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Effect of plant-based feed ingredients on osmoregulation in the Atlantic salmon lens

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ABSTRACT

Lenses of adult Atlantic salmon fed with a plant oil and plant protein-based diet (plant diet) were compared to lenses of fish fed a diet based on traditional marine ingredients (marine diet) with respect to biochemical composition and functionality *ex vivo*. After 12 months of feeding, plant diet-fed fish had smaller lenses with higher water contents and lower concentrations of histidine (His) and N-acetylhistidine (NAH) than fish fed with the marine diet. Cataract development in both dietary groups was minimal and no differences between the groups were observed. Lens fatty acid and lipid class composition differed minimally, although a significant increase in linoleic acid was observed. The lenses were examined for their ability to withstand osmotic disturbances *ex vivo*. Culture in hypoosmotic and hyperosmotic media led to increase and decrease of lens volume, respectively. Lenses from 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 lenses from marine diet-fed fish. Culture in hypoosmotic medium resulted in higher cataract scores than in control and hyperosmotic medium. mRNA expression of selected genes, including glutathione peroxidase 4 and SPARC (secreted protein acidic and rich in cysteine), was affected by diet and osmotic treatment. It can be concluded that lenses of farmed Atlantic salmon are affected by the diet composition, both in biochemical composition and physiological functionality in relation to osmoregulation.

1. Introduction

Cataracts, defined as opacities of the eye lens (Hargis, 1991), occur frequently in farmed Atlantic salmon and are a major problem for the aquaculture industry, both ethically and economically (Menzies et al., 2002). The aquaculture industry is rapidly growing worldwide and increasing amounts of feed raw materials are thus needed. Traditionally, farmed Atlantic salmon (*Salmo salar*) are fed diets based on ingredients of marine origin, including fish meal and fish oil (Tacon and Metian, 2008). The limited availability of marine raw materials, however, has created both economic and ethical pressures to find alternative, non-marine sources of feed (Torstensen et al., 2008; Turchini et al., 2009). Plant raw materials are both readily available and cheaper, and can substitute for both marine protein and lipid sources.

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). In addition, a recent study has indicated a link between the use of vegetable oils in the feed and severe outbreaks of cataract in adult Atlantic salmon (Waagbø et al., 2004). However, in general, little effort has so far been made to investigate the consequences of the use of vegetable lipids and proteins in the diet on lens function and cataractogenesis in Atlantic salmon.

Plant proteins generally contain lower concentrations of the essential amino acid histidine (His), compared to marine animal derived proteins (NRC, 1993). Elevated levels of His in the fish diet have been found to be reflected in the lens concentration of the His-derivative N-acetylhistidine (NAH), and to prevent or slow the progression of cataract development in Atlantic salmon (Breck, 2004; Breck et al., 2003; 2005a; b; Tröβe et al., 2008; 2009). The mechanism by which NAH protects the lens, however, is not clear. One of the possible functions of NAH in the lens is to act as an osmolyte. It has been previously suggested that NAH has a role in lens water homeostasis in goldfish (*Carassius auratus*) (Baslow, 1998) and in Atlantic salmon (Breck, 2004; Breck et al., 2005b).

Osmotic stress and impaired osmoregulatory capacity have been proposed as risk factors for cataract development in farmed (Breck and Sveier, 2001) and wild (Bjerkås et al., 2003) Atlantic salmon. Reversible osmotic cataracts have been observed in salmonids directly after transfer from freshwater to seawater (Iwata et al., 1987; Breck and Sveier, 2001), and in farmed sea bass (*Dicentrarchus labrax*) after heavy rain caused a decrease in seawater osmolality (Bjerkås et al., 2000). It has also been proposed that prolonged osmotic stress

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through fluctuating water salinity might finally lead to the formation of irreversible cataracts (Bjerkås and Sveier, 2004).

The aim of the present study was to assess to what extent new plant-based feeds may affect the biochemical composition and the osmoregulatory capacity of the Atlantic salmon lens, compared to a traditional marine diet. By use of an *ex vivo* whole organ lens culture model, we investigated the ability of lenses dissected from salmon with different dietary lipid and protein background to withstand hypoosmotic and hyperosmotic challenge.

2. Materials and methods

2.1. Fish feeding trial and lens sampling

Adult Atlantic salmon were reared in seawater tanks under controlled experimental conditions for 12 months. The water temperature was kept constant at 8.9 ± 0.1 °C. Mean water salinity was 34.9 g L^{-1}, and oxygen saturation in the outlet water was never less than 80%. The final mean weight of the fish in the four dietary groups ranged from 3.3 to 4 kg. Fish used for the lens culture experiment were fed two different diets in triplicate tanks. The feed ingredients, diet compositions and energy contents are given in Table 1. The marine diet (M) was based on traditional marine feed sources, while in the plant diet (P), 80% of the total protein content was substituted by plant proteins, and 70% of the total lipid was substituted by a vegetable oil mix. As a consequence, the amino acid composition differed between the two diets, and the ratio of essential amino acids to non-essential amino acids was lower in the plant diet P (Torstensen et al., 2008). The concentration of the essential amino acid histidine was 48% lower in diet P (7.4 g kg^{-1}) compared to diet M (14.3 g kg^{-1}). The total lipid content was the same in both diets, but diet P had increased percentages of fatty acids typically enriched in plant oils (18:3n-3, 18:2n-6 and 18:1n-9), and decreased levels of typical "marine" fatty acids like n-3 highly unsaturated fatty acids (HUFA) and long chain monoenoic fatty acids like 20:1 and 22:1 (Torstensen et al., 2008). However, based on the present recommendations for Atlantic salmon nutrition, the minimum requirements for all nutrients were covered (NRC, 1993).

At the end of the trial, fish length and weight were measured and cataract status was determined. Where lenses were sampled for either biochemical analysis or lens culture experiments, the fish were anaesthetised with benzocaine (7 g L^{-1}) and killed by a blow to the head. After an incision along the corneal margin, the cornea was peeled back and the lens lifted out with a glass loop. Connecting muscle and ligaments were severed. Lenses intended for biochemical analysis were frozen in liquid nitrogen and stored at −80 °C. Lenses intended for culture were incubated in antibiotic wash as described in Section 2.3.

Table 1
Feed ingredients (g kg^{-1}), proximate feed composition (g kg^{-1}), and energy (kJ g^{-1}) in the marine diet (M) and the plant diet (P).

	Diet M	Diet P
Feed ingredients (g kg^{-1})		
Wheat (Statkorn, Norway)	157	124
Wheat gluten (Cerestar Scandianavia AS, Denmark)	–	150
Corn gluten (Cargill, USA)	–	150
Soybean meal extracted (Denofa, Norway)	–	110
Krill meal (Aker Seafoods Antartic ASA, Norway)	–	50
LT South American (Consortio, Peru)	560	120
Linseed oil (Elbe Fetthandel GmbH, Germany)	–	36
Palm oil (Denofa, Norway)	–	61
Rapeseed oil (Emmelev AS, Denmark)	–	110
Fish oil Nordic (Nordsildmel, Norway)	280	86
Vitamins and minerals	3	3
Proximate feed composition (g kg^{-1})		
Crude fat	343	328
Crude protein	422	426
Ash	67.2	54.5
Starch	91.2	86.6
Dry matter	923	932
Rest of dry matter	0.4	36.9
Energy (kJ g^{-1})	251	253

2.2. Assessment of cataract status

Cataract status was measured by slit lamp biomicroscope inspection (Wall and Bjerkås, 1999). The cataract score of each lens was assessed on a scale from zero (clear lens) to four (completely opaque lens), with a possible maximum score of eight per fish (Wall and Bjerkås, 1999).

2.3. Lens culture and osmotic challenge

The culture medium used was EMEM (Minimum Essential Medium Eagle, M2645, Sigma-Aldrich, St. Louis, USA) adjusted to one of three osmolalities: control (321 mOsm kg^{-1}), hyperosmotic (399 mOsm kg^{-1}), or hypoosmotic (237 mOsm kg^{-1}). The osmolality of the control medium was chosen to equate to the aqueous humour osmolality *in vivo*, previously measured by (Breck et al., 2005a). Medium osmolality was adjusted by addition of either NaCl or de-ionised Milli-Q water (Millipore, Billerica, USA). Gentamycin (Sigma-Aldrich) was added to the medium at a concentration of 1 μL/mL. Medium osmolality was measured using a vapour pressure osmometer (Fiske One-Ten Osmometer, Fiske Associates, Norwood, MA, USA).

The lenses were dissected as described in Section 2.1 and incubated in 1% (v/v) penicillin–streptomycin–amphotericin B (Lonza, Basel, Switzerland) in control medium for one hour before they were transferred to control medium (3 mL) in 12-well culture plates and incubated at 8 °C. The medium was changed after 24 h and the lenses were cultured for a further 4 days without medium exchange before osmotic challenges were performed.

To initiate osmotic challenge experiments, 10–12 lenses from each dietary group were assigned to one of the following treatments: control (continued culture in control medium), hyperosmotic (culture in hyperosmotic medium), or hypoosmotic (culture in hypoosmotic medium). Over the following 4 days the culture medium was removed and stored, and fresh medium (control, hyperosmotic or hypoosmotic) added daily. Medium samples were stored at 4 ºC for LDH measurement or at −20 °C for other analyses. At day 4, the cultured lenses were cleaned by rolling on filter paper, frozen on dry ice, and stored at −80 °C.

Lenses were back-lit by an LED ring light (Schott, Mainz, Germany) against a black background and imaged with a digital camera (Olympus, Hamburg, Germany) mounted at a constant distance. Measurements of lens diameter were carried out by use of the Olympus CellB software (Olympus Soft Imaging Solutions GmbH 1986–2006; www.olympus-europe.com).

2.4. Biochemical analysis

The lens water content was obtained from the difference between wet weight and dry weight. To dry the lenses they were kept for 16–18 h at 103–105 °C and then cooled for 30 min in a desiccator.

Lens lipids were extracted by homogenisation in chloroform/methanol (2:1, v/v). Lens lipid class composition was analysed by high-performance thin-layer chromatography (HPTLC) as described by Jordal et al. (2007). Lens fatty acid composition was analysed by gas chromatography (GC) as described by Torstensen et al. (2008).

Histidine (His) and N-acetylhistidine (NAH) concentrations in lenses and culture medium were analysed by isocratic reverse phase HPLC with UV absorbance at 210 nm, using external standard calibration, as previously described by Breck et al. (2005a). No NAH

was detected in fresh culture medium, thus all NAH detected in lens culture medium samples was assumed to origin from the lenses. Histidine concentrations in fresh control, hypoosmotic, and hyperosmotic media were 0.236, 0.232, and 0.242 μmol mL^{-1}, respectively.

Lactate dehydrogenase (LDH; EC 1.1.1.27) activity in the culture medium was determined by a UV kinetic enzymatic method using a MAXMAT PL clinical analyser and reagents (MAXMAT S.A., Montpellier, France). LDH catalyses the reaction of pyruvate to lactate, using NADH as a hydrogen donor. A volume of 3 μL of each sample was added to 200 μL of a reagent mix containing 0.32 μmol pyruvate and 0.052 μmol NADH (RM LADH0126V, MAXMAT S.A.). The conversion rate of the LDH-catalysed reaction was measured by the decrease in the optical density (OD) at 340 nm and the enzyme activity calculated in U L^{-1}.

2.5. RNA isolation and quantitative real-time PCR (qRT-PCR)

RNA was isolated from whole lenses using the QIAGEN EZ1 RNA Universal Tissue Kit (QIAGEN, Hilden, Germany), according to the EZ1 RNA Handbook (2nd edition April 2008). The lenses were homogenised in QIAzol Lysis Reagent in a Precellys 24 bead mill (Bertin Technologies, Montigny-le-Bretonneux, France). After phase separation with chloroform, the RNA was purified automatically by a QIAGEN BioRobot EZ1 according to the user manual (Version 1.1, July 2005), applying magnetic bead technology. DNA contamination was eliminated from the samples by DNase treatment (QIAGEN RNase-Free DNase Set).

Total lens RNA (500 ng) was reverse transcribed to cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). Each RNA sample was reverse transcribed in duplicate. A standard curve composed of a six-point twofold serial dilution (1000–31.25 ng) of a pool of all RNA samples was run in triplicate to calculate real-time PCR efficiencies for each gene.

The genes assayed in the present experiment were selected based on the outcome of a recent microarray study on histidine-related cataracts in Atlantic salmon (Tröβe et al., 2009). Primer pairs for anserinase (Ans), betaine aldehyde dehydrogenase (BA-DH), voltage-dependent anion-selective channel protein 1 (VDACP1), and HSP90-beta were designed using Primer Express 2.0 software (Applied Biosystems). The primers for Fatty acid binding protein 2 (FABP2), Heat shock cognate 70 kDa protein (HSC71), Phospholipid hydroperoxide glutathione peroxidase (GPX4), and SPARC were designed using Primer3Plus (Untergasser et al., 2007) as described by Tröβe et al. (2009). Isoform-specific primers were used to amplify sodium/potassium-transporting ATPase subunit α1C (ATPA1C) (Nilsen et al., 2007). Heat shock cognate 70 kDa protein (HSC71), Voltage-dependent anion-selective channel protein 1 (VDACP1), and HSP90-beta were used as reference genes to normalise the data. A list of the PCR primers used is given in Table 2.

All cDNA samples were diluted 1:1 in Milli-Q water before real-time PCR. Real-time PCR was carried out on 384-well-plates in a reaction volume of 10 μL, containing 1x Light Cycler 480 SYBR Green I Master (Roche Applied Science, Basel, Switzerland), gene-specific primers (0.5 μM each), and 2 μL cDNA template. A melting curve analysis was applied to confirm the amplification of the expected gene-specific product.

Crossing point (C_P) values calculated by the Lightcycler 480 Software (Roche Applied Science, Basel, Switzerland) were further converted into quantities using gene-specific efficiency values calculated from the standard curves according to the *geNorm* manual (Vandesompele et al., 2002). Dividing the mean of the quantities for each sample by a normalisation factor led to mean normalised expression (MNE) values for the particular genes. The normalisation factor was determined from three reference genes (VDACP1, HSC71, and HSP90-beta) using the *geNorm* VBA applet for Microsoft Excel version 3.4 (http://medgen.ugent.be/~jvdesomp/genorm) (Vandesompele et al., 2002).

2.6. Statistics

T-test, 2-way ANOVA, and repeated measurements ANOVA were used to test for differences between the two dietary groups, effects of dietary groups and osmotic treatments, and effects of dietary groups and osmotic treatments over a four-day experimental period, respectively. Non-parametric tests (Mann–Whitney test or Kruskal–Wallis ANOVA) were used to compare cataract scores in different groups, and otherwise when the underlying assumptions for parametric testing were not met. Two software packages were used to analyse the data, GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA), and the Statistica data analysis software system version 7.1. (StatSoft Inc., Tulsa, OK, USA).

3. Results

At the end of the 12 month feeding trial, fish fed with the plant-based diet (P-fish) were significantly smaller (ca. 9%) compared to fish fed with the marine diet (M-fish) (Table 3). The P-fish also had significantly smaller lenses than the M-fish (*t*-test, $p = 0.004$; Table 3). Interestingly, however, the lens water content was slightly higher in lenses from P-fish compared to M-fish (Mann–Whitney test, $p = 0.01$; Table 3).

To investigate whether the different fatty acid compositions of the diets were reflected in the composition of the lenses, lens lipids and fatty acids were analysed. The total lipid concentration (sum of lipids in mg g^{-1} lens wet mass) in P-lenses was 11% greater than in M-lenses (*t*-test, $p = 0.02$; Table 4). The relative lipid compositions (as a percentage of total lipid content) of lenses from the two dietary groups were, however, very similar. Phosphatidyl inositol (PI) was decreased by 31% in P-lenses (*t*-test, $p = 0.001$), while phosphatidyl

Table 2
Target genes, PCR primers and PCR product sizes.

Gene name	Short name	Accession nr.	Primer forward/reverse	Product size
Betaine aldehyde dehydrogenase	BA-DH	CB496407	CCCCATCTCCACAAACACAGT AGGGTTCAAGATGTCAGGCATT	91
Heat shock cognate 70 kDa protein	HSC71	CA767816	ACCTCGTTGCACTTCTCCAG GCAGCGTGACAAGGTCTCTT	138
HSP90-beta	HSP90B	AF135117	CTCTGGGATGAGCTCCTCACA CCTTTGACCTCTTTGAGAACAAGAA	98
Na,K-ATPase α1C	ATPA1C	AY692145	AGGGAGACGTACTACTAGAAAGCAT CAGAACTTAAAATTCCGAGCAGCAA	85
Phospholipid hydroperoxide glutathione peroxidase	GPX4	CB505439	GGCTGTTCCTTCATCCACTT GCCAGGTACAGAGGTGGAAA	126
SPARC	SPARC	CA052160	CCAAGGCGATGTACTTGTCA GGTTCCTGTCCCACACAGAG	117
Voltage-dependent anion-selective channel protein 1	VDACP1	CB490880	CCTCACCTCAACCAAAGCACAT AGGCAAGGAACACAACAACAAA	121

Table 3
Mean body weights, lens wet weights, lens water contents, lens histidine (His) and N-acetylhistidine (NAH) concentrations in the two dietary groups (M, marine diet; P, plant diet). The data are given as means of triplicate tanks for each dietary group with standard error (SE) and the number of fish per dietary group (N). P-values are given, and the applied statistical tests are indicated by superscript letters ([a], *t*-test; [b], Mann–Whitney test).

	M			P			*p*-value
	Mean	SE	N	Mean	SE	N	
Fish mass (g)	4297	279	9	3393	199	8	0.02[a]
Lens wet mass (g)	0.144	0.003	9	0.124	0.005	8	0.004[a]
Lens water content (%)	51.5	0.09	9	52.6	0.35	8	0.01[b]
Lens His (μmol g^{-1})	1.34	0.07	15	0.83	0.07	15	0.00001[a]
Lens NAH (μmol g^{-1})	14.36	0.16	15	6.23	0.76	15	<0.00001[b]
Cataract score (sum of both eyes)	0.58	0.15	24	1.05	0.33	19	ns[b]

serine (PS) was increased by 12% (*t*-test, $p = 0.005$). The proportional composition of different fatty acids was also very similar between M- and P-lenses (Table 5). Only linoleic acid (18:2n-6) was significantly increased from below detection level in M-lenses to 0.4% in P-lenses (*t*-test, $p = 0.002$).

An important feature of plant protein-based diets is the reduction in the level of His compared to marine diets, with diet P containing approximately half as much His as diet M (7.4 g kg^{-1} versus 14.3 g kg^{-1}). At the end of the 12 month feeding trial, P-lenses had a significantly lower (38%) concentration of His (*t*-test, $p = 0.00001$), and likewise a significantly lower (57%) concentration of NAH (Mann–Whitney test, $p < 0.00001$) than M-lenses (Table 3). Cataract severity was low in both dietary groups, and no significant difference was observed between the groups (Mann–Whitney test). The mean cataract score for P-fish was 1.05 compared to 0.58 for M-fish (Table 3). The cataract prevalence was 63% in P-fish and 46% in M-fish; this difference was, however, not significant due to high variation within the replicate tanks (*t*-test). Where cataract was observed, there were differences in the type of cataract, with perinuclear or deep cortical changes more frequently observed in lenses of P-fish compared to M-fish.

In order to explore the possible links between lens osmoregulation and levels of NAH, an *ex vivo* lens culture model was applied to investigate the responses of Atlantic salmon lenses from M-fish and P-fish to osmotic challenge. Changes in lens size and efflux of NAH and His were measured over a 4 day period (Fig. 1). For the lenses cultured in control medium, the lens size was relatively constant during the experimental period in both dietary groups (Fig. 1A). Under hypoosmotic treatment, a volume increase was observed in both dietary groups. However, a larger volume increase was seen in the P-lenses after 24 h (*t*-test, $p = 0.046$; Fig. 1B). After 3 days exposure volumes in both groups had returned to baseline. In contrast, hyperosmotically treated lenses from both dietary groups showed a decrease in volume, without a significant difference between the dietary groups (repeated measurements ANOVA; Fig. 1C).

To elucidate the possible role of His and NAH as osmolytes in the salmon lens, their efflux in response to osmotic challenge was measured (Fig. 1D–I). Under all conditions, NAH efflux from M-lenses was higher than from P-lenses on the first three days (Mann–Whitney test, $p < 0.05$). Supporting its role as an osmolyte, an increase in NAH efflux was observed when lenses of both dietary backgrounds were exposed to hypoosmotic conditions (Fig. 1E). Efflux was maximal during the first 24 h but had returned to a level close to baseline after 4 days exposure. Of significance, the efflux of NAH from P-lenses was approximately 2.5 fold less than that of the M-lenses. Unlike with NAH, no consistent relationship between histidine efflux, osmolality and dietary group was observed (Fig. 1G–I). To test whether differences in lens volume or lens surface area between the dietary groups were responsible for the differences in NAH efflux, corrected values for the lens NAH concentrations (c_{NAH}/V_{lens} and $c_{NAH}/A_{o\ lens}$) were calculated. This correction did, however, not affect the results (data not shown).

After 9 days of culture, including exposure to osmotic challenge for 4 days, lenses were analysed for NAH and His content. Fig. 2A and B shows the lens concentrations of NAH and His after the culture experiment. Under all conditions, NAH concentrations remained significantly higher in M-lenses compared to P-lenses (2-way ANOVA and Tukey test, $p = 0.001$; Fig. 2A), while the lens His concentrations were similar in both dietary groups (Fig. 2B). Both NAH and His concentrations were significantly affected by the osmotic treatment (2-way ANOVA and Tukey test, $p = 0.00004$ for NAH, and

Table 4
Concentrations of lipid classes in lenses of salmon fed a marine diet (M) or an experimental plant-based diet (P). Means and standard errors (SE) are given as mg lipid per g lens wet mass. *t*-test was used to test for significant differences between the two groups, and *p*-values are stated. $N = 3$ for all groups. PC, phosphatidyl choline; PS, phosphatidyl serine; PI, phosphatidyl inositol; PE, phosphatidyl ethanolamine; CHOL, cholesterol; FFA, free fatty acids; PL, polar lipids; NL, neutral lipids.

Lipid class	M		P		*p*-value
	Mean	SE	Mean	SE	
PC	1.72	0.08	1.94	0.05	0.083
PS	0.50	0.01	0.63	0.02	0.008
PI	0.26	0.01	0.18	0.01	0.006
PE	1.25	0.04	1.34	0.05	0.210
CHOL	1.05	0.04	1.18	0.02	0.046
FFA	0.50	0.03	0.56	0.04	0.279
Sum PL	3.73	0.05	4.10	0.08	0.018
Sum NL	1.55	0.07	1.74	0.06	0.101
Total Sum	5.28	0.05	5.84	0.14	0.022

Table 5
Relative concentrations of fatty acid classes in lenses of salmon fed a marine diet (M) or an experimental plant-based diet (P). Means and standard errors (SE) are given as percentages of the total lens fatty acid concentrations. *t*-test was used to test for significant differences between the two groups, and *p*-values are stated. $N = 3$ for all groups.

Fatty acid class	M		P		*p*-value
	Mean	SE	Mean	SE	
14:0	9.47	0.26	8.43	0.45	0.117
16:0	14.00	0.61	13.03	0.15	0.197
16:1n-9	4.13	0.19	4.37	0.17	0.403
16:1n-7	5.60	0.15	4.97	0.41	0.221
17:0	1.33	0.09	1.43	0.03	0.349
18:0	5.87	0.37	6.50	0.21	0.211
18:1n-11	0.07	0.07	0.00	0.00	0.374
18:1n-9	20.30	0.40	20.10	1.27	0.888
18:1n-7	3.37	0.09	3.10	0.15	0.205
16:4n-3	3.27	0.17	3.50	0.21	0.431
18:2n-6	0.00	0.00	0.40	0.06	0.002
20:1n-9	0.13	0.13	0.33	0.17	0.402
20:3n-6	0.00	0.00	0.13	0.07	0.116
20:4n-6	1.10	0.06	1.27	0.18	0.420
20:5n-3	4.40	0.76	4.40	0.64	1.000
22:5n-3	1.27	0.23	1.40	0.29	0.738
22:6n-3	13.87	2.66	17.03	3.71	0.526
Sum unidentified	11.87	1.76	9.60	2.16	0.462
Sum identified	88.13	1.76	90.40	2.16	0.462
Sum saturated	30.67	1.19	29.40	0.57	0.391
Sum 16:1	9.67	0.15	9.33	0.55	0.589
Sum 18:1	23.73	0.52	23.20	1.42	0.742
Sum 20:1	0.13	0.13	0.33	0.17	0.402
Sum monoenes	33.53	0.72	32.87	2.11	0.780
Sum polyenes	23.97	3.60	28.17	4.63	0.514
Sum n-3	22.83	3.51	26.37	4.42	0.566
Sum n-6	1.10	0.06	1.77	0.24	0.054
n-3/n-6	19.93	1.95	14.67	0.58	0.061

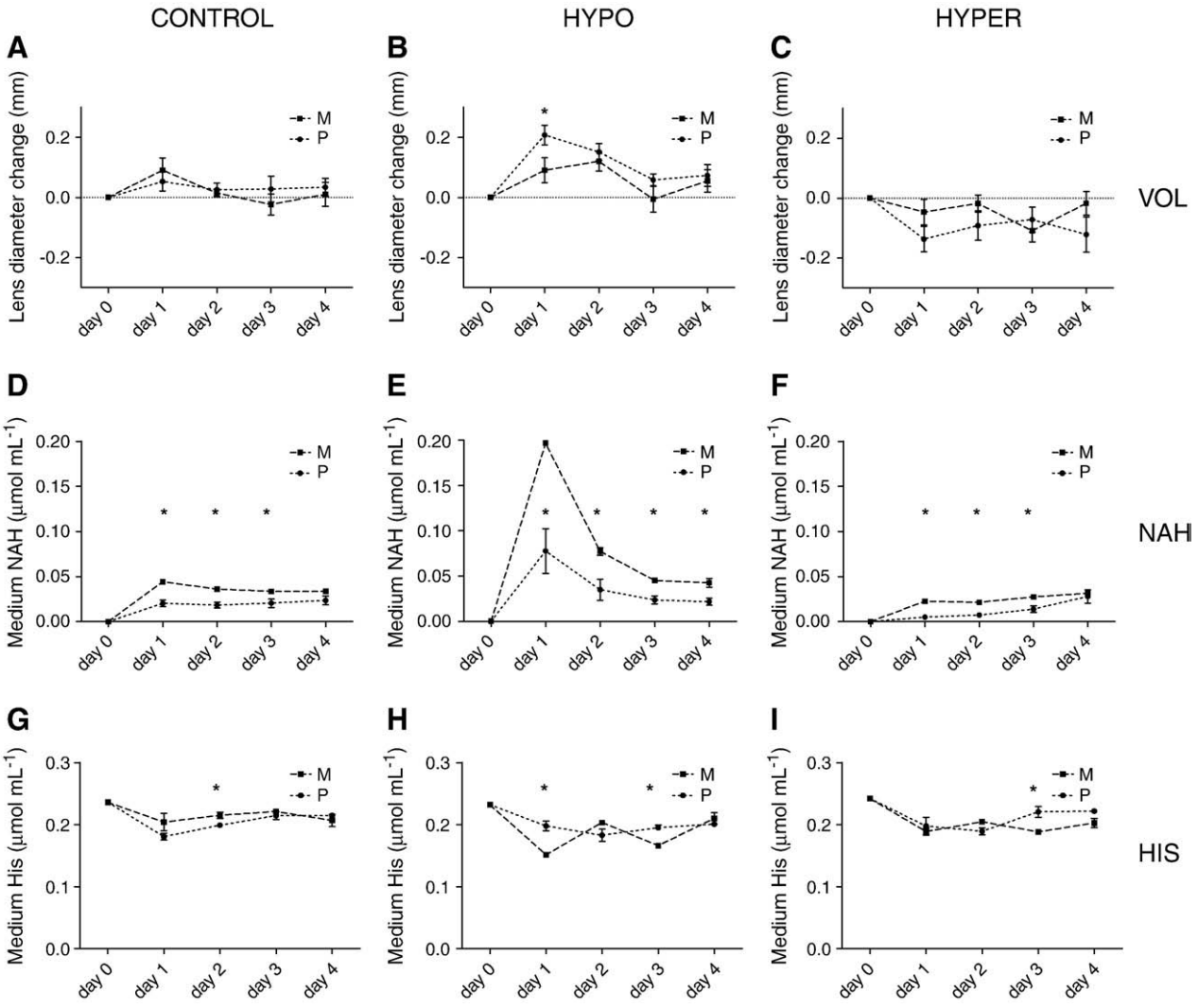

Fig. 1. Responses of cultured Atlantic salmon lenses to osmotic challenge *ex vivo*. The graphs show the status before start of the experiment at day 0 and on the four successive treatment days. The volume changes of the lenses cultured in control (A), hypoosmotic (B), and hyperosmotic (C) medium were 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. NAH and His measurements on day 0 were made in fresh culture medium in which no lenses had previously been cultured. Means for M-lenses are shown as squares and means for P-lenses are shown as dots. Error bars show the standard error of the mean (SE). The asterisks (*) indicate a significant difference between the two dietary groups at the indicated day (*t*-test or Mann–Whitney test, $p<0.05$). $N=10$–12 for lens diameter measurements, and $N=3$–4 for medium NAH and His measurements.

$p=0.0002$ for His). For both dietary groups, the lens NAH concentrations were lower in the hypoosmotic treatment group compared to the control and hyperosmotic treatment groups. Lens His concentrations on the other hand were, for both dietary groups, lower in the control and hypoosmotic treatment groups compared to the hyperosmotic treatment group. There was no significant interaction between the effects of osmotic treatment and the dietary background of the lenses on NAH or His concentrations (2-way ANOVA and Tukey test). Fig. 2C and D shows the accumulation rates of NAH and His in the lenses, where the NAH and His concentrations at the end of the culture experiment are given as percentages of the mean concentrations in M-lenses and P-lenses measured before the culture experiment (given in Table 3). The P-lenses accumulated NAH and His at a higher rate than M-lenses under all conditions. Lenses challenged by hypoosmotic conditions did not accumulate as much NAH as lenses in control and hyperosmotic medium. In hypoosmotic treated M-lenses, the NAH concentrations were lower than before the culture experiment. The His accumulation rates were highest for lenses cultured in hyperosmotic medium, and similar under hypoosmotic and control conditions.

We have shown here that lenses react to osmotic challenge *ex vivo* by change of size and release of NAH into hypoosmotic culture medium. To investigate the possible role of osmotic challenge as a risk factor for cataract formation in lenses with different dietary background, lens opacities in the cultured lenses were assessed at the end of the culture experiment (Fig. 3). The cataract scores were significantly affected by the osmotic treatment (Kruskal–Wallis ANOVA, $p=0.01$). For both dietary groups, the hypoosmotically challenged lenses had higher mean cataract scores than lenses cultured in control or hyperosmotic medium. There was no significant difference between the two dietary groups (Mann–Whitney test), although the P-lenses had slightly higher mean cataract scores in the control and hypoosmotic treatment group. Lactate dehydrogenase (LDH) activity in culture medium samples was used as a marker for cell membrane damage. No significant amounts of LDH activity were found in the culture medium samples, and no differences between dietary background of the lenses or the osmotic treatments were seen (2-way ANOVA).

The expression levels of selected genes previously identified to be responsive to His-related cataract in a microarray study (Trößе et al.,

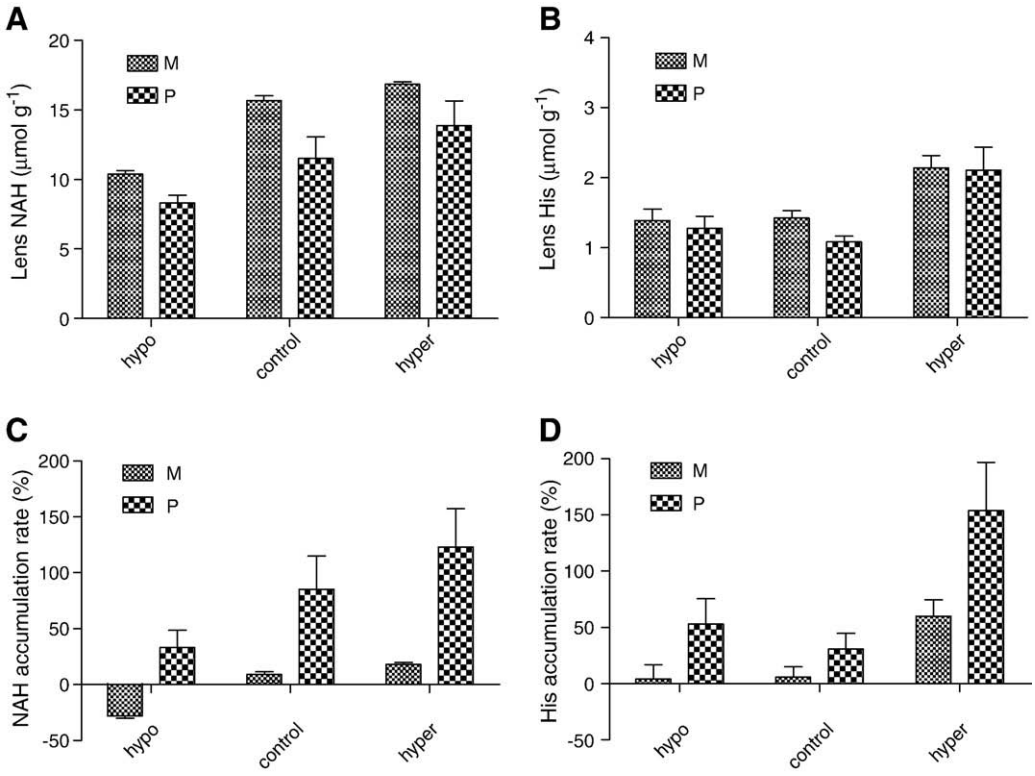

Fig. 2. NAH and His status in lenses of Atlantic salmon fed a marine diet (M) or a plant diet (P), after four days of culture in hypoosmotic (hypo), control or hyperosmotic (hyper) medium. The mean concentrations of NAH (A) and His (B) in the lenses are given in μmol g^{-1}. The mean accumulation rates of NAH (C) and His (D) in the cultured lenses are expressed as percentages of the original mean values in M-lenses and P-lenses measured before the culture experiment (given in Table 3). The standard error of the mean (SE) is shown by error bars. $N = 3–4$.

2009) were measured by qRT-PCR in the cultured lenses after four days of osmotic challenge. Expression levels of phospholipid hydroperoxidase (glutathione peroxidase 4, GPX4; an antioxidant enzyme) and SPARC (Secreted Protein Acidic and Rich in Cysteine; an extracellular matrix protein previously shown to be involved in cataract development) were significantly affected by the dietary background of the lenses (Fig. 4A and B). GPX4 levels were significantly lower in the M-lenses than in P-lenses (Mann–Whitney test, $p = 0.0003$), while SPARC levels were significantly higher in M-lenses than in P-lenses (Mann–Whitney test, $p = 0.009$). The difference in betaine aldehyde dehydrogenase (BA-DH; coding for an enzyme involved in the synthesis of the osmolyte betaine) expression was not significant (Mann–Whitney test, $p = 0.07$; Fig. 4C). However, BA-DH expression levels were significantly affected by osmotic treatment (Kruskal–Wallis ANOVA and Dunn's multiple comparison test, $p = 0.02$; Fig. 5 A), with higher expression levels in the lenses cultured in hyperosmotic compared to hypoosmotic medium. There were no significant changes in the expression of sodium potassium ATPase α1C (ATPA1C; a sodium potassium exchanging pump involved in lens ion homeostasis) and SPARC following osmotic challenge. However, both genes show a trend for higher expression under hypoosmotic conditions compared to hyperosmotic conditions (Fig. 5B and C).

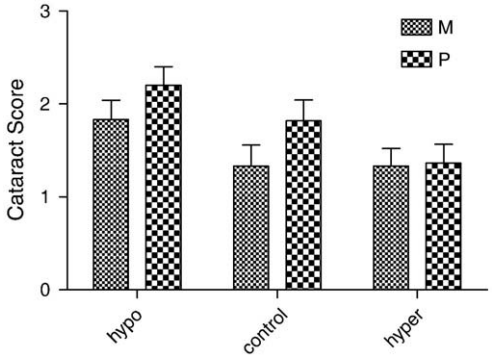

Fig. 3. Mean cataract scores (on a scale from 0 for clear lenses to 4 for completely opaque lenses) of lenses of Atlantic salmon fed a marine diet (M) or a plant diet (P), after four days of culture in hypoosmotic (hypo), control (con), or hyperosmotic (hyper) medium. The standard error of the mean (SE) is shown by error bars. $N = 10–12$.

4. Discussion

In this study, the effects of feeding Atlantic salmon a plant-based diet compared to a marine-based diet, with a specific emphasis on lens biochemistry, physiology and function, have been investigated, since dietary status has previously been shown to be a factor in cataract development.

Despite the large differences in feed fatty acid composition in the present study, only linoleic acid (18:2n-6) was found at a higher concentration in P-lenses than in M-lenses. Diet P contained 13% linoleic acid, while diet M contained 2%. The relative amount of linoleic acid in the lens reflected this difference; however, lens linoleic acid concentrations were an order of magnitude lower than in the diet (0.4% in P-lenses versus not detected in M-lenses). The similar fatty acid profile in M and P-lenses suggests a strict regulation of the fatty acid composition in the salmon lens, compared to other tissues. This hypothesis is further strengthened by the outcomes of similar feeding studies comparing the fatty acid compositions of Atlantic salmon heart, muscle and liver to eye tissues (Bell et al., 1991; Brodtkorb et al., 1997).

In the study of Waagbø et al. (2004), feeding plant oil based diets also resulted in elevated levels of linoleic and other n-6 acids in the lens and corresponded with in a massive increase in the incidence of

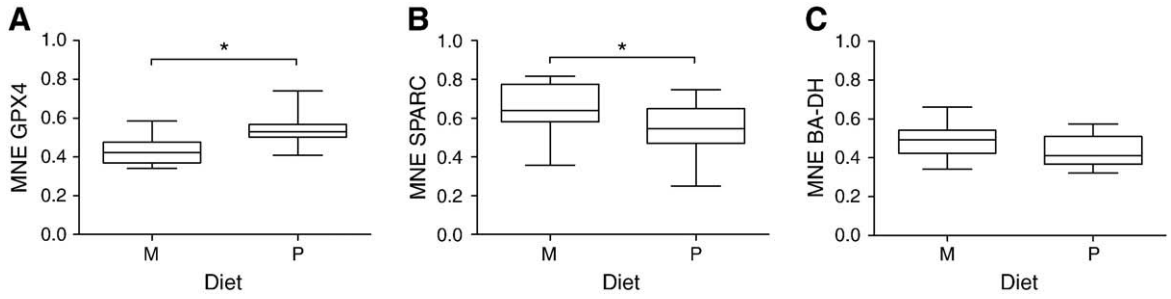

Fig. 4. Mean normalised expression (MNE) levels of the selected genes glutathione peroxidase 4 (A), SPARC (B), and betaine aldehyde dehydrogenase (C), in lenses from fish fed the marine diet (M) or a diet based on plant lipids and proteins (P). The lenses had been cultured in media with different osmolalities for four days. Boxes show the 25–75% percentiles, with the horizontal line indicating the median. The whiskers show the minimum and maximum values. An asterisk (*) shows a significant difference between the two dietary groups (Mann–Whitney test, $p<0.01$). $N=18$.

cataract. Furthermore, in another feeding study with Atlantic salmon, elevated levels of linoleic and other n-6 acids were found in cataractous compared to clear lenses (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). These findings might indicate that feeding high levels of dietary plant lipids may be a risk factor for cataract development in Atlantic salmon, although, as discussed below, no such effect was seen in the present study. Mechanisms whereby membrane lipid composition could affect salmon lens function have not been investigated, although it is known that that it can influence the function of membrane bound proteins, such as Na, K-ATPase (Mizuno et al., 1981; Yeagle et al., 1988; Vemuri and Philipson, 1989). Whilst the activity of Na,K-ATPase was not measured in the present study, expression of Na,K-ATPase α1C mRNA expression levels were not affected by the different diets.

In the current study, there was no significant negative effect of the plant diet with regards to cataract formation. Certain changes in the deeper layers of the lens were, however, more frequently observed in P-lenses, although, the localisation of these changes suggests development at an early stage in the seawater phase. The findings of the present study are contrary to a recent feeding trial (Waagbø et al., 2004), where Atlantic salmon reared in sea water net pens and fed a diet containing the same lipid composition as in the present study developed severe cataracts. Under normal unregulated conditions in the sea, elevated or fluctuating water temperatures have been considered a risk factor for cataract development in farmed fish (Bjerkås et al., 2001). As poikilotherms, fish adapt their membrane lipids not only to dietary lipids, but also to ambient water temperature (homeoviscous adaptations) (Jobling and Bendiksen, 2003). Consequently, temperature is an important environmental variable that should be considered an interacting factor in future lipid fish feeding and lens *ex vivo* culture studies. The results of this study, however, indicate that feeding fish a plant-based diet has little effect on the lipid composition of the lens and hence we conclude that it is unlikely that cataract development would be due to a mechanism linked to lipid composition. Since both the temperature and salinity of the fish rearing tanks were controlled during the study, a possible explanation for the low incidence of cataract during the trial, even in fish on a low histidine diet, is that the stresses, including osmotic stresses, to which sea reared fish would normally be exposed were absent from the study.

The lenses of fish fed the plant-based diet were found to have decreased levels of both histidine and *N*-acetylhistidine. The hypothesis that NAH could be an osmolyte in the salmon lens is strengthened by the observed NAH efflux from lenses incubated in hypoosmotic culture medium (Fig. 1E). Also the NAH efflux clearly depends on the lens NAH concentration. The larger pool of lens NAH as a consequence of higher dietary His levels enables C-lenses to release more NAH into the culture medium when exposed to hypoosmotic conditions, so preventing excessive water uptake and damaging volume changes. At low lens NAH concentrations (i.e. insufficient dietary His supply), the lens may utilise other osmolytes. Breck et al. (2005a), for example, showed that the sum of free amino acids in Atlantic salmon lenses was relatively constant, despite variations in the concentration of NAH induced by different diets. The results of the present study suggest, however, that utilisation of NAH might be more efficient in lens osmoregulation than efflux of other amino acids to compensate for low His and NAH concentrations, as the P-lenses showed a greater volume change than did the M-lenses. The difference in lens NAH concentration between the two dietary groups does not, however, explain why P-lenses were more prone to shrinking in hyperosmotic culture medium than C-lenses, although, the slightly greater water content of the P-lenses would be a contributing factor.

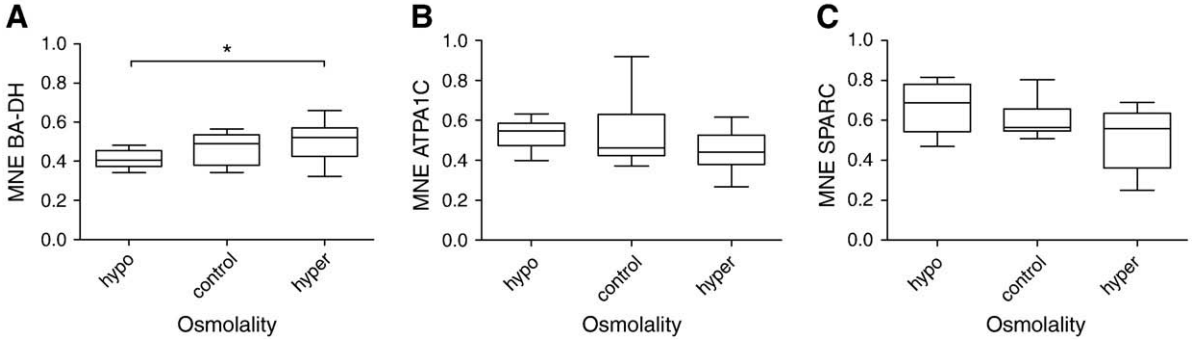

Fig. 5. Mean normalised expression (MNE) levels of the selected genes betaine aldehyde dehydrogenase (A), sodium potassium ATPase α1C (B), and SPARC (C), in lenses from fish fed the marine diet (M) or a diet based on plant lipids and proteins (P). The lenses had been cultured in hypoosmotic (hypo), control (con), or hyperosmotic (hyper) medium for four days. Boxes show the 25–75% percentiles, with the horizontal line indicating the median. The whiskers show the minimum and maximum values. An asterisk (*) shows a significant difference between the two indicated osmotic treatments (Kruskal–Wallis ANOVA and Dunn's multiple comparison test, $p<0.05$). N = 12.

The increase in lens NAH concentrations in culture shows that lenses actively synthesize NAH from His. The net NAH synthesis rates over the nine days of culture were similar in M-lenses and P-lenses, and somewhat higher in lenses cultured in hyperosmotic medium (data not shown). As can be seen in Fig. 2C and D, P-lenses accumulated NAH and His at higher rates than M-lenses, apparently to compensate for the originally low lens NAH and His levels, while M-lenses might have reached a level of NAH saturation. Surprisingly, hypoosmotically challenged P-lenses were still able to accumulate NAH despite also releasing it into the medium. In hypoosmotic treated M-lenses, which had the highest NAH efflux, the NAH concentrations after the culture experiment were lower than before. However, even after 9 days of culture in His-supplemented medium, and with higher accumulation rates in P-lenses than in M-lenses, the NAH concentrations in P-lenses were still lower than in M-lenses, while the His concentrations were similar (Fig. 2A and B). In both dietary groups lens NAH levels were lower in lenses exposed to the hypoosmotic conditions.

Dietary His-dependent cataracts have been shown to affect the expression of a large number of genes in the Atlantic salmon lens (Tröβe et al., 2009). Based on the outcome of this genome-wide screening study, lenses from the present lens culture experiment were analysed for expression levels of selected relevant genes. In the present study, the expression level of GPX4 mRNA was lower in the M-lenses compared to the P-lenses, while the opposite effect was seen for BA-DH and SPARC. GPX4 is the only antioxidant enzyme capable of reducing phospholipid hydroperoxides within membranes and lipoproteins (Ursini et al., 1985), and may protect against the toxicity of ingested lipid hydroperoxides. Plant oils contain generally higher levels of endogenous antioxidants compared to fish oil, and fish oil is also more prone to oxidation due to high levels of n-3 highly unsaturated fatty acids (HUFA) (Ng et al., 2004). Thus, pro-oxidative conditions and a higher demand for GPX4 activity and thus higher GPX4 mRNA expression levels would be expected in M-lenses compared to P-lenses. Indeed, a study by (Sneddon et al., 2003) showed that in human endothelial cells, GPX4 mRNA levels were increased by an essential n-3 fatty acid contained in fish oil, docosahexaenoic acid (DHA). In contrast, and similar to our observations, the transcription levels of other lens antioxidant enzymes were decreased in lenses of Atlantic salmon parr exposed to pro-oxidative conditions caused by elevated dissolved oxygen (Waagbø et al., 2008). Further research is needed to understand the antioxidant response in Atlantic salmon lens with regards to lipid oxidation.

BA-DH mRNA expression levels were increased in hyperosmotically challenged lenses and decreased in hypoosmotically challenged lenses, compared to the unchallenged controls. BA-DH catalyses the synthesis of betaine, which has been shown to act as an osmolyte in bacteria (Sutherland et al., 1986), mammals (Zhang et al., 1996) and fish (Virtanen et al., 1989; Duston, 1993). Increased expression of BA-DH in a hyperosmotic environment, and thus increased betaine synthesis, could contribute to the osmoregulation of the salmon lens. However, the relationship between osmoregulatory ability and NAH shown by this study suggests that betaine would only play a minor role. Na,K-ATPase α1C and SPARC mRNA expression levels were increased in hypoosmotic and decreased in hyperosmotic challenged lenses. Na,K-ATPase is crucial to maintain the sodium homeostasis in the lens and is therefore an object of intense investigation in human cataract research (Delamere and Tamiya, 2004). Expression levels of the multifunctional extracellular matrix protein SPARC have been shown to be increased in cataractous human lenses when compared to normal lenses (Kantorow et al., 1998; Hawse et al., 2003). Both SPARC and Na,K-ATPase α1C mRNA levels were increased in lenses of Atlantic salmon fed a low-His diet compared to a higher-His diet, where the low-His fed fish were more susceptible to cataract development (Tröβe et al., 2009). Increased expression of SPARC and Na,K-ATPase α1C in hypoosmotically challenged lenses might thus indicate that hypoosmotic stress is one of the risk factors contributing to cataract development in Atlantic salmon in the seawater phase.

It can be concluded that although there were small differences in lens size and biochemical composition, the plant diet had no effect on cataract development in adult Atlantic salmon reared under the stable experimental conditions of this study. Although volume regulation in P-lenses was slightly impaired, lenses of both dietary groups were largely able to maintain transparency under osmotic stress. Repeated osmotic challenge, however, over a longer period of time, caused by naturally occurring fluctuations in water salinity, has been proposed as a factor leading to cataract formation in Atlantic salmon (Bjerkås and Sveier, 2004). This situation might be comparable to lenses of diabetic humans, where repeated osmotic stress of minor severity finally leads to an exhaustion of the osmoregulatory system and formation of cataracts (Chan et al., 2008). *Ex vivo* salmon lenses responded to hypoosmotic and hyperosmotic treatment by lens volume change, NAH efflux, and changes in gene expression. The lenses from fish fed a traditional marine diet had a higher capacity to osmoregulate than lenses from fish fed a plant-based diet. Reduced lens osmoregulatory capacity may be associated with increased risk for cataract development under unfavourable farming conditions for Atlantic salmon.

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