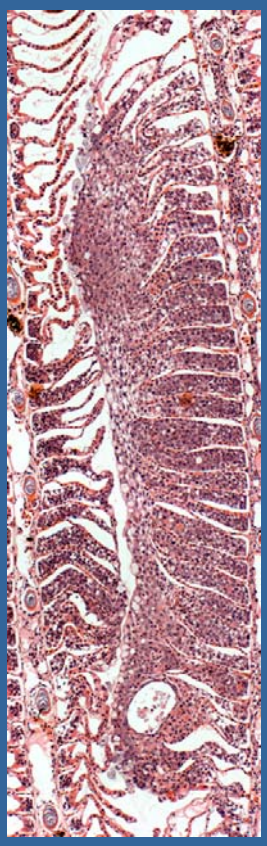
Final Report

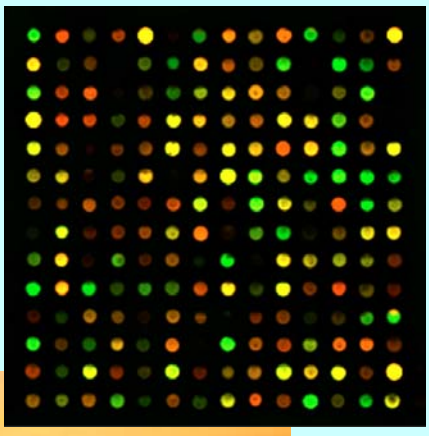
Aquafin CRC - Atlantic Salmon Aquaculture Subprogram:
Use of immunomodulation to improve fish performance in Australian temperate water finfish culture

Barbara Nowak, Andrew Bridle, Kally Gross, Benita Vincent,
Neil Young and Richard Morrison

October 2007

Aquafin CRC Project 3.4.2 (2)
(FRDC Project No. 2004/210)





Aquafin CRC - Atlantic Salmon Aquaculture Subprogram: Use of immunomodulation to improve fish performance in Australian temperate water finfish culture

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OBJECTIVES:

1. To evaluate the use of immunostimulants for control of AGD of Atlantic salmon.
2. To investigate the role of inflammation in AGD of Atlantic salmon
3. To test the effectiveness of vaccination against AGD using crude or partially purified antigens.
4. To investigate the role of antibodies in AGD.

Non-Technical Summary

OUTCOMES ACHIEVED

This project has increased our knowledge and understanding of Amoebic Gill Disease (AGD) and, in particular, our understanding of immune response in Amoebic Gill Disease. We have reviewed literature on the use of immunostimulants in marine grow-out. We have published the first paper on transcriptome profiling in fish parasitic disease. While the results of our project suggest that the performance of immunostimulants in AGD control has been inconsistent, other aspects of our research offer promise. However, these would require further investigation before they could be commercially applied. This project contributed to education and training in the area of fish health. Three PhD students and a postdoctoral research fellow were involved in this project, contributing to human capital development in aquaculture research and policy development.

Before this project our knowledge of immune response in Amoebic Gill Disease (AGD) was fundamentally limited and more information was required to assess the potential for immunomodulators in the management of AGD.

We confirmed that injection of bacterial DNA motif (CpG oligonucleotides) six days before AGD challenge can offer signficant protection to Atlantic salmon (relative percent survival up to 52.5%). However, there was no effect if the fish were challenged immediately post injection with bacterial DNA. This

suggests that while there is a potential benefit from the use of immunostimulants, their application is limited because their efficacy is directly linked to the timing of an outbreak, which can be unpredictable in the field. While fish which survived an initial AGD episode show increased resistance to subsequent AGD infection, in contrast to some diseases this effect cannot be simply explained by the presence of antibodies. The duration of exposure (or number of exposures) appears to be important for the development of serum antibodies. Mucus antibodies could not be detected in Atlantic salmon that survived AGD challenge. Microarray experiments and further gene expression studies suggested that there is a loss of cell-cycle control in AGD lesions. Furthermore, immune pathways are affected since the down-stream effect(s) of the initial inflammatory signals were not detectable. It is possible that this significantly contributes to the extremely high rate of mortality in unmitigated AGD epizootics.

While we have achieved our objectives and answered many of the original questions, new issues have emerged from our research. These include a lack of understanding of the mechanisms of inhibition of inflammatory and immune pathways, significance of antibody response (if any) in AGD, and the potential for vaccine antigen discovery through the use of anti-peptide antibody. The presence and role of a more localised antibody response in the gill mucus or epithelium (currently undetectable) warrants further investigation.

In conclusion, we now have a better understanding of AGD pathogenesis and the reasons why the host immune response is ineffective in this disease. In particular, we have shown that immune pathways are inhibited in Atlantic salmon affected by AGD.

KEYWORDS:
Amoebic Gill Disease, salmon, aquaculture, immunostimulants, inflamation, gene expression, transcriptome analysis

Acknowledgments

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We are grateful to our collaborators, in particular Professor Chris Secombes (University of Aberdeen, Scotland), Professor Ben Koop (University of Victoria, Canada) and Dr Erling Koppang (Norwegian Veterinary College, Norway) for sharing their expertise. We would like to thank the salmon industry for their collaboration and support, in particular David Mitchell (HAC), Adrian Steenholdt (HAC), Jarrod Wells (HAC), Peter Bender (HAC) and Pheroze Jungalwalla (TSGA). This project would not be possible without their help. We would like to thank David Mitchell (HAC) and Peter Bender (HAC) for access to information from farm trials. We would like to thank Dr Peter Montague (Aquafin CRC), Dr Patrick Hone (FRDC), Professor Colin Buxton (TAFI) and Professor Chris Carter (TAFI) for their continuing support during this project. We would like to thank Dr Mark Adams, Dr Phil Crosbie and Michael Attard for all their contributions to this project.

Background

Finfish aquaculture has been a major success of primary industry in Australia in recent years. Driving this success has been the Atlantic salmon industry in Tasmania. Whilst finfish aquaculture is relatively young compared to established livestock industries, aquaculture already makes a significant contribution to the Australian community. For example the Atlantic salmon industry now employs approximately 3000 people directly or indirectly and produces about 22 000 tonnes, worth $250 million (TSGA, 2006/07 data).

Given that disease is production limiting, animal health is naturally considered an extremely important component of any livestock R & D program. There are two fundamental strategies for tackling health issues; (1) prevention and (2) cure.

Before the concept of antibiotic/chemical resistance was raised, curing disease took precedence. However, in light of consistent evidence in the scientific literature, there is a robust argument not to adopt this strategy. Not only has the scientific community recognised the value of the “prevention is better than cure” attitude, but the general public approves this concept on the back of broadened environmental and health awareness. Therefore, disease prevention is justifiably the almost exclusive focus in terms of disease management. Disease prevention can be managed using a suite of strategies modifying the interaction between host, pathogen and environment. Here, we proposed to investigate the potential of improving performance by modifying the host with immunomodulation.

Immunomodulation by definition is any exogenous factor mediating endogenous change in the immune system. Immunomodulation in aquaculture can be unintentional and intentional. Unintentional modulation may be related to stress and management factors. In the case of SBT it may also be due to the original status of the fish at capture.

Intentional immunomodulation includes immunosuppressive (eg. anti-transplant rejection drugs) and immunostimulating (eg. vaccines) factors and in that sense has been used extensively for many decades in managing both human and livestock health. However this project will focus on immunostimulation as a means of disease prevention.

Immunostimulation is the activation of the host-immune system, however, except in the case of vaccination the immune response is not pathogen specific. There is a sound immunological basis for using immunostimulation to influence the immune response of animals, including fish. In order for an animal to mount an immune response to a pathogen it must first have the ability to recognise the pathogen. Given that microbial pathogens evolved first, their hosts have had to develop methods to deal with invading organisms. Pathogens (eg. bacteria & viruses) are built with numerous molecules, however some molecules are shared amongst all these types of organisms.

For example, it is a bit like motor cars built by one manufacturer. The manufacturer builds a number of models of various shapes, sizes and colours but they all possess exactly the same manufacturer's badge. Higher animals have recognised that microbes share these "badges" and developed what is known as pathogen associated molecular pattern receptors (PAMPs). These receptors bind to the shared molecules or in the car example the "badges", activating the immune system.

The scientific community has indirectly known about these receptors predominantly via the effects of Gram negative bacterial lipopolysaccharide (LPS) on the immune system. Recently, these receptors have been identified and characterised in humans and include receptors that recognise microbial polysaccharides (including LPS), DNA, lipoproteins, or peptidoglycan (Heine and Lien 2003, Underhill 2003). Moreover, these receptors are also found in animals such as the fruit fly (*Drosophila melanogaster*). This means that animals within the evolutionary spectrum between humans and Drosophila (including fish) are most likely to possess the same ability to recognise these "badges" through their PAMPs. In fact recent studies have shown that fish possess PAMP genes, although before these discoveries it was clear that the fish immune system could be activated by these microbe-derived molecules. In short, we have a greater understanding of immunostimulants, why they work and how hosts respond to immunostimulants, enhancing the opportunity to harness the benefits of such treatments. In fact, the FRDC Aquatic Animal Health Subprogram has identified immunomodulation and aquatic vertebrate immunology as priorities in the key research areas of "Nature of disease and host-pathogen interaction" and "Aquatic animal health management" and "Surveillance and monitoring".

Immunostimulation is an exciting new concept to Australian aquaculture although it has not been systematically appraised either experimentally or by industry in general. The primary aim of this project was to enhance the performance of Australian temperate water finfish aquaculture through the strategic use of immunomodulators. Certainly the overseas experience with immunostimulants has been encouraging and is regularly reported in the fish farming literature (for example *Fish Farming International*). Despite this success, it should be made clear that immunostimulants are not a panacea for disease in aquaculture and immunostimulants are not effective when used continuously. Accordingly, this project focused on the strategic use of immunostimulants.

AGD significantly affects the Atlantic salmon industry in Tasmania and we have identified three key research areas for the use of immunomodulation to ameliorate amoebic gill disease (AGD) of Atlantic salmon. Immunomodulation may enhance productivity by significantly reducing the number of freshwater baths required to treat AGD. Three lines of inquiry were followed in this project:

a. Preliminary evidence suggests that immunostimulation with the novel CpG oligonucleotides can enhance resistance to AGD (Bridle et al 2003). The existing data set has been expanded by further investigating CpGs.

b. While immunostimulation has been identified as a means of reducing the effect of AGD on Atlantic salmon, studies by a current Aquafin CRC PhD student have led to the hypothesis that in fact it may be the inability of the host to control pro-inflammatory signals upon attachment of amoebae that leads to AGD associated death. A human analogy of this hypothesis would be the induction of a hypersensitive/allergic type reaction by a skin irritant. In the case of humans, topically or orally delivered immunomodulators are often used to control the host reaction. Hence this sub-project also continued investigating the role of inflammatory compounds in AGD. This may provide a possible solution to AGD. We increased our understanding of the inflammatory response in fish and identified the way to new methods of treatment.

c. Crude vaccine trials were included to provide clear evidence that vaccination is possible. While all crude vaccine trials have been unsuccessful so far, antigen dose was low, the adjuvant used was not the most effective, time from vaccination to challenge was usually really short, an antibody immune response to vaccine was not investigated and transfer to sea occurred soon after vaccination. All these factors may have adversely affected the result. There is a need for further crude and partially purified vaccine trials under optimised conditions, including testing of serum antibody to confirm that the vaccine has induced an antibody response.

Our research team has been involved in fish immunology since 1996 and published seventeen refereed publications in this research area. From this project alone, we have published 13 scientific papers with further manuscripts submitted or in preparation. We contribute to teaching during training activities in Australia and Europe (in particular the Netherlands and Denmark), organise workshops (for example Ectoparasites - Immune response and vaccine development, 3rd International Symposium on Fish Vaccinology) and present results at international conferences.

In addition to other related projects, we continue our collaboration with Professor Ben Koop (University of Victoria, DNA microarrays, Atlantic salmon genome project), Professor Chris Secombes (University of Aberdeen, cytokines, travel and living allowance for this collaboration is funded under DEST funding - Innovation Access Program, International Collaboration) and Dr Erling Olaf Koppang (The Norwegian School of Veterinary Science, identification of immune cells, inflammation in Atlantic salmon).

Need

It is impossible to prevent the presence of pathogens in aquaculture systems, particularly in sea-cage grow-out. Most disease outbreaks occur when there is an interaction between pathogens and susceptible fish (for example fish that are immunocompromised due to stress). This will result in lowering the performance of the fish and increase mortalities.

Sustainable aquaculture of finfish requires lowering the risk of disease outbreaks and replacing disease treatment with control strategies. The use of immunomodulators is essential to achieve these goals, in particular in times of increased disease risk or reduced immunocompetence. Our understanding of host-pathogen interactions and immune response allows for the use of appropriate immunomodulators. For example, if a disease is caused by overreaction of the inflammatory response, traditional immunostimulants will not improve the outcomes. Similarly, there is a need to determine correct timing and dose for immunomodulation in mariculture, both in grow-out and hatchery stages. Improved immune response would improve fish performance during grow-out. This is essential for the success of new aquaculture species.

Importantly, immunomodulators are natural products that are derived from microbes so the use of chemical products is avoided. Commercial immunomodulators have been successfully used in aquaculture worldwide, but only experimentally in Australia. For example the oral immunomodulator MicroVital significantly increases survival rates of Atlantic salmon following Vibrosis challenge; and 32% gain in survival rates of salmon fed natural immunomodulators (ß glucans and nucleotides) following exposure to IPN (exotic viral disease) challenge has been achieved in trials in Norway. However, there is little information available for fish species other than salmon. No information is available about the efficacy of these immunomodulators in improving survival of Atlantic salmon affected by Amoebic Gill Disease (AGD). There is a need to develop immunomodulation strategies that are directly applicable to Australian mariculture species, diseases (for example AGD) and unique environmental conditions.

This proposal is part of the FRDC Industry Development Program, Strategy - Aquaculture development - production and production systems. However, this proposal significantly contributed to the Human Capital Development Program, Leadership and Vocational Development. Improved knowledge of immune response and immunomodulators was identified as one of the key research areas for aquatic animal health in Research and Development Plan Aquatic Animal Health Subprogram. Priorities covered by this key research area included immunology in aquatic vertebrates (nature of disease and host-pathogen interactions), immunomodulators (aquatic animal health management) and development of tools for immune status monitoring as a means of implementing health management strategies (surveillance and monitoring). All three elements were included in this proposal. This proposal is consistent with R&D plans for Atlantic Salmon Aquaculture Subprogram. It also addressed the targeted priority: fish health, within Program 2: Industry

Development, Key R&D issues for fisheries and aquaculture in SA, South Australia's Fisheries and Aquaculture Research and Development Strategy 2002-2007. The research focus is within Tasmanian Fisheries and Aquaculture, Aquaculture Strategic Research Plan 1999-2004. This proposal fits in well with the Aquafin CRC strategy and mission by making a significant contribution to achieving sustainable aquaculture in Australia through reduction of economic impact of diseases in farmed fish, development of environmentally friendly approaches to disease management and training aquaculture industry and researchers in the fields of fish immunology.

Objectives

1. To evaluate use of immunostimulants for control of AGD of Atlantic salmon
2. To investigate the role of inflammation in AGD of Atlantic salmon
3. To test the effectiveness of vaccination against AGD using crude or partially purified antigens.
4. To investigate the role of antibodies in AGD

Methods

Objective 1. To evaluate use of immunostimulants for control of AGD of Atlantic salmon

CpGs as an immunostimulant

Experiment 1

Atlantic salmon (≈100 g) were obtained from a commercial hatchery (SALTAS enterprises) and placed in two autonomous, temperature controlled, 4000 L recirculation systems filled with fresh water. Fish were acclimated to 35‰ salinity over 14 d and the water was maintained at 16ºC. Six groups of 25 fish per tank in duplicate tanks were included in the trial. Fish were anaesthetised with clove oil (4.5×10^{-3} % v/v) and intraperitoneally (i.p.) injected with oligodioxynucleotides (50 µg/fish) suspended in 100 µL phosphate buffered saline (PBS, pH 7.4). Oligodioxynucleotides (ODN) were synthetically manufactured and phosphorothioated (Sigma Genosys, Castle Hill, NSW, Australia) to enhance resistance to nuclease digestion. No treatment and PBS only (i.p. injected) groups served as negative controls (Table 1). For identification, fish were tattooed using a Panjet instrument and alcian blue dye. Six days post-injection, *Neoparamoeba* spp. were isolated according to the method described by Morrison et al (2004) and placed in the tanks at a density of 370 cells/L (Figure 1A). The challenge continued until cumulative morbidity reached 70% in any one of the treatment groups. For the description of CpG ODN-mediated resistance, end point analysis was performed using the relative percent survival (RPS) estimate as described by (Jarp and Tverdal 1997). RPS was calculated using the following formula;

RPS = [1 - (% cumulative mortality treated group/% cumulative mortality control group)] x 100

Table 1. Treatment groups used to assess the efficacy of CpG motif-bearing synthetic oligonucleotides in Atlantic salmon challenged with *Neoparamoeba* spp..

	Treatment	ODN	Sequence (5' – 3')
1	No treatment	na	na
2	PBS	na	na
3	CpG	1668	TCCATGACGTTCCTGATGCT
4	Non-CpG	1720	TCCATGAGCTTCCTGATGCT
5	CpG	1681	ACCGATGTCGTTGCCGGTGACG
6	Non-CpG	1740	ACGCATGTGCTTGCGCGTGAGC

Experiment 2

Atlantic salmon (≈100 g) were obtained from a commercial hatchery (SALTAS enterprises) and placed in autonomous, temperature controlled, 650 L recirculation systems filled with fresh water. Fish were acclimated to 35‰ salinity over 14 d and the water was maintained at 16ºC. In three replicate systems, 8 fish per group were assigned to one of four treatment groups;

1. No treatment
2. PBS only injection
3. CpG 1668 ODN
4. Non-CpG 1720 ODN

In another three replicate systems, 8 fish per group were assigned to one of four treatment groups;

1. No treatment
2. PBS only injection
3. CpG 1681 ODN
4. Non-CpG 1740 ODN

Fish were anaesthetised with clove oil (4.5×10^{-3} % v/v) and i.p. injected with ODN as described above. For identification, fish were tattooed using a Panjet instrument and alcian blue dye. For the AGD challenge, *Neoparamoeba* spp. were isolated according to the method described by Morrison et al (2004) and placed in the tanks at a density of 500 cells/L (Figure 1B) immediately after injection with ODN. The challenge was performed as described above.

Objective 2. To investigate role of inflammation in AGD of Atlantic salmon (Morrison et al 2006, Bridle et al 2006, Gross 2007, Morrison et al 2007, Young et al in prep.)

Immune gene expression in AGD affected salmonids (Bridle et al 2006, Morrison et al 2007)

Samples were obtained from laboratory infections and analysed as described by Bridle et al (2006) and Morrison et al (2007). Briefly, gill tissues were taken from normal and AGD-affected Atlantic salmon post-inoculation with *Neoparamoeba* spp.. Gill tissue was flash-frozen in liquid nitrogen and stored at -80ºC until required. In addition, gills from three healthy and three AGD-affected Atlantic salmon at day 38 post-inoculation with *Neoparamoeba* spp. were dissected and placed in RNA*later* RNA Stabilisation Reagent (Qiagen). From each fish, three lengths (approximately 5 mm each) of gill filament from AGD-affected and AGD-unaffected tissue was dissected under a stereo microscope. Total RNA was extracted using an RNeasy RNA extraction kit (Qiagen), a Dounce homogenizer (Wheaton Scientific, Millville, New Jersey, USA) and QIAshredders™ (Qiagen) following the manufacturer's instructions. Total RNA was reverse transcribed using oligo $(dT)_{18}$ and cloned AMV reverse transcriptase (day 2, 5, 8 samples) or SuperScript III (day 38 samples) reverse transcriptase (Invitrogen) according to the manufacturer's instructions (Morrison et al 2007). RT-PCR was conducted in 25 µL reaction volumes consisting of 0.5 µL cDNA, 2.5µL 10× buffer, 0.75 µL $MgCl_2$ (50 mM) 1 µL of each gene specific oligonucleotide (10 µM), nuclease-free water and 0.125 µL Taq polymerase (5 U/µL). Thermal cycling was optimised empirically for each set of primers. qRT-PCR was conducted in triplicate wells using 25 µL reaction volumes consisting of 1 µL cDNA, gene specific oligonucleotides (0.3 µM each), 12.5 µL 2× PCR mastermix (Quantitect™ SYBR® Green PCR kit , Qiagen) and nuclease-free water. Initially the amplification efficiency of each gene was assessed using five 3-fold dilutions of cDNA template. qRT-PCR efficiencies were calculated according to the equation $E = 10^{(-1/S)}$ where S is the slope of a standard curve generated using dilutions of template described above. cDNAs were amplified using the following thermal cycling parameters; 1 cycle of 95°C for 15 minutes followed by 40 cycles of 94°C for 20 s, 56°C for 20 s and 72°C for 20 s. All qRT-PCR reactions were subjected to post-amplification melt-curve analysis and initially PCR products from each

target were analysed by gel electrophoresis and nucleotide sequencing as described above to ensure the appropriate target cDNA was amplified. In addition, sub-samples of reverse transcriptase free cDNA samples were analysed. Amplification data were analysed by a relative method ($\Delta\Delta C_T$) after correction for discrepancy in PCR amplification efficiencies between each gene of interest and the endogenous control (β-actin) using the Relative Expression Software Tool (REST© version 2).

Presence of immune cells in AGD affected fish (Morrison et al 2006a, Gross 2007)

Immunohistochemistry was used to identify immunoglobulin (Ig) positive (Gross 2007) and MHC class II positive cells (Morrison et al 2006a) in normal and AGD affected gills of Atlantic salmon. Briefly, sections were probed with rabbit anti-Atlantic salmon immunoglobulin antibody (a gift from Dr Dina Zilberg) and the distribution of Ig bearing cells in the gills of Atlantic salmon was determined through counts of positive cells (Gross 2007). A rabbit anti-Atlantic salmon MHC II β chain antiserum (Koppang et al, 2003) was used to detect MHC II expressing cells as described by Koppang et al, (2004) and Koppang et al (2003). Samples from laboratory infections were used in this study.

Microarray experiment 1 (Morrison et al 2006b)

The methods are described by Morrison et al (2006b). Briefly, a microarray experiment was designed to comply with minimum information about a microarray experiment (MIAME) guidelines (Brazma et al 2001). A Genomics Research on Atlantic Salmon Project (GRASP) 16K array version 1 microarray chip was used (von Schalburg et al 2005). Gill tissue mRNA obtained from three replicate infected and control fish at each of the sampling points was hybridised (Morrison et al 2006b). Samples were labelled with different fluorophores, and one of the three replicates at each time was reversed (dye flip) to compensate for cyanine fluorophore bias (Morrison et al 2006b). Cluster software was used to analyse differentially expressed genes, the identities of the differentially expressed genes were verified by basic local alignment search tool query and GRASP-designated Gene Ontologies were verified manually by interrogation of UniProtKB/Swiss-Prot database (Morrison et al 2006b). Verification of differential mRNA expression was done using quantitative real-time PCR (qRT-PCR).

Microarray experiment 2 (Young et al in prep.)

Atlantic salmon were sampled at 12, 25 and 36 days post-infection. Gill lesions from AGD positive fish, normal tissue from AGD positive fish and normal tissue from negative controls were collected. Samples from 36 days post-infection were used in this microarray experiment (GRASP 16 K array version 2 microarray chip), and the expression of genes chosen on the basis of the results of the microarray experiment was investigated further in the samples from all sampling points using qRT-PCR. Results were analysed as described above (Morrisson et al 2006b).

Objective 3. To test effectiveness of vaccination against AGD using crude or partially purified antigens (Morrison and Nowak 2005, Morrison et al in prep)

Efficacy of immersion vaccination was investigated in a trial as described by Morrison and Nowak (2005). Briefly, AGD naiveAtlantic salmon (*Salmo salar*) (117 ± 5g, n = 22 fish per treatment) were anaesthetised using AQUI-S (according to the manufacturer's instructions), anchor tagged (Hallprint Pty Ltd., Victor Harbour, Australia) below the dorsal fin for identification and placed into an aerated baths containing 50 L freshwater that contained one of the following; placebo – bath only, wild type amoebae antigens (164900 cell equivalents/L), NP251002 antigens (643889 cell equivalents/L). Fish were bathed for 6 h at 17°C and transferred to a 3000 L recirculation system. Fish were acclimated to seawater (35 ‰) over a 7 d period starting at 20 d post-treatment. At 27 d post-treatment amoebae were scraped from the gills of two AGD affected fish from an experimental AGD infection tank as described by (Zilberg et al., 2001) without mucus digestion. The crude gill preparation was placed in the recirculation system at a concentration of 2867 amoebae/L. During the challenge, mortalities were examined for gross signs of AGD. Percentage of filaments affected by AGD was determined in survivors using histology (Morrison and Nowak 2005).

Objective 4. To investigate role of antibodies in AGD (Vincent et al, 2006, Vincent et al submitted, Vincent et al in prep.)

Detection of anti-*Neoparamoeba* spp. antibodies in serum and mucus

Positive and negative control serum

Serum was obtained from an Atlantic salmon that had been exposed to *Neoparamoeba* spp. and displayed overt signs of resistance. This fish presented a low level of gross gill pathology and prolonged survival in challenge conditions. Serum antibodies bound to wild-type *Neoparamoeba* spp. in an indirect enzyme linked immunosorbent assay (ELISA) and this serum was further characterised and used as a positive control in subsequent Western blot and ELISA assays. Negative control serum was pooled from 5 fish maintained in fresh water and therefore these fish were naive to AGD.

SDS-PAGE and Western blot

To identify the binding activity and specificity of serum antibody against cultured and wild-type amoebae antigens, all serum samples taken (at the end of trial 1, prior to freshwater bathing in trial 2 and at the termination of trial 2) were first processed by Western blot. Initially, pools of serum from 5 fish were screened and sera from pools returning a positive result were subsequently screened individually. Amoebae antigens were reduced in buffer containing β-mercaptoethanol by boiling for 10 min, separated through 6% polyacrylamide gels with 4 x 10^4 cells loaded in each lane (5.5 μg total protein per lane). Antigens were transferred to nitrocellulose membrane (Hybond-C extra, Amersham Biosciences, UK) using a semi-dry transfer apparatus (Hoefer Scientific Instruments, San Francisco, CA). Membranes were blocked in casein solution (Vector, Burlingame, CA, USA). Blocking and antibody incubation steps were for 30 mins and in between incubation steps, membranes were washed 3 x 4 min with tris-buffered saline (TBS, pH 7.2).

Atlantic salmon serum was applied at 1:100 (pooled) and 1:500 (individual). Bound antibodies were detected with rabbit anti-salmon IgM at 1:5000 followed by alkaline phosphatase (AP)-conjugated sheep anti-rabbit IgG (Chemicon, Australia) at 1:5000. For analysis of mucus, antigen concentration was increased to 8 x 10^4 cells per lane and mucus supernatant was diluted 1:1 in casein solution. Bound antibodies were detected with rabbit anti-salmon IgM at 1:500 and AP-conjugated sheep anti-rabbit IgG 1:2000. Mucus collected from 5 Atlantic salmon held only in fresh water was pooled and used as a negative control. Following the final antibody incubation, membranes were washed 3 × in TBS and then in 0.1M tris (pH 9.5) for 5 min. The binding of polyclonal rabbit anti-salmon IgM to Atlantic salmon IgM was initially assessed by western blotting. Normal Atlantic salmon serum and mannan-binding protein (MBP)-purified Atlantic salmon serum IgM were separated through a 12% gel. Proteins were transferred and the membrane was blocked as outlined above. The membrane was probed with polyclonal rabbit anti-salmon IgM and bound antibodies detected with AP-conjugated sheep anti-rabbit IgG as outlined above. All incubation and wash steps were conducted at 20°C. Western blots were developed by enhanced chemiluminescence (ECL) using DuoLuX (Vector), Kodak BioMax Light Film and Kodak GBX developing and fixing reagents (Sigma, Castle Hill, NSW, Australia) following the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA)

The activity of anti-*Neoparamoeba* spp. antibodies was determined by an ELISA. Wild-type *Neoparamoeba* spp. suspended in PBS were sonicated then centrifuged for 10 min at 16 000× *g* and the supernatant stored at –20°C. Protein concentration of the sonicated amoebae was determined by a colorimetric assay (Pierce, Rockford, USA). Optimal conditions for ELISA were determined empirically. Briefly, 96-well flat bottom plates (Sarstedt, Australia) were coated with 50 μL sonicated wild-type *Neoparamoeba* spp. (0.24 μg total protein/well) in coating buffer (50 mM $NaHCO_3$, pH 9.5) at 4°C overnight. Antigen was discarded and wells were blocked for 30 min at 37°C with 0.3 % casein-PBS (Sigma). All serum samples were serially diluted in 0.3 % casein-PBS in duplicate from 1:100 to 1:3200, (50 μL/well) and plates were incubated for 1 h at 20°C. Bound antigen was detected with polyclonal rabbit anti-salmon IgM at 1:500 and horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG at 1:1000 for 30 min at 37°C. The reaction was developed with 50 μL o-phenylenediamine (OPD) (Sigma) and stopped with an equal volume of 3M HCl. Positive and negative control serum was titrated from 1:100 to 1:3200 on each plate in duplicate.

Resistance post-infection and antibody presence (Vincent et al 2006)

Experiments were performed as described by Vincent et al (2006). Briefly, Atlantic salmon with an average weight of 95.2 ± 4.5g were obtained from the Saltas hatchery, Wayatinah, Tasmania and acclimated to 35‰ salinity by multiple sea water exchanges over a 10 d period. This study involved two independent trials. The first exposed Atlantic salmon to *Neoparamoeba* spp. infection at 12°C for a period of 4 weeks and was performed to obtain serum and mucus samples. The second trial was conducted to assess resistance of

Atlantic salmon to AGD challenge. The initial infection period was conducted at 12°C to maintain the infection for 4 weeks at a non-lethal level. This was to ensure that the fish in trial 2 could proceed to challenge and that the survival of these fish was not influenced by a heavy *Neoparamoeba* spp. infection when exposed to challenge condition. To establish infection, gill-derived amoebae were introduced at a concentration of 500 cells/L (Morrison et al 2004).

Fish history and blood sampling (Vincent et al submitted, Vincent et al in prep.)

Serum assessed for anti-*Neoparamoeba* spp. antibodies was taken from a total of 103 AGD-affected and 44 AGD-naive Atlantic salmon. At present, a source of wild-type *Neoparamoeba* spp. are maintained via passage through Atlantic salmon by co-habitation of AGD-naive with AGD-affected Atlantic salmon (UTAS co-habitation tank) at the University of Tasmania, Australia. The AGD-affected Atlantic salmon assessed for serum anti-*Neoparamoeba* spp. antibodies were exposed to wild-type *Neoparamoeba* spp., either in the UTAS co-habitation tank or by inoculation of the fish holding systems with wild-type *Neoparamoeba* spp. (Table 2). AGD-affected fish assessed in the current study included 17 fish collected from the UTAS co-habitation tank, 15 of these were collected as they became moribund from AGD. The remaining two fish from the UTAS co-habitation tank were larger than the tank cohort (80-150 g) and at the time of sampling weighed 580 and 340 g. These fish are here in referred to as fish one and fish two respectively. Due to their size, fish one and fish two were easily observed in the tank and it was estimated that fish one and fish two had been in the UTAS co-habitation tank for six and four months respectively. Fish transferred to this system generally become moribund from AGD within four weeks. Blood was taken from fish one once while fish two was bled four times at 4 week intervals during the 4 month period. A further two groups of AGD-affected Atlantic salmon were assessed for serum anti-*Neoparamoeba* spp. antibodies. These included 23 Atlantic salmon exposed to wild-type *Neoparamoeba* spp (500 cells/L) for 34 days and 63 AGD-affected Atlantic salmon exposed to wild-type *Neoparamoeba* spp. (1152 cells/L) for 72 days in the experiment described by Bridle et al (2005). *Neoparamoeba* spp. are obligate marine organisms, therefore sera from 44 Atlantic salmon maintained only in fresh water was tested to assess if natural antibodies present bound amoeba antigens. The mode and duration of exposure and holding conditions of the fish from which the sera assessed in the current study are summarised in Table 2.

Blood was also obtained from farmed Atlantic salmon after 8, 10 and 13 months of sea-cage culture and assessed for the presence of anti-*Neoparamoeba* spp. (anti-NP) antibodies (Vincent et al submitted).

Table 2. Serum from AGD-affected and AGD-naive Atlantic salmon was assessed for anti-*Neoparamoeba* spp., antibodies. The number of fish sampled, the duration of exposure to wild-type *Neoparamoeba* spp. and fish holding conditions are summarised.

Number of fish	Mode of exposure	Weeks exposed	Salinity (‰)	Water Temp. (°C)
17	Co-habitation	3-24[+]	35	16
23	Inoculation (500 cells/L)	4.8	35	16
63	Inoculation[1] (1152 cells/L)	10.3	35	16
44	Not exposed	N/A	0	14-16

[1] Fish sampled from the experiment described by Bridle et al., [16].
[+] Estimated exposure time.

Results/Discussion

Objective 1. To evaluate use of immunostimulants for control of AGD of Atlantic salmon

CpGs as immunostimulant

Previously, we showed that i.p. injection with ODNs containing unmethylated CpG motifs enhanced resistance of Atlantic salmon to amoebic gill disease (AGD) (Bridle et al 2003). In this study, the reproducibility of this CpG DNA-mediated protection against AGD was assessed. Furthermore, we took advantage of recently completed studies in which 24 ODNs containing CpG motifs were evaluated for their immunostimulatory capacity in Atlantic salmon (Jorgensen et al 2003, Strandskog et al 2007). The best overall performing ODN (CpG 1681) induced proliferation of peripheral blood leucocytes, elicited interferon-like activity from head kidney leucocytes, up-regulated head kidney leucocyte Mx mRNA expression (indirect evidence of IFNα/β activity), and enhanced protection against a challenge with infectious pancreatic necrosis virus (Jorgensen et al 2003, Strandskog et al 2007). This ODN (CpG 1681) was therefore incorporated into the current trial. During trial 1 there was a trend which saw fish in both control groups (no treatment and PBS injection) succumb to AGD-related morbidity faster than their CpG ODN and non-CpG ODN-treated counterparts (Figure 2). When end-point analysis of the relative survival was performed, this effect was clearly evident (Table 3) with the CPG 1668 ODN delivering 48.6% RPS on a combined tank basis. This was compared to 20.8% for the 1668 non-CpG control (1720 ODN). Interestingly, fish treated with the other CpG ODN (1681) performed poorer than fish treated with the non-CpG (1740) control (Table 3).

During the first experiment, fish were treated as described by Bridle et al (2003) which included a 6 day delay between treatment and challenge. Therefore, a second trial was conducted to determine if this delay may reduce the CPG-mediated effect on resistance. Rather than enhancing the resistance to AGD, there were no demonstrable effects of CpG ODN treatment on survival (Figures 3 & 4).

Table 3. Relative survival[1] (%) of CpG ODN treated and *Neoparamoeba* spp. challenged Atlantic salmon.

	Tank 1	Tank 2	Tanks 1 & 2 combined
CpG 1668 ODN	52.5	42.4	48.6
Non-CpG 1720 ODN	15.0	31.2	20.8
CpG 1681 ODN	28.8	37.6	33.3
Non-CpG 1740 ODN	50.0	41.0	45.8

[1] Relative to fish injected with PBS.

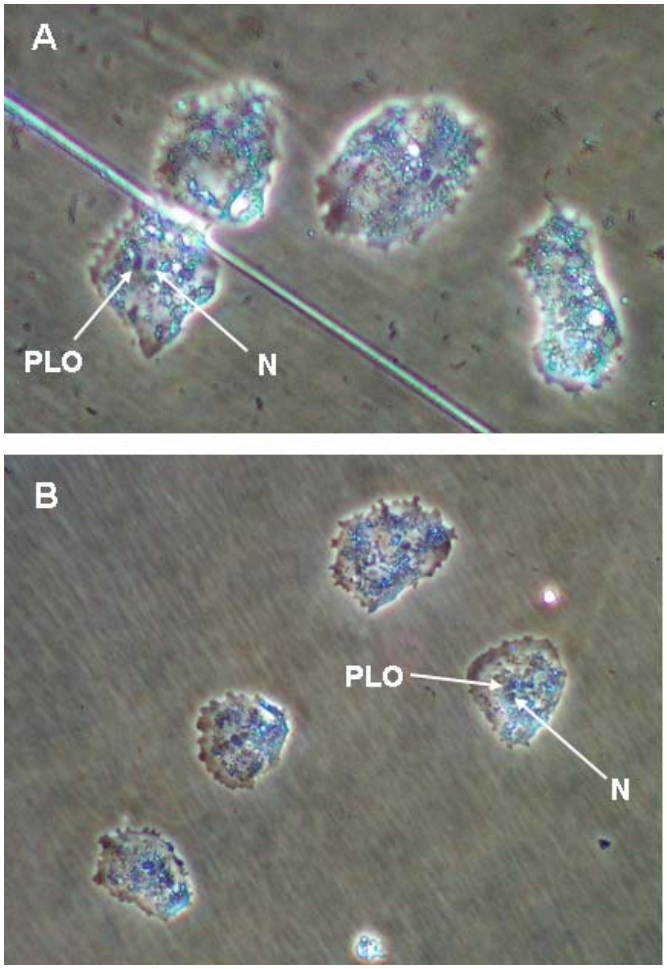

Figure 1. Plastic adhered trophozoites used to induce amoebic gill disease (AGD) in CpG-treated Atlantic salmon. Trophozoites were isolated from AGD-affected Atlantic salmon according to the method of Morrison et al (2004). A – trophozoites used in experiment 1, B – trophozoites used in experiment 2. PLO – *Perkinsiella amoeba*-like organism, N – nucleus.

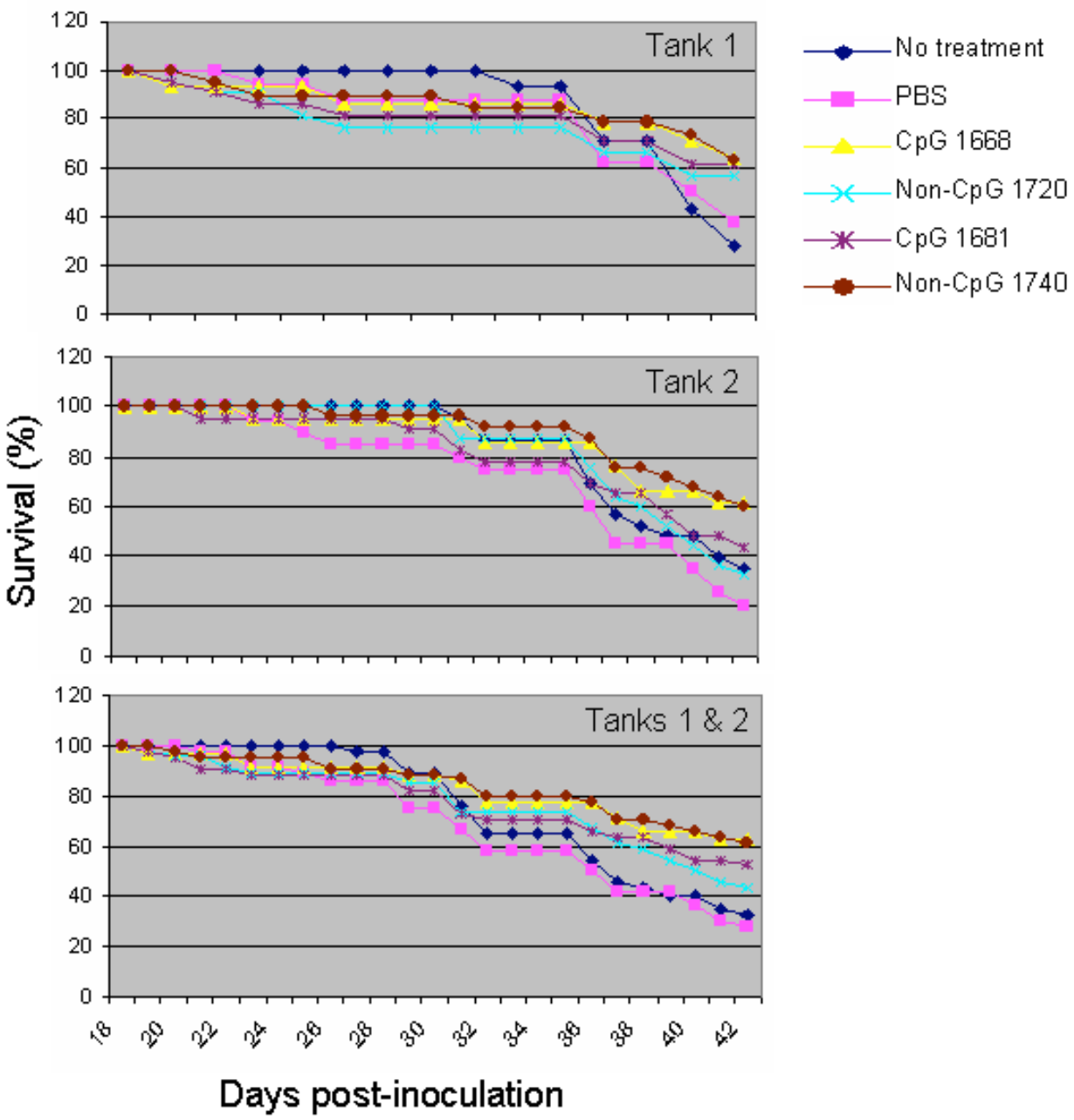

Figure 2. CpG-mediated improvement in the survival of Atlantic salmon against amoebic gill disease (AGD). Atlantic salmon were intraperitoneally injected with oligonucleotides containing CpG (1668 or 1681 ODN) or non CpG (1720 or 1740 ODN) motifs. Six days post-injection, duplicate 4000 L recirculation systems housing the Atlantic salmon were inoculated with 370 cells/L *Neoparamoeba* spp.. Survival of fish is presented on an individual and a combined tank basis. Data are from day 18 post-inoculation when AGD-related morbidity occurred.

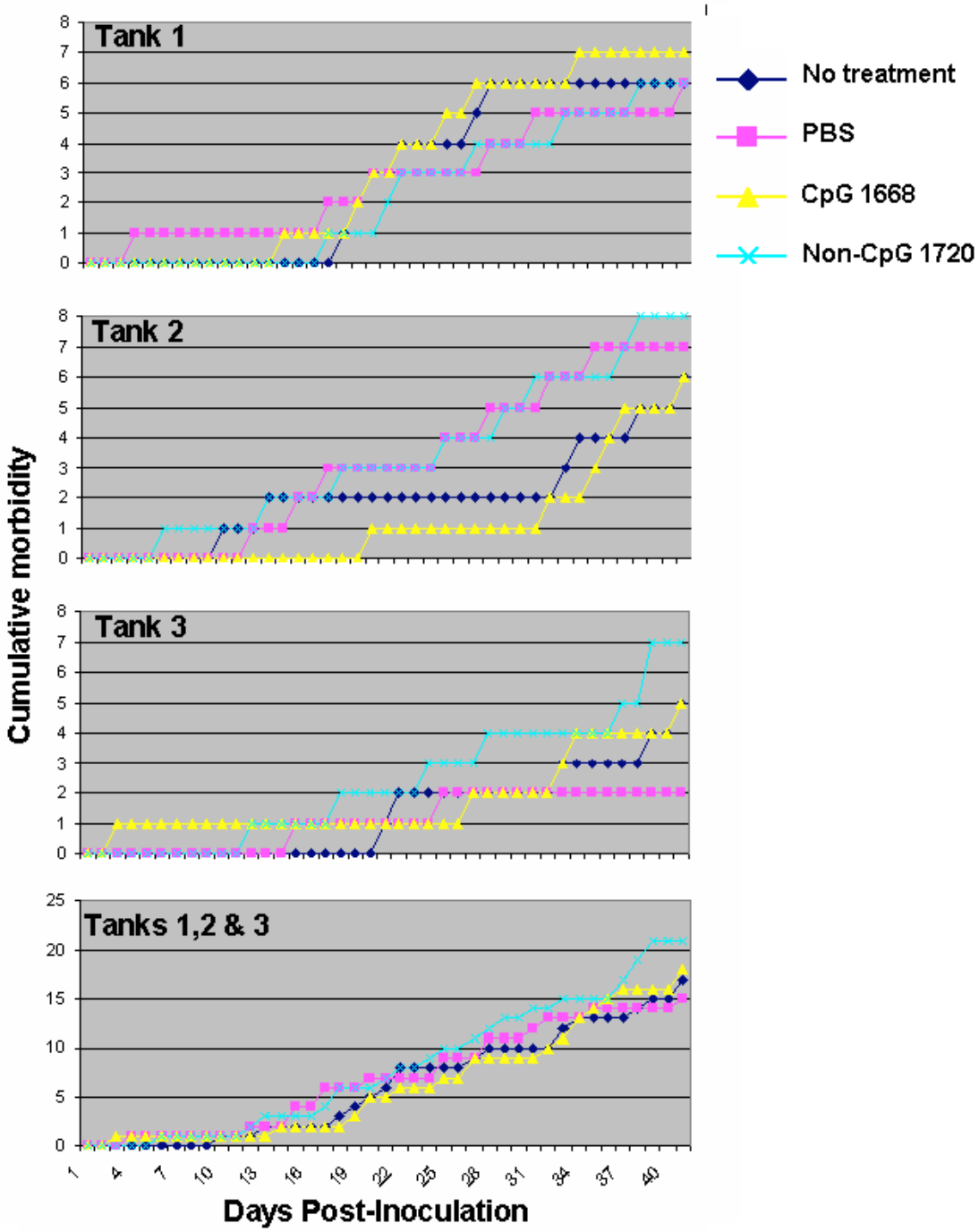

Figure 3. CpG ODN-mediated resistance against amoebic gill disease (AGD) is not reproducible in small recirculation systems. Atlantic salmon were intraperitoneally injected with oligonucleotides containing CpG (1668 ODN) or non CpG (1720 ODN) motifs. Immediately post-injection, triplicate 650 L recirculation systems housing the Atlantic salmon were inoculated with 500 cells/L *Neoparamoeba* spp.. Cumulative morbidity of fish is presented on an individual and a combined tank basis.

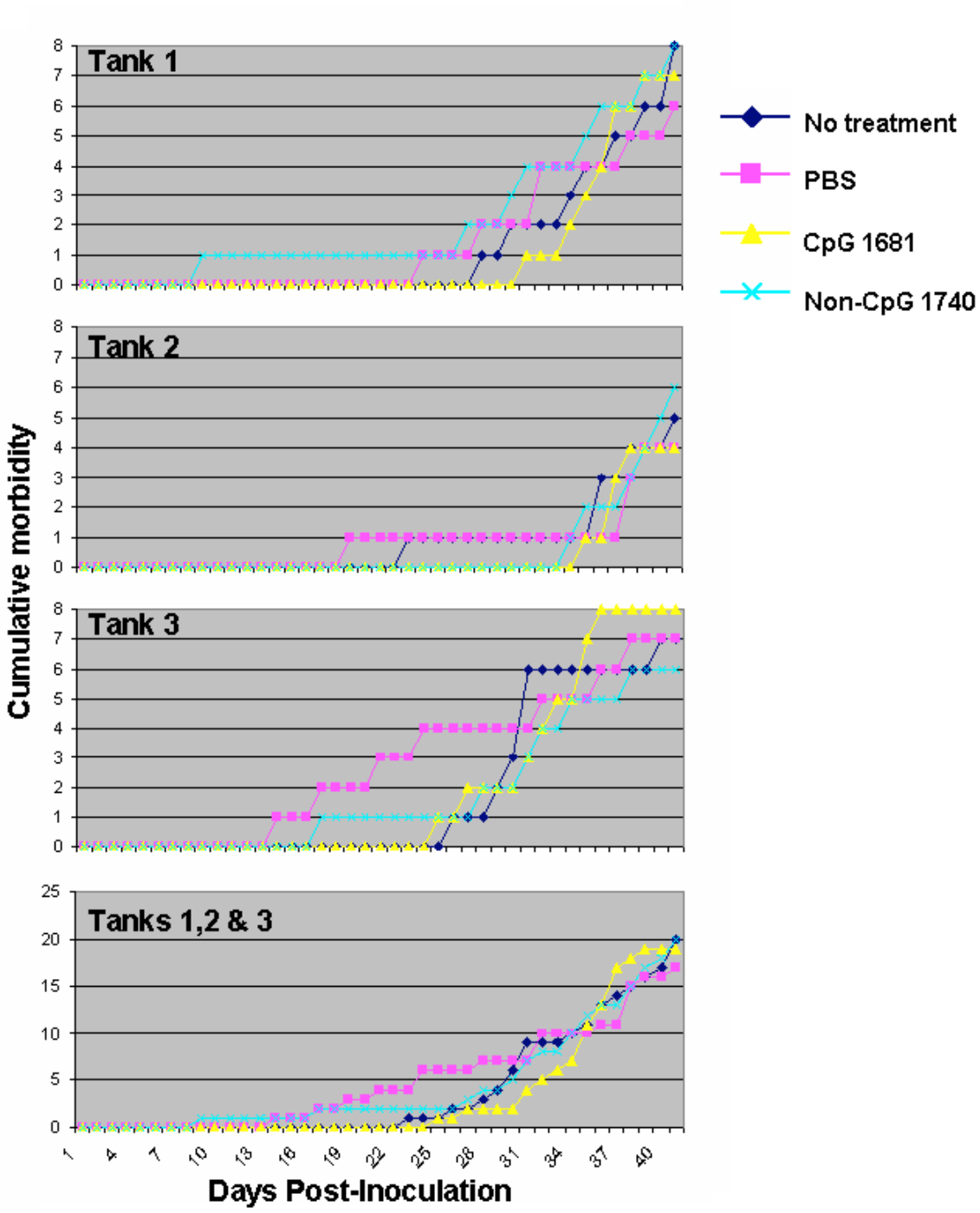

Figure 4. CpG ODN-mediated resistance against amoebic gill disease (AGD) is not reproducible in small recirculation systems. Atlantic salmon were intraperitoneally injected with oligonucleotides containing CpG (1681 ODN) or non CpG (1740 ODN) motifs. Immediately post-injection, triplicate 650 L recirculation systems housing the Atlantic salmon were inoculated with 500 cells/L *Neoparamoeba* spp.. Cumulative morbidity of fish is presented on an individual and a combined tank basis.

These results are in agreement with industry farm trials, which tested the effects of commercially available glucan-enriched diet and nucleotide-containing diet on AGD and gill health. The use of these immunostimulant-containing diets did not result in an increase in freshwater bath interval or improved gill health (D. Mitchell, pers. comm.). This suggests that while immunostimulants can have positive effects in laboratory experiments, their commercial benefits are limited by our knowledge of timing of the disease outbreakand therefore best timing for application of the immunostimulants. We were previously unable to demonstrate an improved survival of fish fed beta-glucans or mixed dietary immunostimulants and challenged with AGD (Nowak et al 2004, Bridle et al 2005).

Objective 2. To investigate role of inflammation in AGD of Atlantic salmon (Morrison et al 2006, Bridle et al 2006, Gross 2007, Morrison et al 2007, Young et al in prep)

Immune gene expression in AGD affected salmonids (Bridle et al 2006, Morrison et al 2007)

Interleukin-1 beta (IL-1β) expression was shown to be consistently induced in gills of AGD affected Atlantic salmon (Bridle et al 2006b, Morrison et al 2007). IL-1β expression was restricted to epithelium in gill lesions (Bridle et al 2006, Morrison et al 2007). Previously, it was shown that rainbow trout up-regulate both IL-1β and iNOS mRNAs in the gill during *Neoparamoeba* spp. infection (Bridle et al 2006a). This led to a hypothesis that IL-1β may induce nuclear factor-kappaB (NF-κB) which in turn modulates iNOS mRNA. In amoebiasis-affected mammals, iNOS up-regulation leads to NO-dependent cellular cytotoxicity against *E. histolytica,* a process that is initiated by TNF-α, together with IFN-γ (Seguin et al 1997). Two Atlantic salmon TNF-α transcripts designated TNF-α1 and TNF-α2 together with their respective genes were cloned and sequenced (Morrison et al 2007). During the early onset of AGD in Atlantic salmon, there were no demonstrable differences in the gill tissue expression of TNF-α1, TNF-α2 nor the interleukin-1 beta (IL-1β), inducible nitric oxide synthase (iNOS) and interferon gamma (IFN-γ) mRNAs compared to tissue from healthy fish (Morrison et al 2007). In Atlantic salmon with advanced AGD, IL-1β but not TNF-α1 or TNF-α2 mRNAs was up-regulated in lesions (Morrison et al 2007). Given that *Neoparamoeba* spp. modulated both TNF-α2 and IL-1β in head kidney leucocytes *in vitro*, it appears either the parasite can influence the cytokine response during infection, or there is ineffective signalling for TNF-α expression, or there are too few cells at the site of infection with the capacity to produce TNF-α (Morrison et al 2007). The lesion-restricted modulation of IL-1β in the absence of any transcriptional change in TNF-α1 and 2, IFN-γ and iNOS indicated heterogeneous modulation of pro-inflammatory cytokines during AGD and supported the notion that significant transcriptional change during AGD occurs in directly affected tissue (Morrison et al 2007). This suggests inhibition of inflammatory process in AGD affected fish, including either an inhibition of IL1β induced NFκβ activation or the process may be inhibited at PRR signalling level. Therefore the biological significance of IL-1β expression in AGD is currently unknown.

Presence of immune cells in AGD affected fish (Morrison et al 2006a, Gross 2007)

There was no significant effect of AGD on the presence of Ig bearing cells. Ig bearing cells were found in all areas of normal uninfected fish gill. Few Ig bearing cells were present in AGD lesions and there was no evidence of the presence of Ig bearing cells in the interlamellar vesicles (Gross 2007). In contrast, an increase in the numbers of MHC II cells was observed in AGD lesions in comparison to normal tissue (Morrison et al 2006a). These cells exhibited increased but variable levels of expression and were predominantly mononuclear cells, possibly macrophages, epithelial cells or dendritic cells (Morrison et al 2006a). These cells were not considered to be B cells, as Ig bearing cells were uncommon in AGD lesions. This presence of MHC class II cells suggests immune cell trafficking and potential contribution of these cells to antigen presenting capacity in fish chronically affected by AGD.

Microarray experiment 1 (Morrison et al 2006b)

Two hundred and six genes, representing 190 unique transcripts were identified as upregulated or downregulated in AGD affected fish (Morrison et al 2006b). Anterior gradient -2 was differentially expressed and its upregulation at 114 and 189 hours post inoculation was restricted to AGD lesions relative to normal tissue from the same gill arch (Morrison et al 2006b). In humans anterior gradient has been implicated in inhibiting tumor suppressor protein p53 (Pohler et al 2004), which was downregulated in AGD lesions (Morrison et al 2006b). Differential expression of transcripts involved in p53 signalling pathway (growth arrest and DNA damage inducible gene (GADD45 beta) – downregulated (2.1) in AGD lesions and Proliferating Cell Nuclear Antigen PCNA – upregulated (2.9) in AGD lesions) suggests a role for p53 in AGD pathogenesis (Morrison et al 2006b). This would explain the hyperplastic reaction in AGD. Furthermore, downregulation of immune response genes (for example mannose-binding protein C, LPS-binding protein and NADPH oxidase cytosolic protein p40phox) was observed post-infection, suggesting potential immune evasion strategy used by the amoebae.

Microarray experiment 2 (Young et al in prep.)

Figure 5 shows ontology of total genes regulated in the experiment, most of the identified genes were responsible for metabolic processes and immune response.

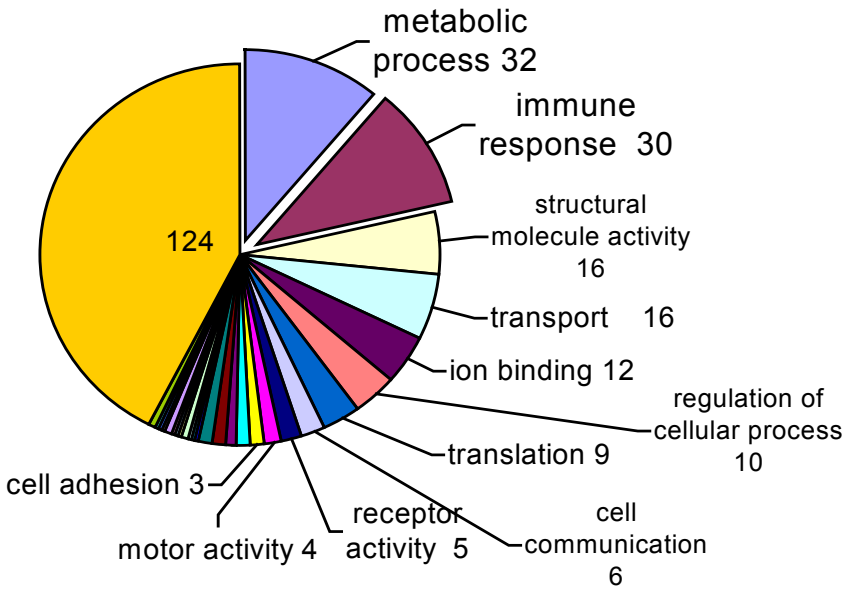

Figure 5. Ontology of total genes regulated in the experiment (Young et al in prep.)

Forty four genes were significantly upregulated and 114 downregulated in the AGD lesions in comparison to normal tissue from the same fish. Genes involved in cytosolic antigen presentation which were downregulated included:

- PA28 (degradation of antigen)
- MHC Class I – UBA, ZE - classical
- β-2- microglobulin (β2M)
- Tapasin binding protein (TAPBP)
- TAP2B – peptide transport to ER

Genes involved in endocytosed antigen presentation which were downregulated included:

- MHC Class II – DAA and DAB
- Invariant chain – 14-1, S25-7, INVX

In contrast to the first microarray experiment, the second experiment focused on later stages of the disease and compared gill lesions with normal tissue. However, despite these differences the results confirmed our previous observations (Morrison et al 2006b, Table 4).

Table 4. Comparison of microarray results

Genes	Whole gill tissue (Morrison et al 2006b)	Lesions only (Young et al in prep.)
Differentially regulated trout protein	2.31 ↑	2.82 ↑
Anterior gradient 2-like protein	2.01 to 2.57 ↑	2.15 to 2.52 ↑
TIMP-2	7.67 ↓	2.32 ↓
Brain protein 44	2.36 ↓	2.12 ↓
Guanine-nucleotide binding protein	2.15 ↓	2.63 to 3.57 ↓
β-2- microglobulin	3.08 ↓	2.06 to 2.56 ↓
Na/K ATPase	2.32 ↓	3.12 to 6.10 ↓

Objective 3. To test effectiveness of vaccination against AGD using crude or partially purified antigens (Morrison and Nowak 2005, Morrison et al in prep)

Atlantic salmon bathed in amoebae antigens from either an avirulent *in vitro* cultured strain of *Neoparamoeba pemaquidensis* or *Neoparamoeba perurans* (wild-type) and challenged with gill-derived amoebae 27 days post-treatment did not show enhanced survival or reduced proportion of AGD-affected gill filaments (Morrison and Nowak 2005). The development of fish vaccines has been predominately based upon vaccination trials with inactivated cell preparations, particularly in the case of bacterial pathogens. This approach has led to the commercialisation of vaccines for bacterial diseases including vibriosis, yersiniosis and furunculosis (Hastein et al 2005). Atlantic salmon develop a serum antibody response after injection with cultured *N. pemaquidensis* (Bryant et al 1995, Akhlaghi et al 1996) or crude wild-type *Neoparamoeba* spp. (Akhlaghi et al 1996). However antibodies were only detected after binding to cultured *N. pemaquidensis* and binding of antibodies to wild-type *Neoparamoeba* spp. antigens was not confirmed. It is now known that only the recently described species *Neoparmoeba perurans* is associated with AGD lesions (Young et al 2007). All cultured gill-derived *Neoparamoeba* spp. tested so far for pathogenicity are avirulent (Kent et al 1988, Howard et al 1993, Findlay 2001, Morrison et al 2005, Vincent et al 2007) and so far attempts to culture *Neoparamoeba perurans* have been unsuccessful (Young et al 2007). The use of cultured antigens for immunisation studies or to characterise the antibody response of Atlantic salmon exposed to *Neoparamoeba* spp. may fail to identify antigens of the predominant agent of AGD, *N. perurans,* that may be relevant *in vivo*. Immunisation of Atlantic salmon with wild-type *Neoparamoeba* spp. did not confer resistance to AGD (Akhlaghi et al 1996, Zilberg and Munday 2001), however immunisations contained very low numbers of wild-type *Neoparamoeba* spp.. Obtaining wild-type *Neoparamoeba* spp. in numbers required for immunisation studies is a limiting factor as the only source is infected host fish. It is difficult to estimate

the amount of wild-type *Neoparamoeba* spp. antigen required to stimulate the development of an antibody response in Atlantic salmon. Rainbow trout immunised with sea lice (*Lepeophtheirus salmonis*) developed a detectable serum antibody response after three immunisations (500 μg protein/immunisation/fish) with adjuvant. To immunise a single fish with *Neoparamoeba* spp. antigens at this protein concentration it would take up to 1 month of isolating wild-type *Neoparamoeba* spp. from the gill tissues of 30-60 AGD-affected Atlantic salmon. This was considered to be unrealistic and logistically impossible, therefore this objective was abandoned and objective 4 was included.

Objective 4. To investigate role of antibodies in AGD

Presence of serum anti-*Neoparamoeba* spp. antibodies (Vincent et al 2006, Vincent et al submitted, Vincent et al in prep.)

Atlantic salmon exposed and subsequently challenged with AGD are more resistant than naive control fish (Vincent et al 2006). Seventy three percent of Atlantic salmon previously exposed to AGD survived to day 35 post-challenge compared to 26% exposed to *Neoparamoeba* spp. for the first time, yet the gill pathology of surviving naive control or previously exposed fish was not significantly different. Development of resistance to AGD is associated with anti-*Neoparamoeba* spp. antibodies that were detectable in serum of 50% of surviving Atlantic salmon previously exposed to AGD. However, anti-*Neoparamoeba* spp. antibodies were not detectable in cutaneous mucus of resistant fish (Vincent et al 2006).

Antibodies that bound wild-type *Neoparamoeba* spp. antigens were detected by Western blotting in the sera of five out of 103 fish (Vincent et al in prep.). These included samples obtained from two fish from the UTAS co-habitation tank (fish one and fish two) and three fish from the experiment described by Bridle et al (2005). Fish one and fish two serum antibodies were specific to wild-type *Neoparamoeba* spp. yet distinctively different binding profiles were produced in Western blot. Binding of serum antibodies of fish one by Western blot produced two bands >200 kDa and serum antibodies of fish two produced a smear across a broad molecular weight range. Sodium periodate oxidation of wild-type antigens was performed to assess the binding of anti-*Neoparamoeba* spp. antibodies to peptide or carbohydrate epitope(s). Antibodies present in the serum of fish one were directed towards epitope(s) that were not sensitive to periodate oxidation while antibodies in the serum of fish two failed to bind periodate-treated wild-type *Neoparamoeba* spp. antigens. Similarly, antibodies present in the three sero-positive fish from the experiment described by Bridle et al (2005) were specific to wild-type *Neoparamoeba* spp. and produced a smear across a broad molecular weight range. In addition, these antibodies failed to bind wild-type *Neoparamoeba* spp. antigens after periodate oxidation. In the experiment described by (Bridle et al 2005), β-glucan diets were administered and there was no effect of diet on the susceptibility of Atlantic salmon to AGD. The sero-positive fish identified here were from the control group and were fed commercial Atlantic salmon feed. The five sero-positive samples were also screened against Atlantic salmon cutaneous mucus supernatant obtained from AGD-naive Atlantic salmon to identify potential cross-reactivity with normal flora residing

in the host mucus and no binding was observed (data not shown). In addition to the normal serum controls ran in parallel with the test sera, sera from a further 44 AGD-naive Atlantic salmon was assessed for presence of natural antibodies that may bind *Neoparamoeba* spp. antigens. Antibodies present in the sera of 44 AGD-naive Atlantic salmon did not bind wild-type or cultured *Neoparamoeba* spp. antigens.

The only samples containing anti-*Neoparamoeba* spp. antibodies with measurable activity according to the method outlined by Arkoosh and Kaattari (1990) by an ELISA were those of fish one and fish two. Due to the larger volume of sera attained from fish two, fish two sera was used as the positive control. Binding of anti-*Neoparamoeba* spp. antibodies present in the serum of fish one produced an optical density similar to the positive control sera. At a serum dilution of 1:100, the mean optical density produced by the AGD-naive serum was 0.19 (± SEM 0.00); at the same serum dilution, antibodies present in the serum of fish one and fish two (the positive control sera) produced optical densities of 0.79 (± SEM 0.03) 0.68 (± SEM 0.02) respectively. Whilst the optical density produced at the serum dilution of 1:100 was higher for fish one, anti-*Neoparamoeba* spp., the antibody activity of both fish one and fish two serum was equal at 7.7 units/μl of serum. Further analysis of anti-*Neoparamoeba* spp. antibodies was restricted to the serum samples from fish one and fish two with measurable antibody activity. Fish one and fish two anti-*Neoparamoeba* spp. antibodies bound cell-surface epitope(s) of wild-type *Neoparamoeba* spp. producing intense fluorescence around the cell margin. These data suggest that some Atlantic salmon can develop a serum antibody response to wild-type *Neoparamoeba* spp. infection, however the development of an antibody response with measurable activity by an ELISA is rare.

For the farmed Atlantic salmon an increase in the proportion of fish developing a detectable antibody response to wild-type *Neoparamoeba* spp. was seen over time in culture (Vincent et al submitted). While the interval between bath treatments increased over time in culture, this corresponded to the seasonal reduction in water temperature at the culture site. A further group of putatively AGD-resistant Atlantic salmon broodstock was sampled at 15 months after transfer to sea and anti-NP antibodies were detected in 81% of these samples. The broodstock did not present any gross gill pathology and had not required freshwater bath treatment for over 250 days. Anti-NP antibodies in all sero-positive fish identified here bound cell-surface carbohydrate antigens yet an antibody titre was not detected in any samples by ELISA (Vincent et al submitted). This is consistent with the paucity of Ig bearing cells in gills during AGD infection (Gross 2007). These results provide further evidence for the development of an antibody response in AGD-affected Atlantic salmon and that carbohydrate epitopes of wild-type *Neoparamoeba* spp. are immunodominant in Atlantic salmon.

Benefits and Adoptions

This project directly benefits the salmon industry by contributing to our understanding of the role of inflammation and immune response in AGD. It has direct implications for AGD management and AGD research, including AGD vaccine development. The benefits include improved knowledge of AGD and new methods which can be applied in future salmon health research. We are currently in the process of preparing a funding proposal for research on yersiniosis, which is based on our experience gained from this project. We have provided an up to date review of the use of immunostimulants in finfish growout, which can be easily used by the aquaculture industry.

This project has indirect benefit for other aquaculture sectors in Australia. It provides research methods and knowledge that can be applied to other fish, particularly those farmed in marine pens. Some of these methods will be applied in research on Southern Bluefin Tuna health.

Aquatic Animal Health research (human capital development) in Australia also benefited from this project through training of one postdoctoral fellow (promoted to research fellow position by University of Tasmania) and three PhD students. This training included extensive international collaborations and research done in leading overseas laboratories. These international collaborations, developed during this project, will continue in future research.

Further Development

While this project has increased our understanding of the effects of immunomodulation on AGD, it has also identified knowledge gaps. These include a lack of understanding of the mechanisms of inhibition of inflammatory and immune pathways, the significance of antibody response (if any) in AGD, and the potential for vaccine antigen discovery through the use of anti-peptide antibody or inhibition of amoeba attachment to the gill epithelium. The potential that a more localised antibody response in the gill mucus or epithelium may play a role in resistance of Atlantic salmon to AGD warrants further investigation.

Results of this project have been widely disseminated throughout the salmon industry through industry meetings, workshops and through the Aquafin CRC and FRDC Salmon Aquaculture Subprogram conferences. The results can be exploited commercially by individual companies if they choose to adopt any of the suggested strategies. The results have been published in peer-reviewed international scientific journals.

Planned Outcomes

1. To increase the sustainability of the aquaculture industry through improved health due to the use of immunostimulants
While the results of our project suggest that the performance of immunostimulants in AGD control has been inconsistent, other aspects of our research offer promise. However, these would require further investigation before they can be commercially applied.

2. To increase the knowledge base of fish immunology
We have signficantly increased knowledge base of fish immunology, in particular our understanding of immune response in Amoebic Gill Disease. We have published the first paper on transcriptome profiling in a fish parasitic disease.

This project significantly contributed to achieving the CRC contract outcome: "Improved treatments and disease management as a result of better understanding of host-pathogen interaction". Through the application of microarrays and functional experiments, this project significantly increased our understanding of host-pathogen interactions. Results of this project provided evidence that immunostimulants, if powerful enough and provided at a correct time, improved survival during AGD challenge by 50%. Furthermore, this project has contributed to antigen discovery for vaccine development.

Conclusions

Objective 1. To evaluate use of immunostimulants for control of AGD of Atlantic salmon

In a laboratory experiment, Atlantic salmon intraperitoneally (i.p.) injected with CpG oligodioxynucleotides (50 µg/fish) six days before AGD challenge, showed RPS ranging from 28.8 to 52.5%. However, this effect was absent if fish were challenged immediately post-injection. This indicates that while there is some potential for the use of immunostimulants, their commercial application is limited as it relies on detailed understanding of the timing of disease outbreaks. These results are consistent with inconclusive results from commercial trials of in-feed immunostimulants.

Objective 2. To investigate role of inflammation in AGD of Atlantic salmon

In Amoebic Gill Disease epithelial cell hyperplasia continues unabated until death suggesting that there may be loss of cell-cycle control. This hypothesis is further supported by down-regulation of tumour suppressor protein p53 gene while anterior gradient-2, a protein that inhibits p53 phosphorylation in humans is up-regulated in AGD lesions. IL-1β is up-regulated in the gills of AGD-affected Atlantic salmon and rainbow trout and may be important in this regard. TNF-α has the capacity to mediate the proliferation of epithelial cells in mammals and also head kidney leucocytes of fish yet in the face of on-going cell division in the gills of AGD-affected fish, there is no evidence that TNF-α may be involved in the propagation of lesions. Since Atlantic salmon head kidney leucocytes produce TNF-α2 mRNA upon stimulation with *Neoparamoeba* spp., the transcriptional quiescence of TNF-α mRNA in AGD-affected tissue may be driven by factors other than immunological refractiveness to the amoebae. This suggests inhibition of inflammatory response in AGD affected fish.

Objective 3. To test effectiveness of vaccination against AGD using crude or partially purified antigens

Atlantic salmon bath inoculated with amoebae antigens from either virulent or avirulent cells did not show enhance protection against subsequent AGD challenge. Bath vaccination did not improve survival of the fish, possibly due to too low dose of antigen or inaccessibility of antigen to the fish's immune system. To immunise a single fish with *Neoparamoeba* spp. antigens at the protein concentration as used in other experimental antiparasitic vaccines developed for fish it would take up to 1 month of isolating wild-type *Neoparamoeba* spp. from the gill tissues of 30-60 AGD-affected Atlantic salmon. Due to the high requirement for amoebae, the vaccination using crude antigen has been considered logistically difficult. This resulted in abandoning this objective and adding objective 4.

Objective 4. To investigate role of antibodies in AGD

Carbohydrate epitope(s) of wild-type *Neoparamoeba* spp. appear to be immunodominant in Atlantic salmon and the development of anti-peptide antibodies specific to wild-type *Neoparamoeba* spp. is, at this point, an isolated finding. The duration of exposure (or multiple exposures) to wild-type *Neoparamoeba* spp. appears to be important for the development of a serum antibody response in AGD-affected Atlantic salmon. This suggests that the identification of peptide candidate vaccine antigens by screening serum against wild-type *Neoparamoeba* spp. is unlikely. However, it is still possible that a more localised antibody response, in the gill mucus or epithelium (so far undetected), may play a role in resistance of Atlantic salmon to AGD.

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Appendix 1

INTELLECTUAL PROPERTY

The intellectual property and valuable information arising from this report are:

1. Copyright in this report

Appendix 2

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Appendix 3

POTENTIAL FOR USE OF IMMUNOSTIMULANTS IN FINFISH GROWOUT

Richard N. Morrison and Barbara F. Nowak

Literature Review

December 2007

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List of abbreviations

↑ - increase
↓ - decrease
→ - no effect
bDNA – bacterial DNA
CHSE –Chinook salmon embryo cells
CL - chemiluminescence
COX-2 – cyclo-oxygenase 2
dph – days post- hatch
EST - expressed sequence tag
FCA – Freund's complete adjuvant
FIA - Freund's incomplete adjuvant
HSP – heat shock protein
HKL – head kidney leucocyte
IFN - interferon
IgM – immunoglobulin M isotype
IHNV - infectious haematopoietic necrosis virus
IL-1β – interleukin-1 beta
IM - intramuscular
IP - intraperitoneal
IPNV - infectious pancreatic necrosis virus
ISAV – infectious salmon anaemia virus
IV - intravascular
LPS - lipopolysaccharide
Mø – macrophage
MH - major histocompatibility
MPO - myeloperoxidase
NCC – non-specific cytotoxic
O^{2-} - superoxide anion
ODN - oligodeoxynucleotides
NBT – nitroblue tetrazolium
PBLs – peripheral blood leucocytes
PMA – phorbol 12-myristate 13-acetate
PRR - pattern recognition receptors
RAG1 – recombinase activating gene-1
RBC – red blood cell (erythrocyte)
SGR – specific growth rate
SHK-1 – Atlantic salmon head kidney cell line
α-SRBC – anti-sheep red blood cell
SRBC – sheep red blood cell
TNFα – tumour necrosis factor-alpha
TO – Atlantic salmon head kidney primary cell line
TLR - toll-like receptor

Introduction

Finfish aquaculture is an important primary industry both in Australia and beyond. Fish grown using intensive production methods can be susceptible to infectious diseases and while antibiotics and other chemicals offer therapeutic benefit, the effects of chemical residues in fish and the surrounding environment are of broad community concern. This concern, together with restrictions in production due to disease, has been the impetus for the identification of novel disease prevention strategies. Logically, research and development have focussed upon vaccines, and the production of efficacious vaccines has largely underpinned substantial growth in aquaculture productivity during the past 20 years (Sommerset et al. 2005). Vaccines offer protection against specific pathogens, yet for many years it has been proposed that the immune system of fish can be stimulated to elicit pan-specific immunity. This is achieved by the administration of so-called "immunostimulants". An immunostimulant according to (Bricknell & Dalmo 2005) is "a naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens". Indeed, many immunostimulants have the capacity to increase the anti-microbial activity of fish leucocytes *in vitro* and increase resistance to disease *in vivo* (frequently reviewed, Table 1). Immunostimulants activate the production of reactive oxygen (O^{2-}) and nitrogen (NO) molecules, lysozyme, complement and bactericidal activities. They also enhance phagocytosis, chemotaxis, cellular cytotoxicity and proliferative (mitogenic) abilities of leucocytes. In addition, immunostimulants up-regulate the mRNA expression of important pro-inflammatory genes such

as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNFα). Together, activation of these immune defence mechanisms may elicit protection against subsequent infections that would otherwise cause chronic or acute disease (Raa 1996). These demonstrable benefits are encouraging and support the immunostimulation concept, yet the question remains as to whether the use of immunostimulants is really beneficial for finfish aquaculture or merely an elegant concept. This review explores the current literature base on immunostimulants with particular emphasis on their potential for use in finfish aquaculture.

Table 1. Reviews on immunostimulants for finfish.

Title of Review	Reference
A review of CpGs and their relevance to aquaculture	(Carrington & Secombes 2006)
Nucleotide nutrition in fish: Current knowledge and future applications	(Li & Gatlin 2006)
Current research on the immunostimulatory effects of CpG oligodeoxynucleotides in fish	(Tassakka & Sakai 2005)
The use of immunostimulants in fish larval aquaculture	(Bricknell & Dalmo 2005)
The mode of action and use of immunostimulants in fish and shellfish farming	(Raa 2001)
The use of immuno-stimulants in fish and shellfish feeds	(Raa 2000)
Current research status of fish immunostimulants	(Sakai 1999)
Immunostimulants in fish diets	(Gannam & Schrock 1999)
Some aspects of the application of immunostimulants and a critical review of methods for their evaluation	(Galeotti 1998)
Adjuvants and immunostimulants for enhancing vaccine potency	(Anderson 1997)
The use of immunostimulatory substances in fish and shellfish farming	(Raa 1996)
Immunostimulants, adjuvants and vaccine carriers in fish: Applications to aquaculture	(Anderson 1992)
The use of immunostimulants to increase resistance of aquatic organisms to microbial infections	(Raa et al. 1990)

Immunostimulants, vaccines and adjuvants

An immunostimulant in the context of this review is considered a substance with the capacity to activate anti-microbial effector mechanisms independent of major histocompatibility (MH) restriction. That is, up-regulation of innate or non-combinatorial immune effectors, independent of antigen recognition by either B (membrane bound immunoglobulin) or T cell receptors. An adjuvant on the other hand is a substance or substances administered together with vaccine antigen(s). Thus an immunostimulant can be an adjuvant and *vice versa*. A vaccine elicits pathogen-specific immunity after the administration of a whole pathogen, a pathogen-derived component, a recombinantly produced pathogen-derived component or a plasmid that encodes a pathogen-derived protein. For the purposes of this review, only immunostimulants and not adjuvants will be assessed.

How do immunostimulants work?

It has been known for decades that microbes activate the immune system yet the mechanisms by which activation occurs have only recently been discovered. Conserved microbial structures are recognised by so-called pattern recognition receptors (PRR) on cells of the immune system. These include toll-like receptors (TLR) as well as others such as Dectin-1, the β-glucans receptor (Brown 2006), macrophage scavenger receptors (Greaves & Gordon 2005) and the mannose receptor (Apostolopoulos & McKenzie 2001). A diverse range of microbial patterns bind TLRs (Figure 1) and the coding sequences for these receptors are well conserved amongst diverse

vertebrates (Roach et al. 2005). Indeed many TLR ortholog encoding cDNAs or expressed sequence tags (EST) have been cloned and sequenced from teleost fishes (Hirono et al. 2004, Phelan et al. 2005, Rodriguez et al. 2005, Tsoi et al. 2006, Tsujita et al. 2006) (Figure 2). In addition, data mining of the fugu genome (Aparicio et al. 2002) has uncovered entire TLR families (Oshiumi et al. 2003, Roach et al. 2005).

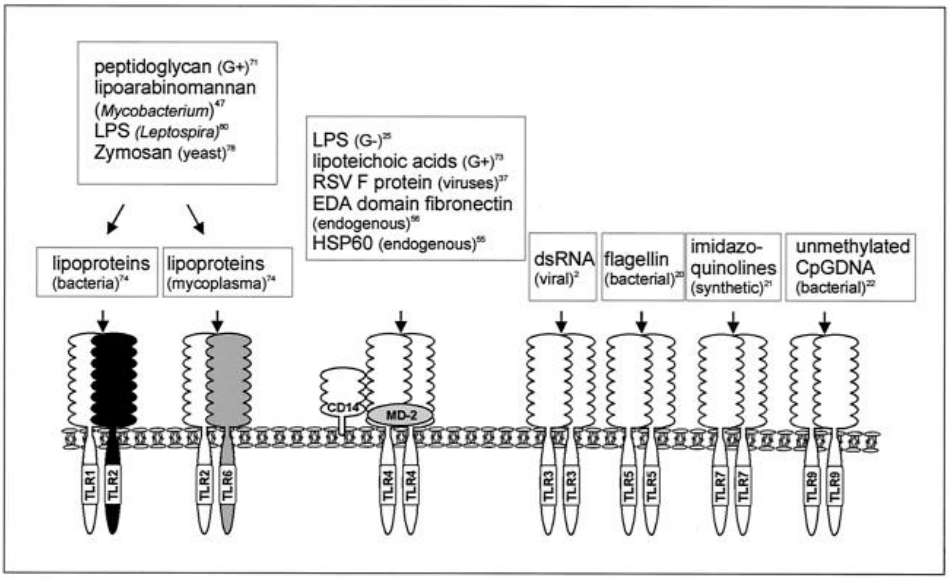

Figure 1. Mammalian toll-like receptors (TLRs) bind a range of microbial-associated molecular patterns. Ten different mammalian TLRs have been described but no ligands of TLR8 and TLR10 are known and no “natural” ligands have been described for TLR7. TLR4 complexes with other molecules like CD14 and MD-2.. LPS - lipopolysaccharide; HSP60 - heat shock protein 60; dsRNA - double-stranded RNA. From (Janssens & Beyaert 2003).

Intracellular signalling downstream of TLRs is extremely complex (Oda & Kitano 2006) (Figure 3). Apart from the TLR3 and one of two TLR4 signalling pathways, the myeloid differentiation primary response gene 88 (MyD88) is central to activation processes (Oda & Kitano 2006). In zebrafish, MyD88 has also been identified and appears to be important in coordinating the innate immune response against bacterial challenge (van der Sar et al. 2006). Therefore not only are there similarities between fish and mammalian TLRs

but also the flow-on effects of receptor engagement. There are exceptions to this notion, notably TLR4-mediated signalling in fish. In mammals lipopolysaccharide (LPS) is a TLR4 agonist and can cause endotoxic shock, a potentially fatal condition (Alexander & Rietschel 2001). Fish have a significantly higher threshold of LPS-mediated activation of leucocytes compared to mammals and are resistant to endotoxic shock (Iliev et al. 2005) despite the presence of TLR4 (Jault et al. 2004). This is speculated to be due to differences in down-stream signalling events. For further information on TLRs with respect to fish and immunostimulants, a comprehensive review was recently published (Bricknell & Dalmo 2005).

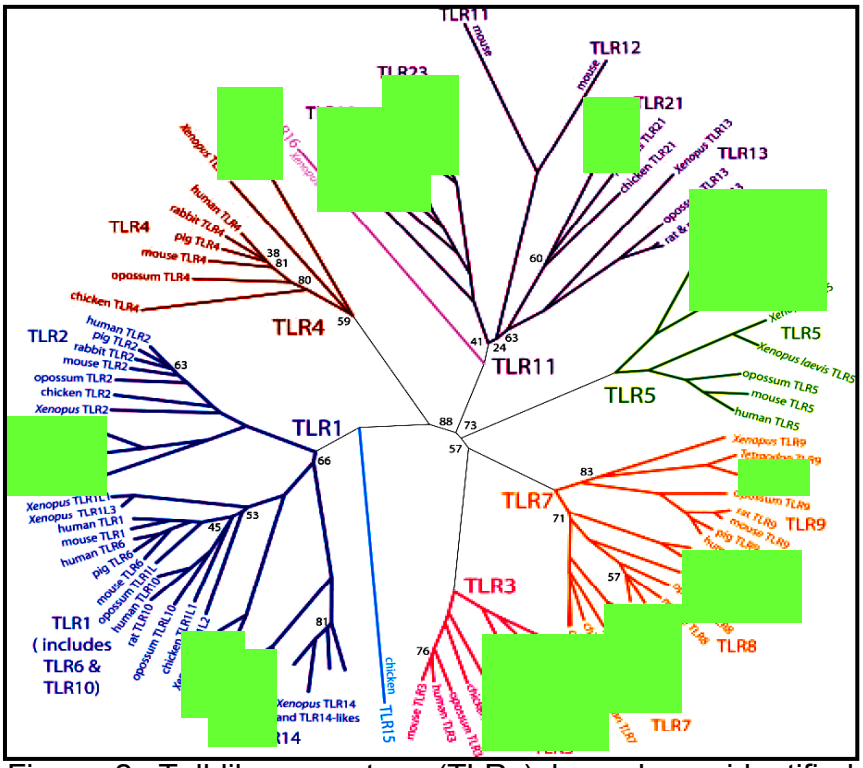

Figure 2. Toll-like receptors (TLRs) have been identified across divergent vertebrates and molecular phylogenetic analysis shows that fish orthologs are closely related to their mammalian TLR counterparts. Teleost TLR sequences have been cloned from marine pufferfish (*Takifugu rubripes*), freshwater pufferfish (*Tetraodon nigroviridis*), zebrafish (*Danio rerio*), goldfish (*Carassius auratus*), Japanese flounder (*Paralichthys olivaceus*) and rainbow trout (*Oncorhynchus mykiss*) and are highlighted in green. From (Roach et al. 2005).

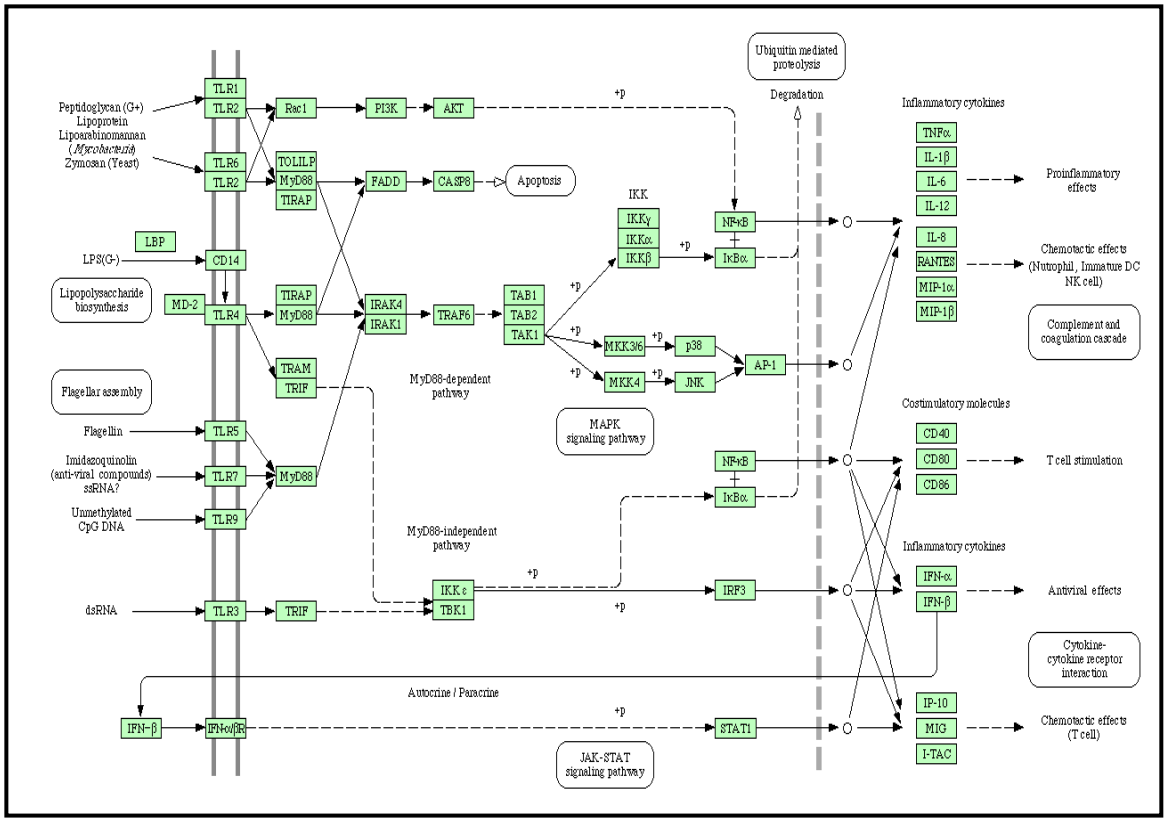

Figure 3. Complexity of toll-like receptor (TLR) signalling in humans. The myeloid differentiation primary response gene 88 (MyD88) is central to most signalling pathways. Pathway from the Kyoto encyclopaedia of genes and genomes (KEGG) at http://www.genome.jp/kegg/pathway/hsa/hsa04620.html.

Natural immunostimulants evaluated empirically in finfish

Diverse natural immunostimulants have been trialled experimentally (Table 2) and for the purposes of this review they have been grouped according to their source.

Bacterial derivatives

Lipopolysaccharide (LPS) is a complex structure forming the outermost shell of Gram negative bacteria. LPS is detected in higher animals by specialised receptors, notably TLR4, CD14 and MD-2. LPS has diverse effects on the

non-specific immune system as well as being a B-cell mitogen. Dalmo & Seljelid (1995) stimulated head kidney macrophages of Atlantic salmon with LPS extracted from *V. anguillarum*. Effects on the immune response included increased phagocytic, pinocytic and acid phosphatase activity and higher intracellular superoxide production. Those results were consistent with Solem et al. (1995), who treated Atlantic salmon macrophages *in vitro* with LPS, enhancing the respiratory burst, phagocytic and bactericidal activities. *In vivo*, *A. salmonicida* LPS has been administered to Atlantic salmon fry as a top-coat on their feed however this treatment had no effect on their resistance to either a *A. salmonicida* or *V. anguillarum* challenge (Guttvik et al. 2002).

Muramyl dipeptide (MDP; N-acetyl-muramyl-Lalanyl-D-isoglutamine), derived from *Mycobacterium* is the ligand of the intracellular Nod2 receptor in mammals (Girardin et al. 2003, Inohara et al. 2003). Nod2 binding leads to activation of the important pro-inflammatory pathway via the transcription factor nuclear factor kappa-B (NF-κB). In rainbow trout, MDP activates head kidney phagocyte production of superoxide anion, phagocytosis and chemotaxis, culminating in enhanced non-specific protection against *A. salmonicida* (Kodama et al. 1993). Most recently, it was shown that MDP activates acidophilic granulocytes of sea bream, enhancing the respiratory burst response, phagocytosis, bactericidal activity and pro-inflammatory cytokine gene expression (Sepulcre et al. 2006). Similarly peptidoglycan, a cell wall component of bacteria stimulated pro-inflammatory cytokine (IL-1β) gene expression in carp (Kono et al. 2002). Bacterial DNA (bDNA) on the other hand, enhanced cytotoxicity of non-specific cytotoxic (NCC) cells of

channel catfish, presumably via the same mechanism as CpG-mediated activation (discussed later) (Oumouna et al. 2002).

Yeast derivatives

Glucan is a polysaccharide polymer of glucose (polyglucose) (Ainsworth et al. 1994). More specifically, β-glucans are branched β -1,3- and β -1,6-linked polyglucoses. They are a significant structural polysaccharide of most yeast and myceloid fungi cell walls. Specialised recognition mechanisms have been developed by higher organisms to detect these cell wall components (Jorgensen et al. 1993a, Jorgensen et al. 1993b). Robertsen et al. (1994), suggested that activation of Atlantic salmon macrophages by glucans occurs by stimulation of a β -glucan receptor (presumably a Dectin-1 ortholog), as the phagocytic ability of macrophages is specifically inhibited by soluble β -1,3-linked glucans. Purified β-glucans and other preparations that contain β-glucans are arguably the most popular immunostimulant in the commercial market place (Table 4).

In terms of the capacity for β-glucans to be immunostimulatory, there are numerous reports of its efficacy after either oral or intraperitoneal (IP) delivery. A report by Yano et al. (1991) discussed the efficacy of oral administration of 10 polysaccharides to enhance protection of carp (*C. carpio*) against bacterial infection. The data implied that greatest protection against *E. tarda* was offered by lentinan, schizophyllan and scleroglucan (1,6 branched-β -1,3-glucans), which suggests that carp may also possess specific glucan receptors. Moreover, protection of carp by lentinan, schizophyllan and

scleroglucan against *Aeromonas hydrophila* was also documented, indicating the non-specific nature of 1,6 branched-β -1,3-glucan immunostimulation.

In a study by Engstad et al. (1992), β-1,3 and β -1,6-linked glucan extract from *Saccharomyces cerevisiae* IP injected into Atlantic salmon heightened lysozyme and complement-mediated haemolytic activity. Similarly, yellowtail (*Seriola quinqueradiata*) displayed heightened serum lysozyme and complement activities after IP injection with schizophyllan and scleroglucan β-1,3-glucans (Matsuyama et al. 1992). Rainbow trout head kidney macrophages stimulated by glucan (β-1,3 and β-1,6-linked yeast glucan) produced superoxide and lysozyme (Jorgensen et al. 1993b). In the same study, macrophages had an increased ability to kill both virulent and avirulent strains of *A. salmonicida* which was correlated to elevated superoxide production.

Atlantic salmon exhibited resistance to *Yersinia ruckeri*, *V. anguillarum* and *V. salmonicida* infection after they were injected IP with M-glucan and challenged with respective pathogens (Robertsen et al. 1990). Likewise, Chen & Ainsworth (1992), found that glucan significantly reduces mortality in challenged fish, however in contrast to that experiment, channel catfish (*I. punctatus*) were challenged by *Edwardsiella ictaluri*. (Yano et al. 1989) observed the effects of 3 different β -1,3-glucans on survival of carp after challenge by *E. tarda*. Schizophyllan, scleroglucan and lentinan all substantially improved carp survival together with increased head kidney leucocyte phagocytic and alternative complement activities.

Phagocytic index, intracellular superoxide production and activity of acid phosphatase were reported to have increased in head kidney macrophages isolated from Atlantic salmon then treated with laminaran (β1-3-glucan) and sulphated laminaran (Dalmo & Seljelid 1995). However, only laminaran-treated cells showed elevated pinocytic activity. Increases in superoxide production, phagocytic activity and myeloperoxidase were also reported to have been enhanced after rainbow trout were fed a commercially produced β-1,3 and β-1,6 glucan (Macrogard®, Table 4). Myeloperoxidase activity, immunoglobulin level and survival when challenged by *A. salmonicicda* increased concurrently (Siwicki et al. 1994). Anderson & Siwicki (1994) challenged brook trout (*Salvelinus fontinalis*) with *A. salmonicida* after immersion in glucan. Mortality was considerably lower in the glucan-treated group than the control group.

Other yeast-derived immunostimulants assessed include whole yeast, polysaccharides and a commercial preparation Vetregard (Table 4) which contains β-glucans, mannan oligosaccharide & peptidoglycan. Siwicki et al. (1994) incorporated the yeasts *Candida utilis* and *Saccharomyces cerevisiae* into rainbow trout diets. Both immunostimulants elevated superoxide, leucocyte and immunoglobulin levels together with phagocytic and myeloperoxidase activities. Mortality in treated fish after challenge by *A. salmonicida* was lower than the control group. Channel catfish (*I. punctatus*) fed diets containing 2.7% *S. cerevisiae* also had an elevated phagocytic

activity (Duncan & Klesius 1996a). However, resistance to challenge by *E. ictaluri* was not enhanced as a result of the immunostimulation. Tilapia (*Oreochromis mossambicus*) treated with a protein-bound polysaccharide preparation from the fungi, *Coriolus versicolor*, also showed an elevated non-specific immune response and resistance to challenge (Park & Jeong 1996).

Animal extracts

Many fractions of animal preparations have been screened in mammals for their immunomodulating ability. Some of those that are successful in eliciting a positive immune response have then been administered to fish in the hope that they will have similar functions as in mammals. Chitin, a polysaccharide that forms a major component of crustacean and insect exoskeletons as well as cell walls of some fungi (Sakai et al. 1990) has been shown to have an immunostimulatory effect in fish. Immunomodulation in rainbow trout after an IP injected dose of chitin was reported by Sakai et al. (1990). Phagocytic activity increased significantly 5 and 7 days post-injection as did CL response 3, 5 and 7 days after stimulation. The LD50 of treatment groups was10-fold higher than the controls. There was no effect on lysozyme and haemolytic activity. Anderson & Siwicki (1994), injected and bathed brook trout (*S. fontinalis*) with Chitosan, a commercially produced chitin based product (Table 4). Protection against *A. salmonicida* infection relative to a control group lasted 7 days. Feed-incorporated Chitosan stimulated several immune functions in rainbow trout, including an elevation in superoxide production, myeloperoxidase, immunoglobulin level and phagocytic and neutrophil killing abilities. Phagocytic activity as indicated by the phagocytic index was

significantly higher than the control group whilst cumulative mortality was considerably lower (Siwicki et al. 1994).

EF203, fermented products of chicken eggs have provided an immunostimulatory effect in mice and pigs, while in rainbow trout, EF203 significantly elevated the chemiluminescent and phagocytic activities as well as the resistance to experimental challenge with *Streptococcus* (Yoshida et al. 1993). Similarly, a glycoprotein fraction of water extract from the abalone, *Haliotis discus hannai* was demonstrated to increase the chemiluminescent, phagocytic and natural killer cell activities and resistance to an experimental challenge by *V. anguillarum* (Sakai et al. 1991). American eels (*Anguilla rostrata*) treated with ETe, an extract of the tunicate, *Ecteinascidia turbinata* were also shown to have an elevated non-specific immune response with the leucocyte binding ability and phagocytic activity increased. However there was suppression of resistance to challenge by *A. hydrophilia* compared to a control group (Sigel et al. 1983). These results were later supported by Davis & Hayasaka (1984) and Stanley & Hayasaka (1995).

Terrestrial plant and algal derivatives

Both terrestrial plants and algal derivatives have immunostimulatory properties in fish. Glycyrrhizin, an aqueous extract of licorice, *Glycyrrhiza glabra* is a glycosylated saponin (surfactact/detergent). It was used as an immunostimulatory substance in rainbow trout, eliciting an elevation in respiratory burst activity (Jang et al. 1995). Sakai (1999a) also described studies by Edahiro et al., (1990, 1991) where orally delivered glycyrrhizin

enhanced resistance of yellowtail against streptococcosis. Other plant-derived preparations described by Jian & Wu (2003) and Venkatalakshmi & Dinakaran (2001) also enhanced protection against *Vibrio alginolyticus* and *Aeromonas hydrophila* respectively. IP injection of a commercial preparation Chevimmun (Table 4) generated an accumulation of leucocytes within the peritoneal cavity and enhanced phagocytic responses (Peddie & Secombes 2003) similar to the response of rainbow trout phagocytes to Finnstim (anhydrous betaine & a beet protein hydrolysate, Table 4).

Stimulants isolated from algae are primarily alginates extracted from various species of macro-algae, however the blue-green alga *Spirulina* has been shown to have immunostimulatory effects when fed to channel catfish (Duncan & Klesius 1996b). Sodium alginate extracted from *Ascophyllum nodosum* was administered to turbot (*Scopthalmus maximus*) via the live food *Artemia*. It increased survival against challenge by *V. anguillarum* compared to a control group (Skjermo et al. 1995). Similarly, Fujiki et al. (1994), found that alginate from the macroalgae *Undaria pinnatifida*, increased resistance in carp to infection by *Edwardsiella tarda*. However, they failed to show the same effect using alginate extract from *Lessonia nigrescens*. It was suggested that the sodium alginates isolated from *U. pinnatifida* stimulated the reticuloendothelial system. Fujiki & Yano (1997), attempted to elucidate the effects of alginate on the non-specific immune system in carp. Alginate enhanced migration of head kidney phagocytes to the peritoneal cavity and the phagocytic ability of these cells was elevated. Yet it did not affect either the activity of the alternative complement pathway or phagocytic and

respiratory burst activities of head kidney phagocytes.

Dietary components

The health and immune response of fish is directly influenced by dietary components or nutrition [reviewed by (Landolt 1989) and (Blazer 1992)]. Immunosuppression in fish fed diets deficient in essential constituents has been well documented. Conversely, an enhanced immune response in fish has been shown by elevating the concentration of specific dietary components above that of the minimum requirements. Immunostimulatory components of the diet have included vitamins C (ascorbic acid) and E (α-tocopherol) whilst vitamin A (retinol acetate) was demonstrated to have no effect on the immune response of Atlantic salmon (Thompson et al. 1994). High levels of vitamin C administered to channel catfish (*I. punctatus)* increased resistance to challenge with *E. tarda* (Durve & Lovell 1982). Likewise rainbow trout had increased resistance to *V. anguillarum* after they were fed high levels of ascorbic acid (Navarre & Halver 1989). Atlantic salmon, fed what was considered to be a high concentration of vitamin C (2.75 g/Kg), displayed enhanced serum complement activity compared to fish fed normal levels of vitamin C (0.31 g/Kg); however, it did not affect superoxide activity and erythrophagocytosis (Hardie et al. 1991). Similarly, the phagocytic activity of channel catfish fed varying concentrations of vitamin C did not show any elevation of phagocytic activity (Li & Lovell 1985, Johnson & Ainsworth 1991). Contrasting those findings, turbot fed vitamin C at 410 and 1180 mg/kg of diet had significantly higher phagocytic activities than a control group (Roberts et al. 1995b). Vitamin C concentrations for these experiments were similar,

suggesting that differences in the immune response may be inherent between these species.

Vitamin E is an antioxidant, important in protecting cell membranes from oxidative damage (Wise et al. 1993b). Hardie et al. (1990) found that vitamin E fed to Atlantic salmon at high levels (800 mg/kg) increased phagocytic activity yet had no effect on superoxide anion production and serum complement and lysozyme activity. Channel catfish fed vitamin E at concentrations of 60 and 2500 mg/kg of diet displayed an increase in erythrocyte oxidative haemolysis and phagocytic activity (Wise et al. 1993b). Vitamin E combined with selenium also activated the non-specific immune response of channel catfish (Wise et al. 1993a) and a commercial vitamin E/selenium-based product (Evestel, Table 4) enhanced protection of rainbow trout against *A. salmonicida* (Siwicki et al. 1994).

Dietary supplementation with nucleotides is becoming common, after it was shown that nucleotides can not only modulate the immune system (Low et al. 2003, Li et al. 2004) but also enhance protection against viral, bacterial and parasitic diseases (Burrells et al. 2001). Other dietary supplements with immunostimulatory properties include Salar-Bec, a multi-vitamin mix (Miles et al. 2001, Table 4) and carotenoids (Amar et al. 2004).

Miscellaneous

Oil-based immunostimulant preparations are reservoirs or depots in that they promote the slow release of stimulants prolonging the immunostimulatory

effect. Freund's complete adjuvant (FCA) is an amalgam of mineral oil and cell walls from mycobacterium such as, *Mycobacterium tuberculosis*, *Mycobacterium butyricum*, *Nocardia* and Corynebacteriae (Anderson 1992, Kajita et al. 1992, Raa 1996). FCA is traditionally used as an adjuvant, co-administered with a vaccine; however FCA alone has immunostimulatory capacity. In a study by Kajita et al. (1992), rainbow trout were IP injected with FCA produced a significant elevation in chemiluminescence (CL), phagocytic and natural killer cell activities. In addition, FCA injected fish were protected against *V. anguillarum* challenge (Kajita et al. 1992).

Table 2. Administration of natural immunostimulants has the potential to stimulate the immune response and protection against viral, bacterial and parasitic pathogens in teleost fish.

Stimulant	Species (size)	Route (conc. or vol.)	Pathogen resistance	Effects of immunostimulant	Reference
Abalone extract	rainbow trout (10 & 50 g)	IP (2 & 10 mg)	↑ *Vibrio anguillarum*	↑ phagocytic, natural killer & CL activities.	(Sakai et al. 1991)
Achyranthes aspera	Indian major carp (200 g)	oral		↑ anti-SRBC antibody response	(Rao et al. 2004)
Achyranthes aspera	Indian major carp (150 g)	oral (0.5%)		↑ anti-chicken RBC antibody response ↑ serum protease inhibitors	(Rao & Chakrabarti 2005)
alginate	carp (24-29 g)	IP (10-40 mg/kg)	↑ *Edwardsiella tarda*		(Fujiki et al. 1994)
alginate	turbot (40 dph)	Oral (via *Artemia*)	↑ *V. anguillarum*		(Skjermo et al. 1995)
alginate	carp (24-32 & 195-231g)	Oral (2.7% w/w)	↑ *E. tarda*	↑ phagocytosis & CL response.	(Fujiki & Yano 1997)
alginate	turbot (larvae from hatch)	bioencapsulated in rotifers from 2 dph		↑ fractional rates of protein synthesis compared to the control, resulting in 3-fold ↑ protein turnover in the larvae. → in feed intake, larval size or survival.	(Conceicao et al. 2001)
alginate (with high mannuronic acid)	Atlantic halibut (first feeding & weaning)	oral via *Artemia*	↑ *V. anguillarum*		(Skjermo & Bergh 2004)
aloe	olive flounder (8.1 g)	oral (0.5%)	↑ *E. tarda*	↑ PBL chemiluminescent response	(Kim et al. 2002)
bDNA	catfish (20-60 g)	*in vitro*		bDNA ↑ NCC cytotoxicity.	(Oumouna et al. 2002)

Stimulant	Species (size)	Route (conc. or vol.)	Pathogen resistance	Effects of immunostimulant	Reference
Brewtech, GroBiotic®	hybrid striped bass (64.5 g & 118 g)	oral (1 or 2%)	→ *Mycobacterium marimun*	May ↑ growth.	(Peng & Gatlin 2005)
carotenoids	rainbow trout (50 g)	oral 100 & 200 mg/kg β - carotene or astaxanthin		→ SGR, feed:gain ratio, O^{2-} & total plasma immunoglobulin. ↑ serum alternative complement activity & phagocytosis. ↑ serum lysozyme.	(Amar et al. 2004)
chevimmun	rainbow trout (100-500 g)	IP (75% preparation)		↑ leucocyte chemotaxis, phagocytosis & respiratory burst activity. ↑ anti-protease activity.	(Peddie & Secombes 2003)
Chinese medicine	yellow croaker (120 g)	oral (0.5, 1.0 & 1.5%)	↑ *Vibrio alginolyticus*	↑ phagocytic, lysozyme & complement activities	(Jian & Wu 2003)
chitin	rainbow trout (10 & 50 g)	IP (100 mg/kg)	↑ *V. anguillarum*	↑ phagocytic activity & CL response	(Sakai et al. 1990)
chitin	gilthead seabream (125 g)	oral (0, 25, 50, 100 mg/kg feed)		→ lysozyme activity. ↑ natural haemolytic complement activity & cytotoxic activity. ↑ respiratory burst & phagocytic activities.	(Esteban et al. 2001)
chitin	gilthead seabream (125 g)	IV (0.1 mg) & IP (1 mg)		IV - → any parameters. IP - ↑ respiratory burst & phagocytic activities. ↑ cytotoxic activity.	(Esteban et al. 2000)
chitosan	Indian major carp (rohu) (29-38 g)	IP (100 µg)		↑ total serum protein, serum albumin, serum globulin, phagocytic index	(Sahoo & Mukherjee 1999)
chitosan	rainbow trout (200 g)	oral (0.5% w/w)		↑ O^{2-} activity, MPO, neutrophil killing ability	(Siwicki et al. 1994)
chitosan	brook trout (100 g)	immersion & IP (100 µg/mL & 100 µg)	Short-term ↑ *A. salmonicida*		(Anderson & Siwicki 1994)
cod milt proteins	Atlantic cod (5 g)	oral (0.1%)	↑ *V. anguillarum*	-	(Pedersen et al. 2004)
C-UP III\ (herbal extract)	tilapia (63-98 g)	oral (0.2%)	↑ *Aeromonas hydrophila*	↑ neutrophil phagocytosis & chemiluminescence	(Chansue et al. 2000b)

Stimulant	Species (size)	Route (conc. or vol.)	Pathogen resistance	Effects of immunostimulant	Reference
EF 203 (fermented chicken egg)	rainbow trout (70 g)	oral (100-1000 mg/kg)	↑ *Streptococcus* sp.	↑ phagocytic activity & CL response	(Yoshida et al. 1993)
Ergosan	rainbow trout (100-500 g)	IP (250 µL of 0, 0.01, 0.1, 1, 5 & 10 mg/mL)		↑ leucocyte chemotaxis, proportion of neutrophils, phagocytosis, respiratory burst & complement activities & expression of IL-1β, IL-8 & TNF-α2.	(Peddie et al. 2002)
ETe (tunicate extract)	American eel	-	↑ *A. hydrophila*	↑ leucocyte binding ability & phagocytic activity	(Sigel et al. 1983)
ETe (tunicate extract)	American eel (250 g)	IP or IV (0.5 mL)	↑ *A. hydrophila*	↑ phagocytic activity, antibody titres	(Davis & Hayasaka 1984)
ETe (tunicate extract)	channel catfish (50-100 g)	IP (0.625-1.25 µg)	↓ *Edwardsiella ictaluri*	↑ phagocytic activity, → antibody titres.	(Stanley & Hayasaka 1995)
Evetsel (vitamin E & selenium)	rainbow trout (200 g)	oral (1%)	↑ *A. salmonicida*	↑ O^{2-}, → MPO activity	(Siwicki et al. 1994)
Finnstim	rainbow trout (200 g)	oral (1.5%)		↑ O^{2-} & MPO activity, → neutrophil killing ability	(Siwicki et al. 1994)
firefly squid (*Watasenia scintillans*)	rainbow trout	*in vitro*		↑ NBT reduction, respiratory burst, mitogen proliferation	(Siwicki et al. 1996)
Freund's complete adjuvant	rainbow trout (10 & 50 g)	IP (0.05 & 0.1 ml)	↑ *V. anguillarum*	↑ CL, phagocytic & natural killer cell activities	(Kajita et al. 1992)
FVP (Manda)	Japanese flounder (260-380g)	oral (3, 6, 15 mg/kg body weight/day for 4 w)		↑ phagocytic activity ↑ lysozyme activity.	(Ashida & Okimasu 2005)
glucan (EcoActiva)	snapper (180 g)	oral (0.1% v/w)		↑ Mø O^{2-} when PMA-stimulated. No ↑ classical or alternative complement activity. ↑ growth rate of fish.	(Cook et al. 2003)

Stimulant	Species (size)	Route (conc. or vol.)	Pathogen resistance	Effects of immunostimulant	Reference
glucan (Vitastim)	chinook salmon	bath & oral (0, 0.01, 0.1, and 1.0% w/w)	↑ *A. salmonicida*		(Nikl et al. 1993)
glucan & mannuronic acid	Senegalese sole (larvae)	oral (bioencapsulated in *Artemia*)		→ survival or growth over 121 d post-hatching. Unplanned disease outbreak occurred @ 44 dph & → in mortality rates between groups.	(Cunha et al. 2003)
glucan, levamisole, vitamin C & vitamin E	Indian major carp (39 g)		↑ *E. tarda*	↑ specific immunity & ↑ resistance in immunocompromised fish. → specific immunity & protection in healthy fish.	(Sahoo & Mukherjee 2002)
glucan, vitamin U & vitamin C	sturgeon hybrid	oral (glucan - 0.1 & 0.5% for 8 w, vitamin U - 100, 200 and 300 ppm for 4 w, vitamin C - 10, 100 & 1000 mg/kg for 20 w)		Glucan ↑ leucocyte activity & ↑ phagocytosis. Vitamin U ↑ phagocytosis & respiratory burst activity of leucocytes. Vitamin C ↑ plasma lysozyme activity.	(Jeney & Jeney 2002)
glucans	channel catfish (20 g)	oral (0.2%)	→ *E. ictaluri*	↑ Mø & neutrophil chemotaxis, phagocytic activity, → antibody titres	(Duncan & Klesius 1996a)
glucans	common carp (30-38 g)	IP (2-10 mg/kg)	↑ *E. tarda*	↑ phagocytic activity, alternate complement activity.	(Yano et al. 1989)
glucans	channel catfish (100 g)	IP (50 & 70 µg)	↑ *E. ictaluri*		(Chen & Ainsworth 1992)
glucans	yellowtail (38-50 g)	IP (2-10 mg/kg)	↑ *Enterococcus seriolicida*	↑ phagocytic activity, serum complement & lysozyme activities.	(Matsuyama et al. 1992)
glucans	African catfish (20 g)	oral (0.1%)		↑ respiratory burst & bactericidal activities	(Yoshida et al. 1995)
glucans	Atlantic salmon (120-220 & 65-110 g)	IP (1 & 0.7 mL)		↑ lysozyme & complement activities	(Engstad et al. 1992)
glucans	rainbow trout (300-400 g)	IP (1 mL)		↑ O^{2-} & bactericidal activity, → lysozyme activity	(Jorgensen et al. 1993b)

Stimulant	Species (size)	Route (conc. or vol.)	Pathogen resistance	Effects of immunostimulant	Reference
glucans	rainbow trout (200 g)	oral (0.2 g/100 g feed)	↑ *A. salmonicida*	↑ O^{2-}, phagocytic & MPO activities & immunoglobulin level.	(Siwicki et al. 1994)
glucans	Atlantic salmon (300-1000 g)	*in vitro*		↑ O^{2-}, phagocytic & acid phosphatase activities	(Dalmo & Seljelid 1995)
glucans	brook trout (100 g)	IP & immersion (100 µg & 100 µg/mL)	↑ *A. salmonicida*		(Anderson & Siwicki 1994)
glucans *(Saccharomyces cerevisiae)*	Atlantic salmon (20-35 g)	IP (2 mg/fish)	↑ *Vibrio salmonicida, Yersinia ruckeri, V. anguillarum*		(Robertsen et al. 1990)
glucans	swordfish, rosy barbs, black tetras (ornamental fish)	oral (0.1 & 1 % for 21 d)	↑ *A. hydrophila* & *Pseudomonas fluorescens*		(Turnau et al. 2000)
glucans	Indian major carp (39 g)	oral (0.1% for 7 d)	↑ *E. tarda, A. hydrophila*	↑ non-specific immunity when fed to immunocompromised fish. No ↑ in specific immunity when fed to immunocompromised fish.	(Sahoo & Mukherjee 2001)
glucans	carp (25-30g)	ip (100, 500, 1000 µg/fish on days 1, 3, & 5)	↑ *A. hydrophila*	↑ total leucocyte count & neutrophil & monocyte populations ↑ after administration of glucan. ↑ Mø O^{2-}.	(Selvaraj et al. 2005)
glucans	Asian catfish	oral (0, 0.1% for 1, 2, or 3 w)	↑ *A. hydrophila*	↑ MPO, lysozyme, O^{2-} &hemagglutination titre. → alternative complement activity & SGR.	(Kumari & Sahoo 2006)
glucans	rainbow trout & steelhead (4.2-23 g)	oral (2 & 4%)	→ *Ceratomyxa shasta*		(Whipple 2002)
glucans	tilapia (83 g)	oral (0, 2 or 10 g/Kg of body weight for 5 d)		↑ TNF-α like protein. ↑ IL-1β, IL-10, IL-12-like proteins. ↑ Mø phagocytic indices.	(Chansue et al. 2000a)
glucans	Indian major carp (35 g)	IP 4 × @ 2 w intervals (0, 5, 10, 15 mg/kg body weight)	↑ *A. hydrophila* & *E. tarda*	↑ in leucocyte count, phagocytic ratio, phagocytic index, lysozyme activity, complement activity & serum	(Misra et al. 2006)

Stimulant	Species (size)	Route (conc. or vol.)	Pathogen resistance	Effects of immunostimulant	Reference
				bactericidal activity.	
glucans	gilthead seabream (5-10 g)	oral (1 & 10 g/Kg feed)	↑ *Photobacterium damselae* subsp. *Piscicida*	.	(Couso et al. 2003)
glucans, vitamin E, vitamin C	sea bass (120, 403, 414 g)	oral (2% glucan, 2% vitamin C, 50 ppm vitamin E)		↑ alternate complement & lysozyme activities.	(Bagni et al. 2000)
glycyrrhizin	rainbow trout (300-500 g)	*in vitro*		↑ respiratory burst activity	(Jang et al. 1995)
IL-1β (short peptides)	rainbow trout (100-500 g)	IP	↑ VHSV	↑ peritoneal leucocyte migration, phagocytosis & intracellular respiratory burst.	(Peddie & Secombes 2003)
lactoferrin	Asian catfish (40 g)	oral (0, 50, 100 & 200 mg/kg)	↑ *A. hydrophila*	↑ serum lysozyme level, O^{2-}.	(Kumari et al. 2003)
lactoferrin	gilthead seabream (150 g)	oral (50, 100, 200 mg/kg feed)		↑ cytotoxic activity. → humoral immune response.	(Esteban et al. 2005)
lactoferrin & vitamin C	Atlantic salmon (74 g)	oral (140 mg lactoferrin/Kg feed & 150 or 1000 mg vitamin C/Kg feed)	→ ISAV & *A. salmonicida*	→ serum or head kidney lysozyme, complement activity, phagocytic CL responses.	(Lygren et al. 1999)
LPS	Atlantic salmon (300-1000 g)	*in vitro*		↑ phagocytic, pinocytic, O^{2-} & acid phosphatase activities	(Dalmo & Seljelid 1995)
LPS	Atlantic salmon	*in vitro* (1-100 µg)		increase in; respiratory burst, phagocytic & bactericidal activities	(Solem et al. 1995)
LPS (*A. salmonicida*)	Atlantic salmon (fry)	oral (0.1%)	↓ *A. salmonicida*	slight ↑ in total Ig.	(Guttvik et al. 2002)
LPS	Atlantic salmon (2.1 Kg)	IP (1 mg/kg bw)		↓ alternative complement activity. Triploid fish took longer to regain complement activity & had a slower onset of the hypoferraemic response following LPS injection suggesting they may be at a disadvantage compared to diploids in their defence	(Langston et al. 2001)

Stimulant	Species (size)	Route (conc. or vol.)	Pathogen resistance	Effects of immunostimulant	Reference
				against bacterial infections.	
lysozyme	carp (100-120 g)	oral lysozyme (IP OTC 10 mg/kg, oxolinic acid 10 mg/kg & lindane 10 mg/kg for immunosuppression)		Lysozyme partially corrected the suppressed antibody response to *Yersinia ruckeri* in antibiotic treated fish.	(Studnicka et al. 2000)
Macrogard, Ergosan	sea bass (80 g)	oral (Macrogard 0.1%, Ergosan 0.5%)		↑ serum complement activity. ↑ serum lysozyme, gill & liver HSP concentration	(Bagni et al. 2005)
medicinal plants	rainbow trout (41 g)	oral (0.1 & 1%)		↑ respiratory burst, phagocytosis & total plasma protein.	(Dugenci et al. 2003)
Mucor circinelloides	gilthead seabream (125 g)	oral (10 g/Kg feed one wild-type & 2 mutant strains)		Slight ↑ specific growth rate. Slight ↑ serum lysozyme activity. ↑ phagocytosis & cytotoxicity.	(Rodriguez et al. 2004)
muramyl dipeptide (MDP)	rainbow trout	IP	↑ *A. salmonicida*	↑ phagocytosis, O^{2-}, chemotaxis	(Kodama et al. 1993)
nisin *(Lactococcus lactis* metabolite)	turbot (40-60 g)	*in vitro* (25, 2.5, 0.25, 0.025 g/mL), *in vivo* (IP with 100 µL of 0.25, 0.025 or 0.0025 µg/mL)		*in vitro* - ↑ head kidney Mø CL. *in vivo* - ↑ serum lysozyme activity. → CL response. → serum NO & serum antibacterial index.	(Villamil et al. 2003)
nucleotides (yeast RNA)	hybrid striped bass	oral (0.5%)	↑ *Streptococcus iniae*	↑ O^{2-}	(Li et al. 2004)
nucleotides	turbot (121 g)	oral (0.4%)		↑ expression of IgM & RAG-1 genes in gills and spleen (↓ expression in kidney). ↓ lysozyme expression in spleen & kidney. ↑ IL-1β in kidney.	(Low et al. 2003)
nucleotides (Optimun)	turbot (121 g)	oral (0.4%)		↑ & ↓ immune-related gene expression	(Low et al. 2003)
nucleotides	rainbow trout (217	oral	↑ *V. anguillarum,*		(Burrells et al. 2001)

Stimulant	Species (size)	Route (conc. or vol.)	Pathogen resistance	Effects of immunostimulant	Reference
	g), Atlantic salmon (53-55 g), coho salmon (100 g), Atlantic salmon (60 g)	(0.03%)	ISAV *Piscirickettsia salmonis, Lepeophtheirus salmonis*		
Ocimum santum (leaf extract)	tilpia (25-30 g)	IP (10, 1, 0.1, 0.01, 0.001, 0.0001 & 0.00001% of 10% leaf extract)		↑ α-SRBC antibody titre & number of activated neutrophils.	(Venkatalakshmi & Dinakaran 2001)
Ocimum santum (leaf extract)	tilapia (25 g)	IP & oral	↑ *A. hydrophila*	IP ↑ α-SRBC antibody titre & neutrophil activity. Oral ↑ antibody response	(Logombal et al. 2000)
peptidoglycan	common carp (120 g)	IP (0.01, 0.1 & 1 mg/fish)		↑ IL-1β & O^{2-}.	(Kono et al. 2002)
peptidoglycan	Japanese flounder (120 g)	IP (1 mg)		↑ head kidney leucocyte O^{2-}.	(Kono & Sakai 2001)
peptidoglycan	turbot (fry)	oral (0.2g/Kg feed)		Feeding for 3 w prior to transportation & stocking stress ↓ mortalities during transport, acclimation & early culture.	(Jin & Xiao-Ling 2004)
polysaccharide	tilapia (30-50 g)	IP (0.1 $mg.g^{-1}$) & oral (0.1 mg/g)	↑ *E. tarda*	↑ phagocytic activity	(Park & Jeong 1996)
polysaccharides (curdlan, inulin, krestin, laminaran, lentinan, levan, schizophylian, selerogiucan, yeast glucan & zymosan)	carp (30-40 g)	oral (2-10 mg/kg)	↑ *E. tarda*	range of levels in survival when challenged	(Yano et al. 1991)
propolis	gilthead seabream (100-200 g)	IP (5 mg), oral (0, 0.1, 10 g/Kg feed)		Limited immunostimulatory effects. IP administration more effective than dietary intake.	(Cuesta et al. 2005)

Stimulant	Species (size)	Route (conc. or vol.)	Pathogen resistance	Effects of immunostimulant	Reference
rIL-1β	rainbow trout (300-500 g)	IP	↑ *A. salmonicida*	↑ peritoneal leucocyte migration & phagocytosis. ↑ expression of systemic IL-1β, COX-2 & lysozyme II mRNAs.	(Hong et al. 2003)
roe lectin	gilthead seabream (65-80 g)	*in vitro* (head kidney cells)		↑ phagocytic activity of Mø.	(Ng et al. 2003)
Salar-bec, Ergosan, Betamak C85, Lysoforte	striped snakehead (66.7 g)	IP (100 µL of 5mg/mL)	↑ *Aphanomyces invadans (=piscicida)*	Salar-bec ↑ resistance to challenge. Serum & Mø of Salar-bec & Ergosan treated fish inhibited *A. invadans* growth.	(Miles et al. 2001)
glucan (schizophyllan)	carp (1 & 69 g) flounder (12 & 54 g)	oral (1 g/kg), fed 2% bw 2×/day, 2 w on experimental diet, 1 w commercial diet, 2 w experimental	↑ *A. hydrophila* (carp), *E. tarda* (flounder)	↑ no. Mø & neutrophils, phagocytic activity & serum lysozyme.	(Kwak et al. 2003)
Selenium & vitamin E	channel catfish (10.9 g)	oral (Selenium 0-0.8 mg/kg &vitamin E 0-240 mg/kg)		↑ respiratory burst activity	(Wise et al. 1993a)
Spirulina platensis	channel catfish (18 g)	oral (2.7%)	→ *E. ictaluri*	↑ chemotaxis, phagocytic activity & antibody titre.	(Duncan & Klesius 1996b)
Titremax	tilapia (40-50 g)	ISB (0.5 mL)		↑ lysozyme activity & number of NBT positive cells from swim bladder, peripheral blood & head kidney	(Chen et al. 1998)
transferrin	goldfish	*in vitro* (75-150 ng)		↑ NO response.	(Stafford et al. 2004)
triiodothyronine (T3 thyroid hormone)	Indian major carp (21.1 g)	oral (0, 1, 5, 10 mg/kg)	↑ *A. hydrophila*	↑ total serum protein & globulin. ↓ albumin:globulin ratio. ↑ growth & O^{2-}.↑ antibody titres against *A. hydrophila*.	(Sahoo 2003)
vetregard	African catfish (20 g)	oral (1 g/Kg)		↑ respiratory burst & bactericidal activities	(Yoshida et al. 1993)

Stimulant	Species (size)	Route (conc. or vol.)	Pathogen resistance	Effects of immunostimulant	Reference
vitamin A	Atlantic salmon (20 g)	oral (0.37-15.0 mg/kg diet)	→ *A. salmonicida*	↑ serum antiprotease activity. → respiratory burst, bactericidal, lysozyme & complement activities.	(Thompson et al. 1994)
vitamin A	seabream (150 g)	oral (0, 50, 150, 300 mg/kg feed for 1, 2,4 or 6 weeks). IP (0, 0.05 & 0.30 mg/100g biomass)		oral - → serum lysozyme, serum MPO, HKL respiratory burst or phagocytosis. ↑ leucocyte MPO.	(Cuesta et al. 2002)
vitamin C	channel catfish (4 g)	oral (0-140 mg)	↑ *E. tarda*		(Durve & Lovell 1982)
vitamin C	channel catfish (3 & 9.7 g)	oral (0-3000 mg/kg diet)	↑ *E. ictaluri*	↑ antibody levels & complement activity. → phagocytic activity	(Li & Lovell 1985)
vitamin C	rainbow trout (1-10 g)	oral (0-2000 mg/kg diet)	↑ *V. anguillarum*	↑ growth & antibody levels	(Navarre & Halver 1989)
vitamin C	Atlantic salmon (20 g)	oral (0.05-2.75 g/Kg)		↑ complement activity. → erythrophagocytosis & O^{2-} activity.	(Hardie et al. 1991)
vitamin C	turbot (26.6 g)	oral (300-2000 mg/kg diet)		↑ serum lysozyme & phagocytic activities. → serum protein or glucose levels	(Roberts et al. 1995b)
vitamin C	channel catfish (320-420 g)	IP (0.625-1.250 µg)		→ bactericidal capability & phagocytic activity	(Johnson & Ainsworth 1991)
vitamin C	catfish (21 g)	oral (90 mg/kg)	↑ *A. hydrophila*	↑ antibody titre when administered with LPS	(Anbarasu & Chandran 2001)
vitamin C	Indian major carp (3 dph)	oral (1000 mg/kg feed)		↑ infiltration of phagocytic cells after injection with FCA	(Sobhana et al. 2002)
vitamin C	gilthead seabream (150 g)	oral [500 (control), 3000 mg/kg for 2, 4, 6, 8 or 10 w]		↑ phagocytic activity, ↑ natural haemolytic complement activity, ↑ respiratory burst.	(Ortuno et al. 1999)
vitamin E	gilthead seabream (150 g)	*in vitro* (0.01-10 µg/ml), oral (0, 600, 1200, 1800 mg/kg feed)		*in vitro*- ↑ natural cytotoxic activity of leucocytes. oral - ↑ natural cytotoxic activity.	(Cuesta et al. 2001)

Stimulant	Species (size)	Route (conc. or vol.)	Pathogen resistance	Effects of immunostimulant	Reference
vitamin E	gilthead seabream (150 g)	oral [100 (control), 600, 1200, 1800 mg/kg feed for 15, 30 or 45 d]		↑ serum alternate complement activity & phagocytosis of head kidney leucocytes.	(Ortuno et al. 2000)
vitamin E	Atlantic salmon (26 g)	oral (7-800 mg/kg)		↑ phagocytic activity. → erythrocyte & leucocyte numbers, O^{2-} , complement & lysozyme activity.	(Hardie et al. 1990)
vitamin E	channel catfish (5.8 g)	oral (0-2500 mg/kg)		↑ resistance to erythrocyte oxidative haemolysis, phagocytic activity & humoral antibody levels. → agglutinating antibody levels	(Wise et al. 1993b)
vitamin E	rainbow trout (54g)	oral (0, 100 or 1000mg supplemental vitamin E/Kg diet)		Fish fed vitamin E deficient diet ↓ growth, ↑ mortality, ↓ complement activity ↑ hepato-somatic index ↑ RBC fragility & ↓ hematocrit	(Pearce et al. 2003)
vitamin E & C	gilthead seabream (12 g)	oral (250 mg/kg)		Vitamin E ↓ effect of density mediated ↓ in serum alternative complement activity	(Montero et al. 1999)
vitamin E & HUFAs	rainbow trout (100 g)	oral (vitamin E - 0, 100, or 1000 mg/kg feed, HUFA - 20 or 48% dietary lipid)		Humoral & cellular immune functions were impaired in the vitamin E deficient diets & improvement in most parameters corresponded to its supplementation.	(Puangkaew et al. 2004)
Yeast	hybrid striped bass	oral (1, 2 & 4%)	↑ *S. iniae*	↑ O^{2-}→ lysozyme activity	(Li & Gatlin 2003)
Yeast (*Saccaromyces cerevisiae*)	rainbow trout (200 g)	oral (2.7%)	↑ *A. salmonicida*	↑ leucocytes, O^{2-}, phagocytic & MPO activity & immunoglobulin titre	(Siwicki et al. 1994)
Yeast (*Saccaromyces cerevisiae*)	channel catfish (20 g)	oral (2.7%)	→ *E. ictaluri*	↑ phagocytic activity. → chemotaxis & antibody titres	(Duncan & Klesius 1996a)

Stimulant	Species (size)	Route (conc. or vol.)	Pathogen resistance	Effects of immunostimulant	Reference
yeast & vitamin C	Indian major carp (rohu) (29-38 g)	oral (yeast - 150 mg/kg feed, vitamin C - 500 mg/kg feed) IP (levamisole - 0.5 mg/kg body weight)		↑ α-SRBC antibody titre, ↑ α-*E. tarda* antibody titre. ↓ blood glucose in levamisole treated fish. ↓ neutrophil:lymphocyte & erythrocyte fragility index.	(Sahoo et al. 1999)
yeast (Brewtech) &/or nucleotides (Optimun)	red drum (1 g)	oral (Brewtech 2%, Optimun 0.2%)	→ *Amyloodinium ocellatum*	→ stress-induced cortisol response	(Li et al. 2005)
yeast (*Candida utilis*)	rainbow trout (200 g)	oral (2.7 g/100 g feed)	↑ *Aeromonas salmonicida*	↑ O^{2-}, MPO & neutrophil killing activities	(Siwicki et al. 1994)
yeast (*Saccharomyces cerevisiae*)	gilthead seabream (175 g)	oral (0 or 10 g/kg feed of whole wild yeast or fks-1 strain for 2, 4, or 6 w)		↓ serum peroxidase & complement activities. ↑ lysozyme activity phagocytosis, respiratory burst activity & natural cytotoxicity.	(Rodriguez et al. 2003)
yeast RNA	carp (100 g)	oral (0.15, 1.5 & 15 mg/fish for 3 days)		↑ NBT activity in kidney cells. ↑ serum complement & lysozyme activity. ↓ in *A. hydrophila* in blood, kidney & liver after IP injection.	(Sakai et al. 2001)
yeast RNA	Indian major carp (13.4 g)	oral (0.1, 0.2 & 0.4%)	↑ *A. hydrophila*	↑ leucocytes, total protein, globulin & ↓ albumin:globulin ratio.	(Choudhury et al. 2005)

Synthetic immunostimulants

Synthetic immunostimulants include chemically synthesised analogues of bacterial or fungal components as well as other synthetic chemicals (Table 3). FK-565 (heptanoyl-y-D-glutamyl-(L)-mesodiaminopimelyl-(D)-alanine) was originally isolated from *Saccharomyces olivaceogriseus* and later synthesised. A single intraperitoneal injection (1 mg/kg) one day before bacterial challenge with *A. salmonicida* enhanced resistance to challenge, stimulated phagocytic cells and restored the immunosuppressive effect of cyclophosphamide (Kitao & Yoshida 1986).

Levamisole is the levo-isomer of tetramisole and has long been recognised in veterinary practice as an anthelminthic in ruminants (Kates et al. 1974, Janssen 1976, Symoens & Rosenthal 1977). Kajita et al. (1990) elucidated the immunomodulatory effects of levamisole on rainbow trout. Fish IP injected with levamisole produced an increase in phagocytic and natural killer cell activities, chemiluminescence responses. Resistance to *V. anguillarum* infection and activation of the alternative complement pathway was also observed. Those results concurred with Jeney & Anderson (1993), who used levamisole to elicit increased oxidative and phagocytic activity of rainbow trout spleens, *in vitro*.

It appears the immunostimulatory function of levamisole is also positive in marine fish. Mulero et al. (1998b), fed gilthead seabream (*Sparus aurata*) a diet containing levamisole. Fish fed 500 mg levamisole/Kg feed had complement activity significantly higher than a control group ten weeks post-

treatment although, complement activities of fish fed lower doses were not affected. Lymphokine production contrasted that of complement activity, with significantly higher levels at the lower dose (250 mg levamisole/Kg feed). Phagocytic and respiratory burst activities were also stimulated. Secondary effects of levamisole administration in seabream included increased resistance to *V. anguillarum* and a faster growth rate. In a later *in vitro* experiment by the same authors, levamisole failed to heighten the respiratory burst, phagocytic and bactericidal activities of gilthead seabream (Mulero et al. 1998a). Concentrations of levamisole (0-100 ng/mL) may have been insufficient to provide adequate stimulation, as the fish immune response to levamisole displays a dose-response relationship (Siwicki et al. 1990).

Sexually mature common carp IP injected with levamisole three times, at three day intervals had increased leucocyte and neutrophil numbers. Neutrophil phagocytic and myeloperoxidase activity improved, as did leucocyte migration and the lysozyme level (Siwicki & Studnicka 1987). Similarly, an oral dose of levamisole activated non-specific responses in juvenile carp. It affected metabolic and phagocytic activity of neutrophils, numbers of phagocytic cells and leucocytes and lysozyme level positively (Siwicki 1989).

Newly hatched carp larvae bathed in levamisole at concentrations ranging from 2 mg/dm^3 to 20 mg/dm^3 were shown to have significantly heavier weight and length than a control group (Siwicki & Korwin-Kossakowski 1988). The authors postulated that the increase in growth performance was presumably a

result of immune stimulation. As the antibody response in carp is not fully functional until between 2 and 10 months (Manning et al. 1982), any performance enhancement in carp would be a result of activation of the non-specific immune system.

Unmethylated cytosine-phosphodiester-guanine (CpG) motifs are rich in bacterial but not vertebrate genomic DNAs (Krieg 2002) and vertebrates have developed mechanisms to identify these motifs using their TLR9 receptor (Figure 1). Oligodeoxynucleotides (ODNs) can be synthesised to contain CpG motifs and indeed the immunostimulatory ability of synthetic ODNs led to the discovery of CpG-mediated immunostimulation (Krieg 2002). Since then, research on CpGs and their receptor TLR9 have received much attention, including those groups working on comparative immunology [see reviews by (Tassakka & Sakai 2005, Carrington & Secombes 2006)]. Synthetic CpGs have a broad spectrum of immunostimulatory abilities in fish including a capacity to up-regulate pro-inflammatory cytokine mRNAs (Jorgensen et al. 2001b), phagocytic activities (Tassakka & Sakai 2002, Lee et al. 2003, Meng et al. 2003, Tassakka & Sakai 2003) and leucocyte proliferation (Tassakka & Sakai 2003, Carrington et al. 2004, Shen et al. 2006). A capacity for CpG-mediated enhanced protection against viral (Jorgensen et al. 2003), bacterial (Lee et al. 2003) and parasitic (Bridle et al. 2003) infections has also been demonstrated.

Table 3. Administration of synthetic immunostimulants has the potential to stimulate the immune response and protection against viral, bacterial and parasitic pathogens in teleost fish.

Stimulant	Species (size)	Route (conc. or vol.)	Pathogen	Effects	Reference
carotenoids (astaxanthin, canthaxanthin & β-carotene)	rainbow trout (140 g)	oral (100 mg/kg)		↑ serum complement & lysozyme activities. ↑ serum & non-specific cytotoxicity & phagocytic activity.	(Amar et al. 2001)
CpG-ODN	flounder *in vitro* 350-400 g, *in vivo* 35-40 g	*in vitro* - added to culture media *in vivo* – (IP)	↑ *E. tarda*	*in vitro* - ↑ CL response in phagocytes. *in vivo* - ↑ respiratory burst activity.	(Lee et al. 2003)
CpG-ODN	Atlantic salmon (80 g)	IP 50 µg/fish	↑ *Neoparamoeba* spp.		(Bridle et al. 2003)
CpG-ODN	catfish (20-60 g)	*in vitro*		↑ NCC cytotoxicity.	(Oumouna et al. 2002)
CpG-ODN	Atlantic salmon (*in vitro* - 500 g, *in vivo* - 50 g)	ip - 1, 10, 50, 100 µg/fish	↑ IPNV	ODN with 6-mer CpG motif showed the highest stimulatory activity & ↑ protection against IPNV.	(Jorgensen et al. 2003)
CpG-ODN	carp (100 g)	*in vitro* (1 µg/µL)		↑ phagocytic & O^{2-} activities, lymphocyte proliferation.	(Tassakka & Sakai 2003)
CpG-ODN	common carp (100 g)	IP (0.1, 1 & 10 µg/fish)		↑ phagocytosis in kidney, serum lysozyme activity.	(Tassakka & Sakai 2002)
CpG-ODN	rainbow trout (200g)	*in vitro* (head kidney cells)		↑ IL-1β expression & interferon-like cytokines in head kidney Mø.	(Jorgensen et al. 2001b)

CpG-ODN	grass carp (600-1000g)	*in vitro* (0.5, 2.5, 5, & 10 µM)		Activated Mø. ↑ O^{2-}, H_2O_2, acid phosphatase & bactericidal activity.	(Meng et al. 2003)
CpG-ODN	Atlantic salmon (500 g)			↑ antiviral cytokine activity. Mø activated by CpG-ODN.	(Jorgensen et al. 2001a)
CpG-ODN	rainbow trout (300g)	*in vitro* (6, 1, 0.6, 0.1, & 0.06 µg/mL)		↑ PBL proliferation. Spleen & head kidney cells did not produce specific antibody to protein antigen.	(Carrington et al. 2004)
CpG-ODN	rainbow trout (300-500 g)	*in vitro* (5 µg/mL)		↑ proliferation in head kidney cells.	(Shen et al. 2006)
FK-565	rainbow trout	*in vitro* & *in vivo*	↑ *A. salmonicida*	↑ phagocytosis	(Kitao & Yoshida 1986)
levamisole	common carp (5.0-6.8 kg)	IP (5-20 mg/kg)		↑ leucocyte numbers, phagocytic, MPO & lysozyme activities, leucocyte migration, & antibody titre	(Siwicki 1987)
levamisole	common carp	bath (10 µg/mL)	↑ *A. hydrophila*	↑chemotaxis, phagocytic & CL activities	(Baba et al. 1993)
levamisole	common carp (larvae)	bath (2-20 mg/dm)		↑ growth rate	(Siwicki & Korwin-Kossakowski 1988)
levamisole	common carp (50-100 g)	oral (3-8 mg/kg)		↑ O^2 & phagocytic & lysozyme activities	(Siwicki 1989)
levamisole	rainbow trout (10 & 50 g)	IP (0.1-5.0 mg/kg)	↑ *V. anguillarum*	↑ phagocytic & natural killer cell, alternate complement, CL activities. → bactericidal activity	(Kajita et al. 1990)
levamisole	rainbow trout (1.0 kg)	*in vitro*		↑ O^{2-} & phagocytic activity	(Jeney & Anderson 1993)
levamisole	gilthead seabream (100 g)	oral (125-500 mg/kg feed)	↑ *V. anguillarum*	↑ phagocytic, complement, lymphokine & respiratory burst activities, growth	(Mulero et al. 1998b)
levamisole	hybrid striped bass	oral (100, 250, 500 &	→*S. iniae*, *A. hydrophila*	↑ intracellular O^{2-}→ extracellular O^{2-}, hematocrit, serum lysozyme,	(Li et al. 2006)

		1000 mg/kg)		agglutinating antibody & peroxidase	
levamisole	Atlantic salmon (100-200 g)	bath (2.5 mg/L)		↑ phagocytic index, phagocytic capacity & phagocytic activity. ↑ O^{2-} & lytic activity in mucus & serum.	(Findlay & Munday 2000)
levamisole	gilthead seabream (200 g)	*in vitro* (0-100 mg/mL)		→ respiratory burst, phagocytic & bactericidal activities	(Mulero et al. 1998a)
levamisole, yeast & vitamin C	Indian major carp (rohu) (29-38 g)	oral (yeast - 150 mg/kg feed, vitamin C - 500 mg/kg feed) IP (levamisole - 0.5 mg/kg body weight)		↑ α-SRBC antibody titre, ↑ α-*E. tarda* antibody titre. ↓ blood glucose in levamisole treated fish. ↓ neutrophil:lymphocyte & erythrocyte fragility index.	(Sahoo et al. 1999)
Poly I:C (synthetic dsRNA)	Atlantic salmon (25-35 g)	IP (0.2 mL of 2mg/mL suspension)	↑ ISAV	↑ Mx protein in liver, stomach, hindgut, head kidney & spleen.	(Jensen et al. 2002)
Poly I:C (synthetic dsRNA) & interferon	Atlantic salmon Mø & fibroblast cells & CHSE	*in vitro*		↑ Type I IFN-like activity. Correlation between Mx protein expression & protection against IPNV in CHSE cells.	(Nygaard et al. 2000)
Poly I:C (synthetic dsRNA) & interferon	Atlantic salmon (SHK-1 & TO cell lines)	*in vitro*	↑ ISAV & IPNV	Minor ↑ protection against ISAV in SHK-1 but not TO cell lines.	(Jensen & Robertsen 2002)

Table 4. Examples of products commercially marketed as immunostimulants or products that contain immunostimulants. Note that components and formulations are largely proprietary.

Product	Description	Supplier
Aquacite[1]	bioflavonoids, immunostimulants & vitamin C	James A Mackie Group
Betabek	vitamins C, E, B1, B2, B6, B12, an extract of a fermented product, mannans & β-glucans.	James A Mackie Group
Betamak C85	brewers yeast	James A Mackie Group
Brewtech®	partially autolyzed brewers yeast	International Ingredient Corporation
Chevimmun	plant extracts	Chevita GmbH
Chitosan	chitin derivative from crustacean exoskeletons	Central Institute of Fisheries Technology, Kochi India
EcoActiva™[2]	partially autolyzed brewers yeast	Carlton & United Breweries
Enhance	↑ ω-3 fatty acids (EPA and DHA), histidine (soluble antioxidant), boosted levels of Vitamin E and Vitamin C, organic selenium, glucans and nucleotides	Ridley Aqua-Feed
Ergosan™	plant extract, alginic acid from *Laminaria digitata*, algal based carrier	Schering Plough Animal Health
Finnstim®	anhydrous betaine & a beet protein hydrolysate	Danisco
lactoferrin	purified from bovine milk	Sigma, DMV International, Morinaga Co. Ltd
Lysoforte™	lysophospholipids	Kemin Industries
MacroGard®	β-glucans	Biotec Pharmacon
Nutra transfer diet	MacroGard® (β1,3-1,6 glucans), Nucleotides, and boosted vitamins & minerals	Skretting (Nutreco)
Optimun	nucleotides	Chemoforma Ltd
Oro glo layer dry	plant extract	Aquaculture Vaccines Ltd
Vetregard	β-glucans, mannan oligosaccharide & peptidoglycan.	Alpharma
Salar-bec	vitamins C, E, B_1, B_2, B_6 & B_{12}	Aquaculture Vaccines Ltd

[1]Patent pending, [2]No longer manufactured.

Factors Affecting the Efficacy of Immunostimulation

Experiments and field trials using immunostimulants are influenced by a number of environmental and physiological variables, such as size of fish, stage of maturation, nutrition, temperature, dose/concentration of stimulant, timing of administration and route of administration. Hence it is difficult and perhaps erroneous to compare results between experiments. In this section the most important factors influencing the efficacy of immunostimulants have been explored.

Temperature

Temperature is considered to be the most important variable that affects the fish immune response (Ellis 1982). As fish are poikilotherms, the effects of temperature on the immune response are expected to be marked with immunosuppression at lower temperatures (within the physiological range) (Miller & Clem 1984, Bly & Clem 1992). However, at low temperatures there appears to be a disparity between the response of each compartment in the immune system with some functions suppressed at low temperatures while others are unaffected (Raa 1996). Within the specific immune system, a temperature-dependent immune response may only occur within a narrow time period, as the secondary response of carp immunised with bovine serum albumin (BSA) was shown to function normally, provided the initial stimulation occurred at high temperatures (Wishkovsky & Avtalion 1982). This finding was in agreement with a later study by Plumb et al. (1986). Furthermore, it appears that if fish are kept at constantly low water temperatures during stimulation, the

immune response lags but its function is not impaired in contract to what was suggested previously (Paterson & Fryer 1974). In a later experiment, carp treated with levamisole and held at two temperatures (12 and 22ºC) (Siwicki 1989) displayed enhancement of the non-specific immune response at both temperatures. This finding may be significant in the use of immunostimulants to enhance the immune response of fish at low temperatures (within the physiological range).

Dose

Traditional chemotherapeutants generally show a linear dose-response relationship, however immunostimulants are efficacious in only a modest range of concentrations (Raa 1996). Overdoses of immunostimulants may be suppressive while underdoses may not be effective. The mechanisms that underpin this are yet to be explained, however Raa (1996) suggested that competition for receptors, overstimulation causing debilitation of the immune system and a homeostasis problem may influence this relationship. Levamisole has a relatively narrow range of immunomodulating efficacy as indicated by a number of studies using various species of fish. The non-specific immune response of rainbow trout was evaluated after spleens were treated with 1, 10 or 100 µg of levamisole *in vitro* (Jeney & Anderson 1993). Neutrophil oxidative ability was heightened at all concentrations but phagocytic activity only responded at the 1 and 10 µg concentrations. Furthermore, the non-specific immune response of rainbow trout spleens treated with levamisole (5, 25 or 50 µg) was suppressed at the highest concentration, as indicated by neutrophil oxidative ability and phagocytic and adherence indexes (Siwicki et al. 1990).

Groups treated with 25 and 50 µg also showed suppression of the specific immune system. These results were supported by Siwicki & Studnicka (1987), who treated carp with levamisole at doses of 5, 10, 15, and 20 mg/kg body weight. The non-specific immune response was elevated at the lower concentrations whilst suppressed at the 15 and 20 mg/kg doses. Dietary components influencing the immune response have been shown to have a typical dose response relationship unlike other immunostimulants. Channel catfish fed a diet containing vitamin C (0-3000 mg/kg diet) were shown to have an elevated immune response only at the highest concentration (Li and Lovell, 1985). A positive correlation between vitamin C concentration and immune response was determined in turbot (Roberts et al. 1995a). Similarly, rainbow trout fed 3 dietary levels of vitamin C were assessed for their resistance to bacterial infection, with increases in survival directly associated to the level of vitamin C (Navarre & Halver 1989). Many other immunostimulants have shown dose-dependent relationships. Atlantic salmon exposed to 1, 10 and 100 µg lipopolysaccharide (LPS) from *A. salmonicida* and other gram negative bacteria exhibited maximal non-specific immune response at the median concentration (Solem et al. 1995). M-glucan (0-1800 µg) injected into the intraperitoneal cavity of Atlantic salmon had maximal effect on resistance to *V. anguillarum* challenge at the 50-200 µg doses (Robertsen et al. 1990). However, results from this experiment should be interpreted tentatively, as there was no replication and fish (control and treatment groups) were kept in the same tank. Other stimulants that have elicited a dose dependent response include glycyrrhizin (Jang et al. 1995), FK-565 (Kitao et al. 1987) and EF203 (Yoshida et al. 1993).

Timing of administration

The timing of administration of immunostimulants is crucial in order to maximise their potential. Immunostimulants and adjuvanted vaccines must be administered prior to the outbreak of disease in order to prevent disease related mortalities. Immunostimulants may be administered before a stressful event such as grading, transporting or during changes in seasonal water temperatures, as they have shown to alleviate stress induced immunosuppression by elevating the immune response back to basal levels (Kitao & Yoshida 1986). Levamisole administered to rainbow trout after or concurrently with a *Y. ruckeri* vaccine increased both the specific and non-specific immune response, however when administered 6, 4 or 2 days prior to vaccination it did not affect the immune response (Anderson et al. 1989). Similarly, administration of the immunostimulant ETe to American eels (*Anguilla rostrata*) 2 days after a challenge provided better protection, yet when fish were treated 2 days before or simultaneously with bacteria, no protection was observed (Davis & Hayasaka 1984).

Route of administration

Reports of effective routes of administrating immunostimulants include bath, immersion/dip, oral and injection (Tables 2 & 3). Administration by injection is widely accepted as the most efficacious route; however this method is not always practical or cost-effective. Comparisons between routes of administration are difficult, as determination of the quantitative uptake of immunostimulant during immersion or bath is difficult. Interpretation of results from such comparisons should be with caution. Brook trout (*S. fontinalis*)

treated with glucan or chitosan by immersion or injection were challenged by a bathing in *A. salmonicida*. All treatment groups showed some protection 1, 2 and 3 days after treatment. Moreover, fish injected with the immunostimulants displayed a higher degree of protection (Anderson & Siwicki 1994).

The side effects of immunostimulants and adjuvants

The overall efficacy of immunostimulants is determined not only by immunological responses but also by the side effects on fish. The importance of elucidating side effects cannot be underestimated, with the success of many potential commercial stimulants depending on these results. Yet, many authors ignore this aspect. Potential side effects include lesions at the site of administration, decreased growth and in extreme cases mortality. Post [1963; cited by (Anderson 1992)], described the side effects of vaccination of rainbow trout with FCA adjuvanted *A. hydrophila*. Fish developed open lesions at the point of injection, rendering them susceptible to secondary infection. This reaction appears typical of injected oil adjuvanted vaccines and indeed there are many reports of these side effects; however there is a paucity of information on the side effects of immunostimulation. Absence of this information presumably suggests that there are no side effects worthy of reporting

Conclusions

Research and development of immunostimlants for finfish has formed a large literature base that in general conclusively demonstrates that immunostimulants have the capacity to be “immunostimulatory”, enhancing protection against

infectious diseases. Therefore in broad terms, these data suggest that immunostimulants offer substantial promise to the aquaculture sector. Upon closer inspection there are limitations to this notion. Whilst evidence of immunostimulant-mediated enhancement of protection is unequivocal, the choice of pathogens used for these challenges is questionable. In most instances, challenges have been performed with pathogens for which efficacious vaccines are commercially available (Table 5). These data therefore merely provide proof-of-concept and are redundant to commercial aquaculture growout operations. There may be a window of opportunity to administer immunostimulants to larval/juvenile fish prior to vaccination although little attention has been paid to this area of research (Bricknell & Dalmo 2005). However, as this review focuses on the use of immunostimulants in grow-out, their application in hatcheries is outside its scope. Where vaccines are not available for finfish growout, immunostimulants may provide some prophylactic support in health management. The cost-benefit of such a treatment should be assessed on a case-by-case basis, but given that efficacy of most immunostimulants is greatest after injection; substantial immunostimulant-mediated improvement in production is required for commercial expediency. Furthermore, appropriate dose and timing must be determined for efficient use of immunostimulants.

Table 5. Immunostimulants enhance protection against various pathogens but commercial vaccines are available for almost all pathogens tested.

Pathogen(s) used for challenge	Commercially available vaccines[1]
Viral pathogens	
IHNV	yes
ISAV	yes
IPNV	yes
VHSV	no[2]
Bacterial pathogens	
Listonella (*Vibrio*) *anguillarum* (various sero & biotypes)	yes
Vibrio salmonicida	yes
Vibrio alginolyticus	no
Aeromonas salmonicida subsp. *salmonicida*	yes
Aeromonas hydrophila	yes
Yersinia ruckeri	yes
Photobacterium piscicida	yes
Enterococcus seriolicida syn. *Lactococcus garvieae*	yes
Streptococcus or S. iniae	yes
Renibacterium salmoninarum	yes
Edwardsiella tarda	yes
Edwardsiella ictaluri	yes
Piscirickettsia salmonis	yes
Pseudomonas fluorescens	no
Fungal pathogens	
Aphanomyces piscicida	no
Parasitic pathogens	
Neoparamoeba spp.	no
Lepeophtheirus salmonis	no
Ichthyophthirius multifiliis	no

[1]Vaccine availability according to (Sommerset et al. 2005). [2]Under clinical trials in commercial conditions (Lorenzen & LaPatra 2005).

Some of the main limitations of immunostimulants are a lack of understanding how different factors affect their usefulness. Knowledge of the effects of dose, duration of administration, frequency of administration, temperature, age/size related response, host health status or physiological status is lacking. That means that application of immunostimulants cannot be easily modified once the circumstances change. Other aspects of host effects, such as effects of genetic polymorphism on efficacy of immunostimulants are also unknown. Genetic variation may explain at least some inconsistencies in published data on immunostimulants (Li & Gatlin, 2006). Additionally, if research was done only on effects of the immunostimulant on the immune response, there is often no

understanding how a particular change in immune response will affect host-pathogen interaction and therefore the disease outcome. Furthermore, some research is done on commercial products for which the exact formulation is unknown.

Efficacious immunostimulants may be identified using a shot-gun strategy (mass screening) or a more systematic approach to locate immune pathways that can be restored or activated to enhance protection against pathogenic challenge. Given the discoveries of PRRs in recent years, the latter approach is becoming more viable. In the mean time, empirical testing of immunostimulants on a case-by case basis is perhaps the most logical approach to applied research in this field. However, this may take long time before an effective immunostimulant is identified. Furthermore, negative results of testing do not provide any information or understanding and are not necessarily a proof that the immunostimulant will not work under all conditions. Finally, this approach will not allow to provide any information how if anhow the application of immunostimulant should be change if ther is any change in environmental conditions or fish status. All this makes the empirical testing approach ineffficient.

Recommendations

Future research on immunostimulants should focus on systematic approaches to identify immune pathways that can be restored or activated to enhance protection against pathogenic challenge. Understanding the disease processes and immune response in the disease is crucial for more strategic use of

immunostimulants. The most beneficial use of immunostimulants would be when vaccines are not available, for example during early life stages or against fungal or parastiic pathogens. Immunostimulants are required that are cost-effective in terms of both manufacture and delivery, and that protect fish against pathogens for which there are no commercial vaccine(s), and there is little evidence of these in the current literature base.

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Appendix 4

PRESENTATIONS RESULTING FROM THIS PROJECT

Bridle, A., Morrison, R. and Nowak B. (2005) Immune response in Amoebic Gill Disease, EAFP 12th International Conference, 11-16 September 2005, Copenhagen, Denmark (presented by B. Nowak).

Morrison, R., Bridle, A., Cooper, G., Koop, B., Nowak, B. (2005) Identification of differentially expressed genes in amoebic gill disease (AGD) affected Atlantic salmon (*Salmo salar* L.) 2nd FRDC Aquatic Animal Health Program Scientific Conference, 26-28 July, Cairns.

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Appendix 5

PUBLICATIONS ARISING FROM THIS PROJECT

1. Morrison RN, Crosbie PBB, Nowak, BF (2004) The induction of laboratory-based amoebic gill disease (AGD) revisited, *Journal of Fish Diseases* **27**: 445-449.

2. Morrison RN, Nowak BF (2005) Bath treatment of Atlantic salmon (*Salmo salar*) with amoebae antigens fails to affect survival to subsequent amoebic gill disease (AGD) challenge, *Bulletin of European Association of Fish Pathologists* **25**: 155-160.

3. Gross KA, Powell MD, Butler R, Morrison RN, Nowak BF (2005) Changes in the innate immune response of Atlantic salmon (*Salmo salar*) exposed to experimental infection with *Neoparamoeba* sp., *Journal of Fish Diseases* **28**: 293-299.

4. Bridle AR, Carter CG, Morrison RN, Nowak BF (2005) The effects of beta-glucan administration on macrophage respiratory burst activity and Atlantic salmon (*Salmo salar* L.) challenged with amoebic gill disease (AGD) - evidence of inherent resistance, J*ournal of Fish Diseases* **28**: 347 - 356

5. Morrison RN, Crosbie P, Adams MB, Cook MT, Nowak BF (2005) Cultured gill derived *Neoparamoeba pemaquidensis* fail to elicit AGD in Atlantic salmon (*Salmo salar*), *Diseases of Aquatic Organisms* **66**: 135-144.

6. Morrison RN, Koppang EO, Hordvik I., Nowak BF (2006) MHC class II^+ cells in the gills of salmon experimentally infected with amoebic gill disease, *Veterinary Immunology and Immunopathology* **109**: 297-303.

7. Bridle AR, Morrison RN, Nowak BF (2006) The expression of immune-regulatory genes in rainbow trout, *Oncorhynchus mykiss*, during an amoebic gill disease (AGD) infection, *Fish and Shellfish Immunology* **20**: 346 - 364.

8. Morrison RN, Cooper GA, Koop BF, Rise ML, Bridle AR, Adams MB, Nowak BF (2006) Transcriptome profiling of the gills of amoebic gill disease (AGD)-affected Atlantic salmon (*Salmo salar* L.) - A role for the tumor suppressor protein p53 in AGD-pathogenesis?, *Physiological Genomics* **26**: 15-34.

9. Vincent BN, Morrison RN, Nowak BF (2006) Amoebic Gill Disease (AGD) affected Atlantic salmon *Salmo salar* L. are resistant to subsequent AGD challenge, *Journal of Fish Diseases* **29**: 549-559.

10. Bridle A, Morrison R, Cupit Cunningham PM, Nowak B (2006) Quantitation of immune response gene expression and cellular localisation of interleukin-1β mRNA in Atlantic salmon, *Salmo salar* L., affected by amoebic gill disease (AGD), *Veterinary Immunology and Immunopathology* **114**: 121-134.

11. Gross K, Alcorn S, Murray A, Morrison R, Nowak B. (2006) *In vivo* interactions between *Neoparamoeba* spp. and salmonid leucocytes, the effects of parasite sonicate on anterior kidney leucocyte function, *Journal of Fish Biology* **69**: 293-300.

12. Morrison RN, Zou J, Secombes CJ, Scapigliatti G, Adams MB, Nowak BF (2007) Molecular cloning and expression analysis of tumor necrosis factor-α in amoebic gill disease (AGD)-affected Atlantic salmon (*Salmo salar* L.), *Fish and Shellfish Immunology* **23**: 1015-1031.

13. Vincent BN, Adams MB, Crosbie PBB, Nowak BF, Morrison RN (2007) Atlantic salmon (*Salmo salar* L.) exposed to cultured gill-derived *Neoparamoeba branchiphila* fail to develop amoebic gill disease (AGD), *Bulletin of the European Association of Fish Pathologists* **27**: 112-115.