Genomic responses of salmonids to pathogenic agents and nutritional interventions

Genomiske responser på patogene agens og foringsmessige intervensjoner hos salmonide

Philosophiae Doctor (PhD) Thesis

Stanko Škugor

Department of Animal and Aquacultural Sciences
Norwegian University of Life Sciences

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Abstract

Aquaculture exposes salmonid fish to diverse stressors, including pathogens and inadequate nutrition. The identification of molecular basis underlying compromised health and growth caused by these factors is confounded by the complexity of induced responses and simultaneous action of multiple stressors. The goal of this thesis was to improve knowledge of several adverse conditions by means of functional genomics. The molecular processes associated with disease resistance and fast growth were investigated with an aid of the salmonid cDNA microarray (SFA.2) enriched with genes involved in stress and immune responses. Genome-wide gene expression profiling was followed with qPCR confirmation of the most important findings. A similar design of experiments was applied. The selection of organs for analyses was based on pilot microarray screening that detected the most informative responses from a wider range of tissue samples. The microarray and qPCR analyses of the transcriptomes revealed the gene expression changes behind the studied phenotypes.

Paper I addressed the molecular determinants underlying high susceptibility to sea lice in Atlantic salmon. Systemic nature of responses and previously unknown role for adaptive immunity were revealed. Alternatively activated macrophages and predominant type 2 differentiation of T helper (Th) cells were observed in response to the parasite. However, this early activation was followed by general immunosuppression in all organs while cellular stress markedly increased in damaged skin. Further, the observed gene expression profile supported the notion of delayed healing of wounds inflicted by sea lice.

Vaccine-dependent determinants of protective responses in Atlantic salmon against the causative agent of furunculosis, *Aeromonas salmonicida* were investigated in Paper II. The results suggested that tight regulation of the inflammatory response is beneficial for the host. The selective induction of complement components in vaccinated survivors was the only observed enhanced effector immune mechanism. Importantly, elevated expression of genes involved in repair and prevention of immune-mediated damages and in clearance of endogenous and exogenous toxic compounds were associated with survival.

In Paper III, the ability of β-glucan lentinan from shiitake mushroom to modulate inflammation induced by injected lipopolysaccharide (LPS) was tested in rainbow trout. Lentinan dampened the expression of numerous proinflammatory genes in spleen whose activation could have detrimental consequences for the host. Interestingly, cross-experimental

comparison revealed remarkable similarities between responses of lentinan fed fish and those observed in salmon resistant to *A. salmonicida* (Paper II) and infectious salmon anemia virus (ISAV) [1]. Thus, avoiding immunopathology by selective suppression of potentially harmful immune responses was predicted for LPS-challenged trout pre-conditioned with lentinan.

Paper IV examined the effects of three dietary regimens based on: 1) partial fish meal replacement by extracted soybean meal, 2) feeding of control diet at reduced ration and 3) the combination of the interventions, feeding of soybean meal based diet at reduced ration. Highly similar transcriptional responses to soy and reduced feeding were revealed while the joint treatment unexpectedly resulted in weaker expression changes of the majority of hepatic genes. The observed changes in a number of metabolic pathways indicated a high level of conservation of responses to restricted feeding between the cold-blooded and warm-blooded vertebrates. The study identified twelve nutrition-responsive candidate biomarkers but no specific differences between groups. Slower growth could be beneficial for health of salmon due to down-regulation of genes involved in inflammation and stress responses.

The performed studies indicated the regulations of gene expression that can determine the ability of fish cells and tissues to sense and adapt to a wide range of challenges. The identified differentially expressed genes are involved in both generalized and specific responses. Notably, the results illustrate highly contextual nature of biological responses exemplified by inflammation, which is regarded as a "double-edged sword". Th2-modified response was linked to high susceptibility to sea lice in salmon (Paper I) while fish with excessive inflammatory responses succumbed to furunculosis (Paper II). Results from Paper III were in line with these findings; detrimental consequences from LPS exposure due to overly activated inflammation could be predicted for fish not fed with lentinan. The three studies with pathogenic agents thus illustrate the paramount importance of properly balanced immune responses and their subsequent regulation. Slower growth might be favourable in this sense, as the attenuated gene expression of cellular stress markers and potentially dangerous inflammatory mediators was shown in Paper IV.

List of papers

I. Local and systemic gene expression responses of Atlantic salmon (*Salmo salar* L.) to infection with the salmon louse (*Lepeophtheirus salmonis*)

Škugor S, Glover KA, Nilsen F, Krasnov A.
BMC Genomics 2008 Oct 23; 9:498

II. Hepatic gene expression profiling reveals protective responses in Atlantic salmon vaccinated against furunculosis

Škugor S, Jorgensen SM, Gjerde B, Krasnov A.
BMC Genomics 2009 Oct 30; 10(1):503

III. Modulation of splenic immune responses to bacterial lipopolysaccharide in rainbow trout (*Oncorhynchus mykiss*) fed lentinan, a beta-glucan from mushroom *Lentinula edodes*

Djordjevic B[1], Škugor S[1], Jørgensen SM, Overland M, Mydland LT, Krasnov A.
Fish Shellfish Immunol 2009 Feb; 26(2):201-9
[1] equally contributing authors

IV. Gene expression profiling reveals similar hepatic responses to restricted feeding and extracted soybean meal in diets for Atlantic salmon (*Salmo salar* L.)

Škugor S, Helland B, Refstie S, Afanasyev S, Vielma J, Krasnov A
Submitted

Abbreviations

ALAS2	5-aminolevulinate synthase
AP-1	Activator protein 1
C/EBPs	CCAAT-enhancer-binding proteins
CD	Cluster of differentiation
cDNA	Complementary DNA
CF	Fishmeal based diet at full ration
CpG	Cytosine-phosphodiester-guanine
CR	Fishmeal based diet at reduced ration
CREB	cAMP response element binding
DNA	Deoxyribonucleic acid
dpi	Days post infection
ECM	Extracellular matrix
ER	Endoplasmic reticulum
i.p.	Intraperitoneally
IFN	Interferon
IL	Interleukin
IL13Rα2	Interleukin 13 receptor a 2
IL-1RA	IL-1 receptor antagonist
ISAV	Infectious salmon anemia virus
JAK	Janus kinase
JNK	Jun N-terminal kinase
LECT2	Leukocyte cell-derived chemotaxin 2
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
PAMP	Pathogen-Associated Molecular Patterns
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E2
PGG-glucan	Poly-[1-6]--D-glucopyranosyl-[1-3]--D-glucopyranose glucan
PRRs	Pattern recognition receptors
qPCR	Real-time quantitative PCR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SF	Extracted soybean meal diet at full ration
SFA.2	Salmonid fish array 2
Sp1	Sp1 transcription factor
SR	Extracted soybean meal diet at reduced ration
STAT	Signal Transducer and Activator of Transcription
TAP	Transporter ATP-binding cassette protein

TGF	Transforming growth factor
Th	T helper
TLR4	Toll-like receptor 4
TNF	Tumor necrosis factor
UPR	Unfolded Protein Response

1. Introduction

Prior to the "omics" revolution, perturbations in biological systems were assessed with functional assays or by determining expression changes in one or few genes or proteins. The establishment of genomic resources promoted development of high-throughput gene expression screening tools for various species [2], which in turn increased power of molecular biology approaches. Expression profiling or transcriptomics examines the levels of mRNAs in a given cell population, commonly with an aid of high density DNA microarrays. Measurement of the genome-wide gene expression profiles in tissue samples by microarrays provides unparalleled opportunities to investigate the changes induced by external stimuli and to gain knowledge of biological variation within populations. Untargeted screening of transcriptomes is free from assumptions of which genes and pathways are involved and thus represents a "hypothesis-free" approach, which is especially useful for studies of poorly explored traits and conditions.

In addition to mechanistic insight, global gene expression profiling is used for the discovery of biomarkers associated with the studied condition. Expression signatures are used in medical and veterinary sciences as diagnostic and prognostic biomarkers and to monitor disease progression and drug response or to design individualised therapies and treatments [3]. The increasing availability of genomic resources for various pathogen species and their hosts enabled the use of functional genomics' tools for studies of infectious diseases by analyzing global variations in gene expression that occur during infection on both sides of the host–pathogen interaction [4]. Such improved understanding of protective responses and pathogenesis is expected to make an essential contribution to vaccine development. Biomarkers are widely used in fish biology for diagnostics of infectious diseases and stress caused with abiotic factors, social interactions and environmental pollution [5-7]. Animal and fish breeding will also benefit from functional genomics research. The traits of interest, such as disease resistance to a specific pathogen, are often difficult and costly to measure. The usual dead-or-alive-based endpoints recorded in most challenge tests are insufficient to adequately assess resistance [8]. Multiple expression profiling enables assessment of resistance with much higher resolution. One of the future challenges in this area is the identification of resistance biomarkers which would decrease and eventually eliminate the need for challenge tests with live pathogens. Furthermore, nutritional transcriptomics will

increase our knowledge of the interaction between physiological and immunological processes on one side and dietary factors on the other [9].

The topics addressed in this thesis were selected by practical importance and need of further research. Farmed salmonids are exposed to a wide range of environmental stressors and pathogens, which negatively effect growth and survival. Farmed fish completely depend on provision of formulated food, while high stock densities increase chance of encounter with pathogens. Upon transfer to sea, smolts are exposed to the physiological stress of osmoregulating in saline waters and may be particularly vulnerable to additional challenges, including reduced access to feed due to newly formed social hierarchy, switch to a different diet and encounter with novel pathogens.

Aquaculture is poised to overtake capture fisheries as a source of food fish. The reported aquaculture production in 2006 was ca 51.7 million tonnes and it is expected that by 2015 production will reach ca. 70 million tonnes and 80–90 million tonnes in 2030 [10]. In order to achieve this ambitious goal, the whole sector will face great challenges. The ability to detect effects of various negative factors and manipulate resistance and growth with an aid of selective breeding, vaccination and nutritional interventions is therefore of great importance for aquaculture. The achievement of this goal requires more efficient diagnostic tools and better understanding of pathogenic and protective responses and of nutritionally induced changes.

Dissection of generalised responses and those that are specifically elicited under the studied conditions were the main research objectives of this thesis. This task is complicated with diversity and complexity of changes in responding organisms. For this reason, the application of genome-wide microarray technology, which makes it possible to monitor a large number of cellular processes simultaneously, is highly relevant in this research area. The four presented studies were based on experiments with dietary stresses and pathogens that confront Atlantic salmon in the aquaculture environment and with vaccines and immunomodulatory agents:

1. Molecular determinants of Atlantic salmon's susceptibility to sea lice. The sea louse (*Lepeophteirus salmonis*) is an ectoparasite that poses a threat both for farmed fish and wild salmonid populations. The knowledge on local and systemic responses and protective and pathogenic reactions was limited at the time when the experiments were initiated.

2. Molecular mechanisms behind the differential protection from vaccination against *Aeromonas salmonicida* in Atlantic salmon. Gram-negative *Aeromonas salmonicida* is a causative agent of furunculosis. Vaccination confers incomplete protection and molecular underlying of defence and pathology are largely unknown.

3. Immunomodulation of responses to lipopolysaccharide (LPS) by dietary β-glucan lentinan. The use of immunostimulants in aquaculture is limited by the abilities to detect their effects on the immune system. Lentinan, extracted from shiitake mushrooms, was evaluated in the rainbow trout LPS inflammation model.

4. Molecular mechanisms behind reduced growth caused by restricted feeding and extractable soybean meal in diets for Atlantic salmon. Adverse effects of dietary factors are well documented but molecular mechanisms behind the associated growth retardation are not fully understood.

A brief background for these topics is presented in the next six chapters.

1.1. Characteristics of the salmon louse infection

Ectoparasitic sea lice are currently the most problematic parasites for the salmon farming industry and wild salmonid populations [11]. The salmon louse, *Lepeophtheirus salmonis* (Krøyer, 1837), is the most important louse species in the North Atlantic, which infects fish in their marine phase; parasitizing has been recorded on salmonid species in the genera *Salmo, Oncorhynchus*, and *Salvelinus*. Chemotherapy and management strategies such as fallowing (single-year class farms) still play an essential role in sea louse control. This appears to be insufficient as only in a period from 2007 to 2009, the number of lice has approximately tripled in Norwegian waters (www.lusedata.no). Additionally, salmon lice from farmed fish serve as reservoirs for the pathogenic *A. salmonicida* bacterium and are implicated as potential vectors for other infectious agents [12]. Hence, alternative strategies to control salmon louse are required, such as the use of functional feeds or breeding programs for parasite-resistant hosts.

The life cycle of salmon louse is complex and consists of ten life-stages: two planktonic nauplii, one infective copepodid, four sessile chalimii and mobile stages that comprise two pre-adult and one adult stage [13]. After the final molt, females mature into adults that continuously produce eggs, which give rise to free-living planktonic stages before settlement on a host. Salmon louse development is strongly dependent on the temperature [14]. The parasite feeds on host mucous, skin tissue and blood; sessile stages feed around the site of attachment while mobile stages move freely over the host's skin. The damage to the skin is mainly superficial during chalimus stages, however, juvenile lice were shown to induce significant changes of immune and stress responses [14]. Pre-adult and adult stages feed more aggressively. Blood then becomes a more important food component, particularly for adult females [15]. This leads to osmoregulatory dysfunctions and may cause mortality, particularly in smolts [16]. Mortality can also occur following the moult into pre-adults even without the development of open lesions [17, 18], and is possibly mediated by PGE_2 produced by lice [19].

The rates of lice development depend on the host species. For instance, the parasites develop faster and produce more eggs on Atlantic salmon than on chinook salmon (*Oncorhynchus tshawytscha*) (see [20] and references therein); this is suggested to be due to differences in hosts' nutritional status. Species-specific immune responses to lice attachment and feeding were demonstrated as well [21-24]. Coho salmon (*Oncorhynchus kisutch*) is the most resistant

species that mounts a quick immune response accompanied with marked skin epithelial hyperplasia [22, 25]. The population of infiltrated immune cells is predominantly composed of neutrophils. In contrast, Atlantic salmon initiates a very mild inflammatory response and shows no apparent tissue responses to attached lice [22, 25]. More pronounced inflammation in pink salmon (*Oncorhynchus gorbuscha*) compared to chum salmon (*Oncorhynchus keta*) was associated with the more rapid lice rejection [23]. Hyperplastic and inflammatory responses in the epidermis and underlying dermal tissues at sites of lice attachment are the accepted biomarkers of resistance. Gene expression studies revealed that sea lice infection induces responses in internal immune organs as well [26, 27]. Intraspecific comparisons revealed some of the involved molecular players associated with the improved chalimus expulsion. Namely, the early gene expression of pro-inflammatory cytokines [interleukin (IL)-1β, IL-8 and tumour necrosis factor-α (TNF-α)] in head kidney was associated with protection [23].

Weak host responses can be due to immune suppression by infection-induced stress. In fact, coho salmon implanted with cortisol showed increased susceptibility to *Lepeophtheirus salmonis* [28]. Johnson and Albright [28] reported that the treatment resulted in the decreased inflammatory response and less epithelial hyperplasia. The fact that host responses develop at sites of attachment as soon as the parasites become detached and at sites in the nearest proximity of sites of active attachment suggests that stress is not the only responsible factor for attenuated tissue responses in Atlantic salmon [29]. In addition, infections with low number of lice can cause changes in the physiology and immunology of the host both in presence and absence of a cortisol stress response [27]. Trypsin proteases and PGE_2 have been identified in the secretions of *L. salmonis* and in the mucus of infected Atlantic salmon [19, 30, 31]. Collectivelly, these observations support the hypothesis that the identified secreted molecules of salmon lice are immunomodulatory and pathogenicity factors. Thus far, it can be concluded that host susceptibility to parasites is due to the suppression of protective immune responses, osmoregulatory dysfunction and stress.

1.2. Vaccination against *Aeromonas salmonicida salmonicida*

All species of salmon, trout, charr and grayling and in addition, a number of non-salmonid hosts are susceptible to infection with the gram-negative bacterium *Aeromonas salmonicida*

[32, 33]. There are at least four subspecies and *A. salmonicida salmonicida* is known as the typical pathogenic strain [34]. It induces furunculosis and causes severe septicaemia, which eventually results in mortality. High water temperatures and physiological stress contribute to the occurrence of disease [33, 35]. The transmission occurs mainly by the pathogen's entry into gills, mouth, anus and skin injuries through contact with infected fish or contaminated water. Mechanisms of pathogenicity include type three secretion system, A layer protein, lipopolysaccharide (LPS), iron binding and outer membrane proteins, proteases and different kinds of toxins [36, 37].

Peracute infections most commonly occur in fingerling fish, which may manifest only limited signs of disease. Individuals with the clinically inapparent (covert) infection may transmit the disease to cohabitant fish [38]. Acute infections usually occur in juvenile and adult fish. The clinical signs encompass one or several of the following symptoms: skin darkening, pop-eye, loss of appetite, rapid gill movement, erratic swimming behaviour, lethargy, hemorrhaging at the base of fins and oral cavity, bloody exudates from anus and finally, the furuncle-like lesions, the specific sign of furunculosis [39, 40]. Development of the furuncles is however most often associated with chronic infections. Hemorrhages of internal organs and enlarged spleen are also among the disease symptoms. The gastrointestinal tract is usually devoid of food and the intestine is often severely congested.

Good management practices, resistant stocks, use of functional feeds and vaccines can minimise losses from furunculosis. Vaccination is an essential strategy that reduced the use of chemicals, especially antibiotics in salmonid aquaculture [41]. Vaccines can be administered orally, by immersion and injection. The first successful vaccination was oral, achieved by feeding chloroform-killed *A. salmonicida* to cutthroat trout (see references in [42]). Immersion vaccines with inactivated bacterial pathogens were developed later. Unfortunately, neither oral nor immersion vaccines confer long and complete protection against *A. salmonicida*. Mortality is reduced but not prevented entirely.

At present, injection is used as the main vaccination strategy in salmon industry. Different suspensions of killed bacteria or bacterins, and purified antigens were tested in injection vaccines, initially without adjuvants [43, 44]. The induced immunity varied in terms of the duration of protection and levels of antibody production. An improvement was achieved when

a combination of bacterins was used [45]. Vaccines without adjuvants require booster to achieve long-term protection, which makes them impractical for commercial aquaculture.

Vaccines with formaldehyde inactivated *A. salmonicida salmonicida* emulsified in adjuvants that enhance the immune responses and are administered as a single injection have proven most effective [46]. At present, vaccines are injected intraperitoneally (i.p.) to anesthetised salmon of ≥35 g in weight at the end of freshwater period shortly before transfer to sea. The use of mineral oil adjuvanted vaccines has major advantages compared to water-based products in terms of protection but unfortunately, they cause severe side effects. Injection site lesions, adhesions between internal organs and reduced appetite and growth have been reported [47-50]. Deviations from the recommended injection point reduce vaccine efficacy while temperature and fish size at vaccination influence the development of intra-abdominal lesions and bone deformities [51]. Interestingly, Berg et al. [52] did not find differences in the antibody response between size groups, or between high and low lesion score fish. Koppang et al. [53] provided evidence for antibody-mediated liver damage induced by adjuvant oil. The authors suggested that the injected amount of vaccine is the critical factor responsible for the observed adverse effects of vaccination.

Polyvalent PHARMAQ vaccines against furunculosis are based on different mixtures of bacterial and viral antigens. Improved protection with polyvalent formulations was reported [54, 55]. In contrast, a more recent study suggests that polyvalent vaccines for Atlantic salmon may result in a less efficient protection, indicative of antigen competition [56]. The formaldehyde inactivation has also been demonstrated to alter important surface antigens [57].

Although currently available vaccines work reasonably well, bacterial antigens and the protective immune responses they elicit are not fully understood. Surface components such as the A-layer protein and LPS, involved in the initial contact between the host and pathogen may play a role of protective antigens in salmon vaccines against furunculosis [58-60]. Lund et al. [59] identified the cellular and extracellular antigens of *A. salmonicida*, including a caseinolytic protease, the A-layer protein and LPS as the dominant factors.

Humoral antibody responses, cell-mediated immune responses and non-specific defence mechanisms that involve humoral and cellular factors are the main lines of protection.

Immunisation with bacterin mixtures most likely induces different responses to the variety of antigens. In addition, oil-adjuvants may contribute to non-specific protection [61] while others dispute this suggestion [60]. Identification of protective mechanisms is difficult due to high complexity of induced responses. Early studies suggested that at least part of the protective immunity is conferred with a serum factor, since passive immunization can protect fish [62]. Midtlyng et al. [63] demonstrated an association between the antibody levels to the A-layer protein and survival in Atlantic salmon, however reports on the relationship between vaccine efficacy and antibody responses to putative protective antigens in various fish species are contradictory. Overall, bacterin-based vaccines do not induce potent cellular responses [64], whilst a live aroA deletion mutant of *A. salmonicida* vaccine was shown to preferentially enhance T-cell over B-cell responses in rainbow trout [65].

Clearly, limited efficiency of vaccination against furunculosis, which significantly reduces mortality but fails to eradicate infection, can be determined with different factors.

1.3. Immunomodulation by β-glucan lentinan

The advances in development and assessment of bioactive nutrients promoted use of dietary supplements that have the ability to modify immune responses. Immunomodulators can be defined as substances that augment and/or reduce certain immune responses thus improving protection against pathogens and stressors. Pre-conditioning by a diet containing such feed additives can prepare the immune system to the threats it may encounter.

β-glucans are abundant in nature and are considered as potent immunomodulators that can be successfully applied orally to fish, e.g. [66-70]. This is a diverse group of polysaccharides composed of D-glucose units joined together in linear chains by 1-3, 1-4 and 1-6 β-bonds. β-glucans from different sources vary in their structure, which influences their physiological functions. Importantly, β-glucans possess conserved Pathogen-Associated Molecular Patterns (PAMP) similar to those found in cell walls of fungal pathogens. Many if not all mushrooms that belong to higher basidiomycetes contain biologically active β-glucans [71].

Lentinan from the shiitake mushroom *Lentinula edodes* produces promising antitumor and antimicrobial effects [72-75]. The main chain of lentinan consists of (1-3)-linked units with (1-6)-branch units linked at, on average, an interval of three main chain units. The average molecular weight of lentinan is 500 kDa [76]. Its biological activity depends on the molecular weight, branching, solubility in water and dose [77, 78].

The receptor(s)-mediated effects of β-glucans are being studied and increasingly understood. The recognition of PAMP is a key aspect of metazoan immunity that relies on germ line encoded molecules called pattern recognition receptors (PRRs). Upon agonist binding, PRRs trigger anti-pathogen signalling cascades. The recognition by receptors for β-glucans initiates innate immunity to combat fungal infections. Glucans are bound and internalized by multiple PRRs on immune cells. Class A scavenger receptors and Type 3 complement receptor are shown to be involved in their recognition [79-81]. A non–Toll-like PRR called dectin-1 was identified as the major receptor that recognises fungal pathogens in mammals [82]. Glucan receptors are widely distributed throughout the body and recognition is not confined to cells of the immune system [83, 84]. Signalling through dectin-1 and other identified β-glucan receptors is probably responsible for the lentinan-mediated effects observed in mammals. In mice, the recognition, at least for some fungal β-glucans, requires co-stimulation with TLR-2 in addition to dectin-1 [85]. Orally administered water-soluble glucans exert biological effects; they are bound by cells of the gastrointestinal tract and translocated into the systemic circulation [86].

Modulation of immune responses is largely attributed to the ability of β-glucans to regulate the production of pro- and anti-inflammatory cytokines by macrophages and T cells. In the vast literature on β-glucans, they are referred to as immunomodulators, immunostimulants and immunosuppressants. For example, β-glucans isolated from *S. cerevisae* activated NF-κB in the human monocyte-like cell line [87] while in neutrophils, NF-κB induction was not accompanied with the production of pro-inflammatory cytokines [88]. A number of studies found that β-glucan enhanced production of the Th1-type cytokines TNF-α, IL-12 and IFN-γ while it reduced the production of the Th2-type cytokines IL-4 and IL-6 in mice and humans [89-91].

Stimulation of production of NO and the cytotoxic activity of macrophages by lentinan was reported [89, 92] and enhanced Th1-responses could explain its beneficial effects in infections with intracellular pathogens [92, 93]. Lentinan influences a range of immune functions such as phagocytosis, respiratory burst activity, lysosome and complement activity [94-97]. The

relative proportion of T-cell subpopulations and their effector functions were shown to be affected by lentinan [98, 99]. On the other side, lentinan reduced induction of circulatory TNF-α and IL-1β with LPS in mice with no effect on IL-6 and IL-10 levels [73]. Yet, in the breast cancer and keratinocyte cell lines lentinan stimulated both Th1 (TNF-α and IL-8) and Th2 (IL-4 and IL-13) cytokines [100]. In conclusion, modulation of innate and adaptive immune responses by lentinan is host-mediated and highly contextual, determined by the cell type and different regulatory influences in the environment.

To date, several studies on lentinan's effects in fish have been reported. Stimulation of the cytokine production (IL-1β) and proliferation of leucocytes by *in vitro* lentinan treatment was observed in common carp [101]. Lentinan increased survival in carp challenged with bacterial pathogens *Edwardsiela tarda* and *Aeromonas hydrophila*, possibly due to the increased phagocytosis and activation of the alternative complement pathway [97, 102]. Survival of vaccinated coho salmon following challenge with *Aeromonas salmonicida* was significantly higher when lentinan was used as an adjuvant [103]. Interestingly, the resistance to the pathogen did not involve stimulation of B-cells. The study of Nikl et al. [103] suggests that in fish, as well as in mammals, β-glucans may have greatest effects on macrophages and the T-cells.

1.4. Lipopolysaccharide induced inflammation in fish

Lipopolysaccharides or endotoxins are complex glycolipid molecules that function as protective components in the outer membrane of virtually all gram negative bacteria. This bacterial group includes important fish pathogens from *Vibrio*, *Aeromonas*, *Pasteurella* and *Edwardsiella* genera [104]. While LPS of different bacteria have different structure, they all contain a lipid part (lipid A) and a polysaccharide region covalently linked to this membrane anchor domain. The polysaccharide region is made of the core oligosaccharide portion (consisting of inner and outer parts) and the O-specific chain. Sugars of the outer core oligosaccharide and the O-specific chain are not present in LPS from several bacterial genera [104].

LPS exhibit exceptionally versatile biological activity through their ability to stimulate cells of diverse eukaryotes ranging from insects to mammals. LPS stand among the most powerful stimulators that mimic infection by gram-negative bacteria; therefore, they have been extensively used to experimentally induce inflammatory responses in mammals [104]. LPS also exert a multitude of biological effects in fish, including various immune responses (reviewed in [105]). Fish are known for their remarkable tolerance to toxic effects of LPS [106]. Challenges with high doses of LPS do not lead to endotoxin-induced mortality in salmonid fish (see references in [105]).

Major lymphoid organs in fish are the head kidney and spleen that, in addition to the heart, intestine and liver, accomplish the uptake and subsequent localisation of LPS. Endothelial cells and macrophages from these organs are mainly responsible for endocytosis of LPS [107-109].

The conserved common architecture of the lipid A domain in LPS represents a PAMP, which is perceived as an indicator of infection by gram-negative bacteria. Dramatic responses to LPS are due to the specific and sensitive recognition of lipid A by numerous cellular and humoral components of innate immunity (reviewed in [104]), some of which have not been found in fish genomes. Toll-like receptor 4 (TLR4) that is critical for the induction of specific immune responses to LPS in mammals, is absent from all the studied fish species with the notable exception of zebrafish, which has two TLR4 orthologues [110]. However, Sepulcre et al. [111] showed that TLR4 is a negative regulator of LPS signalling in zebrafish embryos. Furthermore, TLR4-associated co-stimulatory molecules involved in the activation of the LPS cellular signal transduction in mammals appear to be absent in fish or may perform different functions [112]. Collectively, these findings support the hypothesis that the LPS recognition in fish is different from that of mammals.

Despite the absence of endotoxic shock, LPS is a potent inducer of inflammation in fish. The strongest responses take place in lymphocytes, monocytes/macrophages and possibly, dendritic cells. Interestingly, high pro-inflammatory potency of LPS in stimulated salmon monocytes/macrophages is not correlated with its capacity to induce MHC II-mediated antigen presenting [113, 114]. Instead, LPS appears to stimulate components of the MHC I pathway of antigen processing [115].

Zhou et al. [116] demonstrated that coordinated activities of transcription factors AP-1, AP-2, C/EBPs, NF-κB, CREB and Sp1 in murine spleen correlate with production of pro-inflammatory cytokines. LPS-initiated intracellular transduction cascade in fish also involves the activation of transcription factors AP-1 and NF-kB and different MAPKs, resulting in the transcriptional activation and subsequent release of pro-inflammatory cytokines [111, 113, 114]. TNF-α and IL-1β are among the endogenous mediators released systemically during the early phase of LPS-induced activation of innate immunity in both mammals [104] and fish [114, 117]. These cytokines are involved in diverse paracrine and autocrine mechanisms that initiate a complex network of secondary reactions, including the induction of acute phase proteins [118].

Cytokines recruit and activate immune cells to eliminate invading pathogens [104], but may also cause detrimental effects through the induction of excessive inflammatory responses. Matrix metalloproteinases (collagenases) are involved in the LPS-induced remodelling of head kidney extracellular matrix (ECM) in trout, most likely to enable trafficking of immune cells [114]. LPS was shown to modulate the metabolism of lipid inflammatory mediators in fish as well [119] and the respiratory burst, involved in the killing of microbial organisms [120, 121]. Endotoxin is a thymus-independent antigen capable of activating B lymphocytes into antibody production without T helper lymphocytes [122]. In contrast to mammals, several studies showed that fish respond to LPS with preferential production of antibodies against the polysaccharide O-chain or the core region instead of antibodies against the lipid A portion [59, 123, 124]. The microarray study by MacKenzie et al. [114] revealed that LPS treatment of trout monocyte/macrophage cells down-regulated genes involved in energy metabolism, protein biosynthesis, cytoskeleton biogenesis and other basic cellular functions. In this respect it is noteworthy to mention deleterious effects of LPS on mammalian liver that include depletion of mitochondrial glutathione, mitochondrial DNA damage, increased generation of reactive oxygen species (ROS) and impaired oxidative phosphorylation [125, 126]. However, the hepatic responses to LPS also include activation of genes involved in mitochondrial biogenesis and eventual TNF-α mediated hepatic cell proliferation [127].

In mammals, LPS can trigger pro-apoptotic or pro-survival pathways and the outcomes are cell type specific [128]. Similarly, LPS was shown to promote both proliferation and apoptosis of fish leukocytes [129, 130]. Significant increase in cellular proliferation in the head kidney and the coordinated expression of genes with roles in heme metabolism suggest

activation of hematopoietic, potentially erythropoietic, mechanisms upon LPS stimulation in fish [115, 131]. LPS activation has the capacity to modify the hypothalamo-pituitary-adrenal axis [129, 130, 132]. Modulations of downstream immuno-endocrine interactions were reported in several fish [133-135].

1.5. Feed deprivation in fish

The majority of domesticated animals have food available at all times, that is, they are fed *ad libitum* in order to maximise the production efficiency. However, the access to feed may differ for individuals in the group even in the adequate presence of feed. Given that salmonids exhibit aggressive behaviours and compete for feed, the opportunity for feeding within the confined population is often not uniform [136, 137]. The establishment of size and behavior based social hierarchies results in differences in growth and decreased production efficiency [136].

Responses to reduced feeding in ectothermic and endothermic vertebrates have similar and different features. Overall, the severity of nutrient deprivation required for the initiation of adaptive responses is substantially greater in ectotherms and limited adjustments suffice to sustain normal metabolic functions over protracted periods of complete food absence [138]. Partial deprivation of food (restricted feeding) in mammals and birds quickly initiates metabolic changes similar to those induced by the complete absence of food [138-140]. In carp, hepatic gene expression changed only after approximately two weeks of starvation ([138] and references therein). Lipolysis genes remained unchanged for an even longer period. Protein appears to be well conserved during starvation in carp as well. Hepatic glycogen, on the other hand, was quickly mobilized upon initiation of starvation in carp. The following decline in glycogenolysis was concurrent with an up-regulation of genes encoding glycolytic enzymes. During migration, salmon preferentially use lipid and spare protein until the later migration phase, when lipid stores are almost completely depleted [141]. Tolerance to food deprivation in fish is partly explained with much lower metabolic rates in ectotherms in comparison to endotherms [142]. In addition, many fish species have evolved in habitats characterized by periodic availability of resources.

Different organisms employ various behavioural, physiological and structural regulations to maintain homeostasis at limited feeding [138]. The liver increases expression of genes encoding plasma proteins and enzymes involved in detoxification and metabolism of carbohydrate, protein, lipid, and cholesterol [143]. Hepatic adaptations to food deprivation are essential due to the key role of liver in the distribution of metabolic energy. Lowering of hepatic metabolism accommodates for the more efficient use of limited resources [138, 144].

Many animals reduce reproductive functions when food is limited since reproduction is energy expensive. Reduced availability of food significantly affects the ability of salmonid fish to mature [145]. Inhibition of reproduction by restrictive feeding is related to the changes of hepatic metabolism. Cholesterol is precursor to all steroid hormones and attenuation of cholesterol biosynthesis in liver occurs as part of metabolic adaptations to food deprivation [146]. In addition, steroid hormones are mainly catabolised in liver [147].

The order and dynamics of nutrient utilisation in fish and mammals have similar and different features and depend on the severity of deprivation [138, 148]. Increased hepatic gluconeogenesis and decreased glycolysis are among the key changes observed in mammals [148]. Knowledge on glucose metabolism in fasted fish is limited but overall, changes are similar to those in endothermic vertebrates [149-152]. In mammals under caloric restriction, general reduction in the rates of protein and lipid synthesis and nucleotide metabolism is also seen [148, 153-155]. Caloric restriction has not been thoroughly investigated in fish. More recently, Bower et al. [156] described some of the metabolic adaptation in muscle observed at fasting and re-feeding in trout.

Feed restriction is negatively related to growth rate. However, mild restriction that avoids undernourishment was shown to have positive effects on health and life span in a range of phylogenetically diverse species [157]. Large-scale microarray studies were instrumental for establishing gene expression biomarkers linked to health and lifespan effects of restricted feeding [148, 153, 158]. The decrease of hepatic cell number and mass that occurs after the initiation of either caloric restriction or fasting in mammals is likely to protect against hepatic cancers due to lower rates of macromolecular synthesis and cell division and increased rate of apoptosis [159, 160]. One of anti-cancer factors can be the decrease in lipogenesis as fatty acid biosynthesis is required for the survival of cancer cells [161, 162]. Lifespan extension by caloric restriction is also attributed to sustained decrease in lipogenesis and enhancement of

lipolysis in the liver. Importantly, the down-regulation of hepatic genes encoding inflammatory mediators is observed in response to caloric restriction in mammals [148, 154]. Positive effects of dietary restriction have not been addressed in fish so far.

1.6. Fish meal replacement with soy products in salmonid diets

Feeding high-energy diets with an optimal balance between the macronutrients provides the desired efficiency in terms of both productivity and environmental impact [163, 164]. In near future the output of marine capture fisheries will become insufficient to meet the requirements of rapidly growing aquaculture industry which consumes large quantities of high-quality fishmeal [10]. The need for suitable alternative protein sources is consistently increasing.

Soybean meal is considered to be one of the most promising cost-effective substitutes for fish meal [165]. Diets with up to 34% extracted soybean meal inclusion are readily accepted by Atlantic salmon adapted to fish meal-based diets [166]. The digestibility of crude protein in salmonid diets with soy products varies from 60–70% up to a very high level of 85%, comparable to low-temperature-dried fish meals [167, 168]. The protein content of soybean meal is high (≈50%) and the amino acid composition is relatively favourable [165]. If dietary protein is available in excess, the low methionine content of soy protein is not limiting for fish growth [168].

Value of novel protein sources depends on digestion kinetics, rates of gastric emptying and absorption [169]. Soy proteins have been shown to lower postprandial peripheral delivery of amino acids and their incorporation into proteins in [170], due to the earlier and stronger catabolism of amino acids from soy in visceral organs. Digestion kinetics of soy protein most likely modulates protein deposition in Atlantic salmon as well (Mydland et al, unpublished results).

The limited use of soybean meal in salmon aquaculture industry is due to possible negative impacts of soy-based diets on fish health, growth, and reproductive development. In Atlantic salmon and rainbow trout, soybean meal causes distinct histopathology characterised with inflammation of the distal intestine, in addition to impaired growth and protein utilization

[171]. A proteome study that compared two diets containing proteins derived from soy and non-soy plant sources found soy-induced changes in the hepatic metabolism, including alterations in levels of several stress and structural proteins [172]. The negative effects of soy can be reduced or eliminated by decreasing dietary levels and by treatments that remove lowly digestible carbohydrates and anti-nutrient compounds [173, 174]. The results of feeding soybean products treated in different ways to salmonids are encouraging [166, 175].

Isoflavones and soy proteins contribute to health benefits observed in mammals fed soy-based products [176, 177]. Modest hypocholesterolemic effect is commonly observed in mammals [178]. So called "resistant proteins", which are non-digested remnants of dietary proteins, were shown to function like plant-derived dietary fibers in the intestine [179]. Soy-derived "resistant proteins" appear to be at least partly responsible for the stimulated faecal excretion of cholesterol and bile acids, as shown by Yamatani et al. [180]. Proteins derived from soy have beneficial effects on insulin sensitivity. Improvements in response to nutritional interventions were reported in rats [181], pigs [182], rabbits [183] and monkeys [184] while the study of Bos et al. [170] did not detect changes in glucose, insulin, and glucagon levels in response to soy in humans. Replacing animal protein in the diet with soy protein was shown to slow the progression of kidney disease in patients with diabetic nephropathy [185]. Higher inclusion of soy-based products in human diets is suggested as a strategy to attenuate the expression of hepatic pro-inflammatory proteins [186-188]. Increased generation of ROS associated with cardiovascular diseases was counterbalanced by soy, resulting in improved endothelial function [189]. However, Matthan et al. [190] did not find any effect of several soy products on cardiovascular disease risk factors.

The three most abundant isoflavones found in soy are genistein, daidzein and glycitein, which may act as phytoestrogens by directly binding to estrogen receptors in fish [191]. Modulation of transcription of estrogen-regulated gene products and the effects of soy isoflavones on enzymes involved in steroid metabolism have been reported in mammals (reviewed in [192]). Isoflavones were shown to exert estrogenic, anti-estrogenic or partial agonistic effects depending on the target cell type, isoflavone concentration, age and hormonal status. There are also numerous reports of these compounds exerting their effects in fish. Genistein induced hepatic vitellogenin synthesis [193] and caused a growth-promoting effect similar to that of estradiol-17β [194] in yellow perch (*Perca flavescens*). Feeding genistein, daidzein, and soy-based diets increased plasma vitellogenin in several fish species [195-197]. Ng et al. [198]

suggested that estrogen-like activities of soy isoflavones may be mediated by the inhibition of hepatic and renal degradation of estradiol-17β. The synergistic interaction of pure isoflavones and soymilk matrix was detected by Rando et al. [176] emphasizing the limitations of analyses of isoflavones administered as pure substances in the evaluation of their effects on estrogen receptor signalling.

Obviously, understanding biological alterations induced by dietary soy in salmonid fish requires further investigation.

2. Methodology

To gain a global overview of the immunity, metabolism and regulatory pathways, all studies employed the salmonid SFA2 cDNA microarray platform. SFA2 chip includes 1800 unique clones printed in six spot replicates. The gene composition is adapted to studies of responses to pathogens and stressors; most genes were selected by their functional roles. The four topics included in this thesis had been scarcely investigated. Hence, the general approach was to perform pilot microarray screenings in order to determine tissues with most informative responses and then to proceed with more detailed gene expression analyses including biological replicates. Microarray analyses were followed by the real-time quantitative PCR (qPCR) analyses based on SYBR Green I fluorescent chemistry. The two technologies are based on different principles and concordance of results increases confidence in findings.

Microarray analyses are based on simple procedures. RNA isolated from two samples is reverse transcribed with incorporation of a red and green cyanine dyes. Labels are combined and hybridized to a microarray. Scanning with laser scanner determines amounts of dyes in each spot which provide a measure of gene expression ratios in samples. qPCR is based on the continuous fluorescent detection of amplified DNA during PCR as SYBR Green I emits fluorescence when bound to double-stranded DNA. In contrast to the end-point detection PCR, quantification occurs during the exponential phase of amplification. Relative quantification of gene expression differences between two samples involves normalisation to a reference gene(s). Multiple gene expression profiling followed with data mining was used in all studies to search for common and distinct responses as well as markers of protection/pathology and rapid/retarded growth.

3. Objectives

The four presented studies employed gene expression profiling to improve the knowledge of salmonid fish responses to different stressors under conditions of intensive production. Thus, the objectives of the thesis can be summarised as follows:

I. To search for the gene expression signatures of common and specific responses to different stressors

II. To find the stress-responsive genes and pathways and to elucidate their involvement in beneficial and pathological responses

4. Summary of papers

Paper I

In Paper I, comprehensive gene expression analyses were conducted in order to better understand susceptibility of Atlantic salmon to sea lice infections. Skin, liver, head kidney and spleen were sampled at three time points [3 days post infection (dpi), 22 dpi and 33 dpi]. During this period the complete development of *Lepeophtheirus salmonis*, from copepodids to mobile adult stages was accomplished. Rapid responses occurred in all organs and previously unrecognised role for the T-cell mediated immunity was revealed. Induction of arginase I concurrent with the low expression profile of decoy receptor IL13Rα2 gene in skin of Atlantic salmon implied alternative activation of macrophages and predominant Th2 differentiation of T helper cells in response to the parasite. In fact, it was found that early response involved a mixture of responses as supported by the up-regulation of IL-1 receptor type 1, β2-microglobulin (B2M), IL-12β, CD4 and CD8α. The input of the Th1/Th17 arm in responses to sea lice appeared relatively low however. Th2 cells are known to contribute to B cell activation and antibody production. A group of immunoglobulin-like transcripts showed a similar temporal profile in the three inner organs: up-regulation was detected 3 dpi in the spleen and head kidney and next increase was seen only in spleen 33 dpi. The early immune system activation was followed by attenuation of most responses, thus the inflammation can be classified as Th2-modified. Programed death ligand-1 was identified as the potential negative regulator of T-cell-mediated responses in fish, as shown in mammals. The profile of Unfolded Protein Response (UPR) markers implied activation of the cellular stress responses in damaged skin already 22 dpi. However, TGF-β and IL-10, cytokines involved in healing of tissues and repair were induced only 33 dpi. Up-regulation of major ECM components, laminin and elastin, were also observed only when lice reached their adult stage. The expression of matrix metalloproteinases, involved in the remodelling and destruction of ECM, steadily increased throughout the infection period in all tissues except liver. This suggested an increased risk of self-inflicted tissue damage.

The absence of appropriate inflammatory reaction likely explains susceptibility to sea lice in Atlantic salmon. Together with the expression profile characteristic of delayed wound healing, the findings suggested modulation of host responses by lice.

Paper II

Paper II reports on the search for molecular determinants of vaccine-dependent protective responses in Atlantic salmon against *Aeromonas salmonicida*, the causative agent of furunculosis. Comparison of hepatic expression profiles of challenged naïve and vaccinated fish categorized as susceptible (early mortality) and resistant (survival to the end of challenge) uncovered protective responses associated with vaccination. Infection in all challenged fish was confirmed with finding of *A. salmonicida* DNA in livers and spleens and was further supported with the high expression of genes responsive to bacterial pathogens.

Differences between susceptible and resistant fish were observed in a number of genes that regulate gene expression at various levels: signal transduction, chromatin remodelling, and transcription and mRNA metabolism. NF-κB was equally induced in resistant and susceptible fish. JunB and JunC, components of AP-1, a transcription factor complex with a major role in the regulation of immune responses, showed greater induction in fish with low resistance. Similar pattern was shown by immune cells' markers and chemokine attractants. In addition, elevated expression of genes implied in the regulation of cellular interactions with ECM suggested increased inflammatory infiltration in the liver of susceptible fish. Susceptible fish also showed higher expression of genes encoding cytoskeletal proteins, which are essential for cellular motility. Overall, the gene expression profiles of resistant fish implied pervasive suppression of inflammatory responses, except for the marked induction of genes encoding complement components.

Transcriptomes of the resistant vaccinees were characterized by the down-regulation of genes involved in protein degradation (proteasome components and extracellular proteases) while protease inhibitors showed opposite regulation. Moreover, genes involved in the protection against auto-immune damages and in clearance of endogenous and exogenous toxic compounds were highly induced in this group. Cellular stress was lower in resistant fish as well, as evidenced by expression differences in a number of cellular chaperones, including classical markers of UPR, 78kDa and 94 kDa glucose regulated proteins.

Collectively, the data show that greater control of immunopathology is associated with survival in vaccinated fish exposed to *Aeromonas salmonicida*.

Paper III

β-glucans hold great potential as biological modifiers of immune responses. The study was designed to evaluate the impact of β-glucan lentinan from shiitake mushroom on the immune system of rainbow trout challenged by intraperitoneal injection of LPS. Interestingly, the lower inclusion dietary level of 0.2% produced greater effects than the 0.4% inclusion; the group that received 0.2% lentinan was selected for detailed analyses. Close to 300 genes were significantly affected by LPS, substantial number of which was down-modulated by lentinan. Several genes encoding proteins with binding capacities and transporters of organic compounds were negatively affected by lentinan, including several galectin genes that code for multifunctional proteins with carbohydrate binding capacities. In contrast, the induction of the gene encoding albumin, an abundant plasma protein, was enhanced by lentinan. Collectively, these results indicated a possible neutralization of LPS and its delivery to removal sites, thus reducing loading of LPS onto cells in lentinan fed fish. The group of genes down-modulated by lentinan included STAT1 and STAT3, major transducers of IFN-signaling, and several genes known to be regulated by IFN. Levels of mRNAs coding for a number of pro-inflammatory cytokines, chemokines and their receptors were similarly affected. The observed expression profiles also suggested attenuation of MHC class I antigen presentation and proteasome associated protein degradation. Stronger up-regulation of ROS scavengers, together with 78 kDa and 94 kDa glucose regulated proteins and many other chaperones suggested increased oxidative and cellular stress in fish that did not receive dietary β-glucan. One more putative beneficial effect of lentinan was the reduced leukocyte recruitment.

The absence of mortality endpoints in this study precluded definite conclusions on the beneficial effects of lentinan. Meaningful interpretation was however achieved following the comparisons of global gene expression profiles from the SFA.2 microarray database with this experiment. Due to the large number of identified genes, cross-experimental comparisons revealed convincing similarities between splenic responses of lentinan fed fish and hepatic responses of salmon resistant to *A. salmonicida* (Paper II) and infectious salmon anemia virus (ISAV) [1]. In conclusion, selective attenuation of the detrimental and enhancement of beneficial processes could be predicted for lentinan fed trout. The study represents an approach for the assessment of candidate immunomodulators without the need for challenge with live pathogens.

Paper IV

Paper IV examined the effects of three dietary interventions in comparison to fishmeal based diet given at full ration (CF). The interventions consisted of feeding 1) control diet at reduced ration (CR) 2) partial fish meal replacement with 20% inclusion level of extracted soybean meal at full ration (SF) and 3) the combination of the interventions, feeding of soybean meal based diet at reduced ration (SR). The experiment continued for 54 days and the reduction in growth was as expected (SR>CR>SF>CF). Compared to CF, the thermal growth coefficient decreased in SR by 67%, in CR by 51% and in SF by 22.2%. Liver was selected for analyses as a central metabolic and immune organ, which responds to the alterations of nutritional status. Transcriptional profiling uncovered highly similar hepatic responses to soy (SF) and reduced feeding (CR). Somewhat unexpectedly, the joint treatment (SR) resulted in smallest deviation from CF for the majority of genes. Biphasic pattern implied a hormetic regulation of responses to the studied dietary factors.

Twelve candidate biomarkers were found to have greater regulation in this study than in other experiments from our database. Four of these genes were up-regulated ribosomal proteins whose expression significantly increased with the severity of feeding stress. Future studies can also consider the following markers (all down-regulated) of altered nutritional status: adenosine kinase, an enzyme of nucleotide metabolism, immune-related liver-expressed antimicrobial peptide 2B, a stress responsive N-myc downstream regulated protein and four genes involved in lipid and steroid metabolism. No genes with expression differences between the study groups were found.

Changes in nucleotide, carbohydrate and lipid metabolism were in line with the state of relatively mild nutritional deprivation. Increase in oxidative phosphorylation was also observed. Down-regulation of genes involved in cholesterol and steroid hormone metabolism suggested a possible decline in reproductive functions. Nutritional interventions and retarded growth in this study were associated with the down-regulation of genes involved in oxidative and cellular stress responses, xenobiotic metabolism and inflammation. A small number of IFN- and TNF-dependent genes and genes involved in Jun N-terminal kinase (JNK) and NF-κB signalling were up-regulated however. Results indicated that maximum growth rates may have a negative impact on salmon health.

5. Discussion

5.1. Paper I

The study generated an integrated view of responses to salmon louse in Atlantic salmon. The complexity of this host–pathogen relationship has been recognized for a long time. Lice induce systemic immune responses and modulate the ability of fish to defend against other pathogens. Chalimus stages were shown to alter blood biochemistry and cortisol levels generally increase as parasites develop towards the mobile stages (reviewed in [29, 199]). Integrated physiological functions including those that determine swimming ability are also significantly affected by the parasite [200]. Studies of species-specific differences and immunosuppressive cortisol effects formulated the "inflammation hypothesis", which explains susceptibility with cellular infiltration and tissue response in the affected skin.

Paper I adds to this knowledge and systematically classifies responses to salmon louse as predominantly mediated by the Th2 type of immunity, or more precisely by Th2-modified or Th2-like response. In fact, the observed responses implied a contribution of other Th cell subsets. Modest input from other inflammatory pathways was derived from the comparison to the study of Iliev et al. [113], performed with the same microarray platform (see Appendix Figure 1). The study addressed *in vitro* responses of leukocytes to classical PAMPs (CpGs and LPS) which typically induce Th1 type responses. The assumption that the inflammatory signature in the affected skin of Atlantic salmon largely reflects the composition of the leukocytic infiltrate justified the comparison to the profile of PAMP-induced transcriptional responses in the population of isolated leukocytes. In contrast to stimulated leukocytes, damaged skin did not show regulation of NF-κB pathway, TNF receptors and genes involved in oxidative burst (e.g. cytochrome B-245). However, a panel of genes implicated in inflammation were up-regulated in both experiments. Responses to lice and PAMP overlap on the levels that are relatively distant from perception and first stages of signal transduction.

The findings are in concordance with the rich body of research, which shows that the mixture of Th cell mediated responses is the norm rather than exception [201, 202]. Another lecture from mammalian parasitology is that the evasion of adaptive immunity is indispensable to parasite survival [201, 203]. Thus, hyporeactivity and polarization of the immune response towards a Th2 type could be a common characteristic of infections with metazoan parasites in

mammals (e.g. [204]) and fish. Protective responses to sea lice are likely to involve the pro-inflammatory Th1 and possibly Th17 axes while the contribution of the Th2 response, which typically develops in response to parasites, could be important in the regulation of wound healing and tissue repair. The emerging picture suggests that protection against lice in Atlantic salmon implies prevention of immunosuppression and steering of Th-mediated responses.

5.2. Paper II

At present, commercially available vaccines against furunculosis provide only partial protection from disease and associated mortality. Development of new, more efficient vaccines is required. Modified live vaccines replicate within the host, which results in a strong cellular immune response, in addition to stimulated antibody and mucosal immunity [205]. DNA vaccines also possess characteristics that make them attractive for the aquaculture industry [206]. On the other side, it is important to elucidate factors that determine efficiency of currently available vaccines. Both naïve and vaccinated salmon show high variation in resistance to the pathogen. Vaccination against furunculosis is supplemented with the selection for increased resistance to *A. salmonicida*. High additive genetic variation in resistance to furunculosis was consistently shown in challenge tests and in field tests (see references in [207]). More recently, Gjerde et al. (unpublished results) found a substantial genetic component behind the differential resistance to furunculosis in vaccinated fish. The molecular factors underlying vaccine protection were addressed in Paper II.

Expression profiling identified some of the players involved in vaccine-dependent protection. Genes from the adaptive arm of immunity showed no difference between resistant and susceptible fish. Events downstream from the establishment of antibody-antigen complexes involve activation of the complement system. Quite fittingly, a coherent induction of genes encoding effectors of the complement system was revealed in resistant vaccinees. This was concurrent with a marked induction of a number of their negative regulators. Hence, mobilisation of complement together with the induction of mechanisms which protect host tissues from complement damages correlated with survival. Due to limited knowledge of the complement system in fish, it remained unknown however, which of the complement pathways was responsible for protection.

It could be concluded from the gene expression data that survival relies on the ability of vaccinated hosts to actively eliminate bacteria, tight control of inflammation, efficient clearance and prevention of self-inflicted tissue damage. Though indirectly evidenced, results suggested that furunculosis in Atlantic salmon possesses a strong immunopathological component.

5.3. Paper III

Lentinan has attracted much attention in the clinical practice as it provides benefits in diverse settings ranging from cancer to viral, bacterial and fungal infectious diseases (see references in Chapter 1.3.). As opposed to yeast-derived β-glucans, the potential of lentinan in aquaculture has not been explored much. Because differences exist among β-glucans isolated from different sources, it is essential to independently evaluate their biological activity before suggestions are made for the use of a particular β-glucan.

A somewhat unexpected finding was that lentinan acted predominantly as an anti-inflammatory agent in rainbow trout injected with LPS, as the expression of many genes known to be involved in immunopathology showed lowered profile in the group receiving lentinan than compared to control.

The majority of β-glucan studies provide evidences for their potent immunostimulating abilities. Indeed, so far, the only known glucan without a trace of pro-inflammatory cytokine stimulation is PGG-glucan [88]. In a study by Poutsiaka et al. [208], β-glucan Zymocel induced anti-inflammatory IL-1RA without IL-1β production, leading to the proposal that β-glucans confer protection via anti-inflammatory effects. This view was supported by the findings of Luhm et al. [209] who showed that yeast β-glucan opposed LPS-induced response in human leukocytes by shifting a pro- to an anti-inflammatory phenotype. Paper III demonstrates a similar mode of action for a different β-glucan compound and in a non-mammalian vertebrate model. Thus, the described transcriptome changes may actually reflect some of the typical biological consequences of a β-glucan. The data support the conclusion that lentinan modulates trout splenic responses with suppression of potentially detrimental processes while large part of immune functions remains unchanged.

5.4. Paper IV

Study reported in Paper IV addressed the question whether gene expression profiling can distinguish between nutritional interventions that have negative impact on growth. Retarded growth is an ultimate biomarker of undernourishment, however it is not descriptive of the type and nature of the nutritional stress in question. Experiment included restricted feeding, feeding with a diet containing extracted soybean meal and combination of these treatments. One of the research tasks was to explain the adverse effects of soy on salmon growth.

Microarray analyses found very similar gene expression changes in the groups. Soy-induced effects were nearly identical to those induced by restricted feeding. Although specific biomarkers for each intervention were not detected, the study improved understanding of the complex interplay between genes and nutritional factors. Numerous differentially expressed genes, representative of the affected metabolic responses (steroid, lipid, and glucose) were identified. The increased expression of ribosomal proteins found in all three groups was in contrast with the observations from a microarray study in rainbow trout completely deprived of food where these genes were repressed [210]. This exemplifies the differences that exist between responses to starvation (involuntary and complete deprivation of food) and restricted feeding (partial deprivation of food) in fish; of note is that the latter is much less studied in fish as opposed to mammals where health benefits induced by partial dietary restriction attract much attention. Dietary restriction was shown to have numerous positive effects on animals ranging from nematodes to long-living mammals as long as it does not cause malnutrition [211]. The findings of Paper IV imply that increased expression of numerous immune-related genes in CF could in theory translate into health complications for the fastest growing fish. Fast growth is linked to increased incidence of artherosclerosis in salmon [212] and on a molecular level, increased anabolic metabolism was shown to induce inflammatory response in adipocytes of Atlantic salmon, similar to what is observed in obese mammals [213]. Furthermore, low profile of genes involved in oxidative and cellular stress that was associated with growth retardation could be interpreted as beneficial.

A close link between metabolism and immunity has become a hot topic because of the great number of diseases in humans that have a nutritional cause [214]. The finding of a panel of consistently elevated TNF- and IFN-related genes in SF, CR and SR suggests that responses to nutrients and pathogens are highly integrated in fish as well and should be further studied.

5.5. General discussion

The role of high throughput analytical methods is consistently increasing in different areas including fish biology and aquaculture research. Studies of single genes and proteins have limited abilities to provide accurate diagnostics and to shed light on diverse and intricate biological pathways underlying complex traits. Although substantial effort has been put into the development of methods to measure disease resistance, neither single markers nor combination of several immunological parameters have shown high correlation with disease resistance [215]. Consequently, indirect selection for resistance, or growth, has not been applied in any fish breeding program hitherto. Global gene expression profiling technology has developed into an essential tool to detect, predict and better understand protective and pathological responses to environmental stressors and pathogens. The technology allows for the analysis of expression patterns of a large number of genes simultaneously in a sample and is now available for a number of aquacultured species.

5.5.1. Gene expression profiling reveals hormetic responses

In paper III, global expression profiling enabled clear separation of lentinan and LPS effects in the spleen of rainbow trout. It was also found that different magnitude of gene expression changes was induced by the two dietary inclusion levels of lentinan. The lower inclusion level of 0.2% produced more pronounced effects than the 0.4% inclusion (see Appendix Figure 2). It is noteworthy that an optimal effect of lentinan against *Salmonella enteritidis* and *Staphylococcus aureus* was achieved by a smaller dose in a study of Markova et al. [95]. In Paper IV, it was impossible to separate the effects of two studied stressors, dietary extractable soybean meal and reduced feeding. Single or in combination, they elicited very similar gene expression changes although growth was reduced proportionally to the level of the feeding stress, as expected. The expression changes seen in SR, which was the most severe treatment, were smaller than in SF and CR, which could mean that the adaptive abilities of the liver were reduced at highest level of feeding stress.

Papers II and IV show that dose-response relationships can be complex. The observed expression profiles deviated form the simple dose-dependent type of response and could be

classified as biphasic or hormetic [216]. Better knowledge of this type of responses is essential for the development and use of biomarkers in aquaculture. In particular, studies of responses to low exposures are of major practical concern, e.g. when determining the optimum dose of feed additives, or in toxicological studies, when estimating the effects of pollutants. Ideally, J-shaped curve should be assessed within each experiment by including a wider range of levels of studied factors and by adjusting the study design so that the hypothesis of a biphasic response could be properly addressed. The two studies in this thesis illustrate usefulness of the expression profiling by microarrays as a suitable technology that can be used to measure end-points in which hormetic responses might occur.

5.5.2. Cross-experimental comparisons

One of the important advantages of microarrays is possibility to compare expression patterns of the same genes under different conditions. Expression profiles determined in four studies were compared with results of other experiments (≈200) stored in the SFA2 gene expression database. These experiments mainly investigated responses to pathogens and pollutants.

Regulation of genes involved in iron metabolism was expectedly seen in all four studies, particularly in experiments with live pathogens and LPS. The withdrawal of bioavailable iron is a typical response to pathogens which require iron for their growth, referred to as the iron withholding strategy [217]. Low profiles of haemoglobin beta and 5-aminolevulinate synthase (ALAS2) coding genes were observed in internal organs of lice infected fish. This was in line with the finding that Atlantic salmon less resistant to *L. salmonis* had lower level of blood haemoglobin [218]. Reduced levels of free iron and heme could be predicted in fish susceptible to furunculosis. This implied that the iron withholding strategy is protective neither against *A. salmonicida* nor lice. Lentinan modified expression of a number of genes related to iron-metabolism in rainbow trout, e.g. ALAS2 was dramatically down-regulated by dietary β-glucan. Therefore, possible negative effects of lentinan on erythropoesis should be checked before a suggestion for its use can be made.

The group of genes involved in homeostasis of iron, and biosynthesis of heme and heme containing proteins is known to play important roles in a multitude of processes, in addition to

their involvement in the protection against pathogens. Iron and heme catalyse reactions that generate free radicals and thus take part in regulation of the oxidative stress. Iron capture and storage is a primary function of melano-macrophage centres in haemolytic diseases but interestingly, their number increases in response to starvation in fish ([219] and references therein). Iron withholding defence system could be readily triggered whenever there is an increased risk from ROS-induced damage. Indeed, genes implied in iron metabolism were found regulated under the three nutritional interventions in Paper IV. Although the study suggested lower expression of genes from oxidative stress pathways, the definite status of the intracellular redox state is difficult to assess from transcriptional data. Besides, the redox situation in other tissues could be substantially different from the one observed in liver.

Regulation of the intracellular redox status was observed in all four studies. If reactive ROS are produced in excess and the function of antioxidant systems is compromised, damaging reactions with various cell constituents will ensue, leading to impairment of cellular functions. On the other side, ROS produced in a regulated fashion are involved in the control of a wide array of cellular functions. ROS are now firmly placed at the crossroads of opposing pathways that transduce death or pro-survival signals to cells. It is shown that a pro-apoptotic TNF-α initiated pathway, as well as NF-κB-mediated survival are both affected by ROS. The prevention of the TNF-α induced apoptosis by NF-κB involves suppression of the JNK cascade [220]. In addition to controlling cell survival, the aforementioned pathways are crucially involved in the regulation of immune and inflammatory responses. Thereby, ROS are implicated as important mediators of inflammatory responses in fish.

Ample evidence was found for the differential activation of TNF-α and NF-κB mediated inflammatory pathways in tissues under experimental conditions investigated in this thesis. For instance, lower level of oxidative stress in lentinan fed fish was in line with the reduced induction of the iron-binding ferritin, involved in the iron withholding system, and the attenuated expression of TNF-α-dependent genes and genes involved in the activation of JNK. A number of ROS scavengers had expression profile in vaccinated survivors indicative of lower risk from oxidative stress.

Genes coding for endoplasmic reticulum (ER) stress proteins, including a number of heat shock proteins and sensors of UPR (X-box-binding protein-1 and 78 kDa and 94 kDa glucose regulated proteins), to name but a few, were shown to be sensitive markers of perturbations in cellular functions. The performed gene expression profiling identified a set of UPR

intracellular pathways that signal the presence of cellular stress in salmonid fish tissues. UPR is a collection of pathways elicited in response to the compromised ability of ER to perform its functions [221]. Cells actively engaged in immune and metabolic activities, such as hepatocytes and immune cells, transport large amounts of cargo through the ER and are hence ideal for studying the cross-talk of UPR on one side, and inflammation and metabolic alterations on the other. Changes in the ER homeostasis caused by metabolic perturbations or cytokines are shown to trigger ROS production and UPR [221]. Further, activation of UPR is associated with induction of inflammation. The data presented in this thesis imply the link between UPR and the inflammation in fish too, e.g. reduced expression of UPR markers in slowly growing fish was concurrent with the down-regulation of a number of inflammatory mediators (Paper IV).

Interestingly, many similarities were found between splenic gene expression profile (Paper III) and the profiles in Atlantic salmon infected with virus (ISAV) [1] and *A. salmonicida* (Paper II). Genes encoding INF-dependent (JAK/STAT, galectins) and MHC-I-related (B2M, proteasome, TAP, tapasin) proteins and the gene encoding chemokine leukocyte cell-derived chemotaxin 2 (LECT2) were up-regulated in three experiments, but their levels of induction were smaller in the group receiving lentinan and in fish resistant to virus and bacterium. The overlap of hepatic and splenic expression profiles implicates roles for these genes in the same biological pathways, further suggesting that the observed expression profile is a signature of common innate protective responses. These examples demonstrate that identification of differentially expressed genes under different experimental conditions is of great interest.

Comparative analyses of presented studies illustrate the “double-edged sword” nature of immunity. Immune activation confers protection against pathogens if properly controlled. Exaggerated immune responses bring destruction of own tissues as a side effect of elimination of the invaders. The immune system evolved complex mechanisms to control itself so that it does not harm the host.

Obviously, failure to control development of lice in Atlantic salmon is linked to risk of tissue damage. Weak response to lice is associated with chronic infection but little immunopathology at sites of lice attachment and active feeding. Controlling immunopathology helps the survival of sea lice on fish and increases the probability for parasites to complete the life cycle and reproduce. A study by Mustafa et al. [222] revealed that lice infection enhanced susceptibility to the microsporidian *Loma salmonae* in trout.

Thus, exaggerated Th2 during sea lice infection may be the cause of the impairment of protective immunity mounted against concurrent infections. The assessment of cross-regulatory suppression of Th1/Th2/Th17 responses is complicated by the shortage of cell-specific markers for salmonid fish however. An additional caveat is that the induced Th2 response might be a misdirected type of response that does not harm lice and thus only contributes to pathology. In mammals, Th2-initiated healing cascade, if unrestrained, results in pathological fibrotic responses in several chronic parasitic infections [223]. It is not clear if Th2-associated goblet cell hyperplasia and increased production of mucus induced by lice [14, 29; 224] is in any way directly detrimental for the fish though these responses were shown to be non-protective. On the other side, epidermal and dermal fibroplasia is almost completely absent in Atlantic salmon at sites of lice attachment and feeding, in line with the suppressed Th2 response by immunomodulating compounds secreted from lice.

In another scenario, detrimental consequences are induced by uncontrolled activation of immune mechanisms. Efficient and timely elimination of the pathogen is essential for the quick resolution of inflammation because removing the inciting stimuli by activated immune responses substantially impedes further pro-inflammatory mediator synthesis. Findings from Paper II reveal that activation of immune responses is beneficial when in conjunction with stringent control. It was shown that mobilisation of the complement system in resistant fish was in concert with the induction of the multiple regulatory proteins that prevent host damage with complement activation. Endogenous molecules originating from sites of cellular damage induce inflammation. Thus, repair and clearance serve as important pro-resolution/anti-inflammatory mechanisms that lead to the re-establishment of homeostasis and evasion from chronic infections. In conclusion, protection was shown to be an active process that involves engagement of both effector and repair/clearance mechanisms.

Beneficial effects of dietary lentinan (Paper III) were to a large extent attributed to its potent ability to down-modulate processes in splenic tissue that lead to the establishment of pro-inflammatory profile in cells, such as loading of LPS onto cells, its recognition and signal transduction. Eicosanoids, cell adhesion molecules, cytokines, chemokines and other molecules, such as matrix metalloproteinases, involved in the recruitment and migration of leukocytes also take part in uncontrolled inflammatory reactions. Many of the genes encoding these inflammatory mediators had lower profile in lentinan fed fish. Importantly, inflammation was not completely dampened and collectively, these effects can be considered beneficial.

6. Conclusion

Finding of differentially expressed genes enabled discrimination of specific responses to different stressors. In addition, global gene expression profiling improves understanding of molecular processes underlying the phenotypes of resistance and growth in salmonid fish. Mounting appropriate responses to a diversity of stressors is a demanding task imposed on fish in their complex aquaculture environment. The presented findings support the notion that protection from pathogenic agents and high growth are interconnected and dependent on correct kinetics and balance of multiple processes that play roles both in immunity and metabolism.

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Appendix

MathctName	CPG-4	CPG-24	LPS-4	LPS-24	SL-S3	SL-S4
Similar responses to SL and PAMP						
Matrix metalloproteinase 9	2.36	2.41	1.82	1.60	2.28	4.20
Matrix metalloproteinase-13	1.68	2.32	2.11	3.70	4.71	102.50
Tissue inhibitor of metalloproteinase 2	1.86	1.37	1.75	1.77	1.74	10.15
C type lectin receptor B	2.56	6.34	1.54	6.20	1.98	49.74
LECT2 neutrophil chemotactic factor	1.30	2.06	1.27	1.85	1.21	1.92
CCAAT/enhancer binding protein beta	1.52	6.89	2.13	4.31	34.05	24.01
Cofactor required for Sp1 activation	NS	1.79	NS	2.19	1.48	2.01
High-mobility group box 1	2.24	11.45	2.90	10.55	NS	4.08
Transcription factor jun-B-1	3.19	1.25	2.54	1.19	11.24	1.88
Thioredoxin	NS	7.26	NS	1.76	1.46	10.93
TATA-binding protein associated factor 2N	-2.46	-1.93	-1.61	-1.77	-1.85	-2.89
Dermatan-4-sulfotransferase-1-2	-2.17	-1.58	-1.38	-1.64	-1.30	-1.49
Deoxyribonuclease gamma precursor	-1.90	NS	NS	-1.60	-1.39	-2.08
Deoxyribonuclease II-1	1.01	-1.62	NS	-2.00	NS	-1.97
Deoxyribonuclease I-like-2	ND	NS	NS	-3.34	-1.24	-4.05
Serine protease-like protein-2	-2.14	-1.67	-1.51	-1.57	-1.22	-2.02
Quinone oxidoreductase	-2.65	-1.79	-1.52	-1.89	NS	-1.51
SL-specific responses						
Nucleophosmin 1	NS	-1.59	NS	NS	6.57	4.92
DNA-directed RNA polymerase II 23 kDa	NS	-1.54	NS	NS	2.60	4.00
Vacuolar ATP synthase 16 kDa subunit	1.43	NS	1.23	NS	2.29	3.28
Fibrillarin	NS	NS	NS	NS	2.50	3.21
Suppressor of initiator codon mutations	NS	1.34	1.32	1.39	5.02	2.95
Mitogen-activated protein kinase 6	NS	-1.60	NS	NS	2.02	2.10
Cytochrome c-1	2.54	NS	1.92	NS	3.59	2.43
ADP,ATP carrier protein T2	NS	-1.29	NS	NS	1.87	2.34
ADP,ATP carrier protein 3	1.31	NS	NS	1.32	2.08	1.33
Nonspecific cytotoxic cell receptor protein-1	NS	1.71	NS	NS	6.47	2.28
Interleukin 13 receptor alpha-2	NS	NS	NS	ND	-5.69	-4.28
Tumor differentially expressed protein 1	NS	1.47	NS	NS	-1.84	-1.60
Sphingosine 1-phosphate receptor Edg-3	NS	NS	NS	NS	-1.76	-3.59
MHC class I antigen	NS	NS	NS	NS	-1.42	-1.71
Beta-2-microglobulin-1 JB1	1.22	NS	1.15	NS	NS	-2.72
beta-2 microglobulin-1 BA1	NS	NS	NS	NS	-1.22	-3.16
PAMP-specific responses						
CCL4	12.45	18.38	4.83	3.88	-1.45	NS
Tumor necrosis factor receptor superfamily 5	3.86	1.90	2.48	1.69	NS	1.44
Tumor necrosis factor receptor superfamily 9	3.07	3.99	3.62	2.70	NS	NS
Tumor necrosis factor receptor associated	4.98	1.51	3.59	NS	NS	NS
TNF decoy receptor	1.77	30.25	2.35	1.51	NS	NS
Phosphotyrosine independent ligand for the Lck SH2	6.13	3.44	3.20	2.87	NS	NS
High affinity immunoglobulin gamma Fc receptor I	3.44	4.08	1.10	1.89	NS	NS
High affinity immunoglobulin epsilon receptor alpha	2.23	2.96	1.16	1.49	NS	NS
NF-kappaB inhibitor alpha-1	5.14	1.68	3.78	3.13	NS	NS
NF-kappaB inhibitor alpha-3	3.98	2.10	3.72	2.46	NS	NS
Nuclear factor NF-kappa-B	1.74	1.33	1.34	2.40	NS	NS
Nuclear factor kappa-B, subunit 1	1.64	1.43	1.32	2.33	NS	-4.31
C-Jun protein	2.28	3.22	NS	ND	NS	NS
Cytochrome B-245 heavy chain-1	1.54	NS	1.73	2.82	NS	NS
Cytochrome b-245 light chain	1.48	2.48	NS	3.37	NS	NS
Cytochrome B-245 heavy chain-2	NS	NS	1.64	2.21	NS	NS
Tolloid-like protein (nephrosin)	1.62	-2.82	NS	-2.74	NS	NS

Figure 1. Microarray comparison of gene expression in skin directly affected by salmon lice (SL) 22 dpi (S3) and 33 dpi (S4) and head kidney leukocytes at 4 and 24 hours after stimulation with CpG and LPS [113]. Data are log-Expression ratios.

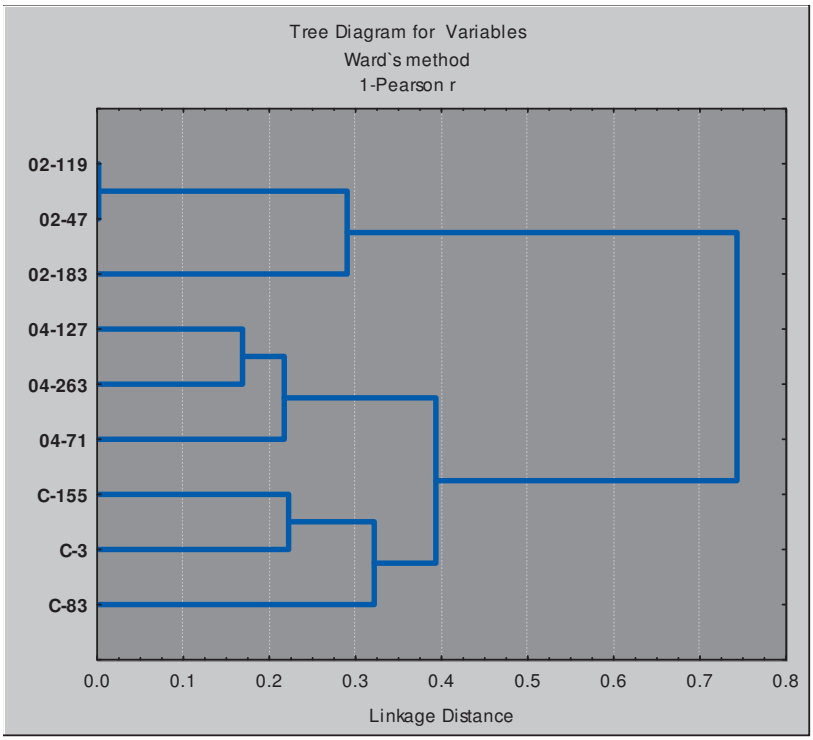

Figure 2. Hierarchical clustering of the gene expression profiles of fish fed 0,2% lentinan (02) and 0,4% lentinan (04) and control (C); three individuals were analyzed in each study group. The log-Expression ratio values of 391 genes with differential expression ($p < 0.01$) in at least one sample were included into analysis. The distance metrics were calculated by Pearson correlation and the tree was built with Ward's method.

Paper I

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Local and systemic gene expression responses of Atlantic salmon (*Salmo salar L.*) to infection with the salmon louse (*Lepeophtheirus salmonis*)

Stanko Skugor*[1], Kevin Alan Glover[2], Frank Nilsen[2,3] and Aleksei Krasnov[1]

Address: [1]Nofima Akvaforsk Fiskeriforskning, P.O.Box 5010, Ås 1430, Norway, [2]Institute of Marine Research, PO Box 1870, Nordnes, 5817 Bergen, Norway and [3]Department of Biology, University of Bergen, Box 7800 N-5020 Bergen, Norway

Email: Stanko Skugor* - Stanko.Skugor@nofima.no; Kevin Alan Glover - kevin.glover@imr.no; Frank Nilsen - fni001@exchange.uib.no; Aleksei Krasnov - Aleksei.Krasnov@nofima.no

* Corresponding author

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Abstract

Background: The salmon louse (SL) is an ectoparasitic caligid crustacean infecting salmonid fishes in the marine environment. SL represents one of the major challenges for farming of salmonids, and veterinary intervention is necessary to combat infection. This study addressed gene expression responses of Atlantic salmon infected with SL, which may account for its high susceptibility.

Results: The effects of SL infection on gene expression in Atlantic salmon were studied throughout the infection period from copepodids at 3 days post infection (dpi) to adult lice (33 dpi). Gene expression was analyzed at three developmental stages in damaged and intact skin, spleen, head kidney and liver, using real-time qPCR and a salmonid cDNA microarray (SFA2). Rapid detection of parasites was indicated by the up-regulation of immunoglobulins in the spleen and head kidney and IL-1 receptor type 1, CD4, beta-2-microglobulin, IL-12β, CD8α and arginase 1 in the intact skin of infected fish. Most immune responses decreased at 22 dpi, however, a second activation was observed at 33 dpi. The observed pattern of gene expression in damaged skin suggested the development of inflammation with signs of Th2-like responses. Involvement of T cells in responses to SL was witnessed with up-regulation of CD4, CD8α and programmed death ligand 1. Signs of hyporesponsive immune cells were seen. Cellular stress was prevalent in damaged skin as seen by highly significant up-regulation of heat shock proteins, other chaperones and mitochondrial proteins. Induction of the major components of extracellular matrix, TGF-β and IL-10 was observed only at the adult stage of SL. Taken together with up-regulation of matrix metalloproteinases (MMP), this classifies the wounds afflicted by SL as chronic. Overall, the gene expression changes suggest a combination of chronic stress, impaired healing and immunomodulation. Steady increase of MMP expression in all tissues except liver was a remarkable feature of SL infected fish.

Conclusion: SL infection in Atlantic salmon is associated with a rapid induction of mixed inflammatory responses, followed by a period of hyporesponsiveness and delayed healing of injuries. Persistent infection may lead to compromised host immunity and tissue self-destruction.

Introduction

The salmon louse (SL), *Lepeophtheirus salmonis*, is a marine ectoparasitic caligid crustacean infecting wild and farmed salmonids of the genera *Salmo*, *Salvelinus* and *Onchorhynchus* [1]. The life cycle consists of two planktonic larval stages, an infectious stage where copepodites attach to a suitable host, 4 immobile chalimus stages where the louse is firmly attached to the host's skin or fins, then 2 free-moving pre-adult stages before the final adult stage [1,2]. Heavy infestations present one of the major problems faced in marine salmon aquaculture and the concomitant rise of epizootics in wild populations is causing much debate about the possible ecological impacts of farmed fish [3,4].

Salmon lice feed on host mucous, skin tissue and blood. Juveniles usually cause only abrasive wounds on the host skin but nevertheless lead to systemic stress responses and modulations of the immune system and physiology (reviewed in [1,5,6]). Host susceptibility differs among the salmonid species [7-10]. Coho salmon (*O. kisutch*) successfully expels parasites during chalimii stages while Atlantic salmon (*S. salar*) fails to initiate inflammation and shows no apparent tissue responses to the attached larvae [8,11]. The ability to expel parasites can be determined with hyperplastic and inflammatory responses in the epidermis and underlying dermal tissues [1] and references therein. Hyperinflammatory phenotype in the resistant coho salmon is manifested within a day post infection and is characterised by a rich neutrophil influx at the site of parasite attachment. The inflammatory infiltrate persists during the whole period of salmon lice development on coho salmon and is accompanied with the pronounced epithelial hyperplasia that in some cases completely encapsulates the parasite. Intraspecific comparisons revealed the association of an early regulation of pro-inflammatory interleukin (IL)-1β, IL-8 and tumour necrosis factor-α (TNF-α) with the improved chalimus expulsion in resistant species, which was attributed to the exaggerated T helper 1-type (Th1) responses (normally targeting viruses and bacteria) [12].

To elucidate the factors that determine high susceptibility of Atlantic salmon to lice and to evaluate the side consequences of infestation we conducted gene expression analyses throughout the life cycle, from copepodids to pre adults. Samples of damaged and intact sites of skin and immune organs (spleen, head kidney and liver) were collected 3 days post infection (dpi), 22 dpi, and 33 dpi; these time-points corresponded to the key stages (respectively copepodids, chalimus III/IV, preadult females and males). Multiple gene expression profiling is especially efficient in studies of scantily investigated conditions that may involve interactions of multiple factors. We used a high density salmonid fish cDNA microarray (SFA2 or immunochip) designed specifically for studies of responses to stressors and pathogens. In comparison with previous version ([13,14], GEO GPL1212), the updated platform was substantially enriched in immune genes (see[15]; GEO GPL6154). Real-time qPCR analyses were performed to validate the microarray results and to expand studies by examination of genes that were not present on the platform.

Results

Responses to salmon lice in skin

Samples of intact skin were collected from the infected salmon within the whole study period, however, it was not possible to sample damaged skin before 22 dpi (see Materials and Methods, Fig. 7). Microarray analyses were designed to contrast the direct and indirect responses of infection with SL. Comparison of infected and intact sites from the same fish (Fig. 1) subtracted systemic responses and revealed the direct effects of parasites. Microarray comparison of intact skin from challenged and control fish was conduced to evaluate the systemic responses, however, expression changes were small (data not shown). The qPCR analyses were performed for damaged and intact skin, using skin from uninfected fish as control (Fig. 2). Both methods revealed profound changes in gene expression post infection.

Microarray analyses found differential expression of a number of genes linked to immunity. Ca-dependent (C-type) lectin receptor B and lectin receptor C, which were up-regulated in SL damaged skin, can take part in a multitude of biological processes. They regulate cellular interactions, including migration and adhesion of antigen presenting cells with lymphocytes [16,17]. In mammals, different dendritic cell (DC) subsets and maturation stages display distinct C-type-lectin profiles, depending on the local microenvironment and pathogen products [18,19]. Matrix metalloproteinases (MMP) 9 and 13 are commonly quiescent at the healthy state and become activated post-injury. These enzymes, working in conjunction with tissue inhibitors of metalloproteinases (TIMPs) (Fig. 1) can be involved in a wide range of processes such as cleavage and activation of cytokines, release of cytokines and growth factors from extracellular matrix (ECM), and establishment of a gradient for migration of cells [20]. Additionally, MMPs play the key role in the remodelling and destruction of ECM [21]. Both MMP9 and MMP13 displayed similar expression profiles, characterized by opposite regulation in the intact and damaged sites (Fig. 1 and Fig. 2D). The CCAAT/enhancer-binding protein (C/EBP) family of transcription factors are involved in positive and negative control of cell proliferation and differentiation and immune responses [22](Fig. 1 and Fig. 2H). High-mobility group box protein, a proinflammatory

Genes	22 dpi	33 dpi
Immune response, apoptosis, regulation of gene expression		
C type lectin receptor B	0.98	5.64
C type lectin receptor C	1.25	1.62
Matrix metalloproteinase 9	1.19	2.07
Matrix metalloproteinase-13	2.23	6.68
Tissue inhibitor of metalloproteinase	0.80	3.34
CCAAT/enhancer binding protein beta	5.09	4.59
High-mobility group box 1	0.28	2.03
LECT2 neutrophil chemotactic factor	0.28	0.94
Annexin A1-1	0.67	1.72
Complement component C8 beta chain	0.82	1.34
X-box binding protein 1	2.28	1.32
Nonspecific cytotoxic cell receptor	2.69	1.19
Mitogen-activated protein kinase 6	1.02	1.07
CD63	0.87	0.84
B-cell translocation gene 1-2	0.80	0.79
X-linked interleukin-1 receptor accessory	0.74	0.32
Cofactor required for Sp1 activation, subunit	0.56	1.01
Nucleophosmin 1	2.72	2.30
DNA-directed RNA polymerase II 23 kDa po	1.38	2.00
Fibrillarin	1.32	1.68
Interleukin 13 receptor alpha-2	-2.51	-2.10
Quinone oxidoreductase	-0.42	-0.59
Beta-2-microglobulin-1 JB1	-0.09	-1.44
beta-2 microglobulin-1 BA1	-0.29	-1.66
Sphingosine 1-phosphate receptor Edg-3	-0.82	-1.84
Serine protease-like protein-1	-0.46	-1.16
Deoxyribonuclease gamma precursor	-0.48	-1.05
Deoxyribonuclease I-like	-0.32	-2.02
G1/S-specific cyclin D2	-0.60	-1.34
Cytochrome P450 27	-1.21	-0.61
All-trans-13,14-dihydroretinol saturase	-0.59	-0.86
TATA-binding protein associated factor 2N	-0.89	-1.53
Prostaglandine D synthase	-0.32	-3.53
5-lipoxygenase activating protein	-0.71	-0.10
Mitochondria, oxidative stress		
ADP,ATP carrier protein 3	1.06	0.41
ADP,ATP carrier protein T2	0.91	1.23
ATP synthase beta chain-1	0.90	0.30
Cytochrome c-1	1.84	1.28
Glutathione peroxidase	0.82	1.62
Thioredoxin	0.54	3.45
Thioredoxin-like protein 4A	-0.66	-4.85

Genes	22 dpi	33 dpi
Protein folding, cellular stress		
14-3-3 B1-like	0.68	1.02
14-3-3C2	1.84	2.48
78 kDa glucose-regulated protein	3.08	0.92
94 kDa glucose-regulated protein	1.04	0.98
Heat shock 60 kDa protein	1.55	1.66
Heat shock 70 kDa protein 1	0.88	0.86
Heat shock 70kD protein 9B	1.93	0.54
Heat shock 70kDa protein 8	0.81	0.93
Heat shock cognate 70 kDa protein	0.92	0.93
Heat shock cognate 71 kDa protein	0.74	0.82
Heat shock protein HSP 90-beta-2	1.59	1.64
T-complex protein 1, alpha subunit	0.45	2.39
T-complex protein 1, gamma subunit	0.32	0.83
T-complex protein 1, subunit 2	1.10	1.30
T-complex protein 1, subunit 5	0.74	1.86
Cytoskeleton, extracellular proteins		
Myosin heavy chain cardiac	-0.38	-3.69
Myosin heavy chain skeletal	0.84	-2.24
Myosin light chain	1.54	-1.72
Parvalbumin alpha-2	0.44	-1.09
Reticulum calcium ATPase	1.06	-1.71
Actin alpha skeletal	1.62	0.63
Actin, cytoplasmic	1.48	1.88
Tropomyosin alpha 3 chain-3	1.54	0.35
Vacuolar ATP synthase 16 kDa	1.19	1.71
Calpactin I light chain	0.30	1.30
Coronin-1B	0.94	1.25
PDZ and LIM domain protein 1	0.89	0.99
Profilin-1	0.69	0.84
Cofilin, muscle isoform	0.91	0.70
ARP2/3 complex 34 kDa subunit	0.75	0.71
Cytokeratin 8	0.70	1.01
Tubulin alpha-3 chain	0.91	0.96
Keratin, type II cytoskeletal 8	0.71	0.92
Tubulin alpha-ubiquitous chain	0.77	0.91
Transgelin	0.69	0.75
Calmodulin-1	0.87	0.57
Cathepsin B	0.79	1.32
Cathepsin S	1.05	1.13
Alpha 2 type I collagen-1	NS	-3.04
Collagen alpha 1(V) chain	0.98	-2.58
Collagen a3(I)-2	0.26	-2.12
Collagen alpha 1(I) chain-1	0.08	-2.08
Collagen a3(I)-1	0.23	-1.66
Hyaluronan and proteoglycan link protein	-0.23	-0.89

Figure 1
Microarray comparison of gene expression in damaged and intact skin of SL infected fish, examples of differentially expressed genes. Pooled samples were analysed, data are log-ER (expression ratios). Significantly up- and down-regulated genes ($p < 0.01$, t-test, 12 spot replicates per gene) are highlighted with red and green scales, NS means not significant.

cytokine with DNA binding properties [23] was up-regulated 33 dpi (Fig 1).

Down-regulation of a decoy receptor IL-13 receptor alpha-2 (IL13Rα2) implied induction of the IL-4/IL-13 axis [24] and possible polarization towards the T helper 2-type (Th2) immune response, a typical response against parasites. The T helper cells (Th) that differentiate into the Th2 subset are characterised by their ability to suppress development of the IL-12 producing Th1 subset [25]. There-

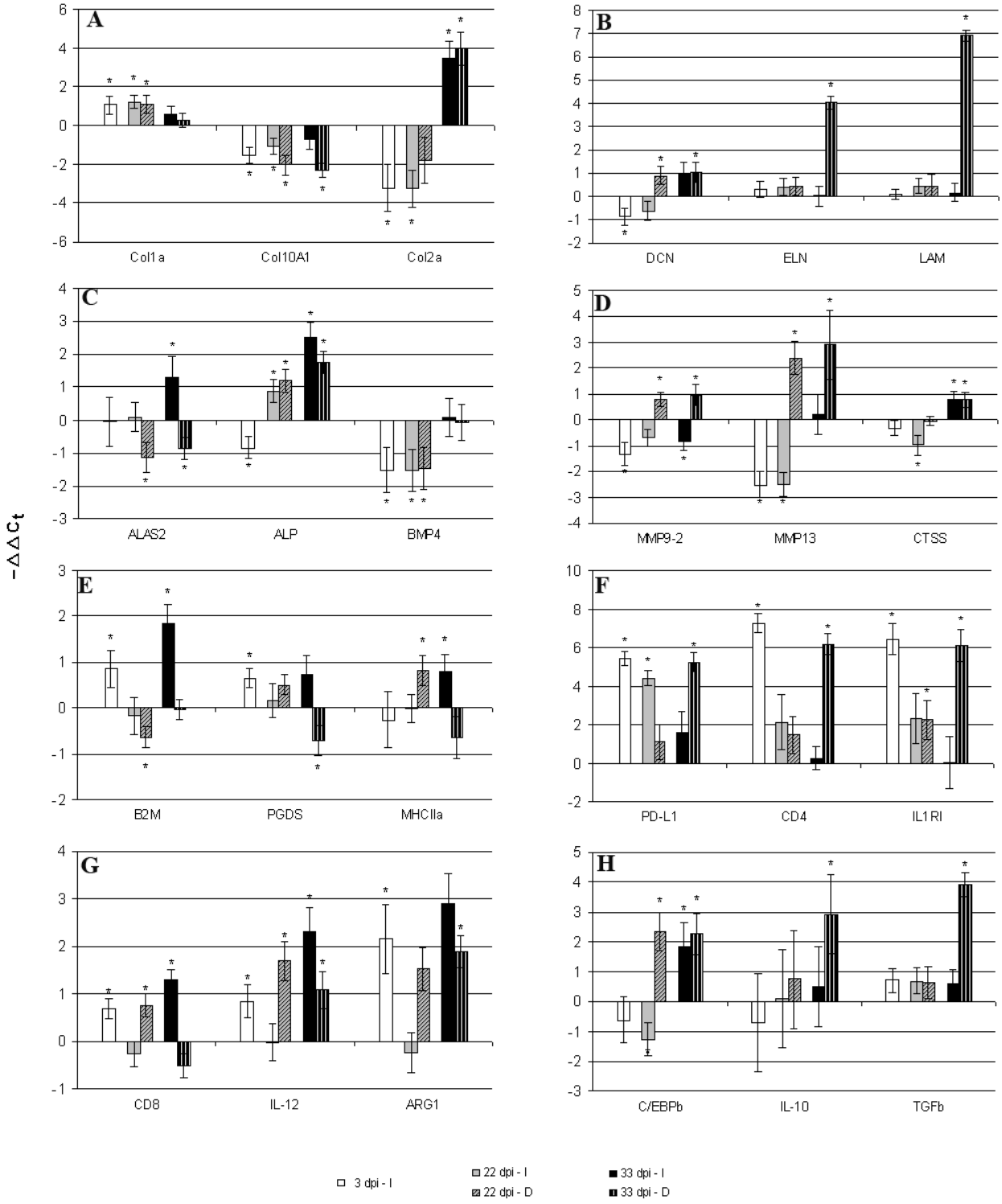

Figure 2 *(see legend on next page)*

Figure 2 *(see previous page)*
Gene expression in skin analyzed with qPCR (individual samples). Data are -ΔΔCt ± SD (n = 6). **A**. collagen (COL) genes: COL1a, COL2a and COL10a **B**. components of the extracellular matrix (ECM): decorin (DCN), elastin (ELN) and laminin (LMN) **C**. 5-aminolevulinate synthase (ALAS2), alkaline phosphatase (ALP) and regulatory bone morphogenic protein (BMP4) **D**. proteases involved in ECM remodelling, matrix metalloproteinases (MMP) 9 and 13 and in antigen presentation, cathepsin S (CTSS) **E**. beta-2-microglobulin-2 JB1 (B2M), a component of the major histocompatibility complex (MHC) class I; MHC class II α chain (MHCIIa) and regulator of inflammation prostaglandine D synthase (PGDS) **F**. T cell-inhibitory programmed death ligand 1 (PD-L1), CD4, marker of T cells and IL-1 receptor type 1 (IL1RI), transducer of pro-inflammatory signals **G**. CD8α, expressed on cytotoxic T cells; IL-12β, produced in Th1 settings and ARG1, marker of the Th2 response H. CCAAT/enhancer-binding protein β (C/EBPb) involved in the control of cell proliferation and differentiation; regulatory cytokines IL-10 and transforming growth factor-β (TGF-β). Significant difference from uninfected control (t-test, $p < 0.05$) is indicated with *. I-intact skin of infected fish, D-skin damaged by sea lice

fore, we included in qPCR analyses the key genes that could give information on the type and dynamics of T cell responses throughout the study period (Fig. 2E, F, G, and 2H). Marked increase of CD4 transcripts 3 dpi at intact sites supports a rapid infiltration of T cells into the skin after the exposure to copepodids (Fig. 2F). High expression of CD4 at injured sites 33 dpi indicates a second wave of T cell migration from lymphoid organs or an increased proliferation in the periphery. CD4+ Th cells are essential intermediaries of the adaptive immune system, which instruct innate effector cells and amplify their responses mainly through the secretion of specific cytokines. IL-1 receptor type 1 (IL1RI) transduces signals from proinflammatory cytokines IL-1β and IL1α and can serve as a marker of a newly described highly inflammatory effector Th subset, Th17 [26]. The IL1RI expression profile was similar to that of CD4 (Fig. 2F). IL1RI was highly responsive to SL induced damage whereas its expression steadily declined in the intact sites after the initial increase 3 dpi. IL-10 can down regulate inflammatory Th responses via a regulatory CD4+ subset, Treg [27]. Both IL-10 and TGF-β are pleiotropic cytokines, which are generally regarded as anti-inflammatory. We observed synchronous up-regulation of IL-10 and TGF-β at 33 dpi (Fig. 2H). Possible differentiation of Th1 was indicated with changes of the transcript levels of IL-12β and beta-2-microglobulin-2 JB1 (B2M), a component of the major histocompatibility complex (MHC) class I (Fig. 2G, E). B2M was down-regulated 22 dpi and interestingly, similar changes were observed in skin of carp infected with ectoparasite *Ichthyophthirius multifiliis* [28]. Changes of CD8α suggested involvement of cytotoxic T cells (Fig. 2G). Arginase 1 (ARG1), a typical marker of alternatively activated macrophages (M2), central downstream effector cell of the Th2 response, implied the pronounced activation of M2 and their rapid recruitment both at the onset and at the end of infection (Fig. 2G). Activation of T cell-related genes 3 dpi was followed with decline 22 dpi and increase at 33 dpi in intact skin (Fig. 2F, G). Sarcoplasmic/endoplasmic reticulum calcium ATPase involved in calcium sequestration was up-regulated 22 dpi in injured areas and then down regulated 33 dpi (Fig. 1). A similar expression pattern was seen in several genes involved in calcium signaling and muscle contraction, including calcium-binding protein parvalbumin α2 and several myosin genes (Fig. 1).

Regulation of a number of genes with known anti-inflammatory actions was observed. Annexins were consistently up-regulated at the damaged sites (Fig. 1) [29]. Increase of prostaglandine D synthase (PGDS) expression 3 dpi was followed with down-regulation 33 dpi at the sites of SL attachment (Fig. 1 and Fig. 2E). PGDS is the key enzyme involved in the synthesis of PGD_2, which is further metabolized to 15-d-PGJ_2, a potent anti-inflammatory mediator [30]. It can inhibit the production of iNOS, TNF-α and IL-1β in macrophages through the inhibition of MAP kinases, nuclear factor kappaB (NFkB) or IkB kinase [31,32]. 15-d-PGJ2 mediates the inhibition of proliferative responses of T cells and induces apoptosis of T cells by a PPAR-γ-dependent mechanism [33].

Accumulation of misfolded proteins in endoplasmic reticulum lumen activates a set of intracellular signalling steps collectively called the unfolded protein response (UPR). UPR is induced by a variety of insults, including nutrient and oxygen deprivation, pathogen infections, changes in redox status and intralumenal calcium (reviewed in [34]). Microarray analyses showed highly significant induction of mitochondrial proteins involved in biosynthesis and transport of ATP, heat shock proteins, 94 kDa glucose-regulated protein (GRP94), 78 kDa glucose regulated protein (GRP78), and X-box binding protein 1 (XBP-1) indicating unfolded protein response (UPR), typical of wounded tissue (Fig. 1).

As expected, massive changes in genes for proteins of ECM took place and they were substantially greater in the damaged sites (Fig. 1 and Fig. 2A, B). In addition to MMPs, several lysosomal proteases, cathepsins were regulated, which was in line with the degradation of tissue (Fig. 1 and Fig. 2D). Secreted cathepsin S (CTSS) is an elastolytic cysteine protease capable of degrading ECM components.

We observed down-regulation of several types of collagens and up-regulation of elastin and laminin in the damaged sites and these changes increased markedly at the late stage of SL development (Fig. 1 and Fig 2A, B). Gene expression changes that can be relevant to remodelling of ECM were not only observed in infected sites. Down-regulation of decorin (DCN) was found in intact skin 3 dpi and 22 dpi (Fig. 2B). Rapid changes were seen in the bone morphogenetic protein (BMP4), which was markedly suppressed until 33 dpi in both intact and injured sites (Fig. 2C). The enzymes involved in modification of extracellular matrix also showed rapid responses (Fig. 2D). Increase of alkaline phosphatase (ALP) activity was reported in mucus of SL infected Atlantic salmon [35] and in regenerated scales of a common goby [36]. In the present study, early decrease of ALP expression at the intact sites was followed with the up-regulation in all areas (Fig. 2C).

Responses to salmon lice in the head kidney, liver and spleen

The gene expression changes in the intact sites of skin suggested rapid systemic responses to parasites. This was supported by the results of analyses in the head kidney, spleen and liver (Fig. 3 and Fig. 4). These organs were included in the study due to their essential roles in immunity. Rapid change was seen in a group of immunoglobulin (IG) like genes, which then returned toward control levels in the head kidney (Fig. 3B). Early increase of the expression of these genes in the spleen declined during chalimus developmental stages, with subsequent return to a similar level to initial by day 33. Changes of MMPs (delayed increase in the head kidney and spleen first noticed at 22 dpi) were one of the most remarkable features of the infected fish (Fig. 3A and Fig. 4A). In our earlier studies, similar MMP profiles were observed in rainbow trout challenged with handling stress [14]. Differential expression of B2M was seen in all studied tissues of SL-infected fish and only in the liver these genes were up-regulated (Fig. 3A). Quinone oxidoreductase and all-trans-13,14-dihydroretinol saturase were progressively down-regulated in the liver while opposite was seen for quinone oxidoreductase in the head kidney and for all-trans-13,14-dihydroretinol saturase in the spleen (Fig 3A). Adenosine deaminase is a regulator of inflammation [37,38], which was down-regulated in liver 22 dpi, while it had a peak in expression at the same stage of infection in the head kidney and spleen (Fig. 3A). C-type mannose binding lectin (MBL1) was up-regulated in liver but only 3 dpi and 33 dpi (Fig. 3A and Fig. 4B). MBL recognises carbohydrates on both foreign organisms and damaged cells and cellular debris and then initiates their removal and local inflammatory responses. The 5-lipoxygenase activating protein was markedly activated in head kidney at all stages but only 33 dpi in spleen (Fig. 3A). This protein is required for the production of leukotrienes, best known for their potent chemotactic and leukocyte-activating effects [39,40]. CXC chemokine receptor (CXCR4) has a potent chemotactic activity for lymphocytes and was shown to inhibit haematopoietic stem cell proliferation [41]. In the present study, up-regulation of CXCR4 in spleen 22 dpi was observed (Fig. 4A). A suit of other chemokines with potential roles in recruitment and activation were regulated in internal organs (CC chemokine SCYA110-1, CC chemokine SCYA110-2, leukocyte cell-derived chemotaxin 2 and macrophage migration inhibitory factor-like) (Fig. 3A).

Co-ordinated changes were seen in the genes involved in metabolism of iron and erythropoiesis. In head kidney and spleen this group was down-regulated within whole study period while in liver, initial decrease was followed with the gradual elevation. Selected representatives for qPCR analyses in spleen were haemoglobin beta chain (HBB) and erythroid-specific 5-aminolevulinate synthase (ALAS2) (Fig. 4A) and heme oxygenase 1 (HMOX1) and HBB in liver (Fig. 4B).

Discussion

Results of this study represent a significant contribution into the understanding of the underlying physiological basis for the high susceptibility of Atlantic salmon to salmon lice and the side effects caused by the infection with this parasite. The ability to reject parasites can be determined with inflammation and healing of wounds, and here, we observed expression changes of genes involved in these processes.

ECM and wound healing

The pathology of Atlantic salmon infected by high numbers of lice is characterised by gross lesions, vast areas of eroded skin on the head and back, necrotic tissue and subepidermal haemorrhaging at margins of lesions (reviewed in [1]). Because of the danger of osmotic shock in aqueous environment, any break in the fish skin must be rapidly repaired. The initial hemostatic event upon breaching of epidermis provides the provisional fibrin-fibronectin wound matrix, which is a framework for cell adhesion, migration, and repair [42]. Maintaining sufficiently high levels of plasma fibronectin, produced in the liver, plays an important role in wound healing [43]. Fibronectin was down-regulated in liver of Atlantic salmon already 3 dpi and continued until 33 dpi, suggesting limited wound healing ability in the afflicted skin (Fig. 3A). Fibronectin is a large adhesive glycoprotein which interacts with cells and transmits signals primarily through integrin receptors expressed on a variety of epidermal cells including keratinocytes, endothelial cells and fibroblasts, allowing them to interweave with the fibrin clot in the wound space [44,45]. In a fish scale-skin culture system, dermal sub-

A

Genes	HK-3dpi	HK-22dpi	HK-33dpi	L-3dpi	L-22dpi	L-33dpi	S-3dpi	S-22dpi	S-33dpi
Matrix metalloproteinase-13	0.42	0.76	0.24	NS	NS	0.36	0.16	1.00	0.24
Matrix metalloproteinase-9	-0.51	0.65	0.54	-0.13	NS	NS	NS	0.94	1.86
Tissue inhibitor of metalloproteinase	-0.55	0.50	0.55	NS	NS	-0.36	NS	0.68	2.10
beta-2 microglobulin-1 BA1	0.31	-1.09	-1.13	0.44	-0.47	1.54	NS	-0.49	-0.55
Beta-2-microglobulin-2 JB1	0.31	-1.11	-0.64	NS	NS	NS	NS	-0.58	-0.55
All-trans-13,14-dihydroretinol saturase	0.94	NS	NS	0.82	0.73	0.21	1.86	1.59	1.57
5-lipoxygenase activating protein	0.68	0.77	0.51	NS	NS	0.33	0.53	NS	0.53
Quinone oxidoreductase	-0.64	0.31	NS	NS	-0.79	-0.96	-0.59	NS	NS
Adenosine deaminase 3	-0.63	0.33	0.28	-0.32	-0.91	-1.10	-1.12	0.56	0.32
Annexin A1-1	-0.67	NS	0.37	NS	NS	NS	NS	-0.52	0.67
C-type mannose-binding lectin	NS	NS	NS	1.78	NS	4.28	NS	NS	-0.31
Macrophage migration inhibitory factor	2.05	-0.90	NS	NS	NS	NS	NS	NS	NS
CC chemokine SCYA110-1	NS	NS	0.36	NS	NS	-0.91	NS	0.88	0.67
CC chemokine SCYA110-2	-0.96	NS	1.85	-0.19	NS	-0.41	-0.56	0.72	3.14
Leukocyte cell-derived chemotaxin	NS	-0.42	NS	-0.23	-0.78	0.85	0.23	-0.15	0.09
Chemokine receptor CXCR4	NS	NS	NS	NS	NS	NS	0.10	1.32	0.46
Fibronectin precursor	NS	NS	NS	-0.44	-0.49	-1.41	NS	NS	NS
Cathepsin B-2	-0.19	-0.52	NS	NS	NS	0.56	0.22	-0.10	0.68
Cathepsin S	0.55	0.31	0.13	NS	NS	NS	0.17	-0.27	NS
Interferon regulatory factor 1-1	NS	-0.29	NS	NS	NS	NS	0.43	-0.46	NS
Liver-expressed antimicrobial peptide 2B	NS	NS	NS	0.94	0.36	0.90	NS	NS	NS
Lysozyme C precursor	-0.16	-0.89	-0.86	-0.99	NS	NS	NS	-0.51	-1.36
All-trans-13,14-dihydroretinol saturase	-0.04	NS	NS	-1.17	-0.27	0.30	-0.60	0.54	0.77
ATP-binding cassette D3	NS	NS	NS	NS	NS	0.51	NS	0.89	1.98
ATP-binding cassette transporter 1	NS	NS	NS	NS	NS	-0.96	NS	0.60	0.54
ATP-binding cassette, sub-family F	NS	NS	NS	NS	0.50	NS	-0.62	-0.90	-0.71
5-aminolevulinate synthase	0.24	-0.41	-0.32	-1.15	NS	NS	-0.52	-0.79	-1.07
Integrin beta-1-binding protein	NS	-0.44	-0.31	NS	0.37	NS	-0.97	-0.98	-0.65

B

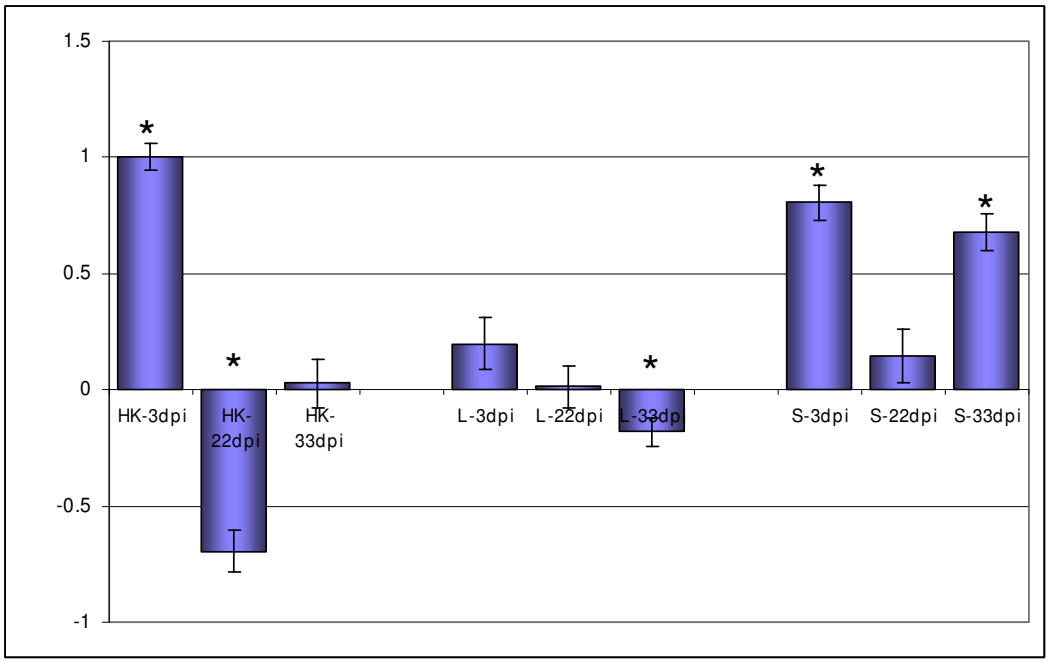

Figure 3

Microarray analyses of gene expression in head kidney (HK), liver (L) and spleen (SP). Pooled samples were analysed using uninfected fish as a common reference. **A** – log-expression ratios. Significantly up- and down-regulated genes ($p < 0.05$, t-test, 12 spot replicates per gene) are highlighted with red and green scales, NS is not significant. **B**: mean expression profiles of nine immunoglobulins designated by the Unigene clusters and most similar mammalian genes: Omy 9391 (Ig kappa chain V-III region VG), Omy 416 (Ig kappa chain V-IV region JI), Omy 23312 (Ig kappa chain V-IV region B17-1), Omy 9391 (Ig kappa chain C region), Omy 30091 (Ig kappa chain V-IV region Len), Ssa 709 (Ig kappa chain V-IV region B17-2), Ssa 78 (Ig heavy chain V-III region HIL), Omy 11287 (Ig mu heavy chain disease protein), Ssa 709 (Ig kappa chain V-I region WEA) Data are mean log-expression ratios ± SE, significant differences from zero ($p < 0.05$) are indicated with *.

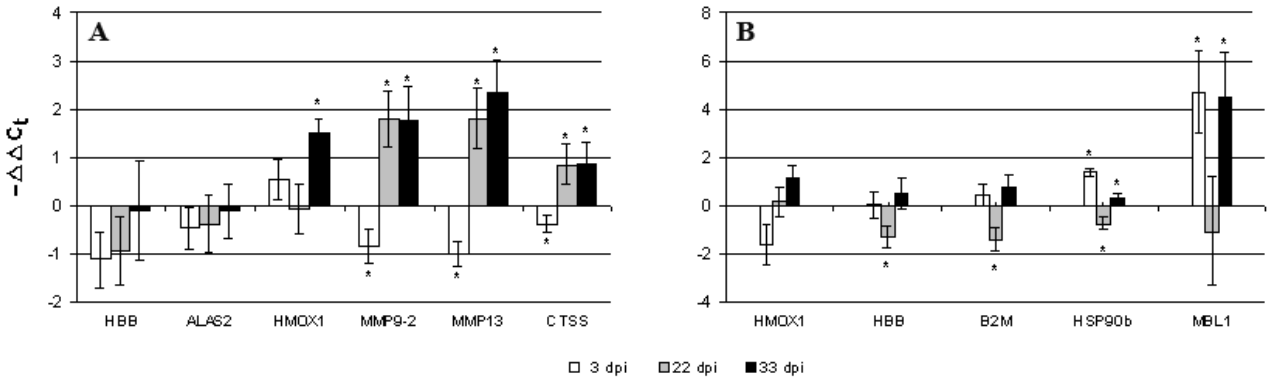

Figure 4

Gene expression in spleen(A)and liver (B) analyzed with qPCR (individual samples). Data are -ΔΔCt ± SD (n = 6). Significant difference from uninfected control (t-test, $p < 0.05$) is indicated with *. **A**: spleen. Haemoglobin beta (HBB) and 5-aminolevulinate synthase (ALAS2) were selected for qPCR analyses as the representatives of genes involved in erythropoiesis and metabolism of iron. Two metalloproteinases (MMP) 9 and 13 engage in ECM remodelling and cathepsin S (CTSS) may indicate activation of the Th1-related adaptive immunity **B**: liver. Genes selected for qPCR analyses: heme oxygenase 1 (HMOX1) and HBB, involved in iron metabolism; beta-2-microglobulin-2 JB1 (B2M), an indicator of Th1 responses; heat shock protein 90 β (HSP90b), implied in cellular stresses; C-type mannose binding lectin (MBL1), receptor possibly involved in the recognition of SL-derived antigens. Significant difference from uninfected control (t-test, $p < 0.05$) is indicated with *.

strates such as fibronectin and type I collagen were able to initiate migration of keratinocytes and epidermal outgrowth even in the absence of growth factors [46]. In the present study, expression profiles of genes encoding ECM components were also similar to profiles characteristic for slowly repairing injuries [47-49]. This included down-regulation of several collagens at the end of experiment. A slight but consistent up-regulation of COL1a was detected by qPCR, however, COL10a was stably down-regulated as well as COL2a at 3 dpi and 22 dpi. A relatively low induction of decorin, a regulator of assembly of collagen fibrils [50,51] and TGF-β activity [52,53], was seen 22 dpi and similar changes were observed in ALP. However marked up-regulation of major ECM proteins elastin and laminin was seen only 33 dpi as well as induction of COL2a. Noteworthy, slow reparation of ECM was in parallel with stable up-regulation of MMP9 and MMP13 in the damaged sites, whose excessive activity may contribute to the development of chronic wounds [54].

Delayed wound healing could be accounted for by the insufficient expression of several regulatory proteins. Increase of TGF-β, an essential stimulator required for ECM development [55], was seen not earlier than 33 dpi. Actions of TGF-β, which is released from platelets and macrophages immediately after injury, largely depend on the presence of fibronectin and other ECM components [53] and vice versa. It attracts neutrophils, macrophages, and fibroblasts, which in turn release more TGF-β. Relatively modest changes in matrix composition were shown to have major effects on cell responses and growth [56], including self-renewal [57]. We studied the expression of a developmentally important gene, BMP4, another TGF superfamily member, which is known to be up-regulated in undifferentiated stem-like cells [58] and to play an important role in skin homeostasis [59]. This gene was markedly down-regulated until 22 dpi. Collectively, these gene expression profiles of skin ECM and MMPs classify the wounds afflicted by SL as chronic, due to the significantly protracted duration and deregulation of events in the healing cascade. Together with opposite expression changes of several genes in the intact and damaged sites (MMPs, C/EBPb) this could indicate a modulatory activity of SL and/or constant damage inflicted by the growing parasites. The ability of SL to reduce the protective responses has been reported by several groups. Firth et al. (2000) [60] characterised low molecular proteases (LMW) secreted by *L. salmonis* onto the surface of the fish as trypsins, which are known to be used by many parasites for invasion and to suppress immune responses in their hosts [61]. Fast et al. [11] correlated the reduced respiratory burst and phagocytosis in macrophages of infected Atlantic salmon and rainbow trout with the appearance of LMW bands in the mucus.

Inflammatory responses

The rate of wound healing and the ability to reject parasites could also be explained by the characteristics of inflammatory responses to SL. Inflammation is regarded as a two-edged sword since destructive alterations are

closely associated with the subsequent reparation of damaged tissues and often involve the same or closely related molecular mechanisms and cellular elements. Hence, suppression of inflammation may result in chronicity. In SL infected coho salmon, tissues develop pronounced epidermal hyperplasia at the sites of attachment, which is accompanied with abundant cellular infiltration within the dermis beneath chalimii [1,8]. Inflammatory infiltrate consists mostly of neutrophils. Cellular debris and phagocyte neutrophils are abound at the early phases of infection whereas macrophages and a small number of lymphocytes appear later. Collectively, the findings in these studies are in line with our results showing restricted tissue inflammatory changes at both copepodid and chalimus stages. Early up-regulation of immunoglobulin-like genes in the head kidney and spleen, in addition to a panel of immune genes in the intact areas of skin, indicated a rapid activation of the systemic antiparasitic responses. In SL infected skin, up-regulation of Sp1 cofactor, a partner of NFkB [62], provided indirect evidence for possible activation of the NFkB pathway. An inflammatory state could also explain the decreased expression of quinone oxidoreductase in SL skin 33 dpi, as was similarly observed in mammalian cells [63]. However, the input of NFkB pathway in responses to SL was relativley low. At low level of NFkB activation, T cells develop an anergic state through Ca2+ signalling [64]. In this respect, it is noteworthy to mention that SL infection induced a number of Ca2+ regulatory genes, e.g. calcineurin, calmodulin and calpactain I light chain (Fig. 1B).

Restricted inflammatory responses in SL damaged skin were in parallel with massive up-regulation of chaperones indicative of the induction of UPR. In addition, increased levels of genes regulating mitochondrial proteins were observed. This has been previously observed in relation to responses to handling stress [14] and acute toxicity [13,65] in rainbow trout. Such opposite regulation of stress and immune responses is well known. Cortisol, the most common marker of stress in fish is widely used for anti-inflammatory therapy. Increased levels of cortisol that were reported in lice infected salmon [12,35,66] can at least partly account for the lack of strong inflammation. Steady increase of MMPs in all organs except liver was a remarkable feature of SL infected salmon. In salmonid fish these genes are up-regulated with both stress and inflammatory agents [14,67]. Prolonged stress, infection and combination of these two can result in chronic degradation of ECM.

What immune cells can be involved in responses to SL?

Microarray analyses revealed signs of inflammation but did not indicate which immune cells could have taken part in responses to SL. To address this, a set of markers was included in the qPCR analyses. ARG1, a marker of alternatively activated macrophages (M2) [68] with an important role in tissue remodelling and wound healing [69], was up-regulated 3 and 33 dpi but down-regulated 22 dpi at the intact sites. In contrast to classically activated macrophages (M1), M2 are induced by Th2 cytokines IL-4 and IL-13, that are prevalent at parasitic infections and in wound settings [70,71]. The maintenance of M2 requires the adaptive arm of the immune system [72]. Therefore, decreased ARG1 expression 22 dpi suggested the possibility of insufficient signaling from the responsible T cell subset (Th2) at this stage.

Responses to parasites are often described in terms of Th1/Th2 dichotomy but recent studies have shown that host-pathogen interactions are more complex. A novel T cell effector subset Th17, characterised by the production of IL-17 was identified as well as a regulatory T cell subset (Treg), its signature cytokines being inhibitory IL-10 and/or TGF-β [73]. In mammals Th1, Th2 and Th17 reciprocally regulate the development and function of each other, while Treg cells suppress all three subsets [74,75]. The regulatory cytokines control inflammation and thus protect against immunopathology, but, in so doing, they reduce the effectiveness of immune mechanisms responsible for the expulsion of the parasites. Th2-dependent immune effector mechanisms are diverse and include fibroblast recruitment to the damaged tissue and collagen production, smooth muscle hypercontractivity and accelerated epithelial cell turnover [70,71,76]. Numerous studies of fish-parasite interactions described physiological responses, typically induced in a Th2 setting, e.g. increased mucus production and goblet cell hyperplasia in *Gyrodactilus* and SL infection models [1,5,77].

It is becoming increasingly evident that a superimposition of polarized response profiles in parasitic infections is the norm rather than the exception [75,78]. Concurrent regulation of markers of opposing immune responses in this study adds to this notion, e.g. early T cell response occurred in both the CD4+ and CD8+ T cell compartment (Fig. 2F, G). We visualized the map of immune responses which can accommodate for any particular combination of activated T cell responses (Fig. 5). In this map, each of the four major T cell-mediated responses can overlap with any other one and more than a combination of two is possible. Although Th1, Th2, Th17 and Treg are regulated with the lineage-specific sets of cytokines and respond to different targets, they can coexist in combinations. Th1 and Th2 responses can overlap with anti-inflammatory Treg or, quite the opposite, with the Th17 responses.

A specific set of innate effector cells is summoned by each of these T cell subsets. The newly described Th17 developmental pathway is characterised by the rapid neutrophilia [79]. The Th17 is thought to be an ancient lineage highly

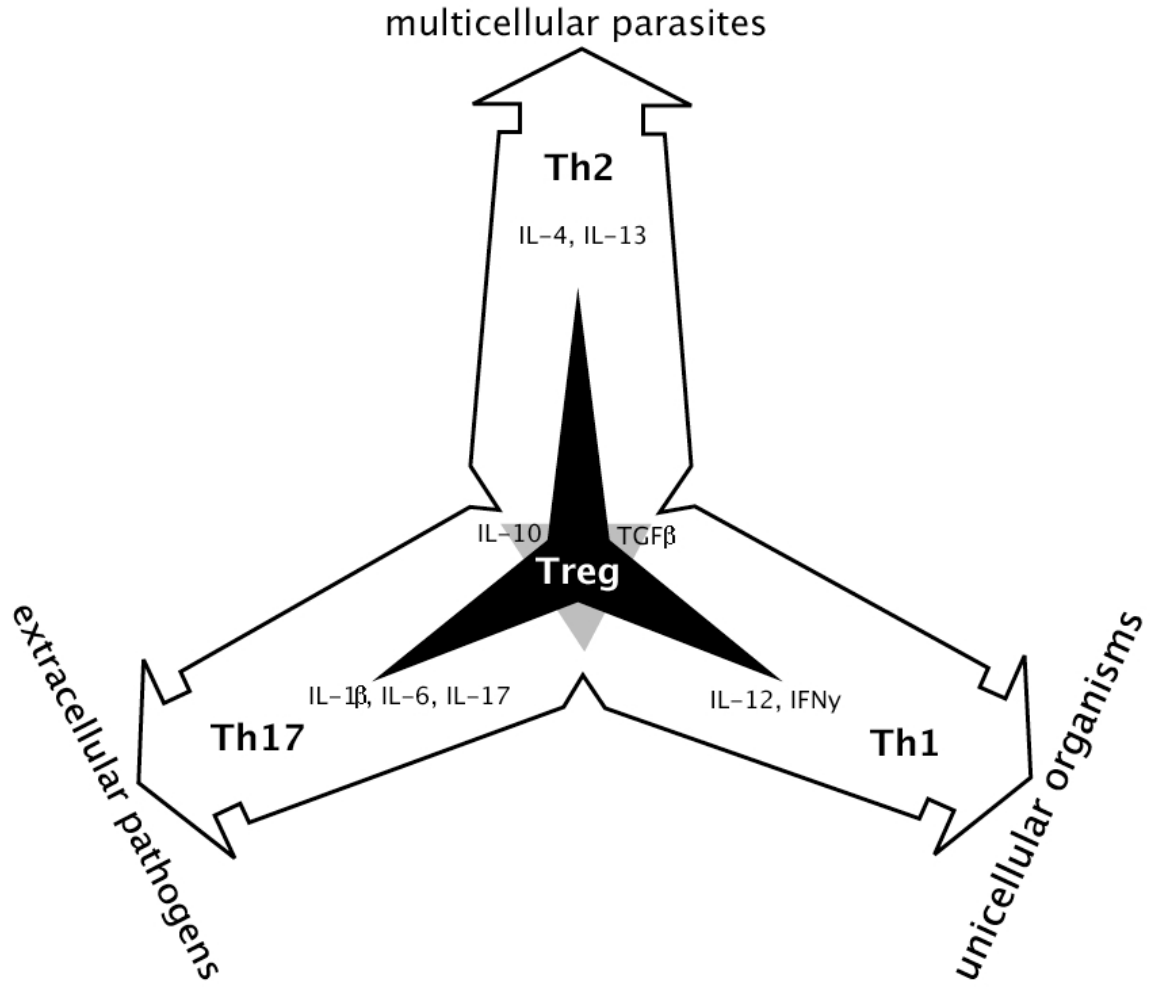

Figure 5

A map of T cell-mediated responses to pathogens. Three effector subsets, Th1, Th2, Th17, and the regulatory Treg are characterized by distinct cytokine profiles [73]. All three pro-inflammatory subsets reciprocally antagonize each other as indicated with a grey triangle in the centre where they were shown to overlap. Treg cells, represented with a black three-pointed star superimposed at the centre of the figure, inhibit all three Th subsets, thus preventing excessive inflammatory responses. T cell-mediated responses represent combined immune responses, which include both innate and adaptive components. Immune response to most bacterial and viral pathogens is generally pro-inflammatory. The Th1 cells secrete interferon γ (IFNγ) and IL-12, which protect against viral infections and other intracellular pathogens. Th17 is a highly pro-inflammatory arm characterised by rapid induction of neutrophils at the inflamed tissue and requires IL-1 and IL-6 for its activation. In contrast, parasitic infections drive Th2 immune responses characterized by production of IL-4 and IL-13, which mediate elimination of multicellular parasites. In addition to driving polarized Th2 responses, parasitic infections are associated with the induction of Tregs and immunoregulatory IL-10, which can induce immune anergy. The resultant effect of this is that parasitic infections can be characterized by an overall down-regulated immune system and therefore modified Th2-response, termed Th2-like. Because many cytokines can be produced and utilised by a number of different cells (IL-10 being a good example [106]), it is clear that multiple cell types may contribute to the regulation of the type and extent of inflammation. Thus, immune regulation likely depends on the specific combination of different T cells called upon during an infection than on a clear predominance of one response profile.

conserved in all vertebrates including the jawless lamprey [80,81]. This highly pro-inflammatory CD4+ Th subset plays an important role in the immediate responses to injuries with high risk of necrosis [82], and in protection against extracellular pathogens which are not efficiently cleared by Th1-type and Th2-type immunity [83]. The outcome of parasitic infections may be determined by the balance of pro-inflammatory and regulatory immune

responses. Our observations indicate that reactions to SL in Atlantic salmon are consistent with the bias towards the regulatory/Th2-like responses.

Identification of genes involved in the Th1/Th2 axis was greatly enhanced with the sequencing of several fish genomes [80,84]. The cytokine networks are becoming increasingly better known in fish. Overall, fish possess a repertoire of cytokines, which is similar to mammalian [85,86], including recently cloned IL-6 [87,88], IL-12 [89] and IL-10 [90-93]. First Th2 type interleukin, IL-4 was cloned in 2007 [94]. Also recently, the master regulators of Th1 and Th2 development, T-bet and GATA-3, respectively, were described in fish [95,96]. Cytokine profiles alone are insufficient for the accurate assignment of T cells to lineages given that cytokines may work in different ways. For example TGF-β induces development of both Treg and Th17 in mice and suppresses Treg development in humans [97]. One may anticipate much greater differences between mammals and teleost fish.

Rapid involvement of T cells in response to infection in skin was implied already 3 dpi. However, the delayed healing in SL infected Atlantic salmon may indicate an impaired or modified, Th2-like response. Long lived parasites often cause chronic infections via the induction of Treg cells and concomitant down-regulation of protective Th responses [71]. The involvement of a Treg-like subset, as implied by the up-regulation of TGF-β and IL-10 at 33 dpi, was not seen in the chronic phase of SL infection. This coincided with the molting of pre-adults into mobile adult stages. Though probably down modulatory towards the effector Th arms in fish, IL-10 and TGF-β may benefit the host at this stage due to the reduced damage caused by inflammatory reactions and/or enhanced healing response. The augmented healing would not harm SL either, as most chalimii molted into mobile life stages 33 dpi and maintenance of an open wound to aid in feeding may not have been necessary. We also observed the regulations of gene expression in the internal organs, which were consistent with changes in the skin. Early activation of the humoral response, as evidenced by the transcriptional wave of immunoglobulin-like genes, was followed by general hyporesponsiveness during immobile SL stages 22 dpi (Fig. 3B and 3A). Expression profiles of immunoglobulins partially reverted 33 dpi only in the spleen.

Possible roles of cells and the regulatory network are presented in Figure 6. Interestingly, the expression profile of programmed death ligand 1 (PD-L1), a negative co-stimulatory signal of T cell activation [98], was similar to that of CD4. The differential regulation of all-trans-13,14-dihydroretinol saturase in skin, liver and spleen also implies a dynamic regulation of immune cells through retinol signalling (Fig. 1B and Fig. 3A). Vitamin-A deficiency is known to induce immune abnormalities in T-cell subsets [99-101]. Retinoic acid signalling was recently shown to inhibit Th17 and promote Treg differentiation [102]. Interestingly, two structurally novel protein families with a high affinity to retinol and fatty acids, potentially playing a role in modifying inflammatory environment were identified in parasitic nematodes [103,104].

The skin expression profile of cytochrome P450 27, which ties together retinoid, PPARγ and LXR signaling [105] implies the down regulation of a whole regulatory network based on natural/endogenous ligands: retinoids and modified fatty acids and prostanoids (Fig. 1B). Of note is the observation that all-trans-13,14-dihydroretinol saturase is down regulated in skin and liver but from 22 dpi becomes up-regulated in spleen. In addition, 5-lipoxygenase binding protein, involved in the generation of leukotrienes, another set of lipid mediators of immunity was up-regulated throughout the study period in head kidney. Such expression profiles may stem from the fact that skin is under the direct and stronger modulation by the parasite than the internal organs.

Conclusion

Initial infection of Atlantic salmon with SL is associated with rapid sensing and induction of mixed inflammatory responses. A combination of restricted inflammation, which can be due to hyporesponsiveness of the immune cells, and delayed healing of injuries, can account for the limited ability to reject parasites. Persistent infection of Atlantic salmon with SL implies compromised immunity and self-destruction of tissues. Development of markers for different subsets of salmon T cells will greatly enhance the opportunity to study responses of Atlantic salmon to SL, and other parasites.

Materials and methods

Challenge test

Salmon used for this experiment originated from the Aqua Gen AS (previously referred to as NLA) strain. Salmon smolts of mixed and unknown family background were transferred to Institute of Marine Research in Bergen, transferred to full salinity saltwater, and distributed into two replicate tanks (two control and two for *L. salmonis* infection). Fish were hand fed a commercial diet once daily during the entire experimental period. Water temperature was 10°C ± 1.5°C during the entire experimental period. Egg strings from hatchery reared SL were collected and placed into single incubators. Approximately 75 infectious copepodids/fish were used to infect 40 salmon (20/tank). On the day of infection, the water level in the replicate tanks was reduced to approximately one third of the tanks original volume, the water supply was stopped, and the water was aerated with oxygen. One

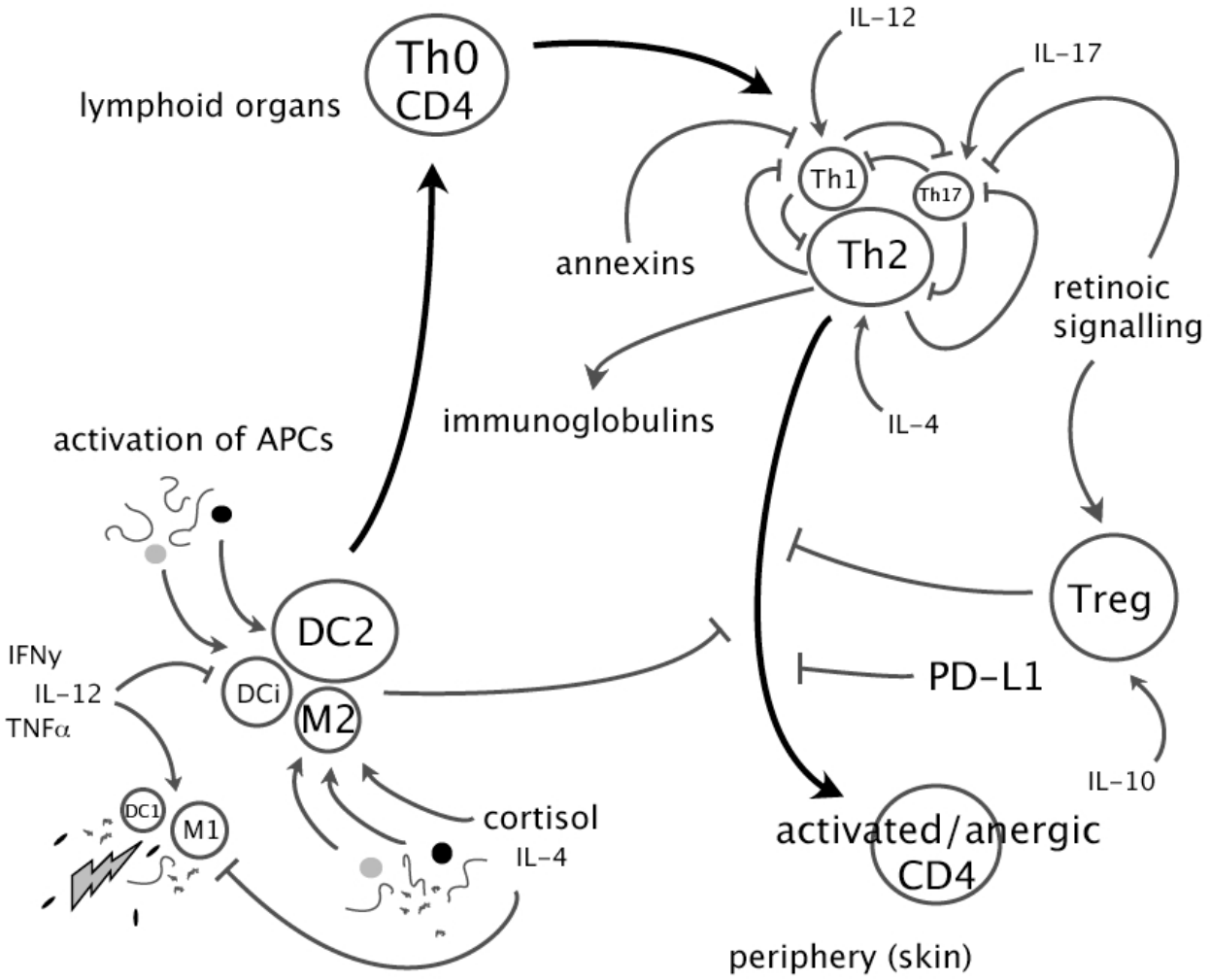

Figure 6

Hypothetical model of responses of immune cells to SL. Classical activation of macrophages (M) and dendritic cells (DC) induces M1 and DC1 phenotypes, which drive CD4+ T cells toward Th1. In contrast, SL may selectively induce C-type lectin receptors and possibly other receptor classes on alternatively activated macrophages (M2), immature DC (DCi) and DC2 to preferentially induce Th2 cells [75]. Parasite antigens are presented to CD4+ T cells in lymphoid tissues. Upon activation, Th2 cells proliferate and induce immunoglobulin production, possibly down-regulating other Th subsets. However, different subsets can coexist and a balanced combination may result in susceptibility or resistance. Activated Th2 cells migrate to the site of SL attachment where they mediate expulsion of chalimus larvae. Antagonism within the Th compartment and suppression by Treg cells can inhibit CD4+ T cell effector functions. Several other mechanisms can have key functions in the shaping of the T cell repertoire, and in regulation of inflammatory responses to SL, including reciprocal Th17 and Treg differentiation mediated by vitamin A derivatives [107] and various anti-inflammatory agents, such as annexins. Programmed death ligand 1 (PD-L1) possibly provides a distinct negative regulatory checkpoint in T cell differentiation [98]. Endogenous products (e.g. cortisol and prostaglandins), cellular debris and SL products are also able to potently influence immune responses [108-110].

hour post infection, the water supply was reinstated and oxygen supply to the tanks removed.

The experimental fish were sampled on the following dates: 21.09.2006 (3 dpi = copepodids), 11.10. 2006 (22 dpi = chalimus III/IV), and 21.10.2006. (33 dpi = pre-adult females and males) (Fig. 7A). At 22 dpi the number of lice per fish ranged from 4 to 20 with mean 12.2 ± 1.8. Individuals with average numbers of parasites were used for analyses. Control fish were sampled in parallel with

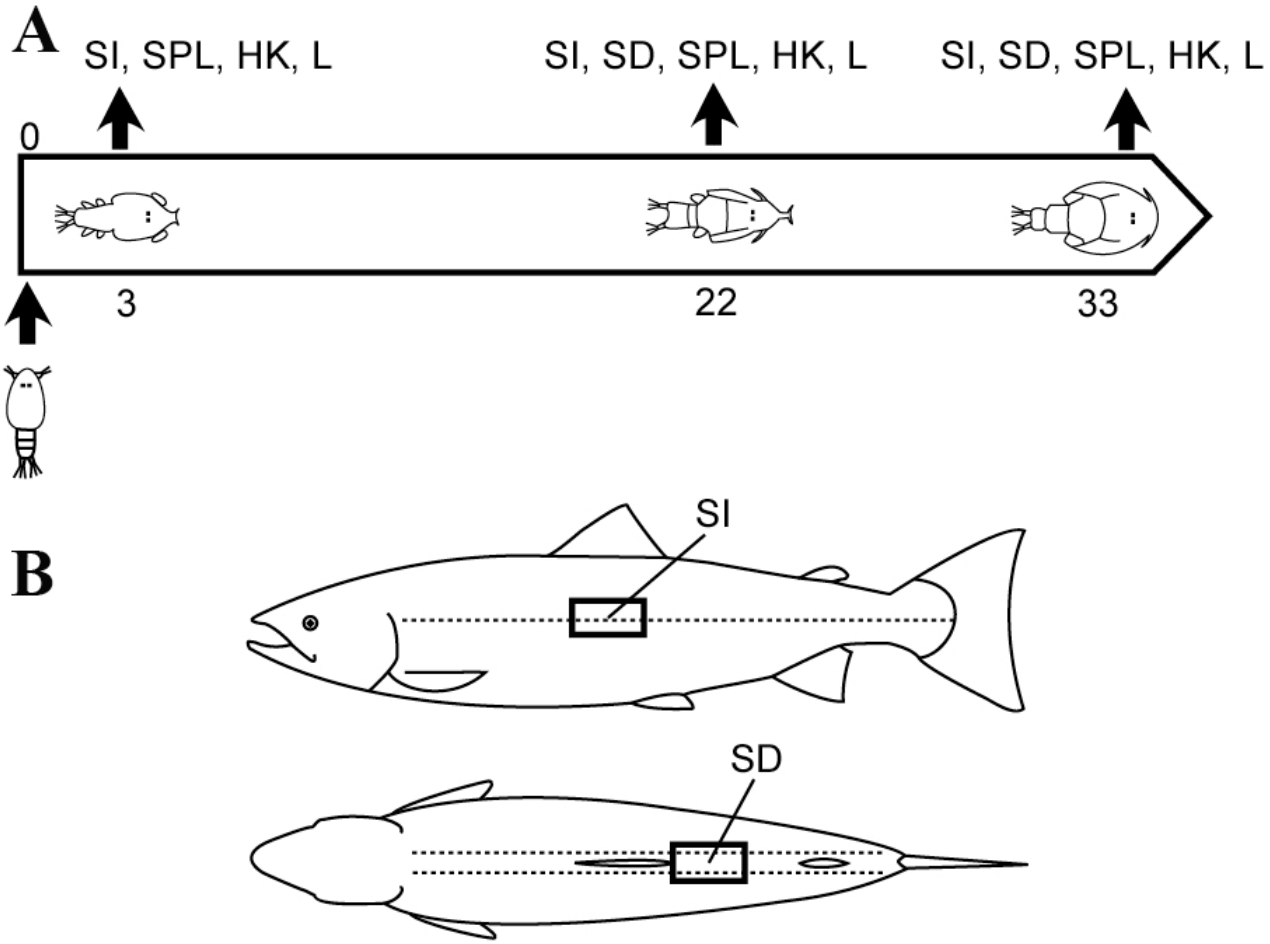

Figure 7
Design of the experiment.A: Two study groups of Atlantic salmon were SL infected fish with approximately 75 copepodids per fish. On days 3, 22 and 33 tissue samples were collected from the control and infected test groups; time-points corresponded to copepodids (3 dpi), chalimus III/IV (22 dpi) and preadult stages (33 dpi). **B**: Due to the small size of SL at the copepodid stage, it was not possible to reliably locate them on fish at the sample taken 3 dpi. Consequently, SL damaged skin was only sampled 22 dpi and 33 dpi. Salmon lice tend to aggregate around fins. Therefore, the area behind the dorsal fin was chosen in order to sample damaged skin. Because attached lice were never found along the lateral line, samples of intact skin of infected fish were taken from the area below the dorsal fin at the intersection with the lateral line. SI – intact skin; SD – damaged skin; SPL – spleen; HK – head kidney; L – liver; dpi – days post infection.

challenged fish. In addition to skin, samples of the head kidney, spleen and liver were collected and preserved in RNALater (Ambion, Austin, TX, USA) (Fig 7B). Lice induced damage to the fish was moderate, and no open wounds were observed on any of the experimental fish. Furthermore, there was no evidence of any secondary infections either on the surface or internal organs for the infected or control fish.

Microarray analyses

The salmonid fish microarray (SFA2, immunochip) includes 1800 unique clones printed each in six spot replicates. The genes were selected by their functional roles and the platform is enriched in a number of functional classes, such as immune response (236 genes), cell communication (291 genes), signal transduction (245 genes), protein catabolism (90 genes) and folding (70 genes). The complete composition of platform and sequences of genes are provided in submission to NCBI GEO (GPL6154). Total RNA was extracted from soft tissues with TriZOL (Invitrogen, Carlsbad, CA, USA), whilst Fibrous tissue kit (Qiagen sciences, Maryland, USA) was used with skin. Total RNA was purified with Pure Link (Invitrogen, Carlsbad, CA, USA). Microarray analyses

Table 1: Real-time qPCR analyses

Target	Primer sequence from 5' to 3'	Amplicon size (bp)	Accesion number
Matrix metalloproteinase 13	F CCAAAAAGAGGGCACCAGATGG R CCAAAAAGAGGGCACCAGATGG	53	DW539943
Matrix metalloproteinase 9	F AGTCTACGGTAGCAGCAATGAAGGC R CGTCAAAGGTCTGGTAGGAGCGTAT	72	CA342769
Cathepsin S	F CGAAGGGAGGTCTGGGAGAGGAAT R GCCCAGGTCATAGGTGTGCATGTC	87	CA355014
Bone morphogenetic protein 4	F TCAAGTTGCCCATAGTCAGT R CACCTGAACTCTACCAACCA	207	CA056395
Alkaline phosphatase	F CTAGTTTGGGTCGTGGTATGT R TGAGGGCATTCTTCAAAGTA	185	CA358635
Heat shock protein 90β-20	F GAACCTCTGCAAGCTCATGAAGGA R ACCAGCCTGTTTGACACAGTCACCT	72	CF752846
Collagen 10α	F TGGTGCTCTTTGACTGCCTGTAA R CATCCTGTGTGTTGCAATATCACA	180	EG837148
Collagen 1α	F AGAGAGGAGTCATGGGACCCGTT R GGGTCCTGGAAGTCCCTGGAAT	155	
Collagen 2α	F TGGTCGTTCTGGAGAGACT R CCTCATGTACCTCAAGGGAT	151	BX865386
Decorin	F GAACCTGGCTAAGCTGGGTCTAA R GAACAGGCTGATGCCAGAGTACAT	256	DQ452069
Elastin	F GAGGCTACAGACCAGGAGGAGTT R TCTGGGTCGGTGGGTTTGTA	226	BU694149
Laminin	F CATGTGACATGGACACAGGAA R CGTCCTCAGCCTCATAGGTGTA	273	DY722974
CCAAT/enhancer binding protein β	F TACGTCCTGGGCTATCCTGAACTGC R CCAGACGAACCGTTGTTGTCCA	140	CA348284
Erythroid 5-aminolevulinate synthas	F CACATGAGACAGCTGCTCCTGGAGA R GCTCCAGCAAGATGTCACACACCT	121	DW580939
Heme oxygenase 1	F AGTCAGTGGAGAGAGACCTGGAGCA R GGTTGTCTTTGCCGATCTGTCTGAG	117	CA363120
Haemoglobin beta chain	F ACAAACGTCAACATGGTCGACTGG R TCTTTCCCCACAGGCCTACGAT	67	NM_001123666
Mannose binding lectin 1	F TCCATTGCACTGGGCGATGC R CACTGCTTCCACCTGAGCCTCA	105	CA349943
Prostaglandin D synthase	F CCTACACCAACCTGAACGCTGATG R ACGCTGGCTGGTGAAGGTGAAG	98	CA352578
MHC class II α chain	F AGTCAGGTGGACCAGGAACAATCA R CTGGAGAACTGGTTGAGGGTGAAA	96	CA379977
CD8α	F CGTCTACAGCTGTGCATCAATCAA R GGCTGTGGTCATTGGTGTAGTC	266	AY693391
IL-12β	F TCTACCTACACGACATTGTCCAGCC R ATCCATCACCTGGCACTTCATCC	62	AJ548830
Arginase 1	F AGCCATGCGTATCAGCCAA AAGGCGATCCACCTCAGTCA	122	EG929369
Programmed death ligand 1	F TCAACGACTCTGGGGTGTACCGATG R TCCACCTCATCTCCACCACGTCTC	133	CA366631
Beta-2-microglobulin	F TCGTTGTACTTGTGCTCATTTACAGC R CAGGGTATTCTTATCTCCAAAGTTGC	107	AF180478
TGF-β	F AATCGGAGAGTTGCTGTGTGCGA R GGGTTGTGGTGCTTATACAGAGCCA	332	EU082211+ AJ007836
IL-1 receptor type 1	F CCAAAAAGAGGGCACCAGATGG R CGTATCGTCTCTCCAACACCTCAGG	126	NM_001123633
CD4	F TGCATTGTTCCTCTCTTCCACAGC R CCGTCCCAAGGTACCATAGTACCAA	128	EG852912
IL-10	F ATGAGGCTAATGACGAGCTGGAGA R GGTGTAGAATGCCTTCGTCCAACA	54	EF165028
Eukaryotic translation initiation factor 3 subunit 6	F GTCGCCGTACCAGCAGGTGATT R CGTGGGCCATCTTCTTCTCGA	92	CX040383

were conduced in pooled samples with equal inputs of RNA from six individuals. A dye swap design of hybridization was applied. In analyses of injured skin, the intact sites from the same individuals were used as a control. Microarray comparisons were also conducted with intact skin from challenged and control fish; these data were not reported due to relatively small expression changes. Analyses of head kidney, spleen and liver used the uninfected fish as a reference. Each sample was analyzed with two slides. The control and test samples (20 μg RNA in each) were labelled with respectively Cy3-dUTP and Cy5-dUTP (Amersham Pharmacia, Little Chalfont, UK) for the first slide and dye assignment was reversed for the second slide. The fluorescent dyes were incorporated in cDNA using the SuperScript™ Indirect cDNA Labelling System (Invitrogen, Carlsbad, CA, USA). The cDNA synthesis was performed at 46°C for 3 hours in a 20 μl reaction volume, following RNA degradation with 0.2 M NaOH at 37°C for 15 min and alkaline neutralization with 0.6 M Hepes. Labelled cDNA was purified with Microcon YM30 (Millipore, Bedford, MA, USA). The slides were pretreated with 1% BSA fraction V, 5× SSC, 0.1% SDS (30 min at 50°C) and washed with 2 × SSC (3 min) and 0.2 × SSC (3 min) and hybridized overnight at 60°C in a cocktail containing 1.3 × Denhardt's, 3 × SSC 0.3% SDS, 0.67 μg/μl polyadenylate and 1.4 μg/μl yeast tRNA. After hybridization slides were washed at room temperature in 0.5 × SSC and 0.1% SDS (15 min), 0.5 × SSC and 0.01% SDS (15 min), and twice in 0.06 × SSC (2 and 1 min, respectively). Scanning was performed with GSI Lumonics ScanArray 4000 (PerkinElmer Life Sciences, Zaventem, Belgium) and images were processed with GenePix 6.0 (Axon, Union City, CA, USA). The spots were filtered by criterion $(I\text{-}B)/(S_I+S_B) \geq 0.6$, where I and B are the mean signal and background intensities and S_I, S_B are the standard deviations. Low quality spots were excluded from analysis and genes presented with less than three high quality spots on a slide were discarded. After subtraction of median background from median signal intensities, the expression ratios (ER) were calculated. Lowess normalization was performed first for the whole slide and next for twelve rows and four columns per slide. The differential expression was assessed by difference of the mean log-ER between the slides with reverse labelling (6 spot replicates per gene on each slide, Student's t-test, $p < 0.01$). Complete microarray results are provided as an additional file 1.

Quantitative real-time RT-PCR

The cDNA synthesis was performed on 2 μg of DNAse treated (Turbo DNA-*free*™ (Ambion, Austin, TX, USA) total RNA using TaqMan® Reverse Transcription reagents (Applied Biosystems, Foster City, CA, USA) and random hexamer primers. The PCR primers (Table 1) were designed using the Vector NTI (Invitrogen) and synthesized by Invitrogen. PCR. Efficiency was checked from ten-fold serial dilutions of cDNA for each primer pair (additional file 2). Real-time PCR assays were conduced using 2X SYBR® Green Master Mix (Roche Diagnostics, Mannheim, Germany) in a 12 μl reaction volume, primer concentrations were 0.4–0.6 μM each. PCR was performed in duplicates in 96-well optical plates on Light Cycler 480 (Roche Diagnostics). Relative expression of mRNA was calculated using the $\Delta\Delta C_T$ method; the chosen reference gene for all tissues was eukaryotic translation initiation factor 3 subunit 6 (eIF3S6), which showed no differential expression according to the microarray results. Two more commonly used genes (EF1A and GAPDH) were tested for stability using the GeNorm software, however only eIF3S6 met criteria of stability in the analyzed material. Differences between infected and control fish were analyzed with Student's t-test ($p < 0.05$).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors contributed to the overall experimental design. KG and FN designed and performed the challenge tests, whilst SS and AK carried out the gene expression analyses, data analysis, and produced the first manuscript draft. All authors read, contributed to, and approved the final manuscript.

Additional material

Additional file 1

Complete results of microarray analyses. *Data are log (Expression ratios) and p-values of differential expression (t test).*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-9-498-S1.xls]

Additional file 2

Efficiency of PCR.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-9-498-S2.doc]

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Paper II

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Hepatic gene expression profiling reveals protective responses in Atlantic salmon vaccinated against furunculosis

Stanko Škugor*[1,2], Sven Martin Jørgensen[1], Bjarne Gjerde[1] and Aleksei Krasnov[1]

Address: [1]Nofima Marin, P.O.Box 5010, Ås 1430, Norway and [2]Department of Animal and Aquaculture Sciences, Norwegian University of Life Sciences (UMB), P.O. Box 5003, Ås 1432, Norway

Email: Stanko Škugor* - Stanko.Skugor@nofima.no; Sven Martin Jørgensen - sven.jorgensen@nofima.no; Bjarne Gjerde - bjarne.gjerde@nofima.no; Aleksei Krasnov - Aleksei.Krasnov@nofima.no

* Corresponding author

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Abstract

Background: Furunculosis, a disease caused with gram negative bacteria *Aeromonas salmonicida* produces heavy losses in aquaculture. Vaccination against furunculosis reduces mortality of Atlantic salmon but fails to eradicate infection. Factors that determine high individual variation of vaccination efficiency remain unknown. We used gene expression analyses to search for the correlates of vaccine protection against furunculosis in Atlantic salmon.

Results: Naïve and vaccinated fish were challenged by co-habitance. Fish with symptoms of furunculosis at the onset of mass mortality (LR - low resistance) and survivors (HR - high resistance) were sampled. Hepatic gene expression was analyzed with microarray (SFA2.0 - immunochip) and real-time qPCR. Comparison of LR and HR indicated changes associated with the protection and results obtained with naïve fish were used to find and filter the vaccine-independent responses. Genes involved in recruitment and migration of immune cells changed expression in both directions with greater magnitude in LR. Induction of the regulators of immune responses was either equal (NFkB) or greater (Jun) in LR. Expression levels of proteasome components and extracellular proteases were higher in LR while protease inhibitors were up-regulated in HR. Differences in chaperones and protein adaptors, scavengers of reactive oxygen species and genes for proteins of iron metabolism suggested cellular and oxidative stress in LR. Reduced levels of free iron and heme can be predicted in LR by gene expression profiles with no protection against pathogen. The level of complement regulation was greater in HR, which showed up-regulation of the components of membrane attack complex and the complement proteins that protect the host against the auto-immune damages. HR fish was also characterized with up-regulation of genes for proteins involved in the protection of extracellular matrix, lipid metabolism and clearance of endogenous and exogenous toxic compounds. A number of genes with marked expression difference between HR and LR can be considered as positive and negative correlates of vaccine protection against furunculosis.

Conclusion: Efficiency of vaccination against furunculosis depends largely on the ability of host to neutralize the negative impacts of immune responses combined with efficient clearance and prevention of tissue damages.

Background

Furunculosis caused by *Aeromonas salmonicida* spp *salmonicida* is a bacterial disease affecting salmonid species, including cultured and wild Atlantic salmon (*Salmo salar L.*) (reviewed in [1,2]), salmonids and other fish species [3-7]. Disease may have local and systemic, acute, subacute and chronic forms with diverse symptoms ranging from erratic swimming and slight darkening of skin, to haemorrhage on the abdominal walls, viscera and heart and ulcerative lesions [8]. Furunculosis is caused with non-mobile, aerobic gram negative bacillus *Aeromonas salmonicida*. This is an opportunistic pathogen with diverse strains that are characterized with different virulence. Furunculosis may cause heavy losses in salmon aquaculture due to mortality, decrease of growth rates, feed conversion and fish quality. Large-scale vaccination made it possible to reduce the incidence of disease and the use of antibiotics [9]. At present commercial vaccines against furunculosis are widely used and a number of experimental vaccines has been tried [10,11]. Vaccination decreases mortality of Atlantic salmon but fails to prevent it completely. Difficulties in the development of neutralizing vaccines are most likely accounted for by the high diversity of *A. salmonicida* strains and mechanisms of pathogenicity, which can be determined with various factors including type three secretion system, A layer protein, lipopolysaccharide, iron binding and outer membrane proteins, peptidases and toxins of different nature (reviewed in [2,11]). Knowledge of the virulence factors is far from complete. The limited success of protection against furunculosis is also related to high individual variation of responses to vaccination in Atlantic salmon [12].

Vaccination against furunculosis provides pathogen specific protection [2,9,13]. This indicates an important role of acquired immunity, which is however insufficient for the complete prevention of mortality. Outcomes of disease may depend substantially on the events, which take place after the recognition of pathogen. Activation of B and T cells and the complement system results in mass production of humoral factors that regulate recruitment of immune cells in blood and infected tissues. This results in the orchestration of anti-bacterial defense, including effector mechanisms, neutralization and clearance of exogenous and endogenous toxins, pathogens, damaged cells and their components. Defensive responses form a complex network, which may vary substantially among individuals. There are many possible scenarios with successful and deleterious outcomes. We used multiple gene expression profiling to outline the mechanisms that determine success of vaccine protection against furunculosis in Atlantic salmon and to search for the correlates of protection. Microarray analyses have been performed in fish vaccinated against different pathogens [14-17], however, to our knowledge, this study is the first attempt to search for the correlates of vaccine protection.

Results

Design of analyses

To search for the correlates of vaccine protection against furunculosis, individual samples of fish that survived to the end of challenge test with no apparent symptoms of disease (high resistant, HR) were hybridized with pooled samples of salmon with manifestation of furunculosis (dark skin, lethargy, abnormal swimming behaviour and small haemorrhages at the base of fins) [8] at the onset of mass mortality (low resistance, LR). The same design of hybridization was applied to unvaccinated fish and the results were used to filter the vaccine-independent changes of gene expression (Figure 1). Pathogen was detected with qPCR in the liver and spleen of all analyzed fish though the load was substantially lower in HR (Figure 2). Pilot microarray analyses of different tissues (heart, spleen and liver of vaccinated fish) found greatest differences between HR and LR in the liver and this organ was chosen for the continuation of studies. Real-time qPCR results from all uninfected fish were used to form a calibrator sample in order to verify the microarray results and to determine the direction of gene expression changes. We report only genes that were associated with survival in vaccinated salmon.

Recruitment of immune cells

Up- and down-regulation of the immune cells markers (Figure 3) could be evidence for the changes in the composition of leukocytes in the liver. Mammalian homolog of CD37 (down-regulated) is expressed in mature B and T cells and myeloid cells [18] while CD40 (induced), also found in myeloid and B cells, is in addition expressed in fibroblasts, endothelial cells and in the basal epithelial cells [19]. Genes regulating cell motility also changed in both directions (up-regulated and down-regulated) with greater magnitude of responses seen in LR. Induction of leukocyte cell-derived chemotaxin (LECT2), which attracts neutrophils [20] was markedly greater in LR. Src kinase-associated phosphoprotein 55-related protein (SKAP2) was down-regulated. This protein is involved in signalling activated by interactions between cells and extracellular matrix (ECM) [21]. Microarray analyses revealed lower expression levels in HR of a large group of genes implicated in recruitment and motility of immune cells (Figure 3B). Both annexin A1 (ANXA1) and annexin A3 (ANXA3) showed noticeably lower levels in HR. The anti-inflammatory activity of annexins (ANXAs) is attributed mainly to their ability to interfere with neutrophil extravasation [22,23]. In mammals, ANXA1 is ubiquitously expressed, while ANXA3 has more selective expression patterns and tissue distribution [24]. Migration of cells is a highly regulated process controlled by cellular interactions with ECM. HR showed lower expression levels of integrin binding protein (ITGB1BP3/MIBP), which mediates reduced laminin cell adhesion and inhibition of matrix deposition [25]. Movement of cells involves the

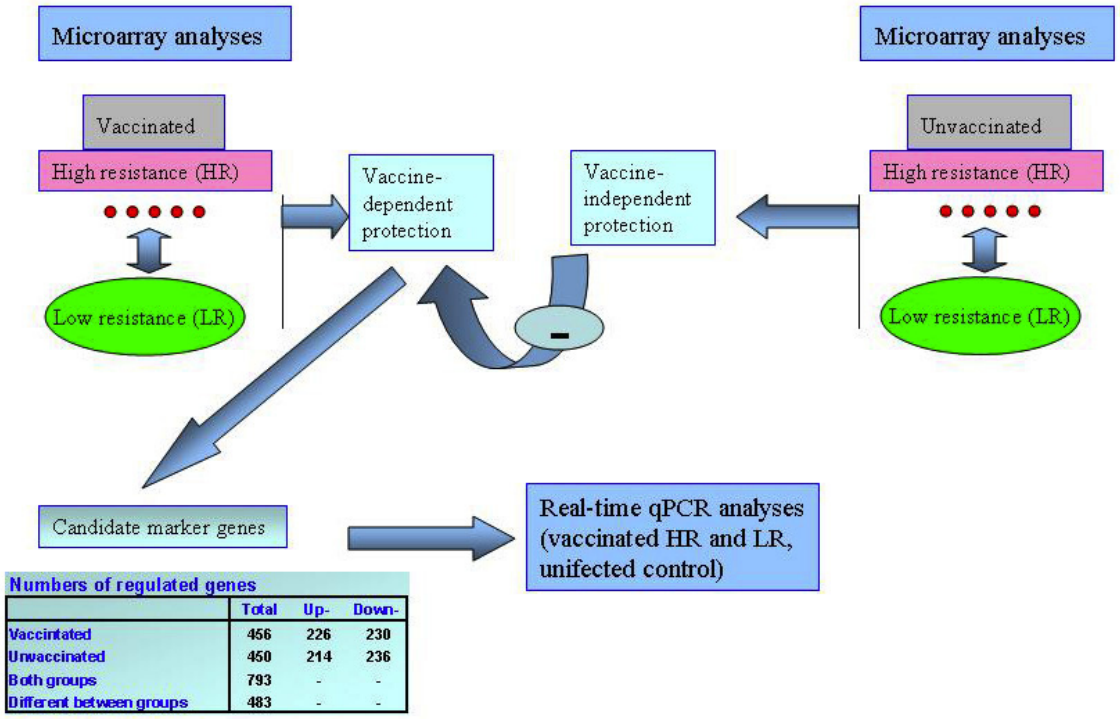

Figure 1

Design of gene expression analyses. Microarray comparison of HR (individual samples) with pooled LR outlined the gene expression changes associated with protection against *A. salmonicida*. Results obtained in naïve, unvaccinated fish indicated vaccine-independent protection and were used for the filtration of data obtained in vaccinated fish. Microarray analyses found genes with expression differences between LR and HR. The real-time qPCR analyses were conduced to verify the microarray results and to compare gene expression in the infected and uninfected fish. The numbers of differentially expressed genes are presented.

cytoskeleton. The 8-kDa dynein light chain (DLC8) is an essential component of the dynein motor complex that provides the driving force for microtubule-based transport within cells [26]. A potential link between cytoskeleton dynamics and gene regulation is implied by the fact that DLC8 binds to the inhibitor of the transcription factor NFkB preventing its translocation to the nucleus [27,28]. Profilin and cofilin that are involved in the restructuring of the actin filaments had lower expression in HR (Figure 3B). Same difference was observed in coronin 1-B, an actin-binding protein required for chemokine-mediated recruitment [29] and efficient cell protrusion and migration [30].

Signal transduction and regulation of gene expression, anti-bacterial effectors

Six genes known for their key roles in closely intervened immune pathways were analyzed with both microarrays and qPCR (Figure 4A). All were up-regulated in the infected fish except for JunC in the HR group. The NFkB complex regulates numerous genes involved in the immune responses to bacteria [31]. Microarray analyses suggested greater expression level of NF-kappaB-p105 (NFkB1) in HR but qPCR analyses did not confirm difference between the study groups. Given a 1000-fold increase of NFkB1 in pathogen challenged fish in comparison with uninfected controls, discrepancies could be accounted for by the limited dynamic range of microarray analyses. Real-time qPCR also did not detect differences between the HR and LR in the expression of NF-kappaB inhibitor alpha (NFKBIA) that retains NFkB in the cytoplasm. Structurally and functionally related c-Jun, JunB and JunD together with the members of Fos and ATF/CREB protein families make up the transcriptional regulator AP-1, which is essential for the cooperative induction of many cytokine genes [32]. AP-1 mediated regulation is cell type specific, depends on the relative abundance of its subunits and presence of other nuclear factors. For exam-

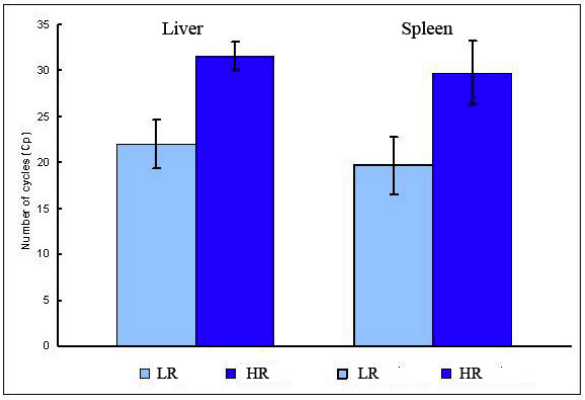

Figure 2
Pathogen loads analysed by real-time qPCR in the liver and spleen of vaccinated salmon. Data are cycle threshold (Ct) ± SD. Differences between LR and HR are significant ($p < 0.001$, ANOVA test, 8 fish per group).

ple, the pleiotropic transcription factor Yin Yang 1 (YY1), whose higher levels were detected in HR by microarray, plays important roles in immune cells [33,34] and cooperates with AP-1 to regulate gene expression [35,36].

Microarray analyses (Figure 4B) showed differences between HR and LR in genes that regulate gene expression at different levels: signal transduction, promoter binding, modification of chromosomes, maturation and maintenance of mRNA. Several genes with known immune functions showed higher expression levels in LR, including the phosphotyrosine independent ligand for Lck SH2 or p62 that regulates activation of NFkB by TNFα [37]. General transcriptional activity seemed repressed in HR in comparison to LR as judged by the profile of TATA-binding protein associated factor 2N (TAF15), involved in the transcription complex assembly and transcription initiation by RNA polymerase II. Supportive of gene silencing in HR was the up-regulation of the histone deacetylase 9 [38]. On the other side, polyadenylate-binding protein 4 (PABPC4) that was markedly over expressed in HR is known for its ability to enhance the stability and translation of cytokine mRNAs [39].

Up-regulation of NFkB and AP-1 by pathogens and cytokines induces mass production of immune mediators and effector proteins. Expression differences in proteosome components and extracellular proteases (nephrosin, matrix metalloproteinase 9 and tissue inhibitor of metalloproteinase) (Figure 5B) could be an evidence for the higher level of protein degradation in LR. On the contrary, improved resistance was associated with the activation of protease inhibitors that protect tissues from damage. Alpha-1-antiproteinase like protein (Antiprot1) was up-regulated in HR while LR showed no difference when compared to uninfected control (Figure 5A).

Cellular and oxidative stress, metabolism of iron

In addition to cytokines and pathogens, NFkB and Jun proteins respond to various cell damaging factors, including free radicals and other genotoxic agents that can cause apoptosis, growth arrest, altered DNA repair or altered differentiation. AP-1 contains cysteine motifs that regulate its activity in response to oxidative stress [40]. NFkB can also activate protection against oxidative and cellular stress by providing anti-apoptotic and proliferation-promoting signals. A suite of chaperones and protein adaptors of different types (heat shock proteins, 14-3-3 proteins, glucose regulated proteins, DnaJ, cyclophilins) were expressed at higher level in LR fish (Figure 5B) and this could be evidence of cellular stress. Genes for proteins involved in regulation of redox status and protection against reactive oxygen species (ROS) also showed differences between the study groups. Five genes from this functional group that had higher expression levels in HR are presented in Figure 5B. In contrast, all genes involved in metabolism of iron had higher expression levels in LR. Transferrin is an extracellular transporter of iron and ferritin stores iron inside cells. Protoheme IX farnesyltransferase is an enzyme of heme biosynthesis while heme oxygenase (HO) plays a key part in heme degradation. Real-time qPCR showed induction of HO in all infected fish, consistent with its potent cytoprotective and anti-inflammatory functions [41], but the expression level was greater in LR (Figure 4A). Hemopexin transports heme to the liver for degradation and hepcidin regulates iron metabolism at different levels. Both genes respond to bacterial pathogens in various fish species [42-44]. Differences between LR and HR were also seen in regulators of cell cycle. Cyclin D showed lower level in HR. This gene links external cues with regulation of cell proliferation and directs entry to G1 phase by phosphorylation of retinoblastoma protein [45]. Our previous studies found marked activation of cyclin D with toxicity and pathogens [46,47].

Complement

The complement is a complex system consisting of 3 pathways (classical, alternative and lectin), which helps to kill and clear pathogens. The complement components are present in plasma as inactive proteins and the biochemical cascade is triggered by recognition of pathogens. In higher vertebrates, the complement pathways are activated in different ways. The C1Q component of C1 complex of the classical pathways (CP) binds to antigen-antibody complexes thus linking the innate and adaptive arms of immunity. C-type mannose-binding lectin (MBL) has the same role in lectin pathway (LP) but unlike C1Q commonly does not require antibody. The alternative pathway (AP) does not include specialized pathogen

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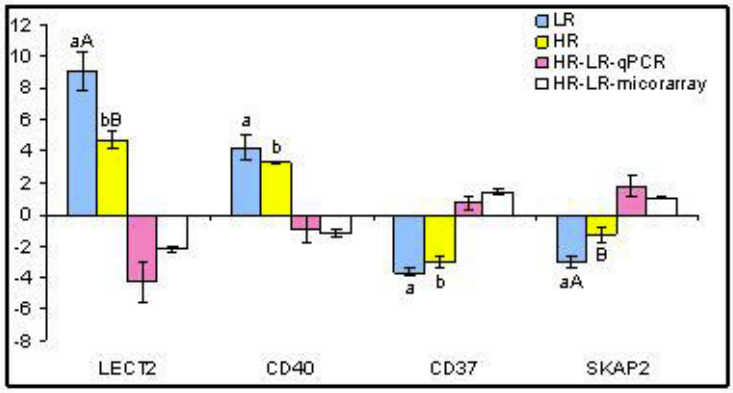

B

Genes	Expression ratio
Markers and regulators of immune cells	
CD63	-1.17 ± 0.13
Chemokine-like factor family member 7	0.66 ± 0.21
CCL4	-0.82 ± 0.17
LECT2 neutrophil chemotactic factor	-1.26 ± 0.14
CC chemokine SCYA110-1	-0.73 ± 0.18
Chemokine receptor-1	1.17 ± 0.21
IL-1 receptor-associated kinase	1.28 ± 0.12
Interleukin enhancer binding factor	-0.86 ± 0.20
Allograft inflammatory factor	-0.78 ± 0.19
Integrin beta-1-binding protein	-1.09 ± 0.27
Annexin A1-2	-1.22 ± 0.09
Annexin A3	-1.31 ± 0.34

Genes	Expression ratio
Cytoskeleton	
Actin cytoplasmic	-0.94 ± 0.06
Profilin-1	-1.29 ± 0.18
Coatomer epsilon subunit 2	-1.20 ± 0.17
Cofilin, muscle isoform	-1.97 ± 0.12
Coronin-1B	-1.68 ± 0.25
Dynein light chain	-1.10 ± 0.17
Gamma crystallin B-1	-0.73 ± 0.13
Myristoylated alanine-rich protein kinase C	-1.16 ± 0.12
Prohibitin-2	-1.33 ± 0.14
Septin 10	-1.48 ± 0.25
Syntenin 1	-1.26 ± 0.24
Thymosin beta-4	-1.36 ± 0.36
Troponin T-3	-1.04 ± 0.18
Tubulin alpha-1 chain	-1.04 ± 0.21
Tubulin alpha-3 chain	-1.18 ± 0.15

Figure 3

Immune cell markers and genes involved in recruitment and migration of immune cells. **A**: Real-time qPCR versus microarray analyses. Results demonstrated concordance in direction of change between the two techniques supporting differential regulation of the gene subset. Data for qPCR are ΔΔCt ± SE of 10 HR and 10 LR versus 8 fish in the unchallenged control and data for microarray are mean $\log_2$-ER ± SE of 6 HR fish hybridized to a pooled sample of 10 LR fish. Differences between HR and LR determined with microarrays and qPCR are shown. Different capital letters (A, B) denote a difference between challenged fish (HR and LR) while small letters (a, b) denote a difference between challenged and control fish (ANOVA, $P < 0.05$). LECT2 - leukocyte cell-derived chemotaxin 2, SKAP2 - Src kinase-associated phosphoprotein. **B**: Microarray results, examples of differentially expressed genes with significant differences between vaccinated and unvaccinated fish (t-test, $n = 6$ and 5, $p < 0.05$). Results for unvaccinated fish are not shown, data are $\log_2$-ER ± SE.

binding proteins. To date, the sensor proteins of CP have not been identified in salmonid fish. We included in analyses the C1Q binding protein (C1QBP), which showed greater expression in HR (Figure 6A). C1QBP is a ubiquitously expressed protein found intracellularly, on the cell surface, in plasma and the extracellular matrix. It interacts with a host of proteins including the globular heads of C1Q molecules, thus potentially modulating the numerous C1Q-mediated functions [48]. The functional analog of mammalian MBL is also unknown. Microarray results suggested down-regulation of a C type MBL in HR. However qPCR analyses found a 1000-fold induction of MBL in HR while there was no difference between LR and the uninfected control. This disagreement can be accounted for by the limited ability of cDNA microarrays to discriminate between transcripts of structurally similar members of multi-gene families. Blastx search of the MBL sequence presented on the microarray (Genbank CA376643) found three rainbow trout and nine Atlantic salmon proteins. Despite considerable sequence divergence (similarity ranged within 38-83%) the salmonid lectins contain highly conserved domains (Figure 7) that, hypothetically,

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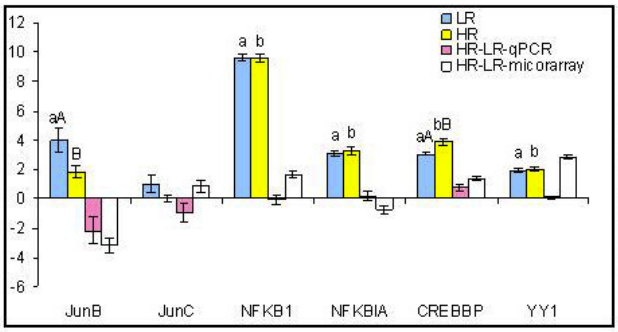

B

Genes	Expression ratio
MAPK/ERK kinase kinase kinase 4-1	0.81 ± 0.16
Phosphotyrosine independent ligand for Lck SH2	-2.01 ± 0.26
GADD45 gamma	-1.76 ± 0.48
CCAAT/enhancer binding protein beta	-1.46 ± 0.17
Chromosome-associated kinesin KIF4A	-1.24 ± 0.15
Nonhistone chromosomal protein HMG-17	-0.87 ± 0.05
TATA-binding protein associated factor 2N	-0.94 ± 0.14
Histone deacetylase 9	0.96 ± 0.08
Splicing factor, arginine/serine-rich 3	-1.87 ± 0.28
Cold inducible RNA binding protein-2	-1.05 ± 0.19
Cellular nucleic acid binding protein	-0.61 ± 0.09
Polyadenylate-binding protein 4	1.07 ± 0.05

Figure 4

Signal transducers and regulators of gene expression. The figure shows expression changes indicative of recruitment and migration of immune cells. A. Real-time qPCR versus microarray analyses. Data for qPCR are ΔΔCt ± SE of 10 HR and 10 LR versus 8 fish in the unchallenged control and data for microarray are mean $\log_2$-ER ± SE of 6 HR fish hybridized to a pooled sample of 10 LR fish. Differences between HR and LR determined with microarrays and qPCR are shown. Different capital letters (A, B) denote a difference between challenged fish (HR and LR) while small letters (a, b) denote a difference between challenged and control fish (ANOVA, $P < 0.05$). NFKB1 - nuclear factor NF-kappa-B p105 subunit, NFKBIA - NF-kappa-B inhibitor alpha, CREBBP - CREB-binding protein, YY1 - YY1 transcription factor. B. Microarray results, examples of differentially expressed genes with significant differences between vaccinated and unvaccinated fish (t-test, n = 6 and 5, $p < 0.05$). Results for unvaccinated fish are not shown, data are $\log_2$-ER ± SE.

may cross-hybridize. The actual number of salmonid MBL-like proteins and their relation to the complement system remain unknown. The genes for other serum components of the complement had greater expression levels in HR. These are serine proteases of lectin (MASP) and alternative pathways (factors B), C3, the convergence point of all complement pathways, C5 and C9, the parts of membrane attack complex. Factor H is a regulator of the alternative pathway while vitronectin (VTN) protects tissues against damages caused by the terminal membrane attack complex. The cell surface receptors of C1Q and C type lectin had higher expression in LR (Figure 6B). We show these genes since theoretically, they may interact with the complement system. However, their roles in salmonid fish are unknown and await exploration.

Clearance and reparation

HR was characterized with markedly higher expression levels of genes that protect tissues from damages, neutralize and remove toxic compounds and products of cell degradation (Figure 8A). Fibronectin (FN1) is an acute phase protein required for protection and reparation of ECM [49]. CYP3A7 can metabolise a number of endogenous and exogenous compounds [50,51] while liver bile salt export pump (ABCB4) mediates transmembrane movement of phosphatidylcholine and cholesterol from liver hepatocytes into bile [52]. MA analyses found a panel of genes involved in clearance, detoxification and reparation of tissues with higher expression levels in HR (Figure 8B). Removal of cholesterol seems to have high importance as evidenced by the profile of ATP-binding cassette transporter 1 (ABCA1), a cholesterol efflux pump in the cellular lipid removal pathway [53]. The intermediary metabolic enzyme alanine-glyoxylate aminotransferase (AGXT) is involved in the detoxification of glyoxylate, a product of amino acid metabolism and purine degradation in Atlantic salmon [54]. Previously we reported up-regulation of this gene in the liver of trout exposed to toxicity [46]. A number of genes with higher expression in HR have roles in the transport and modification of lipids. The exact metabolic function of the up-regulated transporter fatty acid-binding protein-3 (FABP3) in Atlantic salmon's liver remains unclear [55]. Diacylglycerol kinase delta 2 (DGKD) is involved in the conversion of diacylglycerol to produce phosphatidic acid. One more lipid-binding protein, beta-2 glycoprotein I, also known as apolipoprotein H is a precursor of anti-bacterial peptides [56]. Differences between the study groups were observed in several genes encoding growth factors. Melanoma-derived growth regulatory protein (MIA) (down-regulated in HR) mediates detachment of cells from ECM structures enhancing their migratory potential [57]. The up-regulated fibroblast growth factor-20 is a potent mitogen that induces DNA synthesis and cell proliferation [58].

Discussion

Vaccines development is targeted at the complete neutralization of pathogens via binding to antibodies or TCR. However to date this aim has not been achieved for many diseases of Atlantic salmon including furunculosis. Vacci-

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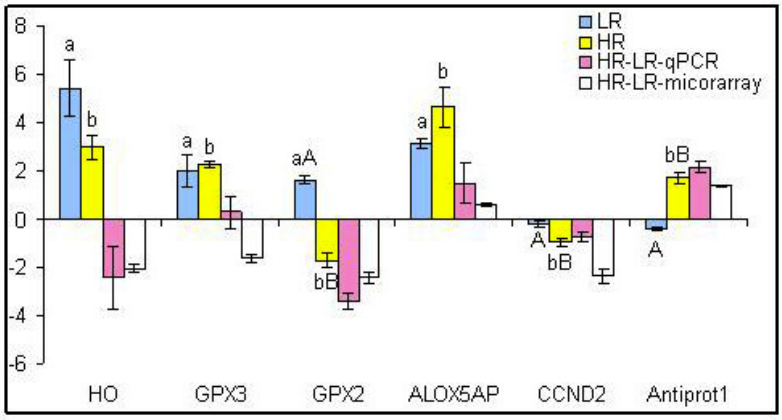

B

Genes	Expression ratio
Metabolism of iron	
Transferrin	-0.39 ± 0.17
Ferritin heavy chain-1	-0.92 ± 0.12
Ferritin heavy chain-2	-0.75 ± 0.14
Hemopexin	-0.52 ± 0.09
Hepcidin 1	-3.51 ± 0.23
Protoheme IX farnesyltransferase	-0.95 ± 0.19
Ceruloplasmin	-0.70 ± 0.08
Protein binding	
Heat shock factor 2-1	0.86 ± 0.22
Heat shock protein HSP 90-beta	-0.67 ± 0.14
78 kDa glucose-regulated protein precursor	-1.98 ± 0.43
94 kDa glucose-regulated protein	-1.81 ± 0.34
14-3-3 B1-like	-1.37 ± 0.31
14-3-3C2	-1.41 ± 0.24
15 kDa selenoprotein	-1.24 ± 0.26
DnaJ homolog subfamily C member 9	-0.83 ± 0.15
DnaJ homolog subfamily B member 11	-1.66 ± 0.39
DnaJ homolog, subfamily C, member 3	-1.18 ± 0.35
Cyclophilin-1	-0.71 ± 0.08
Peptidyl-prolyl cis-trans isomerase 1	-0.78 ± 0.16

Genes	Expression ratio
Protein degradation	
Proteasome subunit alpha type 7-2	-0.75 ± 0.16
26S proteasome non-ATPase regulatory subunit 14	-1.89 ± 0.74
Matrix metalloproteinase-2	0.66 ± 0.08
Matrix metalloproteinase-9	-2.01 ± 0.35
Tolloid-like protein (nephrosin)-1	-1.26 ± 0.16
Tissue inhibitor of metalloproteinase 2	-1.24 ± 0.35
Serine protease hepsin	0.97 ± 0.26
Serine protease-like protein-2	-0.49 ± 0.11
Inter-alpha-trypsin inhibitor heavy chain	0.74 ± 0.13
Metalloproteinase inhibitor 3	1.14 ± 0.25
Cell cycle and responses to oxidative stress	
Cyclin A2	-1.46 ± 0.24
Cyclin G1	-1.10 ± 0.23
Polyposis locus protein 1	-2.16 ± 0.34
Glutathione peroxidase 7	0.90 ± 0.16
Glutathione peroxidase 4	-0.96 ± 0.11
Glutathione S-transferase, mitochondrial	0.75 ± 0.44
Peroxiredoxin 1-2	1.63 ± 0.06
Peroxiredoxin 4	0.61 ± 0.27
Catalase-2	0.85 ± 0.18
Selenoprotein T-1	-1.80 ± 0.27
Transaldolase	-1.82 ± 0.33

Figure 5

Anti-bacterial effectors, markers of cellular and oxidative stress. A. Real-time qPCR versus microarray analyses. Data for qPCR are ΔΔCt ± SE of 10 HR and 10 LR versus 8 fish in the unchallenged control and data for microarray are mean log_2-ER ± SE of 6 HR fish hybridized to a pooled sample of 10 LR fish. Differences between HR and LR determined with microarrays and qPCR are shown. Different capital letters (A, B) denote a difference between challenged fish (HR and LR) while small letters (a, b) denote a difference between challenged and control fish (ANOVA, $P < 0.05$). HO - heme oxygenase; GPX3 - plasma glutathione peroxidase; GPX2 - gastrointestinal glutathione peroxidase; ALOX5AP arachidonate 5-lipoxygenase-activating protein; CCND2 - G1/S-specific cyclin D2; Antiprot1 - alpha-1-antiproteinase-like protein. B. Microarray results, examples of differentially expressed genes with significant differences between vaccinated and unvaccinated fish (t-test, n = 6 and 5, $p < 0.05$). Results for unvaccinated fish are not shown, data are log_2-ER ± SE.

nation reduces mortality but fails to provide a complete protection as confirmed in this study. Vaccinated HR fish that survived to the end of challenge test still showed relatively high levels of infection in the liver and spleen and induction of genes known for strong responses to bacterial pathogens. For example NFkB was induced 1000-fold with respect to the uninfected control. Markedly lower pathogen loads in HR suggested that survival of the vaccinated salmon was most likely determined with the ability to suppress and clear bacteria. Comparisons of gene expression within the groups of vaccinated and naïve fish found large scale differences between HR and LR. It is noteworthy to mention that analyses with vaccinated fish did not find significant role of genes involved in adaptive

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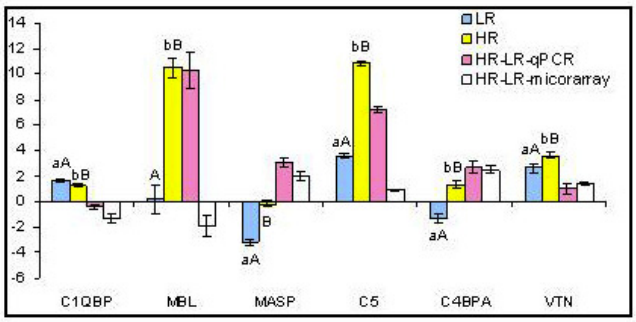


B

Genes	Expression ratio
Similar to C1q	-2.05 ± 0.35
C type lectin receptor B	-1.27 ± 0.14
Complement component C1q receptor	-1.20 ± 0.21
Complement control protein factor I-B	0.58 ± 0.08
Complement factor Bf-1	0.49 ± 0.05
Complement factor B/C2-B	0.33 ± 0.18
Complement factor H-3	1.24 ± 0.28
Complement C3-1	0.58 ± 0.10
Complement component C9	0.51 ± 0.08

Figure 6
Genes involved in the complement cascade. A. Real-time qPCR versus microarray analyses. Data for qPCR are ΔΔCt ± SE of 10 HR and 10 LR versus 8 fish in the unchallenged control and data for microarray are mean $\log_2$-ER ± SE of 6 HR fish hybridized to a pooled sample of 10 LR fish. Differences between HR and LR determined with microarrays and qPCR are shown. Different capital letters (A, B) denote a difference between challenged fish (HR and LR) while small letters (a, b) denote a difference between challenged and control fish (ANOVA, $P < 0.05$). C1QBP - complement component 1, Q subcomponent binding protein; MBL - C-type mannose-binding lectin; MASP - mannan-binding lectin serine protease, C5 - complement component C5, C4BPA - complement component 4 binding protein, alpha, VTN - vitronectin. B. Microarray results, examples of differentially expressed genes with significant differences between vaccinated and unvaccinated fish (t-test, n = 6 and 5, $p < 0.05$). Results for unvaccinated fish are not shown, data are $\log_2$-ER ± SE.

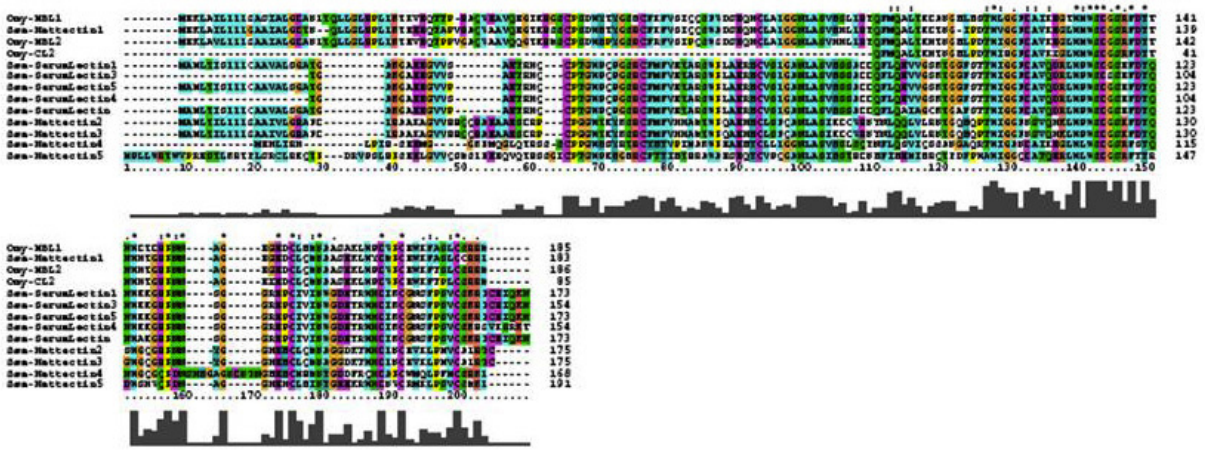
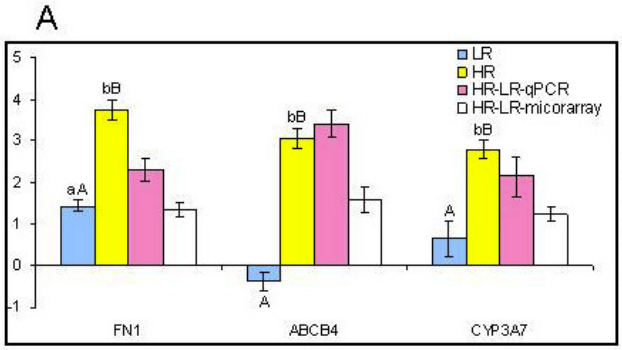


Figure 7
Alignment of rainbow trout and Atlantic salmon lectins. Sequence information includes the Genbank accession number; species is indicated as Omy for *Oncorhynchus mykiss* and Ssa for *Salmo salar*. Omy185132516 is encoded by the transcript spotted on the microarray. The sequences were aligned with ClustalX. D. Alignment of the predicted complement component 1, Q subcomponent binding protein (C1QBP) from three fish species with the human ortholog; species is indicated as Omy for *Oncorhynchus mykiss*, Ssa for *Salmo salar*, Dr for *Danio rerio* and Hs stands for *Homo sapiens*. The sequences are available at the accession numbers: human DQ891331, salmon paralog 1, salmon paralog 2, trout paralog 1 and trout paralog 2. The sequences were aligned with ClustalX.



B

Genes	Expression ratio		
Carboxylesterase HU1a	1.11	±	0.14
Alanine-glyoxylate aminotransferase	1.80	±	0.13
Aldehyde dehydrogenase 7	0.53	±	0.22
ATP-binding cassette transporter 1	0.80	±	0.17
Beta-2-glycoprotein I	1.20	±	0.08
Bone morphogenetic protein 8A	0.73	±	0.06
Delta-6 fatty acid desaturase	0.72	±	0.16
Dermatan-4-sulfotransferase-1-1	0.89	±	0.08
Diacylglycerol kinase delta2	0.51	±	0.09
Epoxide hydrolase 1	0.44	±	0.19
Fatty acid-binding protein-1	1.20	±	0.26
Fibroblast growth factor-20	0.88	±	0.14
Hypoxia-inducible factor prolyl hydroxylase 1	0.76	±	0.07
Mannosidase alpha	0.66	±	0.16
Melanoma derived growth regulatory protein	-0.71	±	0.14
Plasminogen precursor-1	-0.36	±	0.15
Saxitoxin and tetrodotoxin binding protein 1	1.52	±	0.15
Selenoprotein P	2.01	±	0.21
Serum albumin precursor	1.59	±	0.16
UDP-glucuronosyltransferase 1-8	0.89	±	0.23

Figure 8
Genes involved in tissue protection, clearance and reparation. A. Real-time qPCR versus microarray analyses. Data for qPCR are ΔΔCt ± SE of 10 HR and 10 LR versus 8 fish in the unchallenged control and data for microarray are mean log-ER ± SE of 6 HR fish hybridized to a pooled sample of 10 LR fish. Differences between HR and LR determined with microarrays and qPCR are shown. Different capital letters (A, B) denote a difference between challenged fish (HR and LR) while small letters (a, b) denote a difference between challenged and control fish (ANOVA, $P < 0.05$). FN1 - fibronectin, ABCB4 - liver bile salt export pump. B. Microarray results, examples of differentially expressed genes with significant differences between vaccinated and unvaccinated fish (t-test, n = 6 and 5, $p < 0.05$). Results for unvaccinated fish are not shown, data are $\log_2$-ER ± SE.

immune responses. This was in contrast with our studies of salmon challenges with virus (ISAV), which showed clear dependence between resistance evaluated by time of survival and activation of adaptive immunity [59]. Rapid stimulation of Igs was also seen in salmon challenged with the parasite, salmon louse [60]. Apparently, pathogen did not stimulate further activation of adaptive immunity in the vaccinated fish. Therefore one may assume that vaccine-dependent protection of salmon infected with *A. salmonicida* was determined mainly with the events that take place after recognition of the pathogen. Given high complexity and diversity of this network and limited level of knowledge, multiple gene expression profiling provides an efficient approach to search for the protective mechanisms.

The gene expression analyses were designed with focus on the mechanisms of vaccine-dependent protection against furunculosis. Genes with greater expression changes in HR can be regarded as candidate markers of protection while opposite regulation may indicate either pathology or unsuccessful defense. Overall, expression changes that can be interpreted as active anti-bacterial responses tended to be greater in LR. We produced an indirect evidence for larger regulation of immune cells recruitment in LR. More detailed study of the immune cells populations is complicated with shortage of cell-specific markers for salmon. Interaction of recruited and resident cells with pathogen components and cytokines activates signal transducers and other regulators of gene expression. We found increase of several genes known for their key roles in responses to pathogens and the magnitude was either greater in LR or equal in LR and HR (NFkB).

A panel of genes for proteins involved in metabolism of iron had higher expression in LR. These regulations indicate the need to reduce the levels of free inorganic iron and heme. HO was activated in all infected fish but the magnitude was greater in LR. HO is the rate-controlling enzyme of the degradation of heme into iron, carbon monoxide, and biliverdin, which is subsequently converted to antioxidant bilirubin [61]. Extracellular iron ions bind to transferrin, which delivers it into cells. Within cells iron is stored as complex with ferritin. Damage of erythrocytes is one of the symptoms of furunculosis. Heme and iron catalyze production of free radicals through Fenton's reaction thus increasing risk of oxidative stress. Shortage of iron may suppress proliferation of pathogenic bacteria and therefore sequestration of bioavailable iron and heme is regarded as anti-bacterial defense. In case of furunculosis this strategy is obviously unsuccessful.

The complement system was the only group of immune genes that showed strong association with survival and several genes presented in Figure 8A can be considered as

candidate markers of vaccine protection against furunculosis. The complement system is the major link between the effector anti-bacterial mechanisms of adaptive and innate arms of immunity. The role of complement in antibody mediated defense against *A. salmonicida* was demonstrated in rainbow trout. Combination of specific IgM and complement accelerated ingestion of bacteria and ingestion-activated respiratory burst in phagocytes [62]. The complement pathways converge at the level of C3 convertase, which initiates the anti-bacterial effector mechanisms. C3 and the downstream genes, the parts of membrane attack complex had higher expression levels in HR, especially C5. Mobilization of the effector complement mechanisms correlated with survival, however it remained unknown, which of the complement pathways was responsible for these changes. Increase of C1QBP, the negative regulator of CP was greater in LR. This could be regarded as indirect evidence for higher activity of CP in HR, however, the expression difference between the study groups was relatively small. Furthermore, it remains unknown if CP provides the only or the major connection between the complement system and acquired immunity in salmonid fish. Homolog to mammalian C1Q was found in lampreys, primitive vertebrates that lack adaptive immunity [63]. The link between the classical pathway and antibodies appeared in the course of vertebrate evolution but the timing of this event remains undefined. C1Q is the member of a large multi-gene family with diverse functions [64]. Analysis of non-redundant Atlantic salmon mRNA sequences with blastx revealed 17 distinct C1Q related transcripts. To our knowledge, the antigen-antibody complex binding protein of salmonid CP has not been identified so far. In theory, interaction between the fish complement and acquired immunity molecules can be mediated by MP. MBL interacts with immunoglobulins in mammals [65] despite the principal role of CP in recognition of antibody antigen complexes. The qPCR analyses showed dramatic up-regulation of MBL in HR. Given the lack of changes in LR, this gene is one of the most promising markers of the vaccine-dependent protection. However taking into account the presence of multiple lectins with unknown roles in Atlantic salmon, induction of MBL does not necessarily mean activation of MP. Furthermore, MASP showed no increase in HR in comparison with uninfected control. Expression profiles of the complement factors Bf and B/C2-B suggested higher activity of AP in HR, however, differences between HR and LR were minor. Diversity of putative sensor proteins in salmonid fish and limited knowledge on their functions impede interpretation of the gene expression data.

Importantly, HR showed greater expression of factor H, the negative regulator of AP, and vitronectin (VTN), a protein that protects host tissues from the complement damages. Similarly, the transcript encoding alpha antiproteinase-like protein was up-regulated in HR relative to LR. Vaccine-dependent resistance to furunculosis was clearly associated with the abilities to prevent and repair damages from pathogen and immune responses and to neutralize and clear toxic compounds of endogenous and exogenous origin. The genes shown in Figure 8 can be divided in three functional groups - tissue reparation, clearance and xenobiotic metabolism - each represented with a gene whose markedly greater up-regulation in HR was confirmed with two methods. Elevated levels of fibronectin can be beneficial since FN1 is involved in reparation of tissues at the wound contraction stage of wound healing [66]. Due to its opsonic properties, fibronectin takes part in the removal of unwanted substances in the liver by phagocytic Kupffer cells [67]. CYP3A4 metabolizes a strikingly large number of xenobiotics including bacterial toxins and more than half of modern prescription drugs. This gene is down-regulated in inflammatory conditions by a range of cytokines and NFkB [68,69]. Hence individuals with strong innate immune responses may have a higher risk of bacterial intoxication. The role of ABCB4 in bacterial diseases has not been reported so far. One may speculate that the transporter protein that directs phospholipids from liver to bile can be important for clearance of remains of killed cells and bacteria. Transcriptomic comparison of salmon with high and low resistance to pathogen indicated pivotal importance of processes that deserve more attention in studies of fish diseases.

Conclusion

Gene expression analyses revealed significant differences between vaccinated fish with high and low resistance to furunculosis. We did not find strong association between survival and most anti-bacterial responses though HR showed higher expression levels of several complement components. Results suggest that outcomes of vaccination depend largely on the ability of host to prevent the negative impacts of immune responses and to repair damages. This can be illustrated with the inductions of protease inhibitors, negative regulators of complement, genes involved in metabolism of lipids and xenobiotics and growth factors. Studies outlined a number of genes with positive and negative correlation with protection.

Methods

Challenge trials

The unvaccinated Atlantic salmon was from 279 full sib families (the offspring of 140 sires and 279 dams) while the vaccinated fish were a random sample of fish from 150 of the 279 families (the offspring of 87 sires and 150 dams). The families were produced by Salmo Breed AS in November 2006 and were reared in separate trays and tanks at Nofima Marin Sunndalsøra to a body size suitable for individually tagging with pit-tags in July (30 fish/family) and September (15 fish/family) 2007. The 30 fish/family were vaccinated intraperitoneally with a six

component oil adjuvanted vaccine from PHARMAQ on 2 to 4 October 2007.

The pathogen challenge trials were approved by The National Animal Research Authority http://www.fdu.no according to the 'European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (EST 123).

Two groups of fish were infected with furunculosis at VESO Vikan, Norway in two separate tanks; the unvaccinated fish on 2 October 2007 at an average body weight of 30 g and the vaccinated fish on 22 November 2007 at an average body weight of 46 g. Infection was performed by cohabitation of 300 salmon (shedders) injected intraperitoneally with a virulent strain of *A. salmonicida* O2. The trial with unvaccinated fish was terminated after 20 days when cumulative mortality reached 76% while the trial with vaccinated fish, including 100 unvaccinated fish from the same families as controls was terminated after 60 days when cumulative mortality was 39% (vaccinated) and 72% (controls). In both trials liver, spleen and heart were sampled from 10 fish with symptoms of disease (darker colour, unusual swimming behaviour) at 10% cumulative mortality (14 and 22 days after challenge of respectively vaccinated and unvaccinated fish) and 10 fish at the end of trials. These groups were designated respectively as low resistant (LR) and high resistant (HR). Tissue samples were immediately dissected in 6 mm^3 pieces using RNase-free procedures and preserved in >1:10 v/v of RNALater (Ambion, Austin, TX, USA) at 4°C overnight following storage at -20°C. Presence of *A. salmonicida* infection was confirmed on a selection of fish at each stage by inoculating tryptic soy agar with swabs from posterior kidney and incubating at room temperature overnight. In addition, levels of bacteria in liver and spleen from both stages were assessed by PCR (see below).

Detection of pathogen

DNA was extracted from the liver and spleen of vaccinated fish using the Qiagen DNeasy Blood and Tissue kit (Hilden, Germany) according to manufacturer's instructions. Equal amounts of isolated DNA (1.2 μg) were used as templates in 12 μl qPCR reaction volumes. DNA fragment (accession number X64214), reported to be highly specific for *Aeromonas salmonicida* (Hiney et al, 1992) was used to design primers. The cycling conditions were 95°C for 5 min (preincubation), 95°C for 5 sec, 60°C for 15 sec, 72°C for 15 sec (amplification); 95°C for 5 sec, 65°C for 1 min (melting curve); 55 cycles were performed.

Microarray analyses

The salmonid fish microarray (SFA2, immunochip) includes 1800 unique clones printed each in six spot replicates. The complete composition of platform and sequences of genes are provided in submission to NCBI GEO Omnibus (GPL6154). Total RNA was extracted with TriZOL (Invitrogen) and purified with Pure Link (Invitrogen) according to the manufacturer's instructions. RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and RNA integrity assessed using Bioanalyzer (Agilent 2100 Bioanalyzer, Agilent Technologies, Waldbronn, Germany) all samples giving RIN >8 and high purity without DNA contamination. The liver was chosen based on the results of pilot hybridizations with different tissues. Individual HR samples (6 vaccinated fish and 5 naïve fish) were hybridized to pooled LR samples (equal contribution from 6 fish) from the same groups. RNA (20 μg in each sample) was labelled with respectively Cy5-dUTP and Cy3-dUTP (Amersham Pharmacia). The fluorescent dyes were incorporated in cDNA using the SuperScript™ Indirect cDNA Labelling System (Invitrogen, CA, USA). The cDNA synthesis was performed at 46°C for 3 hours in a 20 μl reaction volume, following RNA degradation with 0.2 M NaOH at 37°C for 15 min and alkaline neutralization with 0.6 M Hepes. Labelled cDNA was purified with Microcon YM30 (Millipore). The slides were pretreated with 1% BSA fraction V, 5× SSC, 0.1% SDS (30 min at 50°C) and washed with 2 × SSC (3 min) and 0.2 × SSC (3 min) and hybridized overnight at 60°C in a cocktail containing 1.3 × Denhardt's, 3 × SSC 0.3% SDS, 0.67 μg/μl polyadenylate and 1.4 μg/μl yeast tRNA. After hybridization slides were washed at room temperature in 0.5 × SSC and 0.1% SDS (15 min), 0.5 × SSC and 0.01% SDS (15 min), and twice in 0.06 × SSC (2 and 1 min, respectively). Scanning was performed with GenePix4100A and images were processed with GenePix 6.0 (Molecular Devices). The spots were filtered by criterion $(I\text{-}B)/(S_I+S_B) \quad 0.6$, where I and B are the mean signal and background intensities and S_I, S_B are the standard deviations. Low quality spots were excluded from analysis and genes presented with less than three high quality spots on a slide were discarded. After subtraction of median background from median signal intensities, the expression ratios (ER) were calculated. Lowess normalization was performed first for the whole slide and next for twelve rows and four columns per slide. The differentially expressed genes were selected using a two step procedure. First, technical accuracy was assessed by difference of $\log_2$-ER from zero in six spot replicates (Student's t-test, $p < 0.01$). The mean values were calculated and a single value per fish was used in subsequent analyses. Second, the genes with technically significant changes in at least half of vaccinated and naïve fish were selected and difference between these groups was assessed by biological replicates (t-test, $p < 0.05$). At total, 13 microarrays were used, one for the heart and spleen and 11 for the liver. Complete microarray results are provided in Additional file 1 and the data were submitted to NCBI GEO Omnibus (GSE18120).

Table 1: Real-time qPCR analyses.

Genbank accessions, target	Primer sequence from 5' to 3'	PCR efficiency
CA366296, Src kinase associated phosphoprotein	F GAGGTGCTCCCAGAGGATGACA R CAGTCCCACAAGCCCTGGTAGT	2.0
CA349943, C-type mannose-binding lectin	F TCCATTGCACTGGGCGATGC R CACTGCTTCCACCTGAGCCTCA	1.667
CA361395, Leukocyte antigen CD37	F TGCTGAGACAAGCTTCTTCATGCC R CGACATCGTAGCACTTCCACCAAT	1.696
CA364370, CD40	F CTGTAAACTGCACCCATACTGCGAC R ATGGGCTGAGGCTTGTCTTGTTC	1.716
CU068239, Leukocyte cell-derived chemotaxin 2	F CTGTGTTGTCAGAGTGCGAGATGGT R TACACACAATGTCCAGGCCCTGA	1.899
CA369467, 5-lipoxygenase activating protein	F TCTGAGTCATGCTGTCCGTAGTGGT R CCTCCCTCTCTACCTTCGTTGCAAA	1.751
CA366162, Alpha-1-antiproteinase-like protein	F CCACAAGGCTGTGCTGAGCGTA R TGAGCATGATGGTGTCTGGGAGAG	1.702
CA366315, YY1 transcription factor	F AAAGAAGACGACGCGCCCAG R GGTGTGGAGATGCTTCCTCATCG	1.893
CF752495, Jun B	F CATCAGAAGTCGGCTCGCTGAA R GGTGTCGGTGTGGTAGTGATGACA	1.743
CA341859, NFkB1	F CAGCGTCCTACCAGGCTAAAGAGAT R GCTGTTCGATCCATCCGCACTAT	1.685
CA343143, NFkB inhibitor	F TGGTAACCTTGTGAAGGAGCTGGA R GCTCAGCATGTTCTGTGGCTTCAT	2.00
CA361415, Jun C	F CAGCATGACACTGAACCTGGCTGA R GCAAGTTTGAGGAGCTGCACATCC	2.00
CA345853, Plasma glutathione peroxidase precursor	F CCTTCCAGTACCTGGAGTTGAATGC R CTCATGATTGTCTCCTGGCTCCTGT	1.904
CF753103, Glutathione peroxidase-gastrointestinal	F TGTACCTCAAGGAGAAGCTGCCGT R ATTAAGGCCATGGGATCGTCGC	2.00
CA363120, Heme oxygenase	F TGGGTCTGACCTGGGTCCTCTCAT R GAGGGTGGTTTCAGCGTTGAGC	1.664
CA368533, Vitronectin	F AAGCCCTTCGACGCCTTCCT R CCTCTGATGCCCCACTTGTCGTAG	2.00
CA382259, Complement component 4 binding protein	F TGGTGGAGTATCAGTGTGACAGGCA R GGTGGATTTGGCTCAAACTGTCCT	2.00
CA364804, Complement component 5-2	F AGAACTCTTCCGAGTTGGCATGGT R AGTGATGCTGGGATCCATCTCTGA	1.952
CA366393, Mannan-binding lectin serine peptidase	F TCAGGTGCTGACGGAGAGGTCA R GCACTCTGAATCCCTCTGGTAGGAG	1.971
CA387557, Complement component 1Q binding	F CGGTCTCTCTGGATGATGAGCCATA R CCACATCCACACGACACAGGAGTA	2.00
CA376069, Cytochrome P450 3A27	F CCAACCTGCTGAACGGAATGAA R AGAACTCCTTCACTTCGATGGCCT	2.00
CA378743, Fibronectin	F GCATGTCTGAGACGGGCTTCAA R AGTCACATCGGAAGTGTCCACTGC	2.00
CA384134, G1/S-specific cyclin D2	F CATCAGACCACAGGAGTTGCTGGA R AAGTCATTTGGAGTGACAGCCGC	2.00
CA363965, Liver bile salt export pump	F TACGACACCAACGTAGGTTCCCAGG R GGATCTTAGGGTCGCGGATGATC	1.686
CA385588, CREB binding protein	F CTCCAGCCCAGGCCAACTCC R GGCCAGGCAGGTGAGCTCCT	2.00
X64214, *A. salmonicida* genomic DNA fragment	F GTTTACCACGTAATCTGAATTGTTCTTTTC R ATTGCTTATCGAGGCAGCCAAC	1.890
AJ427629, 18S rRNA	F GCCCTATCAACTTTCGATGGTAC R TTTGGATGTGGTAGCCGTTTCTC	2.00

Quantitative real-time RT-PCR

Twenty five genes were selected for qPCR analyses taking into account their functional roles and the results of microarray analyses (Table 1). All genes were analyzed in vaccinated (experimental) and uninfected (control) fish. Total RNA was extracted and quality assessed as described in the section above. The cDNA synthesis was performed on 0.5 μg of DNAse-treated total RNA (according to manufacturer's protocol for routine DNAse treatment, Turbo DNA-*free*™ (Ambion, Austin, TX, USA) using TaqMan®

Gold Reverse Transcription kit (Applied Biosystems, CA, USA) and oligo dT primers, according to manufacturer's protocol. PCR primers were designed using Vector NTI (Invitrogen) and synthesized by Invitrogen. The amplicon lengths set to be between 50 and 200 bases were checked on 1.5% agarose gel. PCR efficiency was calculated from tenfold serial dilutions of cDNA for each primer pair in triplicates. Real-time PCR assays were conducted using 2× SYBR® Green Master Mix (Roche) in an optimised 12 μl reaction, using 1:10 diluted cDNA, primer concentrations of 0.4-0.6 μM each. PCR was performed in duplicates in 96-well optical plates on Light Cycler 480 (Roche) under the following conditions: 95°C for 5 min (preincubation), 95°C for 5 sec, 60°C for 15 sec, 72°C for 15 sec (amplification), 95°C for 5 sec, 65°C for 1 min (melting curve). 45 cycles were performed. Relative expression of mRNA was evaluated by ΔΔCT. Three commonly used candidate reference genes (18s rRNA, eukaryotic translation initiation factor 3 subunit 6 and elongation factor EF-1a) were tested and 18s rRNA was selected as the reference gene for all samples by the stability criteria. Dependence of gene expression on infection, vaccination and resistance was analyzed with ANOVA ($p < 0.05$).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors contributed to the overall experimental design. BG was responsible for the challenge test. SŠ and SJ carried out the gene expression analyses whilst AK analyzed the results and produced the first manuscript draft. All authors read, contributed to, and approved the final manuscript.

Additional material

Additional file 1

Complete results of microarray analyses. *The data provided represent the gene ratios in salmon with high and low resistance to furunculosis. The table includes log_2 (Expression ratios) and p-values of differential expression (t test).*

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Paper III

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Modulation of splenic immune responses to bacterial lipopolysaccharide in rainbow trout (*Oncorhynchus mykiss*) fed lentinan, a beta-glucan from mushroom *Lentinula edodes*

Brankica Djordjevic [a,1], Stanko Škugor [a,b,*,1], Sven Martin Jørgensen [b], Margareth Øverland [a,c], Liv Torunn Mydland [a,c], Aleksei Krasnov [b]

[a] *Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, P.O. Box 5003, Ås NO-1432, Norway*
[b] *Nofima Akvaforsk Fiskeriforskning AS, P.O. Box 5010, Ås NO-1430, Norway*
[c] *Aquaculture Protein Centre, CoE, Norway*

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ABSTRACT

Immunostimulants (IS) are considered a promising approach for improving resistance to pathogens in fish aquaculture. At present, development of IS are complicated due to limited knowledge on the mechanisms of their action. To assess the use of global gene expression analysis for screening of candidate IS we applied lentinan, a β-glucan from the mushroom *Lentinula edodes*, as a model. After feeding rainbow trout (*Oncorhynchus mykiss*) with lentinan-supplemented (L) and control (C) diets for 37 days, fish were injected with bacterial lipopolysaccharide (LPS), a classical inducer of inflammation. Gene expression was analyzed in LPS-challenged compared to saline-injected fish using a salmonid 1.8 k cDNA microarray (SFA2.0 immunochip) and real-time qPCR. Spleen was selected for data analyses due to highest magnitude of responses and its key role in the fish immune system. A group of genes implicated in acute inflammatory responses was higher induced in C versus L, including IFN-related and TNF-dependent genes (galectins and receptors, signal transducers and transcription factors), genes involved in MHC class I antigen presentation and leukocyte recruitment. A similar trend was observed in metabolism of iron and xenobiotics, markers of oxidative and cellular stress. Interestingly, differences between C and L were similar to those observed between salmon with low and high resistance to infectious salmon anemia virus. Genes with equal responses to LPS in L and C were related to cell communication (cytokines, chemokines and receptors), signal transduction, activation of immune cells, apoptosis, cellular maintenance and energy metabolism. In conclusion, lentinan decreased the expression of genes involved in acute inflammatory reactions to the inflammatory agent while major parts of the immune response remained unchanged. Such effects are expected for IS, which should modify immunity by enhancing beneficial and reducing detrimental responses.

1. Introduction

Immunostimulants (IS) are additives that augment the innate and adaptive immune responses and are regarded as a promising supplement to vaccination and selective breeding, which remain the key strategies for prevention of diseases in fish aquaculture. A number of β-glucans of different origins have been tested with different rates of success as well as other bacterial and plant products (reviewed in [1,2]). The development of IS is complicated due to the limited knowledge on mechanisms of their action. A definitive evaluation of IS efficiency requires challenge with active pathogens but given the high costs and ethical concerns of these experiments, a merely trial and error approach appears inappropriate. Pathogen challenging should be preceded with screening of candidate IS, which assesses their impacts on immunity and predicts efficiency under various conditions, i.e. infections with bacteria and viruses with different modes of action and pathogenesis. Adequate responses to pathogens presume a precise balance between different immunological processes. Therefore, IS should enhance beneficial and decrease adverse responses rather than activate or suppress immunity in general. Target immune functions that require modification remain to be identified in fish. Glucans of different origin have been shown to affect various

* Corresponding author. Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, P.O. Box 5003, Ås NO-1432, Norway. Tel.: +47 93097838; fax: +47 64949502.
E-mail address: stanko.skugor@nofima.no (S. Škugor).
[1] These authors contributed equally to this work.

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immune functions in different aquaculture species. In these studies a suite of immune parameters were used including lysozyme, complement and myeloperoxidase activity, phagocytic index and respiratory burst and antibody titer [3–8]. However, given the high complexity of immune networks and limited repertoire of immune assays, use of multiple gene expression profiling can provide an efficient approach to this problem.

Lentinan, a β-glucan polysaccharide isolated from shiitake, an edible mushroom *Lentinula edodes*, has been tested in numerous clinical studies as an anti-cancer drug and IS (reviewed in [9,10]). The stimulatory effects of lentinan on macrophages [11], leukocytes [12], different types of T cells [13,14] and humoral immune factors [15] have been demonstrated both, in vitro and in vivo. In this study we investigated lentinan as a feed additive for rainbow trout (*Oncorhynchus mykiss*). Injection of bacterial (*Escherichia coli*) lipopolysaccharide (LPS) was applied as a model of inflammation. LPS is the major constituent of the external layer of Gram-negative bacteria and a prototype inducer of inflammation in diverse species. LPS stimulates production of chemokines and pro-inflammatory cytokines, which activate recruitment and differentiation of immune cells and switch on anti-bacterial mechanisms including complement, extracellular proteases and reactive oxygen species (ROS). In mammals LPS causes sepsis and organ failure. Despite markedly greater tolerance in comparison with higher vertebrates [16], fish develop large-scale inflammatory responses to LPS. For gene expression analyses we used real-time qPCR and the salmonid fish microarray (SFA2.0) customized for studying responses to pathogens and environmental stressors [17,18]. The previous version of this platform was successfully used in studies of responses to LPS in isolated macrophages [19], head kidney [20] and ovary [21] of rainbow trout. The new chip provides an improved coverage of immune functions as confirmed in experiments with virus-infected fish [22].

2. Materials and methods

2.1. Fish, diets and experimental conditions

The fish experiment was carried out at the fish laboratory at the Norwegian University of Life Sciences (Ås, Norway). The whole experiment lasted 54 days; with a 14-day adaptation period and a 37-day experimental period where fish were fed the experimental diets. The experimental fish were rainbow trout (*O. mykiss*) with an average start body weight of 641 g ($n = 70$). Fish were in twelve 300 L tanks, four tanks per group (average parameters: water flow 7 L min^{-1}; water temperature 9.4 °C; oxygen saturation 72%). Continuous 24 h illumination was provided for the duration of the whole experiment.

Lentinan was included into manually prepared moist pelleted feed at the levels 0.2% (02L), 0.4% (04L) and 0.0% kg^{-1} diet (C – control; the same diet without additive). Yttrium oxide (Y_2O_3) was included as an inert marker for digestibility estimation. Formulation is shown in Table 1 in Supplementary data. The feed was provided three times per day, distributed manually and fish were fed 2% of their body weight.

After 37 days each dietary group was divided into two, which received injection with either LPS (*E. coli* strain O26:B6; Sigma–Aldrich, St. Louis, USA), or isotonic sodium chloride solution (saline) of injection quality (Fresenius Kabi, Uppsala, Sweden). Challenge with LPS was conducted as described [23]. Briefly, LPS was dissolved in saline in the concentration 1 mg ml^{-1} and was injected intraperitoneally at the dose 3 mg kg^{-1}, while the saline-injected fish received 1 ml $kg^{-1}$of saline. Samples of spleen, liver and head kidney were taken after two days and stored in RNA later (Invitrogen, Carlsbad, CA, USA).

2.2. RNA extraction and cDNA synthesis

Total RNA from tissue was isolated with TRIzol and purified with PureLink Micro-Midi kit (Invitrogen) using manufacturer's protocols and guidelines. Samples were precipitated and stored under ethanol at −70 °C. cDNA synthesis was performed on 125 ng DNAse-treated (Turbo DNA-*free*™, Ambion, Austin, TX, USA) total RNA using TaqMan® Reverse Transcription reagents (Applied Biosystems, Biosystems, Foster City, CA, USA) and random hexamer primers, according to manufacturer's protocol.

2.3. Microarray analyses

The salmonid fish cDNA microarray (SFA2.0 immunochip) contains 1800 unique clones printed each in 6 spot replicates. The genes were selected by their functional roles and the platform is enriched in a number of functional classes such as immune response (236 genes), cell communication (291 genes), signal transduction (245 genes) and receptor activity (126 genes), apoptosis (120 genes), cell cycle (76 genes), protein catabolism (90 genes) and folding (70 genes) and response to oxidative stress (39 genes). The gene composition and sequences are provided in GEO (**GPL6154**).

All hybridizations used fish challenged with LPS as a test and a pooled sample of 6 saline-injected fish from the same study group as a reference. Pilot microarray analyses were conducted to select a study group and tissue for the individual comparisons. Pooled samples of the liver, anterior kidney and spleen from 02L, 04L and C were analyzed; equal amounts of RNA from 6 individuals were combined in each sample. Transcriptome responses in 02L were stronger than in 04L and the gene expression changes were greatest in the spleen. Therefore, we chose spleens from fish fed with 0.2% lentinan for the individual analyses. Microarray analyses described in Section Results included 6 individuals from 02L (hereafter denoted L) and 6 individuals from C, one slide was used per each fish. The test and reference samples (20 μg RNA in each) were labeled with respectively Cy5-dUTP and Cy3-dUTP (Amersham Pharmacia, Little Chalfont, UK) using the SuperScript™ Direct cDNA Labeling System (Invitrogen). The cDNA synthesis was performed at 46 °C for 3 h in a 20 μl reaction volume, followed with RNA degradation with 0.2 M NaOH at 37 °C for 15 min and alkaline neutralization with 0.6 M Hepes. Labeled cDNA was purified with Microcon YM30 (Millipore, Bedford, MA, USA). The slides were pretreated with 1% BSA fraction V, 5 × SSC, 0.1% SDS for 30 min at 50 °C and washed with 2 × SSC for 3 min and 0.2 × SSC for 3 min at room temperature and hybridized overnight at 60 °C in a cocktail containing 1.3 × Denhardt's, 3 × SSC, 0.3% SDS, 0.67 μg μl^{-1} polyadenylate and 1.4 μg μl^{-1} yeast tRNA. After hybridization slides were washed at room temperature in 0.5 × SSC and 0.1% SDS for 15 min, 0.5 × SSC and 0.01% SDS for 15 min, and twice in 0.06 × SSC for 2 and 1 min, respectively. Scanning was performed with GSI Lumonics ScanArray 4000 (PerkinElmer Life Sciences, Zaventem, Belgium) and images were processed with GenePix Pro 6.0 (Axon, Union City, CA, USA). The spots were filtered by criterion $(I-B)/(S_I + S_B) \geq 0.6$, where I and B are the mean signal and background intensities and S_I, S_B are the standard deviations. The low quality spots were excluded from analyses and the genes presented with less than three high quality spots on a slide were discarded. After subtraction of median background from median signal intensities, the expression ratios (ER) were calculated. Lowess normalization was performed first for the whole slide and next for twelve rows and four columns per slide. The data analyses took into account both technical and biological variation. First, each slide was processed separately and the differential expression (DE) was assessed by difference of the mean log-ER from zero (6 spot replicates per each gene, Student's t-test, $p < 0.05$). Next, genes with DE in at least

4 of 6 fish in either 02L or C were chosen for assessment of responses to LPS (deviation of log-ER from zero) in each group and differences between 02L and C (t-test, $p < 0.05$). The complete data are provided in File 1 in Supplementary data.

2.4. Quantitative real-time RT-PCR (qPCR)

Expression of 26 genes was analyzed by real-time qPCR in LPS-injected and saline-injected fish from 02L and C. The same individuals (6 fish per group) were used for the microarray and qPCR analyses. Genes were chosen to represent the key functional classes affected by L. Based on the microarray results, several genes that are not present on the chip were included into analyses (*IL-12b, IFN-a, CD8a, arginase 1 and 2*). The qPCR primers (Table 1) were designed using the Vector NTI software (Invitrogen) and synthesized by Invitrogen. The specificity of PCR amplification was confirmed by agarose gel electrophoresis and melting curve analysis (Tm calling; Light Cycler 480, Roche Diagnostics, Mannheim, Germany). PCR efficiency (E) was determined from tenfold serial dilutions of cDNA for each primer pair [24]. As a common reference gene *18S rRNA* was used, which was previously optimized and validated [25]. Four potential reference genes were tested (*18S rRNA, EF-1a* and two other non-regulated genes from the microarray results; *SEC13-related protein and NADH-ubiquinone oxidoreductase 19 kDa subunit*) using the BestKeeper [26] and GeNorm software [27]; however, only *18S rRNA* met qualifications of stability. The PCR assays were optimized using 2 × SYBR® Green Master Mix (Roche Diagnostics) and varying amounts of cDNA and primer concentrations. Optimal PCR conditions in a 12 μl reaction volume were 3 μl 1:10 diluted cDNA for all assays (1:2000 for *18S rRNA*). Primer

Table 1
Real-time qPCR primers.

Genes	Primers	GenBank	Reference proteins
BPI/LBP	5-GTGAATGGACTCACCGTCACTGACA-3 5-TGTATAGCCAGCTTGCTCCACCAT-3	CA349380	NP_001118057.1
LGALS9	5-GTCCTGTCTATTGCCTTCTCCAACC-3 5-GGTTTCGTTGACCACTGTGTGGA-3	CA353586	NP_001118080.1
ABCD3	5-CCATCGGGAAGATGACGGTCA-3 5- TCTGCTTCTCCCTCAGGTTTCCA-3	CA369192	NP_998647.1[a] (e-114)
ABC-1	5-AGCGGGGGAAACAGTAGCAGGA-3 5-GCCTGGTCTTGAGATTGTGGGTGT-3	CA347504	XP_687304.3 (e-132)
ABCF2	5-CGGGCGGCATGATGTTGGTTA-3 5-CCAAGATGTCCCGGTTCCATTTG-3	CX723047	AAH66505 (e-103)
TNFRSF6B	5-TCTCCTGGTATTTGCGCTCTGTGGT-3 5-TATAAGTCGGTGTGTGAGCGCCTGA-3	CA351440	NP_001118086.1
TNFAIP6	5-ATCTTCACTGTCATCTGGGCCATC-3 5-TTTACGCGATTCCCTGTGGTAGAC-3	CA347334	NP_001035333.2 (e-96)
JAK-1	5-ACACTGATATTGGGCCGTTCTGGA-3 5-CCTCGTCCTCTGCATCTTTACCAAC-3	CA378782	NP_571148.1 (e-52)
MAP3K6	5-CCTGGAGGTGATTCTGCCCATT-3 5-GGTCTCGACTGCGATGGTCTTTAA-3	CA346979	XP_001920578.1 (e-87)
IL1RLP	5-CCAAAAAGAGGGCACCAGATGG-3 5-CGTATCGTCTCTCCAACACCTCAGG-3	CA362179	NP_001117105.1
IFN-α	5-TGCAGTATGCAGAGCGTGTG-3 5-TCTCCTCCCATCTGGTCCAG-3	AJ580911.2	CAE45642
CD8α	5-CGTCTACAGCTGTGCATCAATCAA-3 5-GGCTGTGGTCATTGGTGTAGTC-3	AY693391	NM_001124263.1
IL-12β	5-TCTACCTACACGACATTGTCCAGCC-3 5-ATCCATCACCTGGCACTTCATCC-3	AJ548830	CAD69014
TAPBP	5-CAAGCTATCGTTTCTCGCCTTTACT-3 5-TGGTTTCTCCTGGACAAACCAGCAC-3	CA355065	NP_001118025.1
CD37	5-TGCTGAGACAAGCTTCTTCATGCC-3 5-CGACATCGTAGCACTTCCACCAAT-3	CA361395	NP_001096092.1 (e-51)
RRAGA	5-ACGCCAAGGTCTTCTGCCTGGT-3 5-AAGCCAATGGCCTGGACAGACG-3	CA345232	NP_956008.1 (e-119)
ALAS1	5-CTCCATGATCCAAGGCATCCG-3 5-GGTCTGATTTCAGCAGCAGCTCTC-3	CA365876	NP_958444.1 (e-132)
ALAS2	CACATGAGACAGCTGCTCCTGGAGA GCTCCAGCAAGATGTCACACACCT	DV201251	AAH67149.1 (e-130)
HMOX2	AGTCAGTGGAGAGAGACCTGGAGCA GGTTGTCTTTGCCGATCTGTCTGAG	CA363120	NP_955972.1 (e-77)
CA	GGTGTATGGGGGCCATCAAATC GGAGTGGTCAGGGAGCCATCAT	AB117757	NP_001117693.1
ND4	GCTAGTTTAGCAAATCTGGCCCTCC GCTGTAATTAACGTGCCTAGTCCCG	CX148939	NP_008299.1
PGDS	CCTACACCAACCTGAACGCTGATG ACGCTGGCTGGTGAAGGTGAAG	CA352578	ABY21331.1
NAMPT	5-GACTTCAATTTCCTGCTGGCTA-3 5-CTGCTTGTAATGTGTGACCT-3	CA376673	XP_686052.1 (e-77)
ITGB1BP3	5-GAAGTGCGTTATCGGCATTGGA-3 5-CCACACAACAGTTGGGAAGGTTCT-3	CU063356	NP_942578.1 (e-92)
ARG1	5-AGCCATGCGTATCAGCCAA-3 5-AAGGCGATCCACCTCAGTCA-3	EG929369	DAA01450
ARG2	5-GACAGGCTCGGCATTCAGA-3 5-AAAGACGGGTCCATCGCAT-3	DW581882	AAL17947
18S rRNA	5-GCCCTATCAACTTTCGATGGTAC-3 5-TTTGGATGTGGTAGCCGTTTCTC-3	AJ427629	

[a] Accession numbers of putative zebrafish (*Danio rerio*) homologs are underlined and the e-values of blastx searches are added in brackets.

concentrations were 0.4–0.6 μM. PCR was performed in duplicates (triplicates for E-curves) in 96-well optical plates on Light Cycler 480 (Roche Diagnostics). Running conditions were 5–10 min pre-incubation following 40 cycles of 95 °C for 5 s, 60 °C for 15 s, 72 °C for 15 s. Cycle threshold (C_T) values were calculated using both the fit points and second derivative methods (Roche Diagnostics), with respective rejection of C_T values above 37 and 35. The C_T values of test and reference genes from each individual were compared pairwise and the obtained ΔΔCt values were averaged. Statistics was calculated using Unistat version 5.5 (Unistat, London, UK). Difference between groups was analyzed with ANOVA with subsequent Newman–Keuls test ($p < 0.05$).

3. Results

3.1. Fish data

During the experiment, fish grew to an average final weight of 829 g. There were no significant differences in final weights among groups fed different levels of lentinan. Significant differences ($p = 0.03$) in crude protein digestibility were shown between fish fed 0.2% lentinan compared with 0.4% lentinan and control. Neither feed gross energy nor apparent digestibility for fat, starch, and ash differed between the groups.

3.2. Gene expression analyses

The main goal of this study was to reveal the lentinan-induced alterations associated with modifications of immunity. Comparison of the LPS-treated rainbow trout with saline-injected fish from the same study groups enabled us to subtract the effects that were not related to the responses to the endotoxin. Overall, 319 genes corresponded to the selection criteria (DE in at least 4 of 6 samples in L or C) and of these, 292 genes were significantly affected by LPS (File 1 in Supplementary data). Fig. 1 shows examples of genes with known functions based on gene ontology. To further compress data, we organized the average log-ER values for functionally related genes with highly correlated expression changes (Fig. 2). Microarray and qPCR analyses produced similar results for most genes (Fig. 3) except for one *CXC chemokine and B7-H1*; results for these genes are not shown. Such discrepancies are common for members of multi-gene families and can account for the limited ability of cDNA microarrays to discriminate between structurally similar paralogs. In rainbow trout this problem is aggravated with a large number of duplicate genes with different expression profiles.

L affected several genes encoding proteins with binding capacities and transporters of organic compounds (Figs. 1 and 3). *LBP (LPS binding protein)/BPI (bactericidal/permeability-increasing protein)* was down-regulated, especially in L, while several galectins were higher up-regulated in C compared to L. These highly conserved proteins bind to different ligands with preference to carbohydrates, and can be involved in a wide range of immune-related processes (reviewed in [28–30]). The L-induced changes were greatest in *albumin* (increase) and *ATP-binding cassette D3*, *ABCD3* (decrease). The mammalian ABCD3 encodes for a peroxisomal transporter of long acyl-CoA fatty acids [31]. Two other members of this multi-gene family (*ABCB2 and ABCF2*) were induced by LPS with no difference between groups (Fig. 3).

Transcriptomic studies of LPS-induced changes in mammalian macrophages [32], neutrophils [33], endothelial cells [34] and microglia [35] revealed massive induction of chemokines and cytokines, their receptors and cytokine activated genes, antibacterial factors, cytoskeleton components and proteins involved in responses to oxidative stress. These functional groups of genes also responded to LPS in our study. Differences between L and C groups were observed for IFN-α as well as for IFN- and TNF-dependent genes, including receptors, transducers and effector proteins (Figs. 1 and 3). The roles of putative fish homologs of TNF receptors (*TNFRSF6B and TNFRSF11B*) are unknown. Given the remarkable divergence between members of this superfamily [36], distant similarity to mammalian genes does not allow for an accurate prediction of function in phylogenetically remote species. C showed greater up-regulation of *JAK-1 and STAT-1 and -3* (Figs. 1 and 3), components of the pathway that plays a key role in IFN-signaling in a wide range of conditions, including stimulation with LPS [37]. Interestingly, this was in parallel with down-regulation of *MAP3K6* (Fig. 3), a kinase involved in activation of JNK, another crucial factor in the toll-like receptor (TLR) signaling pathway controlling activation of inflammatory cytokines. Decreased expression in L compared to C was also seen for a signal transducer *RRAGA*, a small GTPase with unknown function (Fig. 3). With respect to the cytokine-dependent proteins up-regulated by L (Figs. 1 and 3), *TNFAIP6* encodes a secretory protein with a hyaluronan-binding domain that takes part in cell–cell and cell–matrix interactions during inflammation [38]. Similarly up-regulated was *IFN-inducible protein 44 (IFI44)* (Fig. 1), which is known to be rapidly induced during viral infections and encodes an intracellular protein with anti-proliferative activity [39].

Microarray analyses showed effects of LPS on several cytokines and chemokines and receptors (Fig. 1). However, differences between study groups was only observed for *LECT2*, a cytokine characterized by extremely high sensitivity to bacterial infections in fish [40]. Responses to LPS were strongest for putative homologs to *SCYA106* (induction) and *SCYA110* (repression), two CC-type chemokines identified in channel catfish, *Ictalurus punctatus* [41]. qPCR results showed a pronounced inhibitory effect of L on *IL-1 receptor-like protein (IL1RLP)* (Fig. 3), a transducer of highly pro-inflammatory pathways regulated by IL-1 and IL-17.

LPS affected a number of genes involved in regulation of inflammation and anti-bacterial effectors. *Adenosine deaminase* and anti-microbial proteins (*lysozyme* and *cystein-rich protein*) were equally down-regulated in both groups (Fig. 1). Genes for two enzymes of eicosanoid metabolism, *PGDS* and *CYPIIJ2* or *arachidonic acid oxygenase* were down-regulated in both groups (Fig. 1), and qPCR analysis revealed significant differences between C and L for *PGDS* (Fig. 3). Decreased expression of *cytochrome P450 27*, which integrates retinoid, PPAR and LXR signaling [42], suggested suppression of a whole regulatory network based on retinoids, modified fatty acids and prostanoids in lentinan fed fish.

Up-regulation of *annexin-1* in L could imply decreased production of eicosanoids through inhibition of phospholipase A2 (Fig. 1). This enzyme releases arachidonic acid from cellular membranes, which is subsequently transformed to prostaglandins, thromboxane and leukotrienes, collectively termed eicosanoids [43].

Chemokines, cytokines and eicosanoids induce resident phagocytes and attract circulatory leukocytes. Annexins are known as negative regulators of neutrophilic recruitment [44]. L suppressed activation of genes for two enzymes of nicotineamide metabolism, which are also known as *pre-B cell enhancing factor* or *visfatin*, and *NAMPT* or *Integrin b-1-binding protein 3*, *ITGB1BP3* (Fig. 3), both involved in recruitment of leukocytes. L enhanced down-regulation of a leukocyte marker *CD9* or *motility-related protein* (Fig. 1). Respiratory burst in macrophages and neutrophils is one of the most important anti-bacterial responses. *Cytochrome B-245*, one of the major components of the ROS-producing system, was equally up-regulated together with *transaldolase* in both groups (Fig. 1). This enzyme of the pentose phosphate pathway generates flux of NADPH, which is rate-limiting for the mitochondrial production of ROS [45]. L enhanced LPS-induced up-regulation of the gene encoding *Ras-related C3 botulinum toxin substrate*, *RAC2* (Fig. 1). This small GTP-binding protein is a component of NADPH oxidase in phagocytic leukocytes, an enzymatic ROS-

Genes	Control	L
Binding and transport of ligands		
*Serum albumin precursor	-0,68 ± 0,24	1,04 ± 0,48
*Galectin like 2	1,40 ± 0,18	0,29 ± 0,23
*Galectin like 1	1,22 ± 0,14	0,44 ± 0,18
Galectin-3 binding protein	-0,58 ± 0,21	-1,16 ± 0,42
C type lectin receptor B	1,44 ± 0,39	0,86 ± 0,29
TNF and IFN-related		
*TNF receptor superfamily member 11B	2,39 ± 0,22	1,08 ± 0,45
TNF receptor superfamily member	0,87 ± 0,08	0,49 ± 0,21
*Tyrosine-protein kinase FRK	1,60 ± 0,36	0,07 ± 0,12
*Signal transducer and activator of transcription 1	1,28 ± 0,17	-0,16 ± 0,53
*Signal transducer and activator of transcription 3	1,11 ± 0,09	0,57 ± 0,25
*Interferon-inducible protein 44-2	2,80 ± 0,50	1,38 ± 0,39
Interferon-inducible protein 44-3	0,77 ± 0,05	0,50 ± 0,13
Interferon-inducible guanylate-binding protein 2	1,28 ± 0,12	1,10 ± 0,26
Interferon regulatory factor 1-1	0,73 ± 0,08	0,57 ± 0,11
Cytokines, chemokines and receptors, signal transducers		
*LECT2 neutrophil chemotactic factor	1,21 ± 0,08	0,58 ± 0,09
CCL4	0,63 ± 0,14	0,42 ± 0,18
CC chemokine SCYA110-2	-2,23 ± 0,24	-1,98 ± 0,47
CC chemokine SCYA106	3,52 ± 0,30	2,86 ± 0,56
High-mobility group box 1	2,04 ± 0,19	2,34 ± 0,40
Cytokine receptor common gamma chain	-0,28 ± 0,29	-0,80 ± 0,23
Receptor-interacting serine/threonine-protein kinase 2	0,54 ± 0,10	0,69 ± 0,14
Interleukin-1 receptor-like protein 2	0,46 ± 0,13	0,87 ± 0,29
Guanylate-binding protein	1,06 ± 0,23	1,03 ± 0,26
Cytokine inducible SH2-containing protein 5	-0,79 ± 0,02	-0,72 ± 0,04
Protein phosphatase 2C gamma isoform	0,49 ± 0,02	0,56 ± 0,08
Other inflammatory regulators, effectors		
*Annexin A1-2	0,10 ± 0,07	0,54 ± 0,13
Adenosine deaminase 3	-0,60 ± 0,22	-1,07 ± 0,18
Cytochrome P450 2J2	-0,04 ± 0,73	-1,09 ± 0,22
Cytochrome P450 27	-0,28 ± 0,27	-0,66 ± 0,16
*High affinity immunoglobulin gamma Fc receptor I	1,00 ± 0,24	-0,19 ± 0,36
*High affinity immunoglobulin epsilon receptor alpha	0,87 ± 0,12	0,35 ± 0,14
Cytochrome B-245 heavy chain-1	0,71 ± 0,11	0,64 ± 0,13
*Ras-related C3 botulinum toxin substrate 2	0,06 ± 0,04	0,70 ± 0,15
*Ornithine decarboxylase antizyme-2	-0,04 ± 0,07	-0,93 ± 0,29
Lysozyme g-3	-0,67 ± 0,22	-0,88 ± 0,35
Cysteine-rich protein 1	-0,98 ± 0,15	-0,86 ± 0,25

Genes	Control	L
Antigen presenttion		
*beta-2 microglobulin-1 BA1	0,68 ± 0,09	-0,16 ± 0,29
*Beta-2-microglobulin-1 JB1	0,81 ± 0,06	0,56 ± 0,12
MHC class 1b antigen	0,88 ± 0,13	0,47 ± 0,07
MHC class I antigen	0,57 ± 0,05	0,67 ± 0,08
*Lymphocyte antigen 75	0,77 ± 0,05	0,37 ± 0,09
MHCII alpha chain	-0,78 ± 0,10	-1,10 ± 0,28
*Transport-associated protein (TAP)	2,04 ± 0,16	0,76 ± 0,39
26S proteasome non-ATPase regulatory subunit	1,01 ± 0,33	0,68 ± 0,10
Proteasome activator complex subunit 2	0,84 ± 0,09	0,81 ± 0,15
*Proteasome subunit beta type 9 precursor	1,22 ± 0,09	0,63 ± 0,13
26S protease regulatory subunit 6B-1	0,64 ± 0,14	0,64 ± 0,17
Cathepsin B-1	0,62 ± 0,13	0,77 ± 0,11
Cathepsin C-3	-0,55 ± 0,09	-1,01 ± 0,22
Cathepsin D-2	0,87 ± 0,14	0,97 ± 0,14
Procathepsin L-1	1,07 ± 0,21	0,62 ± 0,17
Blood cells		
*CD9	-1,26 ± 0,19	-0,71 ± 0,17
Macrophage receptor MARCO	-0,69 ± 0,22	-0,87 ± 0,40
PTB domain adaptor protein CED-6	1,17 ± 0,10	0,94 ± 0,19
B-cell antigen receptor complex associated alpha	-1,66 ± 0,36	-1,30 ± 0,28
B-cell translocation gene 1-2	-0,59 ± 0,39	-1,07 ± 0,34
B-cell antigen receptor complex associated beta	-1,31 ± 0,19	-1,54 ± 0,27
DeltaB	-0,81 ± 0,07	-0,37 ± 0,12
Deltex protein 1	-0,78 ± 0,31	-0,56 ± 0,10
Notch1	-1,08 ± 0,19	-1,16 ± 0,25
Stomatin	1,12 ± 0,24	0,87 ± 0,15
Metabolism of xenobiotics and ROS, stress and apoptosis		
*Ah receptor interacting protein	0,74 ± 0,13	0,34 ± 0,11
*Aldehyde dehydrogenase 1A2	2,30 ± 0,25	1,04 ± 0,32
*ADP-ribosylation factor 4	1,36 ± 0,28	0,64 ± 0,13
Huntingtin	0,04 ± 0,12	-1,29 ± 0,27
Defender against cell death 1	0,51 ± 0,14	0,45 ± 0,15
Deoxyribonuclease II-1	-0,87 ± 0,11	-0,79 ± 0,27
Programmed cell death protein 6	0,38 ± 0,31	0,43 ± 0,19
Ferritin heavy chain-1	1,27 ± 0,11	0,49 ± 0,02

Fig. 1. Microarray gene expression in lentinan fed and control fish treated with LPS. Data are mean log-ER (expression ratio) ± SE from 6 individual spleen samples from each 0,2% lentinan fed fish (L) and control study group (C). Genes that were up- and down-regulated by LPS are highlighted with respectively orange and green scales. Yellow means no significant difference from zero. Significant difference between L and C is marked with *. See File 1 in Supplementary data for complete microarray data.

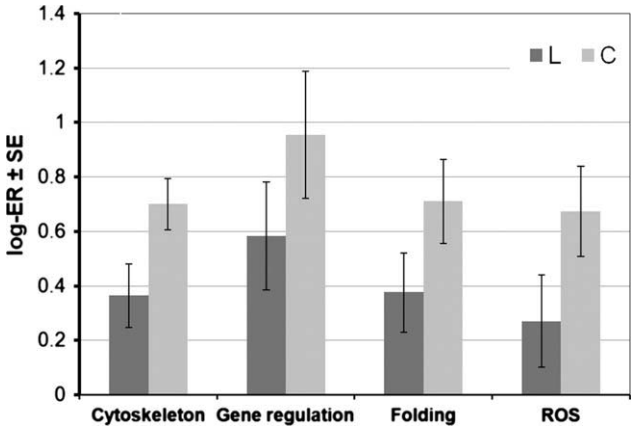

Fig. 2. Functionally related genes with co-ordinated expression. Data are log-ER ± SE; 0,2% lentinan fed fish (L) and control fish (C) are significantly different for all groups ($p < 0.05$). *Cytoskeleton* (*10 genes*): tubulin chain α-3 and 1, ubiquitous; cytokeratin 8-1 and 8-2, myristoylated alanine-rich protein kinase C substrate, ARP2/3 complex 34 kDa, cofilin, actins α and β. *Gene regulation* (*7 genes*): acidic leucine-rich nuclear phosphoprotein 32 A-2, splicing factor 3b, subunit 3, nucleolar protein NAP57, nucleophosmin 1, fibrillarin, histone H14, nucleolar protein Nop56-2. *Protein folding and modification* (*22 genes*): heat shock protein (hsp) 90 kDa-β 1 and 2, hsp 75 kDa, hsp 70 kDa, hsp 70 kDa 9B-2 and 8, hsp 60 kDa 1 and 2, hsp cognate 70 kDa, hsp cognate 40 kDa, AHA1, T-complex protein 1, subunits 1, 2 and 5, DnaJ homologs A2, B11 and C3, 78 kDa and 94 kDa glucose-regulated proteins, 14-3-3C2, peptidyl-prolyl cis-trans isomerase 9, stress 70 protein chaperone. *Responses to oxidative stress* (*ROS*) (*9 genes*): thioredoxin and peroxiredoxin 1, transaldolase, selenoproteins15 kDa and P, [Cu–Zn] superoxide dismutase and catalase, glutathione peroxidase and reductase. See File 1 in Supplementary data for complete microarray data.

generating complex [46]. *RAC2* is also essential for differentiation of T cells [47] and myeloid cells [48]. A number of genes for proteins involved in responses to oxidative stress were up-regulated with LPS while only one scavenger of ROS, an extracellular heparin-binding *selenoprotein P* was down-regulated (Fig. 2). *Catalase* and *mitochondrial glutathione reductase* were enhanced greater in C. Lentinan decreased expression of putative *ornithine decarboxylase antizyme* and non-hepatic *ARG2*, but not *ARG1* (Figs. 1 and 3). Previous studies have shown high arginase activity in the inflammatory milieu [49]. Arginases convert L-arginine to L-ornithine, leading to the synthesis of polyamines via ODC. Alternatively, arginine can be utilised by nitric oxide synthase (NOS), an enzyme responsible for the synthesis of nitric oxide (NO) with roles in the control of blood pressure and cellular defence mechanisms. ARG1, once thought to be liver-specific, may have an important role in generating polyamines essential for cell proliferation and matrix production in inflammation [50].

LPS activates antigen presenting cells (APC), which initiate the development of adaptive immune responses [51]. We observed up-regulation of genes involved in antigen presentation, including *MHC class I antigens*, components of proteasome, *b-2-microglobulin* (*B2M*), *transport associated protein* (*TAP*) and *TAP-binding protein*, *tapasin (TAPBP)* (Figs. 1 and 3). L significantly reduced the increase of *TAP* and one of the two *B2M* genes. Despite apparent activation of antigen presentation, *CD8a*, a marker of cytotoxic T cells, markedly decreased in both groups as well as *IL-12b*, which plays a key part in the differentiation of naive T cells into Th1 cells (Fig. 3). Down-regulation of *MHC class II* was in line with decreased expression of B cell-associated genes including *CD37* and regulators of hematopoiesis (Figs. 1 and 3).

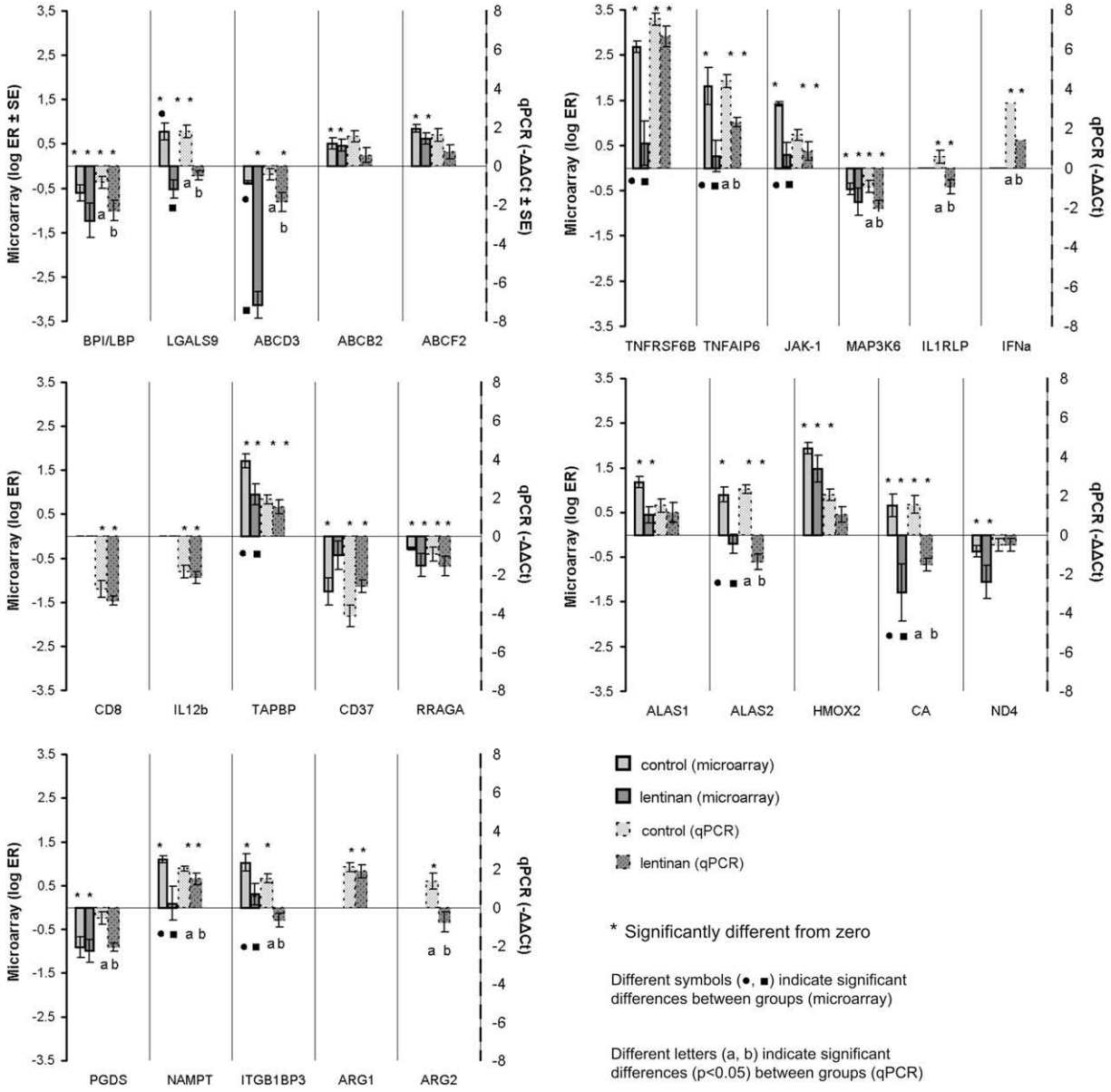

Fig. 3. Real-time qPCR versus microarray analyses. Data for qPCR are ΔΔCt ± SE of 6 LPS-challenged individuals versus 6 saline-injected and data for microarray mean log-ER ± SE of individual spleen samples ($n = 6$). Genes are: LPS binding protein (BPI/LBP), lectin, galactose binding, soluble 9 (VHSV-induced protein-9) (LGALS9); ATP-binding protein D3 (ABCD3); ATP-binding cassette transporter 2 (ABCB2); ATP-binding cassette, sub-family F member 2 (ABCF2); TNF decoy receptor (TNFRSF6B); TNF-inducible protein TSG-6 precursor (TNF- stimulated gene 6 protein) (TNFAIP6); Tyrosine-protein kinase Jak1 (JAK-1); MAPK/ERK kinase kinase 6 (MAP3K6); interleukin-1 receptor-like protein (IL1RLP); interferon α (IFN-α); CD8; interleukin-12b (IL-12β); tapasin, TAP-binding protein (TAPBP); leukocyte antigen CD37 (CD37); similar to GTP-binding protein rag A, Ras-related GTP-binding A (RRAGA); aminolevulinate, delta-, synthetase 1-2 (ALAS1); 5-aminolevulinate synthase (ALAS2); heme oxygenase-2 (HMOX2); carbonic anhydrase (CA); NADH dehydrogenase subunit 4 (ND4); prostaglandine D synthase (PGDS); pre-B cell enhancing factor, nicotinamide phosphoribosyltransferase (NAMPT); muscle-specific β1 integrin binding protein 2 (ITGB1BP3), arginase 1 (ARG1); arginase 2 (ARG2).

In addition to inflammatory responses, L reduced effect of LPS on several functional groups including cytoskeleton, protein folding and regulation of gene expression (Fig. 2). Similar changes were seen in genes for proteins involved in metabolism of xenobiotics (*aldehyde dehydrogenase 1A2 and Ah receptor-interacting protein*) and iron (Figs. 1 and 3). Microarray analyses revealed no difference between the study groups in the up-regulation of *HMOX2*, whereas LPS-induced expression of this gene in L was not statistically significant as measured by qPCR (Fig. 3). On the other hand, effects of LPS on *ferritin* (intracellular storage of iron) were greater in C (Fig. 1) while L changed the expression level of the erythroid-specific isoform of *5-aminolevulinate synthase (ALAS2)* in opposite direction (Fig. 3). In contrast, non-specific mitochondrial *ALAS1* was equally up-regulated in both groups.

4. Discussion

The goal of this study was to examine the impact of lentinan on the immune system of rainbow trout and to develop an approach for selection of candidate IS. We could anticipate immune effects of lentinan but efficient doses were unknown. Doses of glucans applied as feed additives commonly range from 0.09–0.1% [3,6,7] to 1% kg^{-1} diet [52]. We preferred to begin with intermediate high (0.2%) and high (0.4%) and the former had greater impact. Inhibitory effects of high IS doses were discussed earlier, e.g. [3,53]. Digestibility of lentinan in fish is unknown and overall knowledge on absorption and pharmacokinetics of orally administered soluble glucans is limited. However, Rice et al. [54] demonstrated that intestinal epithelial cells actively translocate fungal-derived soluble

glucans into the systemic circulation. Furthermore, the effects of orally administrated glucans on immunity suggest their entry into circulation. Since IS are used to modify responses to infections, their effects can be difficult to evaluate unless the organism is challenged with active or protracted pathogens. Therefore we assessed the effects of lentinan in rainbow trout exposed to the bacterial antigen LPS, a classical inducer of inflammation. IS are expected to enhance beneficial immune responses and down-regulate those that can be detrimental to the host. In this respect, LPS appears to be an appropriate model. Responses to LPS are similar to those caused by pathogenic bacteria. Consequently, the character and magnitude of regulation of immune functions are expected to correlate with the ability to combat infections and to provide an insight into the protective mechanisms. However, excessive responses to LPS develop pathologies with risk of septic shock and organ failure. Though tolerance to LPS in fish is much greater than in mammals and sepsis does not develop even at doses that are lethal to higher vertebrates, a number of adverse effects take place. We chose the spleen for gene expression analyses due to its high responsiveness to LPS and its crucial importance for the fish immune system. Cellular responses to LPS are initiated with binding and transport. Detection of antigen switches on signal transduction, which in turn activates immune effector mechanisms and cell communication via cytokines and chemokines. These processes as well as interactions with humoral and cellular immune factors affect diverse functions and cellular processes, including migration and differentiation of immune cells. We observed responses to LPS at different levels and a substantial part of these was modified by lentinan.

Sensing of LPS is initiated by binding to circulatory LBP [55] and in this respect it is noteworthy to mention that expression of *BPI/LBP* was more strongly down-regulated in L than in C. A complex of LBP and CD14 activates TLR4 [56], which is believed to play a pivotal role in LPS signaling in mammals. The marked differences in the LPS sensing between salmonid fishes and mammals can be due to the absence and/or functional differences of TLR4, which has not been identified to date [57] and accessory molecules including MD2, CD14, TRAM/TICAM2 and LBP. In principle, the role of TLR4 in fish cells can be taken by another protein or proteins which bind to endotoxin and activate signal transduction. Down-regulation of *BPI/LBP* coincided with a marked difference in expression of serum *albumin* between C and L. Increased expression of this multifunctional protein in L could have several beneficial consequences. Albumin is the most abundant circulating scavenger of free oxygen radicals, also known to inhibit iron-induced lipid peroxidation [58]. Moreover, increased expression of albumin in L could enhance neutralization of LPS and its delivery to removal sites, thus reducing loading on cells [56,57]. We also found changes in genes for transporters and carbohydrate-binding proteins, whose functions are not well known in fish. However, our results from other studies suggest that these genes are unlikely to be involved in direct interactions with LPS. For example, *ALD3* was down-regulated in spleen of Atlantic salmon infected with infectious salmon anemia virus (ISAV), while *galectins* were markedly induced across different tissues [22]. The role of ALD3 in responses to pathogens is unknown, however for galectins, several studies have reported their regulation by IFN in mammalian cells (e.g. [59]). A possible dependence of galectins on IFN in salmonid fish may explain their similar responses to ISAV and LPS.

In mammals, TNFα and type I IFN are known to play a key part in LPS signaling and their contribution varies in different cell types. Almost identical effects of LPS and TNFα are reported on endothelial cells [34], while the role of type I IFN was minor in human neutrophils [33]. However, in most LPS-responsive cells a large part of effects is accounted for by the MyD88-independent pathway, which is under control of type I IFN. The results of gene expression analyses suggested that L could down-regulate both pathways in LPS-treated trout. Expression of *IFN-a*, the only known type I IFN in salmonid fish, was significantly higher in C in comparison with L. Similar differences were observed for two *STAT* genes, major transducers of IFN-signaling, and for *IFI44* in addition to several other IFN-dependent genes. Similarly, a panel of *TNF receptors* and TNF-dependent genes were more strongly up-regulated in C than in L. The inflammatory roles of TNFα and type I IFN pathways have not been sufficiently clarified in fish. However, in mammals, combination of LPS and TNFα is considered a major risk factor at exposure to endotoxin [60]. Interestingly, in IFN-β knockout mice, mortality caused by LPS decreased dramatically [61] or was completely eliminated [62].

Not all L-induced effects could be simply explained since many genes could be involved in different processes. Furthermore, expression changes that arise as an indirect result of changes in regulatory networks may not necessarily have functional consequences. For example, STAT-mediated dependence of B2M and TAP on IFN [63] may explain the apparent contradiction between up-regulation of these genes and marked reduction of *CD8a* and *IL-12b*. Nonetheless, a number of observations, albeit inconclusive, support a favourable effect of lentinan. Development of the inflammatory status is associated with recruitment of leukocytes, which together with elimination of pathogens and infected cells cause heavy damages to tissues. In our study, lentinan modified responses of several genes, which together with other functions regulate migration of leukocytes. Cytoskeleton proteins required for cell motility and antigen presentation also showed a weaker response to LPS in L versus C (Fig. 2). These changes can be an evidence for reduced inflammatory responses in lentinan fed fish. However, some aspects of the immune response may have been enhanced in this group. Since ARG2 and NOS share a common substrate, down-regulation of *ARG2*, has the potential to instigate inflammation by shifting arginine metabolism toward NO synthesis at the cost of polyamine production.

Another example of a group involved in diverse processes is genes encoding proteins of iron metabolism. Modification of iron metabolism is important for protection against both microbes and oxidative stress. Decrease of bioavailable iron within cells and in body fluids retards bacterial growth and is referred to as the iron-withholding strategy in innate immunity (reviewed in [63–65]). LPS stimulated *ferritin*, the intracellular iron storage protein, and the induction was stronger in C. Up-regulation of *ferritin* could also be explained with a need for sequestering of free iron, which catalyzes production of ROS through Fenton's reaction. Heme released from the damaged erythrocytes needs to be rapidly removed since it also induces production of free radicals, which can be used by bacteria. Splenic expression of *HMOX2*, a gene for the enzyme catalyzing the degradation of heme into biliverdin, iron and carbon monoxide, was up-regulated in all study groups. In mammals, this gene is induced by LPS, IFN and TNF [64,65]. In addition to degrading toxic heme by HMOX2 into free iron, it is noteworthy that biliverdin may have afforded cytoprotection due to its potent antioxidant properties [64]. To maintain heme at constant levels, degradation should be compensated with biosynthesis. However, up-regulation of *ALAS2*, the key enzyme of heme biosynthesis in erythrocytes, was observed only in C. *ALAS2* and *carbonic anhydrase (CA)* displayed matching expression profiles, characterized by opposite regulation in C and L. In theory, up-regulation of *CA*, the enzyme catalyzing reversible hydration of carbon dioxide, could be evidence for enhanced differentiation of red blood cells in C. However, our results from a large number of other microarray experiments show that in salmonid fish, *CA* is tightly co-regulated with other genes involved in metabolism of iron and heme. Furthermore, equal increase in *stomatin*, an integral protein of erythrocyte membranes, and decrease of several

regulators of hematopoiesis (*deltaB, deltex and notch*), suggested that lentinan did not exert any negative impact on this process (Fig. 1). Changes in genes involved in energy metabolism were also equal in both study groups. We believe that regulation of iron metabolism in C was needed principally for protection against oxidative stress, a notion supported by stronger up-regulation of ROS scavengers in C. This group included genes encoding proteins with well-known protective roles. Induction of *superoxide dismutase* and *catalase*, enzymes that destroy free radicals, has been reported in many studies and appears as one of the most characteristic features of responses to LPS. L also reduced induction of several genes involved in metabolism of xenobiotics, including *Ah receptor-interacting protein*, which is known for its high sensitivity to both LPS and dioxins [65]. Another evidence for reduced stress in L was the stronger response in C observed for genes involved in protein folding.

Results from this study suggested that lentinan prevented acute and potentially dangerous effects of LPS but did not suppress inflammatory responses in general. Importantly, a large number of genes implicated in various immune functions (lectins, cytokines and receptors, signal transducers and anti-bacterial proteins) showed equal changes in L and C. Selective modulation of immunity is an expected action of IS. Obviously more studies will be needed to assess lentinan as a feed additive. Effects of glucans may vary depending on doses, duration of feeding, season and fish species and this may explain seeming contradiction between published results. For example, increase of complement activity was observed in glucan fed hybrid tilapia *Oreochromis niloticus x Oreochromis mossambicus* [8] and sea bass *Dicentrarchus labrax* [4] but not in croaker *Pseudosciaena crocea* [3], Asian catfish *Clarias batrachus* [6] and snapper *Pagrus auratus* [5]. Responses to LPS may also vary substantially with respect to time after challenge. A definite conclusion on the beneficial effects of IS presumes changes in survival and/or performance in fish, and this cannot be obtained through challenge models with inflammatory agents. LPS as well as other compounds with PAMPs does not kill salmonid fish even at high doses, and rapid resolution of inflammation does not allow for detection of changes in nutrition and growth. However, an important advantage of microarrays is the possibility to compare gene expression changes and profiles between different experiments. Our recent study on ISAV-infected salmon focused on differences between individuals with early and late mortality (EM and LM); time of survival was used as a measure of susceptibility and resistance [22]. Interestingly, a number of changes observed in L were identical to those associated with sustained survival in virus-infected fish (File 2 in Supplementary data). These included e.g. lower expression levels of *galectins*, IFN-dependent genes, genes involved in antigen presentation, immune cell communication and cellular stress, while only *ALD3* was down-regulated in both studies.

5. Conclusions

In our view, results of this study justify the use of global gene expression analysis for screening of candidate IS. From previous studies, effects of lentinan on the immune system of rainbow trout could be anticipated but lacked evidence which would be difficult to obtain without high-throughput methods. Microarray analyses showed that lentinan could modify potentially dangerous effects of LPS, i.e. activation of TNF- and IFN-signaling, oxidative and cellular stress, while major parts of the immune response remained unchanged. A general drawback with models based on treatment with PAMPs for evaluating effects of IS, is the lack of endpoints needed for definite conclusions. We resolved this by comparing data with expression profiles from studies with active pathogens, which revealed that changes induced by lentinan could be associated with improved protection. Additional studies will be required to clarify the optimum doses and duration of feeding with lentinan.

Acknowledgements

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Appendix. Supplementary data

Supplementary material associated with this article ca be found in the online version, at doi:10.1016/j.fsi.2008.10.012.

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Paper IV

Gene expression profiling reveals similar hepatic responses to restricted feeding and extracted soybean meal in diets for Atlantic salmon (*Salmo salar* L.)

Stanko Skugor[*1,2], Barbara Grisdale-Helland[1,3], Ståle Refstie[1,3], Sergey Afanasyev[1,4], Jouni Vielma[5], Aleksei Krasnov[1]

[1]Nofima Marin, P.O.B. 5010, 1432 Ås, Norway

[2]Department of Animal and Aquaculture Sciences, Norwegian University of Life Sciences (UMB), P.O. Box 5003, Ås 1432, Norway

[3]Aquaculture Protein Centre, CoE, Norway

[4]Sechenov Institute of Evolutionary Physiology and Biochemistry, St Petersburg 194223, Russia

[5]Finnish Game and Fisheries Institute, P.O.B2 00791 Helsinki, Finland

[*]Corresponding author to who reprints requests should be sent

Running head: Transcriptomic effects of restricted feeding and soy

Key words: Atlantic salmon, growth, soy, reduced ratio, liver, microarray

Abstract

We assessed gene expression analyses for studies and diagnostics of nutritionally-induced growth reduction in Atlantic salmon. The four experimental treatments consisted of feeding a fishmeal-based control diet or one including 20% extracted soybean meal, at full or reduced rations for 54 days. Compared with the fish fed the control diet at full ration, the thermal growth coefficient decreased in the group fed with the control diet at reduced ratio by 51%, the soy diet at full ration by 22.2% and the soy diet at reduced ration by 67%. The hepatic gene expression changes were analyzed with cDNA microarray and qPCR. Transcriptomic responses to soy and reduced feeding were highly similar, consistently with the decreased intake of the soy diet. Unexpectedly, joint treatment produced smaller expression changes than single treatments. A search across our gene expression database showed 12 genes with greater regulation in this experiment than in other studies that used the same microarray platform. These included four up-regulated ribosomal proteins and four down-regulated genes for lipid and steroid metabolism. Growth reduction was associated with coordinated down-regulation of genes involved in oxidative and cellular stress responses, xenobiotic metabolism and protein degradation. To conclude, this pilot study revealed significant hepatic gene expression changes induced with nutrition. The responses weakened at the most severe underfeeding however, and the detection of specific markers was problematic. High expression of stress-related genes in the control group suggests that maximum growth rates can be associated with health problems in salmon.

Introduction

Success of commercial Atlantic salmon aquaculture presumes production of large size fish in relatively short time periods. Rapid growth of fish is essential; retardation of growth causes heavy economical losses. Growth rate is a highly complex trait, determined with a combination of genetic merit, health and physiological status of fish, multiple environmental factors and nutrition. Reduced growth rates of farmed fish are observed periodically and often remain unexplained (1). Aquaculture requires tools to assess possible reasons for decreases in growth rates and this needs comparative studies of responses to different stressors. We report results of a pilot experiment that assessed hepatic gene expression profiling in order to study the growth decrease caused by nutritional interventions: restricted feeding, soy-based diet and a combination of these treatments.

Growth rate is negatively affected by food deprivation at all levels of reduction. In mammals and birds, restricted feeding rapidly initiates metabolic changes similar to those caused by fasting (2,3) and (4). A cause-and-effect relationship between gene expression and positive health and lifespan effects of restricted feeding has been established (5, 6). Ectotherms are characterized with much greater tolerance to nutrient deprivation than mammals and birds (4), and even protracted periods of complete food absence do not cause irreversible changes in teleost fish, e.g. (7). Compared with other vertebrates, the severity of nutrient deprivation required for the initiation of adaptive responses in fish is substantially higher, but responses are similar (8, 9, 4).

Soybean meals are extensively used as substitutes for high-quality fish meal in feeds for aquaculture species. The replacement of fish meal with soy products however, causes adverse effects on growth and nutrient retention. The amino acid profile of soy protein is well balanced, except for a low level of methionine (10). The extent of negative effects depends on the purity of the soy protein, level of inclusion and treatment applied for neutralization of anti-nutritional factors (11, 12). Soy products may contain compounds that interfere with the digestive processes in fish, including phytic acid, proteinase inhibitors, agglutinin lectins, oligosaccharides, non-starch polysaccharides and isoflavones (12). High levels of soy decrease appetite and feed intake, which can be due to the inferior palatability or intestinal dysfunctions (13, 14).

We used the salmonid fish cDNA microarray SFA2 (15, 16) for screening and selection of genes, which were further analyzed with real-time qPCR. The study addressed the questions*:* is it possible to find gene expression changes that are specific for the growth reducing nutritional interventions, and are transcriptomic responses to restricted feeding and soy protein similar?

Materials and Methods

Diets and feeding trial

Two iso-nitrogenous, extruded diets, a control diet based on fish meal, and a soybean meal diet containing 43% fish meal and 20% extracted soybean meal, were manufactured by Nofima Ingrediens (Fyllingsdalen, Bergen, Norway) (Table 1). The feed particle size was 3 mm. The diets were analyzed for dry matter (DM) (105 °C, until constant weight), crude protein (N x 6.25; Kjeltec Auto System, Tecator, Höganäs, Sweden), crude lipid after HCl hydrolysis (Soxtec HT6; Tecator, Höganäs, Sweden) and ash (550 °C, overnight).

Two months before the start of the trial, Atlantic salmon (*Salmo salar* L.) were individually tagged (Passive Integrated Transponder, Trovan Ltd., U.K.). At the start of the trial, the fish were fasted for 2 days, individually weighed (297 ± 28 g, mean ± S.D.), and then groups of 59 fish were randomly distributed to four 1-m^2 tanks. The tanks were supplied with seawater (11.7 ± 1.2 °C) and were designed to accommodate collection of waste feed from the effluent water in wire mesh boxes. The fish were held under continuous light and were fed using automatic band-feeders for 54 days. Two tanks of fish were fed either the control or soy diet, in excess. The two other tanks of fish were fed rations at levels approximating 40% of the intake of the full-fed group for each diet. The waste feed was collected daily, and approximate feed intake for each tank was calculated by taking into account the waste feed level and the percentage recovery of dry matter from the diet in the system (17). The rations were adjusted every 3 days based on intake level. After the trial, the waste feed was analyzed for DM content and actual feed intake for each tank was recalculated. The tanks were checked daily for dead fish. The oxygen saturation level for all tanks was maintained over 85%. Water temperature was measured daily. At the end of the growth trial, the fish were fasted for 2 days and then individually weighed. After 12 further days of feeding and a 2-day fast, individuals with growth rates similar to the average within their respective group were selected for gene expression analyses. The study groups were designated as CF (control diet, full ration), CR (control diet, reduced ration), SF (soy diet, full ration) and SR (soy diet, reduced ration).

Calculations

Relative feed intake (RFI), % body weight (BW) per day: 100 X (dry feed intake (FI)) X $((BW_0 + BW_1)/2)^{-1}$ X $(\text{days fed})^{-1}$, where BW_0 and BW_1 are initial and final body weights, respectively.
Thermal growth coefficient (TGC): 1000 X $(BW_1^{1/3} - BW_0^{1/3})$ X $(\sum T)^{-1}$, where $\sum T$ is the sum day-degrees Celsius (18).
Feed efficiency ratio (FER): wet BW gain X FI^{-1}.

Microarrays

The salmonid fish microarray (SFA2) includes 1800 unique clones printed each in six spot replicates. The complete composition of the platform and sequences of genes are provided in submission to NCBI GEO Omnibus (GPL6154). Total RNA from tissue was extracted with TRIzol and purified with Pure Link (Invitrogen, Carlsbad, CA, USA). The liver was chosen based on the results of pilot hybridizations with different tissues. Individual samples from SF, CR and SR (5 fish from each group; one microarray per fish) were hybridized to CF (pooled control; equal contribution from 6 fish). Test and control RNA (20 µg in each sample) were labeled with Cy5-dUTP and Cy3-dUTP (GE Healthcare, Sweden), respectively. The fluorescent dyes were incorporated in cDNA using the SuperScript™ Indirect cDNA Labeling System (Invitrogen, Carlsbad, CA, USA). The cDNA synthesis was performed at 46°C for 3 hours in a 20 µl reaction volume, followed by RNA degradation with 0.2M NaOH at 37°C for 15 min and alkaline neutralization with 0.6 M Hepes. Labeled cDNA was purified with Microcon YM30 (Millipore, Bedford, MA, USA). The slides were pretreated with 1% BSA fraction V, 5 × SSC, 0.1% SDS (30 min at 50°C), washed with 2 × SSC (3 min) and 0.2 × SSC (3 min) and hybridized overnight at 60°C in a cocktail containing 1.3 × Denhardt's, 3 × SSC, 0.3% SDS, 0.67 µg/µl polyadenylate and 1.4 µg/µl yeast tRNA. After hybridization, slides were washed at room temperature in 0.5 x SSC and 0.1% SDS (15 min), 0.5 x SSC and 0.01% SDS (15 min), and twice in 0.06 x SSC (2 and 1 min, respectively). Scanning was performed with GenePix4100A and images were processed with GenePix 6.0 (Molecular Devices, Sunnyvale, CA, USA). The spots were filtered by criterion $(I-B)/(SI+SB) \geq 0.6$, where I and B are the mean signal and background intensities and SI and SB are the standard deviations. Low quality spots were excluded from analysis and genes presented with less than three high quality spots on a slide were discarded. After subtraction of median background from median signal intensities, the

log_2-expression ratios were calculated. Lowess normalization was performed first for the whole slide and next for twelve rows and four columns per slide. Statistical analyses included two stages, assessing both technical errors and biological variation. First, differential expression was evaluated in each sample by difference from zero of the mean log_2-expression ratio (6 spot replicates per gene, Student's t-test, $p<0.01$). Each gene with a technically significant difference from reference in more than half of analyzed individuals, in at least one of the study groups, was selected. Next, biological variation was analyzed. Differences between groups were assessed with ANOVA followed with Newman-Keuls test ($p < 0.05$). The groups of functionally related genes with similar changes were represented with mean log_2-expression ratio values. To find genes with preferential responses to feeding stress, we compared proportions of samples with differential expression in the present study with results from 185 experiments in our gene expression database. Differences were assessed with Fisher's exact probability ($p < 0.05$). The complete microarray data are provided in the supplementary file.

Quantitative real time PCR

The cDNA synthesis was performed on 0.5 µg DNAse-treated total RNA (Turbo DNA-freeTM, Ambion, Austin, TX, USA) using TaqMan® Gold Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) and oligo dT primers. PCR primers were designed using Vector NTI (Invitrogen) and synthesized by Invitrogen. The amplicon lengths set to be between 50 and 200 bases were checked on 1.5% agarose gel. PCR efficiency was calculated from tenfold serial dilutions of cDNA for each primer pair in triplicates. Real-time PCR assays were conducted using 2X SYBR® Green Master Mix (Roche Diagnostics, Mannheim, Germany) in an optimized 12 µl reaction volume, using 1:10 diluted cDNA, with primer concentrations of 0.4-0.6 µM. PCR was performed in duplicate in 96-well optical plates on Light Cycler 480 (Roche Diagnostics, Mannheim, Germany) under the following conditions: 95°C for 5 min (pre-incubation), 95°C for 5 sec, 60°C for 15 sec, 72°C for 15 sec (amplification), 95°C for 5 sec, and 65°C for 1 min (melting curve). 45 cycles were performed. Relative expression of mRNA was evaluated by ΔΔCT. Geometric average of eight reference genes selected by results of microarray analyses (18S rRNA, RNA polymerase II, eukaryotic translation initiation factor 3 subunit 6, NADH dehydrogenase 1 alpha subcomplex subunit 8, 70 kDa peroxisomal membrane protein, prostaglandine D synthase

and NADH dehydrogenase subunit 4, elongation factor 1 alpha) was used. Results were analyzed with ANOVA followed by Duncan's and Dunnett's test ($p < 0.05$).

Results

Fish fed the control diet *ad libitum* had almost doubled in weight and had the highest growth rates (TGC, 2.43), followed by those fed the soybean meal diet at full ration (1.89) (Fig. 1; Table 2). The 64% reduction in feed intake reduced growth rates by 51% in the CR group. Compared with CF, the combination of both the soy diet and the reduced ration decreased TGC in the fish by 67%. Feed efficiency was highest in the CR group (1.36) and lowest in the SF group (1.07).

Microarray analyses revealed high consistency and similarity of the transcriptomic changes in all analyzed fish. To compare the biological replicates we selected 484 genes with significant changes in at least one study group. Correlation of the expression profiles was strong within CR and SF (mean Pearson r was 0.76 and 0.71, respectively), being close to correlations between the groups (mean r was 0.68, Fig. 2A). Higher variation within SR might be related to a markedly smaller number of differentially expressed genes (Fig. 2B). Many genes that responded to single treatments were insensitive to the combination of treatments, and close to zero values increased noise. Correlations by 183 genes with significant changes in all three groups were similar in all study groups (Fig. 2A). The transcriptomic responses to restricted feeding and soy were comparable by magnitude. Genes changed expression in the same direction, either up- or down-regulation in all study groups, and we did not find genes with markedly different responses to the treatments (Fig. 3,4). Several functional groups showed coordinated expression changes (Fig. 4). The mean expression changes in these groups were relatively small but highly significant due to strong correlation and low variance of expression profiles; most of them showed no differences between the treatments. The qPCR analyses of 20 genes were carried out to verify the microarray results (Fig. 5) and to include genes not represented on the SFA2 platform (Fig. 6). qPCR showed the decrease of expression changes in genes of the fish fed the soy diet at the low ration level. In general, both methods produced similar results except for hepcidin (HPX) and glutathione S-transferase theta 1 (GSTT1). There was a tendency for greater variation in the qPCR data, especially in the SF fish and the geometric mean of eight reference genes was used to stabilize the results. Higher stability of microarray results is most likely accounted for by the global normalization that compensates for fluctuations of individual genes.

To search for candidate markers we used our gene expression database; SR was excluded due to small expression changes. Significantly higher probability of responses to feeding stress in comparison with other treatments was found in 12 genes (Table 4). The list included four up-regulated ribosomal proteins, five down-regulated genes encoding proteins involved in steroid and lipid metabolism, adenosine kinase, an enzyme of nucleotide metabolism, an antimicrobial peptide with predicted immune functions and stress responsive N-myc downstream regulated protein. All these functional groups showed responses to the nutritional interventions (Fig. 3-5). Four of 12 genes were analyzed with microarrays and real time qPCR with satisfactory concordance between the two methods (Fig. 5).

Ribosomal proteins were the only functional group whose up-regulation significantly increased with the severity of feeding stress (Fig. 4). The changes in the translational machinery coincided with slight increases in several proteasome components and down-regulation of lysosomal proteases. High density lipoprotein binding protein or vigilin, HDLBP, which is found in the nucleus and cytoplasm of all eukaryotic cells, can be implicated in both protein biosynthesis and metabolism of lipids and steroids. HDLBP has high affinity for RNA and elevated expression levels in cells with increased translational activity (19). Besides, HDLBP protects cells from excessive accumulation of cholesterol (20). In our study, down-regulation of HDLBP (Fig. 5) was in line with the decreased expression of a suite of genes involved in various aspects of lipid and steroid metabolism (Fig. 3, 5). Liver is a major site of steroid hormone catabolism and bile acid production. Very-long-chain acyl-CoA synthetase (VLACS), which takes part in lipid and possibly bile acid metabolism (21) and two more genes with roles in these pathways, an aldo-keto reductase (AKR) and 3-oxo-5-beta-steroid 4-dehydrogenase (AKR1D1), and 17ß-hydroxysteroid dehydrogenase 4 (HSD17B4), were down-regulated (Fig. 5). HSD17B4, a dehydrogenase/reductase family member has also important functions in the modification of fatty and bile acids (see references in (22)). AKR1D1 is involved in the degradation of steroid hormones and plays an important role in bile acid biosynthesis (23). All interventions suppressed steroid receptor membrane associated progesterone receptor component 2 (PGRMC2). Reduced expression was also found in genes encoding two enzymes of eicosanoid metabolism, cytochrome P450 2K4 (CYP2W1), which metabolizes arachidonic acid, and CYPIVF3 or leukotriene-B(4) omega-hydroxylase (Fig. 4). In contrast, arachidonate 5-lipoxygenase (ALOX5), which transforms fatty acids in leukotrienes was up-regulated. Microarray analyses found decreases

in a number of genes involved in transport and modification of lipids and cholesterol. Apolipoprotein A-IV (APOA4) takes part in chylomicron metabolism; whereas, lysosomal acid lipase/cholesteryl ester hydrolase (LIPA) is associated with the uptake of lipoprotein particles and following lysosomal lipolysis and cholesterol transformation to bile acids (24).

Changes were seen in a number of other metabolic pathways. Genes encoding proteins involved in the mitochondrial electron transport and oxidative phosphorylation were up-regulated in all study groups (Fig. 4). Adenosine kinase (decreased in all groups, Fig. 3) phosphorylates adenosine to AMP, thus regulating concentrations of extracellular adenosine and intracellular adenine nucleotides. Several more genes for enzymes of nucleotide metabolism were either slightly up-regulated (ectonucleoside triphosphate diphosphohydrolase and adenosine deaminase) or down-regulated (beta-ureidopropionase). Liver plays a key role in the regulation of glucose homeostasis and as expected, genes involved in glucose metabolism were affected. Three glycolytic genes were down-regulated (Fig. 3); whereas, transcripts encoding two key gluconeogenic enzymes showed bi-directional responses. The greatest difference between glucose-6-phosphatase (G6PC) (up-regulated) and fructose-1,6-bisphosphatase isozyme (FBP2) (down-regulated) was seen in SF fish.
Down-regulation of UDP-glucuronosyltransferase (UGT1A8) may be associated with a decrease in steroid metabolism. This enzyme transforms small lipophilic compounds, such as steroids, bilirubin and hormones, into water-soluble, excretable metabolites (25). Besides, glucuronidation is essential for the detoxification and excretion of exogenous compounds. It is noteworthy that several more genes involved in detoxification were down-regulated (Fig. 4). This group included alcohol dehydrogenase (NADP+) that is involved in the reduction of biogenic and xenobiotic aldehydes (26). Predicted attenuation of xenobiotic metabolism was in parallel with down-regulation of genes involved in protection against reactive oxygen species (ROS). In addition, there was a trend towards reduced expression of genes involved in homeostasis of iron, and biosynthesis of heme and heme-containing proteins, including hemoglobins and catalase. Three genes from this group were included in qPCR analyses (Fig. 5). Carbonic anhydrase catalyses reversible hydration of carbon dioxide. In our studies, this gene has consistently shown high correlation with hemoglobins. Hemopexin encodes an acute phase protein that takes part in heme trafficking to the liver and is involved in protection from oxidative stress. The heme-containing enzyme catalase degrades hydrogen peroxide, thus being involved in metabolism of ROS. The down-regulation of ROS scavengers was in parallel

with decreased expression of 12 genes encoding chaperones from several multi-gene families. Notably, this group included 94 kDa glucose-regulated protein (GRP94), a marker of unfolded protein response that senses protein overload in the endoplasmic reticulum.

Microarray results suggested that nutritional interventions suppressed a suite of immune-related genes, except for IFN- and TNF-dependent genes (Fig. 3 and 4). Analyses of individual immune related genes (Fig. 5, 6) found changes in both directions. Down-regulation was observed in an opsonic glycoprotein fibronectin (FN), an acute phase protein produced mainly in the liver, liver-specific antimicrobial protein, LEAP-2B, MHCI class I antigen and programmed death ligand 1 (PD-L1/B7-H1), a negative regulator of T-cell responses. Up-regulated Src kinase-associated phosphoprotein 55-related protein (SKAP2) is an adaptor protein involved in the integrin-mediated adhesion and related functions in immune cells (27). Our recent studies showed the important role of a liver bile salt export pump, ABCB4 and alpha-1-antiproteinase-like protein (Antiprot1) in protection against a bacterial disease furunculosis (28). Arachidonate 5-lipoxygenase (ALOX5) transforms fatty acids in both proinflammatory and antiinflammatory leukotrienes.

Discussion

To use gene expression analyses for diagnostics of nutritional stress, it is necessary to find responses that ideally, discriminate different stressors in a dose-dependent manner. In our pilot study we observed sizeable and consistent gene expression changes associated with restricted feeding and/or the inclusion of extracted soybean meal in the diet. Individual variations were relatively small when analyzed with microarrays, which commonly produce more stable results in comparison with qPCR. The genes that responded to the treatments changed expression in the same directions (up- or down-regulation) in all study groups. It was difficult however, to find specific markers for diagnostics of the feeding stress explored in this study. To search for the candidate genes we used a simple approach, previously useful in our studies of responses to aquatic contaminants (29) and viruses (15). Results produced in this study were compared with the gene expression database to find genes with higher probability of differential expression under the applied treatments and significance was assessed with exact Fisher's probability. Several genes were found, but their specificity remains uncertain. Four of 12 genes shown in Table 3 encode ribosomal proteins. The SFA2 platform includes 69 ribosomal proteins that have shown highly coordinated expression changes in different experiments. In contrast to the repression of ribosomal protein genes in starved rainbow trout (30), increased expression of 43 ribosomal proteins was found in all groups of this study. Similar changes were observed in response to different stressors, such as water borne contaminants with different chemical structures and modes of action (31). Other candidate markers are involved in diverse processes and have also shown differential expression under diverse conditions. Both microarray and qPCR analyses showed marked down-regulation of a gene encoding LEAP-2B, an antimicrobial protein with unknown properties and functions. Given high sensitivity of fish antimicrobial proteins to diverse stressors (32), this gene can be considered a promising marker.

A large part of the gene expression changes were well in line with regulation occurring in undernourished condition. FN is a sensitive index of nutritional state since its plasma levels fall within a few days of restricted feeding, e.g. (33). The FN changes in CR and SF were in line with this report, whereas, slight induction of FN in SR was shown with qPCR. The initiation of caloric restriction in mammals induces the hepatic expression of the urea cycle enzyme arginase (ARG) that utilises arginine as substrate (5). No changes were observed in ARG1, whereas ARG2 was

significantly down-regulated in SF. A decrease in adenosine kinase in all groups could be evidence for disrupted recycling between adenosine and AMP and reductions in all three adenine nucleotides (34). This might affect mitochondrial capacity, since metabolic flux rate in hepatic mitochondria is proportional to the available intracellular adenine nucleotides. Increased expression of genes for the components in the electron transport chain (Fig. 4) can probably be regarded as a compensatory regulation. Changes in nucleotide metabolism were further corroborated with up-regulation of ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1), a membrane-protein which hydrolyzes extracellular ATP and ADP to AMP. AMP is converted to adenosine, which is further degraded by adenosine deaminase (up-regulated in CF and SR). Association between reduction of hepatic nucleotide metabolism and caloric restriction was reported by Spindler and Dhahbi (35). The canonical response to energy restriction in mammals involves adjustments in the activity of key gluconeogenic and glycolytic enzymes in liver (36), as also reported in sea bream (8, 9). Gluconeogenesis is known to maintain plasma glucose concentrations over long periods of fasting in salmonids (37). G6PC, which was up-regulated in all study groups, responds to energy restriction in both mammals (2) and fish (38, 8). Down-regulation of several genes encoding glycolytic enzymes was consistent with a decrease in glycolytic activity under restrictive dietary regimens, as demonstrated in mammals (3) and fish (39). Amino acids are used as an energy source after depletion of hepatic glycogen and lipid stores in starved fish (40, 4). In the present study, there was no evidence for increased protein catabolism. A slight stimulation of several proteasome components was observed, but cathepsins were down-regulated (Fig. 4).

The gene expression analyses did not discriminate responses to different nutritional interventions in this study. Though several genes showed small differences between CR and SF, in general, responses to feed restriction and soy were highly similar. Lower growth rates of salmonid fish fed diets with high levels of soy can be due to the presence of physiologically active compounds with different modes of actions, improper nutrient balance, decreased feed intake and impaired absorption of nutrients. Based on our transcriptomic studies of the responses of salmonids to chemical stressors (29), we have outlined highly sensitive markers that detect even weak toxic impacts, such as presence of low levels of oxidized lipids (manuscript under preparation). There were no signs of toxicity in the soy-fed salmon however, and genes involved in detoxification processes were equally suppressed by all interventions (Fig. 4). We observed expression changes of genes for proteins

involved in steroid metabolism, but no differences were found between CR and SF. Soy contains isoflavones (phytoestrogens) that mimic the actions of endogenous steroid hormones, even at low concentrations found in food (41), but there was no stimulation of estrogen responsive genes. Both ERß2 and ERα1, the most abundant estrogen receptor transcripts in the liver of rainbow trout (42), were down-regulated in our study. Adverse effects of soy on growth can be due to deficiencies of methionine and bioavailable iron in soy (10, 43). We did not however, find differences in genes for proteins involved in iron metabolism (equal down-regulation in all groups, Fig. 4) or amino acid metabolism. The non-starch carbohydrate fraction from soy could also negatively affect digestibility of nutrients through binding to bile acids, by obstructing the digestive enzymes' action and by increasing the movement of substrates in the intestine (reviewed in(12)). In salmon fed with the soy diet at full ration both feed intake and feed efficiency were lower than in control. Mild undernourishment most likely explains similarity of the gene expression profiles in CR and SF.

Down-regulation of genes for proteins involved in steroid and lipid metabolism was one of the most consistent trends in this study. In mammals, caloric restriction lowers hepatic expression of genes involved in fatty acid biosynthesis (44, 4, 45), decreases endogenous synthesis of cholesterol and rapidly attenuates hepatic synthesis of bile acids (46, 44). Consumption of soy also results in modest reductions in cholesterol and lipid blood concentrations, attributed to the combined action of soy proteins and isoflavones (47). We observed marked down-regulation of several genes with key roles in biosynthesis, modification and transport of lipids. The decreased expression of lipase/cholesteryl ester hydrolase, high density lipoprotein binding protein and very-long-chain acyl-CoA synthetase suggested reduced cholesterol metabolism in all treatments. Together with down-regulation of AKR1D1 and HSD17B4, this could affect hepatic bile biosynthesis and bile-dependent events including digestion and absorption of fats and fat-soluble vitamins. Attenuation of cholesterol biosynthesis in liver could inhibit steroidogenesis and in this respect, it is noteworthy to mention down-regulation of HSD17B4, as well as progesterone and estrogen receptors. Bromage et al. (48) showed in several salmonid species that restricted nutrition, especially at an early stage of gonadal development, reduced the percentage of maturing individuals in the following spawning season. Microarray analyses revealed increased expression of a gene from the estrogen-related receptor family comprised of orphan nuclear receptors, which regulate different aspects of cellular energy

homeostasis (49). In addition to reproductive functions, hepatic steroid signaling is involved in the control of whole body energy metabolism (50).

Diagnostics of adverse conditions presumes dose-dependent changes or greater deviations at high levels of stressors. In this study, gene expression differences between the control (CF) and the group with the lowest growth rates (SR) were smallest by magnitude. Many genes responded to restricted feeding and soy, but not to combination of these treatments. Attenuated responses to stronger stressors have been reported in different research areas, including toxicology, radiology and exercise physiology. Collectively, bi-phasic dose-responses are referred to as hormesis. Hormesis presumes positive effects of weak stressors, explained with activation and training of protective mechanisms; restricted feeding can be interpreted in light of this concept (51). Growth retardation in aquaculture fish is commonly regarded as only negative. One may ask however if maximum growth rates achieved at high rations are healthy for salmon.

Microarray analyses found activation of genes for the components of the mitochondrial electron transport chain, in parallel with decreased production of endogenous ROS scavengers, and in theory, this might mean a higher risk of oxidative stress. Salem et al. (30) and Drew et al. (52) also observed down-regulation of a panel of antioxidant genes in starved rainbow trout and zebrafish, respectively. In our study, control fish had higher expression levels of genes for proteins of iron and heme metabolism. Given that iron and heme are potent catalysts of reactions that generate free radicals, these genes are also involved in regulation of redox status. Restricted feeding however, has consistently been demonstrated to lower the rate of ROS generation in mitochondria in mammalian models (reviewed in (53)). The observed down-regulation of scavengers could be due to lower production of ROS and other types of free radicals in underfed fish. Similar trends in a large number of chaperones suggested cellular stress in salmon that received control feed at maximum ration in this study. This was in line with down-regulation of endoplasmic reticulum and cytoplasmic chaperones by caloric restriction in mammals (reviewed in (6)).

Additional evidence for the possibility of oxidative stress in fish with reduced growth was a weak but consistent up-regulation of serine/threonine protein kinases MAP4K4-1 and 2, MAP3K5 and misshapen-like kinase 1 (MINK1), all members of the JNK cascade. Similar changes were seen in

other components of this pathway: c-Fos (part of AP1 complex), p65, the subunit of NFkB transcription factor and IkB kinase complex-associated protein, a scaffold protein that regulates three kinases in the NFkB signaling cascade. These genes are components of the complex network that integrates and coordinates responses to oxidative stress, apoptosis and inflammation. In mice, long- as well as short-term caloric restriction dramatically increases apoptosis in liver, leading to a significantly lower incidence of tumors (6). Possible activation of the multiple apoptotic pathways in salmon with reduced growth was evidenced with the up-regulation of pro-death p53; whereas, genes with anti-apoptotic roles (Bax inhibitor-1, BCL2/adenovirus E1B 19kDa interacting protein 2 and defender against cell death 1) were down-regulated, except for the BAG-family molecular chaperone regulator-4 (Fig. 3). Increased expression was observed in several TNF- and IFN-dependent genes, including TNF receptor superfamily member 19 that mediates activation of both NFkB and JNK. TNFα signaling cascade has a dual role in liver as it can induce both cell-death and proliferation of hepatocytes (54).

Apart from slight induction of TNF- and IFN-related genes that are involved in a magnitude of processes, most likely including the regulation of metabolism (55), there was a consistent trend towards reduced expression of immune genes, especially in salmon with moderate retardation of growth. Reduction in the expression of inflammatory markers in liver has been reported in response to restricted calorie intake in mice (56, 57) and soy-based diets in mammals (58, 59, 10). Down-regulation was observed in specialized immune genes, such as antigen presenting MHC1b, STAT, a key mediator of IFN signaling, allograft inflammatory factor and the complement components (Fig. 4). A number of genes shown in Fig. 3 have diverse roles, including immune responses. In mammals, obesity, probably the worst consequence of excessive feed consumption, is often associated with the chronic inflammatory status leading to various health problems. Immunosuppression and anti-cancer effects caused by modest feed deprivation are well documented (35), and prevention of age-related dysfunctions of the immune system by caloric restriction was reported both in short- and long-lived animals (60). Reduced expression of immune genes in absence of pathogens may mean a lower risk of autoimmune disorders in salmon.

To summarize, the gene expression analyses found consistent changes caused with nutritional interventions in Atlantic salmon. Use of this approach for diagnostics is questionable however, due

to the relatively low specificity of responses and their attenuation at strong retardation of growth. Our results suggest that restricted feeding may have both beneficial and negative health consequences. The observed gene expression changes permit different scenarios, and conclusions cannot be made without functional studies.

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Table legends

Table 1. Formulation and chemical composition of the experimental diets

Table 2. Primers used for qPCR analyses

Table 3. Feed intake, growth and feed efficiency of the study groups

Table 4. Candidate markers of feeding stress in the liver of Atlantic salmon

Table 1. Formulation and chemical composition of the experimental diets

Diet code	Control	Soy
Ingredients (g kg^{-1})		
Fish meal	560	433
Wheat	183	101
NorSeaOil	233	242
Extracted soybean meal		200
Vitamin mix[1]	20	20
Mineral mix[1]	4	4
Chemical composition (g kg^{-1})		
Dry matter (DM)	939	943
In DM		
Crude protein	452	434
Crude lipid	296	318
Ash	98	88

[1]As described by (61)

Table 2. Primers used for qPCR analyses.

Target: Gene name, symbol and Genbank accession	PRIMER(Forward, Reverse)
Arginase 1, ARG1, EG929369	AGCCATGCGTATCAGCCAA AAGGCGATCCACCTCAGTCA
Arginase 2, ARG2, DW581882	GACAGGCTCGGCATTCAGA AAAGACGGGTCCATCGCAT
Carbonyl reductase, DCXR, CA342225	GTCACCCCCGACCAGTTTGA CGCCTGGCTGGAGATGTTCA
Liver expressed antimicrobial protein, LEAP-2B, CA387966	CCACAGCAAAGCAACGCAAG CGTTCCAGTAGCTTGGTGCCCT
Programmed death ligand 1 PD-L1/B7-H7, CA366631	TCAACGACTCTGGGGTGTACCGATG TCCACCTCATCTCCACCACGTCTC
MHCI class I antigen, MHC Ia, CA359060	CGCATGATGCATAACAGCACTG AGCTGTACTGGTCTGTGCCCTTT
Carbonyc anhydrase, CA, EL561323	GGTGTATGGGGGCCATCAAATC GGAGTGGTCAGGGAGCCATCAT
Arachidonate 5-lypoxygenase, ALOX5, CA346166	TTGTGTGGGACGTGGTGTGTATC AGGTCCTGCATCCCAAAGCT
Membrane associated progesterone receptor, PGRMC2, BX082826	AGAAGGAGGCCCTGAAAGACAC TTTGAGTGAACTGGGTCTCCCA
Fibronectin precursor, FN, CA378743	GCATGTCTGAGACGGGCTTCAA AGTCACATCGGAAGTGTCCACTGC
Glutathione S-transferase theta 1, GSTT1, CA361754	TTTGGCTCAGGCTCTTGATTCC GAGTTTCAGAGAGCCTTCCAGGTC
Catalase, CAT, EL560112	CCAGATGTGGGCCGCTACAA TCTGGCGCTCCTCCTCATTC
Hemopexin, HPX, CA363230	ATCACCTGGGGCATGTTGACG TCCAGCTGGATGTCCTTGGG
Ribosomal protein S15, RPS15, CX143557	CAAGCAGCAGTCCCTCCTGA GACCATGTCCCTGAGGTGAGTCT
Liver bile salt export pump, ABCB4, CA363965	TACGACACCAACGTAGGTTCCCAGG GGATCTTAGGGTCGCGGATGATC

Alpha-1-antiproteinase-like protein, Antiprot1, CA366162	CCACAAGGCTGTGCTGAGCGTA TGAGCATGATGGTGTCTGGGAGAG
High density lipoprotein binding protein, HDLBP, CA372345	GAAACAGCTGCTCTCTCTGGCC CGCCTCCTTTACCGATCAGG
Very long acyl-Coa synthetase, VLACS, CA371001	TGGAGACCTGCTGAAGATAGGCC GGGTGAGGATGTCAGCTACTTCAGT
Estradiol 17 beta dehydrogenase, HSD17B4, CA370123	GGATCGTTCCTTTGGCAGAAC TGGTTCCAGGCAGCTCTAGTGAC
Src kinase-associated phosphoprotein 55 related protein, SKAP2, CA366296	GAGGTGCTCCCAGAGGATGACA CAGTCCCACAAGCCCTGGTAGT
Reference: Gene name, symbol and Genbank accession	**PRIMER SEQUENCE (Forward, Reverse)**
18S rRNA, AJ427629	GCCCTATCAACTTTCGATGGTAC TTTGGATGTGGTAGCCGTTTCTC
RNA polymerase II , RPOLII, CA049789	TAACGCCTGCCTCTTCACGTTGA ATGAGGGACCTTGTAGCCAGCAA
Eukaryotic translation initiation factor 3 subunit 6, eIF3S6, CX040383	GTCGCCGTACCAGCAGGTGATT CGTGGGCCATCTTCTTCTCGA
NADH dehydrogenase 1 alpha subcomplex subunit 8, NDUA8, NM_001160582	TCTGTCGCTGGGAGGAGAAGGA GTCCAGGCAGGTCCGATACTCTGT
70 kDa peroxisomal membrane protein, PMP70, CA369192	CCATCGGGAAGATGACGGTCA TCTGCTTCTCCCTCAGGTTTCCA
Prostaglandine D synthase, PGDS, CA352578	CCTACACCAACCTGAACGCTGATG ACGCTGGCTGGTGAAGGTGAAG
NADH dehydrogenase subunit 4, ND4, DQ288271	GCTAGTTTAGCAAATCTGGCCCTCC GCTGTAATTAACGTGCCTAGTCCCG
Elongation factor 1 alpha, EIF1a, AF321836	CACCACCGGCCATCTGATCTACAA TCAGCAGCCTCCTTCTCGAACTTC

Table 3. Feed intake, growth and feed efficiency of the study groups

Study group[1]	CF	SF	CR	SR
Start weights, g (mean ± S.D., n=59)	299±27	290±26	293±29	304±28
Final weights, g (mean ± S.D., n=59)	561±82	480±57	404±40	382±76
Relative feed intake, % body weight d^{-1}	0.98	0.85	0.43	0.34
Thermal growth coefficient (mean ± S.D., n=59)	2.43±0.55	1.89±0.37	1.18±0.34	0.81±0.62
Feed efficiency	1.16	1.07	1.36	1.24

Table 4. Candidate markers of feeding stress in the liver of Atlantic salmon

Genes	This study	Other studies[a]	P-values[b]
3-oxo-5-beta-steroid 4-dehydrogenase[c]	5/10[c]	18/143[d]	0.030
Estradiol 17 beta-dehydrogenase 4[c]	5/10	14/180	0.0061
High density lipoprotein-binding protein[c]	7/10	46/186	0.045
Very-long-chain acyl-CoA synthetase[c]	6/10	19/180	0.0049
Cytochrome P450 2K4-1	8/10	33/179	0.0058
40S ribosomal protein S15-2[c]	7/10	22/177	0.0029
40S ribosomal protein S21	6/10	27/184	0.0164
60S ribosomal protein L18	7/10	25/186	0.0039
60S ribosomal protein L7a-2	7/10	44/186	0.0383
Adenosine kinase 3	8/10	51/183	0.0343
Liver-expressed antimicrobial peptide 2B[c]	10/10	40/143	0.0088
N-myc downstream regulated protein	9/10	38/185	0.0038

[a]Studies addressed responses to toxicity and pathogens in the liver of salmonid fish (15),(62),(63) and (61).

[b]Exact Fisher's probability

[c]Genes analyzed with real-time qPCR (see Table 4)

[d]Number of samples with differential expression / total number of samples.

Figure legends

Fig. 1. Growth in the study groups. Data are mean thermal growth coefficients (TGC) and error bars represent standard deviation (n = 59). Fish with TGC values in between horizontal marks were used for gene expression analyses.

Fig. 2. Summary of the transcriptomic responses in the study groups. **A**: correlation of the expression profiles (log_2 – Expression Ratios) within groups (CR, SF and SR) and between groups (CR-SF, CR-SR and SF-SR). Data are mean Pearson r ± SE. **B**: numbers of genes that met criteria of differential expression: significance ($p < 0.05$ or < 0.01 in at least 3 of 5 fish, mean fold change > 1.4 or 2).

Fig. 3. Examples of differentially expressed genes (microarrays analyses). Data are mean log_2-expression ratios, n = 5 in each group. Significant differences from control are indicated with color scale presenting log_2-expression ratios or fold changes (in parentheses).

Fig. 4. Expression changes in functional groups revealed with microarray analyses. Data are mean log_2-expression ratios ± SE. Proteasome: ubuquitins, ubiquitin ligase and conjugating protein. Proteases: cathepsins B, C, D, L1 and L2, nephrosin. Mitochondria: cytochrome b, cytochrome c oxidase subunits, NADH dehydrogenase subunits 4 and 5-1, NADH-ubiquinone oxidoreductase 20 kDa subunit, ATP synthase beta chain. Ribosomal proteins: 16 components of 40S subunit and 27 components of the 60S ribosomal subunit. Responses to stress and oxidative stress (ROS): oxidative-stress responsive, catalase, superoxide dismutase (Cu-Zn), glutathione peroxidase, glutathione S-transferase theta, peroxiredoxins 1, 6, thioredoxin-like protein 4A, selenium-binding protein 1 and selenoprotein T, serum/glucocorticoid-regulated kinase and N-myc downstream regulated protein-1. Chaperones: heat shock 60, 70 and 90 proteins, heat shock cognate 70 kDa, stress 70 protein chaperone, 94 kDa glucose-regulated protein, 15 kDa selenoprotein and DnaJ homologs. Xenobiotic metabolism: alanine-glyoxylate aminotransferase, alcohol dehydrogenase (NADP+), aldehyde dehydrogenase 9 A1, epoxide hydrolase 1, UDP-glucose 4-epimerase, UDP-glucuronosyltransferase. IFN-dependent and TNF-dependent: IFN regulatory factors 1, 4 and regulators 1, 2, guanylate-binding protein, ligand superfamily member 13B, receptor superfamily member 19, receptor). Complement: components 3, 5, 6 and 8, factors B/C2-B, Bf2 and H, serine like proteins. Iron and heme: 5-aminolevulinate synthase, carbonic anhydrase, beta globin, hemoglobin alpha and beta chains, hemopexin.

Fig. 5. Genes analyzed with microarrays and real-time qPCR. Microarray data are mean log_2-expression ratio ± SE; n = 5 animals in each group. qPCR results are mean -ddCt ± SE; n = 6 in all study groups. Difference from control denoted with an asterisk was assessed with Duncan's t test ($P < 0.05$) and difference between the groups was based on homogenous subsets of multi-comparison Dunnett's test (letters in common denote same homogenous subset, $P < 0.05$).

Fig. 6. Genes analysed with real-time qPCR. Data are mean -ddCt ± SE; n = 6 in all study groups. Difference from control denoted with an asterisk was assessed with Duncan's t test ($P < 0.05$) and difference between the groups was based on homogenous subsets of multi-comparison Dunnett's test (letters in common denote same homogenous subset, $P < 0.05$).

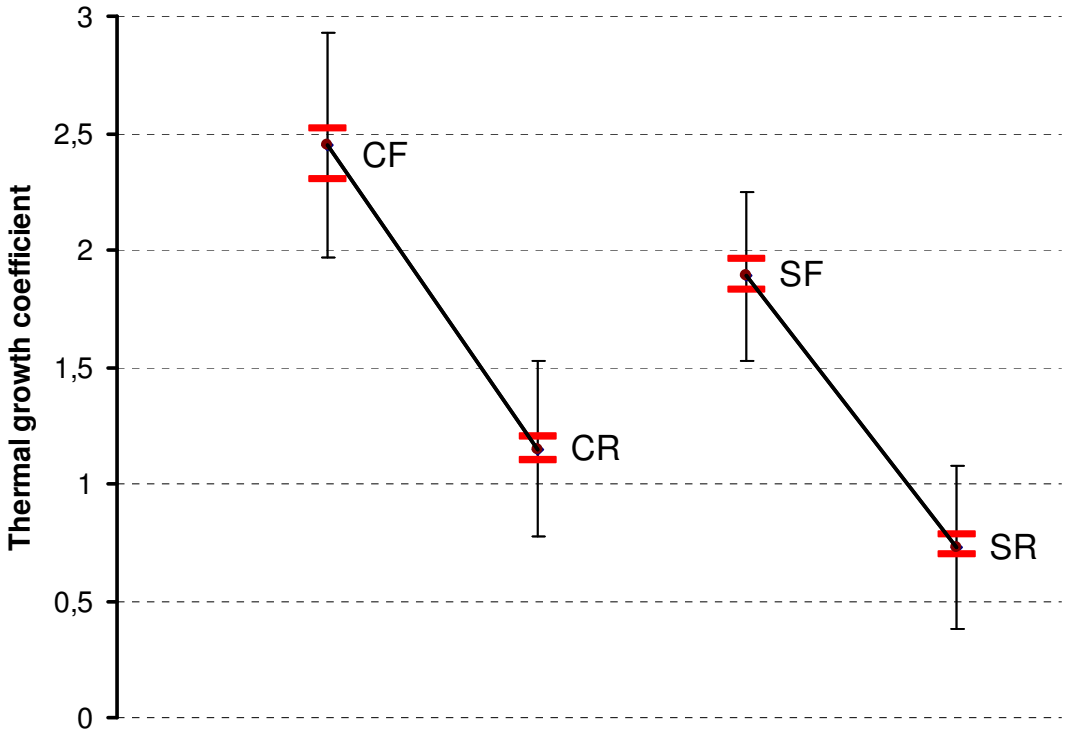
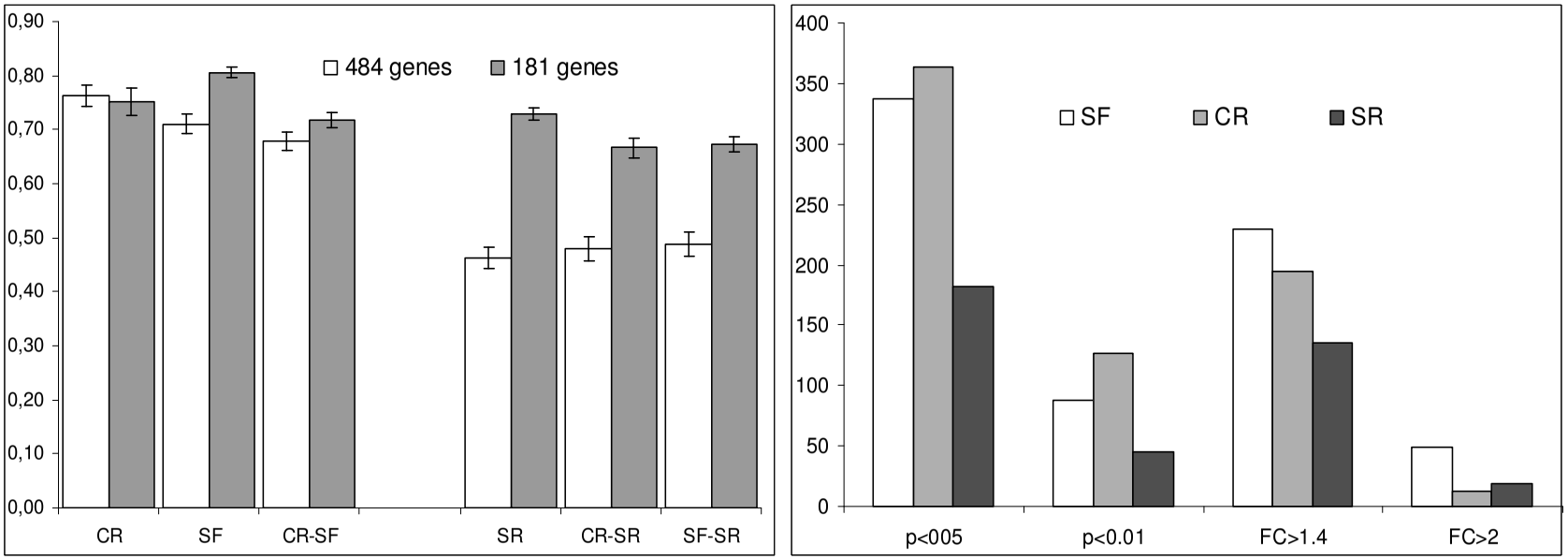


Fig.1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SF
CR-SF
SR
CR-SR
SF-SR
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350
300
250
200
150
100
50
0
SF
CR
SR
p<005
p<0.01
FC>1.4
FC>2

Fig.2

ID	GENES	CR	SF	SR
	Carbohydrate metabolism			
DV200529	Fructose-bisphosphate aldolase A	0,67	0,38	0,21
DV670949	Beta enolase	-0,52	-0,53	-0,51
DV670773	Glyceraldehyde-3-phosphate dehydrogenase	-0,24	-0,36	-0,59
BX869335	Fructose-1,6-bisphosphatase isozyme	-0,53	-0,79	-0,31
CU070548	Glucose-6-phosphatase	0,49	0,86	0,10
	Lipid and cholesterol metabolism and signaling			
CA377953	3-oxo-5-beta-steroid 4-dehydrogenase	-0,73	-1,04	-0,69
CA365100	Delta-6 fatty acid desaturase	-1,08	-1,61	-0,42
DY737446	Apolipoprotein A-IV	-0,62	-0,19	-0,51
CA359906	Leukotriene-B(4) omega- hydroxylase	-0,38	-1,51	-0,21
EV384586	Cytochrome P450 2K4-2	-0,59	-0,61	-0,55
CA369597	Lysosomal acid lipase/cholesteryl ester hydrolase	-0,73	-0,98	0,05
CA383629	Estrogen receptor-2	-0,81	-0,24	-0,42
CA361379	Estrogen receptor-1	-0,53	-0,58	-0,10
CA366558	Steroid hormone receptor ERR2	0,64	0,32	0,19
	Nucleotide metabolism			
EV377799	Beta-ureidopropionase	-0,52	-1,19	-0,88
EG923615	Adenosine kinase	-1,11	-1,21	-0,92
CA358107	Ectonucleoside triphosphate diphosphohydrolase	0,46	0,39	-0,47
EV378274	Adenosine deaminase	0,53	0,42	0,19
CT563975	ADP,ATP carrier protein T2	0,33	0,37	0,16
	Apoptosis			
CU066444	Caspase recruitment domain protein 4	0,70	0,40	0,49
CA370435	Caspase 2	0,28	0,72	0,36
CA366462	Apoptosis regulator Bcl-X	0,43	0,58	0,47
CA344820	Baculoviral IAP repeat-containing protein 2	0,51	0,55	0,23
CA346488	p53-like transcription factor	0,31	0,58	0,31
CA348325	BAG-family molecular chaperone regulator	0,63	0,33	-0,02
CA367619	Programmed cell death protein 8, mitochondrial precursor	-0,60	-0,46	-0,36
CA377672	Defender against cell death 1	-0,32	-0,49	-0,77
CA363935	BCL2/adenovirus E1B 19-kDa protein-interacting protein 2	-0,73	-1,00	-0,43
CA367787	Bax inhibitor-1	-0,74	-1,50	-0,26

ID	GENES	CR	SF	SR
	JNK, NFkB signaling			
CA343485	C-Fos protein	0,06	0,54	0,56
CA357582	MAPK/ERK kinase kinase kinase 4	0,31	0,40	0,13
CA346979	MAPK/ERK kinase kinase 5	0,33	0,20	0,30
CA368189	MAPK/ERK kinase kinase 6	0,41	0,33	0,26
CA347260	Transcription factor p65	0,60	0,45	0,25
CA384063	IkappaB kinase complex-associated protein	0,64	0,91	0,84
	Immune functions			
CA043257	MHC class 1b antigen	-0,60	-2,05	-0,63
BT073792	beta-2 microglobulin-1 BA1	-0,39	-0,40	-1,64
CA357761	Galectin-3	-0,73	-1,19	0,09
CX141783	Acute phase protein	-0,29	-0,91	-0,54
CA361151	Annexin A1	-0,57	-0,87	0,08
CA365039	CD63	-0,54	-0,88	-0,78
CA361724	Signal transducer and activator of transcription 3	-0,45	-1,01	-0,14
CA369653	Cellular nucleic acid binding protein	-0,76	-0,83	-0,36
CA374135	CCL4	-0,38	-0,80	0,22
CA382425	B-cell translocation gene 1	-0,44	-0,71	-0,54
CA383795	Allograft inflammatory factor-1	-0,64	-0,62	0,10
EV384755	Ras-related C3 botulinum toxin substrate	-0,68	-1,10	-0,21
CA355782	Suppressor of cytokine signaling	0,61	0,63	0,33
CX148602	CCAAT/enhancer binding protein delta	0,10	0,76	1,43
	Miscelanious			
CA379854	Inositol 1,4,5-trisphosphate receptor type 1	0,77	0,71	0,52
CA349627	Hypoxia-inducible factor 1 alpha	-0,41	-0,66	-0,38
EL552816	ADP-ribosylation factor 4	-0,59	-1,09	-0,86
CU071980	Gap junction alpha-3 protein	-0,52	-0,85	-1,53

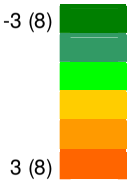
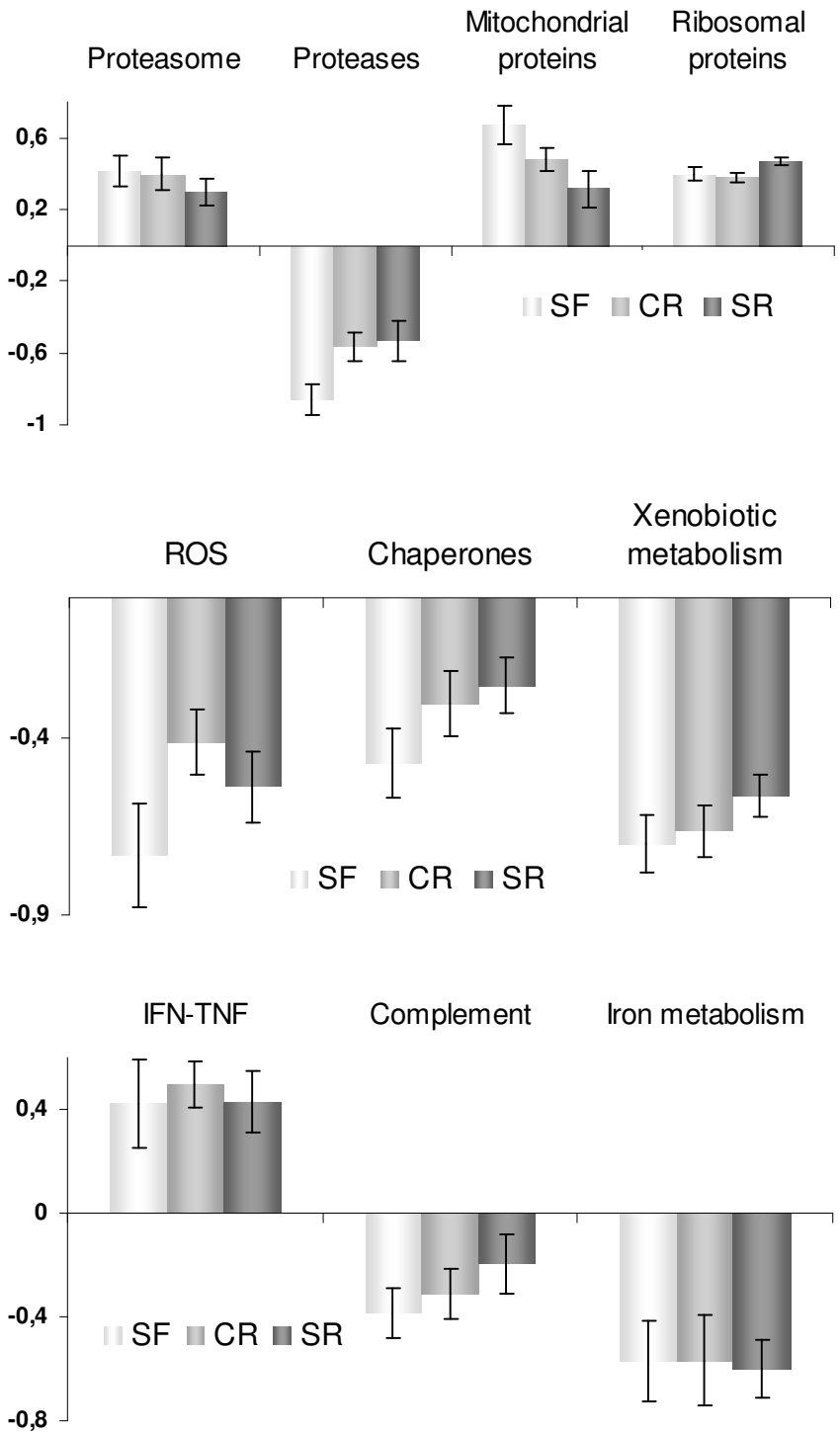


Fig.3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Complement
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Fig.4

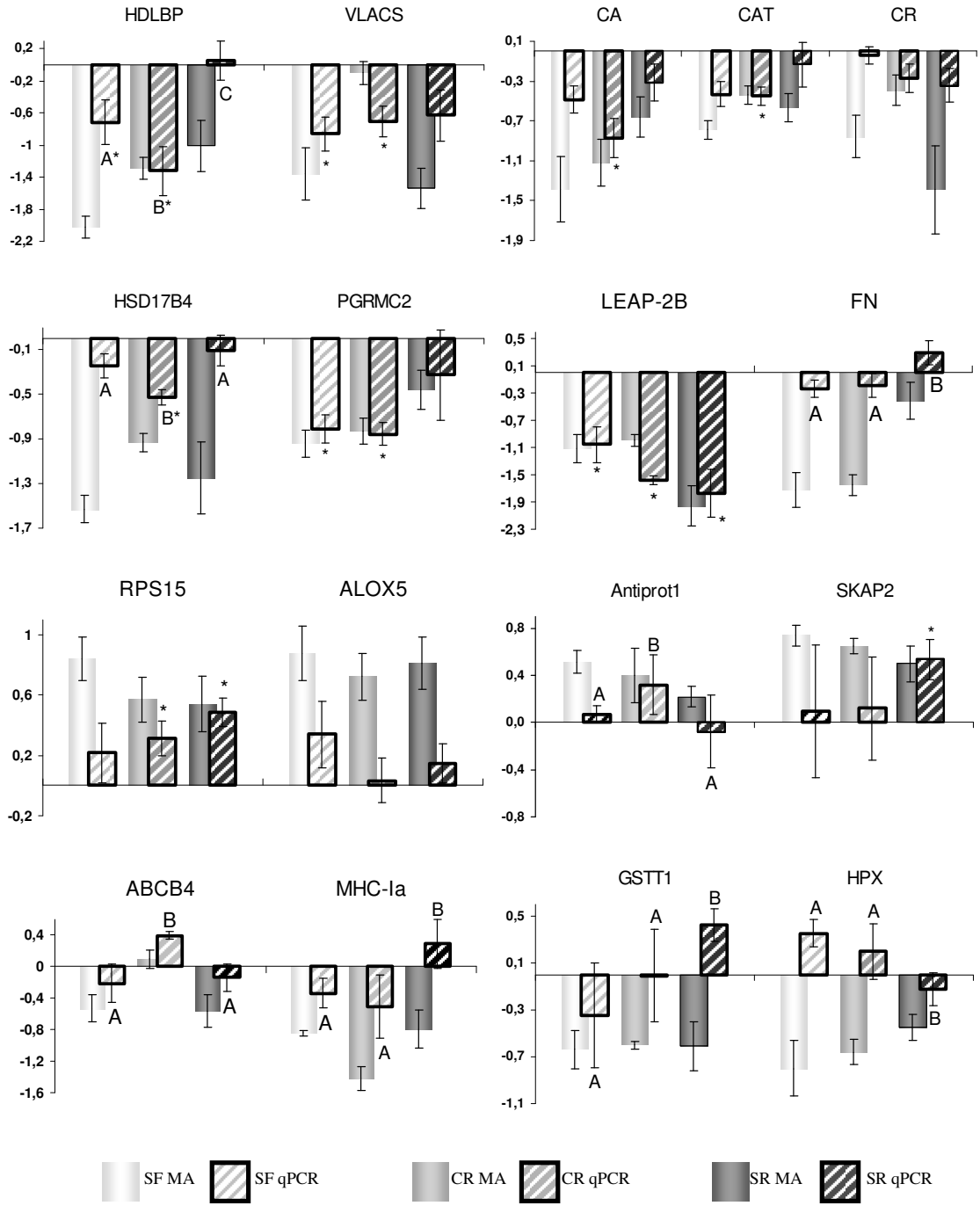
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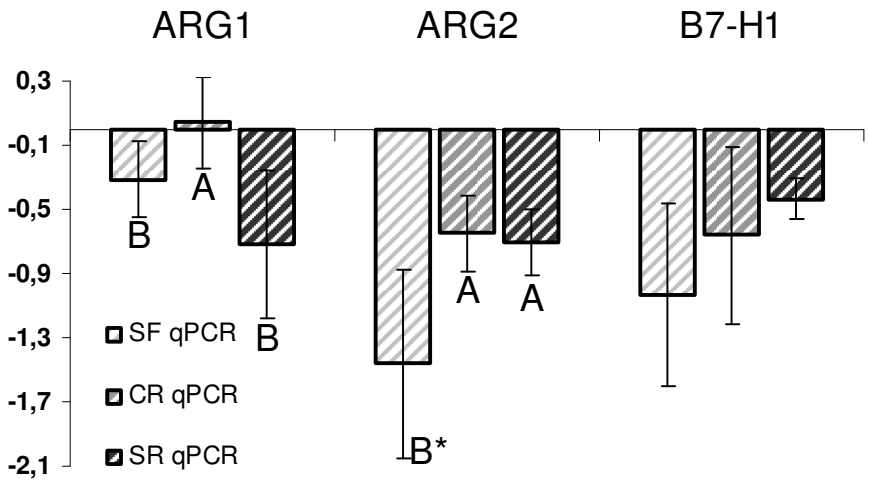
Fig.5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B
A
B
A
A
B*

Fig.6