Genetically modified plants as fish feed ingredients

Roundup Ready® soy, MON810 maize, Atlantic salmon, zebrafish

Nini Hedberg Sissener

Dissertation for the degree philosophiae doctor (PhD)
at the University of Bergen

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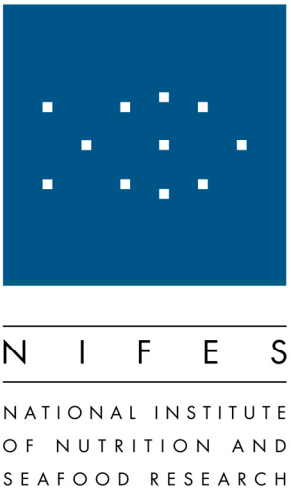

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Scientific environment

My PhD thesis was completed at the National Institute of Nutrition and Seafood Research (NIFES) in Bergen, Norway, under the supervision of Dr. Gro-Ingunn Hemre. Co-supervision was also provided by Dr. Monica Sanden (NIFES). I have been part of the research programme Aquaculture Nutrition. Ernst Morten Hevrøy and Andreas Nordgreen from the research programme have been involved in some of the work.

My work has been part of the project "Genetically modified plant products (GMPP) in feed for Atlantic salmon – A follow-up study on physiological responses and DNA traceability". The project group here at NIFES has consisted of Dr. Gro-Ingunn Hemre, Dr. Monica Sanden and master student Elisabeth Eie. Anne-Marie Bakke, Åshild Krogdahl and Jinni Gu from the Norwegian School of Veterinary Science (NVH), and Knut G. Berdal, Lene E. Johannessen and Christer Wiik-Nielsen from the National Veterinary Institute (NVI) are also involved in this project. The work reported in this thesis has been done in collaboration with them.

The proteomics work was carried out at the Aberdeen Proteome Facility, which is part of the School of Medical Sciences at the University of Aberdeen. This work was done in collaboration with Dr. Sam Martin from the School of Biological Sciences, University of Aberdeen and Dr. Phil Cash from the Division of Applied Medicine, School of Medical Sciences.

Acknowledgements

First and foremost, I would like to thank my main supervisor Gro-Ingunn Hemre for excellent supervision, support and encouragement along the way. Monica Sanden also made valuable contributions, both in planning, practical work and fruitful discussions during the "GMO-morning coffees". Anne-Katrine Lundebye Haldorsen and Ernst Hevrøy gave constructive feedback in the writing process of the thesis. Also, thanks to all my co-authors on the papers for their efforts, constructive criticism and invaluable input for the papers of this thesis.

Thanks to Dr. Sam Martin for accepting our invitation for collaboration, for organizing my very pleasant and rewarding stay in Aberdeen, and to both him and Dr. Phil Cash for advice and input during the proteomics work. Evelyn Argo gave excellent day-to-day technical assistance with the gel work, being extremely helpful, patient and always smiling. Also thanks to Elizabeth Stewart for help with the protein identifications. Much appreciated technical assistance here at NIFES was provided by Jaap Wessels, Eva Mykkeltvedt, Leikny Fjeldstad and Elin Krohnstad. Thanks to Ivar Helge Matre and Lise Dyrhovden technical assistance during the salmon feeding trial at the Institute of Marine Research, Matredal.

Also I would like to thank all the other PhD students and other colleagues at NIFES for their support, and for all the good times and social events outside of work.

The project was supported by the Norwegian Research Council, grant no. 172151. Work carried out at Aberdeen Proteomics Unit is supported in part by a grant from the BBSRC. Thanks to the Monsanto Company for kindly supplying the Roundup Ready® soybeans and MON810 maize as well as their near-isogenic maternal lines.

Bergen, May, 2009

Nini Hedberg Sissener

Abstract

Commonly used plant ingredients in salmon feed such as soybeans and maize are increasingly grown as genetically modified (GM) varieties. The question arises whether these are equally nutritious and safe for the fish as conventional varieties. The target species of this thesis is Atlantic salmon (*Salmo salar*), while zebrafish (*Danio rerio*) is used as a potential model. Extensive evaluations of performance, health, organ function and stress response were carried out in a 7-month salmon feeding trial with GM soy (25%). A smaller study was conducted on zebrafish evaluating both GM soy and GM maize as feed ingredients, including tracing of dietary DNA in fish tissues.

Feeding salmon GM soy did not affect growth, body composition, haematology or weights of liver, spleen, head-kidney and proximal intestine, compared to non-GM soy. Distal intestine was larger in the GM group at one sampling point, but not at the end of the trial. Mid intestine was consistently smaller in the GM group. Typical soybean meal (SBM) -induced alterations in the distal intestine were seen in both groups. However, GM fed fish had shorter mucosal fold height at one sampling point, and more mucosal fold fusion overall in the trial, which could indicate slightly more pronounced SBM-induced changes. In liver, glycogen deposits were lower in salmon fed GM soy. Apart from this, no morphological differences were detected in any organs. Of parameters related to health, there were no differences in haematology, proportion of leukocytes types, lysozyme concentrations in spleen and head-kidney, or concentrations of plasma enzymes. Plasma triacylglycerol (TAG) was higher in the GM group overall in the trial, while no diet effects were seen on plasma glucose and protein. Fish in both diet groups responded similarly to seawater transfer; they adapted well and rapidly regulated chloride ion content in plasma back to basal levels. Similarly, there were no differences in stress response between the two diet groups. Proteomic comparisons of livers from fish in the two diet groups exhibited small fold differences, and differentially expressed proteins all had a high false discovery rate. The diet groups were not distinguishable by principal component

analysis, suggesting that other sources of variation such as individual or technical variation were more prominent than the difference between fish fed GM and non-GM soy.

Concluding from the salmon trial, minor diet effects were seen. In parameters where reference values are found in the literature, both diet groups were within the normal range. The present results did not confirm observations from previous trials of increased spleen size and lysozyme levels. The response in plasma TAG was opposite of what has been observed previously, suggesting that this response was not caused by the genetic modification, but by other factors like small variations in anti-nutrients. No indications of adverse health effects and no difference in performance during 7 months, lead to the conclusion that GM soy appears to be an equally good feed ingredient as non-GM soy at a 25% inclusion level, which is higher than what is used commercially at present.

The use of zebrafish as a model greatly reduces the cost and duration of trials evaluating GM plants as feed ingredients. Soy and maize of GM and non-GM varieties were used in a 2x2 factorial design, in a 20-day feeding trial. Fish fed GM maize had better growth, lower mRNA level of *superoxide dismutase-1* (*SOD-1*) in liver and a tendency towards lower level of *heat shock protein (HSP)70* compared to fish fed non-GM maize. These results are very different to a previous salmon study using the exact same batch of maize, which could limit the usefulness of zebrafish as a model. With GM soy, there was no effect on growth, but there were significant interaction effects between soy variety (whether GM or not) and sex on total RNA yield from liver and *SOD-1* level in liver. These might have been caused by isoflavones.

Results from the zebrafish trial were inconclusive, both on use of GM maize in feeds and zebrafish as a model. Tracing of dietary DNA, however, was successfully accomplished with results corresponding well with work done in other species.

Contents

List of publications

1. **Nini H. Sissener, Monica Sanden, Anne-Marie Bakke, Åshild Krogdahl, Gro-Ingunn Hemre.** A long term trial with Atlantic salmon (*Salmo salar* L.) fed genetically modified soy; focusing general health and performance before, during and after the parr-smolt transformation. **Aquaculture (in press), doi: 10.1016/j.aquaculture.2009.05.002**

2. **Nini H. Sissener, Anne-Marie Bakke, Jinni Gu, Michael H. Penn, Elisabeth Eie, Åshild Krogdahl, Monica Sanden, Gro-Ingunn Hemre**. An assessment of organ and intestinal histomorphology and cellular stress response in Atlantic salmon (*Salmo salar* L.) fed genetically modified soy. **Manuscript.**

3. **Nini H. Sissener, Samuel A.M. Martin, Phillip Cash, Ernst M. Hevrøy, Monica Sanden, Gro-Ingunn Hemre.** Proteomic profiling of liver from Atlantic salmon (*Salmo salar*) fed genetically modified soy compared to the near-isogenic non-GM line. **Marine Biotechnology (submitted).**

4. **Nini H. Sissener, Lene E. Johannessen, Ernst M. Hevrøy, Christer R. Wiik-Nielsen, Knut G. Berdal, Andreas Nordgreen, Gro-Ingunn Hemre.** Zebrafish (*Danio rerio*) as a model for investigating the safety of genetically modified feed ingredients (soy and maize); performance, stress response and uptake of dietary DNA sequences. **British Journal of Nutrition (submitted)**

The style of the papers is that required by the different journals.

In the thesis, the papers are referred to as paper 1, 2 etc.

Abbreviations

1DE – one-dimensional electrophoresis
2D-DIGE – two-dimensional digital image gel electrophoresis
AB – apoptotic bodies
ADP – adenosine di-phosphate
ALAT – alanine aminotransferase
ANF – Anti nutritional factor
ANOVA – analysis of variance
AOAC – Association of Analytical Communities
ARP – acidic ribosomal phosphoprotein
ASAT – aspartate aminotransferase
ATP – adenosine tri-phosphate
BCA – bicinchoninic acid
BLAST – basic local assignment tool
Bw – body weight
CaMV – cauliflower mosaic virus
CAT – catalase
CCB – Collodial Coomassie Blue
cDNA – complementary DNA
Ct – cycle threshold
CTAB – Cetyl Trimethyl Ammonium Bromide
CuZnSOD – Copper Zink Superoxide Dismutase (SOD-1)
DG –Deposit of glycogen
DHA – decosahexaenoic acid
DI – distal intestine
DISI – distal intestine somatic index
DNA – deoxyribonucleic acid
DTT – dithiolthreitol
EDTA – ethylenediaminetetraacetic acid
EF1α – elongation factor 1-alpha
EFSA – European Food Safety Authority
ENP – enterocyte nucleus position
EPA – eicosapentaenoic acid
EPSPS – 5-Enolpyruvylshikimate-3-phosphate synthase
ER – endoplasmatic reticulum
EROD – Ethoxyresorufin-O-deethylation
ESI – electrospray ionization
EST – expressed sequence tag
EV – enterocyte vacuolization
e-value – expectancy value
FAO – Food and Agriculture Organization (of the United Nations)
FCR – Feed conversion ratio
FDR – False discovery rate
FFSBM – full fat soybean meal
FI – feed intake
Fl – fork length
FWER – Family wise error rate
GCMS – gas chromatography mass spectrometry
GLC – gas liquid chromatography
GM – genetically modified

GPx – glutathione peroxidase
GRASP – Genomic research on all salmon
h – hours
Hb – Haemoglobin
HCB – hexachlorobenzene
HCH – hexachlorcyclohexane
Hct – Haematocrit
H&E – haematoxylin and eosin
HIS – hepato- somatic index
HKSI – head kidney somatic index
HPLC – high performance liquid chromatography
HPRT – hypoxanthine guanine phosphoribosyl transferase
HSP – Heat Shock Protein
ICP-MS – inductively coupled plasma mass spectrometry
ILSI – International Life Sciences Institute
IPG – immobilized pH gradient
K – condition factor
kDa – kiloDaltons
LC – MS/MS – Liquid Chromatography – Tandem Mass Spectrometry
LDH – lactate dehydrogenase
LI – liver
LOD – limit of detection
LOQ – limit of quantification
LPC –lamina propria cellularity
LW – lamina width
MALDI-TOF – matrix assisted laser desorption ionization – time of flight
MCH – mean cell haemoglobin
MCHC – mean cell haemoglobin concentration
MCV – mean cell volume
MF – mitotic figure
MFF – mucosal fold fusion
MFH – mucosal fold height
MISI – mid intestine somatic index
MM – melanomacrophage
MMC – melanomacrophage centre
MNP – mean probable number
MnSOD – Manganese Superoxide Dismutase (SOD-2)
mRNA – messenger RNA
NADH – Nicotinamide adenine dinucleotide dehydrogenase
NCBI – the National Centre for Biotechnology Information
NOS – nopaline synthase
NRC – National Research Council
OECD – Organization of Economic Cooperation and Development
PCA – principle component analysis
PCR – polymerase chain reaction
PEP – phosphoenolpyruvate
PFU – PCR forming units
pI – isoelectric point
PISI – proximal intestine somatic index
qPCR – quantitative PCR
RBC – red blood cell
REST© - relative expression software tool
RIN – RNA integrity number

RNA – ribonucleic acid
Rpl13α – ribosomal protein L13α
RRS® - Roundup Ready® soy
RT – reverse transcription
Rubisco – ribulose-1,5-bisphosphate carboxylase
SBM – Soybean meal
SD – standard deviation
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM – standard error of the mean
SGLT – sodium-glucose linked transporter
SmC – submucosa cellularity
SmW – submucosa width
SOD – superoxide dismutase
SPC – soy protein concentrate
SPI – soy protein isolate
SSI – spleen somatic index
TAG – triacylglycerol
TPI – triosephosphate isomerase
TPP – thymidine phosphorylase precursor
WBCC – white blood cell count

1. Theoretical Background

The scope of this background chapter has been limited to themes of direct relevance to the work carried out for this thesis. Environmental, political and socio-economic aspects of the use of genetically modified (GM) plants have thus been omitted to a large extent. I start by giving a briefly introduction to GM plants, some issues of safety assessment and previous results on the use of GM plants in fish diets. Then, I explore the feed ingredients of interest in this thesis, soy and maize, focusing on their role in salmon feeds and details of the GM varieties used in this work, Roundup Ready® soy (RRS®) and MON810 maize. Finally, I give a brief description of the fish species, including the rationale for choosing Atlantic salmon (*Salmo salar*) as a target species, -and of the response parameters that were selected.

1.1 Introduction

1.1.1 Genetically modified plants

Genetic engineering, has been successfully employed to introduce new properties in crop plants. There are two commonly used ways in which GM plants are made, the biolistic/ physical method (microprojectile bombardment system), and the biological method (*Agrobacterium*-mediated transformation). In the former, the DNA segment to be inserted is coated on metal particles which are then accelerated into plant tissue or cells, where the DNA is integrated into the plant genome (Hansen & Wright, 1999). The biological method takes advantage of the fact that *Agrobacterium tumefaciens* infects plants by integrating a segment of plasmid DNA, known as T-DNA (transfer-DNA), into the chromosomal DNA of plant cells (Gelvin, 2003). The gene of interest can thus be inserted into the T-DNA region, and *Agrobacterium* will act as a vector for genetic engineering (Gelvin, 2003). Integration of the novel DNA into the plant genome happens through illegitimate recombination (non-homologous

end-joining) rather than homologous (or site directed) recombination (Hansen & Wright, 1999). Transgene insertion is an imprecise and poorly understood event (Somers & Makarevitch, 2004). Introduction of superfluous DNA, and deletions and rearrangements of host DNA at the insertion site are common occurrences, especially when the particle bombardment method is used (Latham *et al.*, 2006). This random insertion might disrupt endogenous gene expression, causing unintended changes in levels of macro- or micronutrients, anti-nutritional factors (ANFs) or toxicants (Cellini *et al.*, 2004). However, the phenomenon of unintended changes is not unique to GM plants, but occurs in traditional plant breeding as well (Kuiper *et al.*, 2003; Larkin & Harrigan, 2007).

Since 1996, the adoption of GM crops has increased rapidly. In USA, which is the world's largest soybean producer, over 80% of the soybean production is now GM (Dill, 2005). In addition to the USA, the main GM producing countries are Argentina, Brazil, Canada, India and China, between them they have about 95% of the global GM crop area (James, 2007). The main GM crops are soybean (51%), maize (31%), cotton (13%) and canola (5%), modified for herbicide tolerance (63%), stacked traits (19%) or insect resistance (18%) (James, 2007). Stacked traits is the fastest growing group (66% growth from 2006 to 2007), most commonly combining herbicide tolerance with insect resistance, but also combining new output traits with one or both of these.

GM plants modified for traits such as insect resistance and herbicide tolerance are often referred to as "first generation". They often only have changed expression of a single protein, and the aim is to not cause any changes in the nutritional value compared to conventional non-modified varieties. The "second generation" are GM plants with more elaborate modifications, and the intent is often to change nutrient composition. Attempts to tailor nutrient composition are likely to give unintended effects, as further changes are made in metabolic pathways (Larkin & Harrigan, 2007). This may cause altered levels of undesirable substances, e.g. ANFs, and reduced growth in experimental animals (Böhme *et al.*, 2005; Böhme *et al.*, 2007).

1.1.2 Safety assessment of GM plants

Several concerns have been raised regarding the safety of transgenic plants. According to Kuiper *et al.* (2001), a comprehensive safety assessment includes toxicity and allergenicity testing of the novel protein, molecular characterization of the insert, evaluation of potential unintended/secondary effects and potential for horizontal gene transfer to other species. Both RRS® and MON810 used in this work have been approved as feed ingredients both in Norway and the EU. However, they are presently not being used by the Norwegian fish feed industry (Anon., 2009).

Safety testing of whole foods is problematic, as high doses cannot be given to experimental animals without introducing nutritional imbalances, and thus safety margins for intake are hard to establish (Kuiper & Kleter, 2003). Furthermore, allergenicity and toxicity testing of the novel protein is often conducted with protein produced in yeast or bacterial cultures, rather than isolated directly from the plant, this can cause differences in protein folding, glycosylation and phosphorylation (Kuiper & Kleter, 2003). A changed glycosylation pattern of a transgenic protein when expressed in GM peas has been shown to cause hypersensitivity in mice that was not observed with the native form of the protein in beans (Prescott *et al.*, 2005).

To cope with the particular nature of safety assessment of GM food/ feed (as compared to food additives or contaminants) the term "substantial equivalence" was coined by the Organisation for Economic Co-operation and Development (OECD). This concept is based on comparison of the GM plant to conventional plants with a history of safe use, and is the first step in the safety assessment (OECD, 1993). This can be described as a comparative approach, and is based on chemical analysis (EFSA, 2006). However, a chemical comparison alone has limitations, as it will often not include all known compounds, and certainly not unknown components in the plant. Non-targeted profiling of the transcriptome, proteome or metabolome are promising strategies for the detection of unintended effects, but need to be developed and validated (Kuiper *et al.*, 2003; Le Gall *et al.*, 2003; OECD, 2003; Coll *et al.*, 2008). Levels of ANFs causing adverse effects in salmon and interaction between

different ANFs are largely unknown (Francis *et al.*, 2001a), complicating the evaluation if small compositional changes are seen. Additionally, presently unknown ANFs or metabolites that might affect fish health will never be detected with a targeted, chemical approach. There is no general consensus on how and when feeding trials should be performed (Knudsen & Poulsen, 2007; EFSA, 2008).

Another issue is how to interpret differences that might be found between treatments in feeding trials. Although a response parameter is significantly different from the control group, this does not necessarily mean that it represents an adverse effect; it can simply be an adaptive response. Adaptive responses are reversible, of limited duration and differ from adverse effects by not causing injury (EFSA, 2008). Adverse or toxic responses can be reversible or irreversible, but are characterised by causing injury or damage to the animal (EFSA, 2008). It is essential to have in depth knowledge on the normal physiology of the test animal to be able to assess whether observed values are outside the normal physiological range. Further, there is the issue of false discoveries, when many different parameters are tested there is a greater risk that significant changes occur due to chance alone. Rather than only focusing on the statistical significance of isolated findings, one should make a more holistic evaluation. On the other hand, real effects might not be detected due to insufficient power of the experiment, with either too low replication or too large variance in the data material. Wilson *et al.* (2001) provide a list of questions for assessing the biological relevance of differences detected in toxicological studies, which could also be useful for GM safety assessment;

-Are the trends dose-related?

-Are they reproducible?

-Is there a relationship to other findings?

-Does the magnitude of the differences suggest that they are biologically important?

1.1.3 GM plant products in fish diets

With increasingly limited availability of fish meal, the inclusion of alternative protein sources in feeds for carnivorous fish species is a necessity (FAO, 2005). As a substantial, and increasing, proportion of the soybeans and maize offered on the world market are of GM varieties, one must look into the feasibility of using these in fish feeds. At present, the salmon industry in Norway and the EU do not use GM feed ingredients, but fish feed producers find it increasingly difficult to obtain guaranteed non-modified varieties of certain plant products (Kaushik & Hemre, 2008).

A limited number of fish feeding trials to assess GM plants as feed ingredients have been conducted (summarized in Table 1). The studies vary in their focus, as well as fish species, inclusion level and type of GM ingredients and duration of the trials. Some studies have utilized the maternal near-isogenic line of the GM plants in their control diet while others have used an un-related, conventional variety. In many studies, a fishmeal reference diet has also been included in the design to establish the normal levels of the measured parameters.

In the first fish feeding trial conducted with RRS®, catfish (*Ictalurus punctatus*) fingerlings (3g) were fed a diet containing 45% GM soybean (of the total diet) or the near-isogenic maternal line for 10 weeks (Hammond *et al.*, 1996). Fish grew to about 22g during the trial, and growth, survival, feed conversion and fillet composition exhibited no differences between the diet groups. Similarly, no differences were observed in growth, feed performance and whole body composition of rainbow trout (*Onchyhuncus mykiss*) fed RRS® for 3 months, at 15 and 30% of the total diet (Chainark *et al.*, 2006). No further health or performance parameters were measured, as the primary focus was on tracing DNA sequences in muscle.

Table 1 Overview of fish feeding trials published in the scientific literature with genetically modified (GM) plant products. Only studies evaluating the quality of GM plants as feed ingredients are included.

GM plant	Fish species	GM (%)	Duration	Main effects*	Reference(s)
RRS®	Catfish (*Ictalurus punctatus*)	45%	10 weeks	No differences	(Hammond *et al.*, 1996)
RR canola	Rainbow trout (*Onchyhuncus mykiss*)	5,10,15, 20%	**	Protein retention ↑	(Brown *et al.*, 2003)
GM lupin	Red seabream (*Pagrus auratus*)	60%	40 days	Growth ↑	(Glencross *et al.*, 2003)
RRS®	Atlantic salmon (*Salmo salar*)	17%	3 months	Spleen size ↑ Lysozyme ↑	(Hemre *et al.*, 2005; Bakke-McKellep *et al.*, 2007a)
MON810 RRS®	Atlantic salmon	12.1% 12.5%	8 months	No differences Plasma TAG ↓ Cell proliferation ↓ Glucose uptake ↑	(Sanden *et al.*, 2005; Sanden *et al.*, 2006; Bakke-McKellep *et al.*, 2008)
RRS®	Rainbow trout	15, 30%	3 months	No differences	(Chainark *et al.*, 2006)
MON810	Atlantic salmon	15, 30%	82 days	Feed intake ↓ Growth ↓ Liver size ↑ Distal intestine↑ Glucose uptake ↑ SOD ↑ CAT↓ Granulocytes↑	(Hemre *et al.*, 2007; Sagstad *et al.*, 2007)
RRS®	Atlantic salmon	15, 30%	28 days	Spleen size ↑ Plasma TAG ↓	(Sagstad *et al.*, 2008)

* List of differences between fish fed GM to fish fed non-GM plants; (↑) indicates that the parameter in question was higher in the GM fed fish, while (↓) indicates lower values in the GM-group. Further details on the changes and confounding factors in the studies are discussed in the text. **Duration of study is not clear from the paper. Roundup Ready® soy, RRS®; TAG, triacylglycerol; SOD, superoxide dismutase; CAT, catalase.

A 3-month study was conducted with Atlantic salmon fed RRS® (17% inclusion), and a diet with conventional soy and a fishmeal diet as controls (Hemre *et al.*, 2005; Bakke-McKellep *et al.*, 2007a). Relative size of the spleen was larger in the GM compared to the non-GM group, but neither were statistically different from the fishmeal control (Hemre *et al.*, 2005). There were no differences in growth, nutrient utilization or in other organ indices. Haematological parameters, plasma nutrient concentrations and leakage of organ specific enzymes to the plasma compartment showed similar values in all dietary groups (Hemre *et al.*, 2005). There was a tendency towards increased lysozyme activity in the head kidney of the GM fed fish ($p=0.06$), but no differences in soy-induced inflammations or major histocompatibility complex were detected in the distal intestine, nor in lysozyme or immunoglobulin levels in other tissues, or EROD (Ethoxyresorufin-O-deethylation) activity in liver (Bakke-McKellep *et al.*, 2007a). As the maternal line was not used as control, differences in the soy cultivars were suggested as a potential confounding factor.

The spleen was also significantly larger in GM versus non-GM fed fish in a 28-day trial with 15 and 30% inclusion of RRS®, with the maternal soy-line used in the control diets (Sagstad *et al.*, 2008). Another difference observed in this study was lower plasma triacylglycerol (TAG) levels in the GM-fed fish; a slight difference in saponin levels between the two soy qualities was suggested as the cause. There were also differences in growth, feed utilization and transcription of mRNAs coding for stress proteins between the soy-fed fish and fish fed the fishmeal reference diet, but these were independent of the soy being GM or not.

In a study conducted on first feeding Atlantic salmon for 8 months 12.5 % hybrid RRS® and 12.1% hybrid MON810 were used in the GM diets, and commercial soy and maize and a fishmeal diet were used as controls (Sanden *et al.*, 2006). Plasma TAG was found to be lower in the GM-soy fed fish compared to those fed conventional soy. Spleen weights were not reported. No differences due to GM soy or maize were seen on growth or nutrient retention (Sanden *et al.*, 2006). Investigating the intestinal tract of these fish, Sanden *et al.* (2005) reported higher cell

proliferation in fish fed the non-GM soy diet compared to GM soy. Both were elevated compared to the fishmeal reference diet. No differences were observed in intestinal indices. Bakke-McKellep *et al.* (2008) investigated active intestinal glucose-uptake and SGLT1 (sodium-glucose linked transporter) protein levels in the pyloric caeca, and found the highest levels with GM soy, intermediate levels with non-GM soy and lowest with the fishmeal diet. No differences were observed in lysozyme levels, immunoglobulin M or various digestive enzymes in the intestinal tract.

Atlantic salmon post-smolt fed GM maize (MON810) at 15% and 30% of the total diet had a significant reduction in feed intake, growth rate and final weight compared to those fed non-GM maize (Hemre *et al.*, 2007). Further, significant differences were revealed in organ indices; liver and distal intestine were both larger in the fish fed GM maize. Some differences were observed in head-kidney and spleen somatic index, but these were not dose-related, and not significantly different between the GM and non-GM groups as a whole. The same organs were evaluated histologically, but no morphological differences were seen. Maltase activity in the mid-intestinal segment of fish fed the high GM diet was higher than the high non-GM group. Uptake of glucose in pyloric caeca was also significantly higher in fish fed the GM diet. Fish health was also evaluated focusing on stress- and immune-response biomarkers (Sagstad *et al.*, 2007). Superoxide dismutase (SOD) had higher activity in liver and distal intestine, while catalase (CAT) showed significantly lower liver activity in fish fed GM maize. Heat shock protein 70 (HSP70) was significantly higher in the liver of fish fed GM maize compared to the fishmeal reference diet, while the non-GM maize group exhibited intermediate levels. The differences in activity/protein level of CAT, SOD and HSP70 were not reflected in levels of the mRNA coding for these proteins. Differential counts of white blood cells revealed a significantly higher proportion of granulocytes in the blood of fish fed GM maize. The authors suggested changes in the immune response and a mild stress response. Altered liver metabolism was also indicated based on the higher liver index and changes of CAT, HSP70 and SOD.

Lupin modified to contain increased of methionine had a beneficial effect on growth of juvenile red seabream (*Pagrus auratus*), but only when fed a protein restrictive diet, and would thus be of little practical value (Glencross *et al.*, 2003). Two lines of glyphosate tolerant canola were tested in feed for rainbow trout; one line was equivalent to non-modified canola judged by fish performance, while the other gave increased protein retention, increased body protein and moisture and reduced body lipid (Brown *et al.*, 2003). This was most likely due to higher protein content in that canola line (4% higher than the non-GM line), which does not seem to have been balanced for in the formulation of the diets (although information on this, as well as compositional analysis of the diets were lacking).

Additionally, some studies have investigated the uptake of transgenic DNA sequences in fish (Sanden *et al.*, 2004; Nielsen *et al.*, 2005; Nielsen *et al.*, 2006; Sanden *et al.*, 2007; Chainark *et al.*, 2008). These will not be discussed in detail, as tracing of dietary DNA constitutes a minor part of this thesis. In a study where Atlantic salmon were force-fed high concentrations of DNA fragments, uptake was observed in liver, kidney and blood, with the highest concentrations in liver and kidney (Nielsen *et al.*, 2005). Intravenously injected DNA fragments were detected in muscle, liver, kidney and blood in another study (Nielsen *et al.*, 2006). By means of *in situ* hybridization, transgenic DNA was identified in intestinal cells of fish fed GM soy, although only in a few of the samples examined (Sanden *et al.*, 2007). Chainark *et al.*(2008) found DNA fragments of the transgenic promoter from GM soybeans in the leukocytes, head kidney and muscle of rainbow trout fed a GM soy diet. These studies were all conducted on Atlantic salmon and rainbow trout, thus our work was conducted to see if the response in zebrafish was similar. A further dimension was added to the study by also including analysis of the rubisco (ribulose-1,5-bisphosphate carboxylase) gene which is present in large copy numbers in soybean and maize and is often used as a marker for the uptake of plant DNA in animals.

1.2 Target ingredients

1.2.1 Use of soybean products in salmon diets

Soybean (*Glycine max*) has high protein content and a relatively well balanced amino acid profile, making it a suitable ingredient for fish feeds. On the negative side, there is a long list of ANFs present in soybean, such as proteinase inhibitors, lectins, phytic acid, saponins, phytoestrogens (isoflavones), antivitamins, phytosterols, oligo-saccharides and allergens (Tacon, 1997; Francis *et al.*, 2001a).

Heat-labile ANFs generally do not pose a problem in commercial salmon feeds, as heat treatment occurs during the feed extrusion process, and for many soy products also during processing (Refstie, 2007). Extrusion is cooking under pressure, exposing the ingredients simultaneously to moisture and elevated temperature. This moist heat treatment eliminates most of the trypsin inhibitors and agglutinating lectins, while phytic acid is also reduced (Alonso *et al.*, 2000; Romarheim *et al.*, 2005).

The nutritional qualities of soy products are closely related to the processing procedure used (see Table 2), which affects protein content and levels of heat-stable ANFs. Full fat soybean meal (FFSBM) is the least processed while in soybean meal (SBM) much of the lipid fraction has been removed. These meals can either be dehulled (outer covering of the seeds removed) or not, the former slightly increasing protein content. In diets for Atlantic salmon FFSBM and SBM cause reductions in feed intake, growth and lipid digestibility at high inclusion levels (Olli *et al.*, 1994; Olli *et al.*, 1995; Krogdahl *et al.*, 2003). Additionally, inflammatory responses in the distal intestine (enteritis) are observed, and seem to be caused by alcohol-soluble components in soy, such as saponins, phytosterols, isoflavones, oligosaccharides and/or other unknown components (Van den Ingh *et al.*, 1991; Baeverfjord & Krogdahl, 1996; Van den Ingh *et al.*, 1996). Studies conducted on Chinnok salmon (*Onchyrhyncus tshawytscha*) and rainbow trout indicate that soyasaponins are responsible for growth depression and partly for the enteritis caused by intake of

soybean products (Bureau *et al.*, 1998), while studies on Atlantic salmon indicate that interaction between different ANFs are important in the latter (Knudsen *et al.*, 2007). Most of the studies have been conducted with SBM rather than FFSBM, but these generally contain the same levels of ANFs relative to protein fraction, thus one can assume similar responses to similar replacement levels at a crude protein basis (Olli *et al.*, 1994).

Table 2 Overview of different soy qualities used in fish feeds, their processing methods and selected parameters affecting their suitability for inclusion in salmon feeds.

	FFSBM	**SBM**	**SPC**
Processing	Grinded whole beans, can be de-hulled or not.	Solvent extracted with hexane (which removes most lipid)	Alcohol extracted (which reduces the carbohydrate fraction).
Protein content	42-45%[1]	50-54%[1]	65-70% [1]
Heat-stable ANFs	Oligosaccharides Saponins Phytosterols Isoflavones Phytic acid Antigens Unknowns??	Oligosaccharides Saponins Phytosterols Isoflavones Phytic acid Antigens Unknowns??	Phytic acid Unknowns??
Maximum replacement*	13%[2]	20%[3]	75%[4]

Full fat soybean meal, FFSBM; Soybean meal, SBM; Soy protein concentrate, SPC. *Percentage of crude protein that has been replaced with the different soy qualities without affecting growth in grow-out stages of Atlantic salmon. [1]Lusas and Riaz (1995). [2]Hemre *et al*. (2005). [3]Olli *et al*. (1995). [4]Storebakken *et al*. (2000).

Soy protein isolate (SPI) has a very high protein content and most fibres are removed, but still contains the alcohol-soluble components that negatively affect the fish, e.g. saponins are concentrated as the protein fraction is increased (Ireland & Dziedzic, 1986). As SPI is an expensive product it is mainly used by the food industry, not in feed for production animals. Soy protein concentrate (SPC) has lower protein content than SPI, but is alcohol extracted and thus contains low levels of alcohol-soluble ANFs. Up to 75% crude protein replacement with SPC has been shown to support

equal growth in Atlantic salmon compared to fish meal based diets, but phytase treatment appears beneficial (Storebakken *et al.*, 1998; Storebakken *et al.*, 2000). In rainbow trout, successful 100% substitution of fish meal with SPC has been reported (Kaushik *et al.*, 1995; Gaylord *et al.*, 2006), but rainbow trout seems to adapt better to soy-based diets than Atlantic salmon (Refstie *et al.*, 2000).

Variable results exist on the levels of soy which cause adverse effects even with the same quality of soy, and some studies report growth depression at the mentioned inclusion levels in Table 2 or even lower, both for SPC (Brown *et al.*, 1997) and SBM (Krogdahl *et al.*, 2003). This is probably due to different diet formulations used, and variable levels of ANFs which are often not analysed and vary between soy cultivars. Further, fish in different life stages show variable tolerance to plant ingredients (Dersjant-Li, 2002). At high replacement levels, care must be taken that diets meet the methionine requirement, and that feed is palatable and well accepted by the fish. One might also consider incorporation of or pre-treatment with phytase to increase the phosphorus availability. Even at levels where growth depression is not seen, responses in the intestine have been observed (Krogdahl *et al.*, 2003; Sanden *et al.*, 2005), which might raise animal welfare issues.

Despite these problems, soy is commonly used in commercial feeds at low to moderate inclusion levels. Compared to other plant proteins, the amino acid profile is relatively good and can be adjusted for low methionine by adding crystalline amino acids, or mixing different sources of plant material. The various soy products are also widely available. The amount of cake/ meal produced from oilseeds globally was 170 million tonnes in 2000, compared to less than 7 million tonnes for fishmeal, and whilst fishmeal production has remained relatively constant over the past 20 years, the production of oilseed meals is increasing (FAO, 2004). Among the oilseeds produced in the world, soybean constitutes 39% (Oil-World-statistics, 2005/2006), and has a lower oil and higher meal (protein) fraction than most other oilseed types.

1.2.2 Roundup Ready® soybean

Most of the GM soy grown is RRS® (modification event GTS 40-3-2), which has been on the US market since 1996 (James, 2007). The transgenic protein expressed in RRS® is 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium sp*. strain CP4 (CP4 EPSPS) (Padgette *et al.*, 1995). The CP4 EPSPS enzyme confers resistance to glyophasate (*N*-phosphonomethyl-glycine) (see Figure 1), the active ingredient in the non-selective herbicide Roundup. In addition, the inserted sequence contains the promoter cauliflower mosaic virus (CaMV) 35S, the EPSPS chloroplast transit peptide and a part of the 3´non-translated region of the nopaline synthase (NOS) terminator gene (Padgette *et al.*, 1995). The insert has later been found to also contain a portion of truncated CP4 EPSPS coding sequence and a DNA segment of unknown origin, indicating rearrangements (Windels *et al.*, 2001). The particle acceleration method was used for inserting the transgene (Padgette et al. 1995).

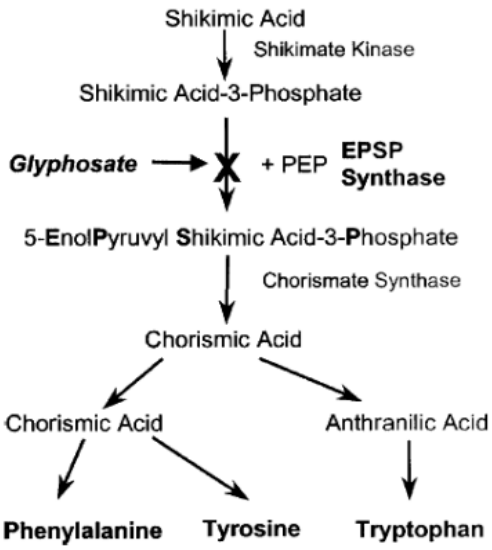

Figure 1. The mode of action of glyphosate, adapted from Dill (2005). Glyphosate harms plants by inhibiting the shikimate pathway, which produces aromatic amino acids. The enzyme EPSPS catalyzes the transfer of the enolpyruvyl moiety of PEP (phosphoenolpyruvate) to shikimate-3-phosphate. Glyphosate is competitive to PEP-binding to EPSPS. Roundup® plants have inserted an EPSPS-gene of bacterial origin (*Agrobacterium*), which is insensitive to glyphosate.

No toxic effects of CP4 EPSPS were detected in mice fed high doses and this protein does not hold typical characteristics of a food allergen (Harrison *et al.*, 1996). The compositional equivalence of RRS® to its non-transgenic parental line has been assessed by measuring proximate composition, amino- and fatty- acid profiles and

selected ANFs (Padgette *et al.*, 1996). Results on whole seeds, toasted and non-toasted defatted meals, protein concentrates, protein isolates, refined oils and soybean lecithin from GM and non-GM soy were comparable and within ranges reported for conventional soybeans. Some minor statistically significant differences were considered biologically unimportant by the authors (Padgette *et al.*, 1996). Further, RRS® sprayed with glyphosate and RRS® grown in different locations over three generations were also compositionally equivalent to non-modified soy (Taylor *et al.*, 1999; McCann *et al.*, 2005).

1.2.3 Use of maize in salmon diets

Maize (*Zea mays*) can be included in fish diets as a starch source. Starches, pectins and hemicelluloses have pellet-binding capacities (Krogdahl *et al.*, 2005), and are thus included in extruded fish feed for the sake of technical qualities. Used at low inclusion levels starch also results in protein sparing for salmonids (Hemre *et al.*, 1995a; Grisdale-Helland & Helland, 1997; Hemre *et al.*, 2002b; Krogdahl *et al.*, 2004) and even have growth-promoting effects compared with no starch inclusion (Hemre *et al.*, 1995a). As the level of starch in the feed increases, starch digestibility decreases (Aksnes, 1995; Arnesen *et al.*, 1995; Krogdahl *et al.*, 2004). The optimum level of carbohydrate for Atlantic salmon is about 10% both for smolts (Hemre *et al.*, 1995a) and during the grow-out phase (Aksnes, 1995). Maize starch performs similarly when compared to other carbohydrate sources in feed for Atlantic salmon (Arnesen *et al.*, 1995; Hemre & Hansen, 1998; Young *et al.*, 2006).

Maize gluten is a protein substance that remains after removal of most of the starch, and can be used as a protein source in fish feeds. Maize gluten is high in protein (65-69%) (OECD, 2002), but has a poor amino acid profile considering the requirements of fish, with low levels of lysine and arginine (Aslaksen *et al.*, 2007). However, maize has a low content of ANFs, apart from phytic acid (Cowieson, 2005). Gomes *et al.* (1995) found that among a range of different plant protein sources tested in rainbow trout, maize gluten had among the best values for apparent protein and lipid

digestibility. In this study maize gluten was included at 25% in the total diet (together with other plant ingredients, together substituting 66% of the fishmeal) and there was no negative effect on growth compared to a fishmeal diet. In Atlantic salmon, Aslaksen *et al.* (2007) achieved comparable protein digestibility to a fishmeal diet at 20% inclusion of maize gluten, and better digestibility than soybean, rapeseed, sunflower and oat diets. Additionally, no morphological changes were observed in the distal intestine. Mundheim *et al.* (2004) showed decreased growth with increasing substitution of fishmeal with a 2:1 mix of corn gluten and full fat soybean meal, but the experimental design does not allow us to ascertain whether one or both ingredients were responsible. In another salmon study, up to 64% substitution of fishmeal with maize gluten did not affect growth, feed conversion or protein digestibility, despite a doubling of fish weight during the trial (Mente *et al.*, 2003).

1.2.4 Bt-maize; YieldGard® (modification event MON810)

MON810 is one of several modification events producing Bt-maize, and has a gene introduced coding for the Cry1Ab-delta-endotoxin from *Bacillus thuringiensis.* This maize variety was commercialized in the U.S. in 1997 (Sanders *et al.*, 1998). Produced by microprojectile bombardement, it contains the CaMV 35S promoter and a HSP70 maize intron to control transcription of the Cry1Ab gene (Sanders *et al.*, 1998). Characterization of the genome-transgene junction sequences suggests that rearrangement involving the inserted gene has occurred (Hernández *et al.*, 2003). The transgenic protein has insecticidal properties and controls lepidopteran pests, such as the European corn borer (*Ostrinia numbilalis*). Cry1Ab acts by binding to specific receptors in the mid intestine of target insects, causing damage to the intestinal wall and resulting in death (Knowles, 1994). Administered to mice in a dose of 4000mg/kg in the feed, the Cry1Ab protein caused no signs of acute toxicity (Sanders *et al.*, 1998). Further, there was no homology with known allergenic proteins (Sanders *et al.*, 1998). However, Cry-proteins have the potential to elicit allergic responses when Bt sprays are used for pesticide control (Bernstein *et al.*, 1999), and

the GM Starlink corn expressing Cry9c was taken off the market due to concerns about allergenicity (Bucchini & Goldman, 2002).

Substantial equivalence to non-modified varieties has been shown for Bt-maize TC6275 (Cry1F) and for glyphosate tolerant corn (Sidhu *et al.*, 2000; Ridley *et al.*, 2002; Herman *et al.*, 2004). In MON810, proximate composition, fatty acid profile, calcium and phosphorus levels were similar to the non-modified, maternal line (Sanders *et al.*, 1998). Some differences were observed in levels of different amino acids, but these were not consistent across season and growth locations, thus not considered to be of biological relevance (Sanders *et al.*, 1998). However, ten Bt corn hybrids, including four MON810 hybrids were found to have higher lignin (major structural component of plant cells) content than their near-isogenic lines (Saxena & Stotzky, 2001).

Additionally, reduced use of insecticides and often reduced levels of fumonisin (a mycotoxin) in corn kernels can result in differences in nutritional value of Bt versus conventional maize (Munkvold *et al.*, 1997). Although large variability in mycotoxin is observed, the general trend is reduced levels in Bt varieties compared to conventional maize varieties, due to better resistance against *Fusarium spp*. as insect damage can predispose for contamination (e.g. Dowd, 2000; Bakan *et al.*, 2002; Papst *et al.*, 2005). This has been used to explain higher weight gain in the GM fed group in some feeding studies with various production animals fed Bt maize (reviewed by Flachowsky *et al.*, 2005). Mycotoxins are secondary metabolites produced by fungi (molds), and include a range of chemically diverse toxins. Consequences of mycotoxins in feed to production animals can be reduced growth (due to reduction in feed intake and inhibition of protein synthesis), effects on the endo- and exocrine system and suppression of the immune system (Yiannikouris & Jouany, 2002).

1.3 Fish species

1.3.1 Atlantic salmon; target species

Atlantic salmon is an important aquaculture species with a global production of 1.3 million tonnes in 2006 (FAO, 2007), and the target species of this work. Commercial salmon grow-out feeds typically contain around 35% protein, 35% lipid and 10% starch. As feed comprise 50-60% of the total production cost (Anon., 2008), it is of tremendous importance that the feed support optimal growth. Salmon is a carnivorous fish, with an intestine adapted to utilise an energy-dense diet rich in protein and low in carbohydrates (Buddington *et al.*, 1997). This causes a range of problems when plant materials are included in the feed. As salmon are so sensitive to plant components, e.g. ANFs in soy, GM plants might cause problems not observed in other animal species that are more tolerant to these ingredients. It is to a large extent unknown which ANFs cause problems, at what levels and what the interaction effects between them are (Francis *et al.*, 2001a), thus comprehensive chemical analyses of the plant materials would not enable us to conclude whether there would be a negative effect.

Soybean meal-induced enteritis in salmon increases the permeability of the distal intestine (Nordrum *et al.*, 2000), which might have an effect on uptake of dietary DNA, and thus salmon could serve as an interesting model in this regard. Additionally, fish have higher uptake of macromolecules over the intestine compared to mammals (Sire & Vernier, 1992).

Atlantic salmon is anadromous, spending early life stages in freshwater before migrating to seawater as smolts and then returning to freshwater to spawn. The parr-smolt transformation process comprises a range of preparatory adaptations for seawater entry, involving changes such as increased hypoosmotic regulatory ability, hormonal-, metabolic-, morphological and behavioural changes, and is a particularly sensitive stage in the life cycle (for review: Hoar, 1988; Boeuf, 1993). Enzyme

activity of Na^+K^+ATPase in the gills is the most reliable indicator of smolt status (Handeland & Stefansson, 2001). Seawater challenges of 24 or 96 hours followed by measuring survival and / or the chloride (Cl^-) content in plasma are also commonly used. Successful accomplishment of the parr-smolt transformation and correct timing of seawater transfer are important issues in aquaculture.

1.3.2 Zebrafish; model species

The zebrafish is a commonly used model organism in developmental, molecular and toxicological studies (Driever *et al.*, 1994; Fishman, 2001; Penberthy *et al.*, 2002; Hill *et al.*, 2005). This is not the case in nutritional studies as little work has been done, and not even basic nutritional requirements are published (Lawrence, 2007). However, by using zebrafish as a model organism, duration and cost of feeding trials can be greatly reduced. When working with GM ingredients it can be challenging to obtain proper controls (e.g. the near-isogenic motherline of the GM variety), thus it is advantageous that only small amounts are required to conduct a feeding trial. Furthermore, the fact that the zebrafish is omnivorous (Buddington *et al.*, 1997) means that more plant (GM) ingredients can be included in the diets without compromising fish health.

To what extent zebrafish can serve as a suitable model for Atlantic salmon in dietary studies is unclear, as zebrafish unlike salmon are warm-water, omnivorous fish that lives in freshwater. However, there might be similar physiological responses.

1.4 Response parameters

1.4.1 Selection of response parameters

A logical starting point in assessing the use of RRS® and MON810 in fish diets is to follow up on some results from trials referred to in section 2.1.3. It is unclear whether the differences observed in those experiments were due to the genetic modification

per se or to confounding factors such as differences in ANFs, especially when using a conventional, non-related maize or soy strain as control. Use of the near-isogenic maternal line should decrease these differences to a minimum (ILSI, 2003; EFSA, 2006). Results from those trials will not be repeated here, only some brief background information on the different organs and biological functions and some further reasons for investigating these will be given.

Furthermore, there could be effects that were not detected in these trials as the "right" parameters were not measured. When searching for responses to potential unintended changes in the GM plants, one does not know what one is looking for, thus transcriptomics or proteomics could be useful. These have been described as discovery driven (as opposed to hypothesis driven) research, as they are used to screen for differences in mRNA or protein expression levels in a particular tissue on a global scale (Aebersold *et al.*, 2000). They require no *a priori* assumptions regarding which pathways or processes might be affected (Aebersold *et al.*, 2000). As many pathways are monitored simultaneously for changes, one could possibly detect many inter-related changes, and it might be easier to assess the biological meaning than for isolated findings. The term "proteome" was coined in 1995, referring to the study of the protein complement of the genome (Wilkins *et al.*, 1996) and the field of proteomics has since seen immense developments, increasing the reproducibility, throughput and successful application also for non-model species (Fey & Larsen, 2001; Griffin *et al.*, 2001).

The use of sensitive early biomarkers will allow us to detect responses at lower doses or after shorter duration of exposure; sublethal biochemical and physiological responses precede toxicity and disease (Depledge, 1994). Early response parameters need to be assessed as they may impact on fish welfare, and can also be early signs of adverse effects with growth reduction and reduced fish health in the long term. The majority of feeding studies that have been conducted with GM feed ingredients have focused solely on production parameters (such as growth, retention and carcass yield) in trials of limited duration (Aumaitre, 2004; Flachowsky *et al.*, 2005).

1.4.2 Organ function and development

Organ indices can be useful to identify organs where alterations occur. This can give us clues about dietary imbalances or differences between dietary groups, but more in-depth analyses must be carried out to determine the cause of alterations. Changes in liver index have been observed with chemical pollutants and with elevated levels or deficiency of vitamin A (Berntssen *et al.*, 1997; Ørnsrud *et al.*, 2002; Hemre *et al.*, 2004), confirming the usefulness of organ indices. The liver is the main metabolic organ, and plays an important role in detoxification of compounds from the intestine and general circulation as well as in the immune response (Morin *et al.*, 1993; Benninghoff & Williams, 2008; Tintos *et al.*, 2008). The dominating cell type is hepatocytes and there is a rich supply of blood vessels and blood filled sinusoids. Hepatocytes of Atlantic salmon are large, regularly shape cells with typical central nuclei, and with moderate cytoplasmic glycogen content. In the fish liver there are no indications of specialized zones carrying out different metabolic tasks as we find in the mammalian liver (Kryvi & Totland, 1997). Nutritionally related changes in the liver proteome have been shown in rainbow trout due to feeding status (Martin *et al.*, 2001) and diet ingredients (Martin *et al.*, 2003; Vilhelmsson *et al.*, 2004). Proteomic analysis of liver has also been used in assessing RRS® in feed for mice (Malatesta *et al.*, 2008a).

The fish intestine serves multiple functions; digestion and absorption, endocrine regulation of appetite and digestion, electrolyte balance (osmoregulation), and immune function (Buddington *et al.*, 1997). The intestine can be divided into three parts; the proximal intestine from which the pyloric caeca originate, the mid intestine and the distal intestine. Fish lack a distinct colon, as nutrient uptake occurs along the entire intestine. However, there is a gradient with the highest rates of absorption in the proximal part and lowest in the distal (Collie, 1985; Bakke-McKellep *et al.*, 2000). Uptake of nutrients through the enterocytes (transcellular route) happens by three mechanisms; passive diffusion, ion-gradient independent carriers (facilitated diffusion) and carriers coupled to the ion-gradient (active transport) (Collie &

Ferraris, 1995). Uptake of glucose is Na^+-dependent and happens with a transporter similar to SGLT-1 in mammals (Collie & Ferraris, 1995). Absorption of amino acids happens by passive diffusion, facilitated diffusion and active transport (Mailliard *et al.*, 1995). In the distal intestine, large peptides or intact proteins can be taken up by endocytosis (Sire & Vernier, 1992). The paracellular route (diffusion of molecules between epithelial cells) is often related to situations such as intestinal pathologies or lesions of the gut surface caused by dietary components or stress (Sire & Vernier, 1992; Sundell *et al.*, 2003). The permeability of the intestine can also be affected by ANFs such as lectins, saponins or trypsin inhibitors (McLean *et al.*, 1990; Bureau *et al.*, 1998; Gatlin *et al.*, 2007). Consequently, increased permeability in the distal intestine and decreased carrier-mediated transport of glucose and amino acids were observed in salmon fed a SBM-diet (Nordrum *et al.*, 2000). Salinity also affects intestinal permeability; salmonids in seawater seems to depend more on carrier-mediated transport of nutrients than in freshwater (Collie, 1985; Nordrum *et al.*, 2000).

Diets containing SBM can cause enteritis in the distal intestine of Atlantic salmon, and decrease both total weight and mucosal fold height (Van den Ingh *et al.*, 1991; Baeverfjord & Krogdahl, 1996; Nordrum *et al.*, 2000). Morphological alterations of the distal intestine caused by enteritis include (1) widening and shortening of the intestinal folds; (2) loss of supranuclear vacuolization in the absorptive cells (enterocytes) in the intestinal epithelium; and (3) cellular infiltration of a mixed leucocyte population in the central lamina propria within the intestinal folds and in submucosa (Baeverfjord & Krogdahl, 1996). These responses seem to be time- and dose dependent, and rapidly disappear when the intestinal tract is no longer exposed to the causatory agent(s) present in SBM (Baeverfjord & Krogdahl, 1996; Krogdahl *et al.*, 2003; Uran, 2008).

The spleen consists of red and white pulp, which produces red blood cells and leukocytes, respectively (Kryvi & Totland, 1997). The head kidney is the anterior part of the kidney which does not have excretory functions, but mainly consists of

haematopoietic tissue where red blood cells, monocytes and granulocytes are produced (Kryvi & Totland, 1997). In rainbow trout, kidney was found to be the tissue with the highest concentration of lysozyme activity, followed by intestine and spleen (Lie *et al.*, 1989). Lysozyme is part of the innate immune response and is an enzyme which aids in damaging bacterial cell walls, primarily of Gram-positive bacteria (Magnadóttir, 2006). Lysozyme is present in mucous, lymphoid tissue, plasma and other bodily fluids. The innate immune response can be affected by handling, stress and dietary components. Increased lysozyme activity was found in the intestine of Atlantic salmon fed soybean molasses (Krogdahl *et al.*, 2000), and also in plasma of rainbow trout exposed to handling stress (Demers & Bayne, 1997).

1.4.3 Haematology and plasma parameters

The screening of haematological parameters is a sensitive, but not very specific, measure of fish health (Blaxhall & Daisley, 1973). Blood parameters in fish have been shown to be influenced by dietary composition and additives (Hemre *et al.*, 1995b; Rehulka, 2000; Rehulka & Parova, 2000) and by metal toxicity (Kotsanis *et al.*, 2000). Infectious diseases and stress also affect fish haematology (summarized by Sandnes *et al.*, 1988). Plasma nutrients can be used to compare nutritional status between experimental groups, and plasma enzymes for health screening. The enzyme LDH (lactate dehydrogenase) catalyses the interconversion of pyruvate (the final product of glycolysis) and lactate, the direction depending on supply of oxygen. LDH is abundant in red blood cells and many tissues, and elevated levels in plasma can indicate haemolysis or leakage from tissues (Jones, 1988). In salmon, LDH was affected by dietary starch source (Hemre & Hansen, 1998). The transaminases ALAT (alanine aminotransferase) and ASAT (aspartate aminotransferase) are located within tissue cells and have no known function in plasma. When all membranes are intact, these are present in plasma only in low concentrations (Tietz, 1976). Higher concentrations are indicative of cell damage, and plasma levels of these enzymes have proven useful in diagnosis of liver and kidney diseases in fish (Racicot *et al.*, 1975). Normal ranges of blood parameters, plasma enzymes and nutrients have been

established for Atlantic salmon (Sandnes *et al.*, 1988). Haematological and plasma parameters can also be affected by stress, which will be discussed in the next section.

1.4.4 Stress response in fish

Stress response in often divided into what happens on the physiological and at the cellular level. Stress can be an adaptive response, the goal of which is to re-establish homeostasis (at a cellular and physiological level), but severe or chronic stress can be maladaptive and have negative consequences for fish (Iwama *et al.*, 1999). Physiological stress responses in fish are characterized by an increase in stress hormones followed by physiological changes in the animal, such as increased glucose production and changes in haematology (reviewed by Iwarma *et al.*, 1998). Connections between physiological and cellular stress response are complex, and not well understood (Iwama *et al.*, 1999; Ackerman *et al.*, 2000). None of the stress-indicators used at present are 100% suitable in reflecting the state of stress in fish (Iwama *et al.*, 2004), thus a combination of different indicators may give a more complete picture.

Heat shock proteins (HSPs) are important components of the cellular stress response as they work to maintain protein integrity and function in the cell, and are elevated in response to a variety of stressors in fish (Iwarma *et al.*, 1998). As molecular chaperones HSPs have important functions also in unstressed cells in the folding, assembly, secretion and regulation of proteins, but may be upregulated to cope with stress-induced denaturation of proteins (Feder & Hofmann, 1999). HSPs are classified according to their size (molecular mass in kilodaltons, kDa), three of the major families that have been studied extensively are; HSP90 (85-90 kDa), HSP70 (68-73 kDa) and low-molecular mass HSPs (16-30 kDa) (Iwama *et al.*, 2004). Different HSPs, even within each family, can differ in inducibility, intracellular localization and function. Which HSPs are affected and to what extent varies among different stressors and tissues (Lele *et al.*, 1997; Stephensen *et al.*, 2002). Fish liver seems to be a sensitive tissue to HSP-response (Iwama *et al.*, 1999). However,

alterations in HSP levels can be difficult to interpret, as mild stress which induces HSP expression improves the ability of the fish to cope with more severe stress later on (summarized in Iwama *et al.*, 1999; Basu *et al.*, 2002). However, Mazur (1996) found that prior handling, which resulted in rise in cortisol levels, reduced the HSP70 and HSP30 response to subsequent heat shock.

Oxidative stress is also part of the cellular stress response and is caused by imbalance between the production of free radicals and the ability of the cells to neutralize these. This can cause oxidative damage to cellular components, such as oxidation of proteins, DNA and steroid components and peroxidation of unsaturated lipids in cell membranes. Free radicals are reactive compounds resulting from the metabolism in cells, which are neutralized by antioxidants. As important components in the defence against oxidative stress, antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) have been detected in most fish species examined (Ken *et al.*, 2003; Martinez-Àlvares *et al.*, 2005). There are two isoforms of SOD, CuZnSOD (SOD-1) is the soluble form that is present in the cytoplasm and nucleus, while MnSOD (SOD-2) is found in the mitochondria. SOD catalyzes the dismutation (chemical reaction in which an element is simultaneously reduced and oxidized to form two different products) of superoxide into oxygen and hydrogen peroxide (H_2O_2). The function of GPx is to reduce lipid hydroperoxides and H_2O_2 (produced by SOD) to alcohols and water, respectively. In mammals there are several different isozymes with different cellular locations, of which GPx-1 is the most abundant, but these have not been well investigated in fish (Stephensen *et al.*, 2002). Antioxidant enzyme levels in fish can be affected by dietary lipid content, antioxidants, starch sources and food deprivation (Mourente *et al.*, 2002; Pascual *et al.*, 2003; Morales *et al.*, 2004; Rueda-Jasso *et al.*, 2004). Responses to various xenobiotics, including pesticides, have been observed in many fish species, although which enzyme is altered and to what extent is highly variable between different species and pollutants (reviewed by Martinez-Àlvares *et al.*, 2005). In most studies, the enzymes are measured in the fish liver.

2. Aims:

- To evaluate effects of RRS® compared to its near-isogenic maternal line on performance and health in Atlantic salmon with a long-term study (7 months), using a relatively high inclusion level of GM soy (25%) (paper 1, 2 and 3). This includes: (1) follow up on inconclusive results from earlier trials; (2) assess GM soy as a feed ingredient in situations that might be particularly challenging for the fish, such as the sensitive parr-smolt transformation and during stress, albeit remaining within what fish will likely experience in a culture situation; and (3) evaluate if commonly observed responses in salmon to SBM inclusion in the diet might be more or less severe when the soy used is GM.

- To use proteomic profiling of liver as a tool to detect potential responses to RRS® as a feed ingredient in salmon (paper 3).

- To conduct a feeding trial on zebrafish with RRS® and Bt-maize, to investigate growth, stress response and uptake of dietary DNA and evaluate the utility of zebrafish as a model in fish feeding studies with GM ingredients (paper 4).

3. Discussion of methods

Many of the chemical methods used in this thesis are standard analytical procedures, which are described in the respective papers; these will not be discussed here. In this section, I will focus, firstly, on weaknesses/strengths of the two feeding trials, including the diets, experimental design, sampling and statistical considerations. Secondly, considerations made in the laboratory work and the data analyses of mRNA levels and proteomics are discussed. An attempt is made not to repeat too much of what is already described and discussed in the respective papers, but mainly to supplement these with additional information.

3.1 Feeding trials

3.1.1 Diets

Roundup Ready® soy, MON810 maize and their near-isogenic maternal lines were obtained from the Monsanto Company (St. Louis, USA). The ingredients were received as whole soybeans and whole maize kernels, and were ground to FFSBM and a maize meal, respectively. During processing and feed production, the non-GM variety was processed first whenever possible to reduce the risk of contamination of GM material to non-GM diets. The diets, both in the salmon and the zebrafish study, were designed to include the maximum amount of GM ingredients possible, thus were high in plant materials and indigestible residues, limiting the levels of protein and lipid obtained in the feed. For the salmon study, one of the aims was to evaluate if commonly observed SBM-effects in the intestine differed between fish fed non-GM and GM soy (paper 2), thus soy levels needed to be sufficiently high to induce these, but at a level where normal growth could still be achieved (Olli *et al.*, 1995; Krogdahl *et al.*, 2003). In the zebrafish diets, high levels of both soy and maize were included; as zebrafish are omnivorous they were expected to tolerate higher total plant levels.

3.1.2 Experimental design

The salmon trial had a simple design with only two diet groups (non-GM and GM soy), and 4 replicate tanks per diet. The trial lasted 7 months, to be able to evaluate long term effects. Four samplings were conducted, both in the fresh- and seawater stages, to follow development of the fish through the parr-smolt transformation. To assess the ability of fish in the two dietary groups to adapt and to maintain/ re-establish homeostasis when transferred from freshwater to seawater, samples were collected just before and after transfer. When three weeks remained of the trial, a cross-over design was implemented in to investigate reversibility of potential effects. At the final sampling, a "stress test" was conducted to compare stress response between the dietary groups. The evaluation of stress response and sampling just after seawater transfer were conducted to see if the diet groups also performed similarly under difficult conditions where dietary imbalances or inadequacies might be more apparent. Despite these attempts to "provoke" effects, none of the conditions were outside what salmon are expected to meet in an aquaculture setting. No fishmeal reference diet was included in the design, as effects of including soy in diets for Atlantic salmon are well studied and described, and this would greatly increase the cost of the trial, probably without contributing anything to the resolution of potential "GM effects".

When the data from the cross-over were analysed, it became apparent that there were systematic differences between fish that had been fin clipped and switched to the opposite diet group and fish that had not. These differences were unrelated to GM content, but affected diverse parameters such as relative sizes of head kidney and proximal- and mid-intestine, RBC, MCV and plasma glucose. Thus, the utility of this kind of design seems severely limited when treatment differences are small, as these will easily be masked by differences caused by implementing the cross-over itself. We would need access to twice as many tanks to implement a more appropriate cross-over design, or we would need individual pit-tagging of fish which the fish were too small for at the beginning of this trial. It is uncertain whether these cross-over effects

were due to the anaesthetic, fin-clipping, moving of tanks or a combination of these factors. No effect of fin-clipping of the adipose fin was found on growth or survival of rainbow trout (Gjerde & Refstie, 1988), while anaesthesia has been found to induce stress responses in fish (Ortuño *et al.*, 2002; Olsvik *et al.*, 2007).

In the zebrafish growth trial we used a 2x2 factorial design, with two factors (maize and soy variety), and two levels within each factor (non-GM and GM). All possible combinations were used in our four experimental diets, meaning that we could study the possible effects of maize and soy variety separately, as well as possible interactions between the two. Thus, although each experimental diet only had 3 replicate tanks, each ingredient being studied (e.g. GM soy) had 6 replicate tanks. The variables used for statistical testing were maize and soy variety, not the four individual dietary groups. Sex was included as a factor in the statistical model for parameters on which it was found to exert a significant effect. Combining GM soy and GM maize in the same diet also allowed for a higher total inclusion of GM ingredients. A reference diet was included in the design, as the protocol for making the experimental diets had not been tried on zebrafish before, and little information is published on nutritional requirements (Lawrence, 2007). Sampling was performed at the end of the feeding period.

3.1.3 Sampling

The same samples were always collected by the same person for consistency. In salmon, blood haematocrit was immediately measured on site, while the remainder of the blood samples were kept on ice/ in the fridge at 4°C until they were analysed for RBC and Hb within 48 hours. Tests done in our laboratory have shown that measurements of RBC and Hb give the same results for five days after the blood has been withdrawn, provided that anti-coagulating agent is added and the samples are refrigerated and not frozen at any point. Plasma was flash frozen after being separated by centrifugation at 4°C, to preserve enzymes until analysis. Salmon tissue samples for mRNA transcription and proteomic analyses were quickly dissected out

and immediately flash frozen in liquid nitrogen to maintain RNA/ protein quality. For the zebrafish study, the head and tail were cut off and the remainder of the body was fixed in a 10x volume of RNA*later*® (Ambion, Austin, USA) for mRNA analysis, while the fish for tracing of dietary DNA were frozen whole in liquid nitrogen. Fish were stored for up to two weeks in RNA*later*® (4°C) before dissection and RNA isolation from liver was performed. However, this storage was not the cause of the degraded RNA in this study, as test fish sampled in a similar way and isolated the next day exhibited a similar variability in RNA quality. Thus, the degradation of RNA seems to have happened either just after sampling (possibly it took too long for the RNA*later*® to completely penetrate the tissue and protect from RNA degradation), or during RNA isolation. The fact that one batch that was isolated together stood out in terms of having especially poor quality, supports the latter hypothesis. However, quality was variable from sample to sample across all batches isolated. Samples of poor quality were discarded and not used for further analyses (see section 4.2.1).

The salmon were fed until sampling (continuous feeding with automated feeders), as SBM-induced enteritis in Atlantic salmon is quickly reversed when the fish is no longer exposed to SBM and fasting causes a rapid decrease in intestinal tissue mass, protein and enzyme capacities (Baeverfjord & Krogdahl, 1996; Krogdahl & Bakke-McKellep, 2005). This was not desirable as we wanted to investigate possible intestinal changes related to GM soy. For the zebrafish, each tank was fed 3 hours before planned sampling of that particular tank, to avoid differences in feeding status. For the stress test and seawater transfer (salmon trial), time lags were also used between onset of stressor/ transfer of each tank so that every tank was sampled at the same time intervals after stress/ transfer.

3.1.4 Statistical analysis

When animals are penned together and share the same food source, each pen (in our case fish tank) and not each individual, represents the experimental unit (ILSI, 2003). Thus, when performing statistical analysis on the data one either has to use the tank

means (or pooled samples), or use a mixed model/ nested ANOVA (with tank as random effect) to account for the fact that individual fish taken from the same tank are pseudoreplicates (Zar, 1999). The latter approach was used for most of the parameters investigated in this thesis. Nested ANOVA maintains both the between-tank and within-tank variability in the analysis, the latter is lost when tank means or pooled samples are used (Ruohonen, 1998; Ling & Cotter, 2003). The nested test will often be more powerful in resolving treatment differences, as statistical power in this design can be increased either by increasing the number of tanks or the number of individual measurements made from each tank, albeit to different extents (Ruohonen, 1998; Zar, 1999).

It was necessary to pool plasma samples from each tank to obtain sufficient amount of plasma for of all the nutrients and enzymes analyses. Individual samples were only collected at seawater transfer (paper 1), as the original plan here was to analyse chloride ion content only (more analyses were conducted when this turned out to be possible). Plasma TAG was significantly different between the two diet groups at this sampling (and overall in the whole experiment), but not at each of the later samplings when plasma samples were pooled. Figure 2 giving the mean values and 95% confidence intervals of plasma TAG at each sampling, demonstrates the low statistical power obtained from pooled samples (evident by large confidence intervals). At the seawater transfer sampling, more fish were sampled and the magnitude of the difference in plasma TAG was slightly larger than at the following samplings, thus we cannot know if individual samples would have lead to significant differences also at these samplings. However, in hindsight, individual samples would have been preferable. This especially applies to the stress test (paper 2), where only 3 replicate tanks per diet obtained at each time point after stress, thus giving low resolution of both stress effects and potential diet effects for plasma nutrients and enzymes.

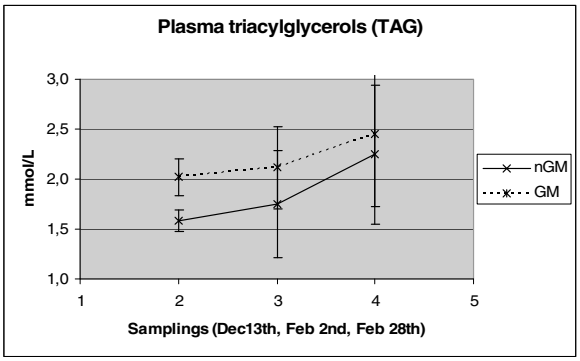

Figure 2 Plasma TAG in Atlantic salmon fed non-GM (nGM) and GM soy. The graph shows the mean value in each group for three consecutive samplings with 95% confidence intervals as error bars.

Another issue regarding the statistical analyses is the presence of confounding variables. Increased variability in the data material will lead to decreased resolution of treatment effects. However, if factors contributing to variability in the data material are known, they can be accounted for in the statistical model (Zar, 1999). This was the case for sex differences in the zebrafish study (paper 4). The effects of maize variety (non-GM versus GM) on growth and *SOD-1* expression were not detected before the sex of the fish was included in the model, as large variation in the data due to sex differences obscured the diet differences. For some of the mRNA data, the RIN value also had to be taken into account (see section 3.2.1). Sex differences have been reported in hepatic HSP70 response and in plasma glucose in juvenile Chinook salmon (Afonso *et al.*, 2003) and in RBC, Hb, Hct, MCV, MCH and MCHC in immature rainbow trout (Rehulka *et al.*, 2004). These are all parameters that were measured in our salmon study (paper 1 and 2), and if there were sex differences in any of them, we could have increased the statistical power of our data by accounting for these. However, the gonads were not developed yet in these fish, thus sex would be difficult to determine.

Particular considerations made in the statistical analyses of mRNA and proteomics data are discussed in the respective sections (3.2.4 and 3.3.2).

3.2 mRNA analysis

3.2.1 RNA quality

High RNA quality is essential to obtain reliable mRNA transcription results. There are two aspects of RNA quality; sample purity and RNA integrity. Purity is assessed by the 260/280 absorbance ratio (protein contamination) and the 260/230 ratio (contamination by organic compounds). The purity can be improved by diluting the sample, or by ethanol extraction. RNA integrity, on the other hand, cannot be improved; sampling procedures must be optimized to minimize degradation in the first place. Integrity can be assessed with a Bioanalyzer (Agilent Techonologies, Palo Alto, USA). In the channels of the bioanalyzer chip, RNA samples are separated according to their molecular weight and then detected by laser-induced fluorescence (Schroeder *et al.*, 2006). The ratio between the 28S and 18S peaks or manual interpretation of the spectra have previously been used to assess integrity, but these methods are inconsistent (Imbeaud *et al.*, 2005; Schroeder *et al.*, 2006). Thus, an algorithm has been developed that gives a RIN number based on the electrophoresis spectrum for each sample, to objectively assess RNA quality in a consistent manner between different laboratories (Schroeder *et al.*, 2006). A RIN value of 10 means that absolutely no degradation has occurred, while a value of 0 means that the sample is completely degraded. RNA degradation can affect Ct-values, giving lower Ct-values (thus higher apparent mRNA levels) in samples with better RNA integrity (Bustin & Nolan, 2004; Imbeaud *et al.*, 2005; Fleige *et al.*, 2006). This can also be the case after normalization to reference genes (Fleige *et al.*, 2006). A RIN-value higher than 5 is recommended for RT-PCR, while higher than 8 is even better to reduce variability introduced in the data by variable RNA quality (Fleige & Pfaffl, 2006; Fleige *et al.*, 2006). Further, amplicon sizes below 200bp are recommended (Fleige *et al.*, 2006).

In my experiments, RNA quality was good in the salmon samples, but variable in the zebrafish samples. Different sampling methods and RNA isolation protocols were

used for the two trials (see papers for details). Ethanol precipitation was performed on the zebrafish samples, as their purity was found insufficient. Further, samples with a RIN value below 6 were discarded and for all genes correlation between the RIN number and the normalized relative expression in the samples was tested. Based on these results, the RIN value was included as a covariate in the statistical model for three of the five genes. The fact that not all the genes were affected might mean that some RNA sequences are more prone to degradation than others.

3.2.2 Primer design

For the primers that were designed for this work, a minimum of three primer pairs were made and tested for each gene. Conserved regions for primer design were located using the sequence alignment tool ClustalW2 on sequences from different species (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and primers were either designed from mRNA sequences or intron segments were avoided by locating open reading frames (http://www.ncbi.nlm.nih.gov/gorf/). Primer Express® 2.0 (Applied biosystems, Foster City, USA) was used for design, and BLAST searches were performed in the NCBI database to check specificity. Primers were tested with a one-step RT (reverse transcriptase)-PCR kit (Qiagen, Hilden, Germany) on a GeneAmp® PCR 9700 machine (Applied Biosystems), followed by electrophoresis (100V for 1 hour) in 2% agarose gel next to a 50bp ladder to ensure that a single product of the expected size was amplified. SybrSafe (Invitrogen, Life Technologies, USA) was used in the gels to allow visualization of the amplification products, which were photographed with a Gel Doc 2000 (Bio-Rad laboratories, Hercules, CA, USA). Further, RT followed by real-time PCR of dilution curves for calculation of efficiency and melting curve analysis was done on GeneAmp® PCR 9700 machine and LightCycler® 480 System (Roche Applied Science, Indianapolis, USA) with SYBR® Green I chemistry. The best primer pair was selected for each gene, or new primers were designed if none were deemed acceptable.

3.2.3 Normalization to reference genes

Relative quantification is commonly used in analysis of PCR data, and depends on normalization of target genes to stably transcribed reference genes. This is done to control for variations in the initial amount of material, and possible differences in inhibitors and amplification efficiency among samples (Bustin *et al.*, 2005). However, transcription of reference gene mRNA may also vary, and choice of reference genes must be validated for each situation to ensure that they are not affected by the experimental treatment (Bustin *et al.*, 2005; Huggett *et al.*, 2005). Use of the "wrong" reference gene can give false results regarding the up- and down-regulation of the target genes (Tricarico *et al.*, 2002). One needs at least three reference genes to assess their stability by comparing expression patterns. NormFinder (Andersen *et al.*, 2004) and geNorm (Vandesompele *et al.*, 2002) are used in the evaluation of reference genes, both have their strengths and weaknesses (Bustin *et al.*, 2005). When stable reference genes are identified, a normalization factor obtained by geometric averaging of these is used for normalization of the data (Bustin, 2002; Vandesompele *et al.*, 2002).

Hence, for my work, potential reference genes that had shown good stability in salmon and zebrafish, respectively, were selected from the literature. Three or four potential reference genes were run in each case, so their stability could be evaluated for my specific experimental situation. Based on these results, all the tested reference genes were included in the normalization index in all three cases (paper 2, 3 and 4).

3.2.4 Statistical analysis of mRNA data

There have been discussions on how to perform statistical analysis of gene transcription data, as the normalized relative quantities are ratios and thus cannot be expected to have a normal distribution (Pfaffl, 2001). However, if one log-transforms the quantities, a normal distribution will often be achieved (needs to be verified by a normality test), thus one might consider standard parametrical procedures. The other option is non-parametrical statistics, such as Kruskal-Wallis ANOVA or permutation

tests. In the latter category, REST© (relative expression software tool), has been specifically developed for handling gene transcription data (Pfaffl *et al.*, 2002). This software compares treatment groups by a pair-wise fixed reallocation randomization test, which randomly re-assigns the data to the treatment groups. The number of times a difference occurs that is equal to or larger than what is seen in the data is used to calculate the p-value. No distributional assumptions are made in this test; the sole assumption is that treatments are randomly allocated. The amplification efficiency of each gene is taken into account and you can normalize target genes to multiple reference genes. However, there are limitations; a nested design is not possible (tank effects are not taken into account), interplate variability if samples are spread on more than one plate are not corrected for, and confounding factors cannot be included in the statistical model.

Both REST©2005/REST©2008 (Corbett Life Science) and ANOVA with log-transformed values were used on the data in paper 2 and 3 (Table 3). The two methods yielded similar results; the only clear deviation was HSP27 in the liver. Here, the p-values from ANOVA were much closer (0.21/ 0.016) to the permutation test if the tank effect was not included (no nesting), suggesting some tank influence, although the tank effect was not significant. In paper 4, sex of the fish and RIN value needed to be accounted for in the statistical model for some of the genes, thus I chose to use only ANOVA on log-transformed values for these data.

Table 3. P-values obtained with REST and nested ANOVA of log(2) of normalized relative expression. Heat shock protein (HSP)70 from paper 2 is not included as these data were not normally distributed even after log(2) transformation.

Paper	Gene	p-value, REST	p-value, ANOVA
Paper 2	HSP27 (intestine)	0.82/ 0.002*	0.86/ 0.023
Paper 2	HSP27 (liver)	0.11/ 0.006	0.44/ 0.11
Paper 3	Calreticulin	0.27	0.30
Paper 3	Alpha-enolase	0.53	0.46
Paper 3	TPI	0.54	0.51
Paper 3	TPP	0.37	0.32

Triosephosphate isomerase, TPI; Thymidine phosphorylase precursor, TPP. *diet/ time.

3.3 Proteomics

The proteomic analyses were completed at the Aberdeen Proteome Facility, in collaboration with Dr. Sam Martin, who has experience in using proteomic tools in dietary studies with salmonids (Martin *et al.*, 2001; Martin *et al.*, 2003; Vilhelmsson *et al.*, 2004; Martin *et al.*, 2007) and Dr. Phil Cash, who has extensive experience in proteomics (e.g. Cash, 1998; Cash, 2000; Cash & Kroll, 2003; Cash, 2008). This section is focused on 2D gel electrophoresis and data analysis. Protein identification is not included, as this topic is thoroughly discussed in paper 3.

3.3.1 Gel electrophoresis

Initially, all samples were run on 1D gels, to ensure consistent quality and determine sample loading amount for subsequent 2D gels (Cash *et al.*, 1995). Then, optimization was carried out using small 2D gels before running the experimental gels described in paper 4. These preliminary gels were run to ensure that we would get a good resolution of the proteins in two dimensions, before the more expensive large gels were run. They were run with and without prior RNase and DNase treatment of the protein samples to determine whether to include this step in the protocol or not. Immobilized pH gradient (IPG) 7cm strips (pH 4-7, GE Healthcare) and precast Zoom®gels run in a SureLock™ kit with NuPAGE®Mops buffer (all Invitrogen) were used for the first and second dimension, respectively. Similar procedures were used as for the experimental gels described in paper 4. A molecular weight marker (SeeBlue® Plus2 Pre-Stained Standard, Invitrogen) was used.

From the experimental gels, a total of 781 protein spots were used for analysis. This clearly does not represent a "total view" of liver protein expression, and is due to limitations of the 2D-gel method. Hydrophobic proteins do not readily dissolve in the buffers, very alkaline or acidic proteins are outside the chosen pH-range of the IPG-strip, and weakly expressed proteins are not detected due to limitations in linear range (Fey & Larsen, 2001). Fractionation of the protein sample and use of several IPG

strips of different pH range for each sample could have been used to improve this situation, but this would require more gels per sample. Additionally, the fractionation process introduces an additional source of variation for later quantification (Fey & Larsen, 2001). The goal of my study was not to detect as many proteins as possible, but rather to have good statistical power to resolve differences between diet groups. The selected pH-range was based on previous experience of Dr. Sam Martin from rainbow trout liver; he had found few proteins with isoelectric point below 4, and poor resolution of individual proteins above 7. We chose to maximize biological replication, as this is more useful than technical replication to increase precision of results (Chich *et al.*, 2007). Nearly 800 proteins represent a substantial proportion of the activity in the liver, and should be sufficient to detect any major metabolic disturbances caused by the diet.

There are continuous developments in the field of proteomics, and a recent improvement in 2D gels is the 2D-DIGE (digital image gel electrophoresis) technology. With 2D-DIGE, up to three samples (for example from two treatment groups and a reference sample that is run in all your gels) are labelled with fluorescent dyes and run together in the same gel. Due to the elimination of technical gel to gel variation, 2D-DIGE offers increased sensitivity (Marouga *et al.*, 2005), which will increase the usefulness of proteomics when small fold differences are of interest. The 2D-DIGE technology would have been preferable to use for my study, but was unfortunately not available at the time in the laboratory where I was working.

3.3.2 Data analysis

When a large number of hypotheses are tested (or the same hypothesis of no difference is tested for a large number of individual spots), both false positives and false negatives will occur (Chich *et al.*, 2007). There are two approaches for dealing with false positives; the family-wise error rate (FWER, also known as Bonferroni correction) and the false discovery rate (FDR) (Benjamini & Hochberg, 1995). With FWER, we set the significance threshold for each hypothesis so low that there is only

5% chance of making a single mistake, which results in very few false positives, but is very strict and leads to many false negatives. A trade off between missed findings and false discoveries is more easily made with the FDR, which is the ratio of false positives to rejected hypotheses (ratio of significant spots expected to be false discoveries, often denoted q). The cut-off point is thus selected at the level of false discoveries one is willing to accept within a particular experimental design (Chich *et al.*, 2007). The protein spots that were significantly different between the two diet groups in my study (paper 4) had a large probability of being false discoveries. However, all the necessary information for the readers to critically evaluate the results are given in the article and although these data cannot be used to draw firm conclusions about affected pathways, they might serve as a starting point for further studies.

The level of replication I used in my study is hard to evaluate, as there are no good methods for calculating power or sample size for proteomic experiments (Kim *et al.*, 2004). Statistical power depends on sample size (replication), variation and magnitude of difference between the treatment groups. As the observed variation was different for each spot on the gel, one cannot define the necessary number of replicates to be able to detect differences of a specific magnitude. Thus, the power calculations done in this experiment were different from spot to spot.

4. Discussion of results

The aim of this discussion is to combine the results from all four papers that are part of this dissertation, and discuss them in relation to the stated aims. I will start with an evaluation of the diets, followed by discussions on growth performance, intestinal-, liver- and immune function, health and stress in fish fed GM-diet. As most of the work was conducted on salmon and GM soy, this will be the main focus, but the zebrafish results, both with GM soy and GM maize, is brought into the discussion whenever applicable. Furthermore, use of proteomics as a tool to elucidate effects of GM feed ingredients to salmon will be discussed, as well as detection of dietary DNA in fish tissues and the use of zebrafish as a model.

Differences between sampling time points, stress responses that were similar between dietary groups, comparison of the experimental diets to the commercial reference in the zebrafish trial etc., are issues that are unrelated to the GM content of the diets, which is the focus of this thesis. These are discussed in the respective papers, and will not be discussed any further here.

4.1 Feed ingredients and diets

The near-isogenic maternal line grown under similar conditions as the GM crop, which we were able to obtain for these trials, is the recommended control (ILSI, 2003; EFSA, 2006). However, there were differences in the proximate composition of the soy lines used in both the salmon and the zebrafish trial (a separate batch for each of the two trials, both received from Monsanto Company, St. Louis, USA). In both cases crude protein level was higher and lipid level lower in the GM compared to the non-GM soy, within that batch. Additionally, there were differences between the two batches. However, all values were within the range of conventional soybeans (OECD, 2001, ILSI Crop Composition Database: www.cropcomposition.org). Macronutrient compositions in the non-GM and GM maize used in the zebrafish trial

were similar, however, this does not exclude differences in ANFs, mycotoxins etc. Variations in nutrient composition between Bt-maize and the near-isogenic maternal line have been observed by others (Aulrich *et al.*, 2001). This shows that it almost impossible to obtain completely identical plant cultivars for comparison, despite having the maternal line. The question then arises on how to interpret minor differences found in measured parameters in the experimental animals. How can one distinguish whether these are due to the genetic modification *per se*, or due to unrelated differences in soy cultivars that might vary from one growing season or location to the next? Faced with this dilemma, it might be a preferable approach to use several commercial non-GM cultivars as controls in the safety assessments of GM ingredients. Thus, one could establish if the parameters measured in animals fed GM plants are within the normal range of animals fed unmodified crops (ILSI, 2003). The disadvantage is that this would greatly increase the cost of trials, as well as the use of experimental animals.

Extensive nutritional analyses were carried out to ensure compositional equivalence of the diets. Still, there will always be compounds in the feed that are not analysed and small differences in the dietary ingredients exist that cannot be balanced in the feed formulation. The soy used in the zebrafish trial has previously been used in a salmon trial, and was analysed for a long list of ANFs (Sagstad *et al.*, 2008), but not for phytoestrogens which I have suggested might cause some effects observed in zebrafish (paper 4). ANFs were not analyzed in the soy used in the present salmon trial, due to the expense associated with the analyses and that it would not be possible to balance the dietary composition of these anyway. Additionally, the difference in amount of "residue" was 0.6% between the GM and non-GM soy, covering the difference in ANF and fiber contents, and several of the methods used to identify various ANFs (e.g. saponins) have a high variation. The maize used in the zebrafish trial has previously been analyzed for phytic acid (Sagstad *et al.*, 2007), and maize is known to have a low content of other ANFs (Cowieson, 2005). Mycotoxins, which I have suggested as a possible explanation for some differences seen in the zebrafish trial, were not analysed. Unexplained differences between fish fed GM and non-GM

maize have also been seen in a previous salmon trial (Hemre *et al.*, 2007; Sagstad *et al.*, 2007), thus it would be interesting to see if mycotoxins might cause these effects. There are numerous different mycotoxins that can potentially be present in the maize/ feeds and there is limited knowledge regarding the effects of mycotoxins on fish. Mycotoxin effects in a dose-response design have been evaluated in catfish (Lumlertdacha *et al.*, 1995), but only in much higher doses than what normally occurrs in maize (Yiannikouris & Jouany, 2002).

Neither the maize nor the soy used in the zebrafish trial contained detectable residues of the herbicides analysed. However, glyphosate, which is the most likely candidate to have been used on the GM soy, was not measured as we do not have this analytical method available. Glyphosate is quickly broken down into a range of different degradation products, which complicates analysis (Kudzin *et al.*, 2002), but is reported to be a benign herbicide with low toxicity to vertebrates (Williams *et al.*, 2000; Cerdeira & Duke, 2006).

4.2 Growth performance

Growth performance is of tremendous economic importance in aquaculture operations, and is thus an important factor in the evaluation of GM feed ingredients. No overall differences in growth, body composition or condition factor were observed between the two diet groups in the salmon trial (paper 1). Significantly higher weight of the GM fed fish at a single sampling point followed by significantly higher growth rate in the other group until the next sampling, suggests that this was a random sampling effect. Similarly, whether the soy was GM or not had no effect on growth in the zebrafish trial (paper 4). As the effect on feed intake was not supported by changes in growth or feed conversion, it seems to be of little biological relevance. Due to the small particle size excess feed could not be collected. Although we tried to avoid overfeeding as best we could by patient hand feeding to satiation, some inaccuracy in the feed intake estimates would be expected. This lack of effect on

growth of RRS® is supported by earlier salmon trials (Hemre *et al.*, 2005; Sanden *et al.*, 2006; Sagstad *et al.*, 2008), and also trials with catfish and rainbow trout (Hammond *et al.*, 1996; Chainark *et al.*, 2006).

Maize variety, on the other hand, affected zebrafish growth; better growth was achieved with GM maize. Condition factor was similar for all dietary groups, while retention was not measured due to limited sample material. In a study with Atlantic salmon fed the exact same batch of GM maize as used in this study, reduced growth was seen in the GM group, caused by a somewhat lower feed intake (Hemre *et al.*, 2007). The feed intake in the zebrafish trial was not affected by GM maize, but as already mentioned, feed intake estimates are not considered completely reliable. Further, the weight was measured on an individual basis and analysed taking the sex of the zebrafish into account, while feed intake was estimated on a tank basis, giving lower statistical power.

The discrepancy of our results with those obtained by Hemre *et al.* (2007) is hard to explain, but could be due to differences in species, stage in the life cycle, duration of the trials, maize inclusion levels or different storage time of the maize. The salmon study was performed in 2004, while the current zebrafish study was conducted more than two years later. However, the maize was stored at -30°C, therefore assumed to be of acceptable quality after this long storage. Different levels of mycotoxins have been used to explain higher weight gain in the GM fed group in three studies with broilers, broiler chicks and piglets respectively (reviewed by Flachowsky *et al.*, 2005). "Hotspots" of mycotoxin contamination, with the toxins not being homogenously distributed, are common, both in the field and during storage (Lopez-Garcia *et al.*, 1999), which might have caused the differences between the two studies. Different methods of feed processing were not likely to have affected mycotoxin levels, as they are chemically stable and not inactivated by heat treatment (EFSA, 2004a, 2004b).

Concluding on growth performance, both feeding trials in this thesis as well as earlier studies show no effect of RRS®, while results on MON810 maize are unclear and should be investigated further.

4.3 Intestinal function

The gastro-intestinal tract is the first site of exposure to GM feed ingredients, thus potential diet effects might be seen here. Soy in the feed is known to affect particularly the distal intestine in salmon (Van den Ingh *et al.*, 1991; Baeverfjord & Krogdahl, 1996; Nordrum *et al.*, 2000), and it would be interesting to see if the severity of these alterations change when the soy is GM. As evaluations of the intestine were only carried out in the salmon trial, the following discussion is based on that experiment.

The relative sizes of the intestinal segments (proximal-, mid- and distal intestine) were measured at all samplings (paper 1). No differences between the dietary groups were seen on the proximal intestine. An effect on the distal intestine was only observed at one sampling; there was no similar trend at the other samplings and no difference overall in the trial. This suggests either a random occurrence or an adaptive response in that particular developmental stage (early seawater). Adaptive responses are characterised by being reversible and of limited duration, and are not considered to be adverse (EFSA, 2008). The parr-smolt transformation is a period where substantial changes occur in the intestine, which is a major osmoregulatory organ and is changing its role from preventing inflow of water in the freshwater stage to actively absorbing ions and water in the seawater stage (Sundell *et al.*, 2003).

In contrast to the distal intestine, the lower weight of the mid intestine in the GM group exhibited a consistent pattern through all samplings and was significant overall in the experiment. Explanations are not evident in the existing data material, and no differences between the dietary groups were detected by histological evaluation (paper2). No similar effect on the mid intestine has been observed in other studies with RRS® fed to Atlantic salmon with either a non-GM commercial soy line (Hemre *et al.*, 2005) or the maternal line as control (Sagstad *et al.*, 2008). According to EFSA (2008), differences are neither considered to be of any toxicological relevance nor to represent adverse effects as long as the values are within the normal physiology of the species. Intestinal indices of Atlantic salmon have been reported both in the

freshwater and early seawater phase (Hemre *et al.*, 2005; Sanden *et al.*, 2005), but as these change so rapidly through development, comparisons are hard to make.

Inflammatory responses were observed in the distal intestine in both dietary groups at all three time points (paper 2), consistent with typical SBM-induced changes previously (enteritis) described in Atlantic salmon (Van den Ingh *et al.*, 1991; Baeverfjord & Krogdahl, 1996; Van den Ingh *et al.*, 1996; Nordrum *et al.*, 2000; Bakke-McKellep *et al.*, 2007b). The observations that mucosal fold heights were lower (significantly so at one sampling) and the degree of mucosal fold fusion more pronounced in fish fed GM compared to non-GM soy may indicate that the GM variety caused elevated inflammation status. These differences could be caused by differences in ANF concentrations, as ANFs are known to vary extensively between different strains of soy (OECD, 2001) and can also vary between GM-soy and its near-isogenic maternal line (Sagstad et al, 2008). Salmon fed diets with 20% SBM grown in 6 different locations exhibited significant variations in all enteritis-related parameters evaluated in the distal intestine (Uran *et al.*, 2008), confirming that differences between soy strains can affect the severity of changes. Nor can differences in feed intake (not measured in this trial) be entirely ruled out as a contributing factor. No differences in proliferation (mitotic bodies) and apoptosis were observed between the diet groups, in contrast to previous findings that a larger number of cells were undergoing proliferation when fish were fed conventional compared to GM soy (Sanden *et al.*, 2005). However, different detection methods were utilized, and the near-isogenic line was not used in the former study.

Concluding on intestinal responses, this is the organ where most diet-related changes were detected, confirming its sensitivity to dietary components and the utility of investigating responses in the intestine. However, the histological observations of the distal intestine were consistent with typical SBM-induced changes. These changes appeared in both diet groups, and the slightly more pronounced response could easily have been caused by small differences in ANFs. The differences between the diet groups cannot be characterized as adverse.

4.4 Metabolism and liver function

No differences in liver indices were observed at any sampling in the salmon trial (paper 1). In the zebrafish trial, livers were not weighed, as the fish were fixed directly in RNA*later*® for subsequent RNA isolation (paper 4). However, the concentrations of the resulting RNA samples (obtained from the homogenization of the whole liver in a fixed volume) were measured, and can probably serve as a gross measure of relative liver size between the diet groups. The liver RNA yield tended to be higher in fish fed non-GM compared to GM soy ($p=0.06$), and there was a significant interaction effect between soy variety and sex ($p=0.003$). The females in the non-GM soy group seemed to have larger livers than the females in the GM soy group, while this was not the case for the males. There was also an interaction effect between soy variety and sex on liver *SOD-1*, with lowered transcription levels in females but not males fed GM soy. These interactions indicate a difference between the non-GM and GM soy affecting the male and female zebrafish differently, for instance by affecting sex hormones.

Soy isoflavones are phytoestrogens known to affect sex hormones and reproduction in mammals (Bennetts *et al.*, 1946; Adlercreutz *et al.*, 1987; Setchell *et al.*, 1987; Duncan *et al.*, 1999), and have been shown to induce vitellogenesis in sturgeon (*Acipenser baeri*) but not in rainbow trout (Pelissero *et al.*, 1991; Latonnelle *et al.*, 2002). Kaushik *et al*. (1995) observed a trend (not significant) of increased circulating vitellogenin in rainbow trout fed increasing levels of soy, while plasma vitellogenin was increased in catfish fed soybean compared to a control diet (Kelly & Green, 2006). The commercial zebrafish diet we used as a reference (Adult zebrafish diet, Harlan Teklad, Madison, USA) does not contain alfalfa or soy protein in order to minimize phytoestrogens, according to the manufacturer.

Soy isoflavones are products of the shikimate pathway, which is the target of the herbicide glyphosate. It has been hypothesized that isoflavone content may be reduced in glyphosate tolerant soy (like RRS®) (Duke *et al.*, 2003). However, differences have not been found when comparing glyphosate treated to hand-weeded

RRS® (Duke *et al.*, 2003), nor when comparing RRS® to its non-modified maternal soy line (Padgette *et al.*, 1996; Taylor *et al.*, 1999; McCann *et al.*, 2005). Generally, there are huge variations in isoflavone content between locations and growing seasons, and the variability in conventional soybeans seem to be more affected by environmental factors than by genetics (OECD, 2001). Isoflavone levels were not measured in our diets, and thus it remains speculation whether they are to blame for the observed effects. If they are, the fact that others have not found isoflavone levels to differ between GM and non-GM soy, shows that this is at least not a consistent difference between RRS® and conventional varieties, although it might have been different in this particular case (possibly depending on when and in what amounts herbicides were applied, or on random differences). Another hypothesis for this effect can be herbicide residues, as the herbicide Roundup has been shown to affect estrogen synthesis in mammalian cell lines (Richard *et al.*, 2005). The reason why no effect on liver size was observed in the salmon study could be either due to the fact that the salmon were juvenile and not going into sexual maturation and/ or differences in the soy batches used for the respective trials.

The soy batch used in the zebrafish trial has previously been used in a salmon trial where lower levels of plasma TAG in the GM fed fish were observed (Sagstad *et al.*, 2008), while in the present salmon trial plasma TAG was higher in the GM group. The discrepancy suggests that the effect on TAG was not a GM-effect, but rather related to variations in levels of anti-nutritional factors, antigens, metabolites or other unknown factors in the plants. Replacement of animal protein with soy protein has been shown to reduce plasma cholesterol and TAG in mammals (Iritani *et al.*, 1986; Carroll & Kurowska, 1995). The same is the case with soy substituting fishmeal in diets for rainbow trout and seabass (*Dicentrarchus labrax*) (Kaushik *et al.*, 1995; Dias *et al.*, 2005). Among the proposed candidates for causing the hypocholesterolemic effects of soy are isoflavones, saponins (Potter, 1995) and phytosterols (Orzechowski *et al.*, 2002). Saponins were higher in the GM group with lower TAG in the study by Sagstad et al. (2008), while isoflavones and phytosterols were neither measured in that study nor in the current study.

There are reports that alcohol-soluble components in soy (which include saponins, isoflavones, phytosterols and oligosaccharides) reduce lipid digestibility in Atlantic salmon (Olli & Krogdahl, 1995), and elevated muscle cholesterol has been observed in tilapia (*Oreochromic niloticus*) given *Quillaja* saponin mixture in the diet (Francis *et al.*, 2001b). Kaushik et al. (1995) reported decreased cholesterol both with SPC and soyflour, however, there was a stronger effect with the latter. Isoflavone content was analysed in both soy ingredients and shown to be less than half in the SPC compared to the soyflour. However, this does not necessarily mean that isoflavones are to blame, as other alcohol soluble ANFs such as phytosterols and saponins presumably were reduced as well. Despite the difference between the two diet groups in our salmon study, the TAG levels for both groups were within the range of values reported for healthy salmon of similar size (Hemre *et al.*, 2002a; Nordgarden *et al.*, 2002), not suggesting any adverse metabolic effect. Further, no differences in plasma glucose or proteins were observed at any of the samplings.

Liver protein expression in salmon was screened by proteomics to reveal potential effects on metabolism or other processes in the liver (paper 3). No clear results were obtained, as the detected fold differences between the diet groups of individual protein spots were minor and the number of statistically significant spots was not higher than what could have been expected to occur by chance alone. The fold changes were very low compared to liver proteome studies investigating substitution of fishmeal with plant protein and comparing different plant proteins in rainbow trout (Martin *et al.*, 2003; Vilhelmsson *et al.*, 2004) or evaluation of GM soy fed to mice (Malatesta *et al.*, 2008a). Furthermore, the dietary groups were not distinguishable by principal component analysis (PCA), which explores structures in the data matrix and displays the data according to the main sources of variance. This indicates that other sources of variation, such as individual or technical variation, were more prominent than dietary effects. Thus, the proteomics data could not detect any biologically meaningful differences between the livers of salmon fed non-GM or GM diets for 7 months.

The FDR of 0.44, observed for the most significant spots in this study, cannot support conclusions of an adverse diet effect. However, the results could be used to identify potentially interesting proteins for further investigation or comparison with other studies or measured parameters. The identified protein spot up-regulated in the GM group was calreticulin and the two down-regulated spots were thymidine phosphorylase precursor (TPP)/ alpha-enolase and triosephosphate isomerase (TPI). In the case of TPP and alpha-enolase that were identified from the same spot, it is not possible to determine whether one or both of these proteins contributed to the down-regulation of this spot. The level of mRNA coding for these four proteins were not differentially regulated, which either could indicate that they were in fact random occurrences (false discoveries) or it could reflect the low correlation often observed between protein and mRNA levels (Anderson & Seilhammer, 1997; Pratt *et al.*, 2002; Heijne *et al.*, 2003; Conrads *et al.*, 2005; Kuo *et al.*, 2005; Link *et al.*, 2006). Thymidine phosphorylase plays a role in nucleic acid homeostasis, participating in the pathways of purine and pyrimidine metabolism, is involved in angiogenesis and is elevated in tumours and chronically inflamed tissues (Brown & Bicknell, 1998). Alpha-enolase (also known as enolase-1) is a glycolytic enzyme present in most tissues, and is one of the most abundantly expressed cytosolic proteins (Pancholi, 2001). Calreticulin performs two major functions in the endoplasmatic reticulum (ER) of non-muscle cells; chaperoning and regulation of Ca^{2+} homeostasis through modulating storage and transport, and is induced by a large range of stressors (Michalak *et al.*, 1999).

The down-regulation of TPI we observed in our study corresponds well with the fact that TPI was also down-regulated in liver of mice fed 14% RRS® compared to a commercial soy line as control during a two-year study (Malatesta *et al.*, 2008a). The 2.2 fold change observed in that study was greater than the 1.3 fold change in our data, but this may be due to the species investigated or duration of the trial. This highlights TPI as an interesting protein that should be investigated in further studies, with possible cross-species relevance. TPI is a glycolytic enzyme that catalyzes the conversion between glyceraldehyde 3-phosphate and dihydroxyacetone phosphate,

and it is essential for efficient energy production by being involved in several metabolic pathways (Knowles, 1991). In an earlier feeding trial with Atlantic salmon, GM soy affected intestinal glucose absorption compared to non-GM (non-maternal line) (Bakke-McKellep *et al.*, 2008), which may again affect liver glucose metabolism.

Further, histological evaluation of livers from the salmon trial showed no structural alterations in either dietary group (paper 2). The sole difference observed between the dietary groups was decreased glycogen deposits in liver of GM fed fish. This may indicate a mild effect on metabolism, also supported by the higher plasma TAG levels in the GM fed group (paper 1). Our evaluation of salmon liver showed minor changes compared to studies on mice fed GM soy, revealing nuclear modification of hepatocytes and indications of increased metabolic rate in early life stages and possibly an accelerated ageing during senescence (Malatesta *et al.*, 2002; Malatesta *et al.*, 2005; Malatesta *et al.*, 2008a). Additionally, glycogen deposits in liver of mice fed GM and non-GM soy were not reported to be different (Malatesta *et al.*, 2008a), thus there seems to be few similarities with our results, apart from the previously mentioned down-regulation of TPI. The origin of the soy used in the mice studies was not described, but the control was not the maternal line, suggesting that the GM and non-GM soy were obtained from different sources and thus grown under different conditions. Further, an *in vitro* study suggested that glyphosate residues might be to blame for the observed effects (Malatesta *et al.*, 2008b). Glyphosate affects liver metabolism and increases leakage of ALAT and ASAT, both in rats (Benedetti *et al.*, 2004) and various fish species (Nešković *et al.*, 1996; Szarek *et al.*, 2000; Jiraungkoorskul *et al.*, 2003), although the fish were exposed by immersion rather than oral intake. Apart from the lowest concentration used in the rat study, concentrations were far above what has been measured in soy plants and grains (Arregui *et al.*, 2003). Increased leakage of ALAT and ASAT to the plasma compartment was not observed in our salmon study (paper 1), as will also be discussed in the next section, giving no indication of liver damage.

Minimal effects on the abundance of individual liver proteins suggest that GM soy can be used in fish feeds with no adverse effect on liver function in salmon. Combined with no difference in growth and that plasma TAG was within the normal range and the difference between the diet groups is likely not related to the genetic modification, one can conclude that there are no adverse effects on metabolism. However, the difference in TPI in the salmon liver, differences in glycogen content and the sex-interaction effects observed in the zebrafish may be interesting to investigate further and potential effects on reproduction and sexual maturation should be evaluated.

4.5 Health and immune function

This discussion is based on the salmon trial, as no health assessments were made of the zebrafish. The spleen and head-kidney, which are both important lymphoid organs in fish, exhibited no diet related alterations when evaluated histologically (paper 2). Further, there were no diet related differences in the relative size of these organs at any sampling (paper 1), thus not supporting earlier findings of larger spleen in salmon fed GM soy (Hemre *et al.*, 2005; Sagstad *et al.*, 2008). Lysozyme levels in both spleen and head-kidney were similar between the diet groups (paper 1). This is in agreement with Bakke-McKellep *et al*. (2008), who found no differences in lysozyme levels between salmon fed GM and non-GM soy, while another study showed a tendency toward elevated lysozyme in head kidney of salmon fed GM soy (Bakke-McKellep *et al.*, 2007a).

Further, there was no effect on the proportion of lymphocytes and granulocytes in the blood (paper 1), contrary to what has been shown in salmon fed Bt-maize (Sagstad *et al.*, 2007). The plasma enzymes ALAT, ASAT and LDH were not different between the dietary groups (paper 1). Despite deviation from reference values reported in adult salmon (Sandnes *et al.*, 1988), the values found in the present study were similar to those reported in salmon at the same developmental stage (Hemre & Hansen,

1998), indicating healthy liver and kidney function in both dietary groups. Finally, haematological parameters were not affected by GM soy.

In this study, GM soy did not cause any effects on fish health that differed from conventional soy. The lack of confirmation of observations from previous studies (spleen size and lysozyme level in head kidney), suggests that these were caused by other factors than the genetic modification.

4.6 Stress response and seawater transfer

During the course of the salmon trial, the fish were exposed to demanding situations, where the ability to maintain or rapidly re-establish homeostasis was compared between diet groups. The parr-smolt transformation is a physiologically challenging and energetically demanding adaptation process, thus additional strain on the fish by diet might become apparent. However, rapid adaptation after seawater transfer was seen in fish fed both the GM and the non-GM diet (paper 1), based on Na^+K^+-ATPase activity in the gill tissue and plasma concentration of chloride ions after transfer, both widely used methods to describe smolt development (McCormick *et al.*, 2002).

Nearly all the haematological parameters measured in the present study were significantly affected both by seawater-transfer, cross-over of dietary groups and the stress-test (paper 1 and 2, details on the changes are further discussed in the respective papers), supporting that these serve as a sensitive, although not very specific, measure of fish health (Blaxhall & Daisley, 1973). Yet, there were no effects on blood parameters of fish being fed GM compared to non-GM soy over a period of 7 months, neither in the freshwater nor the seawater stages. Furthermore, all responses in blood parameter were similar between the dietary groups, indicating that fish fed non-GM or GM soy were equally affected in the different situations and had equal ability to re-establish homeostasis after stress. Similar responses in the two dietary groups to the stress test on plasma nutrients and the levels of mRNA coding for the stress proteins HSP27 and HSP70 in liver and distal intestine (paper 2), further

support this view. The finding of the present salmon study that there was no dietary effect on HSP70 transcription in the liver is in agreement with the data from zebrafish fed non-GM or GM soy (paper 4) and earlier results in salmon (Sagstad *et al.*, 2008).

The zebrafish were not challenged with additional stress, but if there were negative effects of being fed GM plants, this may have constituted a source of stress. Significantly increased *SOD-1* mRNA level in liver of fish fed non-GM maize was found (paper 4). In a previous salmon study with the exact same batch of maize, *SOD-1* level in liver was equal between the diet groups, but total SOD enzyme activity (includes both the soluble isoform, SOD-1, and SOD-2 in mitochondria) was higher both in liver and distal intestine in the GM fed salmon (Sagstad *et al.*, 2007). Further, the apparent increase (although not significant) in *HSP70* level in the liver of non-GM maize fed zebrafish, differ from the previous study by Sagstad *et al*. (2007). In the latter study, no differences were seen at the mRNA level, while HSP70 protein was increased in the liver of salmon fed the GM maize compared to a reference diet, while the non-GM maize diet exhibited intermediate levels. Species differences may explain the different responses in salmon and zebrafish. Additionally, both the length of the trials and of the life-cycles of salmon and zebrafish are very different, complicating comparisons. Neither the observed differences in *SOD-1* nor in growth in the zebrafish study would have been revealed without taking sex into account, which was not determined in the salmon study.

The transgenic protein Cry1Ab was not detected in the diets, probably due to denaturation during heat treatment of the feeds (De Luis *et al.*, 2008). This would possibly exclude effects of the Cry1Ab-toxin, but there might also be other unintended differences between the GM and non-GM maize. As for the growth differences, mycotoxins could be a possible confounding factor. Various mycotoxins have been shown to increase *HSP70* level in rat brain, placenta of pregnant rats, foetal rat liver and cell cultures (Sehata *et al.*, 2004a; Sehata *et al.*, 2004b; El Golli *et al.*, 2006), and increased *SOD-1* in rat brain (Sehata *et al.*, 2004a), but decreased in the liver of pregnant rats (Sehata *et al.*, 2004b).

4.7 Proteomics as a tool

Routine dentification of proteins from a 2D-gel depends on the quality and availability of databases. As the peptide mass can change drastically if only one or a few amino acids are different between species, it can be challenging to work with peptide mass fingerprinting in other species than "model species". Although the use of tandem mass spectrometry, where partial amino acid sequences are derived, have improved the situation, sequence availability in databases is still crucial. The four protein spots excised from the 2D gel in our study were identified with a high level of confidence (paper 3). Although this was a limited number of spots, the success rate probably reflects the increased sequence information available and technological advances, making proteomics increasingly useful for non-model species. This is definitely promising for further proteomics work with Atlantic salmon.

Screening methods such as proteomics have clear advantages compared to selecting a limited number of parameters to measure, as genetic modification of plants can result in unintended effects that are hard to guess *a priori,* and thus hard to predict what the consequences for the fish might be. There are also problems inherent in such an approach. When comparing such a large number of different proteins for changes in expression, there are bound to be random differences due to normal biological variation. The minor differences (small fold changes and high false discovery rates) observed in the proteomic liver analysis in paper 4, reflected the similar performance of the two diet groups in all other measured parameters. These data greatly increase my confidence in the conclusion that feeding salmon diets containing RRS® rather than conventional soy causes no adverse effects.

It would potentially be more interesting to use proteomics on tissues from fish where other dietary effects were more apparent, such as from a trial with GM maize fed to zebrafish or salmon. In a situation of clear differences between diet groups, differentially expressed proteins might be used to identify affected pathways, and proteomics would probably be more useful than in the current situation of minor dietary differences. However, differences detected by proteomics cannot be identified

as adverse, as the quantification is only relative, and we do not have knowledge about the normal ranges of various liver proteins. Targeted studies will be required to determine what observed differences might mean for the organism, and whether or not they constitute adverse effects.

4.8 Tracing of dietary DNA

The fact that dietary DNA was detected in all investigated tissues of the zebrafish (paper 4), support previous studies which have shown that dietary DNA withstands fish feed processing (Sanden *et al.*, 2004; Chainark *et al.*, 2008) and can be taken up in the intestine and distributed to various tissues of the fish (Nielsen *et al.*, 2005; Sanden *et al.*, 2007; Chainark *et al.*, 2008). There are similar findings of DNA stability and uptake in the digestive tract from studies with other animals (Schubbert *et al.*, 1997; Doerfler & Schubbert, 1998; Schubbert *et al.*, 1998; Einspanier *et al.*, 2001; Reuter & Aulrich, 2003; Tony *et al.*, 2003; Mazza *et al.*, 2005; Rossi *et al.*, 2005; Sharma *et al.*, 2006). Although transgenic DNA has been the focus of many studies investigating the fate of DNA in the gastro-intestinal tract, persistence and intestinal uptake of DNA is not a specific property of transgenic DNA. This was shown by detection of the endogenous rubisco gene from maize and soy in the present zebrafish trial, and has also been shown by others (Hohlweg & Doerfler, 2001; Phipps *et al.*, 2003; Reuter & Aulrich, 2003). The high copy numbers of the rubisco genes is probably the reason why these genes were detected more frequently than RRS® and MON810 DNA fragments. The lack of difference in uptake of rubisco between the dietary groups, -shows that the GM insert does not modify the uptake of other dietary DNA fragments. This is in agreement with Mazza *et al*. (2005) who found no difference in uptake of specific maize genes when comparing piglets fed non-GM or MON810 maize. Similar findings have also been reported by others (Reuter & Aulrich, 2003; Rossi *et al.*, 2005).

Presence of DNA fragments from MON810 in the intestine may be explained by residual feed, and it cannot be ruled out that the presence of MON810 DNA in one liver sample could be due to contamination from the intestine during sampling of the zebrafish. Contrary to the higher level of the RRS® than MON810 DNA fragments in the diets, only MON810 and not RRS® was detected in the intestine. This is possibly due to some sequence specificity in the DNA uptake, which would also indicate that the DNA fragments from the intestine had been taken up by the fish and were not from residual feed, or alternatively, there might be differences in stability of different DNA fragments in the intestine, dependent on DNA sequence, methylation patterns or similar. The feed matrix can also influence the persistence and stability of DNA in the intestine (Straub *et al.*, 1999; Palka-Santini *et al.*, 2003). The present results give no reason to suspect that transgenic sequences are taken up more frequently than regular plant DNA, or that this uptake causes any negative effects for the fish. However, the mechanisms and possible function of dietary DNA uptake in the intestine are unknown, as are potential negative or positive consequences of this uptake.

4.9 Zebrafish as a model

The attempt to use zebrafish as a model for the evaluation of GM ingredients showed promise, but also revealed some challenges. Dietary effects (even differences in growth, which is often considered to be a late response) were observed after only 20 days of feeding, which makes zebrafish feeding trials rapid and inexpensive compared to salmon trials. Only small amounts of the feed ingredients were required to for the experiment, which is a huge advantage as availability of pure varieties of the GM in question is limited. If several conventional varieties were to be used as controls, the expenses of the feeding trials would increase, especially studies on salmon, but not as much for zebrafish trials. In regard to tracing of dietary DNA, results in zebrafish corresponded well with what has been reported both in salmon and in other species (see section 4.8).

However, when comparing results from the zebrafish trial to a previous salmon trial performed with the exact same batch of maize, the results corresponded poorly, suggesting that we cannot use zebrafish and automatically conclude on what the effects would be in salmon. Further, parameters commonly evaluated in fish nutrition studies cannot always be measured in zebrafish. For organ indices, haematology and plasma analyses, the small size of the zebrafish presents an obvious problem. Large sex differences were revealed, and as zebrafish early go into sexual maturation, this cannot easily be avoided if a doubling of weight is wanted during the trial. However, the short life-span and early maturation of zebrafish can also present opportunities, as shown by the soy variety/ sex interaction effects revealed in paper 4. Furthermore, molecular tools are more abundant for zebrafish, as the entire genome is sequenced and gene annotation and protein databases are better developed. Thus, one could use zebrafish to screen for potential molecular markers that would later have to be verified for use in salmon.

5. Conclusions

- Roundup Ready® soy seems to be an equally good feed ingredient for Atlantic salmon as non-GM soy, based on fish performance and health. Alterations observed in previous studies with GM soy fed to salmon were not confirmed in this work. This lack of reproducibility indicates that confounding factors, such as variation in ANFs, might cause these effects. No effect of GM soy was detected in stress response or ability to cope with seawater transfer. Slight differences were observed in the severity of SBM-induced changes in the intestine, but these were restricted to effects typically observed in SBM-fed salmon. Several feeding studies have now been conducted and numerous parameters investigated, many of which are considered to be early biomarkers of effects on fish health and performance. Despite some unexplained differences between the dietary groups, no adverse effects of feeding GM soy have been identified in salmon.

- Proteomics showed potential as a tool in the safety assessment of GM ingredients, although this would probably be more apparent if fish experiencing more dietary effects (such as salmon or zebrafish fed GM maize) were investigated.

- Bt-maize (MON810) resulted in better growth and there were indications of reduced stress levels compared to non-GM maize in the zebrafish trial. These results are inconsistent with an earlier salmon trial, thus further studies are warranted to conclude on MON810 maize. Even a slight growth depression can have considerable economic consequences in aquaculture, and stress can have implications for animal welfare. The difference in results between RRS® and Bt-maize both in this trial and in previous work, clearly shows that one cannot take results from the safety assessment of one GM plant and extrapolate to conclude on any other GM ingredient. The results on tracing of dietary DNA give no reason to suspect that uptake in the fish intestine of transgenic DNA is

more frequent than uptake of conventional DNA, although differences seem to exist in either the degradation or the uptake frequency between different DNA sequences. While the results on detection of dietary DNA indicate that zebrafish can be a useful model species, this is more unclear regarding the evaluation of diet ingredients.

6. Future directions

- Adequate control diets already present a challenge in GM safety assessments, but this problem will be increasing in the future. Stacked event GM plants (crosses of two or several modification events) and "second generation" GM plants with intended changes in nutrient composition, are becoming more widespread (James, 2007). This adds new issues to the safety assessment, as a comparators for substantial equivalence are not available and more unintended effects can be expected (OECD, 2003; Flachowsky *et al.*, 2005). This might also provide plant feed ingredients that are better suited for aquafeeds in terms of their amino- (e.g. maize with enhanced lysine content) and fatty acid compositions or the presence of ANFs, thus nutritional benefits also need to be evaluated.

- The major focus of this thesis was on soy, with a minor focus on MON810 maize. Further studies are required on maize, as there were clear dietary effects and contradictory results have been observed between trials.

- Of the 19 GM plants that are presently approved to be used as feed ingredients in Norway, only two (RRS® and MON810) have been tested on salmon. The difference in results for these two support individual assessment also of other GM varieties. Case-by-case assessment has been advocated by others (Kuiper *et al.*, 2001; Larkin & Harrigan, 2007), and is the norm both in Norway (Food Safety Authority), and the EU (European Food Safety Authority). New GM varieties need to be tested, and the development of suitable model systems to reduce cost and duration of trials would aid in this work.

- Screening techniques such as micro array and proteomics should be used both on fish fed Bt-maize and in the evaluation of new GM ingredients, particularly those with intentional changes in nutrient composition, to identify possible unintended effects and identify affected pathways for further study.

- Increased knowledge about some of the possible confounding factors (such as differences in levels of ANFs, mycotoxins and herbicide residues), and how they affect fish would help distinguish these effects from potential "GM effects".

- Despite numerous studies showing that intestinal uptake of dietary DNA occurs, and that DNA can be detected in various organs for some time after feeding. However, the potential function of this uptake and potential consequences, negative or positive, are unknown. Increased knowledge in this area would be useful for elucidating potential consequences of transgenic DNA, if any.

- Possible impact of fish feed remnants and fish feces containing GM plant material on the surrounding marine environment, including marine crustaceans, has not been investigated. The freshwater crustacean *Daphnia magna* fed MON810 compared to non-GM maize exhibited higher mortality, lower proportion of females reaching sexual maturation and an overall lower egg production (Bøhn *et al.*, 2008). This maize was not heat treated, and feed extrusion would denature transgenic Cry-proteins, possibly excluding harm to aqueous crustaceans. However, this should be investigated, and might be a relevant issue particularly in areas of the world where moist aquafeeds are commonly used.

7. References

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