cataracts were observed, but cataract formation was not affected by inclusion of plant feed ingredients (Paper IV).

5. In the *ex vivo* whole-organ lens culture model applied, lenses from fish fed the diets with highest plant lipid inclusion had a decreased osmoregulatory capacity compared to lenses from fish fed the marine control diet or the diet with lower plant lipid inclusion, as assessed by lens volume change. NAH efflux under hypoosmotic conditions was observed (Paper IV).

7. Future perspectives

The work described in the present thesis prepares the ground for a better understanding of the mechanisms of cataract formation in Atlantic salmon. Elevated dietary His levels have been shown to have a cataract mitigating effect in adult Atlantic salmon growers under practical conditions. Although the plant protein containing diets in the feeding trial described in Paper IV were low in His and subsequently lens NAH concentrations were low in the respective dietary groups, no severe cataracts occurred during the trial. The stable experimental conditions might have masked a possible increased susceptibility for cataracts in these groups. A feeding experiment under practical farming conditions in sea water net pens, allowing for better growth and natural temperature fluctuations might have been more appropriate to study His-related cataract formation. Moreover, due to the composition of the four diets in Paper IV it was not possible to separate the effects of dietary plant lipids and plant proteins completely. Feeding trials with experimental diets only differing in one of the factors: lipid source, protein source and His content would be needed to clearly determine the effects of the single factors.

In the fish feeding trial described in Paper II and III, an increase in water temperature was most likely the critical stimulating factor for cataract formation. More research on the possible cataract-stimulating effect of temperature changes has to be done to reach the goal of controlled experimental induction of cataracts and to survey temperature as a risk factor for cataract formation in aquaculture. Such studies should also take into account different dietary lipid compositions, to explore possible bottlenecks in the regulation of the lens membrane composition that may lead to cataract formation. Research on temperature changes might also prove useful to meet the challenges the aquaculture industry faces in the future by increased water temperatures due to global climate changes.

The use of whole lenses for expression analysis in Paper I, III and IV may have masked some potential effects, as the lens consists of two cell types with partly different functions. More clear results could possibly be obtained if only epithelial cells or only fibre cells would be used in future studies on lens gene expression. One could go even further and dissect cells from different locations in the lens, to investigate spatial patterns of gene expression, e.g. using laser-capture microdissection.

Lens epithelial cell lines are commonly used in human cataract research. Primary cell cultures or a cell line of Atlantic salmon lens cells could be established and used to test risk factors for cataract formation *in vitro*, similarly to the *ex vivo* whole-organ culture applied in Paper IV. Compared to whole-organ culture, the advantage of primary cell cultures or cell lines is that the number of experimental animals could be further reduced as the amount of cells obtained from one lens might be sufficient for multiple experiments or experimental treatment groups.

The lens transcriptome screening presented in Paper III gave some indications about the molecular mechanisms involved in cataract formation in Atlantic salmon. However, further work is needed to fully explore the results of this study. A general problem of gene expression studies is that mRNA abundance is not in all cases equivalent to protein abundance or activity, as the cell possesses various regulation mechanisms on the level of transcription, translation and posttranslational modification. Thus, in future studies, analysis of the mRNA expression levels of interesting candidates should be accompanied by studies on the protein level, e.g. applying Western blot or immunoprecipitation techniques and studies of enzyme activity where applicable. Fluorescence labelling of proteins could be applied to reveal their spatial expression patterns in the lens.

In Paper III, two groups of Atlantic salmon that had developed cataracts of different severity due to feeding different levels of dietary His were compared. Due to this experimental design, it was not possible to distinguish between His-related metabolic changes and effects caused by cataractous changes in the lens. To explore the “pure”

molecular mechanisms of cataract formation, fish reared under the same conditions and fed the same diets, but at different stages of cataract formation, could be compared. The gained knowledge of these basic mechanisms would enable a more comparable approach including different species and types of cataracts. Combined with what is known about the functions of NAH and His in the Atlantic salmon lens, this could lead to a better understanding of the mechanisms of His-related cataracts in Atlantic salmon could be created.

Based on the expression pattern relative to cataract progression, several transcripts were proposed as possible expression markers for early cataract formation in Paper III and Table 1. To verify their function as molecular markers, the differential expression of these transcripts - and other potential candidate genes deduced from the literature - should be tested in lenses that will develop cataract, but before visible cataract formation is observed. Adequate experimental material already exists in lenses from the same feeding trial as the lenses used in Paper III, but from a previous sampling, and is ready to be analysed by qRT-PCR. Once approved, it would be possible to apply analysis of the expression markers to lenses from the feeding trial used for Paper IV, where severe cataract formation was expected but did not occur. Such an approach could give further indications on the risk for cataract formation in Atlantic salmon by using dietary plant raw materials. Established expression markers for early cataract diagnosis could help to shorten the duration of future experiments, saving both time and money.

Due to limitations in time, only a small number of potentially affected transcripts could be tested by qRT-PCR in lenses cultured under different osmotic conditions in Paper IV. Other promising candidates involved in cellular ion and water homeostasis would, for example, include the Na,K,Cl-cotransporter, the various isoforms of Na,K-ATPase and aquaporin 0 and 1.

The function of NAH as a lens osmolyte has been confirmed in Paper IV, based on the observation of NAH efflux from lenses under hypoosmotical conditions. Lens protein and NAH turnover have been investigated in Atlantic salmon smolt (Breck et

al. 2005b). To further explore the lens His and NAH-dependent osmoregulatory system, an attempt could be made to characterise the molecular transport mechanisms of His uptake and NAH efflux, as well as the regulation of enzymatic NAH synthesis in the lens and NAH cleavage in the aqueous humour.

Another interesting task would be the investigation of the possible role of betaine as an osmolyte in the Atlantic salmon lens. A transcript coding for the enzyme catalysing the synthesis of betaine, betaine aldehyde dehydrogenase (BA-DH), was found to be expressed in the lens (Paper III). Based on this finding, the mRNA expression of BA-DH was analysed in cultured lenses in Paper IV and was found to be differentially regulated in response to hyperosmotic and hypoosmotic treatment. The next steps to confirm a role of betaine as a lens osmolyte would be to measure the concentration of betaine in Atlantic salmon lenses, preferably exposed to hyperosmotic and hypoosmotic conditions similar to the model applied in Paper IV, as well as in aqueous humour/culture medium, together with the investigation of the betaine transport system.

In light of the possible similarities between cataract formation in Atlantic salmon and in diabetic human lenses, the potential function of lens NAH in antioxidation and antiglycation could be further investigated. A model of oxidative stress on cultured Atlantic salmon lenses, employing hydrogen peroxide (H_2O_2) in the culture medium, was successfully established by Remø (2009). This model could be used in connection with markers for the oxidative status, such as the ratio of oxidised to total glutathione and lipid peroxidation markers, to investigate the antioxidative capacity in lenses with different NAH concentrations. Similarly, the effects of lens NAH concentrations on lens protein glycation could be investigated applying a modified lens culture model, where glucose is added to the culture medium.

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Appendix 1

Significance analysis of microarrays (SAM) ranked gene list.

The transcripts are ranked according to the significance of differentially expression. The table contains all transcripts with a q-value under 31%. Transcripts with a q-value under 5% are regarded significantly differentially expressed between the two dietary groups. d[i]: SAM score for a transcript i; de[i]: expected SAM score for a transcript i. * E: category of "early" regulated transcripts; L: category of "late" regulated transcripts.

Rank	Accession nr.	Transcript name	d[i]	de[i]	Fold Change	q-value	Regulation category *
1	CA054659	Fatty acid-binding protein. intestinal	3.59	2.93	-1.58	0.00	L
2	CB498606	Fatty acid-binding protein. intestinal	3.33	2.67	-1.47	0.00	L
3	CB486047	Stathmin	3.18	2.55	-1.24	0.00	L
4	CA059668	Oncorhynchus mykiss SYPG1 (SYPG1). PHF1 (PHF1). and RGL2 (RGL2) genes. complete cds; DNaseII pseudogene. complete sequence; LGN-like. PBX2 (PBX2). NOTCH-like. TAP1 (TAP1). and BRD2 (BRD2) genes. complete cds; and MHCII-alpha and Raftlin-like pseudogenes. complete sequence	3.16	2.48	-1.29	0.00	L
5	CA051480	UNKNOWN	3.13	2.41	-1.44	0.00	L
6	CB515802	ATP synthase gamma chain. mitochondrial precursor	-3.11	-2.93	1.30	0.00	L
7	CA054838	Plasma retinol-binding protein II	-3.11	-2.70	1.53	0.00	L
8	CA039925	Oncorhynchus mykiss SYPG1 (SYPG1). PHF1 (PHF1). and RGL2 (RGL2) genes. complete cds; DNaseII pseudogene. complete sequence; LGN-like. PBX2 (PBX2). NOTCH-like. TAP1 (TAP1). and BRD2 (BRD2) genes. complete cds; and MHCII-alpha and Raftlin-like pseudogenes. complete sequence	3.10	2.36	-1.25	0.00	L
9	CB498358	Fatty acid-binding protein. intestinal	3.00	2.32	-1.51	6.05	L
10	CB509992	Lipocalin precursor	2.96	2.28	-1.45	6.05	L
11	CB493612	ATP synthase a chain	-2.95	-2.57	1.32	8.25	L
12	CB511332	Fatty acid-binding protein. intestinal	2.91	2.25	-1.43	6.05	L
13	CA056964	Oncorhynchus mykiss mRNA for sex hormone-binding globulin. complete cds	2.90	2.22	-1.30	6.05	L
14	CA043176	Lipocalin precursor	2.87	2.19	-1.31	6.05	L
15	CK990493	Cystatin-B	2.86	2.17	-1.33	6.05	L
16	CA043660	Nuclear receptor 0B2	-2.85	-2.48	1.35	9.07	L
17	CA052954	Polyadenylate-binding protein 1	-2.82	-2.42	1.22	9.07	L

18	CA062758	PREDICTED: similar to endoU protein [Monodelphis domestica]	2.79	2.15	-1.29	9.55	L
19	CB493454	Lipocalin precursor	2.77	2.12	-1.40	9.55	L
20	CB491705	Probable RNA-directed DNA polymerase from transposon BS	-2.75	-2.37	1.28	9.07	L
21	CA055371	GTP-binding protein SAR1a	-2.69	-2.33	1.31	12.10	L
22	CA063402	Phosphoinositide-3-kinase-interacting protein 1 precursor	-2.65	-2.29	1.20	12.10	L
23	CA040156	Integral membrane protein GPR137B	-2.64	-2.26	1.25	12.10	L
24	CB492836	Lipocalin precursor	2.63	2.10	-1.35	14.51	L
25	CA063511	Transaldolase	2.62	2.09	-1.30	14.51	L
26	CB497796	Cystatin precursor	-2.62	-2.23	1.32	12.10	L
27	CB515635	G1 to S phase transition protein 1 homolog	-2.60	-2.20	1.27	12.10	L
28	CA047249	Excluded (Chimera)	-2.59	-2.18	1.50	12.10	L
29	BU965678	NADH-ubiquinone oxidoreductase chain 3	-2.59	-2.15	1.21	12.10	L
30	CB509557	Heat shock cognate 70 kDa protein	-2.59	-2.13	1.33	12.10	L
31	CB514949	Tubulin beta-1 chain	2.58	2.07	-1.20	14.63	L
32	CA044961	Mu-crystallin homolog	-2.58	-2.11	1.25	14.17	L
33	CA063027	Transaldolase	2.53	2.05	-1.27	15.49	L
34	CA054109	Oncorhynchus mykiss IgH.A locus. partial sequence	2.52	2.04	-1.20	15.49	L
35	CA062534	PREDICTED: Danio rerio hypothetical LOC556254 (LOC556254). mRNA	-2.51	-2.09	1.24	15.88	L
36	CB512299	Nascent polypeptide-associated complex subunit alpha	-2.50	-2.07	1.21	15.88	L
37	CA057637	Importin-7	2.50	2.02	-1.18	15.49	L
38	CK990533	Keratin. type II cytoskeletal 8	-2.49	-2.06	1.46	15.88	L
39	CK991278	UNKNOWN	2.48	2.01	-1.23	15.49	L
40	CN44253 4	Cytochrome c oxidase subunit 2	-2.48	-2.04	1.24	15.88	L
41	CA055608	Splicing factor. arginine/serine-rich 16	2.47	1.99	-1.21	15.49	L
42	CA054143	Hematological and neurological expressed 1-like protein	2.45	1.98	-1.16	17.28	L
43	CB492201	Cytochrome c oxidase subunit 2	-2.42	-2.03	1.19	17.75	L
44	CK990254	Human G protein-coupled receptor (GPR2) gene. partial cds	-2.41	-2.01	1.23	17.75	L
45	CB488287	SPARC precursor	-2.41	-2.00	1.46	17.75	E
46	CA060846	UNKNOWN	-2.41	-1.98	1.31	17.75	L
47	CB492503	Nicotinamide riboside kinase 2	-2.40	-1.97	1.25	18.14	L
48	CA063671	UNKNOWN	-2.40	-1.96	1.57	18.14	L
49	CB514299	UNKNOWN	2.40	1.97	-1.24	17.79	L
50	CA050613	Lysosomal-associated transmembrane protein 4A	-2.39	-1.95	1.22	18.14	L
51	CB505283	UNKNOWN	2.39	1.96	-1.20	17.79	L
52	CN44255 8	Cytochrome c oxidase subunit 3	-2.37	-1.93	1.19	18.83	L
53	CA039532	Beta crystallin A3-1	-2.36	-1.92	1.11	18.83	L
54	CK990833	Cystatin-B	2.34	1.94	-1.34	19.44	L
55	CA769320	Fatty acid-binding protein. intestinal	2.34	1.93	-1.37	19.44	L
56	CA058772	Mus musculus FL10 mRNA. complete cds	2.34	1.92	-1.14	19.44	L
57	CA055797	Elongation factor 1-gamma	-2.33	-1.91	1.14	19.66	L
58	CB510751	UNKNOWN	-2.32	-1.90	1.19	19.66	E
59	CB493904	Endothelin-converting enzyme 1	2.32	1.91	-1.30	19.99	E
60	CK991184	Casein kinase II subunit alpha	-2.31	-1.89	1.17	19.66	L
61	CB502110	Calpain-2 catalytic subunit	-2.30	-1.88	1.21	20.82	L

62	CB512096	40S ribosomal protein SA	2.30	1.90	-1.23	20.16	L
63	CA046921	Excluded (Chimera)	2.30	1.89	-1.20	20.16	L
64	CA062809	Polyadenylate-binding protein 4	-2.29	-1.87	1.24	20.93	E
65	CN44251 9	Cytochrome c oxidase subunit 2	-2.28	-1.87	1.25	20.93	L
66	CA041001	UNKNOWN	2.27	1.88	-1.17	21.13	E
67	CA045398	Oncorhynchus mykiss 28S ribosomal RNA gene. partial sequence	2.27	1.87	-1.19	21.13	L
68	CN44254 5	Cytochrome c oxidase subunit 3	-2.27	-1.86	1.26	21.04	E
69	CK990781	Calpain-2 catalytic subunit precursor	-2.26	-1.85	1.20	21.04	L
70	CA041846	UNKNOWN	-2.25	-1.84	1.15	21.72	L
71	CA046171	Excluded (Chimera)	-2.24	-1.83	1.14	21.72	L
72	CB505609	Salmo salar calcium polyvalent cation receptor 3 mRNA. complete cds	2.24	1.86	-1.14	21.13	L
73	CA054600	UNKNOWN	2.24	1.85	-1.20	21.13	L
74	CB496359	Anaphase-promoting complex subunit CDC26	-2.23	-1.82	1.23	22.98	L
75	CA042437	UNKNOWN	-2.23	-1.82	1.25	22.98	L
76	CB500378	UNKNOWN	-2.22	-1.81	1.23	23.56	L
77	CA041959	Cold-inducible RNA-binding protein	-2.21	-1.80	1.22	23.56	L
78	CB497061	LIM domain only protein 7	-2.20	-1.79	1.19	24.42	L
79	CB490880	Voltage-dependent anion-selective channel protein 1	2.18	1.84	-1.18	23.81	L
80	CA036947	Cystatin-B	2.18	1.84	-1.28	23.81	L
81	CA059691	Legumain precursor	2.18	1.83	-1.21	24.64	L
82	CA064204	Protein S100-B	2.15	1.82	-1.27	27.66	L
83	CA064185	Glutaredoxin-1	2.14	1.81	-1.18	28.42	L
84	CB496934	Zea mays clone Contig482.F mRNA sequence	-2.14	-1.79	1.21	28.08	L
85	CB493115	Cystatin-B	2.13	1.81	-1.28	28.48	L
86	CA051588	UNKNOWN	2.13	1.80	-1.23	28.48	L
87	CB497305	Elastase-2A precursor	-2.13	-1.78	1.14	28.15	L
88	CB492976	Phosphate carrier protein. mitochondrial precursor	-2.12	-1.77	1.17	28.86	L
89	CB499214	Glutamine synthetase. mitochondrial precursor	2.10	1.79	-1.23	30.24	L
90	CB493575	FK506-binding protein 1A	-2.09	-1.77	1.23	29.91	L
91	CB509522	TSC22 domain family protein 4	-2.09	-1.76	1.28	29.91	L
92	CB494682	Protein kinase C and casein kinase substrate in neurons protein 3	-2.09	-1.75	1.23	29.92	L
93	CB515741	UNKNOWN	2.09	1.78	-1.16	30.24	L
94	CB505763	GDP-L-fucose synthetase	-2.09	-1.75	1.24	29.92	L

Appendix 2

Concentrations of lipid classes in lenses of Atlantic salmon fed diets containing 100% fish meal (FM) and fish oil (FO), or the indicated percentages of plant protein (PP) and vegetable oil (VO) instead of FM and FO.

Means and standard errors (SE) are given as mg lipid/g lens wet weight. ANOVA and Tukey test were used to test for significant differences between the dietary groups and p-values are stated. Different letters show a significant difference between the respective dietary groups. N=3 for all groups. PC, phosphatidyl choline; PS, phosphatidyl serine; PI, phosphatidyl inositol; PE, phosphatidyl ethanol; CHOL, cholesterol; FFA, free fatty acids; PL, polar lipids; NL, neutral lipids.

	FMFO			**80PP35VO**			**40PP70VO**			**80PP70VO**			
	Mean	**SE**		**Mean**	**SE**		**Mean**	**SE**		**Mean**	**SE**		
PC	20.14	0.41		20.12	0.07		20.29	0.16		19.57	0.28		ns
PS	7.24	0.11		7.97	0.16		8.07	0.45		8.10	0.10		ns
PI	4.17	0.11	a	3.72	0.32	ab	3.46	0.32	ab	2.86	0.11	b	p=0.03
PE	20.49	0.06		20.01	0.07		20.39	0.44		20.23	0.33		ns
DAG	2.44	0.13		2.74	0.13		2.35	0.16		2.56	0.20		ns
CHOL	29.62	0.32		29.84	0.27		30.21	0.61		30.36	0.52		ns
FFA	15.90	0.35		15.60	0.45		15.23	0.43		16.32	0.48		ns
Sum PL	52.04	0.53		51.82	0.44		52.22	0.88		50.75	0.49		ns
Sum NL	47.96	0.53		48.18	0.44		47.78	0.88		49.25	0.49		ns

Appendix 3

Relative concentrations of fatty acid classes in lenses of Atlantic salmon fed diets containing 100% fish meal (FM) and fish oil (FO), or the indicated percentages of plant protein (PP) and vegetable oil (VO) instead of FM and FO.

Means and standard errors (SE) are given as percentages of the total lens fatty acid concentrations. ANOVA and Tukey test were used to test for significant differences between the dietary groups and p-values are stated. Different letters show a significant difference between the respective dietary groups. N=3 for all groups.

	FMFO			80PP35VO			40PP70VO			80PP70VO			
	Mean	SE		Mean	SE		Mean	SE		Mean	SE		
14:0	9.47	0.26		8.30	0.21		9.07	0.47		8.43	0.45		ns
16:0	14.00	0.61		12.97	0.18		13.60	0.55		13.03	0.15		ns
16:1n-9	4.13	0.19		4.30	0.17		4.30	0.10		4.37	0.17		ns
16:1n-7	5.60	0.15		5.23	0.47		5.80	0.21		4.97	0.41		ns
17:0	1.33	0.09		1.33	0.03		1.37	0.03		1.43	0.03		ns
18:0	5.87	0.37		6.33	0.18		6.07	0.24		6.50	0.21		ns
18:1n-11	0.07	0.07		0.03	0.03		0.07	0.07		0.00	0.00		ns
18:1n-9	20.30	0.40		20.13	0.70		21.30	0.53		20.10	1.27		ns
18:1n-7	3.37	0.09		3.33	0.15		3.40	0.10		3.10	0.15		ns
16:4n-3	3.27	0.17		3.47	0.20		3.30	0.17		3.50	0.21		ns
18:2n-6	0.00	0.00		0.17	0.09		0.33	0.03		0.40	0.06		p=0.04
20:1n-9	0.13	0.13		0.30	0.15		0.30	0.17		0.33	0.17		ns
20:3n-6	0.00	0.00		0.00	0.00		0.07	0.07		0.13	0.07		ns
20:4n-6	1.10	0.06		1.27	0.09		1.20	0.12		1.27	0.18		ns
22:1n-11	0.00	0.00		0.00	0.00		0.07	0.07		0.00	0.00		ns
20:5n-3	4.40	0.76		4.40	0.55		3.70	0.55		4.40	0.64		ns
22:5n-3	1.27	0.23		1.37	0.20		1.13	0.23		1.40	0.29		ns
22:6n-3	13.87	2.66		16.57	2.50		13.40	2.57		17.03	3.71		ns
SUM unidentified	11.87	1.76		10.43	1.23		11.60	1.47		9.60	2.16		ns
SUM identified	88.13	1.76		89.57	1.23		88.40	1.47		90.40	2.16		ns
SUM saturated	30.67	1.19		28.97	0.23		30.07	1.29		29.40	0.57		ns
SUM 16:1	9.67	0.15		9.53	0.67		10.07	0.30		9.33	0.55		ns
SUM 18:1	23.73	0.52		23.50	0.87		24.73	0.70		23.20	1.42		ns
SUM 20:1	0.13	0.13		0.30	0.15		0.30	0.17		0.33	0.17		ns
SUM 22:1	0.00	0.00		0.00	0.00		0.07	0.07		0.00	0.00		ns
SUM monoenes	33.53	0.72		33.30	1.57		35.17	0.99		32.87	2.11		ns
SUM n-3	22.83	3.51		25.87	2.89		21.53	3.23		26.37	4.42		ns
SUM n-6	1.10	0.06		1.43	0.09		1.63	0.20		1.77	0.24		ns
SUM polyenes	23.97	3.60		27.27	2.89		23.13	3.44		28.17	4.63		ns
n-3 / n-6	19.93	1.95	a	18.23	2.24	ab	13.17	0.48	b	14.67	0.58	ab	p=0.02