



UNIVERSITY  
OF TASMANIA

**USING THE MUCOSAL RESPONSE TO  
RECOMBINANT *Neoparamoeba perurans*  
ATTACHMENT PROTEINS TO DESIGN  
AN EXPERIMENTAL VACCINE AGAINST  
AMOEBIC GILL DISEASE (AGD)**

by

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Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Institute for Marine and Antarctic Studies

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# APPROVALS

Doctor of Philosophy Dissertation

Mucosal immune responses to *Neoparamoeba perurans*

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## ABBREVIATIONS

|          |  |
|----------|--|
| 2-D      | two dimensional                                |
| AGD      | amoebic gill disease                           |
| ANOVA    | analysis of variance                           |
| ASC      | antibody secreting cell                        |
| BCA      | bicinchoninic acid                             |
| BLAST    | basic local alignment search tool              |
| bp       | base pair                                      |
| BSA      | bovine serum albumin                           |
| b.w.     | body weight                                    |
| CTL      | cytotoxic T lymphocyte                         |
| d        | day  |
| df       | degrees of freedom                             |
| DNA      | deoxyribonucleic acid                          |
| DNP      | dinitrophenol                                  |
| ELISA    | enzyme-linked immunosorbent assay              |
| FCA      | Freund's complete adjuvant                     |
| FIA      | Freund's incomplete adjuvant                   |
| FITC     | fluorescein isothiocyanate                     |
| G        | gauge  |
| <i>g</i> | gravity  |
| <i>g</i> | gram   |
| h        | hour   |
| HRP      | horseradish peroxidase                         |
| HSWB     | high salt wash buffer                          |
| ICC      | immunocytochemistry                            |
| IgG      | immunoglobulin G                               |
| IgM      | immunoglobulin M                               |
| IgT      | immunoglobulin T                               |
| i.p.     | intraperitoneal                                |
| kDa      | kilodalton                                     |
| KLH      | keyhole limpet haemocyanin                     |
| L        | litre  |
| L-15     | L-15 Medium (Leibovitz) for cell culture       |
| LB       | Luria Bertani media                            |
| LC MS/MS | liquid chromatography tandem mass spectrometry |
| LSWB     | low salt wash buffer                           |
| M        | mol  |
| mAb      | monoclonal antibody                            |
| MBP      | mannose-binding protein                        |
| mg       | milligram                                      |
| MHC      | Major histocompatibility complex               |
| min      | minute   |
| mL       | millilitre                                     |
| mm       | millimetre                                     |
| mM       | micromole                                      |
| mRNA     | messenger ribonucleic acid                     |

## Abbreviations

---

|          |   |
|----------|---|
| n        | number of samples   |
| nm       | nanometer   |
| NAPS     | nucleic acid preservation solution                                  |
| NCBI     | National Centre for Biotechnology Information                       |
| NCMCRS   | National Centre for Marine Conservation and Resource Sustainability |
| OD       | optical density   |
| OIE      | World Organization for Animal Health                                |
| p.a.     | peranal   |
| PAMP     | pathogen associated molecular pattern                               |
| PBS      | phosphate-buffered saline   |
| PCR      | polymerase chain reaction   |
| PRR      | pattern recognition receptor  |
| PSN      | penicillin – streptomycin – neomycin                                |
| PVDF     | polyvinylidene difluoride   |
| s        | second  |
| SD       | standard deviation  |
| SDS-PAGE | sodium dodecyl sulphate - polyacrylamide gel electrophoresis        |
| SE       | standard error  |
| <i>r</i> | recombinant   |
| RNA      | ribonucleic acid  |
| rpm      | revolutions per minute  |
| RT       | room temperature  |
| TBS      | tris-buffered saline  |
| TLR      | Toll- like receptor   |
| TMB      | 3,3',5,5'-tetramethyl benzidine                                     |
| V        | Volts   |
| W        | Watts   |
| WB       | western blot  |
| x        | times   |
| µm       | micrometre  |
| µL       | microlitre  |

## EXECUTIVE SUMMARY

Amoebic gill disease (AGD) is the main disease affecting the Tasmanian salmonid industry and the condition has also been described in other major salmon and trout producing countries. AGD is caused by *Neoparamoeba perurans*, and outbreaks of the disease appear during the marine grow-out phase, in particular when water temperature rises. Some characterisation of the host immune response against the parasite has been achieved through gene expression studies and through others investigations which focused on antibody responses against *N. perurans*, particularly IgM. A variety of treatments have been tested, but currently the only treatment option widely used in Tasmania is freshwater bathing, which represent a high economic burden for the industry. Therefore, the development of a vaccine remains a high priority for salmon producers and different types of vaccines have been previously tested against AGD without success.

In order to develop a potentially successful vaccine strategy, a better understanding of the antibody immune response associated with the disease is necessary. To address this general objective, the followings aims were studied in this thesis:

- Investigate the mucosal and systemic immune response of Atlantic salmon against *N. perurans*, the causative agent of AGD.
- Investigate mucosal and systemic anti-*N. perurans* antibody responses to a recombinant putative attachment protein of the amoeba, first identified by the generation of a cDNA library from the parasite.
- Investigate vaccine formulations for AGD, using the recombinant protein described above.
- Investigate other mucosal components potentially involved in the host response against *N. perurans*.

This thesis presents the results obtained from several different experiments aimed at addressing the above stated aims. Firstly, an experiment where the immune responses of Atlantic salmon were assessed at transcription and antibody production levels, after repeated infections with *N. perurans*. Secondly, an experiment where immune responses were assessed after a single infection and fish were fed commercially

developed diets containing immunostimulants. We showed that antibody levels do not always correlate with mRNA transcription levels identified in AGD gill lesions, which is possibly explained by weak correlations existing between protein and mRNA abundances in cells and tissues. Additionally, we demonstrated that the use of immunostimulants containing diets did not affect the levels of serum or skin mucus IgM and were unable to induce IgM and IgT transcription at the site of AGD infection.

Following from this experiment; the systemic and mucosal immune responses of Atlantic salmon were studied using two protein-hapten antigens. This study aimed at evaluating the best delivery method of antigens to be used in the testing of a vaccine candidate in subsequent experiments. The results showed that i.p. injection of immunogens emulsified in FCA was the best delivery method for inducing systemic and mucosal antibody responses.

We described the production of a recombinant protein named *r22C03*, identified as a mannose-binding protein-like (MBP-like) similar to attachment factors of other amoebae, and a putative attachment factor of *N. perurans*. This protein was capable of inducing systemic and mucosal antibody responses against the amoebae and both systemic and mucosal antibodies produced were able to bind the surface of formalin-fixed *N. perurans*. The recombinant protein was then tested as a vaccine candidate against AGD, following the rationale that by using functional antibodies present in mucosal surfaces, the putative attachment factor of *N. perurans* might be blocked and the severity of AGD could potentially be reduced. Fish were immunised with *r22C03* using two different vaccination strategies and then challenged with the parasite. A strong antibody response against the recombinant protein was observed in serum and mucosal surfaces of vaccinated salmon, but no differences in survival curves or size of lesion in the gills were observed. However, a concurrent infection with *Yersinia ruckeri* was present during the experiment, and even though the simultaneous presentation of both pathogens could represent a situation more closely related to infection patterns observed on commercial farms, survival results obtained after the parasite challenge had to be examined with caution in the context of vaccine efficacy against *N. perurans*.

## **Executive Summary**

Following from the unsuccessful challenge, nanoLC-MS/MS and proteomics analyses were used on skin and gill mucus of AGD-affected fish, as a tool to identify the changes in the proteome of mucus after repeated infection with amoebae. Proteins that have been previously related to gene expression in AGD-affected gills as well as proteins that have not been previously described in AGD-affected fish were identified and it was proposed that future research should focus on better understanding the role these components play in the response against infection with *N. perurans*.

This thesis provided further understanding into the mucosal responses to AGD. However, the role mucosal antibodies play in responses against AGD cannot be completely comprehended until the study of IgT responses in AGD-affected fish can be completed, as it has been hampered by the lack of available reagents. Finally, adjuvants that have been designed specifically to elicit mucosal responses need to be fully tested in AGD vaccine formulations.

**CHAPTER 1.**

**GENERAL  
INTRODUCTION**

## 1.1 SALMONID AQUACULTURE INDUSTRY IN TASMANIA

Aquaculture is arguably one of the fastest growing food-producing sectors, accounting for more than 40% of the total fish available for consumption worldwide [1]. The aquaculture industry in Australia is considered small by world standards, but has been growing at rates over 10% annually since 1990s and is mainly based in regional areas [2, 3]. The national aquaculture industry was valued at AUD \$1.1 billion in 2011-12, representing 84,605 t of products (Figure 1.1). Tasmania accounts for the largest share (30%) of gross production value within the country [4], with the largest production sector being salmonid culture which is valued at AUD 503 million (2011-12) and accounts for more than 94% of the total state aquaculture production.

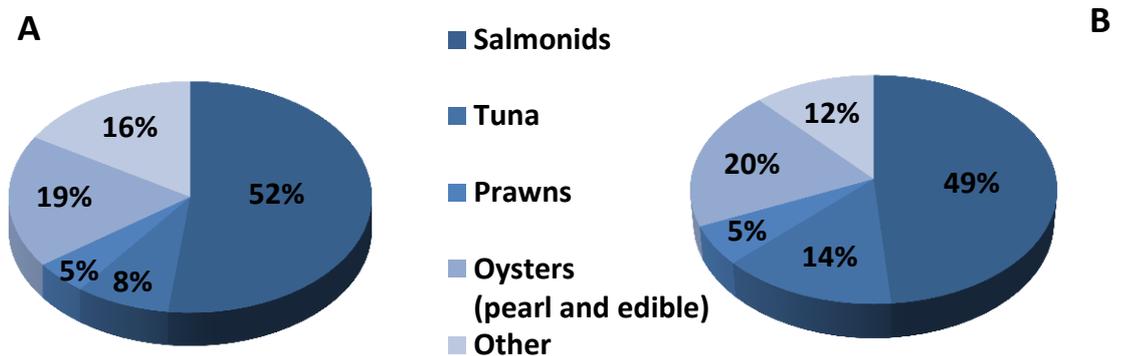


Figure 1.1 Percentage of Australian aquaculture production 2011–12, for total production in weight (A) and for total production value (B). Modified from [4].

Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), are the main species currently cultured in Tasmania, and are ranked among the highest valued (per kg) fin fish species worldwide [5]. In Tasmania, the Atlantic salmon stock originated from a New South Wales hatchery (Gaden Hatchery), where fish were initially introduced from Canada in the mid-1960s, for the Snowy Mountains power scheme lakes. Later during the 1960s, imports of salmonid genetic material were banned by the Commonwealth Government, as they represented a risk of introduction of exotic diseases into Australia [6]. On the other hand, rainbow trout, which are also exotic to Australia, were introduced from the northwest coast of America, and wild populations have developed after initial introduction [6].

In the mid-1980s, a report to the Tasmanian Fisheries Development Authority determined that salmon farming could be effectively developed in Tasmania [7]. Fertilised Atlantic salmon eggs were acquired from the Gaden Hatchery (Thredbo River, Jindabyne, New South Wales) in 1984 [7]. As a result of this initial import, a sea farm was established at Dover in Tasmania's southeast and a hatchery was started at Wayatinah, in central Tasmania [7].

Currently, five different companies form part of the Tasmanian Salmonid Growers Association. These companies are responsible for hatchery, sea farming and processing operations [7]. At this time, small salmon are produced in freshwater hatcheries and are moved gradually through brackish waters to the ocean. The marine grow-out phase of salmon takes place in cages located in the ocean or in estuaries. Farming operations for the marine grow-out phase of Atlantic salmon are located in 3 areas of Tasmania: the majority of the farms are in south-eastern region, in proximity to the D'Entrecasteaux Channel, Huon River, Port Esperance and Tasman peninsula; in the north-west area in Macquarie Harbour and one farm in the north of the state, located in the Tamar estuary [6]. Different water salinity and water exchange conditions are observed in the different grow-out areas, and therefore a variety of pathogens affect the salmon industry in Tasmania.

## **1.2 DISEASES AFFECTING SALMON CULTURE IN TASMANIA**

Most of the fin fish diseases listed as reportable by the OIE are exotic to Australia [8], and only a few conditions, which are not OIE-listed, are of concern for the Tasmanian industry. The main endemic diseases are marine aeromonas disease (MAS), vibriosis, rickettsiosis, yersiniosis and amoebic gill disease (AGD). Atypical strains of *Aeromonas salmonicida*, have been described to cause MAS in salmonids in Tasmania [9]. The presentation of MAS is similar to furunculosis, with Atlantic salmon developing skin ulcerations, which are often contaminated with secondary bacterial infections [9]. Cases are commonly observed on farms located in Macquarie Harbour, and outbreaks generally occur when water temperature is above 10°C and after handling, when fish are more susceptible due to damage of mucosal layer and skin. A carrier-state is also recognised in survivors which, without showing any signs of disease, can continue to infect the susceptible population [9].

Vibriosis, caused by *Vibrio anguillarum*, is a major cause of mortality in marine farmed fish worldwide [10]. In Tasmania however, it mainly affects the production of ocean trout, a term describing rainbow trout grown-out in sea cages. Only one isolation report of this pathogen is available in Tasmania from outbreaks of clinical vibriosis, with only one bacterial serotype (serovar O1) isolated to date [11]. Both vibriosis and MAS have been significantly controlled with the use of a bivalent vaccine for atypical *Aeromonas salmonicida* and *Vibrio anguillarum* (AnguiMonas) which was developed in 2006 by the Tasmanian Government, in collaboration with the salmonid industry [12].

A rickettsia-like organism (RLO) was detected in 2001 in Atlantic salmon in Tasmania [13], and even though it shares some morphological characteristics to *Piscirickettsia salmonis*, the causative agent of piscirickettsiosis in other salmon producing countries, it differs at the genetic level [13]. The clinical signs and gross pathology associated with RLO infection in Tasmanian farmed salmon are similar to those associated with piscirickettsiosis [14]. Periodic outbreaks of the disease have occurred since 2001, with a significant and widely spread event taking place in 2006 on south-eastern marine farms, which led to the recent development of a commercial-ready vaccine against this agent [15].

Yersiniosis is a condition affecting salmonids in the southern hemisphere, which is caused by non-Hagerman strains of the gram-negative bacterium *Yersinia ruckeri* [16]. In Tasmania, even though most of Atlantic salmon are currently vaccinated with a commercial preparation against the pathogen, a large mortality event in a hatchery occurred in 2007 [17], which led to a shortage of stock for the whole industry during that year. The pathogen has also been associated with mortalities in salmon smolts, particularly 3-6 weeks post transfer to seawater cages [16]. The weekly mortalities in sea cages do not surpass 1%, but a carrier condition has been described for the pathogen which can cause severe outbreaks under stress conditions, including after transfer to marine environment [16, 18, 19].

Regardless of the impact bacterial diseases have on the Tasmanian salmonid industry, they have been mostly controlled with the use of vaccines produced with local antigens [12, 15]. In contrast, AGD remains as the most significant disease in the marine grow-out phase in the state, and it is responsible for almost 20% of the production costs, mainly due to control strategies used by the industry and mortalities caused by the parasite.

### **1.3 AMOEBIC GILL DISEASE**

When salmonid culture was established in Tasmania in the mid-1980s, fish were kept for the first time in full-strength salinity water during summer months, and fish exhibited severe respiratory distress, decline in feeding rates and mortalities [20]. The disease affected mainly young fish in their first season at sea, and mortalities were estimated at up to 2% daily on sea farms when sea temperatures raised above 12°C [20]. During subsequent years, the disease became known as AGD and was reported in Chile, Ireland, France, U.S.A., New Zealand and Spain, affecting a range of farmed and native marine fish species [21-30] (Table 1.1). During the last 15 years the range of the disease has increased and AGD has also been reported from Mediterranean areas, and in other countries such as Japan, South Africa, Scotland and Norway [31-41] (Table 1.1), emerging as an important global fish health issue for cultured marine fish.

**Table 1.1 Different fish species and areas where AGD has been reported and *N. perurans* presence has been confirmed.**

| <b>Species</b>                                     | <b>Area</b>         | <b>AGD reported</b> | <b><i>N. perurans</i> confirmed</b> | <b>Ref</b>   |
|--|---------------------|---------------------|-------------------------------------|--------------|
| Atlantic salmon ( <i>Salmo salar</i> )             | Tasmania, Australia | 1990                | 2008                                | [20, 37]     |
|  | Chile               | 1993; 1999;         | 2011                                | [26, 28, 34] |
|  | France              | 1995; 1999          |                                     | [23, 26]     |
|  | Ireland             | 1996; 1997          | 2008                                | [22, 29, 37] |
|  | Spain               | 2001                |                                     | [21]         |
|  | WA, U.S.A.          | 2001                | 2008; 2010                          | [30, 37, 39] |
|  | Norway              | 2008                | 2008                                | [41]         |
|  | Scotland            | 2008                | 2008                                | [37]         |
|  | South Africa        | 2014                | 2014                                | [33]         |
| Coho salmon ( <i>Oncorhynchus kisutch</i> )        | WA, U.S.A.          | 1988                |                                     | [24]         |
| Rainbow trout ( <i>Oncorhynchus mykiss</i> )       | Tasmania,           | 1990                | 2008                                | [20, 37]     |
|  | France              | 1995                |                                     | [23]         |
| Chinook salmon ( <i>Oncorhynchus tshawytscha</i> ) | New Zealand         | 1993                | 2008                                | [28, 37]     |
| Brown trout ( <i>Salmo trutta</i> )                | France              | 1995                |                                     | [23]         |
| Turbot ( <i>Psetta maxima</i> )                    | Spain               | 1995; 1998          | 2008                                | [25, 27, 37] |
|  | South Africa        | 2014                | 2014                                | [33]         |
| European seabass ( <i>Dicentrarchus labrax</i> )   | Mediterranean       | 2000                |                                     | [31]         |
| Sharpsnout seabream ( <i>Diplodus puntazzo</i> )   | Mediterranean       | 2000                |                                     | [31]         |
| Mediterranean seabream ( <i>Sparus aurata</i> )    | Greece              | 2002                |                                     | [32]         |
| Blue warehou ( <i>Seriolella brama</i> )           | Tasmania            | 2008                | 2008                                | [36]         |
| Ayu ( <i>Plecoclossus altivelis</i> )              | Japan               | 2010                | 2010                                | [38]         |
| Ballan wrasse ( <i>Labrus bergylta</i> )           | Norway              | 2013                | 2013                                | [40]         |

The causative agent of AGD was initially recognised as *Paramoeba* sp. [20, 23, 24, 27], but subsequent studies reclassified the putative agent as two members of the *Neoparamoeba* genus [31], which were isolated and cultured from gills of AGD-affected fish [42]. These organism were present in the sediments surrounding salmon farms [43, 44], though never in large quantities in the water column [20] and were also identified by immunohistochemistry in gills of non-salmonid fish species in proximity to sea cages [36].

However, attempts to test the pathogenicity of clonal, cultured *Neoparamoeba pemaquidensis* and *N. branchiphila*. by re-infecting fish under experimental conditions were unsuccessful [24, 45], and in 2007, Young and colleagues [46] were able to confidently identify the aetiological agent as a new species named *Neoparamoeba perurans*, using 18S and 28S ribosomal RNA phylogenetic analyses. These researchers demonstrated that this was the exclusive causative agent of AGD cases around the world [37]. Since then, AGD has emerged as an important disease affecting commercial scale salmon farms in several countries (Table 1.1), causing significant stock losses [34, 41, 47, 48].

The classic AGD presentation is restricted to the gills, characterised by an excessive production of mucus and typical white, raised lesions in the surface of these organs [20]. A “gill score”, which ranges from 0-5, has been generated from macroscopic field observations of these lesions, as a gross measure of the degree of host response to the presence of *N. perurans* and as an indication of the severity of infection [49]. The severity of gill scores in routine checks performed on farms determines the requirement for freshwater bathing to control the disease [50], and gill scores have also been used to assess the effectiveness of potential treatments and immunisation regimes in controlled experiments [51-53]. At microscopic level, the gill lesions are a result of acute multifocal hyperplasia of the respiratory epithelium, which leads to lamellar fusion and reduced respiratory surface area, and the amoebae can be observed often around the margins of the lesions [50].

AGD has probably become one of the most studied gill diseases in fish; a complete description of the cellular components of the lesions and their pathological progression [50, 54-57] and characterisation of the respiratory and cardiovascular effects of the disease [58-65] have been carried out, as well as the study of potential treatment strategies [52, 66-69] and the use of selective breeding programs [70, 71] to reduce the effects of AGD. Numerous studies have also shed some insight on the host immune responses against AGD, generating a certain level of understanding of the innate and specific immune processes against the pathogen.

## **1.4 IMMUNE SYSTEM OF TELEOSTS**

The immune system is a group of biological structures and processes in an organism that protect against disease and comprises various organs and tissues which support different cell populations responsible for the specific and non-specific immune defences [72]. It must be able to detect a wide variety of agents including viruses, bacteria and parasitic antigens and must be able to differentiate between these pathogens and the organisms' healthy tissue.

Teleosts immune system includes two primary lymphoid organs; the thymus and the head-kidney, which may also serve as a secondary lymphoid organ together with the spleen [73]. The kidney is the equivalent of the bone marrow in vertebrates and is the largest site of haematopoiesis [74]. The thymus is responsible for the production and accumulation of T-cells [73] and in most teleosts is located near the gill cavity and in permanent continuity with the pharyngeal epithelium. The spleen is the major peripheral lymphoid organ in jawed vertebrates, in charge of filtering blood to facilitate the entrapment and processing of antigens by immune cells [75]. However, splenic lymphoid tissue in teleosts is poorly developed, mainly due to the abundant lymphohaemopoietic tissue in kidney [75]. In addition, a mucosa-associated lymphoid tissue or MALT has also been described in teleosts [76]. The three main compartments of this mucosal associated tissue are the gut associated lymphoid tissue, which is scattered along the intestine and includes the lamina propria and the intraepithelial compartments; the skin associated lymphoid tissue and the gill associated lymphoid tissue, comprising the gills and the recently described interbranchial lymphoid tissue or ILT [76, 77]. The ILT presents characteristics of a secondary lymphoid organ and contains abundant T-cells embedded in a meshwork of epithelial cells, with very few B-cells [73].

The immune system is typically divided into two main functional branches: innate or non-specific and adaptive or specific immune systems and teleosts possess elements of both. The innate immune system is phylogenetically old, with some form of it presumably present in all multicellular organisms [78, 79]. Many of its components are more active and more diverse in fish than their counterparts in mammals [80]. It represents the first line of defence during the initial hours of infection and its

responses are independent of previous exposures and therefore are similar regardless of the type of infection [81, 82]. Three main compartments form part of the innate immune system: the mucosal surfaces and the cellular and humoral components [83]. The mucosal surfaces act as a mechanical barrier, and several immune related factors are present in their mucosal secretions (see section 1.6 of this Chapter). The humoral factors of the innate immune response are expressed in soluble form, and include the complement system with its three activation pathways (classical, alternative and lectin), antimicrobial peptides, lysozyme and natural antibodies [78, 81]. These innate molecules are generally identified as secreted pattern recognition receptors or PRRs, which are able to bind prevalent biological patterns present in pathogens, denominated pathogen associated molecular patterns (PAMPs) [83]. Using the PRRs the innate immune system is able to recognise potentially pathogenic substances from harmless ones, based on their particular molecular signatures [79]. The PRRs are relatively few and transmitted vertically, which reflects the evolutionary conserved characteristics of this response [80].

The cellular component of the fish innate immune system includes mainly phagocytic cells (monocyte/macrophages and granulocytes) and non-specific cytotoxic cells [80, 83]. Non-specific cytotoxic cells kill a wide variety of target cells including tumour cells, virally infected cells and protozoan parasites [81]. They are morphologically variable and when stimulated by either receptor-bound PAMPs or by antigen uptake, cytotoxic cells in fish can generate respiratory burst activity, which involves the release of bactericidal reactive oxygen species, nitric oxide and other factors; and produce a range of different cytokines which are cell communicators and responsible for function of the innate and adaptive immune responses [81, 83].

Within the PRRs, Toll-like receptors (TLRs) represent one of the most studied families of innate immune receptors that can detect infections [84]. TLRs are cell-associated transmembrane proteins that recognise PAMPs and induce immune effector molecules, triggering and regulate the subsequent networks of innate immune responses [85, 86]. They consist of an extracellular domain of TLRs or LRR (26 leucine-rich repeats), a transmembrane domain and an intracellular C-terminus with a Toll/IL-1 receptor domain. LRRs have a large structural diversity, allowing a

broad range of antigens to be recognized by the TLRs (for a Reviews see ref. [84]). In mammals, two major TLR signalling pathways have been described: MyD88-dependent and MyD88-independent. The activation of these pathways is induced by interactions between different ligands and the LRRs, and through cascades of intracellular signalling, they lead ultimately to the production of pro-inflammatory cytokines, such as interleukin (IL) and tumour necrosis factor (TNF) for the dependent pathway and type I interferon (INF) in the independent pathway [84, 86]. It is currently understood that TLRs can recruit different cytoplasmic adaptors within a cell type or alternatively, the same type of TLR signal in different cell types can alter the response [87]. TLRs can be found in the cell surface, recognising microbial lipids, sugars and proteomes, or in different intracellular compartments, recognising nucleic acid-derivatives from viral or bacterial origin. There is a high level of structural similarity between the key features and factors involved in the cascade signalling of fish and mammalian TLR systems. Nevertheless, significant distinct features and a large diversity have been described for the fish TLRs, possibly due to the diverse evolutionary history and different environments inhabited by fish [84, 87]. TLRs have been described in more than a dozen teleosts species, and during the past decade at least 17 different types have been discovered through genome research in bony fishes, with six molecules not having been previously described in mammals. Some fish's TLRs are true orthologues of their mammalian counterpart, while orthologues of some of the mammalian receptors have not been found in fish. Only a few ligands are currently known for the TLRs described in fish [84, 87]. Further research to identify all the TLR ligands of fish as well as the downstream factors of the signalling cascades is required to understand how they can direct the subsequent adaptive response through induction of different cytokine cascades.

The adaptive immune system appeared later in evolution and is only present in cartilaginous and bony fish, amphibians, reptiles, birds, and mammals [79]. The evolution of three main traits indicate the appearance of this adaptive system: presence of the thymus, the B and T-lymphocytes and the RAG (recombination activation gene) enzymes, which are responsible for generating the great variety of immunoglobulins, including B- and T-cell receptors, through gene reorganisation [83]. The adaptive immune system also comprises humoral and cellular factors.

During an immune response, antigen presenting cells (APC) endocytose antigens, which are then processed and carried from the site of infection to other lymphoid tissues or organs, where the antigen presentation occurs [88]. The antigens are presented to the cellular factors (lymphocytes) of the adaptive branch: T and B-cells. A central component of the adaptive immune response in the antigen presentation process is the major histocompatibility complex (MHC). The MHC is a multigene family which encodes receptor molecules that are able to recognise, bind and present foreign peptides to specialist immune cells, in order to commence an immune response [89]. The highly polymorphic MHC glycoproteins are expressed in the cell surface and consist of an immunoglobulin “stalk”, binding the molecule to the cell surface, and a “basket” receptor, called peptide binding region (PBR). This area is responsible for antigen presentation to the T-cells, and an adequate match between the antigenic peptide, the PBR and the T-cell receptor will initiate an immune cascade. Although MHC binding sites show a certain degree of specificity, they are able to bind numerous different peptides [89, 90]. There are two classes of distinct MHC molecules with different structural and functional characteristics: MHC class I are expressed in surfaces of almost all nucleated cells, and are responsible for presenting endogenously synthesised peptide antigens, such as those originated from intracellular pathogens like viruses, to CD8<sup>+</sup> T-cells, or cytotoxic T-cells. On the other hand, MHC class II molecules are specifically expressed in antigen presenting cells (B-cells, macrophages and activated T-cells), and are responsible for presenting opsonised and degraded pathogen components (i.e. exogenously acquired peptide antigens) to CD4<sup>+</sup> T-cells (or helper T-cells) [83, 90, 91]. Interestingly, the organisation of MHC genes in teleosts differs from that described in humans and mice. The mammalian MHC loci are closely linked, whereas there is a lack of linkage between the classical class I and class II genes in fish, possibly allowing for independent divergence between classes [90, 92, 93]. Associations between certain MHC haplotypes or certain alleles and increased disease resistance have been found, mainly due to the highly polymorphic nature of the MHC [94].

The interaction between the peptide-MHC complex and the T-cell receptor (TCR) is essential to initiate both humoral and cell-mediated responses, by the generation of activated T-cells. Besides the MHC-peptide-TCR interaction, T-cells have additional

membrane molecules, such as the co-receptors CD8 and CD4, which bind to domains of the MHC and strengthen the interaction between TCR and peptide-MHC complex. When naïve T-cells are activated, they undergo through proliferation and differentiation into different effector T-cell populations and can also generate memory responses, producing a pool of cells that can expand in future encounters with the same antigen [83]. The effector populations are responsible for the cellular component of the adaptive immune response, and conduct specific functions such as cytokine secretion and B-cell help (helper CD4<sup>+</sup> T-cells) or cytotoxic killing activity (cytotoxic CD8<sup>+</sup> T-cells) [90].

Cytokines play a major role in the differentiation of T-cells after the antigen recognition in mammals, which define if the subsequent immune response will be beneficial or detrimental for the host [95]. In mammals, IL-2 production increases after T-cell activation, and this particular cytokine is key in the development of naïve CD8<sup>+</sup> cells into effector T-cells, and modulates expression of receptors for other cytokines in differentiation of helper T-cells, promoting development of different subsets [90, 95]. IL-2 has been cloned from rainbow trout and a recombinant form has been shown to increase transcription of IFN- $\gamma$  and IL-2 itself [96].

Naïve CD8<sup>+</sup> cells are incapable of killing target cells, and they do not express IL-2 or IL-2 receptors. They are activated through antigen contact, which induces expression of IL-2 receptor and production of IL-2 as cytokine. This activation is completed by the presence of additional IL-2, secreted by Th1 cells. Hence, proliferation and differentiation of both, cytotoxic effector T-cells and Th1 cells is dependent on the presence of IL-2 [90]. Once activated, these cytotoxic T lymphocytes (CTL) can recognise and eliminate almost any altered cell that present antigens in MHC class I molecules from endogenous peptides, such as viruses and tumours. After the TCR recognises the antigen-MHC class I complex, a CTL-target cell conjugate is formed, the CTL releases cytotoxic granules into the target cell and promptly detaches, leaving the target cell to die by apoptosis. The CTL is then recycled and can attack new target cells. After clearance of antigens, the levels of IL-2 decline inducing apoptosis of Th1 cells and CTLs, therefore reducing non-specific tissue damage due to the inflammatory response [90].

Four different subsets of activated helper T-cells (Th1, Th2, Th17 and induced T-regulatory (T-reg) cells) have been described in mammals, each one producing a different panel of cytokines [95]. There is increasing molecular and cellular evidence of the presence of these subsets in fish. Th1 subset in mammals is responsible for classic cell-mediated functions, such as activation of CTLs; IL-2, IL-12 and IFN- $\gamma$  are the critical cytokines for the development of Th1 cells and this subset secretes mainly IL-2, TNF and IFN- $\gamma$ . Most of the components required for Th1 development have been described in fish [90, 95]. Th2 subset is specialised in immune responses against extracellular parasites. This subset typically secretes the cytokines IL-4, IL-5, IL-9, IL-13 and IL-25 and requires IL-4 for development. The process of differentiation of Th2 is not yet fully elucidated, and some of the components described in the mammalian response have not been described yet in fish. Th17 subset specialises in neutrophil mediated responses against extracellular microbes, by expression of IL-17A, IL-17B and IL-22, which in turn induce expression of various chemokines and antimicrobial peptides. Cytokines involved in Th7 differentiation include IL-6, IL-21, IL23, IL-1 $\beta$  and TGF- $\beta$ . The major components of mammalian Th17 cell development are present in teleost fish. Finally, the T-reg subset regulates and suppresses immune responses against self and foreign antigens. The mechanisms behaving the T-reg suppression are still not fully understood, but there are several mammalian components which have been cloned from different fish species. It is yet to be cleared if the functions of these Th subsets are totally equivalent to those known in mammals, but accumulating evidence suggests that there are numerous similarities. For a full review of the helper T-cells subsets and cytokines involved in the processes in fish see Ref. [95].

B-cells are responsible for the humoral adaptive immune response [78], although their phagocytic activity has been demonstrated in rainbow trout [97]. B-cells can be activated by thymus-independent molecules, such as bacterial cell lipopolysaccharide, or polysaccharides with repeating units, requiring the assistance of an APC. B-cells can also be activated by thymus-dependent molecules like proteins, where the antigen must be processed by the APC and presented to a helper T-cell, which directly contacts the B-cell and elaborates interleukins that provide signals for B-cell differentiation [98]. Upon recognition and activation, B-cells can

differentiate into long lasting memory cells or plasma cells, responsible for producing antibodies or immunoglobulins [83]. Immunoglobulins are the key humoral component of the adaptive immune system, and are found in soluble form (antibodies) in serum and mucosal secretions, or on the surface of B-cells (membrane-bound form) as receptors [76]. In teleost, three different immunoglobulin isotypes have been described: IgM, responsible for systemic responses and discovered decades ago [99], IgD with no clear activity defined yet [100], and IgZ/IgT which has been identified as the mucosal immunoglobulin [101, 102]. Immunoglobulins have various effector functions, which include neutralisation, agglutination, opsonisation and complement-mediated functions [98]. Core components of both the innate and adaptive immune responses are used by fish to control parasite infections. A significant research effort has been made to characterise the host response against *N. perurans*, and yet it is not fully understood (for a review see Ref. [103]).

### **1.5 IMMUNE RESPONSES AGAINST *Neoparamoeba perurans***

The innate components of the immune system are the first obstacle that pathogens have to confront when they contact the host. Initial studies on the innate immune response of Atlantic salmon affected by AGD, showed that the enzyme lysozyme level in serum was not different between AGD-affected and control fish for up to 11 d after exposure to the parasite [104]. However a more recent study found a 4-fold increase in serum lysozyme levels in AGD-affected fish 31 d after infection [105], accompanied by an increase in the percentage of blood neutrophils and monocytes. Nevertheless, the lack of non-infected control fish in the latter experiment makes the interpretation of the results difficult, and it is not clear if this change is only due to the infection and not to other external factors. Another component of the innate immune system studied were phagocytes. Respiratory burst of anterior kidney phagocytes was suppressed as early as 8 d and 11 d post infection with the parasite when compared to naïve controls [104], and phagocyte function has been also reported as suppressed 19 and 26 d following a repetitive infection with the parasite [106].

Host immune responses against *N. perurans* have mainly been studied at the transcriptional level, using either real-time RT-PCR or cDNA microarrays [107-110] as sequences for immune-related genes have become increasingly available [111]. Most of the transcriptional responses to AGD are restricted to the lesions observed in the gills [109]. Initially, there was consensus that a general down-regulation in immune-related genes was present in these lesions. This down-regulation included various genes associated with MHC-I and MHC-II antigen presentation pathways, as well as immunoglobulins transcripts at 36 d post infection [109], however an up-regulation of IL-1 $\beta$  [107, 110] was observed from 7 d after infection. Using 2-D quantitative RT-PCR, a method for mapping transcriptional responses in tissues, a recent study demonstrated that this general down-regulation is not present in early stages of AGD infection, and that it could potentially be an artefact of sampling [112]. These researchers showed that *N. perurans* is indeed capable of inducing a classical inflammatory response in AGD-affected Atlantic salmon, with possible infiltration and involvement of immune cells in AGD lesions, as observed by Adams and Nowak [54]. An increase in transcription of different B-cell and T-cell markers, such as MHC-II, IgM, IgT, MHC-II, TCR, CD4 and CD8 was reported in the gill lesions of AGD-affected fish 10 d post-infection [112].

Immunoglobulins are the main components of the humoral adaptive immune response and antibody responses against *N. perurans* in Atlantic salmon have been documented by various studies [113-116]. These have primarily measured the serum levels of IgM, the main immunoglobulin involved in systemic immune responses in AGD-affected fish. One study demonstrated that resistance of Atlantic salmon against AGD was associated with anti-amoeba antibodies present in serum [117]. Additionally, the percentage of seroconversion in Atlantic salmon has been shown to increase following various cycles of natural AGD infection and freshwater bath treatments in a commercial culture setting [114], but the magnitude of this seroconversion was not reported. In contrast, measurement and characterisation of antibody responses against AGD in mucosal surfaces of Atlantic salmon has proven difficult, with only one study showing the presence of IgM in mucus obtained from skin [115]. However, recent studies have demonstrated that IgT, also called IgZ in some species [102], is the immunoglobulin isotype specialised in mucosal responses

at least in rainbow trout [97, 118]. Even though this immunoglobulin isotype has been described at the transcriptional level in Atlantic salmon [119], there are no functional reports in this species. Nevertheless, the protective nature of any antibody response against *N. perurans* remains unclear, and further studies particularly at the mucosal level are necessary for a better understanding of the roles of different immunoglobulin types and other molecules on the response against the parasite.

## **1.6 STUDY OF MUCOSAL CONSTITUENTS IN FISH AFFECTED BY AGD**

The mucus layer covering the external surfaces of fish has multiple functions which include modulating respiration, ionic and osmotic regulation and defence against diseases (as reviewed by Ref. [120]). This mucous barrier is mainly composed of water, ions and mucins, which are high molecular weight glycoproteins. Mucus also includes a large variety of other proteins and immune-related secretions, including IgM and IgT, complement factors, lysozyme, proteases and antimicrobial peptides, which act as a first line of defence against potential pathogens present in the environment [121].

Augmented mucus production is one of the classical signs of AGD [20]. However, the characterisation of mucus composition of AGD-affected fish has not been performed extensively. Skin and gill mucus were studied in three salmonids in response to *N. perurans* infection [122]. This study documented coordinated biochemical changes in skin and gill mucus induced by the gill parasite in Atlantic salmon and brown trout (*Salmo trutta*), whereas only a localised response in the gill mucus was observed in rainbow trout. In the case of immune responses against the parasite, Ig detection at mucosal level has only been described once, and involved the detection of the IgM isotype in skin mucus [115].

Due to the non-cellular nature of this matrix and the presence of large quantities of inhibiting substances, transcriptional and immunological studies, which have been used in the past to characterise the host response to AGD, lack the capacity to study the variety of immune molecules present in mucus. In this context, proteomics allows the study of the entire range of proteins expressed spatially and temporally by a cell, organ or organism under specific physiological conditions [123], and in the context

of aquaculture it has the potential to aid in the search of antigenic proteins, detection of differentially regulated proteins and characterisation of biologically active proteins in physiological processes [123]. As opposed to genomics, proteomics can provide information of post-transcriptional and post-translational regulation, and therefore data about an organism's physiological state, which could have been missed by the transcriptome [124]. Proteomics has therefore emerged as an exceptional experimental tool for study of mucus and it could be of use to obtain better insight into the protein expression in mucosal surfaces of fish affected by AGD.

Recent studies have used proteomic approaches to identify the role that specific mucus substances might have in the pathogenesis of diseases. Two dimensional (2-D) gel electrophoresis coupled with mass spectrometry is the classical approach used in proteomic studies, and previous research has employed it effectively in analyses of mucus proteomes of fish affected by parasites [125, 126]. However, the 2-D electrophoresis approach presents certain limitations, such as variations between gels, which makes difficult to distinguish between system and biological variations; and the inability to detect the full spectrum of proteins present in a sample, which is necessary for the comprehensive analysis of biological material [127].

New gel free approaches, such as high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS), have become useful in the study of mucus [128], as they represent less expensive alternatives to the traditional 2-D method and allow deeper proteome coverage, particularly for high-throughput proteomics research [124, 129]. However, one issue preventing the extensive use of this technique in samples such as mucus is the lack of an amplification method for proteins, which implies that the characterisation of low-copy number substances, such as antibodies in mucus [97, 130] is still problematic, and therefore other immunological methods such as ELISA are still necessary to complement the information obtained. Proteomics has not been used yet to study mucus of AGD-affected fish, and it is logical to think that it has a great potential in refining the understanding of the possible mucus composition variations in the diseased fish and might permit the identification of new molecules of interest in the host response.

## **1.7 PREVIOUS VACCINATION APPROACHES AGAINST AGD**

In addition to research into the immune responses of salmonids against *N. perurans*, a variety of treatments alternatives have been tested [52, 53, 67-69, 131, 132]. Even though dependent on the presence of water sources, freshwater bathing remains the most commonly used strategy in Tasmania, [131] and alternatively the use of hydrogen peroxide has shown relatively good results against the pathogen in other countries [48]. However, due to the high cost and labour associated with bathing practices, the development of a vaccine against AGD is still one of the research areas of importance for the industry. Vaccines can have a significant positive impact on reducing costs, as was demonstrated back in the late 1980s and 1990s, when they were introduced in Norway for bacterial diseases of Atlantic salmon, resulting in a rapid decline of antibiotics consumption and mortalities [133]. However, the development of vaccines against parasites for many animal species has proven quite challenging [134], and up until late 2013, a commercial vaccine against any parasitic condition affecting commercially cultured fish was yet to be licensed [133]. This lack of success has also been the case with several AGD vaccine attempts. Different types of vaccines have been previously tried against AGD: those involving whole or partially fragmented amoebic antigens, DNA vaccines and those comprised of specifically identified epitopes (Table 1.2).

Before the causative agent of AGD was correctly identified [46], Akhlaghi and colleagues immunised Atlantic salmon with sonicated extracts of cultured *Neoparamoeba* sp. emulsified with Freund's complete adjuvant (FCA), via intraperitoneal (i.p.) injection [135]. Antigens were immunogenic in rainbow trout and Atlantic salmon; but natural infection by cohabitation resulted in successful transmission of the disease. In a subsequent study, salmon were immunised via different routes (i.p. injection or per-anal intubation), using live or sonicated *Neoparamoeba* antigens, from cultured and "wild-type" strains [136]. None of the different preparations had an effect on AGD severity, measured as the number of gill patches observed following immunisation.

A carbohydrate epitope, characterised as a high molecular weight antigen in the surface of *Neoparamoeba* spp., was also tested as a potential vaccine candidate in

salmon [115]. Even though this molecule produced a significant serum and mucus antibody response following an initial immunisation and subsequent booster, no protection against AGD was achieved in the immunised fish, and rather it may have had an immunosuppressive effect.

More recently, different DNA vaccine formulations have been tested. These potential vaccines have shown up to 44% protection in laboratory based challenges [137], when survival curves were analysed. Further studies with DNA vaccines only provided significantly less pathology determined by lower gill scores in vaccinated fish [51], which is a common assessment method used by the industry; however the results were not consistent between different trials and did not comprise the relative percent survival for different groups or measurement of antibody response.

**Table 1.2 Different vaccination approaches against AGD in Atlantic salmon.**

| <b>Immunisation</b>  |                    |               |                      |                  |           | <b>Challenge</b>            |                           |   |              | <b>Response</b>     | <b>Ref</b> |
|--|--------------------|---------------|----------------------|------------------|-----------|-----------------------------|---------------------------|---|--------------|---------------------|------------|
| <b>Antigens</b>  | Route <sup>A</sup> | Dose          | Booster <sup>B</sup> | N                | Adjuvant  | Type                        | Time                      | Antibody <sup>C</sup>                                 | Time         | Protection          |            |
| <i>Neoparamoeba</i> sp.<br><b>PA-016 strain</b><br>(cultured-sonicated)  | inj                | 1 mg          | NO                   | 25               | FCA       | N/A                         | N/A                       | Sera (pooled):3 x higher at 1:64 dilution             |              | N/A                 | [135]      |
| <b>Crude amoebic antigen from gill mucus</b><br>(sonicated)              | inj                | 0.5 mL        | NO                   | 15               | FCA       | N/A                         | N/A                       | 40% seropositive<br>4.5 x higher                      | 6 weeks p.i. | N/A                 | [135]      |
| <i>Neoparamoeba</i> sp.<br><b>whole cells</b>                            | i.p.               | 3,800 cells   | NO                   | 80               | NO        |                             | 30 d p.i.<br>(failed)     | 53% seropositive<br>OD not higher than control        |              |                     |            |
| <i>Neoparamoeba</i> sp.<br><b>whole cells (sonicated)</b>                | i.p.               | 1 mg          | NO                   | 80               | FCA       | Cohabitation x 2            | 45 d p.i.<br>(successful) | 58 and 46% seropositive<br>OD not higher than control | 60 d p.i.    | No (high mortality) | [135]      |
| <i>Neoparamoeba</i> sp<br><b>crude antigen, with or without adjuvant</b> | i.p.               | 1 mg<br>10 mg | NO                   | 40 x 2<br>40 x 2 | FCA       | Natural infection (in farm) | 2.5 months p.i.           | Not measured  |              | NO                  | [135]      |
| <i>Neoparamoeba</i> sp.<br><b>harvested from gills (sonicated)</b>       | i.p.<br>p.a.       | 2,000 cells   | 3 weeks              | 40<br>40         | Montanide | Cohabitation                | 2 weeks post booster      | Not measured  |              | NO                  | [136]      |
| <i>Neoparamoeba</i> sp.<br><b>harvested from gills (live)</b>            | i.p.<br>p.a.       |               |                      | 40<br>40         |           |                             |                           |   |              |                     |            |
| <i>Neoparamoeba</i> sp.<br>(wild-sonicated)                              | i.p.               | 20,000 cells  | 3 weeks              | 40               | Montanide | Cohabitation                | 2 weeks post booster      | Not measured  |              | NO                  | [136]      |
| <i>Neoparamoeba</i> sp.<br>(cultured-sonicated)                          |                    |               |                      | 40               |           |                             |                           |   |              |                     |            |

**Continuation Table 1.2**

| <b>Immunisation</b>  |                    |           |                      |               | <b>Challenge</b>        |                             |                   | <b>Response</b>  |   |                       | <b>Ref</b> |
|--|--------------------|-----------|----------------------|---------------|-------------------------|-----------------------------|-------------------|--|---|-----------------------|------------|
| <b>Antigens</b>  | Route <sup>A</sup> | Dose      | Booster <sup>B</sup> | N             | Adjuvant                | Type                        | Time              | Antibody <sup>C</sup>  | Time  | Protection            |            |
| <b>High molecular weight antigen (HMWA) from infective <i>Neoparamoeba</i> sp.</b> | i.p.               | 50 µg     | 35 d                 | 13            | FCA                     | Experimental<br>500 cells/L | 75 d p.i.         | Sera:<br>100 x higher<br>1,000 x higher<br>(10/13 seropositive)<br>10,000 x higher<br>Mucus:<br>3/5 seropositive | 35 d p.i.<br>49 d p.i.<br><br>105 d p.i.<br>(after challenge) | NO                    | [115]      |
| <b>3 fractions of a 1100 clone DNA EL (&gt;200 clones each)</b>                    | i.m.               | 10 µg DNA | 14 d                 | 50 x 3        | NO                      | Experimental<br>500 cells/L | 14 d post booster | Not measured   |   | Fraction #2<br>RPS 36 | [137]      |
| <b>Fraction #2 DNA EL</b>  |                    |           |                      | 16            |                         |                             |                   |  |   | NO                    |            |
| <b>DNA of 6 targeted antigens</b>  | i.m.               | 14 µg DNA | NO                   | 16            | NO                      | Experimental<br>500 cells/L | 14 d post booster | 2 – 14 x higher  | 8 weeks p.i.  | RPS 33-53             | [137]      |
| <b>DNA of 6 targeted genes (2 vectors) Extra group with molecular adjuvant</b>     | i.m.               | 6 µg DNA  | 14 d                 | 100 x2<br>100 | NO<br>1 µg DNA<br>IL-1β | Experimental<br>500 cells/L | 14 d post booster | Not measured   |   | RPS 19-28             | [137]      |
| <b>Mix of DNA from 6 targeted genes</b>  | i.m.               | 6 µg DNA  | 14 d                 | 120 x 2       | NO                      | Experimental<br>500 cells/L | 14 d post booster | Not measured   |   | RPS 38-41             | [137]      |
| <b>Mix of 6 rproteins from targeted genes</b>                                      | i.p.               | 1 µg each |                      |               | Aluminium oxide         |                             |                   |  |   | RPS 32                |            |

N/A = not available; p.i. = post primary immunisation; EL= Expression library; <sup>A</sup> = i.m. = intramuscular injection, i.p. = intraperitoneal injection, p.a. = peranal intubation, inj = unspecified injection route; <sup>B</sup> = Booster time after first immunisation; <sup>C</sup> = Increase of antibody responses compared to experimental control; RPS= relative percent survival, calculated as  $RPS = (1 - (\% \text{ vaccinated mortalities} / \% \text{ control mortalities})) \times 100$ .

The criteria developed by Amend [138] to test the potency of vaccines in fish (Table 1.3), have been used as a method that allows the evaluation of vaccines under controlled challenge conditions. Their main advantage is the possibility of comparing potency outcomes between challenge experiments by using the relative percent survival results, as long as the test conditions are well defined [138]. However challenge testing of vaccine effectiveness and safety can involve the use of large numbers of animals and considerable distress [139]. Validation of alternative methods, such as evaluating protective antibody responses generated by vaccines, has been proposed as a possible strategy to reduce, refine, and replace the use of animals in vaccine potency testing [139].

**Table 1.3 Criteria for potency testing of vaccines (elaborated by Amend [138]).**

- 
- 1.** Duplicate groups with at least 25 fish (50 total) for each treatment group.
  - 2.** Fish should be maintained for 25-60 d before challenge.
  - 3.** When using bath challenge method, 2 levels of infection should be used.
  - 4.** All fish are pathologically inspected for specific infection.
  - 5.** Non-specific mortality or infections following challenge should not exceed 10% within each group.
  - 6.** The variation between duplicate groups should not exceed 20%.
  - 7.** The infection rate in the control treatment group should be higher or equal than 60%.
  - 8.** The infection rate in the vaccinated group should be less or equal than 24%.
  - 9.** If infection levels as those detailed in 6. and 7. above are not met in one of the challenge levels, the results of the two challenges can be combined if the infection rate is less or equal than 25% in both vaccinated groups, and evaluated as above.
  - 10.** Unsatisfactory results are defined by failure to meet any of the criteria above.
- 

It is now widely accepted that a successful vaccination approach requires the induction of a strong and long-lasting memory T-cell response, which has the potential to generate a large and efficient population of effector cells [140]. APCs in particular dendritic cells in mammals, are considered the limiting step in the generation of an adequate adaptive immune response, as the generation of memory

and effector cells depends on the co-stimulatory signals provided by these APCs to naïve T-cell [141, 142]. Adjuvants represent one alternative to improve vaccine effectiveness, as they can potentially increase antigen uptake and presentation by APCs, induce a “danger” signal via PRR signalling and provide co-stimulatory signals for lymphocyte activation [143]. The majority of vaccines used in aquaculture are delivered by injection, using oil adjuvants and these represent the most effective method of fish vaccination implemented to date [133]. However, understanding cellular signalling and stimulation of the fish immune response can provide useful tools to evaluate whether vaccines are stimulating the appropriate immune pathways.

In the context of AGD, while gene expression studies have provided useful information about regulation of different immune pathways, there is still a need for reagents that allow identification of cellular response’s markers. Immunohistochemical analyses of gills from AGD affected fish have shown the presence of MHC<sup>+</sup> class II cells within lesions, indicative of trafficking of immune cells in the area [144]; and a preliminary study has shown that a certain MHC allele present in Atlantic salmon is associated to lower severity of AGD infection [94]. However, studies on Th responses of AGD affected fish have not been conducted yet and their nature remains unclear, as the more significative cytokine signal reported to date, an increase in transcription of the Th-17 associated IL-1 $\beta$  molecule, might be due to an increase expression of the hyperplastic epithelial cells characteristic of AGD lesions [112]. Expansion of knowledge in this area could be beneficial for the development of an efficacious targeted vaccine.

On the other hand, mucosal vaccination strategies represent a very promising approach for protection of fish against mucosal pathogens, in addition to the advantages presented by the reduced labour required for their application and lower stress levels induced in fish, but their development is based on a better understanding of mucosal immunity and memory responses. Most of the previous vaccination studies have focused on the survival of fish to AGD after vaccination, without taking into account antibody and transcriptional responses. However, it is also important to understand the host antibody responses against the antigen used in vaccination. Therefore, the measurement of antibody levels, the study of their functionality in the

mucosal surfaces in contact with *N. perurans* and their effects on the vaccine success, formed an important component of the research conducted for this thesis.

## **1.8 AIMS AND THESIS STRUCTURE**

AGD has been the subject of a considerable amount of research, and as mentioned before, the development of a vaccine still remains as a high priority for the industry. A better understanding of the antibody immune response associated with the disease is necessary to develop a potentially successful vaccine strategy. Therefore, a series of experiments were conducted in this thesis, aiming to:

1. Investigate the mucosal and systemic immune response of Atlantic salmon against *N. perurans*, the causative agent of AGD.
2. Investigate mucosal and systemic anti-*N. perurans* antibody responses to a recombinant putative attachment protein of the amoeba, first identified by the generation of a cDNA library from the parasite.
3. Investigate vaccine formulations for AGD, using the recombinant protein described above.
4. Investigate other mucosal components potentially involved in the response against *N. perurans*.

Five research chapters were developed as part of this thesis to fulfil the above stated aims:

Chapter 2 describes the results obtained from an experiment where the antibody responses of Atlantic salmon were assessed at transcription and antibody levels, after repeated infections with *N. perurans*; and a second experiment, where immune responses were assessed after a single infection, and fish were fed commercially developed diets containing immunostimulants.

Chapter 3 describes the results of two concomitant studies that assessed the systemic and mucosal immune responses of Atlantic salmon exposed to two protein-hapten antigens, delivered using different strategies, aiming at evaluating the best delivery method for subsequent experiments.

Chapter 4 describes the process involved in generating a recombinant protein identified as a putative attachment factor of *N. perurans* and the results of an

experiment which tested the effects of this protein as an immunogenic factor in systemic and mucosal antibody responses against the amoeba.

Chapter 5 describes a vaccination and challenge trial with AGD, using the recombinant protein described in Chapter 4 via different delivery strategies. The implications of a co-infection with *Y. ruckeri* during the trial are also discussed.

Chapter 6 presents a proteomics study carried out on skin and gill mucus of AGD-affected fish, which aimed to identify the changes in the proteome of mucus after repeated infections with amoebae.

Research Chapters (2-6) are either published or were prepared as manuscripts to be submitted for publication, and therefore some textual overlap between those Chapters was unavoidable. In addition, Chapter 1 (this Chapter) is a general introduction to salmonid culture worldwide and in Tasmania, an explanation of the main diseases affecting salmon in this state and the importance of AGD; it also introduces the subjects developed in the research Chapters. More detailed information about each research Chapter is included in each particular introduction.

Chapter 7 is a general discussion that synthesises the most significant findings from the research Chapters and places them in context with the current understanding of the disease. The referencing style of the journal *Fish and Shellfish Immunology* has been adopted for this thesis, and a single list of references is included at the end of the document.

**CHAPTER 2.**

**EFFECTS OF IMMUNOSTIMULANTS  
AND REPEATED INFECTIONS  
WITH AMOEBIC GILL DISEASE ON ANTIBODY  
LEVELS AND IMMUNE GENE EXPRESSION IN  
ATLANTIC SALMON (*Salmo salar*)**

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**KEYWORDS**

Atlantic salmon, Amoebic gill disease, immunostimulants, antibody response,  
immune gene expression

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## 2.1 ABSTRACT

Amoebic gill disease (AGD) is the main health problem for the salmon industry in Tasmania, Australia and is now reported in most salmon producing countries. Antibody and gene expression responses to the pathogen, *Neoparamoeba perurans*, have been studied following primary exposure; however, the effects of sequential reinfection, which can often occur during net-pen culture of salmon, remain unclear. The effects of immunostimulants on the survival and immune response to AGD have been studied, but their influence on systemic and mucosal antibody levels or immunoglobulin (Ig) transcription is unknown. Herein, we assessed the antibody responses as well as Ig transcription in the gills of Atlantic salmon sequentially exposed to AGD. After four successive AGD challenges, no significant differences in plasma or skin mucus levels of IgM were observed between AGD-naïve and challenged fish. However, IgM gene expression in gill lesions of AGD-affected fish increased up to 31 d after infection, while no changes in IgT, TCR and CD8 transcription were observed. Changes at IgM transcription level did not match the lack of antibody response in mucus, which is possibly explained by weak correlations existing between protein and mRNA abundances in cells and tissues. In the second experiment, we investigated the Ig response to AGD at the transcriptional as well as antibody production level in salmon fed two commercial diets formulated with immunostimulants. The immunostimulant diets did not affect the levels of serum or skin mucus IgM and did not induce IgM or IgT transcription at the site of AGD infection, but both IgM and IgT transcriptions were increased in the gills of AGD-naïve fish and TCR transcription was decreased in AGD lesions. Therefore, further research is required to test potential impacts of different doses of immunostimulants and/or different delivery routes.

## 2.2 INTRODUCTION

Amoebic gill disease is an important disease affecting salmonids worldwide [22, 34, 37, 41], and is the main condition threatening the marine grow-out phase of Atlantic salmon culture in Australia. The causative agent is *Neoparamoeba perurans* [46], a ubiquitous amphizoic marine amoeba. The main signs of the disease are lethargy and respiratory distress and outbreaks can lead to high mortalities [20]. The characteristic

disease presentation is restricted to the gills, where excessive mucus production and white raised lesions can be observed. Histologically, these lesions are characterised by hyperplasia of epithelial cells, with extensive lamellar fusion, formation of large vesicles, and loss of normal gill structure with limited response of immune related cells [20, 54, 55]. The only commercial treatment is freshwater bathing of the affected fish [20], which requires significant infrastructure and is labour intensive. Due to the high costs associated with this disease and subsequent treatment strategies, significant efforts have been directed at the prevention of AGD through the use of immunostimulant diets [105, 145, 146], selective breeding programs [70, 71] and vaccine development [51, 136, 137, 147]. Furthermore, numerous studies have focused on attempting to understand the innate and specific immune processes to the disease (for a review see Ref. [103]).

Studies focusing on antibody responses against *N. perurans* in Atlantic salmon have measured the presence of immunoglobulin M (IgM) [113-116] which is known to occur in systemic and mucosal responses [76]. There is no clear evidence that these antibody responses are protective and they have typically been measured only after a primary AGD infection. However, during commercial culture of Atlantic salmon and following three rounds of natural AGD infection and freshwater bath treatments, the percentage of seroconversion was shown to increase in AGD-affected fish, but the magnitude of the antibody response was not documented [114]. A new class of Ig, known as IgT, was described in rainbow trout (*Oncorhynchus mykiss*) in 2005 [101]. Since then, it has been shown to play an important role in mucosal responses in the gut [97], the skin [118] and the gill [148, 149] of this species. Unfortunately, reagents required to measure the IgT levels in Atlantic salmon are not readily available and, in our experience, the antibodies raised against rainbow trout IgT [97, 149] do not react against this molecule in Atlantic salmon.

Since measuring direct antibody responses in mucosal surfaces of Atlantic salmon have proven difficult, some characterisation of the host immune response against the parasite has been achieved using gene expression [107-110]. Most gene expression studies have focused on a single challenge with AGD, and therefore there is no information available on the immune response at transcriptome level in fish after

multiple rounds of infection. Until recently, the majority of the transcriptional responses to AGD were measured in lesions and characterised by a general down-regulation of immune-related genes, including various genes associated with MHC-I and MHC-II antigen presentation as well as IgM and IgT heavy chain transcripts [109], with the exception of an up-regulation of IL-1 $\beta$  [107, 110]. Recently, researchers in our group were able to demonstrate an increase in gill gene expression of different immune cell markers, namely MHC-I, MHC-II, IgM, IgT, TNF- $\alpha$ , TCR, CD4 and CD8, which varied according to lesion severity in AGD-affected fish [112]. This has shown that *N. perurans* does indeed induce a classical inflammatory response in AGD-affected Atlantic salmon providing evidence of the infiltration and involvement of immune cells in and surrounding AGD lesions.

Effects of several immunostimulants on the immune response against AGD have been tested with mixed results (as reviewed by Ref. [103]). Oral administration is the preferred delivery route for immunostimulants due to the low level of stress induced, the possibility for mass administration regardless of fish size and the success rate obtained with a range of pathogens and immunostimulatory substances (as reviewed by Ref. [150]). In the case of AGD,  $\beta$ -glucans have been shown to stimulate the respiratory burst activity of Atlantic salmon head kidney macrophages *in vitro*, but when incorporated into diets fed to Atlantic salmon, they failed to have the same effect *in vivo* and did not increase the resistance to the disease [146]. Oligodeoxynucleotides (ODNs) containing cytosine-phosphodiester-guanine (CpG) motifs have been demonstrated to increase the resistance to AGD, significantly decreasing mortalities in challenged fish when administered by i.p. injection [145], but they are yet to be tested using other delivery methods. There is no information available yet about the effects of orally administered immunostimulants on systemic and mucosal antibody levels and their mRNA expression in relation to AGD.

The aim of this study was to assess the systemic and mucosal antibody responses and both IgM and IgT gene expression in the gills of fish that had been sequentially exposed to AGD (i.e. four consecutive challenges), as this experimental model resembles more closely the progression of the infection under commercial conditions. Since previous evidence has shown that the levels of seroprevalence in Atlantic

salmon increase with successive infections and freshwater bathing cycles in a commercial setting [114], we aimed at determining if this increase in seroprevalence correlates with an actual increase in the levels of antibodies in the serum and mucus of AGD-affected fish, and with their transcription levels. Additionally, we investigated the effect that commercial diets, formulated with immunostimulants, have on the Ig response against the pathogen in Atlantic salmon, both at the gene expression level as well as the antibody production level. The use of immunostimulatory diets that could control the effects of AGD would be beneficial for the salmon farming industry.

## **2.3 MATERIALS AND METHODS**

### **2.3.1 Effects of repeated AGD exposure on antibody levels and transcription**

#### *2.3.1.1 Fish*

Atlantic salmon (n=100) with average body weight of 162.75 g (SD 35.86 g) were obtained from a commercial farm and held at the Aquaculture Centre, University of Tasmania. Animals were held for 10 d prior to the experiment and for the duration of the trial in a seawater recirculation system consisting of 4 x 1,000 L glass fronted tanks, and maintained at 15±1°C. Two tanks were assigned for infection and two tanks were used as negative controls. This project was approved under UTAS Animal ethics committee approval number A0009717.

Fish from the 2 infection tanks were initially exposed to 150 amoebae/L, and re-exposed to the parasite at the same density 5, 8 and 14 weeks later, to emulate a recurrent infection. Between infections, fish from both infection and control tanks were freshwater (<5 ppt salinity) bathed for approximately 5.5 h to limit the disease progress as per industry practice. Infections were initiated using *N. perurans* harvested from an ongoing infection tank located at University of Tasmania, following procedures described previously [151]. In brief, amoebae were left to attach to Petri dishes, washed and incubated at 18°C overnight, covered in seawater with antibiotics (ampicillin at 9.6 µg/mL and oxolinic acid at 20 µg/mL), harvested and counted using a haemocytometer, placed into 1 L sterile seawater and added to the infection tanks.

### *2.3.1.2 Sampling procedures*

Surviving fish were collected at 18 weeks after the original infection (4 weeks after the last infection, n=24 for AGD-affected; n=19 for non-AGD affected fish) and anaesthetised in 0.1 mg/L clove oil diluted in 10 L of freshwater.

Skin mucus was scraped from both sides (flanks) of the fish using the blunt edge of a scalpel blade and transferred into 2 mL microcentrifuge tubes containing 1 mL “mucus buffer (2 mM PMSF, 10 mM EDTA, 0.02% sodium azide in 0.85% saline with 10 µL anti-protease cocktail (Sigma Aldrich, Sydney, NSW, Australia). On the same day, the mucus samples were centrifuged at 15,000 x g for 1 h, the supernatant collected and frozen at -80°C.

Fish were bled from the caudal vein, and blood was aliquoted into heparinised tubes for collection of whole blood and plasma or into clean tubes for sera. Whole blood was centrifuged at 1,000 x g for 10 min, plasma retrieved and frozen at -80°C.

After mucus and blood collection, fish were killed by an overdose of anaesthetic. Perfusion of the organs was performed with heparinised 0.9% physiological saline (Baxter, Deerfield, IL, USA) via puncture of the bulbous arteriosus as previously specified [152] to remove any remaining blood, until the gills were white. The gill basket was carefully removed and four hemibranchs were placed into 20 mL mucus buffer. Another two hemibranchs were placed into 25 mL of nucleic acid preservation solution (NAPS, 4 M ammonium sulphate, 25 mM sodium citrate, 10 mM EDTA; pH 5.5) as described elsewhere [153]. All the reagents were kept on ice during collection. Gill arches in NAPS were stored for 24 h at 4°C and then transferred to -20°C.

## **2.3.2 Effects of immunostimulatory diets on antibody responses to AGD**

### *2.3.2.1 Fish*

Atlantic salmon (n=380) with average body weight 150.74 g (S.D. 34.11) were obtained from a commercial hatchery and maintained at the Aquaculture Centre at the University of Tasmania. Fish were kept in a 6,000 L recirculating system, comprised of multiple 250 L fibre glass tanks (n=25 fish per tank), in 20 ppt salinity water and 14°C for a week; and then acclimated to full strength salinity and 16°C

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over two weeks. Fish were maintained for 10 weeks before challenge under a continuous 24 h light regime, and water quality was checked daily, with temperature ranging  $16\pm 0.5^{\circ}\text{C}$  over the study.

During acclimation and challenge, fish were fed three times daily to satiation with experimental diets under development by a commercial feed company. Following an initial acclimation diet, a control diet (A) was compared to two experimental diets (B and C) which were similar with the exception of immunostimulant inclusion. Feed consumption levels were evaluated in a parallel study [105]. The immunostimulants used contained various functional ingredients based on beta 1,3-1,6 glucans and nucleotides, as well as essential oils, organic acids and antioxidants. The proximate composition of each diet is shown in Table 2.1

Fish were challenged once with *N. perurans* collected and harvested as described for experiment 1. The total number of challenge amoebae (500 cells/L) was obtained in successive isolations over 3 d and added to tanks accordingly. This project was approved under UTAS Animal ethics committee approval number A00012143.

**Table 2.1 Proximate composition of the four diets used to feed Atlantic salmon in Experiment 2.**

| <b>Diet</b>     | <b>Acclimation</b> | <b>Control<br/>(A)</b> | <b>Experimental<br/>(B)</b> | <b>Experimental<br/>(C)</b> |
|-----------------|--------------------|------------------------|-----------------------------|-----------------------------|
| <b>Moisture</b> | 6.4                | 5.7                    | 6.6                         | 6.3                         |
| <b>Protein</b>  | 46                 | 46.3                   | 46.3                        | 47.2                        |
| <b>Fat</b>      | 26.7               | 29.6                   | 26.9                        | 29                          |
| <b>Ash</b>      | 4.90               | 4.70                   | 5.10                        | 5.00                        |

### 2.3.2.2 Sampling procedures

Mucus and blood were obtained before the challenge and 4.5 weeks later when mortality had reached 60% in one of the treatment groups and the experiment was terminated. This percentage is generally used as an endpoint for challenge experiments, following the guidelines established by the Animal Ethics Committee of the University and by previous studies [138]. Twelve fish from each diet, at each time point, were anaesthetised as described in 2.3.1.2. As the mucus collection method used in the previous experiment proved to be time consuming, for this experiment

individual fish were placed in a 1 L plastic bag and the skin in their flanks massaged for 30 s to harvest mucus; 250  $\mu$ L of antiprotease cocktail (Sigma Aldrich) were added immediately and mixed in with the mucus, which was kept on ice. Mucus samples were transferred to 5 mL plastic tubes within 4 h of collection, vortexed vigorously, passed 20-30 x through a 3 mL syringe with a 25 G needle, centrifuged at 10,000 x  $g$  and 4°C for 10 min and supernatant collected. Blood was drawn from the caudal vein and left to clot in a 1.5 mL microcentrifuge tube overnight at 4°C. Serum was obtained after centrifuging the samples at 1,500 x  $g$  for 10 min at 4°C. Samples were stored at -80°C.

### **2.3.3 Enzyme-linked immunosorbent assay (ELISA)**

The activity of anti-*N. perurans* antibodies in serum, plasma and gill and skin mucus was determined by an ELISA. The coating antigen was sonicated *N. perurans*, collected and harvested as described in section 2.3.1.1. A single point dilution ELISA was run for samples (in duplicate), optimizing coating antigen and sample concentrations as previously described [152]. Optimum concentration for amoebic antigen was 4.8  $\mu$ g/mL, with serum/plasma optimum dilution set at 1:200 and mucus at 1:2. A positive standard curve was used in every ELISA plate, which consisted of a pool of sera from fish selected for high antibody levels from a previous experiment, aliquoted and maintained at -20°C. This standard was used in triplicate 2-fold dilutions (1:50 to 1:6,400) as a reference curve for the serum ELISA, or in 1:2 diluted mucus spiked with standard, as the reference curve for the mucus ELISA. Negative controls, included in duplicate in each plate at the same dilution as the samples, consisted of a pool of sera or mucus from fish not used in this experiment that had been kept in freshwater and therefore naïve to AGD.

Plates were coated overnight at 4°C with antigen (100  $\mu$ L/well), then washed 3 x with low salt wash buffer (2.42 g Trisma base, 22.22 g sodium chloride, 0.5 mL Tween 20 in 1 L distilled water, pH 7.3) and non-specific sites were blocked at 18°C for 2 h with 2% casein (Sigma Aldrich) in TBS (10 mM Tris, 150 mM sodium chloride, pH 7.5). Following another three washes, diluted serum or mucus were incubated for 1.5 h, followed by a mAb against salmon IgM heavy chain conjugated with HRP (Cedarlane, Burlington, ON, Canada) at 1:1,000 dilution for 1 h. After

incubation with each sample and antibody, plates were washed 5 x with high salt wash buffer (2.42 g Trisma base, 29.22 g sodium chloride, 1 mL Tween 20 in 1 L distilled water, pH 7.7). Samples and antibody were added at 100 µL/well, diluted in 0.3% casein in TBS and incubated at 18°C. Bound antibody was visualised by adding TMB One Solution (Promega, Fitchburg, WI, USA) for 10 min at RT and stopped with 2 M H<sub>2</sub>SO<sub>4</sub> solution. Optical density (OD) readings were measured at 450 nm in a Rainbow Thermo plate reader (Tecan Group Ltd., Männedorf, Switzerland).

Sample ODs from ELISA analyses were corrected by subtracting the mean OD of the negative controls in the same plate. ELISA results were reported as levels of antibody units derived from the standard positive curve on individual plates, considering a dilution of 1:50 as 100 antibody units, 1:100 dilution as 50 antibody units and so on. A four parameter logistic function was used to determine the antibody values [152]. This model has shown good results for calculations of total antibody in ELISA analyses when compared to other methods [154]. Calculations were done in GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego CA, USA). Antibody unit values for mucus were expressed per mg of protein in the sample; protein in mucus was measured with a Micro BCA<sup>TM</sup> Protein Assay Reagent Kit (Pierce, Scoresby, VIC, Australia).

### **2.3.4 Gene Expression**

#### *2.3.4.1 RNA extraction and cDNA synthesis*

Gill samples that had been preserved in 5 volumes of NAPS at -20°C, were excised from sites presenting AGD lesions macroscopically, as well as sites without evident lesions, from AGD-affected and non-AGD affected fish. They were classified as gills from non-AGD affected salmon (AGD (-), lesion (-)) and from AGD-affected salmon in areas with no apparent lesion (AGD (+), lesion (-)) or with typical AGD lesion (AGD (+), lesion (+)). Approximately 5 mg of sample was removed from the preservation solution and cells were lysed using an extraction buffer (4 M Urea, 1% SDS and 2 x PBS), containing 5 units of proteinase K. To accelerate digestion, samples were homogenised with pestle and incubated on ice for 15 min. RNA was phase separated from eluted total nucleic acids by addition of 1 mL of TRI reagent® (Sigma-Aldrich) and followed by 50 µL BAN® phase separation reagent (Molecular

Research Centre, Cincinnati, OH, USA) as outlined by the manufacturer. RNA was then precipitated from the supernatant by adding one volume of isopropanol and centrifugation at 16,000 x g for 10 min. The nucleic acid pellet was then rinsed twice with 75% ethanol and resuspended in 50 µL water with 20 mM DTT at 55°C for 5 min. Complete removal of DNA was carried out by adding 1 x Baseline-ZERO™ DNase reaction buffer with 2 units of Baseline-ZERO™ DNase (Epicentre Biotechnologies, Madison WI, USA) for 30 min at 37°C. RNA was quantified by using a Qubit® 2.0 Fluorometer (Life Technologies), and quality was visualised on a 1% formaldehyde denaturing agarose gel using RNA EZvision dye (Amresco, Sydney, Australia) as per manufacturer's instructions [153]. A portion of RNA (1 µg) from all samples was reversed transcribed using a cDNA Synthesis Kit (Bioline, NSW, Australia) with Oligo (dT)<sub>18</sub> primer mix. A portion of remaining RNA from each sample was pooled and 1 µg was reversed transcribed in triplicates for use in preparing real-time qPCR standards. Three additional reactions (1 µg) of pooled RNA without reverse transcriptase were used as no-reverse transcription controls.

#### *2.3.4.2 Real-time qPCR*

Extracted and transcribed cDNA was used to measure the expression of IgT and IgM, as well as T-cell receptor  $\alpha$ -chain (TCR) and CD8 genes by SYBR green chemistry, in reference to two housekeeping genes –  $\beta$ -actin and elongation factor 1 $\alpha$  (EF1 $\alpha$ ) – using the primers listed in Table 2.2. Primers for IgT were designed with Beacon Designer™ 7.8 (Premier Biosoft, CA, USA) and Geneious® 6 software ([www.geneious.com](http://www.geneious.com)). All real-time qPCR analyses were conducted on a iQ™5 Multicolour Real-Time PCR detection system (Bio-Rad, NSW, Australia) with efficiency and linearity of standard curves held to between 85-105% and 0.98-1.00, respectively for all assays. Each PCR reaction was performed in 10 µL volumes consisting of: 1 µL cDNA template, 2 x Sensifast™ + SYBR® mastermix (Bioline), forward and reverse primers (200 nM of each) and molecular grade water. Samples were analysed in duplicates with a 5-step, 4-fold dilution series of the pooled cDNA included into each plate, to calculate efficiency and linearity. Real-time qPCR was run under the following conditions: initial DNA polymerase activation at 95°C for 2

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min, then 40 cycles of 95°C for 10 s, 55°C for 30 s and 72°C for 20 s. At the end of the protocol, a melt curve analysis was run to ensure specificity of amplification.

**Table 2.2 Oligonucleotide primers used in real-time qPCR experiments.**

| Target mRNA        | Primer | Sequence (5' →3')        |
|--------------------|--------|--------------------------|
| <b>β-actin</b>     | F      | TTGCGGTATCCACGAGAC       |
|                    | R      | TAGAGGGAGCCAGAGAGG       |
| <b>EF1a</b>        | F      | TGATTGTGCTGTGCTTA        |
|                    | R      | AACGCTTCTGGCTGTAGG       |
| <b>IgM</b>         | F      | TGAGGAGAACTGTGGGCTACACT  |
|                    | R      | TGTTAATGACCACTGAATGTGCAT |
| <b>IgT</b>         | F      | TGCTCAGTCCGTCTCTCT       |
|                    | R      | ATAATTGTCTTCGCCCACCTT    |
| <b>TCR-α chain</b> | F      | GTCTGACTCTGCTGTGTA       |
|                    | R      | GTGTAGTAGGATGGCTCAT      |
| <b>CD8</b>         | F      | AAGACAACGCTGGAATGG       |
|                    | R      | TATCTGCTCCTCGCTGAA       |

### 2.3.5 Statistical analyses

To assess differences in plasma and mucus antibody responses measured by ELISA before and after challenges in the repeated infection experiment, a Mann-Whitney by ranks test was used to compare samples since data were not normally distributed. A one-way ANOVA was used, followed by Tukey's test for the immunostimulatory diet experiment, when the effect of the diet was considered, before and after challenge. Levene's test was used to verify equality of variances for all the tests. Statistical analyses were performed in Graphpad Prism 5.01 (Graphpad Software) with a *P*-value<0.05 acknowledging significant results.

Statistical analyses for gene expression data were carried out as previously described [153]. The real-time qPCR data were analysed with qBasePLUS software (Biogazelle, Zwijnaarde, Belgium) as described elsewhere [155], where mRNA expression was normalised using the mean expressions of the two reference genes [156]. The normalised relative quantity (NRQ) was calculated for each gene in each assay replicate and scaled to the AGD-naïve group. Data from grouped biological replicates were used for statistical comparisons, following a  $\log_{10}$  transformation as previously suggested [157]. Induction of IgT, IgM, TCR and CD8 was compared between types of samples using a 1-way ANOVA in qBasePLUS, followed by Tukey's test comparison for the repeated infection experiment. For data from the immunostimulatory diet experiment, an initial 2-way ANOVA was used with diet and AGD-status as factors to evaluate the gene transcription level in Graphpad Prism 5.01; and then a 1-way ANOVA followed a Tukey's test was used to assess the effect of the diets on gene transcription within gill sample types in qBasePLUS.

## **2.4 RESULTS**

### **2.4.1 Effects of repeated AGD infection on antibody levels and transcription**

For plasma, the median IgM level in non-AGD affected fish was similar to that of fish after four challenges (Mann-Whitney  $U=165.0$ ,  $df\ 39$ ,  $P=0.126$ ), with only 1 fish after the four challenges showing antibody levels above 100 units. No increase in the IgM levels was found in the skin mucus after the challenges ( $U=214.5$ ,  $df\ 38$ ,  $P=0.472$ ). With the exception of 2 fish, all individuals presented IgM levels close to 0 in both groups. In gill mucus, IgM levels were not detectable for most of the fish from both groups (non-AGD affected and after four challenges), and therefore no statistical analysis was performed (Figure 2.1). The fish with a high antibody response in skin mucus in the non AGD group (2.6 units) actually had a high serum response also (34 units). However, in the case of the group challenged 4 consecutive times, the fish with high response in mucus (4.9 units) did not have the highest response in serum, but the antibody level was still high (41 units).

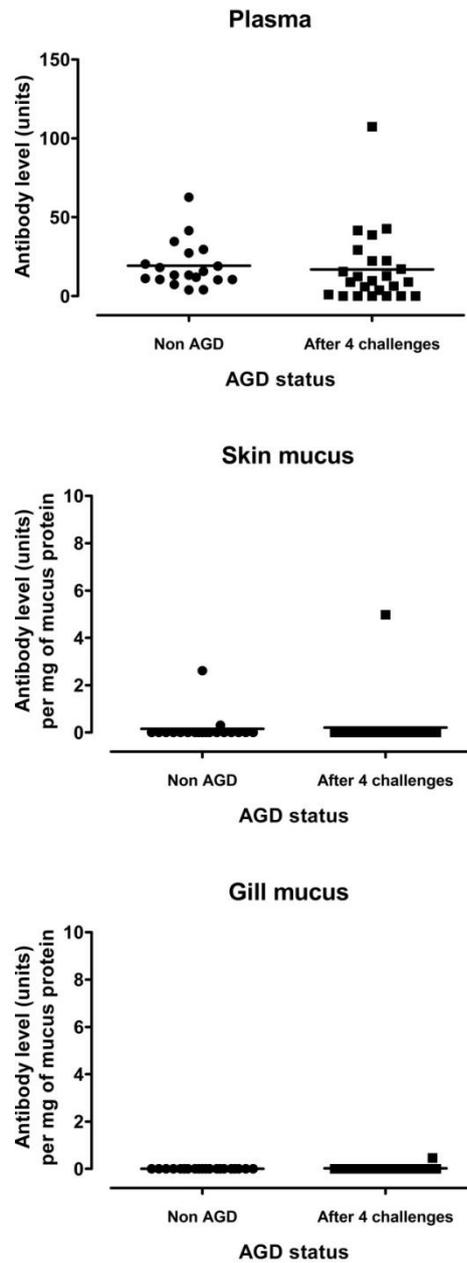


Figure 2.1 Antibody (IgM) levels (units) in plasma and IgM levels in skin mucus and gill mucus corrected by the level of mucus protein, from Atlantic salmon (*Salmo salar*) from experiment 1, which tested the effects of repeated AGD infection. Dots (•) represent fish which have not been affected by AGD (n=19) while squares (■) represent fish that have been subjected to four consecutive challenges with the disease (n=24) for a total period of 18 weeks. Dots/squares indicate individual values and bars represent group means.

Immune-related genes were constitutively expressed in the gill samples of non-AGD affected salmon (Figure 2.2). In contrast to the lack of change observed for IgM level between mucus of AGD-infected and control fish, IgM transcription was significantly up-regulated in AGD-affected gill areas of Atlantic salmon (n=22), following four consecutive infections ( $F=6.551$ , d.f. 2,59,  $P=0.003$ ), when compared to gill samples of non-AGD affected salmon (n=18) or to gill samples obtained from AGD-affected salmon but from areas with no lesion (n=22). However, the change observed for IgM in AGD lesions was less than 2-fold. Alternatively, IgT expression in gills was not significantly different among AGD-affected gill of Atlantic salmon (n=22), gill samples of non-AGD affected salmon (n=18) or gills obtained from AGD-affected salmon but from areas with no lesion (n=22;  $F=0.90$ , d.f. 2,59,  $P=0.412$ ). Additionally transcription of CD8 ( $F=3.125$ , df 2,31,  $P=0.585$ ) and TCR ( $F=3.187$ , df 2,31,  $P=0.055$ ) were not significantly different among the gills from AGD-naïve (n=10) and AGD-affected salmon, with (n=11) or without lesion (n=13) (Figure 2.2).

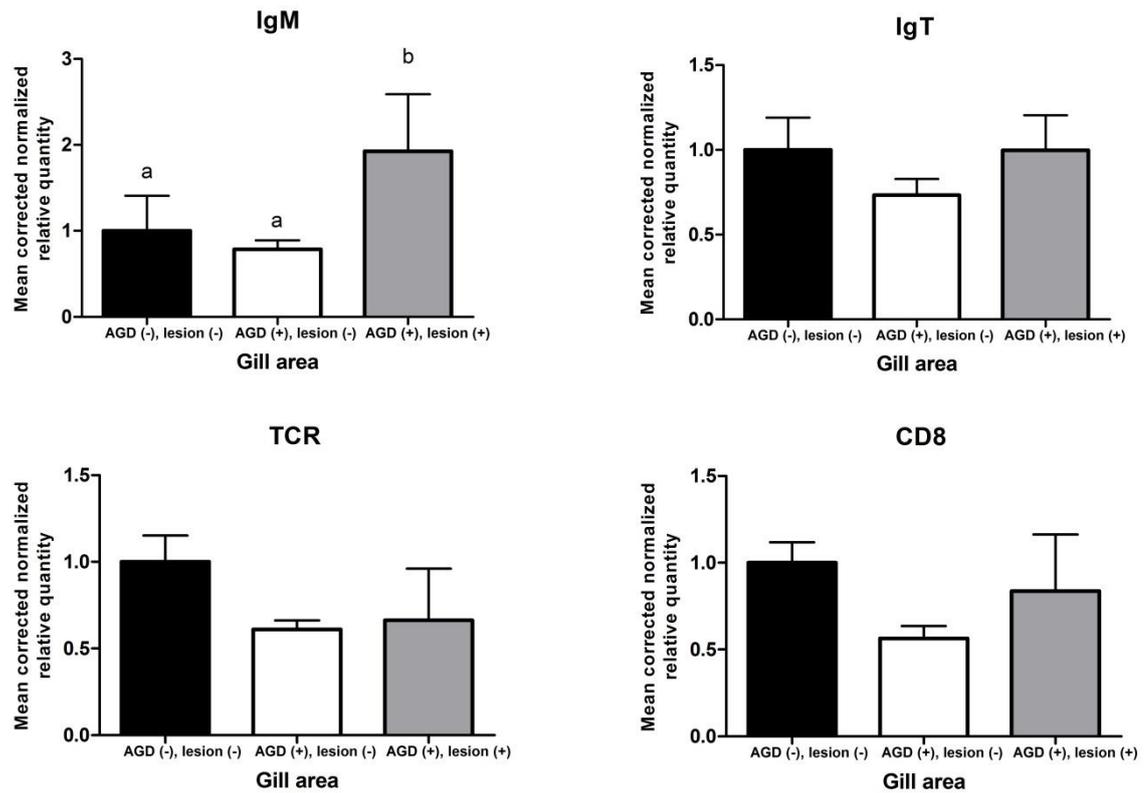


Figure 2.2 Quantitative RT-PCR analysis of immune-related gene expression in gill of Atlantic salmon (*Salmo salar*) from experiment 1, which tested the effects of repeated AGD infection. Three different gill samples were analysed: gills from non-AGD affected salmon (n=10-19) and from AGD-affected salmon in areas with no apparent lesion (n=13-22) or with typical AGD lesion (n=10-22). Bars represent mean values (+S.E.). Different letters represent significant differences by one-way ANOVA ( $P < 0.05$ ).

**2.4.2 Effects of immunostimulatory diets on antibody responses to AGD**

The feed consumption levels, which were between 1.1 and 1.25% of b.w. per day, were not significantly different among the diets before and during challenge, however the level of consumption dropped for all diets during the last week of challenge to levels below 1% of b.w. per day [105].

The antibody levels of serum were not significantly affected by the immunostimulants added to the treatment diets either before ( $F=2.415$ ,  $df$  2,36,  $P=0.105$ ) or after challenge ( $F=1.525$ ,  $df$  2,36,  $P=0.233$ ). Similarly in skin mucus, there was no effect of the immunostimulants on the level of IgM detected before ( $F=0.239$ ,  $df$  2,34,  $P=0.788$ ) or after challenge ( $F=0.066$ ,  $df$  2,35,  $P=0.935$ ). There were a few skin mucus samples that showed a high level of IgM (>50 units) obtained from fish after the challenge from all 3 diets (Figure 2.3). All those fish with more than 50 antibody units per mg of mucus protein after one AGD challenge ( $n=3$ ), also showed serum antibody levels above 10 units.

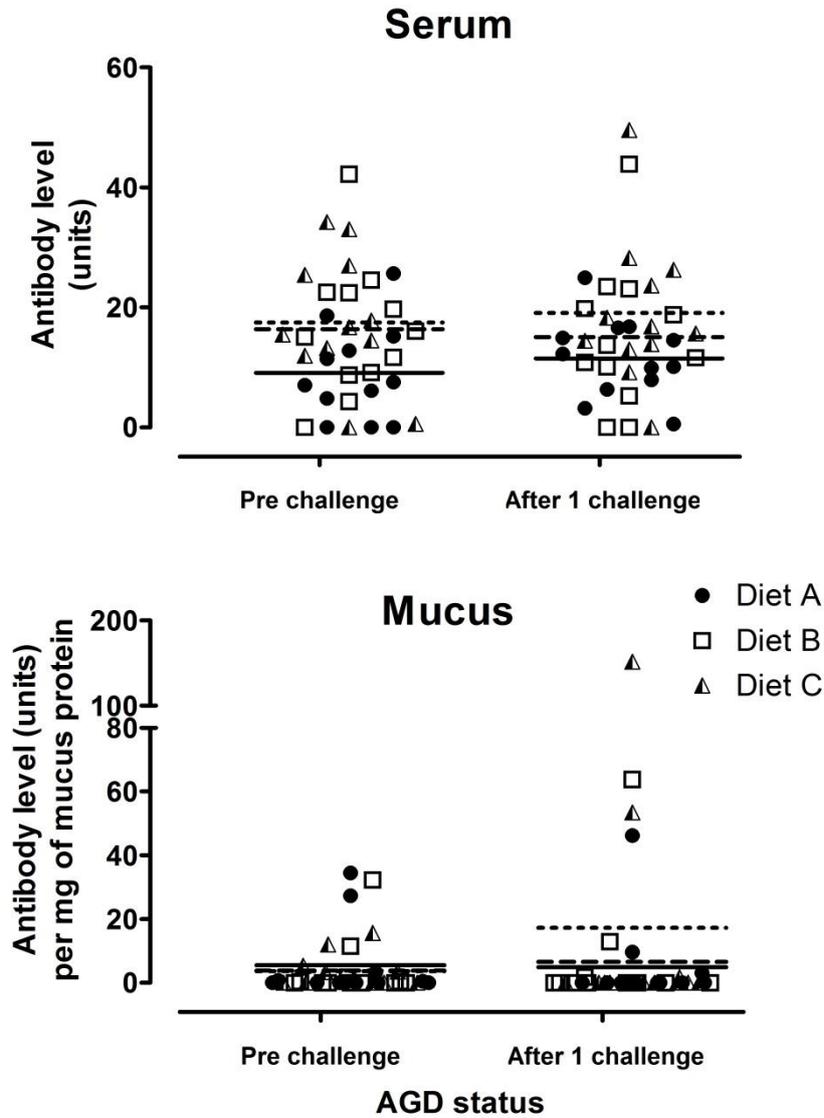


Figure 2.3 Antibody (IgM) levels (units) in serum and skin mucus of Atlantic salmon (*Salmo salar*) from experiment 2, which tested the effects of immunostimulatory diets on responses to AGD. Bars represent mean (n=12 for each group).

To further investigate the effects of immunostimulants included in commercial diets on the immune response of AGD-affected Atlantic salmon, the expression of four different immune-related genes was evaluated. An initial two-way ANOVA using diet and AGD-status as factors, showed a significant effect of AGD-status on the transcription of all four genes and no significant interaction between the two factors in the transcription levels of any of the four genes (Table 2.3). For IgM, IgT, TCR and CD8 average transcription levels were lower in gill samples obtained from AGD-affected fish when compared to AGD-naïve gills (Figure 2.4). Results were similar to those from the first experiment, as changes in IgM transcription levels related to AGD-status did not correlate with the lack of change in IgM levels observed in mucus.

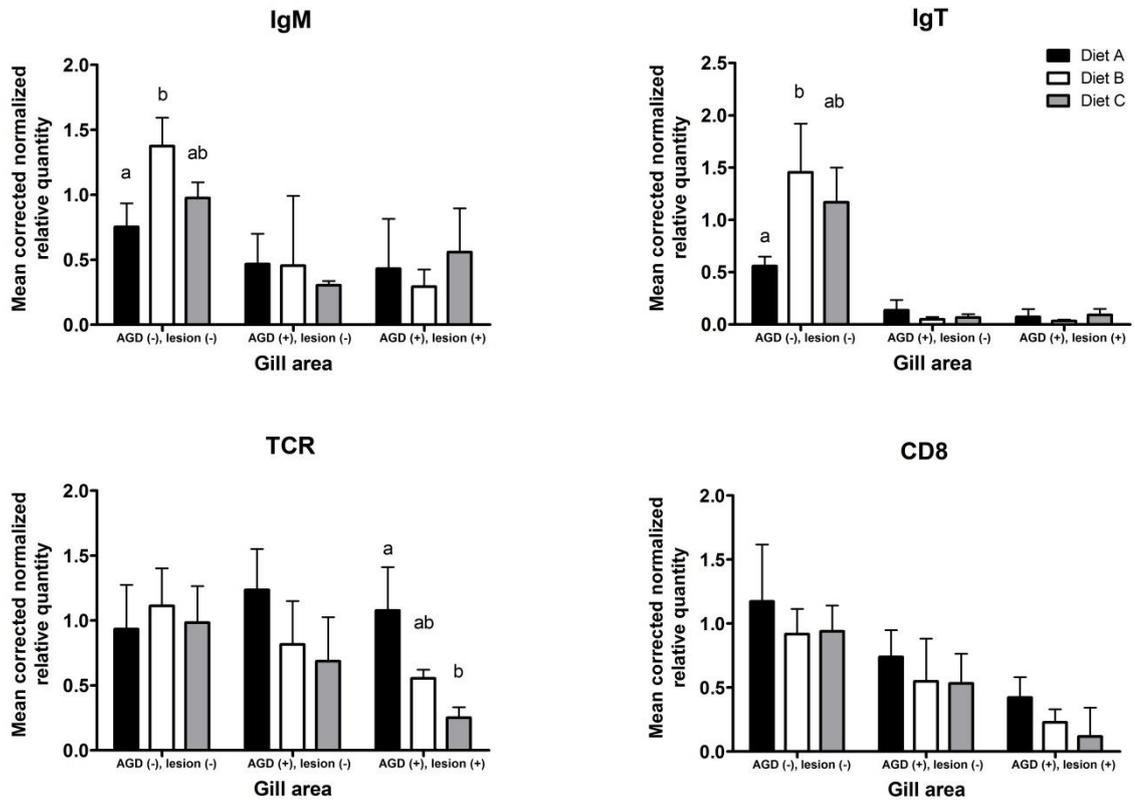
Following these results, the effect of diet on transcription levels was evaluated for each gill sample individually (Figure 2.4). When assessing the effects of diets on the level of IgM mRNA expression in the gill, the only significant difference was observed in the gill samples obtained from AGD-naïve salmon (Figure 2.4), where diet B induced a significant increase in expression levels of IgM mRNA ( $F=3.981$ ,  $df$  2,17,  $P=0.038$ ) compared to the control diet. This effect was not observed in the gills obtained from AGD-affected salmon.

A similar result was observed for IgT mRNA levels. Diet B only had an effect on IgT mRNA expression levels in gill samples from AGD-naïve fish, ( $F=4.667$ ,  $df$  2,17,  $P=0.024$ ). Diets B and C did not affect the levels of expression of IgT in gill samples from AGD-affected fish, with or without lesion (Figure 2.4).

**Table 2.3** Two-way ANOVA results for transcription of immune related genes of Atlantic salmon (*Salmo salar*) from experiment 2, which tested the effects of immunostimulatory diets on responses to AGD. Values in bold show significant results ( $P < 0.005$ ).

| Factor             | IgM         |             |              | IgT          |             |                  | TCR         |             |              | CD8         |             |              |
|--------------------|-------------|-------------|--------------|--------------|-------------|------------------|-------------|-------------|--------------|-------------|-------------|--------------|
|                    | F           | df          | P            | F            | df          | P                | F           | df          | P            | F           | df          | P            |
| <b>Interaction</b> | 1.10        | 4,51        | 0.366        | 2.12         | 4,50        | 0.093            | 1.59        | 4,50        | 0.192        | 0.33        | 4,44        | 0.859        |
| <b>Diet</b>        | 0.03        | 2,51        | 0.975        | 0.67         | 2,50        | 0.519            | <b>3.84</b> | <b>2,50</b> | <b>0.028</b> | 1.50        | 2,44        | 0.235        |
| <b>AGD-status*</b> | <b>7.56</b> | <b>2,51</b> | <b>0.001</b> | <b>44.10</b> | <b>2,50</b> | <b>&lt;0.001</b> | <b>3.69</b> | <b>2,50</b> | <b>0.032</b> | <b>7.34</b> | <b>2,44</b> | <b>0.002</b> |

\* AGD-status identifies the three types of gill samples obtained: gills from non-AGD affected salmon (AGD (-), lesion (-)) and from AGD-affected salmon in areas with no apparent lesion (AGD (+), lesion (-)) or with typical AGD lesion (AGD (+), lesion (+)).



**Figure 2.4** Quantitative RT-PCR analysis of immune-related gene expression in gill of Atlantic salmon (*Salmo salar*) from experiment 2, which tested the effects of immunostimulatory diets on responses to AGD. Diet A represents a commercial formulation and diets B and C incorporated immunostimulants. Three different gill samples were analysed: gill from non-AGD affected salmon and from AGD-affected salmon in areas with no apparent lesion or with typical AGD lesion (n=15-21 for each gill area). Bars represent mean values (+S.E.) Different letters represent significant differences by one-way ANOVA ( $P < 0.05$ ).

The expression levels of TCR and CD8, which are recognised T-cell response markers, were not affected by the diet with immunostimulants in AGD-naïve fish. Further, the different diets did not cause any significant differences ( $P>0.05$ ) in CD8 mRNA expression in the gills of salmon, affected or not by AGD.

As it can be observed in Figure 2.4, the levels of expression of TCR in the gills of those fish fed with three diet formulations were similar across the gill samples with no lesion (AGD-affected or not). However, there was a significant down-regulation in the TCR expression level in AGD-affected gills with lesions in those fish fed diets B and C ( $F=5.181$ ,  $df\ 2,16$ ,  $P=0.018$ ).

## **2.5 DISCUSSION**

Gene expression analyses were used to detect differences in expression of IgM and IgT classes and other immune-related genes, namely CD8 and TCR. Following infection of Atlantic salmon with AGD in four consecutive occasions, mRNA expression of IgM was significantly up-regulated in the AGD lesions compared to no lesion samples from AGD-affected fish and to gill samples from AGD-naïve fish. However, this up-regulation was only close to a 2-fold increase. Additionally, no differences were observed in the expression of IgT, CD8, and TCR. In the present study, samples were obtained 31 d after the last infection of the multiple challenge, and their gene expression profile was not in agreement with the coordinated down-regulation of immune genes, including IgM, IgT heavy chain and TCR, observed by Young and colleagues 36 d after a single infection [109]. IgM and other immune related genes have been shown to be either up- or down-regulated in AGD lesions [109, 112], depending on the sampling time after infection. Investigators in our group have recently demonstrated that B-cell markers such as IgM are up-regulated in AGD lesions classified as “medium” in severity, but not in those classified as “severe” [112], in early stages of infection. However, this was accompanied by a generalised up-regulation in gene expression across different cellular markers, including IgT, TCR, CD4 and CD8, in the gill lesions of AGD-affected fish 10 d after infection [32]. On the other hand, the down-regulation has been documented in cases where samples were collected after 30 d of infection.[109]

The lack of change in the expression profiles of IgT, TCR and CD8 could be due to various reasons. Firstly, the percentage of lesion sampled macroscopically could affect the transcription response obtained. The severity of AGD lesions is a function of lesion age [55] and during this lesion development the cellular diversity shifts as the lesion comprises more undifferentiated epithelial cells. Work by our group [112] suggests that as the lesion matures and the lesion comprises more undifferentiated cells, fewer immune cells infiltrate the lesion and that this likely explains the down-regulation in immune related gene transcription in more mature AGD lesions. In the present study if the majority of samples collected represented “severe” lesions, these areas would exclude the cell types -T and B-cells- which express these genes, explaining the apparent slight down-regulation of the transcription profiles of the immune-related genes when compared to non AGD-affected controls. Therefore, it is likely that changes or lack of them in transcription levels for these genes depend on the amount and severity of lesion in each sample. Secondly, it is possible that after an initial change in transcription level following infection, the expression levels of these markers returned to normal levels 31 d post-infection. In carp (*Cyprinus carpio*) affected by *Ichthyophthirius multifiliis* (Ich), an increase in IgM expression, as well as other immune genes, namely C3, MHC-II and iNOS, was observed in skin samples up to 6 d post-infection, but their expression returned to levels similar to non-infected fish by 26 d post-infection [158]. However, sequential sampling would have been required to monitor transcriptome changes over time. It is also possible that a multiple challenge with AGD was only capable of stimulating IgM gene up-regulation for an extended period and not the expression of genes related to T-cells, similar to what was observed in the gills of trout exposed multiple times to Ichtheronts, where IgM and IgT, but not CD8, were shown to be up-regulated after immunisation and challenge [149]. IgT has been shown to be the main Ig isotype responding and attaching to Ich in trout skin [118], and therefore IgT up-regulation at the gene level is expected; however the presence of an IgT response in AGD lesions is yet to be described.

Even though previous evidence indicated increased seroprevalence in Atlantic salmon with successive infections and freshwater bathing cycles [114], our results showed that this did not correlate with higher antibody levels. Using ELISA, we

could not detect an increase in antibody levels against *N. perurans* in fish that were exposed to the infection four consecutive times, when compared to their controls. Antibody levels in plasma and mucus remained very low in all fish sampled, with only some higher values in plasma and skin mucus but not in gill mucus. Regarding antibody responses, contrasting results have been reported in other parasitic diseases in fish, where consecutive infections have been studied. Using immunohistochemistry to measure the number of IgT<sup>+</sup> and IgM<sup>+</sup> cells in gills of rainbow trout affected by the ciliate Ich, it has been shown that the number of IgT<sup>+</sup> and IgM<sup>+</sup> cells did not differ between fish that were previously immunised with the parasite and those exposed by the first time [149]. However, subsequent studies have shown that plasma antibody levels are 2.3 times higher in fish infected four times with Ich compared to fish only infected once [148]. Similarly, IgT concentration in skin mucus of rainbow trout was greater in fish that underwent consecutive challenges with Ich than in trout after only a single infection, when compared to uninfected control fish [148]. IgM levels remained similar in the different groups, supporting that IgT was the main Ig responding against Ich infection in the skin [118]. In the case of Ich infection, high antibody responses are likely due to the intimate contact of this parasite with the fish epidermis. Ich theronts attach to gills of fish and penetrate through the surface mucus, gill epithelia and into the basement membrane, generating epithelial cell proliferation and infiltration of neutrophils and lymphocytes (as reviewed by Ref. [159]). In contrast, *N. perurans* is mainly associated with the margin of AGD lesions, frequently adhered to by macrophages, and within the lesions, only undifferentiated hyperplastic epithelial cells represent the largest cell population [55]. As mentioned above, the presence of IgT in association with AGD lesions and *N. perurans* is yet to be determined. Previous experience in our laboratory have demonstrated that one of the available anti-trout IgT antibodies [97] does not seem to recognise this molecule in Atlantic salmon (data not shown), and due to this technical limitation, it was impossible to assess the level of this Ig isotype in our study.

There was a significant effect of the immunostimulants diets on the levels of expression of the B-cell related genes. When data were analysed within each gill sample type (e.g. AGD-naïve or AGD-affected with or without lesion), it was clear

that the immunostimulatory diets were supporting an increase in IgM and IgT expression in gills of non-AGD affected fish; however they did not induce a similar response in AGD-affected animals. Similar levels of TCR and CD8 expression were observed across diets for each type of gill sample; however, there was a significant decrease of TCR expression in AGD lesions in fish fed diet C.  $\beta$ -glucans represent one group of immunostimulants that increased antibody levels in a few fish species after oral administration [88], however there is a lack of understanding of their effects on adaptive immune response of fish at gene expression level. There is some speculation that the putative role  $\beta$ -glucans may have on mucosal disease resistance is due to its ability to induce a Th17 response and chemokines [88].

There was no significant effect of diets containing immunostimulants on the IgM levels of serum and skin mucus.  $\beta$ -glucans given orally to rainbow trout as immunostimulants had a significant effect on serum antibody response after immunisation with a bacterial pathogen [160]. However, dietary incorporation of these compounds failed to increase AGD resistance in infected fish and did not stimulate respiratory burst activity of head kidney macrophages and serum lysozyme production *in vivo* [146]. Additionally, antibody responses against AGD have shown to be inconsistent in serum regarding the percentage of seroconversion and they have not been recorded in mucus following AGD infections [23, 113-117, 135, 161]. Further research should be carried on immunisation of fish with AGD antigens and the potential effects dietary immunostimulants such as  $\beta$ -glucans, could have on systemic and mucosal antibody levels.

The changes in transcription levels of IgM in gills for both experiments did not correspond to the differences in IgM levels in mucus. In the experiment that tested the effects of repeated AGD infection, IgM expression was up-regulated in AGD-lesions, while no changes in IgM levels were found in gill or skin mucus. Similarly, in the experiment that tested the effects of immunostimulatory diets on responses to AGD, only gills obtained from AGD-naïve fish showed changes in transcription of IgM influenced by the administration of diet B, while no differences were observed in mucus IgM levels among fish fed the three different diets. Only a few studies have previously conducted parallel assessments of gene expression and levels of Ig

molecules in fish [149, 162-164]. They have mostly used immunohistochemical staining to localise antibody producing cells expressing Ig, rather than measuring antibody levels, and similar to our findings, the transcription levels of IgM and IgT have not always represented the level of effector molecules. In rainbow trout larvae, IgM and IgT mRNA transcripts were observed in excess of 4-fold change straight after hatching, however there were no cells positive for IgM at any time in thymus and different mucosal organs, while IgT positive staining was only observed as a weak reaction in gill mucus 38 d post-hatching [162]. In the same species, transcripts of IgM and IgT were up-regulated 47 d after immunisation and 4 d after challenge with Ich, but no difference was found in the number of IgM<sup>+</sup> and IgT<sup>+</sup> cells in gills at the same sampling time [149]. In contrast, a correlation between the levels of IgM gene expression and IgM<sup>+</sup> cells in posterior intestine of gilthead sea bream (*Sparus aurata*) has been demonstrated [164]. The abundance of a mRNA transcript is often an indicator of whether or not a protein is detectable within the cells or tissue [165]. However, in most of the organisms studied to date, the protein abundance is only weakly correlated to the transcription abundance, with multi-cellular organisms displaying the lowest correlations between protein and mRNA concentrations (~0.4 in average) [166]. These moderate correlation levels suggest that mRNA expression might be sometimes an useful, but not very accurate tool for predicting protein expression levels [167], in particular in single time point experiments, where the gene and protein relationships are often poor [168]. It has been proposed that regulation at the level of mRNA serves as a switch (i.e presence/absence), whereas downstream regulation works as fine tuning mechanisms for protein abundance [165]. In a cell, ratios between protein and mRNA could be mainly determined by translation and protein degradation, thus some proteins may be rapidly translated but not very stable, producing lower final concentrations [165, 166]. These weak correlations between effector molecules and gene expression could explain the disparities observed in the present study between IgM transcription and antibody levels measured in mucus. Nevertheless, these correlation scenarios have been proposed for organisms in a physiological steady-state, and research is still incomplete in perturbed systems, such as those subjected to stress or diseases, but

early results show that correlations between mRNA and protein abundance do not always hold in these distressed systems [165].

In conclusion, we have determined that the systemic and mucosal IgM levels in Atlantic salmon affected with AGD were not higher after multiple infections with *N. perurans*. However, we found evidence that multiple infections stimulate an increase of IgM gene expression in the gill of AGD-affected fish up to 31 d after infection, while no changes in IgT, TCR and CD8 were observed. This was possibly due to different amounts and severity of lesions sampled or alternatively, due to the return of transcription levels to normal after a long period post-exposure.

Both classes of Ig were affected at the gene level by the presence of immunostimulants in the diet; but this was only observed in naïve fish before infection. To our knowledge, no other targeted approaches have been carried out on the effects of immunostimulants on adaptive immune response at gene expression level in Atlantic salmon affected by marine parasites and therefore, further research is required to test their potential impacts in different doses and/or in different application regimes. Finally, changes observed at transcription level for IgM did not correspond to the lack of differences in IgM levels measured in mucus in the present study, which is possibly explained by weak correlations existing between protein and mRNA abundances in cells and tissues. Even though this study provided a baseline understanding of mucosal responses against AGD and their relationship with gene expression, further research is warranted involving mucosal responses against the parasite, in particularly with regard to IgT, and their potential role in AGD vaccine research.

## **2.6 ACKNOWLEDGEMENTS**

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**CHAPTER 3.**

**EFFECT OF IMMUNISATION  
ROUTE ON MUCOSAL  
AND SYSTEMIC IMMUNE  
RESPONSE IN  
ATLANTIC SALMON (*Salmo salar*)**

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**KEYWORDS**

Atlantic salmon, mucosal immunity, haptent-antigens, immunisation

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### 3.1 ABSTRACT

This study aimed to assess systemic and mucosal immune responses of Atlantic salmon (*Salmo salar*) exposed to two protein-hapten antigens - dinitrophenol (DNP) and fluorescein isothiocyanate (FITC) each conjugated with keyhole limpet haemocyanin (KLH) - administered using different delivery strategies. Fish were exposed to the antigens through different routes, and were given a booster 4 weeks post initial exposure. Both systemic and mucosal antibody responses were measured for a period of 12 weeks using an Enzyme-linked immunosorbent assay (ELISA). Only fish exposed to both antigens via intraperitoneal (i.p.) injection showed increased systemic antibody response starting 6 weeks post immunisation. No treatment was able to produce a mucosal antibody response; however there was an increase in antibody levels in the tissue supernatant from skin explants obtained 12 weeks post immunisation from fish injected with FITC. Western blots probed with serum and culture supernatant from skin explants showed a specific response against the antigens. In conclusion, i.p. injection of hapten-antigen in Atlantic salmon was the best delivery route for inducing an antibody response against these antigens in this species. Even though i.p. injection did not induce an increase in antibody levels in the skin mucus, there was an increased systemic antibody response and an apparent increase of antibody production in mucosal tissues as demonstrated by the increased level of specific antibody levels in supernatants from the tissue explants.

### 3.2 INTRODUCTION

It is known that immune responses to antigens can vary due to a large number of factors, for example temperature, route of application and fish species [169]. Previous work on rainbow trout (*Oncorhynchus mykiss*), has shown that intramuscular and intraperitoneal (i.p.) injection of antigens results in limited antibody response in mucus, in contrast to the strong serum antibody response observed using the same methods [170]. In sea bass (*Dicentrarchus labrax*) the gill has been identified as the major organ producing antibody secreting cells (ASC) following direct immersion vaccination [171]. However, there is no similar information available for Atlantic salmon, since little work has been done related to mucosal immunity in this species. The administration of model antigens would allow

an initial evaluation of how mucosal responses are induced through different vaccination procedures used normally in the industry (i.e. injection, immersion and oral vaccination) [172].

Studies associating systemic and mucosal antibody responses with a number of different antigens have been previously conducted in several fish species. Large proteins such as human gamma globulin [173] or gonad cell extracts [174] have shown to induce increases in mucosal responses, as well as increases in systemic antibodies. Similarly, smaller haptens such as fluorescein isothiocyanate (FITC) and dinitrophenol (DNP) have been also used as model antigens when conjugated with larger molecules such as KLH [170, 175-179].

Good antibody responses in rainbow trout to FITC-KLH conjugates have been shown elsewhere [170, 180]. Swan et al. [170] reported increased mucus and serum antibody responses in trout injected with FITC-KLH via i.p., only when this molecule was administered in conjunction with Freund's complete adjuvant (FCA). Similar results were obtained by Drennan et al. [174] as early as 6 weeks in serum but not until 12 weeks in mucus in white sturgeon (*Acipenser trasmontanus*) injected with FITC-KLH.

DNP conjugated with different larger molecules has also been used as antigen, producing increased antibody titres in systemic [178, 181] and mucosal responses [179]. Lobb [179] showed that the secretory immune system of channel catfish (*Ictalurus punctatus*) can be stimulated by external antigens, since an increase in antibody titres in mucus but not in serum was observed 16 weeks after bath exposure to DNP-horse serum albumin. DNP-KLH has been shown to increase serum antibody levels in carp [181] and rainbow trout [178].

This study aimed to assess the systemic and mucosal immune responses of Atlantic salmon exposed to protein-hapten antigens (DNP and FITC conjugated with KLH) administered by different delivery strategies. The results of this will provide initial information on Atlantic salmon responses to different immunisation routes, and will be used as an indication on the best methods to use for future vaccination trials aimed at generating a mucosal humoral response.

### **3.3 MATERIALS AND METHODS**

#### **3.3.1 Fish**

Atlantic salmon with average body weight of 166.38 g (SD 50.23 g) and average fork length of 258.02 mm (SD 21.67) were obtained from a commercial farm and held at the Aquaculture Centre, University of Tasmania. Animals were acclimated for 10 d prior to the initiation of the experiment and held for the duration of the trial in two separate 4,000 L freshwater recirculation systems, each one containing 72 fish. Systems were maintained between 14 – 15.8°C, pH 7.0. Water quality and temperature were assessed daily and 50% water exchanges occurred weekly. Salmon were fed a commercial diet equivalent to 1.5% of their body weight as a daily ration twice a day during acclimatisation and experimental period. This design was similar to that used for rainbow trout [170], which is a closely related species. To identify different treatments, fish were tattooed with alcian blue in their ventral dermis with a particular pattern for each treatment. Alcian blue has been used as a tagging method showing high retention rates in salmonids [182]. This project was approved under the University of Tasmania Animal Ethics Committee approval number A0011493.

During the 12 week study, a few mortalities were observed after sampling in both experiments, most likely due to handling stress, and therefore different numbers of fish were obtained from each group (Table 3.1). However, animals were observed to be generally healthy and increases in average fork length and weight were noted (data not shown).

### Effect of immunisation route on immune responses in Atlantic salmon

**Table 3.1** Number (*n*) of Atlantic salmon (*Salmo salar*) in each treatment group. Treatments included fish immunised via intraperitoneal injection with Freund's complete adjuvant (IP), peranal intubation with Freund's incomplete adjuvant (PA) or immersion of gills and cranial end of fish (GILLS) with fluorescein isothiocyanate conjugated with keyhole limpet haemocyanin (FITC) or with dinitrophenol conjugated with keyhole limpet (DNP). Controls were sham exposed through the same routes with phosphate buffered saline (PBS). Primary and booster immunisations were given at week 0 and week 4.

| WEEK | FITC EXPERIMENT |             |                |            |            |               | DNP EXPERIMENT |            |               |            |            |              |
|------|-----------------|-------------|----------------|------------|------------|---------------|----------------|------------|---------------|------------|------------|--------------|
|      | FITC-<br>IP     | FITC-<br>PA | FITC-<br>GILLS | PBS-<br>IP | PBS-<br>PA | PBS-<br>GILLS | DNP-<br>IP     | DNP-<br>PA | DNP-<br>GILLS | PBS-<br>IP | PBS-<br>PA | PBS-<br>GILL |
|      | <i>n</i>        | <i>n</i>    | <i>n</i>       | <i>n</i>   | <i>n</i>   | <i>n</i>      | <i>n</i>       | <i>n</i>   | <i>n</i>      | <i>n</i>   | <i>n</i>   | <i>n</i>     |
| 0    | 12              | 12          | 12             | 12         | 12         | 12            | 12             | 12         | 12            | 12         | 12         | 12           |
| 2    | 10              | 12          | 12             | 12         | 12         | 12            | 12             | 12         | 12            | 10         | 12         | 12           |
| 4    | 10              | 12          | 11             | 11         | 11         | 10            | 11             | 11         | 12            | 10         | 11         | 11           |
| 6    | 10              | 11          | 11             | 11         | 10         | 10            | 11             | 9          | 12            | 10         | 11         | 11           |
| 8    | 8               | 11          | 10             | 10         | 10         | 9             | 11             | 9          | 11            | 10         | 11         | 9            |
| 12   | 7               | 10          | 10             | 8          | 8          | 9             | 10             | 9          | 11            | 10         | 11         | 9            |

#### 3.3.2 Antigen

Two different hapten-protein antigens (FITC-KLH and DNP-KLH) were used in two different experiments. Both antigens were tested as FITC-KLH has been proven to induce response in rainbow trout [170, 180], and conjugated DNP has been shown to generate an increase in mucus antibody production when exposed via immersion [179].

A FluoroTag™ FITC Conjugation Kit (Sigma Aldrich, St. Louis, Mo, USA) was used to conjugate this hapten to KLH (Merck KGaA, Darmstadt, Germany) following the manufacturer's instructions. In brief, 10 mg of FITC and 20 mg of KLH were diluted in 0.1M carbonate – bicarbonate buffer (0.4 ml and 1.6 mL respectively) and combined by adding the FITC solution drop wise into the KLH solution. The solution was mixed overnight at 4°C in a rotational mixer protected from light. The FITC-KLH conjugate was recovered by elution at room temperature (RT) through a 3.5 mL Sephadex G-25M gel filtration column. Fractions containing the conjugate were pooled and the FITC to protein (KLH) molar ratio (F/P) was determined spectrophotometrically. F/P equalled 9.70 using the equation:  $F/P = [A_{495} \times C] / [A_{280} - (0.35 \times A_{495})]$ , where C is a constant, calculated as 2.87 for FITC [176] and  $A_x$  represent the absorption of the conjugate at x nm. This conjugate was stored at 4°C until used.

The second hapten-protein conjugate, DNP-KLH, was obtained as a crystallised form (Merck KGaA) in a DNP/protein molecular ratio of 623.0 and was prepared just before each use by dissolving 5 mg of the conjugate in 0.7 mL warm distilled water and then into 1.8 mL PBS, combining in a rotational mixer at RT for 1 h. The protein concentration of both conjugates was determined using a Micro BCA™ Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). Commercially obtained coating antigens were used for the ELISA.

### **3.3.3 Treatments**

Two experiments -each testing the effects of one of the hapten-antigens- were carried out simultaneously. Groups of 12 fish were exposed through three different routes to each hapten-antigen: 1) hapten conjugated with KLH emulsified with FCA and administered via i.p. injection (groups FITC-IP and DNP-IP), 2) hapten conjugated with KLH emulsified with Freund's incomplete adjuvant (FIA) and administered by per-anal (p.a.) intubation (FITC-PA and DNP-PA) and 3) immersion of anterior part of fish including opercula for 1 min in hapten-KLH which was dissolved in phosphate buffered saline (PBS; 0.02M phosphate, 0.15 M sodium chloride; 0.876 g sodium dihydrogen phosphate dihydrate, 2.56 g sodium phosphate dibasic dihydrate, 8.77 g sodium chloride in 1 L distilled water, pH 7.3) (FITC-GILLS and DNP-GILLS). Control groups for each experiment included fish exposed to 1) PBS with FCA via i.p. injection (PBS-IP), 2) PBS with FIA through p.a. intubation (PBS-PA) and 3) immersion of anterior part of fish including opercula in PBS for 1 min (PBS-GILLS). Fish immunised via i.p. injection or p.a. intubation were given 200 µL of a solution with a 1:1 ratio of inoculum - with a concentration of 2 mg/mL of hapten-KLH - and adjuvant, in order to obtain a 1 mg/mL solution. This administration dose has been determined to induce an effect in antibody levels in rainbow trout [170]. For the immersion treatment a solution of 60 µg of antigen-KLH/mL of PBS was prepared, following methods by [179]. Fish were exposed to antigens at week 0 and a booster was given at week 4, during which FIA was used instead of FCA for i.p. administration (Table 3.2). FIA was used in the p.a. intubation preparations to provide viscosity to the solution and not strictly as an adjuvant.

## Effect of immunisation route on immune responses in Atlantic salmon

Table 3.2 Experimental design for immunisation of Atlantic salmon (*Salmo salar*) with two different hapten-antigens conjugates: FITC-KLH and DNP-KLH.

| Treatment  | Delivery route        | Abbreviation | n      | Initial Dose and week 4 booster                       |
|--|-----------------------|--------------|--------|---|
| FITC-KLH<br>+ Freund's complete adjuvant (FCA)           | IP injection          | FITC-IP      | 12     | 200 µL (1:1 FCA† with inocula at 2 mg/mL of FITC-KLH) |
| FITC-KLH<br>+ Freund's incomplete adjuvant (FIA)         | PA<br>Intubation      | FITC-PA      | 12     | 200 µL (1:1 FIA with inocula at 2 mg/mL of FITC-KLH)  |
| FITC-KLH<br>+ diluted in phosphate buffered saline (PBS) | Immersion<br>of gills | FITC-GILLS   | 12     | 60 µg of FITC-KLH/mL of PBS for 1 min                 |
| DNP-KLH + FA   | IP injection          | DNP-IP       | 12     | 200 µL (1:1 FCA† with inocula at 2 mg/mL of DNP-KLH)  |
| DNP-KLH + FIA  | PA<br>Intubation      | DNP-PA       | 12     | 200 µL (1:1 FIA with inocula at 2 mg/mL of DNP-KLH)   |
| DNP-KLH in PBS   | Immersion<br>of gills | DNP-GILLS    | 12     | 60 µg of DNP-KLH/mL of PBS for 1 min                  |
| PBS + FCA*   | IP injection          | PBS-IP       | 12 x 2 | 200 µL (1:1 FCA† with PBS)                            |
| PBS + FIA*   | PA<br>Intubation      | PBS-PA       | 12 x 2 | 200 µL (1:1 FIA with PBS)                             |
| PBS*   | Immersion<br>of gills | PBS-GILLS    | 12 x 2 | PBS for 1 min   |

\* Each experiment for each hapten-antigen (FITC-KLH and DNP-KLH) had its own control groups.

† Booster at week 4 was emulsified with Freund's incomplete adjuvant (FIA).

### 3.3.4 Serum and cutaneous mucus sampling

Mucus and blood were obtained from all fish at week 0 and every 2 weeks thereafter. Fish were anaesthetised before treatments and sampling using 1 mL clove oil diluted in 10 L of freshwater. Individual fish were placed in 1 L plastic bags and their skin massaged for approximately 30 s in order to harvest their mucus, 250 µL of antiprotease cocktail (Sigma Aldrich) was added immediately and mixed in with the mucus inside the bags, which were placed on ice. Mucus samples were transferred to 5 mL plastic tubes within 4 h of collection and frozen overnight at -20°C. Samples were then thawed at 4°C, vortexed vigorously, centrifuged at 10,000 x g at 4°C for 10 min and supernatant collected. Similar collection methods have been used successfully in the past to measure mucosal Ig from several species [170, 174, 183]. Blood was obtained from the caudal vein using 1 mL syringes with 25 G needles and

left to clot in 1.5 mL microcentrifuge tubes overnight at 4°C. Serum was obtained after centrifugation at 1,500 x g for 10 min at 4°C. Both serum and mucus supernatant were stored at -80°C.

### **3.3.5 Tissue explants and supernatant**

A method developed by Xu and Klesius [184] to measure the cutaneous antibody response in tissue explants and proven to work in Rainbow trout [170], was modified and used in the present study. During the final sampling at week 12, skin, gills and intestine samples were collected. After collection of mucus and blood, fish were killed by an overdose of anaesthetic and bled as much as possible as specified. Perfusion of the organs via puncture of the bulbous arteriosus with 0.9% physiological saline (Baxter, Deerfield, IL, USA) containing 1 IU of heparin/mL was performed until the gills were white to remove any remaining blood from the organs and ensure that the antibodies present in the culture media were not systemic. Skin was peeled off the muscle on the right side of the animal, from the operculum to the adipose fin, intestine was obtained from the stomach to the anus and the gill arches were excised. All three samples were then washed thoroughly for 1 min in Hank's balanced salt solution containing 0.5% chlorhexidine and transferred immediately to L-15 media (Sigma Aldrich) with 10% bovine foetal serum, 1 x Glutamax<sup>TM</sup> and 2 x PSN antibiotic mix (Gibco, Grand island, NY, USA)(L-15, 2 x PSN). In a laminar flow chamber, all excess tissue was removed from the samples including remnants of muscle in the skin, fat and connective tissue from intestine and cartilage from the gill arches; then samples were washed again with L-15, 2 x PSN and weighed. Tissue samples were then placed in individual wells of a Corning® Costar® 12-well tissue culture plate (Corning, NY, USA) with fresh 1.5 mL of L-15 media, 1 x Glutamax<sup>TM</sup> and 1 x PSN antibiotic mix (L-15, 1 x PSN) and incubated at 18°C for 72 h. The culture media were removed from the plates, centrifuged at 1,500 x g for 10 min at 4°C, the supernatant collected and stored at -80°C.

### **3.3.6 ELISA**

An ELISA was developed to measure the presence of antibodies against FITC or DNP haptens in the serum and mucus of fish and in the culture media supernatant collected from the tissue cultures. The coating antigen was either FITC or DNP

coupled with BSA. A DNP-BSA (Merck) with a molar ratio of 36.0 was used for DNP ELISAs, while FITC-BSA (Invitrogen, Carlsbad, CA, USA) with a ratio of 6 moles of FITC to 1 mol of BSA was employed for the FITC-BSA ELISAs. Samples from serum, mucus or the supernatant obtained from the skin, gills and intestine explants were analysed. The secondary antibody was a monoclonal antibody (mAb) against rainbow trout/Atlantic salmon IgM (Aquatic Diagnostics, Stirling, UK) used at a 1:100 dilution and the tertiary antibody an affinity purified goat anti-mouse IgG (whole molecule) conjugated to horseradish peroxidase (Sigma Aldrich) diluted to 1:1,000.

Preliminary assays using a checkerboard technique were run to optimise the coating antigen and sample concentrations. Antigen was serially diluted (two-fold) in carbonate buffer (1.59 g sodium carbonate, 2.93 g sodium bicarbonate in 1 L distilled water, pH 9.6) and serum, mucus and tissue culture supernatant samples in PBS. A wide range of antigen and sample dilutions were tested. A single dilution ELISA was run for all the samples choosing one dilution of each sample as optimal. A reference standard curve was used in every ELISA plate. Sera from fish selected for relatively high antibody levels from week 12 were used to prepare the standard for each hapten-antigen. A pool of these sera was made, aliquoted and maintained at -20°C. This standard was used in triplicate two-fold dilutions (1:100 to 1:6,400) in PBS for the serum ELISA or serum diluted in 1:1 mucus-PBS or in 1:5 L-15 1 x PSN in PBS, as the reference curve for the mucus or tissue culture supernatant ELISA. This method was chosen since single-point dilution ELISA tests have been used for measuring antibody responses in salmonids [170]. It has also been observed that single-point dilution ELISA when performed with a serially diluted serum standard in the same plate produce results (i.e. antibody units) that correlate with those obtained from end-point dilution ELISA [185].

Negative controls, included in each plate as duplicates in the same dilution as the samples, consisted of either a pool of sera from all experimental animals at week 0, a pool of equal amounts of mucus from all fish at week 0 or just L-15,1 x PSN for serum, mucus and tissue supernatant ELISA tests, respectively.

Optimal serum dilution was set at 1:200, mucus at 1:1, and supernatant from excised skin, gill and intestine at a 1:5 dilution. All samples were processed in duplicate after the optimisation. The plates were coated overnight at 4°C with the appropriate antigen (for DNP-BSA and FITC-BSA coating was set at 5 µg/mL; 100 µL/well), then washed 3 x with low salt wash buffer (2.42 g Tris base, 22.22 g sodium chloride, 0.5 mL Tween 20, pH 7.3) and non-specific sites were blocked with 250 µL/well of 5% skim milk in distilled water at 18°C for 2 h. Following another 3 washes with low salt buffer, serum, mucus or tissue culture supernatant diluted in PBS were added and incubated for 3 h at 18°C. Then 1:100 mAb in 1% BSA in PBS was added and incubated for 60 min at 18°C. Finally the tertiary antibody was added diluted 1:1,000 in 1% BSA in low salt wash buffer and incubated for 1 h at 18°C. Samples and antibodies were added at 100 µL/well. After incubation with each antibody, plates were washed 5 x with high salt wash buffer (2.42 g Tris base, 29.22 g sodium chloride, 1 mL Tween 20, pH 7.7). Bound antibody was visualised by adding 100 µL of chromogen substrate, 3,3',5,5'-tetramethylbenzidine (TMB One Solution, Promega, Fitchburg, WI, USA), which was incubated for 10 min at RT and stopped with 50 µL of 2 M H<sub>2</sub>SO<sub>4</sub> solution in distilled water. OD readings were measured at 450 nm in a Rainbow Thermo plate reader (Tecan Group Ltd., Männedorf, Switzerland).

Samples ODs were corrected by subtracting the mean OD of the negative controls in the same plate. ELISA results were then reported as levels of antibody units calculated from the standard curve of positive control on each plate, considering a dilution of 1:100 of the positive control as 100 antibody units, 1:200 dilution as 50 antibody units and so on, to account for differences between plates. A four parameter logistic function was used:  $OD = a + \frac{d - a}{1 + (\text{dilution} / c)^b}$ , where the four parameters were a and d (minimum and maximum asymptotes, respectively), c (the inflection point in the curve) and b (slope of the curve). This model has shown good results for calculations of total antibody in ELISA analyses when compared to other methods [154]. Calculations were done using the SigmaPlot 11.0 software (Systat software, Inc., Chicago, IL, USA). Antibody unit values for mucus were expressed per mg of protein in the sample; protein was measured with a Micro BCA<sup>TM</sup> Protein Assay Reagent Kit (Pierce).

**3.3.7 Western blot (WB) against FITC and DNP haptens and chemiluminescent detection**

WB were performed to assess specificity of responses for the haptens and cross reactivity with the BSA conjugate. Pooled serum, mucus and tissue supernatants were obtained from fish of the i.p. injected groups sampled at weeks 8 or 12 that showed high antibody levels in the ELISA. Hapten-BSA conjugates were characterised by SDS-PAGE using NuPAGE® Novex® Bis-Tris Mini Gels (Invitrogen). In brief, 6.5 µl of each antigen conjugated with BSA or BSA only samples at a concentration of 1 mg/mL were mixed with 3.5 µL of NuPAGE® LDS sample buffer 4 x (Invitrogen), heated for 10 min at 70°C and run in the gel at 200 V for 35 min. The samples were transferred to a PVDF membrane by electrophoresis for 6 min at 200 V using a rapid iBlot® Dry Blotting System (Invitrogen). The membrane was blocked with 5% dry milk in PBS at 4°C overnight and washed 3 x with low salt wash buffer. To detect the antigen bands, samples were diluted in PBS at 1:1,600 for serum, between 1:10 and 1:200 for mucus and tissue supernatants and incubated with the membrane for 3 h. To optimise the skin explant supernatant blot, Ig was isolated by using an Immobilized MBP affinity column (Pierce, Rockford, IL, USA) following the manufacturer's instructions. Briefly, supernatant was diluted 1:1 in Binding buffer (10 mM Tris, 1.25 M sodium chloride, 0.02% sodium azide, 20 mM calcium chloride, pH 7.4) and run in the column at 4°C. The column was then washed with binding buffer and moved to room temperature. Individual 3 mL fractions were eluted using elution buffer (10 mM Tris, 1.25 M sodium chloride, 0.02% sodium azide, 2 mM EDTA, pH 7.4), collected and their Ig content assessed by monitoring their absorbance at 280 nm using a UV 1201 UV-VIS Spectrophotometer (Shimadzu Corporation, Kyoto, Japan). For mucus, previous attempts have been made to purify IgM using an Immobilized MBP affinity column, similar to what was used for the tissue supernatant. However, due to the consistency of the mucus samples, the column blocked rapidly and it was not possible to purify IgM.

Membranes were then incubated with mouse mAb anti-salmon IgM (Aquatic Diagnostics) at 1:5,000 in 1% BSA-PBS and goat anti-mouse IgG horseradish peroxidase (Sigma-Aldrich) in 1% BSA in low salt wash buffer at 1:10,000.

Membranes were washed 5 x with high salt wash buffer in between incubations. Incubation steps for mAb anti-salmon IgM and goat anti-mouse IgG were carried out for 1 h and all incubation and washing steps were conducted at RT. WB were developed in a dark chamber with ECL Plus Western Blotting 40:1 Detection solution (GE Healthcare, Chalfont St Giles, UK) for 5 min and chemoluminescence was detected with a Hyperfilm ECL sheet (GE Healthcare).

### **3.3.8 Statistical analysis**

One-way analysis of variance (ANOVA) was used to assess statistical differences among treatment groups (IP v/s PA v/s GILLS), as well as measuring differences between each treatment group and its PBS control, at each sampling time for serum and mucus supernatant antibody responses, and only in samples obtained at week 12 for tissue culture supernatant from skin, gill and intestine. When differences were detected, post hoc analysis was performed using pair-wise comparisons with Tukey's test. Only differences among treatment groups or between each treatment and its control group were recorded for all sample types at each week. IBM SPSS Statistics version 19 software (IBM SPSS Inc, Armonk, NY, USA) was used for the analyses. Data had to be log transformed for serum and squared-root transformed for mucus and tissue supernatants to conform to the ANOVA assumption of homogeneity of variance. Differences were considered significant at  $P$ -values  $<0.05$ .

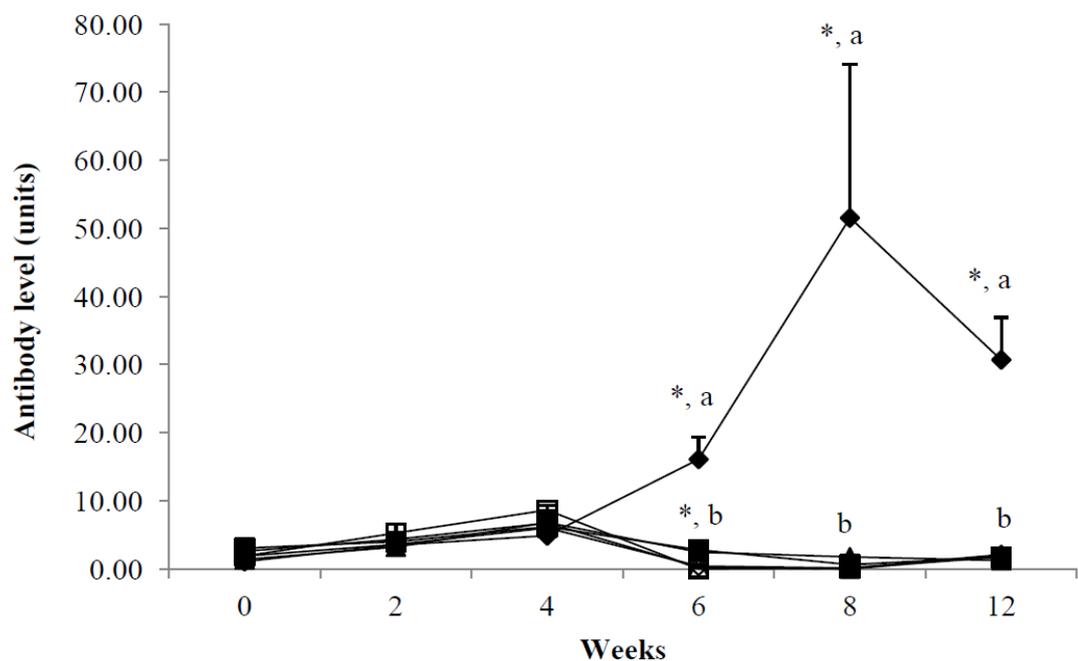
## **3.4 RESULTS**

### **3.4.1 Anti-FITC serum antibodies in ELISA**

During weeks 6, 8 and 12 post initial immunisation, the FITC-IP inoculated group showed a significant increase in the antibody unit levels when compared to their controls and the other treatments groups. The antibody level of this group was nearly 8 x that of the relative control group at week 6 ( $F=39.653$ ;  $df$  5,57;  $P<0.001$ ), but increases over 50-fold ( $F=15.859$ ;  $df$  5,53;  $P<0.001$ ) and over 30 x ( $F=14.847$ ;  $df$  5,46;  $P<0.001$ ) were observed during week 8 and week 12, respectively (Figure 3.1). In addition, FITC-PA and FITC-GILLS groups presented slight but significantly higher antibody levels than their relative control groups during week 6 (Figure 3.1). No differences in serum antibody unit levels were observed among treatments and

## Effect of immunisation route on immune responses in Atlantic salmon

between each treatment and its control group from week 0 to 4 ( $F=1.114$ ;  $df\ 5,67$ ;  $P=0.362$  for week 0;  $F=0.559$ ;  $df\ 5,65$ ;  $P=0.731$  for week 2 and  $F=1.854$ ;  $df\ 5,60$ ;  $P=0.116$  for week 4).



**Figure 3.1.** Antibody levels (units) present in Atlantic salmon (*Salmo salar*) serum, against FITC measured by ELISA. Serum dilution was 1:100. Treatment groups and controls were immunised as explained in Table 3.1 with fluorescein isothiocyanate conjugated with keyhole limpet haemocyanin (FITC). (♦) FITC-IP; (◇) PBS-IP; (■) FITC-PA; (□) PBS-PA; (▲) FITC-GILLS; (△) PBS-GILLS. Significant differences ( $P<0.05$ ) are reported between each treatment and its control group (\*) or among FITC treatments (a,b) at each time point.

**3.4.2 Anti-FITC mucus antibodies in ELISA**

During the collection of the samples, between 1.5 and 5 mL of mucus were obtained from each individual fish. Some mucus samples were contaminated by blood or faeces and therefore were removed from the analyses since they showed higher antibody unit levels (data not shown). No differences were found among treatments or between treatments and their controls for the first 6 weeks ( $F=1.614$ ;  $df$  5,64;  $P=0.169$  for week 0;  $F=0.894$ ;  $df$  5,60;  $P=0.491$  for week 2;  $F=0.518$ ;  $df$  5,59;  $P=0.762$  for week 4;  $F=1.812$ ;  $df$  5,56;  $P=0.125$  for week 6). During week 8, there was a significant difference in the levels of antibody units per mg of protein between all treatments ( $F=3.360$ ;  $df$  5,49;  $P=0.011$ ), however the post hoc test did not reveal where these differences existed, probably due to the Tukey's test capacity to control the family-wise Type I error rate, by decreasing each pair-wise comparisons rate [186]. Only during week 12, the group treated with FITC-IP showed a significant increase in antibody levels per mg of protein when compared to the other treatment groups ( $F=14.847$ ;  $df$  5,46;  $P<0.001$ ) but the level of antibody units per mg of protein did not differ from its control group (Figure 3.2).

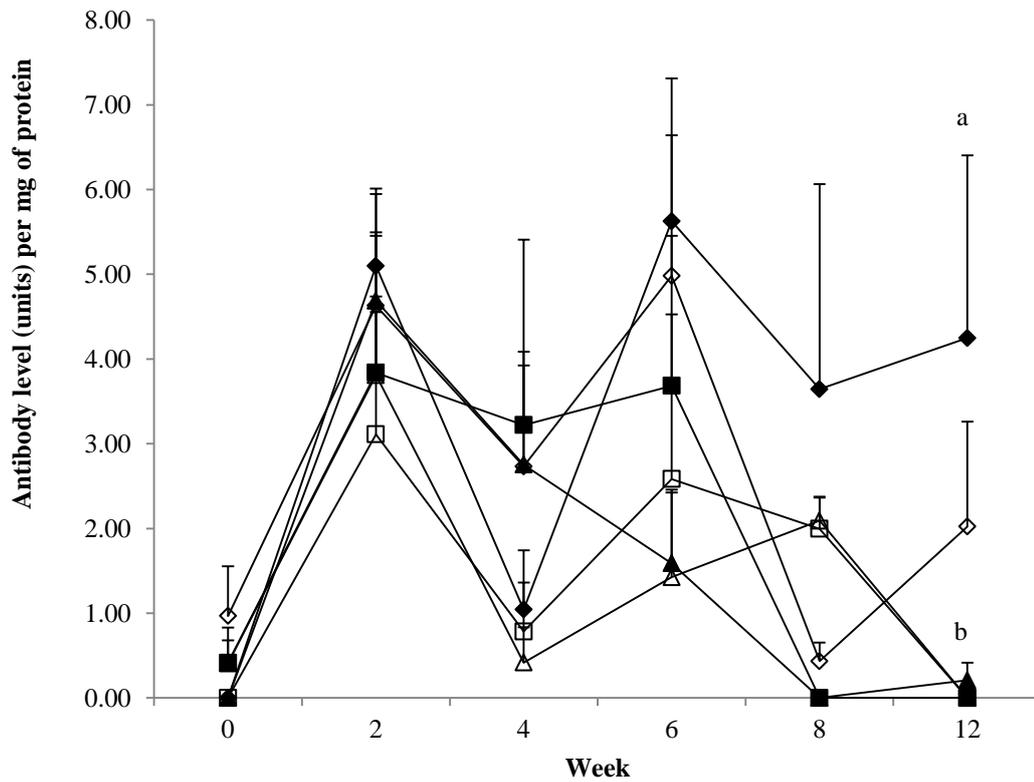


Figure 3.2. Mucus antibody levels (units) per mg of protein present in Atlantic salmon (*Salmo salar*) mucus, against FITC measured by ELISA. Mucus dilution was 1:1. Treatment groups and controls were immunised as explained in Table 3.2 with fluorescein isothiocyanate conjugated with keyhole limpet haemocyanin (FITC). (◆) FITC-IP; (◇) PBS-IP; (■) FITC-PA; (□) PBS-PA; (▲) FITC-GILLS; (△) PBS-GILLS. Significant differences ( $P < 0.05$ ) are reported between each treatment and its control group (\*) or among FITC treatments (a,b) at each time point.

**3.4.3 Anti-FITC tissue supernatant antibodies in ELISA**

The production of antibodies against FITC in tissues excised from salmon at week 12 was significantly higher in skin of fish from the FITC-IP group, when compared to its control group and the other treatment groups ( $F=6.425$ ;  $df\ 5,30$ ;  $P<0.001$ ) (Figure 3.3). Average antibody level was 4-fold higher in the supernatant collected from FITC-IP group skin than in supernatant from any other group. No other differences were observed for skin explants.

Anti-FITC antibody levels in supernatant from gill explants were generally lower than those observed in the supernatant from skin (Figure 3.3), and no significant differences were observed between treatments ( $F=1.335$ ;  $df\ 5,30$ ;  $P=0.277$ ). Similarly, no differences were observed in the level of antibody units between treatments in supernatant from intestine explants ( $F=1$ ;  $df\ 5,30$ ;  $P=0.435$ ). However, for both gills and intestine, the group immunised with FITC-IP showed a larger average than any other group.

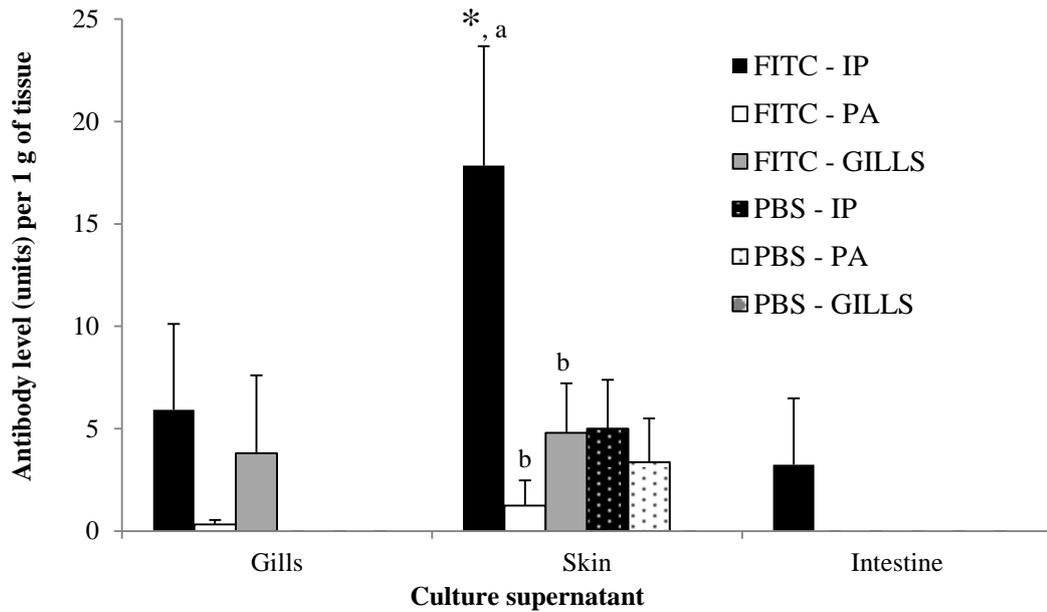


Figure 3.3. Antibody levels (units) present in supernatant from tissue explants obtained from Atlantic salmon (*Salmo salar*) against FITC, measured by ELISA. Supernatant dilution was 1:5. Fish were immunised as explained in Table 3.2 with fluorescein isothiocyanate conjugated with keyhole limpet haemocyanin (FITC) 8 and 12 weeks before obtaining the samples. Tissues were excised and incubated for 72 h in L-15 media supplemented with 10% bovine foetal serum, 2 x PSN antibiotic mix and 1 x Glutamax<sup>TM</sup>. Significant differences ( $P < 0.05$ ) are reported between each treatment and its control group (\*) or among treatments (a,b) for each tissue.

#### 3.4.4 Anti-DNP serum antibodies in ELISA

A similar pattern to the anti-FITC antibodies in serum was observed with anti-DNP serum antibodies. Significant differences in the mean serum antibodies against DNP were only observed for the DNP-IP group and its control during weeks 6, 8 and 12 of the experiment (Figure 3.4). At 6 weeks post immunisation, the DNP-IP group antibody units levels were almost double that of its control group and the DNP-PA group ( $F=3.217$ ;  $df\ 5,59$ ;  $P=0.012$ ). During weeks 8 and 12, the DNP-IP group fish had serum antibody units levels considerably higher than the control group and both DNP-PA and DNP-GILLS ( $F=12.41$ ;  $df\ 5,55$ ;  $P<0.001$  for week 8 and  $F=7.782$ ;  $df\ 5,54$ ;  $P<0.001$  for week 12). At the beginning of the experiment (week 0), the DNP-IP group showed an elevated average serum antibody level when compared to the other treatment groups (DNP-PA and DNP-GILLS) but was not different to its control group (PBS-IP) ( $F=3.952$ ;  $df\ 5,66$ ;  $P=0.003$ ). During week 2, the only significant difference observed in the mean antibody levels was for the control group PBS-IP which showed an increase when compared to the treatment ( $F=5.406$ ;  $df\ 5,65$ ;  $P<0.001$ ). No differences were observed during week 4 ( $F=1.388$ ;  $df\ 5,61$ ;  $P=0.241$ ).

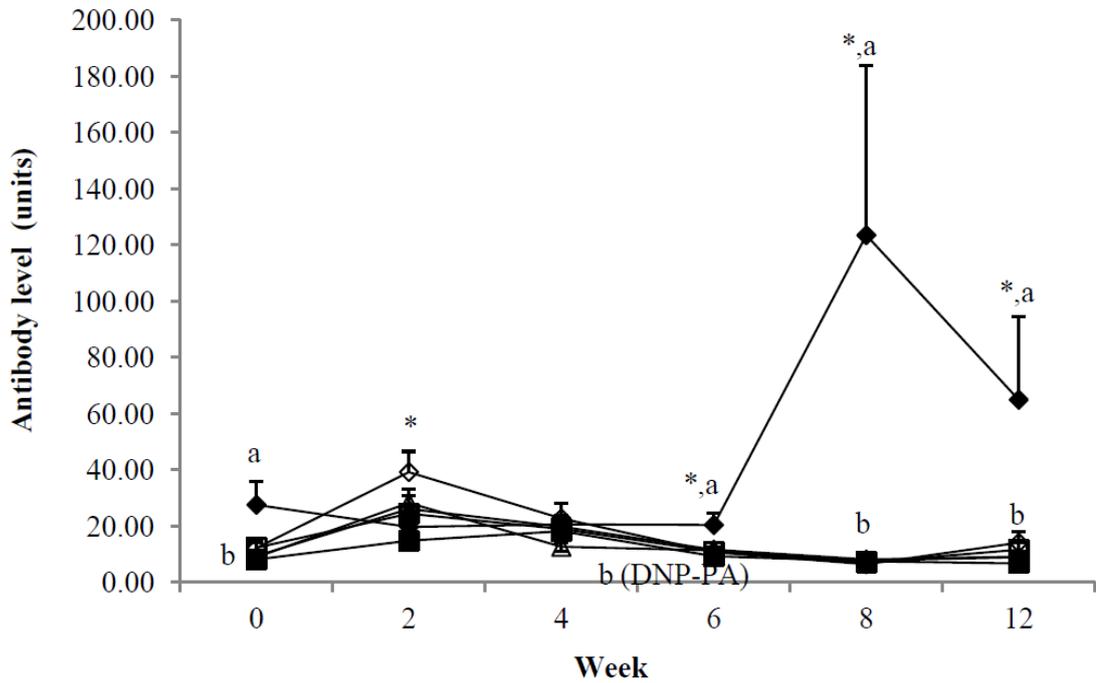
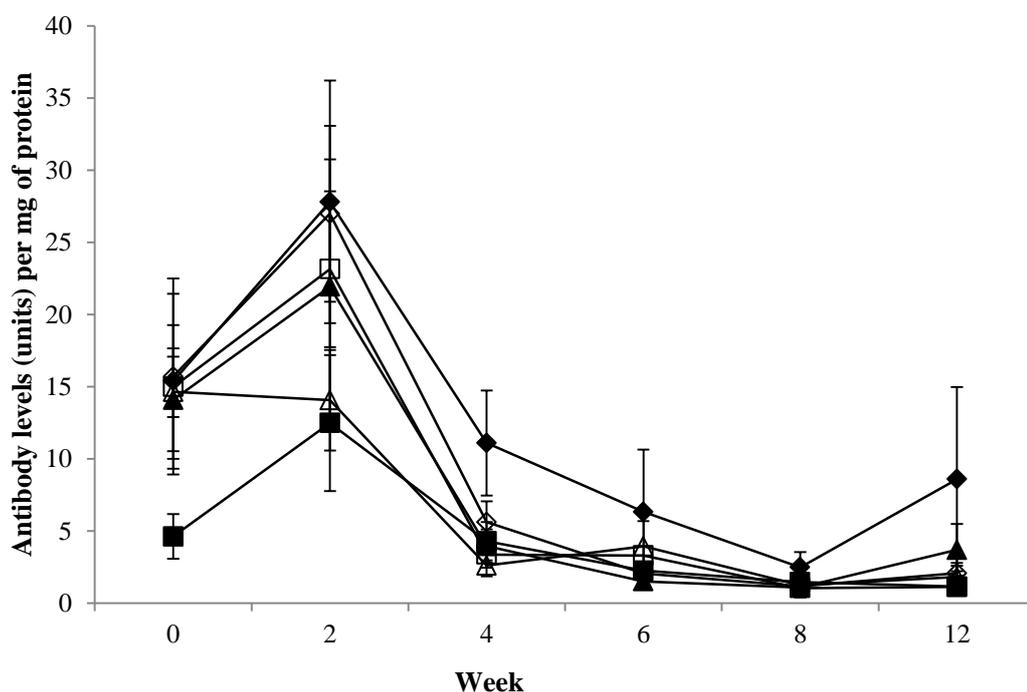


Figure 3.4. Antibody levels (units) present in Atlantic salmon (*Salmo salar*) serum, against DNP measured by ELISA. Serum dilution was 1:100. Fish were immunised as explained in Table 3.2 with dinitrophenol conjugated with keyhole limpet haemocyanin (DNP). (◆) DNP-IP; (◇) PBS-IP; (■) DNP-PA; (□) PBS-PA; (▲) DNP-GILLS; (△) PBS-GILLS. Significant differences ( $P < 0.05$ ) are reported between each treatment and its control group (\*) or among DNP treatments (a,b) at each time point.

### 3.4.5 Anti-DNP mucus antibodies in ELISA

No differences were observed in the average mucus antibody levels per mg of mucus protein in any of the treatment groups and their controls at any time point or among treatment groups at any time point (Figure 3.5).



**Figure 3.5** Mucus antibody levels (units) per mg of protein present in Atlantic salmon (*Salmo salar*) mucus, against DNP measured by ELISA. Serum dilution was 1:1. Fish were immunised as explained in Table 3.2 with dinitrophenol conjugated with keyhole limpet haemocyanin (DNP). (◆) DNP-IP; (◇) PBS-IP; (■) DNP-PA; (□) PBS-PA; (▲) DNP-GILLS; (△) PBS-GILLS. Significant differences ( $P < 0.05$ ) are reported between each treatment and its control group (\*) or among DNP treatments (a,b) at each time point.

### 3.4.6 Anti-DNP tissue supernatant antibodies in ELISA

The mean antibody levels of the tissue explants of fish exposed to DNP showed no significant differences among treatments after 72 h of culture in media for gills ( $F=0.500$ ;  $df\ 5,30$ ;  $P=0.774$ ) and intestine ( $F=1.000$ ;  $df\ 5,30$ ;  $P=0.435$ ). There was a significant difference in the mean antibody levels in skin ( $F=2.946$ ;  $df\ 5,30$ ;  $P=0.028$ ) when running the one-way ANOVA for all treatments. However, there were no significant differences among all three DNP treatments or between each treatment and its control (Figure 3.6). Control groups exposed to PBS via p.a. intubation and through gill immersion showed larger average levels of antibody units in their culture supernatant, which were not different from their respective treatment groups.

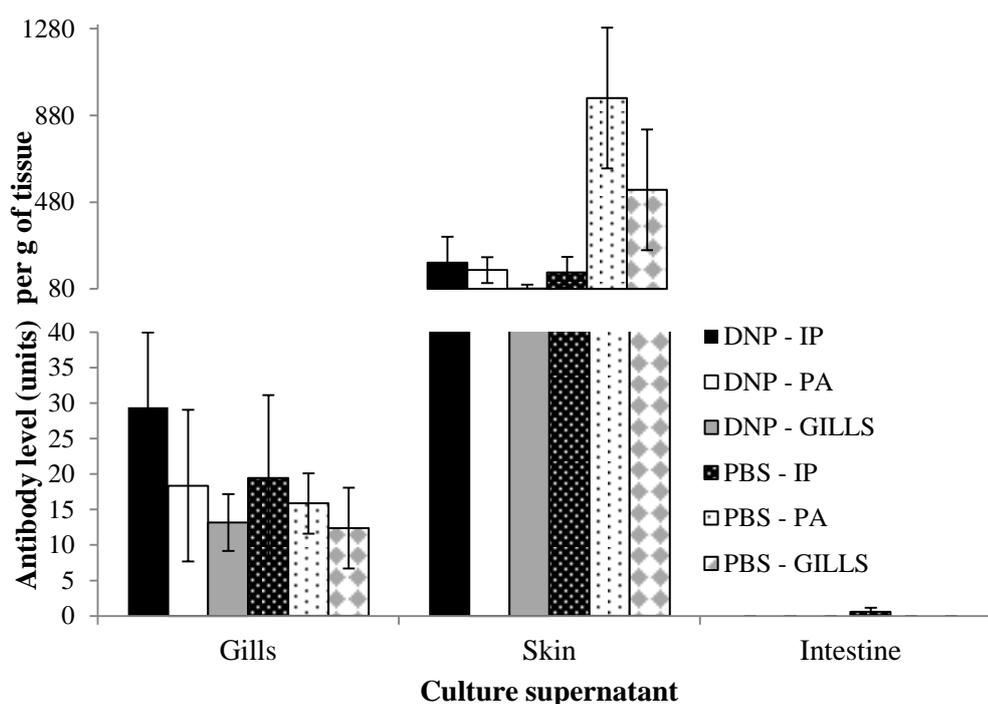


Figure 3.6 Antibody levels (units) present in supernatant from tissue explants obtained from Atlantic salmon (*Salmo salar*) against DNP, measured by ELISA. Supernatant dilution was 1:5. Fish were immunised as explained in Table 3.2 with dinitrophenol conjugated with keyhole limpet haemocyanin (DNP) 8 and 12 weeks before obtaining the samples. Tissues were excised and incubated for 72 h in L-15 media supplemented with 10% bovine foetal serum, 2 x PSN antibiotic mix and 1 x Glutamax<sup>TM</sup>. Significant differences ( $P<0.05$ ) are reported between each treatment and its control group (\*) or among treatments (a,b) for each tissue.

**3.4.7 WB against antigens**

The reactivity of serum obtained from FITC-KLH injected fish via i.p. against FITC was evident in the WB where a signal was observed at around 66 kDa (Figure 3.7, A), which corresponded to the molecular weight of the antigen-BSA conjugates. FITC and DNP are very small molecules (molar mass of 389.3 and 184.106, respectively), which would not increase significantly the molecular mass of BSA. A broad band with a higher molecular weight (between 102 and 150 kDa) was also recognised by the antibodies present in the samples. This band probably represents a mix of native and modified native forms that are produced after heating and cooling of BSA [187]. No signal was observed with negative serum or with any sample in blots containing only BSA (Figure 3.7, B). However, positive mucus and positive tissue culture supernatant samples obtained from the same fish failed to show a positive band reaction, but instead produced high levels of background at all dilutions tested (Figure 3.7, A). When IgM was purified from the skin explant supernatant, a positive band against FITC-BSA was observed in the blot. No bands were detected with negative controls i.e. negative mucus, L-15, 2 x PSN and PBS. A similar pattern was observed for all samples from DNP-KLH i.p. injected fish (data not shown).

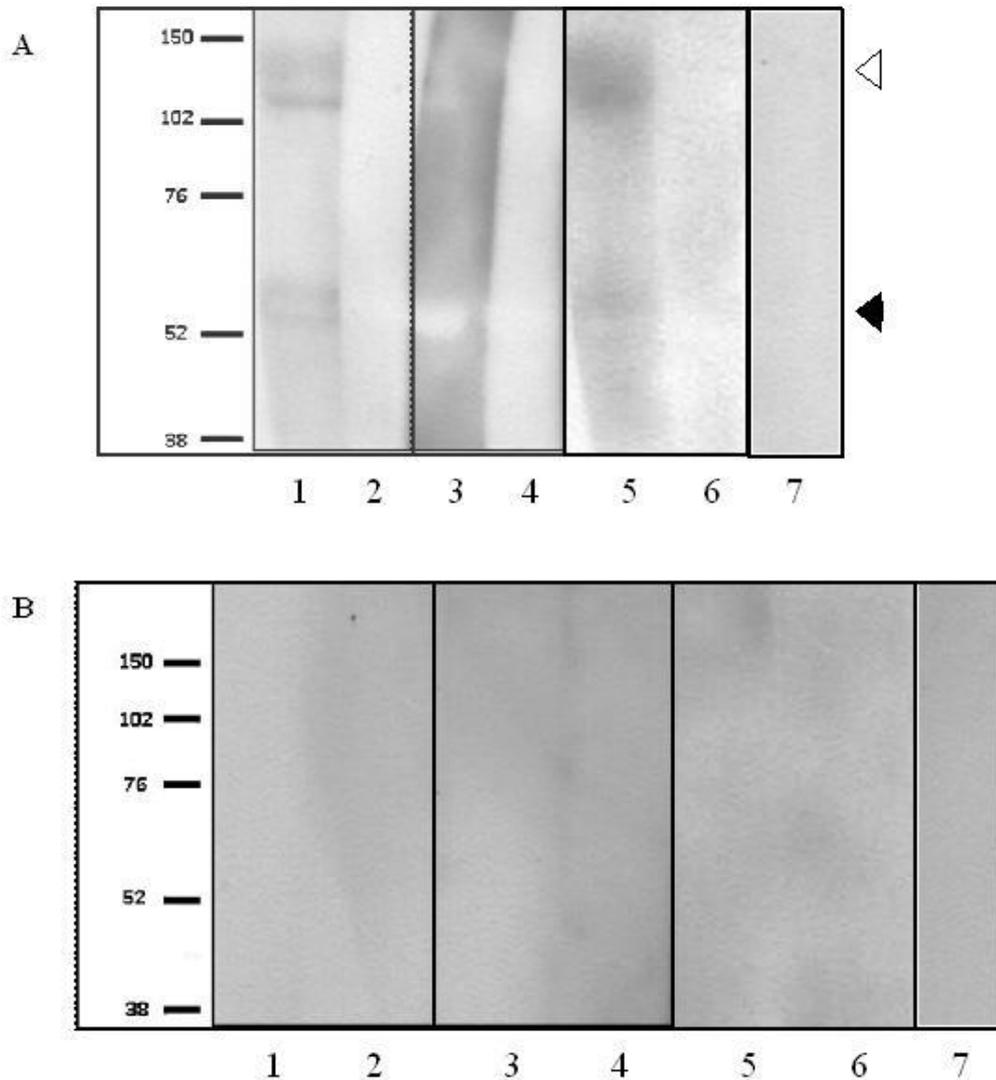


Figure 3.7. Western blot showing reactivity of pooled samples of serum, mucus and tissue supernatants from fish i.p. injected 8 and 12 weeks prior with FITC-KLH against FITC-BSA (A, black arrow head) and BSA only (B). Positive and negative samples tested via ELISA were used. Lanes 1 positive serum, lanes 2 negative serum, lanes 3 positive mucus, lanes 4 negative mucus, lanes 5 positive skin supernatant (purified IgM), lanes 6 culture media only (L-15 media, 1 x Glutamax<sup>TM</sup> and 1 x PSN antibiotic mix) and lanes 7 PBS only. Blots were then probed with mouse anti-salmon IgM mAb at 1:500 and with goat anti-mouse IgG. All samples were electrophoresed under non-reducing conditions. The white arrow head represents mix of native and modified native forms that forms after BSA is heated.

### 3.5 DISCUSSION

To our knowledge, this is the first study conducted on both systemic and mucosal antibody levels in Atlantic salmon and it showed that hapten-antigens can induce a certain level of mucosal response in this species. However there were differences observed between the two antigens used and in relation to other work with rainbow trout.

#### 3.5.1 Systemic antibody production

As expected for the systemic humoral response, both antigens FITC-KLH and DNP-KLH were able to generate an increase in antibody levels when injected with FCA only 6 weeks after the first exposure as shown by ELISA. Western blot results confirmed that the systemic antibodies were specific to FITC and DNP, and did not react against BSA. A similar response has been previously reported in rainbow trout injected with FITC-KLH [170, 175] and DNP-KLH [178], and in carp (*Cyprinus carpio*) immunised with DNP-KLH [181] and white sturgeon [174] exposed to FITC-KLH.

However, since injected vaccines require animal handling which can induce stress, the development and use of vaccines administered orally is desirable [188]. In the present study we targeted the hindgut of salmon as a possible route for antigen delivery, emulating a possible oral vaccine. A direct oral administration was not tested since we lacked an appropriate formulation that protected the antigen from degradation in the stomach. It has been previously demonstrated that salmonids like rainbow trout and brown trout (*Salmo trutta*) are capable of taking up soluble antigens through their hindgut and generate a systemic antibody response [189-191]. In the present study when salmon were exposed to both FITC-KLH and DNP-KLH via p.a. intubation, no significant antibody response was generated in serum over the 12 week period. Contradictory results have been reported previously in fish when exposed to soluble antigens through p.a. exposure. Different studies [175, 176, 189] have demonstrated a marked increase in serum antibody response in rainbow trout post p.a. immunisation with various soluble antigens. However, Swan et al. [170] using a similar dose of hapten-antigen indicated no significant increase in serum response of rainbow trout over a period of 10 weeks, showing that it is likely that the

uptake and processing of antigens through hindgut was inefficient when compared with injection delivery. Bøggwald et al. [188] obtained similar results in Atlantic salmon, where only a very low serum response was observed after p.a. intubation of fish with *Vibrio anguillarum* serotype O1, even though the same antigen generated a considerable response 10 weeks post injection. A recent study in rainbow trout [192] has shown that only 2% of the intraepithelial lymphocytes found scattered between epithelial cells in the guts corresponded to IgM<sup>+</sup> B-cells, which could explain the lack of response. It is likely that in the present study, a lack of response in the fish exposed through p.a. intubation was the reason for the absence of systemic humoral response, since our ELISA test was able to detect the serum antibody response in i.p. injected fish.

Another common form of vaccination used for fish is immersion. It is known that direct exposure to different antigens via immersion in rainbow trout [193], brook trout [194] and barramundi (*Lates calcarifer*) [195] among other species, can induce an increase in systemic antibodies. In the present study, we evaluated the ability of Atlantic salmon to generate a serum response when exposed through immersion to two soluble antigens; however the immersion procedure used was not an effective method to induce a systemic antibody response. The levels of serum antibodies against both haptens DNP and FITC in fish did not change significantly over the course of the experiment. It is important to mention that the immersion treatment only covered the gills and the head of the salmon while the fish were anaesthetised and in an inverted upright position and therefore, it was unlikely that they could have ingested the solutions, leaving the gills as the main route for antigen uptake. As explained by Lobb [179], it is possible that since the fish had been anaesthetised before the exposure, the uptake of antigen through the gills might have been impaired, through a decrease in the opercular movement and respiratory frequency.

### **3.5.2 Mucosal antibody production**

No significant antibody response in skin mucus was induced in fish exposed to DNP-KLH through any exposure route during the course of the experiment or in fish immunised with FITC-KLH through any route until week 8. It has been reported in teleosts that a mucosal antibody response can be generated independently of a

systemic antibody response [175, 179, 196]. Additionally i.p. injection in rainbow trout, white sturgeon, channel catfish, tilapia (*Oreochromis niloticus*), and eel (*Anguilla anguilla*) [170, 174, 180, 197-199], p.a. intubation in eel and African catfish (*Clarias gariepinus*) [198, 200] and immersion in catfish, African catfish, eel and yellow croaker (*Pseudosciaena crocea*) [179, 198, 200, 201] have all demonstrated the ability to induce an increased skin mucus antibody response against different haptens and bacterial antigens. However, in some instances, an antibody response was not observed, which is in agreement with the present study. No antibody response was observed in the skin mucus of Atlantic salmon after immunisation via i.p. injection or p.a. intubation when exposed to four different bacterial antigens [188]. La Frenz et al. [202] showed that rainbow trout immunised by i.p. injection with *Flavobacterium psychrophilum* or its culture supernatant, did not generate any significant skin mucosal antibody response. Rainbow trout also failed to generate a skin mucosal antibody response when exposed to conjugated FITC via p.a. intubation [170], and even immersion has proven to be inefficient at generating a detectable humoral response in skin mucus [173, 197, 202].

Compared with the high concentration of serum Ig, the concentration of Ig present in the skin mucus is very low in salmon and other species [130, 191, 203, 204]. This could explain why the level of antibody units per gram of protein obtained from the skin mucus samples through the ELISA were so low for both haptens during the whole experiment when compared with results from serum. Additionally, it has been further demonstrated that around 50% of the B-cells present in the mucosal surfaces of rainbow trout correspond to IgT<sup>+</sup> IgM<sup>-</sup> B-cells [97], which only produce IgT. However, the IgT responses in the skin have not yet been properly studied, either at the gene or at the protein level [76]. If an IgT response was elicited during this experiment in the skin mucus, it is likely that the anti-IgM mAb used in the ELISA was not able to detect it. Nevertheless, the use of specific antibodies against rainbow trout IgT to detect Atlantic salmon IgT has proven unsuccessful in earlier experiments in our laboratory (unpublished data), limiting its detection. In addition, skin mucus could have interfered with the detection of antibodies in the ELISA, as demonstrated in channel catfish, where only 50% of the Ig were detected in a skin mucus sample [130].

On the other hand, Atlantic salmon immunised only via i.p. injection showed an increase in skin mucus antibody levels during week 12 of the experiment when compared to the other treatments (p.a. intubation and immersion) but not in comparison with their relative control (PBS-FCA i.p. injected). Injection of fish with different antigens has been shown to induce a mucosal antibody response on several occasions [170, 174, 175, 197, 202], but always with the use of an adjuvant. Moreover, a very low or absent antibody response in skin mucus after i.p. injection in both seawater [201] and freshwater [195] acclimated fish has been observed when no adjuvant was used. Furthermore, no antibody response was observed in the skin mucus of Atlantic salmon maintained in water with a salinity of 15‰ after immunisation via i.p. injection with four different bacterial antigens [188] without an adjuvant.

The lack of difference in the mucosal response between fish immunised with both haptens and their control groups exposed to PBS-FCA at every sampling time could be due to an increased non-specific response to the adjuvant used in the antigen formulation. As mentioned before, FCA is a nonspecific stimulator of humoral responses [205]. It is possible that some of the antibody responses observed, particularly in those groups exposed to FCA were just of a non-specific nature, and that is why they were very low, and showed some non-specific reactivity with both hapten antigens in mucus.

### **3.5.3 Tissue explants**

A significant increase in antibodies was measured in the skin explant supernatants of Atlantic salmon injected i.p. with FITC-KLH. And even though there was no significant difference with their controls, gills of fish immunised with both FITC-KLH and DNP-KLH showed a slightly higher level of antibody units in their culture supernatant. These findings are in agreement with previous results for various species [170, 184, 206, 207], and indicate active production of antibodies by localised B-cells, considering that tissues were thoroughly rinsed and all blood was removed by perfusion before the 72 h of culture.

The fact that only salmon which were exposed to FITC-KLH via i.p. injection in this experiment presented an increased antibody response in the tissue explants of

mucosal surfaces shows that there is a relationship between the systemic and mucosal responses. Cain et al. [175] suggested a passive transfer of serum derived antibodies to mucosal sites. More recent studies have shown that it is possible that immunoglobulins present in the serum of fish can be transported to mucosal secretions of either the skin [208] or intestine [97] through a unique polymeric Ig receptor. In this study, it could be speculated that the increase in antibody response observed in supernatant from gills and skin explants might be due to an increase in antibody production by local B-cells and not due to Ig transport from serum to mucus, considering that these tissues were washed and stripped of almost all serum and mucus when processed. If these B-cells were present locally, they might have originated in other organs and then migrated to these tissues, as was previously suggested by Swan et al. (2008), since the increase in antibody production was only observed in fish that were exposed to the antigen via i.p., and not in those which were stimulated in the mucosal areas. However, we did not verify the presence of these cells by immunohistochemistry.

In conclusion, i.p. injection of hapten-antigens in Atlantic salmon has shown to be the best delivery route for inducing an antibody response against two haptens (FITC and DNP) in this species. Even though it did not induce an increase in antibody levels in the skin mucus, there was an apparent increase of antibody production in mucosal tissues as demonstrated by specific antibody levels in supernatants from the tissue explants. The results of the present study helped in elucidating what could be the most appropriate delivery method for prospective vaccines aimed at generating a mucosal humoral response against *Neoparamoeba perurans* the causative agent of Amoebic gill disease (AGD) [46]. This disease is the main disease affecting the Atlantic salmon industry in Tasmania [55]. Blocking the attachment of the amoebae to the gills by means of increasing the production of antibodies in mucus may be an effective way of decreasing the incidence of AGD. Work is currently in progress to identify prospective antigens derived from *N. perurans* that may be administered to salmon and stimulate an adaptive immune response at the site of infection.

**3.6 CONCLUDING REMARKS**

While this paper was on review, Hedfors et al. [209] showed that different commercial available antibodies recognise different IgM isotypes (IgM-A and IgM-B) in different tissues. The mAb used in the present study for ELISA and WB was one of the antibodies that detected the lowest number of IgM<sup>+</sup> B-cells for both isotypes in all tissues examined. This might have had some effect in the capacity of this antibody to detect the total IgM present in our samples, and could be the reason behind some of the low antibody levels detected.

**3.7 ACKNOWLEDGEMENTS**

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## CHAPTER 4.

### ADMINISTRATION OF RECOMBINANT ATTACHMENT PROTEIN (*r22C03*) OF *Neoparamoeba perurans* INDUCES HUMORAL IMMUNE RESPONSE AGAINST THE PARASITE IN ATLANTIC SALMON (*Salmo salar*)

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#### KEYWORDS

Amoebic gill disease; *N. perurans*; recombinant protein; systemic and mucosal antibodies.

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#### 4.1 ABSTRACT

This study investigated the use of a recombinant protein of *Neoparamoeba perurans*, the causative agent of Amoebic gill disease (AGD), as an immunogen to generate systemic and mucosal antibody responses against the parasite. Genes encoding *N. perurans* homologues of mannose-binding protein (MBP) from *Acanthamoeba* spp. have been identified. From these, a *Neoparamoeba* MBP – like EST has been identified and produced as a recombinant fusion protein. Attachment of *N. perurans* to the gill might be reduced by antibody-mediated interference of this protein, but this is dependent on the presence and level of functional antibodies in the mucus. Fish were immunised with the protein via i.p. injection with Freund's complete adjuvant (FCA); and serum and skin mucus samples were collected before and after immunisation. Antibodies (IgM) present in samples were characterised via Western blot and their levels measured with an ELISA. The immunisation was able to induce a systemic IgM response 8 weeks after primary exposure and a mucosal response 4 weeks post initial immunisation, which were specific to the recombinant protein but not to antigens obtained from crude amoebic preparations. However, adherence of the antibodies to the parasite was observed using immunocytochemistry and both, serum and skin mucus IgM, were able to bind the surface of formalin-fixed *N. perurans*. This finding may contribute to further research into the development of a vaccine for AGD.

#### 4.2 INTRODUCTION

Amoebic gill disease (AGD) is a worldwide disease affecting farmed marine fish [37]. Even though the condition affects mainly salmonids [21, 22, 34, 41], it has also been observed in other farmed fish such as turbot (*Scophthalmus maximus*) [25] and juvenile ayu (*Plecoglossus altivelis*) in Japan [35]. AGD is caused by the marine protozoan ectoparasite *Neoparamoeba perurans* [46]. The clinical presentation produces mortalities that may reach up to 50% in total if left untreated [20]. Infected fish show respiratory distress and swim with open opercula [20]; gross pathology of AGD is characterised by raised, multifocal white mucoid patches upon the gills and increased branchial mucus production [20, 22].

In Tasmania, Australia, AGD was described more than two decades ago [210] and it continues to be the main disease affecting the Atlantic salmon (*Salmo salar*) industry [55]. Even though various prophylactic and therapeutic agents have been tested as control methods for the disease [52, 131, 211], freshwater bathing is still the only treatment widely used by the industry. However, this treatment strategy is an increasingly significant economic burden for the Tasmanian salmon industry representing up to 20% of the total production costs, since several baths are required during the warmer period of the grow-out season [21, 212]. Therefore, the development of a vaccine remains a high priority for the local industry.

The process initiating AGD is the attachment of the amoeba to the gill surface, where initial alterations to the epithelium such as desquamation and occasional oedema may occur followed by hyperplastic lesions and fusion of the lamellae after 48 h of infection [56]. Blocking attachment of the amoeba by generating antibodies which react against the attachment proteins could be an effective way of reducing the incidence of AGD and therefore reduce costs for the industry.

Attachment proteins of other species of amoebae are currently being considered as vaccine candidates. In the case of the free-living ubiquitous *Acanthamoeba*, an opportunistic parasite responsible for corneal infections in humans [reviewed by Ref.213], a ~400 kDa mannose-binding protein (MBP) expressed on the surface of the parasite is a major virulence factor responsible for host-parasite interactions [214]. It has been demonstrated that polyclonal antibodies prepared against this MBP inhibit the adhesion of the parasite to the host cell [215].

In the present work, genes encoding *N. perurans* homologues of MBP from *Acanthamoeba* spp. have been identified. From these, a *Neoparamoeba* MBP – like EST has been identified and produced as a recombinant protein. It may be possible to impede attachment of *N. perurans* to the gill by antibody-mediated interference of this protein, but this is dependent on the presence and level of functional antibodies in the gill mucus. To that end, we immunised Atlantic salmon with the recombinant protein. Systemic and mucosal IgM antibodies against this recombinant protein were measured and characterised using ELISA and Western blots (WB), and we observed using epifluorescent microscopy their binding to the surface of *N. perurans*. This

work demonstrates that immunisation with recombinant proteins representative of *N. perurans* cell surface molecules produces functional antibodies. This finding may contribute to further research into the development of a vaccine for AGD.

### 4.3 MATERIALS AND METHODS

#### 4.3.1 *N. perurans* trophozoites

*N. perurans* trophozoites were isolated from infected fish held in a continuous infection tank housed at the Aquaculture centre, University of Tasmania. It has been previously shown through PCR validation [38] that amoebae suspensions obtained from this tank are comprised predominantly of *N. perurans*. Amoebae were isolated through plastic adherence as previously described [151].

#### 4.3.2 Identification and molecular analyses of attachment proteins in *N. perurans*

Amoeba RNA was extracted using TRIzol® reagent (Invitrogen-Life Technologies, Carlsbad, USA) as per the manufacturer's recommendations. A normalised cDNA library was constructed using a Creator™ SMART™ cDNA library construction kit (Clontech, Mountain View, USA) and a Trimmer – direct DSN normalisation kit (Evrogen, Moscow, Russia) as per manufacturer's instructions. Resultant normalised cDNA was cloned into the library vector pTriplEx2 and used to transform electrically competent *Escherichia coli*. Two thousand clones were picked and subjected to 5' direction sequencing at Macrogen ([http://www.macrogen.com/eng/sequencing/sequence\\_main.jsp](http://www.macrogen.com/eng/sequencing/sequence_main.jsp)).

Resultant sequences were trimmed of the vector and subjected to BLAST, tBLASTn, BLASTx and tBLASTx analysis. From the 2,000 sequences, 130 were excluded due to small size and/or bad read. A further 100 sequences were excluded as duplicates representing <7% redundancy in the library. The remaining 1,770 sequences were interrogated against public databases at NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and against known protist pathogen genomic databases (<http://www.sanger.ac.uk/resources/downloads/protozoa/entamoeba.html>). One clone from the normalised library, designated 22C03, following tBLASTx

analysis showed sequence identity with a characteristic C-type lectin carbohydrate binding domain.

### 4.3.3 DNA cloning, sequencing and construction of the expression vector

To facilitate cloning of 22C03 into a bacterial expression factor, it was first amplified by PCR and cloned into the entry vector pENTR (Invitrogen-Life Technologies) as per their protocols to generate an entry clone. The 22C03-pENTR construct was used to transform competent DH10 $\beta$  *E. coli*. Transformation, positive clone selection and plasmid DNA preparation were undertaken as per the manufacturer's protocols. PCR positive clones were subjected to DNA sequencing (BigDye™, PE Applied Biosystems, Foster City, USA) following standard techniques. Once confirmation was completed, culture from one positive clone was used to make a glycerol stock.

22C03 was sub-cloned into the bacterial expression vector pDEST17 (Invitrogen-Life Technologies) from pENTR using the recombination reaction with Gateway® LR Clonase® II enzyme mix (Invitrogen –Life Technologies) as per manufacturer's instructions. Transformed *E. coli* cells carrying the plasmid pDEST17-22C03 were plated on selective Luria-Bertani (LB) plates containing 100  $\mu$ g/mL ampicillin (LB/Amp) and incubated overnight at 37°C. Four clones were randomly picked and grown overnight in LB/Amp medium at 37°C and 200 rpm in an orbital mixer incubator (Ratek, Boronia, Australia). Five microliters of the overnight culture was used as template for PCR confirmation of the insert using the T7 primer (Invitrogen-Life Technologies) and a primer designed 150 bp downstream of the attB2 insertion site. PCR positive clones were subjected to sequencing and analysis using the same primers. A positive clone from the pDEST17-22C03 construct was selected and a glycerol stock made to maintain and propagate the expression clone.

### 4.3.4 Expression and purification of a soluble recombinant 22C03 fusion protein (*r22C03*)

For expression of *r22C03*, BL21 Star (DE3) pLysS *E. coli* cells (Invitrogen-Life Technologies) were used. Transformation of the BL21 Star (DE3) pLysS One Shot cells with pDEST17-22C03 plasmid DNA (100 ng) was performed as per manufacturer's instructions. A random selection of resultant clones were subjected to PCR analysis using the T7 primer sites flanking the insertion sites. Clones with the

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correct insert were grown as a BL21 Star (DE3) pLysS *E. coli* stock culture in 5 mL LB/Amp broth (0.1% glucose, 100 µg/mL ampicillin, 34 µg/mL chloramphenicol) and a subculture from each stock was grown to replace the old stock once a week with an initial dilution of 1:20.

These stocks were streaked onto a LB/Amp plate and incubated overnight at 37°C. A single colony was inoculated into 20 mL of LB/Amp broth. This pre-culture was inoculated in a 1:50 ratio in larger volumes of LB/Amp broth. The bacteria were grown at 37°C with vigorous shaking for 4 h. Expression of recombinant protein was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG; Promega) at 1 mM. To isolate the inclusion bodies, the bacteria were centrifuged at 8,000 x *g* for 30 min, the pellets were washed and resuspended 2 x in TBS (50 mM Tris, 150 mM NaCl, 1mM EDTA, 12 mM 2-mercaptoethanol, pH 7.5) and homogenised using an Ultra-Turrax® T25 (Ika Labortechnik, Staufen, Germany), sonicated 3 x (30 s each) on ice and Triton X-100 added to a final concentration of 0.1 % and incubated at 4°C for 30 min. Inclusion bodies were collected by centrifugation at 8,000 x *g* for 30 min, and washed once more as above but this time adding Triton X-100 to a concentration of 0.5%. The inclusion bodies were then solubilised in 8 M Urea buffer, pH 8.0 (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-HCl, 8 M Urea, 5 mM 2-mercaptoethanol), homogenised, incubated for 1 h at 4°C and centrifuged at 100,000 x *g* for 30 min to remove insoluble material and residual cell debris. The recombinant protein was purified by metal-affinity chromatography with nickel-nitrilotriacetic acid (Ni-NTA) column (Qiagen K.K., Tokyo, Japan). Sequential elution was performed with 8 M Urea buffer, pH 6.3 containing 20, 50, 100 and 200 mM Imidazole. Wash fractions were pooled and filtered and sequentially dialysed into 2 M Urea. Protein concentration was measured using a Micro BCA™ Protein Assay Reagent Kit (Pierce, Scoresby, Australia).

**4.3.5 Experimental animals and treatment**

Atlantic salmon (n=5) with average body weight of 116.38 g (SD 26.71 g) and average fork length of 220.02 mm (SD 21.62) were obtained from a commercial farm. Animals were held at the Aquaculture Centre, University of Tasmania for 10 d prior to the experiment and for the duration of the trial in a 4,000 L freshwater

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recirculation system, which was maintained at 14 – 15.8°C, pH 7.0. Water quality and temperature were assessed daily and 50% water exchanges occurred fortnightly. Fish were fed a commercial diet to satiation twice a day during acclimation and experimental period. This project was authorised under the University of Tasmania Animal Ethics Committee approval number A0012145.

Fish were immunised with *r22C03* via intraperitoneal (i.p.) injection. The antigen was emulsified with Freund's complete adjuvant (FCA, Sigma Aldrich, Sydney, Australia). Fish were given a total of 200 µg of antigen in 200 µL of a solution consisting of a 1:1 ratio of inoculum (2 mg/mL of antigen) and adjuvant, in order to obtain a 1 mg/mL solution. A booster with the same concentration but in Freund's incomplete adjuvant (FIA) was given 4 weeks after the initial immunisation. There is consensus that FCA should be used only for initial immunisations and FIA for subsequent immunisations, since the inclusion of heat-killed bacteria in FCA could have induced a hypersensitivity with granulomatous reaction, while FIA is less toxic, but it mainly stimulates the production of antibody responses [216, 217].

**4.3.6 Serum and cutaneous mucus sampling**

Mucus and blood were obtained from all fish before the first immunisation and at 4, 8, 10 and 12 weeks post immunisation. Fish were anaesthetised before treatments and sampling using a clove oil solution. Individual fish were placed in a 1 L plastic bag, their skin massaged for about 30 s to harvest mucus; 250 µL of antiprotease cocktail (Sigma Aldrich) was added immediately and mixed in with the mucus inside each bag and kept on ice. Mucus samples were transferred to 5 mL plastic tubes within 4 h of collection, vortexed vigorously, passed 20-30 x through a 3 mL syringe with a 25 G needle, centrifuged at 10,000 x *g* and 4°C for 10 min and supernatant collected. Approximately 1 mL of blood was collected from the caudal vein using 1 mL syringes with a 25 G needle and left to clot in a 1.5 mL microcentrifuge tube overnight at 4°C. Serum was obtained after centrifuging the samples at 1,500 x *g* for 10 min at 4°C. Serum and mucus samples were stored at -80°C until used.

**4.3.7 *N. perurans* antigen**

*N. perurans* trophozoites obtained as in section 4.3.1 were used to test whether antibodies generated in serum and mucus recognised epitopes in the parasite. After

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harvesting amoeba from the infection tank, they were concentrated by centrifugation at 500 x g for 5 min and counted using a haemocytometer. Cells were washed twice with PBS (0.02 M phosphate, 0.15 M NaCl; 0.876 g NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 2.56 g Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 8.77 g NaCl in 1 L distilled water, pH 7.3) and used fresh as whole cells or centrifuged again and the pellet frozen at -20°C until used to prepare soluble antigen. Pellets were thawed, diluted in PBS, sonicated 5 x on ice for 20 s at 14 W, centrifuged at 16,000 x g for 10 min and supernatant collected and used as soluble antigen. Protein concentration was measured using a Micro BCA<sup>TM</sup> Protein Assay Kit (Pierce). *N. perurans* antigen was characterised by SDS-PAGE using NuPAGE® Novex® Bis-Tris Mini Gels (Invitrogen-Life Technologies). In brief, 6.5 µL of the antigen were mixed with 2.5 µL of NuPAGE® LDS sample buffer 4 x and 1 µL of NuPAGE® Reducing agent (Invitrogen-Life Technologies) per lane then heated for 10 min at 70°C and run at 200 V for 35 min.

**4.3.8 Enzyme-linked immunosorbent assay (ELISA)**

The activity of anti-*r22C03* antibodies was determined by an ELISA. The coating antigen was either purified *r22C03* or *N. perurans* antigen from section 4.3.7. Single point dilution ELISA, as previously described [152], was performed in duplicate sera or mucus samples. A positive standard, consisting of a mix of sera from fish presenting high antibody levels from the final sampling point, was included in triplicate two-fold dilutions (1:100 to 1:6,400) in every plate. Coating antigen and serum and mucus dilutions were optimised in advance, with optimum concentrations for *r22C03* and *N. perurans* set at 10 and 2.4 µg/mL respectively and serum and mucus dilutions set as optimal at 1:200 and at 1:2 in PBS. Negative controls, run in duplicate, contained a pool of sera or mucus from fish kept in freshwater and therefore naïve to AGD.

Plates were coated overnight at 4°C with each antigen, and non-specific sites blocked with 5% skim milk in distilled water for 2 h at 18°C. Diluted sera or mucus were incubated for 1.5 h. Plates were washed 3 x with low salt wash buffer (LSWB, 2.42 g Trisma base, 22.22 g NaCl, 0.5 mL Tween-20 in 1 L distilled water, pH 7.3) after coating and sample incubation. A mAb against rainbow trout/Atlantic salmon immunoglobulin M (IgM) (Aquatic Diagnostics, Stirling, UK) was diluted 1:100 in

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1% BSA in PBS-T (PBS with 0.1% Tween-20), and incubated for 1 h. An affinity purified goat anti-mouse IgG (whole molecule) conjugated to horseradish peroxidase (Sigma Aldrich), diluted 1:1,000 in 1% BSA in LSBW was then incubated for 1 h. Samples and antibodies were added at 100  $\mu$ L/well and incubated at 18°C. After each incubation, plates were washed 5 x with high salt wash buffer (HSWB, 2.42 g Tris base, 29.22 g NaCl, 1 mL Tween-20 in 1 L distilled water, pH 7.7). Bound antibody was visualised with 100  $\mu$ L TMB One Solution (Promega, Fitchburg, USA), incubated 10 min at RT and stopped with 50  $\mu$ L of 2 M H<sub>2</sub>SO<sub>4</sub> solution. Optical density (OD) readings were measured at 450 nm in a Rainbow Thermo plate reader (Tecan Group Ltd., Männedorf, Switzerland).

Serum and mucus ODs from ELISA were corrected by subtracting mean OD of the negative controls in each plate. ELISA results were reported as levels of antibody units derived from the positive standard curve on each plate, using a four parameter logistic function as described previously [152]. This model has shown good results for calculations of total antibody in ELISA [154]. Function calculations were done using SigmaPlot 11.0 software (Systat Software, Inc., Chicago, USA). Antibody unit values for mucus were expressed per mg of protein in the sample; protein in mucus was measured with a Micro BCA<sup>TM</sup> Protein Assay Kit (Pierce).

**4.3.9 Western blot, dot blot and chemiluminescent detection**

Whole *N. perurans* antigen and *r22C03* were SDS-PAGE characterised with NuPAGE® Novex® Bis-Tris Mini gels, as in section 4.3.7, using *r22C03* (900  $\mu$ g/mL, 6.5  $\mu$ L) or 80,000 *N. perurans* whole cells (approximately 8  $\mu$ g) in 6.5  $\mu$ L PBS per lane. Samples were transferred to PVDF membranes by 6 min electrophoresis at 200 V in an iBlot® Dry Blotting System (Invitrogen-Life Technologies). For WB, membranes were blocked overnight at 4°C with 5% dry milk in PBS and washed 3 x for 30 s with LSBW. To detect antigen bands, serum samples were diluted up to a 1 mL in PBS-T at 1:500 to 1:2,000, and duplicate membranes incubated for 3 h. Then a mouse mAb anti-salmon IgM (Aquatic Diagnostics) at 1:5,000 in 1% BSA PBS-T and a goat anti-mouse IgG horseradish peroxidase (HRP, Sigma-Aldrich) in 1% BSA in LSBW at 1:10,000 were each incubated for 1 h at RT; membranes were washed 5 x for 30 s with HSWB between incubations. The same

**Administration of r22C03 induces immune responses against *N. perurans***

negative control than in ELISA (sera from AGD naïve fish) was used. To test for unspecific binding, supplementary membranes were incubated with samples, primary and secondary antibodies sequentially excluded.

For mucus samples, dot blots instead of WB were used, to have more concentrated antigens on the membrane. It has been shown that the concentration of Ig in mucus could be considerably lower than in serum [130]. PVDF membranes were cut into strips, soaked in 100% methanol for 10 s, distilled water for 5 min and PBS for 10 min and loaded with 2.5 µL of the following: serum diluted 1:400 in PBS, r22C03 (800 µg/mL), amoebae antigen supernatant (200 µg/mL) and PBS in four different dots. Duplicate membranes were blocked overnight in 2% casein in TBS (10 mM Tris-HCl, 150 mM NaCl, pH 7.5). Mucus samples were diluted 1:5 in PBS-T up to 1 mL volume and incubated for 1.5 h at RT. Membranes were incubated for 1 h at RT with a mAb against salmon IgM heavy chain conjugated with HRP (Cedarlane, Burlington, Canada) in 1% casein in TBS at 1:10,000 dilution. Membranes were washed 5 x for 30 s with HSWB after each incubation. Since this experiment did not have fish injected only with adjuvant and no r22C03, a confirmatory dot blot was performed with sera from fish used in another experiment that were immunised with FCA+PBS and 5 weeks later with FIA+PBS (data not shown). Duplicate strips were prepared as explained above, but using FCA antigen instead of *N. perurans* antigen. The FCA antigen consisted of *Mycobacterium tuberculosis* suspended on FCA, which were centrifuged at 13,000 x g for 10 mins, washed with PBS 5 x, sonicated as the amoeba and blotted on membranes. Sera from fish injected only with adjuvants (n=4), a mix of all sera from fish sampled at week 8 (n=5) and only HRP-conjugated antibody were used to probe these strips.

WB and dot blots were developed in a dark chamber by placing ECL Prime Western Blotting 1:1 Detection solution (GE Healthcare, Chalfont St Giles, UK) over the membranes for 5 min. Excess detection solution was removed and a Hyperfilm ECL sheet (GE Healthcare) was placed on top of the blot inside a X-ray film cassette for 2-10 min and developed using Kodak GBX fixer and developer solutions (Sigma Aldrich).

**4.3.10 Sodium periodate oxidation of WB**

WB of amoebic antigens were prepared as above and from the same membrane, adjacent strips were subjected to periodate oxidation as previously described [218] or used as controls. In brief, oxidised strips were incubated in 20 mM sodium periodate (Merck Pty Ltd., Victoria, Australia) and 50 mM NaBH<sub>4</sub> (Sigma Aldrich) or controls incubated only in 50mM NaOAc (Sigma Aldrich) pH 4.5. Membranes were then washed with PBS, blocked, probed and developed with ECL as indicated above.

**4.3.11 Immunocytochemistry**

A *N. perurans* clone [219], identified as clone 4, was used to assess the binding efficacy of antibodies to whole cells. Approximately 10,000 cells suspended in 100 µL of PBS were placed in poly-L-lysine coated microscope slides and left to attach for 1 h at RT. Cells were fixed to the slides with 100 µL of seawater Davidson's fixative (30 parts of 95% ethanol, 20 parts of 37-40% formaldehyde, 10 parts of glacial acetic acid, and 30 parts of seawater) for 30 min. Slides were washed 1 x with PBS and blocked for 1 h with 1% BSA in PBS. Cells were probed with serum (1:10) and mucus (1:2) samples, obtained from fish before and after immunisation with *r22C03*, that showed low or high antibody levels against *r22C03* and *N. perurans* by ELISA. Bound antibodies were detected with a 1:100 mAb against rainbow trout/Atlantic salmon IgM (Aquatic Diagnostics), followed by 1:50 anti-mouse IgG (Fab specific) F(ab')<sub>2</sub> fragment FITC conjugated antibody produced in goat (Sigma Aldrich). Samples and antibodies were diluted in 1% BSA in PBS, added as 50 µL to slides and incubated for 30 min at RT; slides were washed 3 x with PBS following each incubation step. Negative controls included cells probed with southern bluefin tuna (*Thunnus macoyii*) serum followed by a mAb against bluefin tuna (*Thunnus thynnus*) IgM (Aquatic Diagnostics) at the same dilutions as above or samples incubated with one missing reagent at a time (i.e. no serum or mucus or no mAb or no FITC-conjugate). Cells were photographed (Leica DC300F, Leica Microsystems, Wetzlar, Germany) using light and epifluorescent microscopy.

**4.3.12 Statistical analysis**

To assess differences in serum and mucus antibody responses by ELISA among sampling times a one-way analysis of variance (ANOVA) was used. Due to the low

number of replicates (n=5) and the significant differences observed in variances between sampling times as shown by the Levene's test of equality of variances, all data had to be  $\log_{10}$  transformed before analyses. When differences were detected, values were considered significantly different at  $P$ -values  $<0.05$ . IBM SPSS Statistics version 20 software (IBM SPSS Inc., Armonk, NY, USA) was used for the analyses.

## 4.4 RESULTS

### 4.4.1 Protein sequence

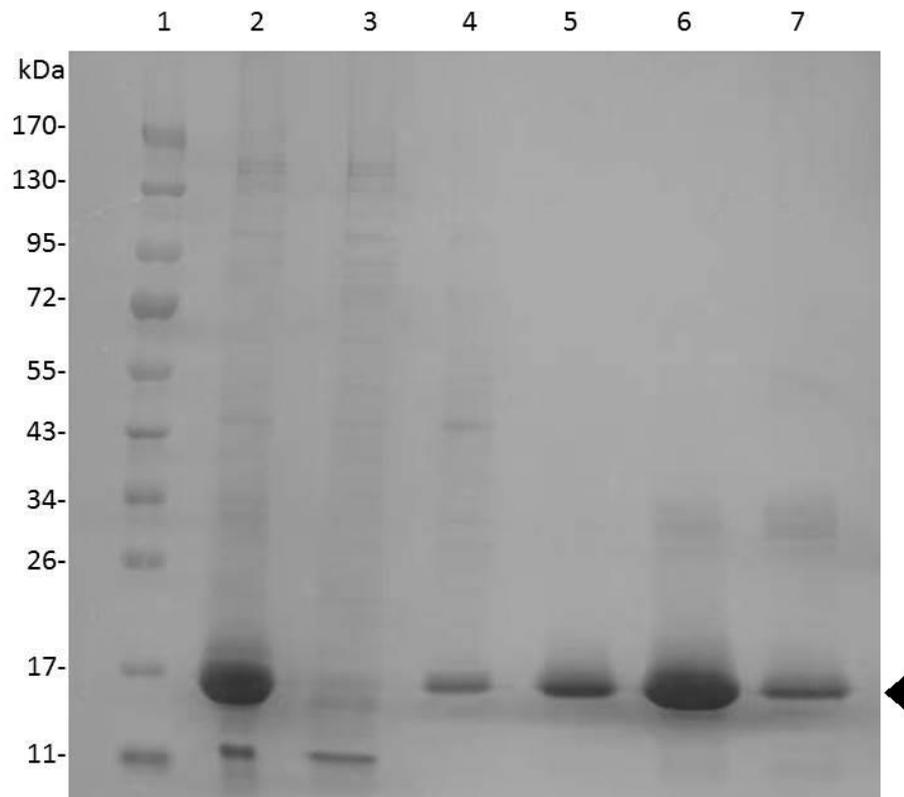
The 22C03 clone, which showed sequence identity with a C-type lectin carbohydrate binding domain, was subjected to further analysis including open reading frame (ORF) determination. Following ORF analyses the *r22C03* EST consisted of a 660 bp open reading frame encoding a 219 aa protein (Figure 4.1). Specifically the DELTA-BLAST analysis showed strong conservation of the C-lectin superfamily between residues 115-210 with a strong conservation of the characteristic ligand binding surface residues in this area. Therefore, this EST became a focus for further investigation as it possibly resembled other C-type surface lectins which pathogenic amoebae species use for cell attachment and initiation of disease [214].

MLKRYPGVRTFLANEASRDVYRGVSVEWISGAKPLARFYDEQGNLLTSETIANLDGNEIDEFFANHGF  
PLRRSELEKETPLIERWNEHTYEIYKFNPLSYSRQFAEAQRMGTEGEEKRGQMLALSSEEEEDFVKD  
# # # # ##### ###  
**LMEKHS**LGTVWLGVS**D**SATEGTWVWF**GGQ**QEAES**PRWAF**GEP**NNAKGA**ENCAVLT**I**HGW**NDV**SCL**TAH**  
**SLVIEFEAT**VPK**AEL**\*

Figure 4.1 Amino acid sequence of recombinant fusion protein *r22C03*. Underlined residues from 2 to 98 represent homology to a Thioredoxin-like fold. Residues in bold represent the C-type lectin carbohydrate recognition domain (residues 78-213). The # above residues 185, 189, 191, 194, 196-99 and 202-204 indicates conserved carbohydrate ligand binding sites.

#### 4.4.2 Expression and purification of a soluble *r22C03* protein by using *E. coli* expression system

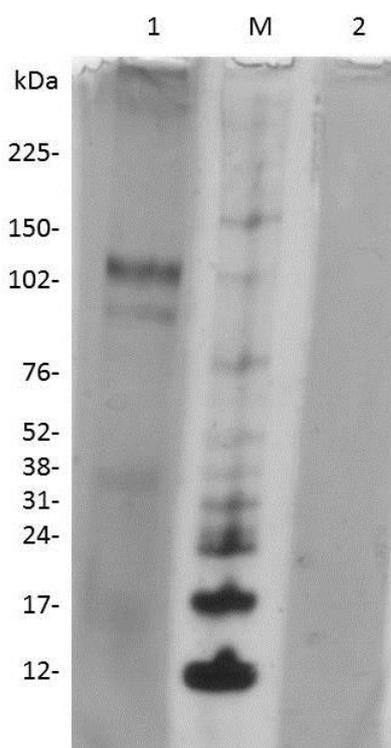
The expressed *r22C03* protein had a molecular mass of about 17 kDa (Figure 4.2, lanes 4-7), which was in accordance with the predicted size. The protein was expressed in inclusion bodies inside the transformed cells, which were solubilised by the denaturing lysis buffer containing 8 M urea. Different fractions obtained from the Ni-NTA column were pooled to obtain the final product (Figure 4.2, Lanes 5, 6 and 7). The protein was then renatured in solution dialysis and the total amount of soluble *r22C03* was close to 2 mg/mL.



**Figure 4.2** Recombinant fusion protein *r22C03* (arrow) solubilised from inclusion bodies and purified using a Ni-NTA Resin. In silver stained SDS-PAGE, the original solubilised *r22C03* before the purification with the Ni-NTA resin could be observed (2), as well as the fraction that did not bind to the resin (3). Eluates after a 20mM imidazole wash (4), 50 mM imidazole wash (5) 100 mM imidazole wash (6) and 200 mM imidazole wash (7) are shown. The last three eluates (5, 6 and 7) were pooled and used as the final purified protein. Molecular weight markers are shown in (1).

#### 4.4.3 *N. perurans* antigens

The antigens obtained from *N. perurans*, after freeze-thaw cycles and sonication, were characterised using SDS-PAGE. *N. perurans* antigens had a concentration close to 200 µg/mL, and therefore approximately 1.3 µg were loaded per lane in gels. When the samples were reduced, the amoebae antigens showed several protein bands: a broad smear of molecular weight larger than 225 kDa, a band of approximately 110 kDa and another close to 90 kDa, a slight band of around 35 kDa and a very slight smear close to 17 kDa (Figure 4.3). In contrast, the non-reduced samples did not show any band pattern.



**Figure 4.3** Silver stained SDS-PAGE gel of *Neoparamoeba perurans* protein lysate. Proteins were obtained through freezing and sonication, run on a 10% SDS-PAGE gel and visualised using silver staining. Reduced (1) and non-reduced (2) antigens are shown. M: molecular weight markers.

#### 4.4.4 Systemic and mucosal antibody levels against *r22C03*

All antibody values were expressed as units above the antibody levels of week 0, which were given a value of 0 units. There was a significant difference in the anti-*r22C03* antibodies levels in serum over time ( $F=16.952$ ,  $df\ 4$ ,  $P=0.002$ ). Serum antibody levels against the immunogen remain close to 0 units for the first four weeks of the experiment, before the booster immunisation was given (Figure 4.4). When serum was obtained 4 weeks following booster immunisation, at week 8, antibody levels in serum against the recombinant protein had increased significantly to over 150 units in average (Figure 4.4). Even though the serum antibody levels remained high for the following four weeks when compared to the initial levels, the average antibody levels of serum obtained during week 10 after immunisation were lower, even though not significantly, than the mean level in serum obtained during week 8 and 12 (Figure 4.4).

In the case of mucus, the antibody levels against *r22C03* increased earlier when corrected for the level of protein in the skin mucus. There was a significant increase ( $F=18.409$ ,  $df\ 4$ ,  $P=0.001$ ) in the mean antibody levels in the skin mucus of salmon after the priming immunisation, showing mean antibody levels close to 50 units four weeks after (Figure 4.4). The increase in antibody levels persisted for other 4 weeks after booster immunisation, and then the average antibody levels of skin mucus against *r22C03* declined significantly (Figure 4.4).

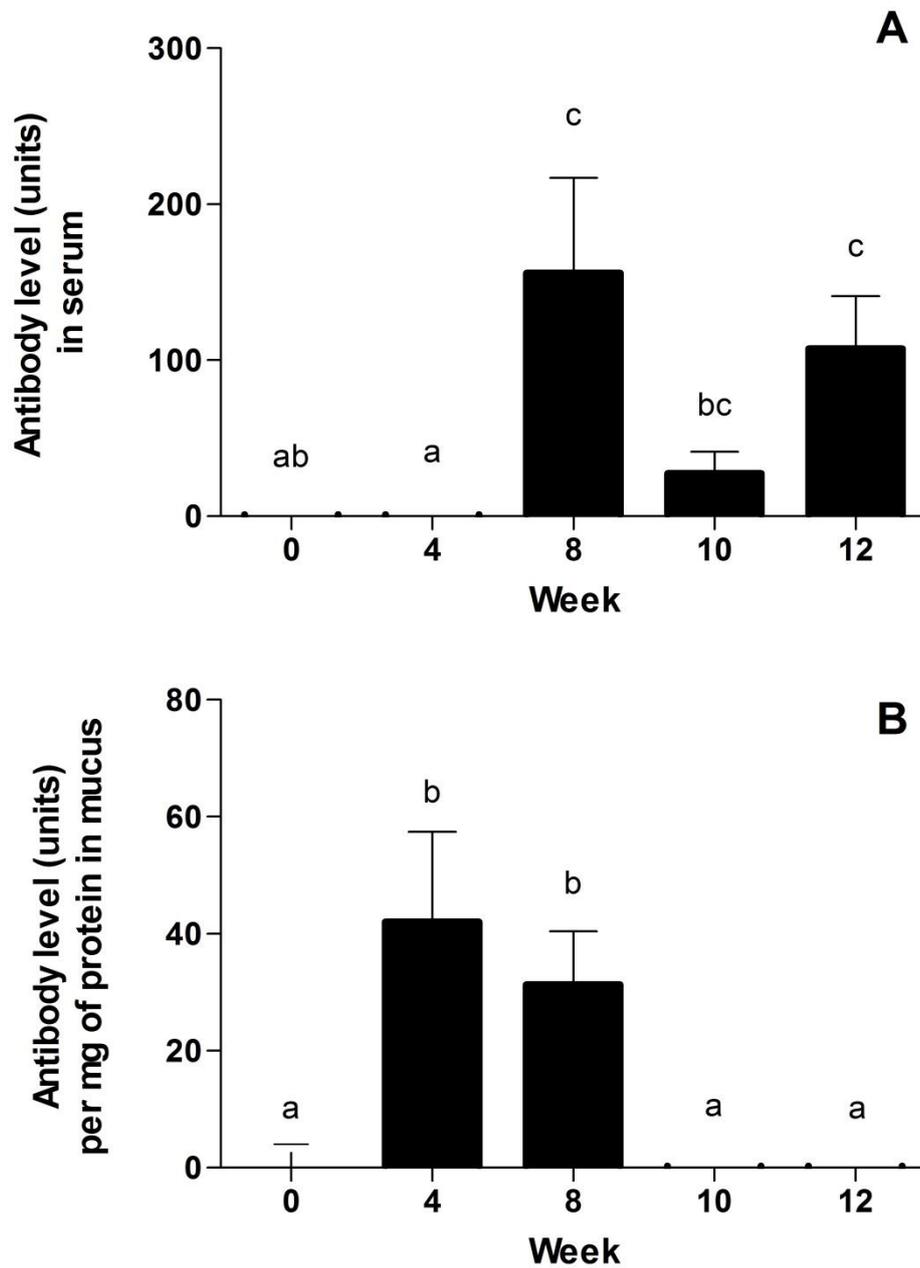


Figure 4.4 Antibody levels (units) against *r22C03* in serum (A) or per gram of protein in skin mucus (B) of Atlantic salmon (*Salmo salar*). Fish were immunised initially with *r22C03* and given a booster immunisation 4 weeks after. Antibody levels were measured by ELISA. Groups labelled with different letters are significantly different of one another by one-way ANOVA ( $P < 0.05$ ).

**4.4.5 Systemic and mucosal antibody levels against *N. perurans* antigens**

Similarly to the response against to r22C03, but in considerably lower magnitude, the antibody levels in serum of Atlantic salmon against amoebae increased significantly 8 weeks after the priming immunisation ( $F=13.723$ ,  $df$  4,  $P=0.008$ ) and after the fish have received the booster immunisation (Figure 4.5). This increase in average antibody levels was maintained in serum obtained 10 and 12 weeks after the initial immunisation, even though the antibody levels against the amoebae antigen dropped, even though not significantly, in serum obtained at week 10.

As in the case of antibodies against the recombinant protein, the antibody levels against *N. perurans* presented a significant increase in skin mucus of Atlantic salmon ( $F=19.070$ ,  $df$  4,  $P=0.001$ ) 4 and 8 weeks after the priming immunisation (Figure 4.5), reaching more than 40 units and close to 30 units, respectively. In the skin mucus samples obtained later during the experiments (weeks 10 and 12), the antibody levels dropped significantly (Figure 4.5), to levels similar to those obtained before immunisation. As opposed to what was observed for the response against r22C03, where the antibody levels were considerably higher in serum than in mucus, antibody levels against *N. perurans* antigens were slightly higher in mucus than in serum.

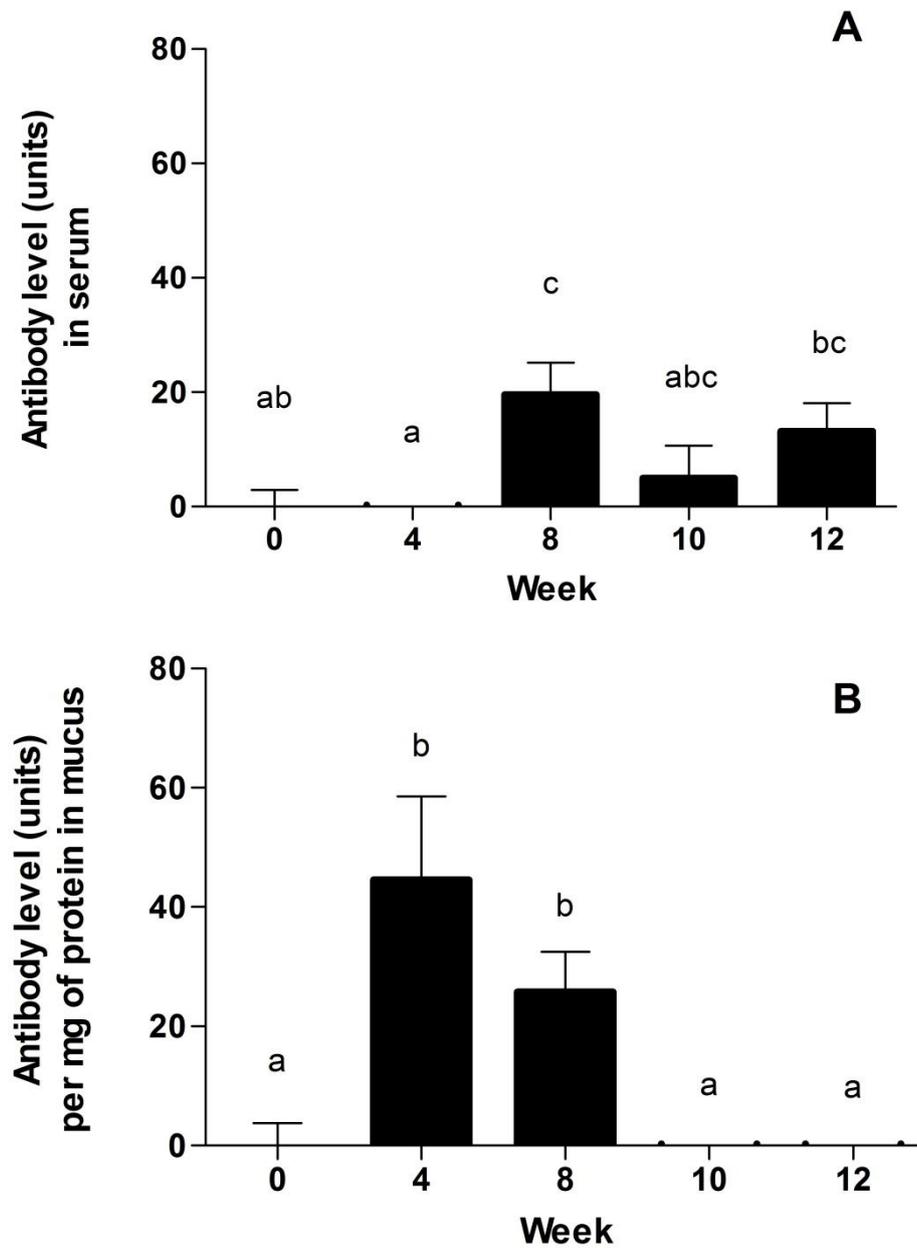
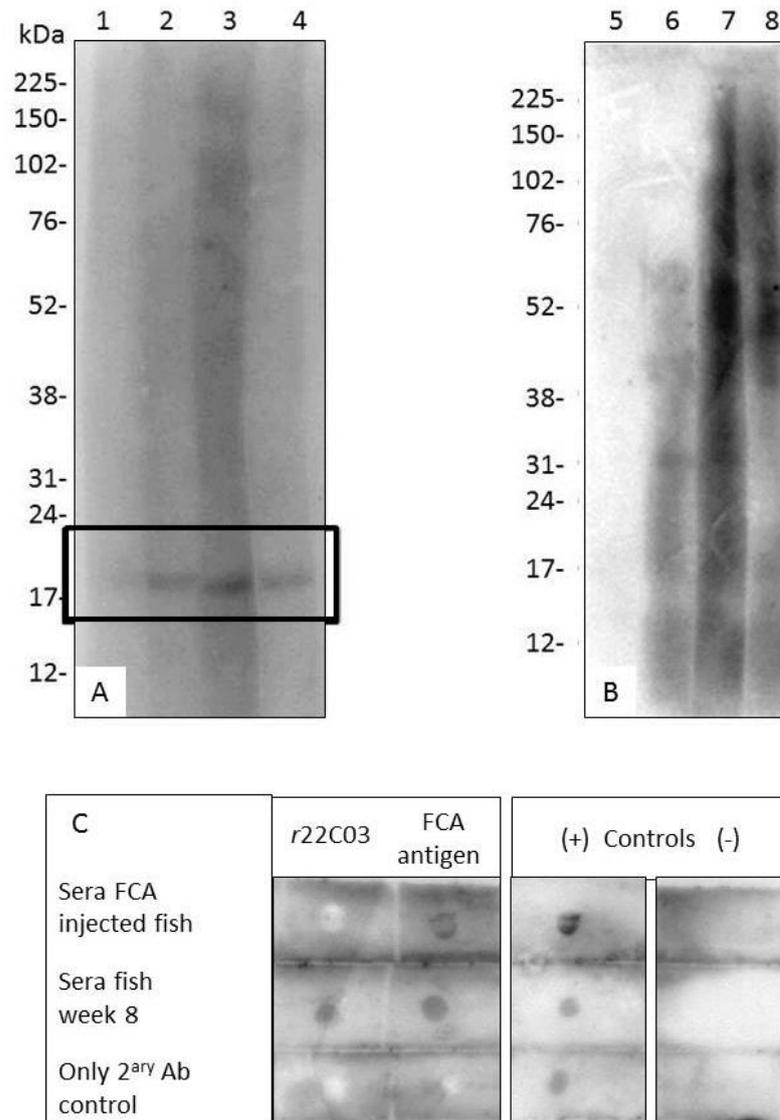


Figure 4.5 Antibody levels (units) against antigens of *Neoparamoeba perurans* in serum (A) or per gram of protein in skin mucus (B) of Atlantic salmon (*Salmo salar*). Fish were immunised initially with *r22C03* and given a booster immunisation 4 weeks after. Antibody levels were measured by ELISA. Groups labelled with different letters are significantly different of one another by one-way ANOVA ( $P < 0.05$ ).

**4.4.6 WB and dot blots**

To corroborate the results obtained with the ELISA, both *r22C03* and *N. perurans* antigens were separated using SDS-PAGE and probed with the different serum and skin mucus samples. Antibodies in serum of fish immunised with *r22C03* bound to a band of approximately 17 kDa, when serum was obtained at 8, 10 and 12 weeks post primary immunisation. However, no antibodies bound to this band in serum samples taken prior to the immunisation (Figure 4.6, A). In contrast, the serum antibodies showed a completely different binding pattern against the amoebae antigens (Figure 4.6, B). A broad molecular range smear could be observed in the WB probed with the serum from the immunised fish in samples obtained 8, 10 and 12 weeks post initial immunisation, but not in the serum of fish sampled before immunisation (Figure 4.6, B). When these membranes were subjected to periodate oxidation, no response was observed after probing with serum from immunised fish. Dot blots performed with sera from salmon injected with FCA and boosted with FIA 5 weeks later (n=4), which were part of another experiment, showed that antibodies in sera from these fish only reacted against the FCA antigen and not against the *r22C03* (Figure 4.6, C).

Administration of *r22C03* induces immune responses against *N. perurans*

**Figure 4.6** Binding of serum antibodies from fish immunised with *r22C03* produced distinctly different profiles against the recombinant protein and the whole *Neoparamoeba perurans* antigens. (A) Anti-*r22C03* antibodies in pooled serum of fish immunised 8, 10 and 12 weeks prior with *r22C03* (n=4-5), reacted to a band of approximately 17 kDa (lanes 2, 3, and 4), but not pooled serum of fish before immunisation (lane 1, n=5). (B) In contrast, binding of serum antibodies of fish immunised with *r22C03* 8, 10 and 12 weeks prior to sampling (n=4-5), produced a smear across a broad molecular range against amoebae antigens (lanes 6, 7 and 8 respectively). Serum obtained from fish before immunisation with *r22C03* did not react against *N. perurans* antigens (lane 5, n=5). Fish were held in freshwater and therefore were AGD-naïve. (C) Sera from fish injected with FCA followed by a booster with FIA 5 weeks later did not show antibody binding to *r22C03* (n=4), but antibodies from these samples did bind to FCA antigen. (+) control was a blot of serum detected only with the secondary antibody. (-) control blot of PBS only probed with samples and secondary antibody.

In the case of mucus, attempts to carry out a WB were unsuccessful, probably in association with lower antibody levels in this sample, as it has been demonstrated in similar species [130]. A dot blot was used to test the specificity of antibodies in the mucus samples. As can be observed in Figure 4.7, the antibodies in the mucus of fish immunised with the *r22C03* reacted clearly against the recombinant protein blot (lane 1, row B) but only very weakly with the blot made of *N. perurans* antigens (lane 1, row C). In contrast, antibodies in mucus obtained from fish before immunisation with *r22C03* did not show any response with either of the antigens (lane 2, rows B and C).

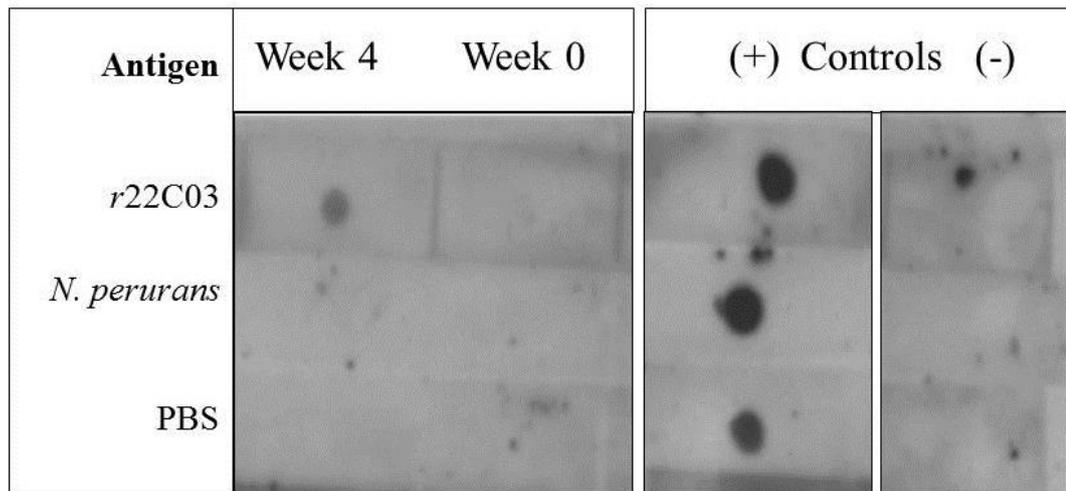
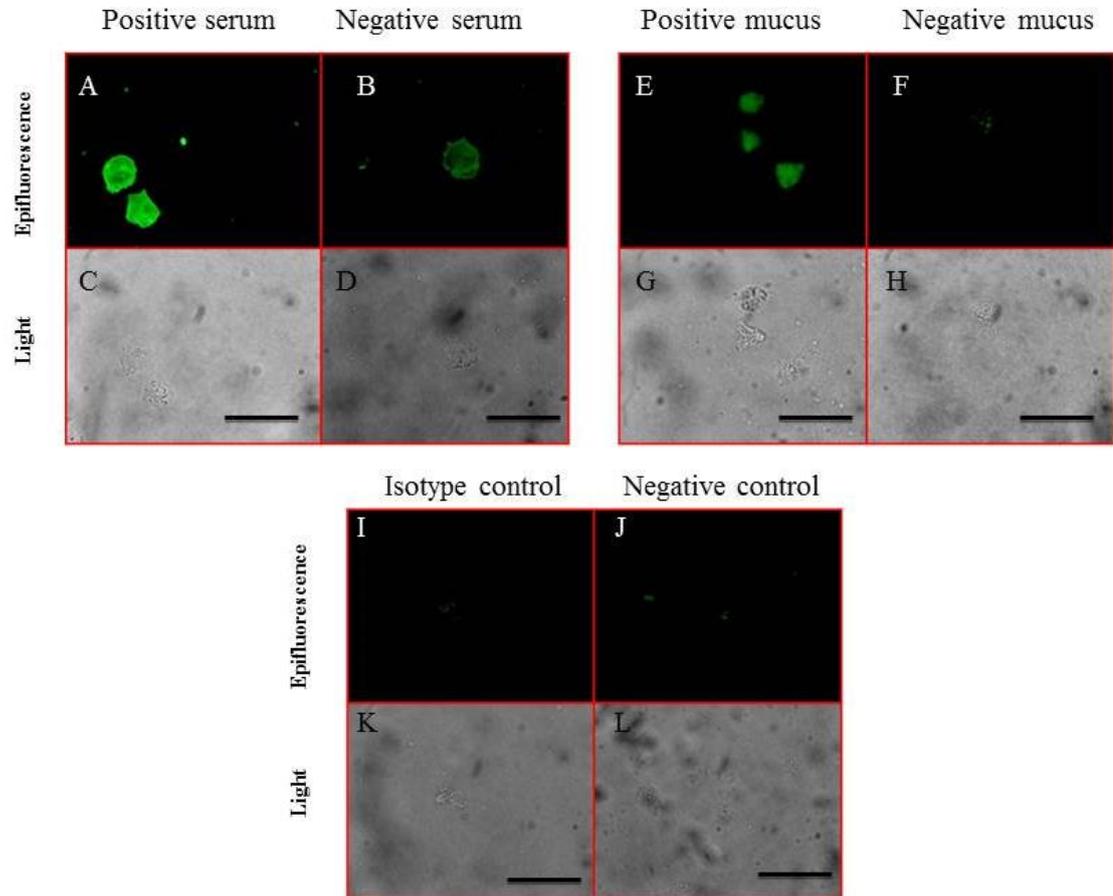


Figure 4.7 Binding of skin mucus antibodies from fish immunised with *r22C03* was strong against the recombinant protein but very weak against the whole *Neoparamoeba perurans* antigens. Anti-*r22C03* antibodies in skin mucus of fish immunised with the recombinant protein 4 weeks prior to sampling, reacted to a blot of recombinant protein *r22C03*, but only very weakly to the blot containing *N. perurans* antigens, these fish had showed the highest absorbance through ELISA. In contrast, skin mucus from fish before immunisation (Week 0) did not react against the recombinant protein *r22C03* or to *N. perurans* antigens. A negative control blotted and probed only with PBS was included. Antigens were diluted in PBS and applied to PVDF membranes. Diluted serum ((+) Control) was applied to the membrane as a positive control to test the secondary mAb; additionally a negative control which was only a PBS blot was also probed with the samples ((-) Control). Serum was pooled from all fish sampled on each date. Fish were held in freshwater and therefore were AGD-naïve.

**4.4.7 Immunocytochemistry**

Serum antibodies to *r22C03* present in fish 8 weeks post immunisation bound *N. perurans* clone 4 cells and generated a high fluorescence intensity (Figure 4.8, A). However, antibodies in serum of fish sampled before immunisation presented some fluorescence background (Figure 4.8, B) but this was lower than that observed in the positive serum samples.

In the case of mucus, antibodies in negative cutaneous mucus - obtained from fish before immunisation with the recombinant protein - did not bind to *N. perurans* cells (Figure 4.8, F). Antibodies in positive cutaneous mucus (4 weeks post-immunisation) did bind to the parasite, generating a low fluorescence (Figure 4.8, E).



**Figure 4.8** Anti-*r22C03* antibodies produced in serum and mucus of immunised Atlantic salmon bind to a cell surface antigen on fixed *Neoparamoeba perurans* trophozoites. *N. perurans* clone 4 trophozoites were fixed and probed with positive salmon serum sampled 8 weeks after immunisation (A and C), with positive skin mucus sampled 4 weeks post immunisation (E and G), negative salmon serum (B and D) and negative skin mucus (F and H) collected from salmon before immunisation. Serum from Southern bluefin tuna (*Thunnus macoyii*) was used as an isotype control (I and K). Cells probed with only PBS (J and L) were used as negative and background control. Positive salmon serum was pooled from 5 fish immunised with *r22C03* 8 weeks prior. Positive skin mucus pooled from 5 fish sampled 4 weeks after immunisation with *r22C03*. Negative serum and skin mucus was obtained from 5 salmon before immunised. All salmon were maintained in freshwater and were therefore AGD-naïve. Scale = 50  $\mu\text{m}$ .

## 4.5 DISCUSSION

Injection of the recombinant protein *r22C03* - a putative attachment factor of *N. perurans* - in conjunction with FCA, was able to induce a systemic and mucosal IgM response in Atlantic salmon against this particular antigen. Moreover, the systemic and mucosal antibodies produced by this immunisation, were able to bind sonicated antigens in ELISA and the surface of *N. perurans*, the causative agent of AGD.

Serum IgM responses against *r22C03* and against the sonicated antigens of *N. perurans* were observed 8 weeks post initial immunisation (4 weeks after the booster immunisation) by ELISA and were maintained until the end of the experiment, 12 weeks post initial immunisation. Systemic antibody responses in salmonids have been observed after similar time intervals following immunisation with model hapten-antigens [170, 175, 180] and proteins [115], therefore the kinetics of the systemic antibody response in the present study was in agreement with previous results.

Even though systemic antibodies against amoebic antigens have been shown following natural AGD infection [23, 114, 117] and after vaccination with amoebic antigens [135], these initial studies failed to demonstrate the presence of mucosal antibodies against the parasite. More recently, the presence of antibodies against amoebic antigens in skin mucus of Atlantic salmon was shown for the first time [115]. A high molecular weight antigen, which represented 15-19% of the total protein of a soluble extract of the amoeba, was identified in the latter study. Fish were immunised via i.p. with this antigen, in conjunction with FCA, and a low but significant level of antibodies was detected in skin mucus, with 60% of fish immunised with the high molecular weight antigen showing a specific mucosal response 105 d post immunisation.

In the present report, antibodies produced in skin mucus against both, *r22C03* and *N. perurans* antigens, showed only a temporary increase in levels measured by ELISA, with IgM levels peaking 4 weeks after the initial immunisation. Interestingly, this peak was earlier than the peak observed in the serum IgM, and moreover, the mucosal IgM response returned to levels close to those detected before the immunisation after only 8 weeks. This is in agreement with previous findings, which

indicate that mucosal responses in fish might develop separately from systemic responses. It has been shown that, in fish immunised with a hapten carrier antigen via i.p. injection, antibody levels in skin mucus can peak earlier and for a shorter period of time than systemic antibody levels, suggesting that antibodies may be produced independently on each site [175]. Additionally, it has been reported in yellow croaker (*Pseudociaena crocea*) that following i.p. injection of inactivated bacteria, systemic and mucosal responses were induced at the same time, and not sequentially as expected if the mucosal response was dependent on the systemic compartment [201]. In the present study, it is unclear why the antibody levels were not detectable in serum before mucus; however, previous studies have demonstrated how mucosal antibodies could peak earlier than systemic antibodies following i.p. injection [175], and these authors pointed out to the possibility of a local secretory immune system involved in local antibody production [220]. The length of the observed response in the present study could also have implications in the duration of the protection generated by *r22C03* against the disease, since the mucosal antibody levels dropped after only a few weeks post immunisation and booster.

In order to characterise the epitopes to which the Atlantic salmon IgM response was directed, the serum samples were used to probe WB; and mucus samples were used to probe dot blots of both, *r22C03* and *N. perurans* antigens. In the case of the serum WB, we observed a faint but specific response against *r22C03*, which was not observed in serum samples obtained before the immunisation. However, when the same serum samples were used to probe WB of amoebic antigens, a broad molecular smear was observed with samples obtained from immunised fish. Recent studies have shown two characteristic binding profiles to amoebic antigens in Atlantic salmon serum: a smear across a broad molecular mass range, which is cleared by periodate oxidation, suggesting a response to carbohydrate epitopes; and a second binding profile showing binding to bands >200 kDa, which are not cleared by oxidation and therefore indicative of putative peptide epitopes [114, 116, 117, 221]. After periodate oxidation, the WB in this study did not show a particular response in serum against the putative attachment protein or any of the amoebic antigens observed in the SDS-PAGE under reducing conditions, suggesting that the amount of this attachment factor in the *N. perurans* samples was very low, and therefore not

detectable by the level of antibodies present in serum. For the dot blots, IgM present in the mucus was once again able to bind faintly to r22C03, but not to the amoebic antigens, confirming perhaps the low amount of antigen present in these samples and demonstrating also the lower antibody concentration present in mucus [130], as opposed to serum antibody levels.

Interestingly, this is the first report in Atlantic salmon of mucosal IgM binding the surface of formalin-fixed *N. perurans*; after the primary immunisation of this fish with the recombinant protein. Antibodies present in mucosal surfaces of channel catfish immune to *Ichthyophthirius multifiliis* are not only able to bind the surface of the protozoan parasite, but also immobilise and reduce the infectivity of the theronts in freshwater [222]. In the case of *N. perurans*, a study conducted using monoclonal anti-*Entamoeba histolytica* Gal/GalNAc antibody, which binds the surface of the parasite, did not find antibodies on the cell surface of the amoeba after cells were suspended in seawater for 1 h [221], but it was not clear if the binding was disrupted by seawater, or if the amoeba may have internalised bound antibodies. It has been proposed that the function of Atlantic salmon antibodies present in external surfaces may be affected by the extreme osmolarity of the marine environment [223]. However, in the present study all fish were kept under freshwater conditions and further characterisation and testing of antibodies found in mucus of fish under seawater conditions are currently being conducted.

Recently, the pivotal role that IgT, but not IgM, has in mucosal immunology has been demonstrated [97, 224]. IgT<sup>+</sup> cells represent the main the B-cell subset in the gut as well as in the gill of rainbow trout. These studies showed that fish surviving an infection induced by bacteria in the gill and parasites in the gill and intestine presented an increase in IgT levels in the gill and intestinal mucus, and the IgT present in gill mucus was capable of binding the pathogens. Unfortunately, the use of the same antibody in our laboratory has proven unsuccessful in recognising IgT in Atlantic salmon and therefore the levels of this Ig isotype could not be measured in the present study. The development of an antibody recognising IgT in Atlantic salmon is warranted and essential for the future study of mucosal responses in this species.

#### **4.6 ACKNOWLEDGMENTS**

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## CHAPTER 5.

# VACCINATION WITH RECOMBINANT PROTEIN (*r22C03*), A PUTATIVE ATTACHMENT FACTOR OF *Neoparamoeba perurans*, AGAINST AGD IN ATLANTIC SALMON (*Salmo salar*) AND IMPLICATIONS OF A CO-INFECTION WITH *Yersinia ruckeri*

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### KEYWORDS

Amoebic gill disease; vaccine; *Salmo salar*; *r22C03*, co-infection, yersiniosis.

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## 5.1 ABSTRACT

Amoebic gill disease (AGD) affects salmonids during the marine grow-out phase in the Tasmanian industry and in other major salmonid producing countries. The bacterial condition yersiniosis can cause high levels of mortality in Atlantic salmon grown in Tasmania post-transfer to seawater, in addition to freshwater outbreaks. A recombinant protein named *r22C03*, previously described as a mannose-binding protein-like (MBP-like) similar to attachment factors of other amoebae, was tested as a vaccine candidate against AGD. Fish were immunised with *r22C03* combined with FCA via i.p. injection, and given a booster five weeks later by either i.p. injection (RP group) or by a dip-immersion (mRP). Fish were challenged twice with *N. perurans*: the initial challenge 16 weeks after primary immunisation was terminated due to presence of ulcerative lesions in the skin of salmon; the second challenge was carried out after five weeks of treatment with oxytetracycline. Antibody levels in serum, in skin mucus and in supernatant from skin and gill explants were measured by ELISA; average size of AGD lesion was recorded from histology sections and survival curves were obtained for the second challenge. Before challenge, *r22C03* induced antibody responses in serum and explants with both vaccination strategies. At the end of the challenge, levels of antibodies were lower than before challenge irrespective of treatment. Both vaccinated groups presented increased serum antibody responses, while only mRP presented antibody responses in skin mucus, and no significant antibody responses were measured in the explants. Antibodies did not confer protection to *N. perurans* infection, as no difference was observed in the survival curves of the vaccinated and control groups, and there was no effect on the gill lesions size. A concurrent infection with *Y. ruckeri* was detected by real-time qPCR in serum of a large proportion of moribund and survivor fish, which probably represented more closely infection patterns observed in commercial settings. However, it could have interfered with the survival results and with the ability of the fish to respond to the amoebae infection.

## 5.2 INTRODUCTION

Tasmanian salmon industry has rapidly grown since its establishment in the mid-1980s, becoming Australia's most valuable individual seafood industry, valued at \$513 million in 2011-12 and producing in excess of 40,000 tonnes [4]. For this high value industry, infectious diseases pose a constant threat to economically successful production.

Amoebic gill disease (AGD) is the main disease affecting the Tasmanian salmonid industry. However, this condition has also been described in other major salmon and trout producing countries [22, 34, 37, 41]. AGD is caused by *Neoparamoeba perurans* [46], a free living and opportunistically parasitic amoeba. The parasite is usually found associated with lesions in the gills [46, 50], which are microscopically characterised by hyperplasia of epithelial cells, lamellar fusion and a restricted response of immune cells [55], and appear grossly as raised white patches on the gills [57]. Outbreaks of the disease appear during the marine grow-out phase and in particular, during the summer months, when water temperature rises [26, 55]. Currently the only treatment option widely used by the Tasmanian industry is freshwater bathing [131]. This practice, which is labour-intensive and stressful for the fish, represents a large percentage of the total production costs, and numbers of gill associated amoebae have been shown to return to initial levels within 10 d of treatment [66]. Decreased feeding rates have also been associated with AGD, and if fish are left untreated, mortalities of up to 50% have been reported in sea cages in Tasmania [20, 114], adding to the AGD related production costs.

Another important disease affecting Atlantic salmon (*Salmo salar*) in Tasmania is yersiniosis. This condition can cause high levels of mortality, as reported in 2007, when approximately half a million juvenile Atlantic salmon died in a hatchery over the course of a few months [17]. In Tasmania, yersiniosis is caused by *Yersinia ruckeri*, the same pathogen that causes enteric red mouth disease (ERM), a severe condition affecting rainbow trout (*Oncorhynchus mykiss*) in the northern hemisphere [16]. However, the Hagerman strain, which is linked to ERM presentation in rainbow trout in the northern hemisphere, is exotic to Tasmania, where only two biotypes of the bacteria are known to occur: serotype O1b, biotype 1 and serotype O1, non-O1b,

biotype 2 [16]. Yersiniosis may also affect Atlantic salmon smolts, commonly 3-6 weeks after the introduction to seawater, causing unspecific external signs which are characteristic of bacteraemia in fish [16]. Increased mortalities, low feeding rates, marked unilateral or bilateral exophthalmos, congestion of the eye's iris and in the base of pectoral and pelvic fins, distended vent and small areas of muscle liquefaction resulting in skin lesions are usually observed [16]. A key factor in the dynamic of the infection, is the establishment of an asymptomatic carrier state in infected fish [18]. The bacteria can remain viable in the intestinal tract and head kidney of the carriers for months, and a cyclical intestinal shedding pattern can develop which can be exacerbated during period of stress, causing regular reinfection and mortality in the population [18].

Vaccination is the best approach the aquaculture industry can utilise to manage and control diseases, and the use of effective vaccines has probably been one of the key factors contributing to the success of intensive salmonid culture [133]. Injection of antigens in conjunction with oil adjuvants have proven to be the most successful approach in fish vaccines to date [133]. Vaccine strategies against parasites remain relatively unsuccessful, even though a large amount of time and resources have been spent on their development [134]. One reason for this relative failure could be the chronic characteristics of parasitic conditions, which often produce ineffective immune responses in the host [134]. Additionally, even though significant progress has been made in the characterisation of fish immune mechanisms and pathways, there are still significant gaps in the knowledge of these functions, in particular regarding toll –like receptors and their ligands and different helper T-cell subsets, and these gaps are different depending on the species. Integrated studies of mechanisms involved in protection are necessary in order to identify optimum antigen candidates, formulation and adjuvants [82, 84, 95, 225].

Early studies of AGD gave a foundation for the possible development of vaccines, since it was shown that successive infections provided fish with certain resistance to the disease, as the number of lesions present in the gills was lower and positive antibody responses were observed in serum samples [23]. Different vaccination approaches have been pursued against AGD in the past, with little success [51, 115,

135-137], and even though some of these studies potentially used the actual causative agent of AGD, this work was performed before it was identified [46]. Due to the high costs associated with AGD treatment, the development of a vaccine remains a high priority of the industry.

In this study, we attempted to test a recombinant protein as potential vaccine candidate, in a laboratory based *N. perurans* challenge. A mannose-binding protein-like (MBP-like) factor, similar to attachment factors of other amoebae species, was identified in the transcriptome of *N. perurans*, and a recombinant fusion MBP-like protein, named *r22C03*, was generated [226]. This particular protein induced the production of systemic and mucosal antibodies capable of binding the surface of *N. perurans* [226]. On this basis, it was assumed that it might be possible to block this putative attachment factor using functional antibodies present in mucosal surfaces, and reduce the severity of AGD. However, it is not clear if antibodies against *N. perurans* offer significant protection (as reviewed by Ref. [103]). In our study, fish were immunised with the recombinant protein using two different vaccination strategies and then challenged with the parasite. A strong antibody response against the recombinant protein was observed in serum and mucosal surfaces of vaccinated salmon. However, a concurrent infection with *Y. ruckeri* was present during the experiment. This could represent more closely the situation observed on commercial farms; nevertheless survival results obtained after the parasite challenge have to be examined with caution in the context of efficacy of the vaccination against *N. perurans*.

## **5.3 MATERIALS AND METHODS**

### **5.3.1 Fish**

Atlantic salmon out of season smolts (n=870) with average body weight 111.65 g (S.D. 26.23) and average body length 219.13 (S.D. 19.69) were obtained from a commercial hatchery and maintained at the Aquaculture Centre at the University of Tasmania. Fish were kept in four individual 4,000 L recirculating systems. For the first 13 weeks, salmon were maintained in freshwater at 15°C for acclimation. Water quality was checked daily, temperature ranged between 15.0-16.5°C (Table 5.1). Fish were maintained under an 18:6 h light/dark regime and fed 2% b.w. divided into 2

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daily rations. This project was approved under the University of Tasmania Animal Ethics Committee approval number A0012145.

**Table 5.1 Timeline for immunisations, challenges and sample collection for the investigation of immunity and protection against AGD induced by recombinant protein *r22C03*.**

| Week  | Treatment  | Salinity (ppt) | T (°C) | Samples taken and procedures                            |
|-------|--|----------------|--------|---|
| 0     | Initial immunisation                             | 0              | 15     | Skin mucus, blood, tissue explants (4 fish per group)   |
| 5     | Booster immunisation and sampling                | 0              | 15     | Skin mucus, blood, tissue explants (4 fish per group)   |
| 9     | Sampling   | 0              | 15     | Skin mucus, blood, tissue explants (4 fish per group)   |
| 12    | Sampling   | 0              | 15     | Skin mucus, blood, tissue explants (4 fish per group)   |
| 14    | Seawater acclimation                             | 25             | 16     | -   |
| 16    | First challenge with <i>N. perurans</i>          | 35             | 16.5   | -   |
| 17    | Freshwater bath (1 d)                            | 0              | 15     | Whole fish for DPIPWE diagnostic                        |
| 19-20 | Antibiotic treatment                             | 20             | 15     | Challenge tank 3 removed from experiment                |
| 20    | Seawater acclimation                             | 25             | 16     | -   |
| 21    | Second challenge with <i>N. perurans</i> (Day 0) | 35             | 16.5   | Blood from moribunds, throughout challenge              |
| 29    | Termination of challenge (Day 58)                | 35             | 16.5   | Skin mucus, blood, tissue explants (all surviving fish) |

### 5.3.2 Immunisation

Fish were immunised with the previously described recombinant protein *r22C03* [226], via i.p. injection initially, and given a booster by either i.p. injection (RP group) or by exposing the fish to the recombinant protein through a dip (mRP) five weeks later (Table 5.2). For the initial i.p. injection, the antigen was emulsified with Freund's complete adjuvant (FCA, Sigma Aldrich, Sydney, NSW, Australia). Fish were given a total of 250 µg of antigen, in 200 µL of a solution consisting of a 2:1 ratio of antigen and FCA. The injection booster was administered at the same concentration but with Freund's incomplete adjuvant (FIA). For the dip booster, fish were immersed for 1 min in a solution containing 50 mg/L of the recombinant protein dissolved in PBS, following concentrations from our previous study [152]. Control treatments included: fish injected with PBS emulsified with FCA and then PBS and FIA (ADJ), and fish injected twice only with PBS (BF), for the RP group; controls for the mRP group included a group injected with PBS and FCA and then

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given a dip in PBS (mADJ), and a group injected initially with PBS and subsequently given a dip in PBS (mBF). PBS only controls were added as handling control for the experiment, to test for potential effects of the adjuvants used. A group of fish was also allocated as an unvaccinated uninfected control (UU) in the uninfected tank (see section 5.3.3). To identify different treatments, fish were tattooed with alcian blue in their ventral dermis, as described elsewhere [152].

**Table 5.2 Experimental design for immunisations of Atlantic salmon (*Salmo salar*) with recombinant protein *r22C03*.**

| Treatment   | Delivery route          | Abbreviation | n    | Initial Dose and booster (week 5)                                      |
|---|-------------------------|--------------|------|--|
| <b><i>r22C03</i> + Freund's complete adjuvant (FCA)</b> | i.p. injection<br>x 2   | RP           | 35*3 | 250 µg (2:1 inocula with FCA†) in 200 µL                               |
| <b><i>r22C03</i> + FCA + dip booster</b>                | i.p. injection<br>+ dip | mRP          | 35*3 | 250 µg (2:1 inocula with FCA) in 200 µL; booster dip at 50 mg/L in PBS |
| <b>PBS + FCA</b>  | i.p. injection<br>x 2   | ADJ          | 35*3 | 200 µL (2:1 PBS with FCA†)   |
| <b>PBS + FCA + dip booster</b>                          | i.p. injection<br>+ dip | mADJ         | 35*3 | 200 µL (2:1 inocula with FCA); booster dip in PBS                      |
| <b>PBS only</b>   | i.p. injection<br>x 2   | BF           | 35*3 | 200 µL PBS   |
| <b>PBS only</b>   | i.p. injection<br>+ dip | mBF          | 35*3 | 200 µL PBS; booster dip in PBS   |
| <b>Unvaccinated uninfected control</b>                  | N/A                     | UU           | 30*1 | Not applicable   |

† Booster at week five was emulsified with Freund's incomplete adjuvant.

#### 5.3.3 *N. perurans* challenge

In preparation for the challenge, salinity and temperature were increased gradually over three weeks to full strength seawater (35 ppt) and 16.5°C. Fish were challenged twice with *N. perurans*: the first challenge which was conducted 16 weeks after the initial immunisation had to be terminated after one week of infection, due to the presence of ulcerative skin lesions in the flanks of fish. The fish were freshwater bathed and brought back to 20 ppt salinity water and 15.5°C for two weeks, as described previously [131].

At this time, a few moribund fish were taken for full post-mortem examination to the Department of Primary Industries, Parks, Water and Environment (DPIPWE). The results of the post-mortem examination revealed the presence of mixed *Vibrio* sp. in skin and gills, which were consistent with normal marine flora. No significant dominant pathogens were isolated from internal organs, and the pathology report indicated a possible overgrowth of normal marine flora secondary to acclimation stressors.

Time and budget constraints prohibited the repetition of the experiment, and therefore a decision was made to treat the concurrent infection with antibiotics. Surviving fish received an antibiotic treatment of 0.83 g oxytetracycline per 10 kg of fish, mixed in with the feed for 15 d, as prescribed by the University of Tasmania's Animal Welfare Officer. A few mortalities were observed during this period, therefore the re-acclimation of fish to seawater was initiated once the mortalities ceased. Over the following two weeks, fish were brought back to full strength salinity and 16.5°C and the second challenge was initiated, which took place five weeks after the first challenge.

For both infections, *N. perurans* were harvested from an ongoing infection tank located at University of Tasmania, following the procedure previously described [151]. In brief, gill arches were obtained from fish and placed over petri dishes covered in seawater, where amoebae were left to attach. The plates were incubated at 18°C overnight with antibiotics (ampicillin at 9.6 µg/mL and oxolinic acid at 20 µg/mL). Amoebae were obtained in successive isolations (over 4 d and 3 d for the first and second challenges, respectively), counted and added to the tanks in 1 L volumes until the 500 amoebae/L dose was reached.

Four tanks were used for the first challenge: three replicates for challenge and one tank as a non-infection control. After the presentation of the ulcerative skin lesions in fish from all four tanks, one of the challenged tanks reached mortalities close to 68% and both vaccinated groups had less than 30% of the initial stock left. Therefore this tank (challenge tank 3) had to be removed from the trial (Table 5.1). For the second infection, two out of the three experimental tanks were challenged and the third tank, which was the same as in the initial challenge, was used as a non-infection control.

#### 5.3.4 Sampling procedure

Mucus and blood were obtained from four fish from each group at four times: at 0 (initial), 5, 9 and 12 weeks post immunisation and before the challenge. Additionally during the second infection, moribund fish in the tanks were also sampled, as well as every surviving individual when the challenge was terminated. Fish were anaesthetised using 1 mL clove oil diluted in 10 L of freshwater or seawater. Skin mucus was collected as previously described [226]. Blood (1 mL) was collected from the caudal vein and left to clot in a 1.5 mL microcentrifuge tube overnight at 4°C. Serum was obtained after centrifuging the samples at 1,500 x *g* for 10 min at 4°C. Mucus supernatant and serum were stored at -80°C.

A method developed by Xu and Klesius [184] to measure the cutaneous antibody response in tissue explants in catfish (*Ictalurus punctatus*) and modified to use in Atlantic salmon [152] was employed in the present study for all fish sampled. In brief, fish were perfused with heparinised saline through the bulbous arteriosus, to rinse all blood from organs, and then the right half of the gill basket and skin were collected, rinsed in Hank's balanced salt solution containing chlorhexidine and transferred immediately to L-15 media (Sigma Aldrich) supplemented with bovine foetal serum, Glutamax<sup>TM</sup> and PSN antibiotic mix (Gibco, Grand island, USA). Organ samples were weighed and placed in individual wells of a tissue culture plate and incubated at 18°C for 72 h in supplemented L-15. Gill and skin explants media from individual wells were removed, centrifuged, supernatant collected and stored at -80°C.

The second left gill arch was obtained from all surviving fish at the end of the challenge; it was rinsed in filtered seawater, fixed in seawater Davidson's fixative for 24 h, transferred to 70% ethanol and processed under routine histological techniques.

#### 5.3.5 Enzyme-linked immunosorbent assay (ELISA)

The coating antigen was the recombinant protein *r22C03*, described elsewhere [226]. Coating antigen and sample concentrations were optimised as previously described [152]. The optimum concentration for the antigen was set at 10 µg/mL. Serum dilution was optimal at 1:200, mucus at 1:2 and tissue explant supernatant at 1:5. A single-point dilution ELISA was run for all samples (in duplicate); a reference

positive standard curve was used in every plate, consisting of a pool of sera from fish presenting high antibody levels, sampled at week 12. The positive standard was used in triplicate two-fold dilutions (1:50 to 1:6,400) in each plate diluted in either PBS, in 1:2 mucus in PBS, or in 1:5 L-15, as the reference curve for the serum, mucus or tissue explant ELISA plates. Negative controls were included in duplicates at the same dilution as samples and included a pool of sera or mucus from fish not used during this experiment but kept in freshwater and therefore AGD-naïve; or L-15 media alone.

Plates were coated overnight at 4°C with the antigen, washed 3 x with low salt wash buffer (LSWB, 2.42 g Trisma base, 22.22 g NaCl, 0.5 mL Tween-20 in 1 L distilled water, pH 7.3) and non-specific sites blocked for 2 h with 250 µL/well of 2% casein (Sigma Aldrich) in TBS (10 mM Tris, 150 mM NaCl, pH7.5). After 3 washes with LSBW, diluted serum, mucus or supernatant were incubated for 1.5 h, followed by a mAb against salmon IgM heavy chain conjugated with HRP (Cedarlane, Burlington, Canada) for 1 h at 1:1,000 dilution. Samples and antibody were added at 100 µL/well in 0.3% casein TBS and incubated at 18°C. After each incubation, plates were washed 5 x with high salt wash buffer (2.42 g Trisma base, 29.22 g NaCl, 1 mL Tween-20 in 1 L distilled water, pH 7.7). Bound antibody was visualised by adding 100 µL TMB One Solution (Promega, Fitchburg, USA), for 10 min at 18°C and stopped with 2 M H<sub>2</sub>SO<sub>4</sub> solution (50 µL). Optical density (OD) readings were measured at 450 nm in a plate reader (Rainbow Thermo Tecan Group Ltd., Männedorf, Switzerland).

Samples ODs from all ELISA analyses were corrected by subtracting the mean OD of the negative controls in the same plate. ELISA results were then reported as levels of antibody units derived from the standard positive curve on each plate, considering a dilution of 1:50 of the positive control as 100 antibody units, 1:100 dilution as 50 antibody units and so on, to account for differences between plates as described previously [152]. Function calculations were done using the GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego CA, USA). Antibody unit values for mucus were expressed per mg of protein in the sample, which was measured with a Micro BCA<sup>TM</sup> Protein Assay Reagent Kit (Pierce, Scoresby, Australia).

### 5.3.6 Assessment of intensity of AGD infection

After gill sections were stained with H&E, they were viewed under a light microscope (Olympus BH2) at 400 x magnification. Individual filaments were assessed for the presence of hyperplastic AGD lesions and the percentage of affected filaments was calculated from the total number of filaments assessed in each gill arch. A filament was only counted when the central venous sinus was visible in at least two-thirds of its length [55]. Average size of lesion was then calculated from the positive filaments only and was defined as the number of interlamellar spaces affected by hyperplasia within each lesion in each gill filament. Only fish that survived the complete course of infection were assessed for intensity of infection, since they were exposed to the parasite for the same length of time (58 d).

### 5.3.7 Analysis of samples for co-infection

#### 5.3.7.1 DNA extractions

After the experiment was terminated, a total of 26 serum samples were analysed for co-infections; from moribund fish before (n=13) or after (n=13) the second amoebae challenge (between 30 and 56 d after *N. perurans* infection). Additionally, 30 samples were analysed from fish surviving until the end of the challenge, 58 d after the second *N. perurans* infection. Sera (100 µL) were obtained as outlined in section 5.3.4 and processed for DNA extraction. Sera were mixed with 900 µL of filter sterilised PBS, and potential bacteria precipitated by centrifugation at 16,000 x *g* for 30 min at 4°C. Supernatant was removed and cells were disrupted using a 1 x TE buffer with 0.3% SDS and 1% Triton-X 100. DNA was then precipitated from the supernatant by adding 7.5 M ammonium acetate, centrifugation at 16,000 x *g* for 10 min and addition of one volume of isopropanol. Samples were left overnight at 4°C and centrifuged another time. The nucleic acid pellet was then rinsed twice with 75% ethanol and resuspended in 30 µL 1 x TE buffer with 0.1% Triton X-100 at 55°C for 5 min.

#### 5.3.7.2 PCR detection

First, targeted PCR amplification of bacterial *rpoB* gene was carried in the extracted DNA from serum, to screen for the presence of any bacterial pathogen. Universal

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primers *rpoB*1698F, 5'-AACATCGGTTTGATCAAC-3' and *rpoB*2041R, 5'-CGTTGCATGTTGGTACCCAT-3' [227] were used. Each PCR reaction was carried in 20 µL volumes consisting of: 2 µL DNA template, 10 µL Sensifast+SYBR mix (Bioline, NSW, Australia), 500 nM of forward and reverse primers and 6 µL molecular grade water. Reactions were carried out in a C1000™ Thermal Cycler (Bio-Rad, NSW, Australia) and cycling conditions consisted of initial activation of DNA polymerase at 95°C for 3 min, and 35 cycles of 94°C for 10 s, 55°C for 30 s and 72°C for 5 s. PCR products were visualised in a 3% agarose gel stained with GelRed™ (Biotium, SA, Australia) run at 75 V for 1.5 h in TBE Buffer (Bioline). Positive controls were also run for DNA extraction and *rpoB* PCR reaction, including cultures of *Aeromonas hydrophila*, *Photobacterium damsela* subsp. *damsela*, *Vibrio alginolyticus*, *V. parahaemolyticus*. Extracted DNA from five positive samples were purified and concentrated using a QiaPrep spin miniprep kit (Qiagen, VIC, Australia), DNA quantified using a Qubit® 2.0 Fluorometer and Quant-iT dsDNA HS assay kit (Life Technologies, VIC, Australia) and submitted for direct PCR sequencing at Macrogen ([http://www.macrogen.com/eng/sequencing/sequence\\_main.jsp](http://www.macrogen.com/eng/sequencing/sequence_main.jsp)).

Extracted DNA was then used to detect the presence of *Y. ruckeri* in sera using a TaqMan probe approach. Real-time qPCR was performed using primers specific to the 16S rRNA gene of *Y. ruckeri* ATCC 29473 strain [16], YrF, 5'-AACCCAGATGGGATTAGCTAGTAA-3', and YrR 5'-GTTTCAGTGCTATTAACACTTAACCC-3', and the 5'HEX- 3'BHQ1-labelled TaqMan™ probe Yr\_HEX 5'-AGCCCACTGGAAGTGGAGACACGGTCC-3'. Real-time qPCR analyses were conducted on a CFX Connect Real-Time PCR Detection System (Bio-Rad). Each PCR reaction consisted of 10 µL volumes containing 2 µL DNA template, 5 µL of MyTaq™ HS mix, 400 nM of each forward and reverse primers, 100 nM of the probe and molecular grade water. Samples were analysed in duplicates with a (+) standard consisting of DNA extraction from *Y. ruckeri*. Real-time qPCR was run under the following conditions: initial DNA polymerase activation at 95°C for 3 min, then 45 cycles of 95°C for 5 s and 60°C for 30 s. Samples were considered as (+) if any amplification was observed.

### 5.3.8 Statistical analyses

To assess differences in serum, mucus and tissue explants antibody levels among groups, a one-way ANOVA was used, followed by a Bonferroni's test of planned comparisons, where vaccine groups were compared against each other and to their particular controls. For those samples obtained before challenge, this test was performed for each individual sampling time. Data had to be log (serum) or square-root (mucus and explants) transformed to meet the assumptions of ANOVA.

A one-way ANOVA was also used to test the differences among treatments when assessing the severity of AGD infection, both for percentage of affected filaments and for average lesion size. Percentage data were arcsine transformed to meet the ANOVA assumptions.

Survival curves were constructed using the Kaplan-Meier estimated survival probabilities. Vaccination and control groups were compared to each other using a log-rank test. Relative percent survival (RPS) for each of the vaccinated and challenged groups (compared to the negative controls) was calculated using the following formula:  $RPS = (1 - (\% \text{ vaccinated mortalities} / \% \text{ control mortalities})) \times 100$  [138].

To test the potential effect of the bacterial infection on the AGD pathogenesis, a correlation between the number of positive filaments or the lesion size and the bacterial load (as  $C_t$  values) and a correlation between average lesion size and bacterial load were carried out. Statistical analyses were performed in Graphpad Prism 5.01 (Graphpad) with a  $P < 0.05$  acknowledging significant results.

## 5.4 RESULTS

### 5.4.1 *r22C03* was able to induce systemic and mucosal antibody responses in both vaccination groups, but at different times before and after challenge

Before the AGD challenge, both vaccination treatment groups (RP and mRP) presented higher antibody levels than their controls in sera. Five and 12 weeks after the initial immunisation, sera of fish from RP group presented significantly higher average antibody levels than its controls ( $P < 0.05$ ), but this value was not different from the mRP mean sera antibody level. Nine weeks after the initial immunisation, only the mRP group presented an average sera antibody level that was significantly

higher ( $P<0.05$ ) than its control groups, while only two samples from the RP group had high antibody values and therefore there was a high variance within the group (Figure 5.1).

Antibody levels in mucus and explants were lower than those observed in sera. No significant differences ( $P>0.05$ ) were observed in the average antibody level between the vaccination groups and their controls at any time in mucus. However, both gill and skin explants presented significantly higher mean antibody levels in the mRP group when compared to its controls 12 weeks after the initial immunisation ( $P<0.05$ ) and additionally, skin explants showed the same difference nine weeks after the immunisation (Figure 5.1).

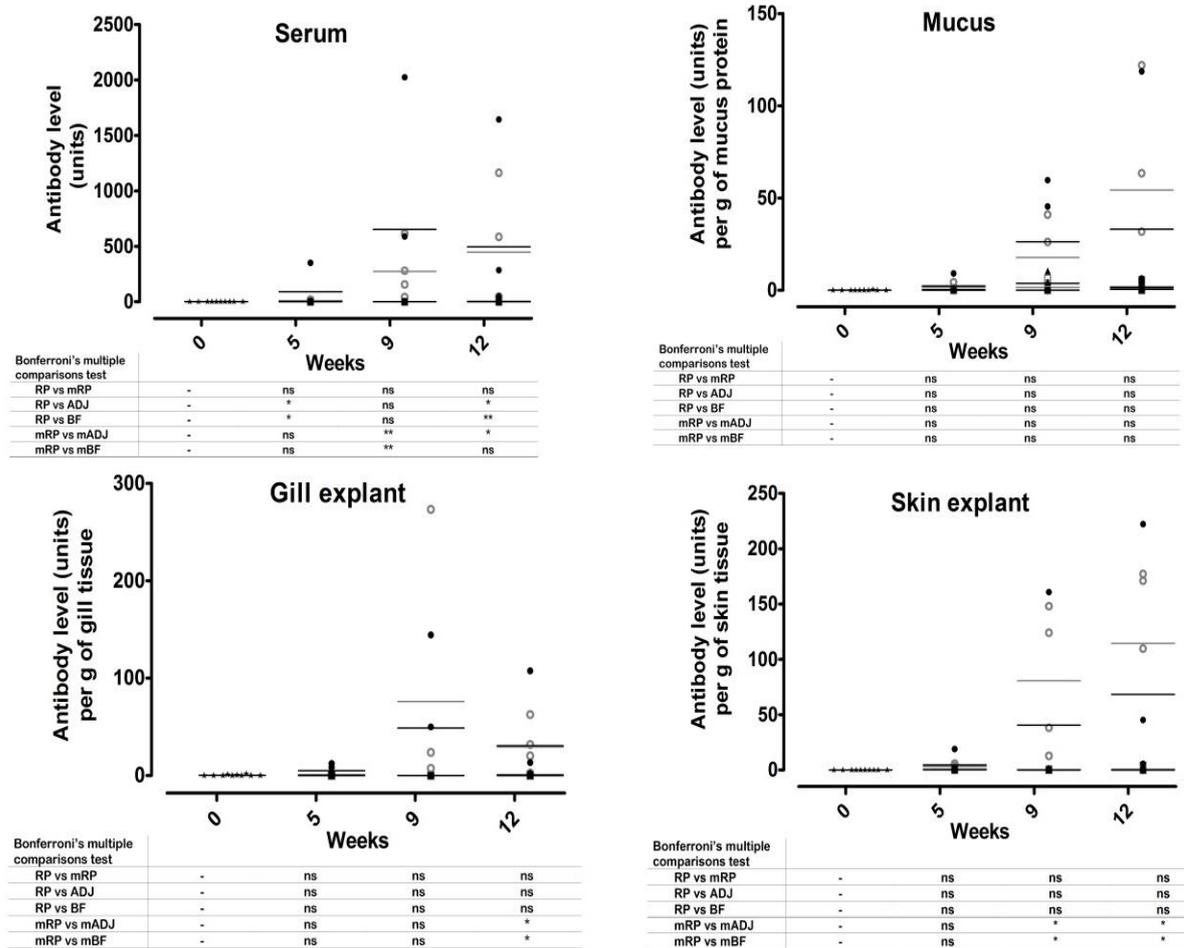


Figure 5.1 Antibody levels (units) in serum, mucus, gill and skin explant of Atlantic salmon against the recombinant protein *r22C03*, before challenge with *Neoparamoeba perurans*. Fish in groups RP and mRP had been immunised with *r22C03* at week 0 and given a booster at week 5. Tables show statistical differences between the vaccinated groups (RP and mRP) and their respective controls for each time point. Groups are identified as: Initial (\*), RP (●); ADJ (■), BF (▲), mRP (○), mADJ (□) and mBF (△). Symbols represent values for individual fish, bars represent averages for each group. n=4 for each treatment at each time point. \*=*P* between 0.05 and 0.01, \*\*=*P*<0.01 by a one-way ANOVA.

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Similarly to pre-challenge observations, ELISA results of all surviving fish at the end of the challenge showed that serum samples had the higher average antibody levels, however levels of antibodies after challenge were overall lower than those observed before challenge irrespective of treatment. Both vaccinated groups presented average serum antibody levels that did not differ significantly from each other but were significantly higher than those observed in any of the control groups (Figure 5.2), ( $F=4.066$ ,  $df\ 5,52$ ,  $P<0.001$ ). The average serum antibody level of the RP group was 202 units, while the average level of the mRP group was close to 176 units. Most of the control groups presented mean antibody levels below 3 units, with the exception of the mBF group which reached an average antibody level close to 13 units.

In the case of skin mucus, only the mRP group had an average antibody level which was significantly higher ( $F=4.178$ ,  $df\ 5,52$ ,  $P=0.046$ ) than one of its controls (Figure 5.2). This group reached an average antibody level of 14 units, with one of the samples showing an antibody level over 70 units. The other vaccination group, RP did not present any differences in the antibody levels when compared to its control groups (Figure 5.2).

No significant differences were observed in the antibody levels present in gill ( $F=5.882$ ,  $df\ 5,23$ ,  $P=0.294$ ) and skin explants ( $F=0.584$ ,  $df\ 5,23$ ,  $P=0.221$ ) at the end of the *N. perurans* challenge. Only two fish, one in each of the vaccinated groups, presented antibody levels that were above 60 units (Figure 5.2).

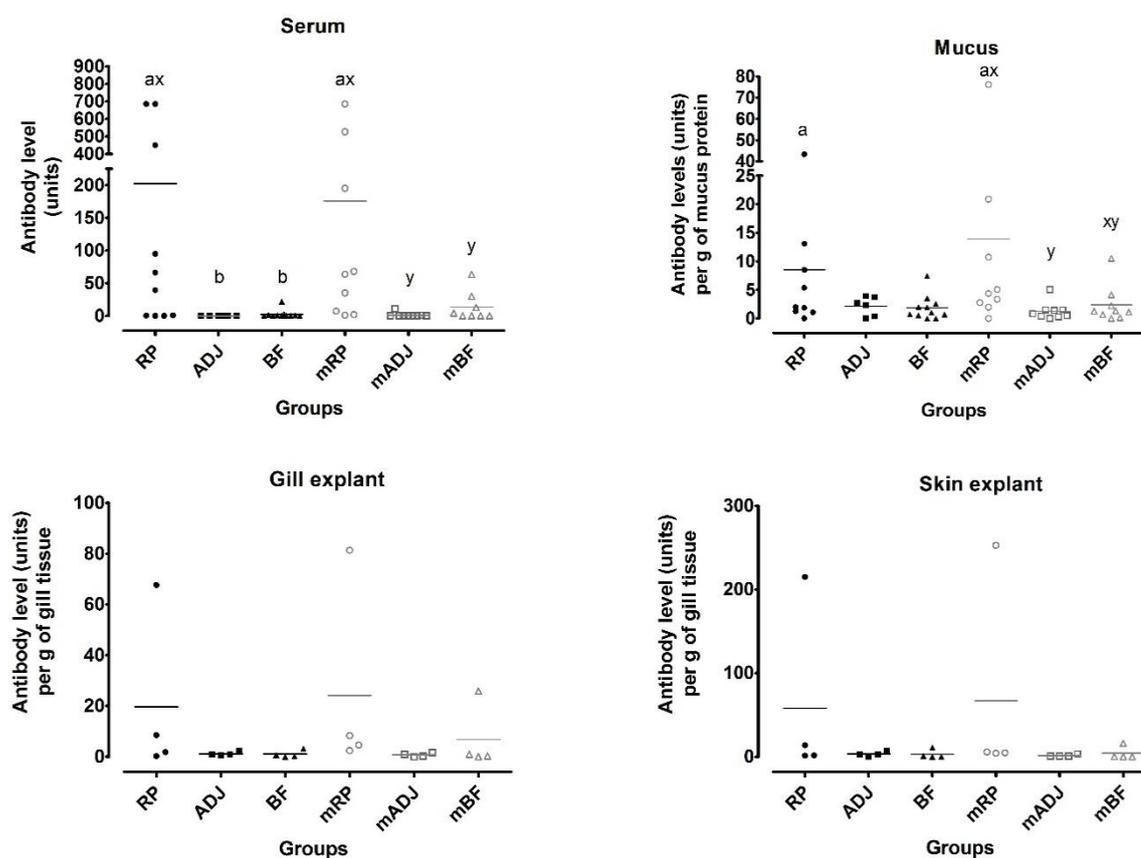


Figure 5.2 Antibody levels (units) in serum, mucus, gill and skin explant of Atlantic salmon against the recombinant protein *r22C03*, after the second challenge with *Neoparamoeba perurans*. Fish in groups RP and mRP had been immunised with *r22C03* 29 weeks prior and given a booster at 24 weeks earlier than sampling. Different letters represent statistical differences between the vaccinated groups (RP and mRP) and with their respective controls by a one-way ANOVA. Symbols represent values for individual fish; bars represent averages for each group.

#### 5.4.2 Vaccination did not affect survival time of AGD-challenged fish

As positive antibody responses were observed in serum and explants 12 weeks post immunisation, fish were acclimated to seawater to initiate a challenge with *N. perurans in vivo*, to test the efficacy of these antibody responses against the amoeba. However, the presence of ulcerative skin lesions and exophthalmos in the fish during the first *N. perurans* challenge, delayed the experiment for five weeks. The mortality curves represent the events observed during the second challenge with *N. perurans* (Figure 5.3).

Morbidity rates were not significantly different between tanks 1 and 2 ( $P < 0.05$ ), therefore data from both tanks were combined to generate the survival curves and to undertake the log rank analysis. After the challenge with *N. perurans*, no difference was observed in the survival times between any of the vaccinated groups and their controls ( $\chi^2 = 0.561$ , df 5,  $P = 0.989$ ). Combined percentage survival for all groups in both infected tanks 58 d after the challenge was between 26% (mBF group) and 33% (BF group) (Figure 5.3, A). The largest RPS (75%) was observed in the mRP vaccinated group when compared to the mBF control (Table 5.3).

Similarly, no differences were found among the survival times of all the treatments groups in the control tank ( $\chi^2 = 8.157$ , df 5,  $P = 0.148$ ). The average survival percentage at the end of the challenge varied between 62% (RP) and 94% (BF). No fish in this group presented any signs of AGD, so it is presumed that these mortalities were associated with the concurrent bacterial infection (Figure 5.3, B).

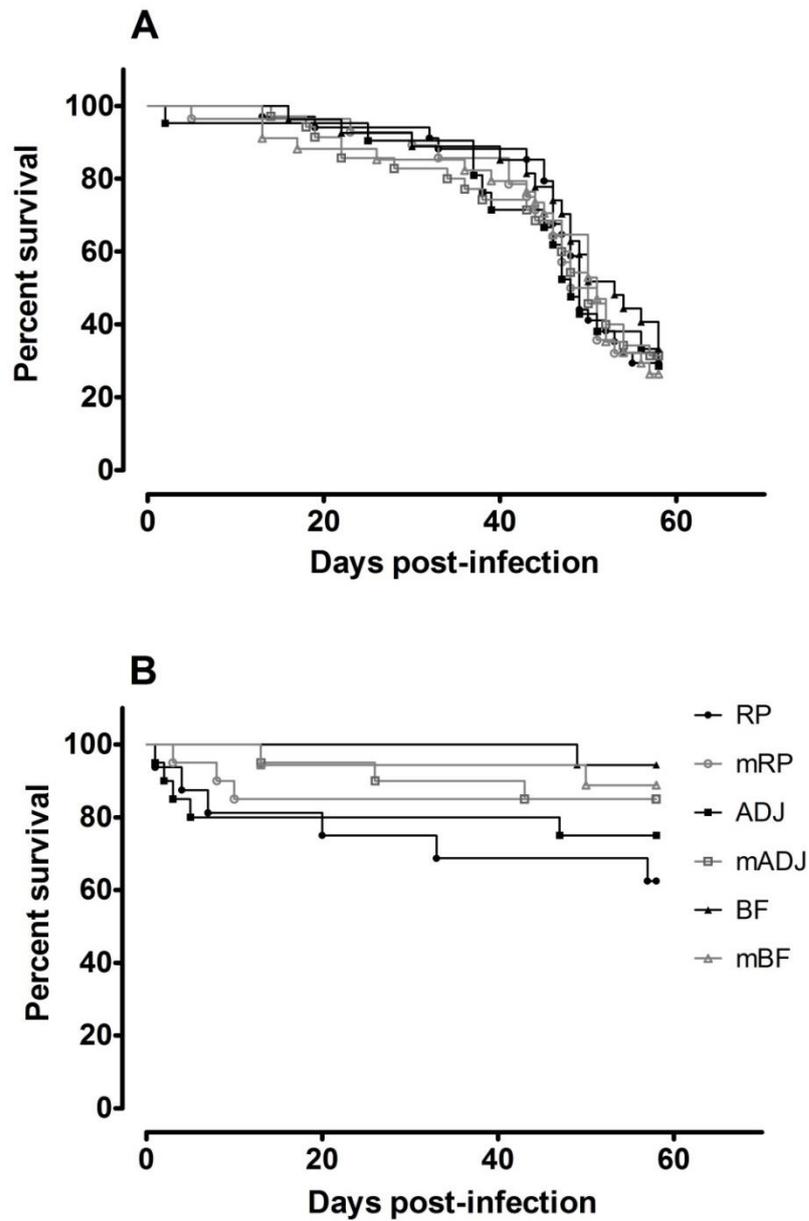


Figure 5.3 Percent survival for Atlantic salmon (*Salmo salar*) vaccinated with *r22C03* after infection with *Neoparamoeba perurans*, in challenged tanks (A) or in a non-infection control tank (B). Fish in groups RP and mRP had been immunised with *r22C03* 21 weeks prior and given a booster at 16 weeks before the challenge.

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**Table 5.3** Relative percent survival (RPS) in each vaccination treatment group at the end of the second AGD challenge.

| Group | RPS vs adjuvant control <sup>1</sup> | RPS vs PBS control <sup>2</sup> |
|-------|--------------------------------------|---------------------------------|
| RP    | 1.2                                  | -5.9                            |
| mRP   | 1.0                                  | 7.7                             |

<sup>1</sup>: RPS vs adjuvant control groups ADJ and mADJ, respectively

<sup>2</sup>: RPS vs buffer control groups BF and mBF, respectively

#### 5.4.3 Vaccination did not have an effect on intensity of AGD infection

There was no significant difference ( $F=0.70$ ,  $df$  5,53,  $P=0.625$ ) among treatment groups for the percentage of filaments affected by AGD at the end of the second challenge (58 d post-infection). The average percentage of AGD-affected filaments varied between 62% for the mRP group and 88% for the mBF group. Interestingly, two fish from the mRP vaccinated group and one fish from the BF control group did not present any affected gill filaments under microscopic examination (Figure 5.4, A). A total of 11 salmon gill samples from the non-infected control tank were also assessed for the presence of AGD lesions, and they were all negative (data not shown).

To obtain a more thorough evaluation of the intensity of infection, the size of individual hyperplastic lesions, measured as the number of interlamellar spaces affected, was also assessed. Similarly to the case of filaments affected, there was no significant differences among the treatments regarding average lesion size ( $F=0.15$ ,  $df$  5,50,  $P=0.98$ ). Average lesion size in AGD-affected fish varied between 9 and 48 interlamellar spaces in all treatment groups (Figure 5.4, B).



#### 5.4.4 Concurrent infection during AGD challenge and *Y. ruckeri* detection

Due to the presence of skin lesions in fish that were not characteristic of AGD, serum of Atlantic salmon was analysed from the presence of other pathogens, after the experiment was terminated. An initial screening of 7 serum samples from moribund fish using primers designed to detect the *rpoB* gene, returned positive results for the presence of bacteria. Hence five of these positive samples were sequenced and results returned positive identifications of *Y. ruckeri*. Therefore, sera were further analysed with specific primers and a TaqMan probe for this pathogen in all moribund and a sample of surviving fish serum samples. Real-time qPCR tests of sera from moribund fish before and after AGD challenge showed that 100% of these fish were positive for *Y. ruckeri* (Table 5.4). A large proportion of these moribund fish (n=10) were from the RP group. In most of the samples,  $C_t$  values were between 32 and 39, with the exception of 1 sample taken at 50 d after *N. perurans* infection, which belonged to a control fish (BF group), and showed a  $C_t$  value of 29.

A total of 30 samples were analysed from surviving fish at the end of the experiment, 58 d after the challenge with *N. perurans* (Table 5.5). Sixteen of these samples were obtained from AGD-challenged fish, while the remaining 14 were obtained from non-challenged controls. Half of the AGD-challenged fish tested positive for the presence of *Y. ruckeri*, while 57% of the non-AGD challenge controls were positive to the presence of the bacterium. In all of the positive samples  $C_t$  values were higher than 36, suggesting that the bacterial concentration in serum was lower than in the moribund fish.

There was no significant correlation between the number of AGD-affected gill filaments and  $C_t$  values for *Y. ruckeri* (Pearson's  $r=-0.009$ ,  $P=0.975$ ) in moribund fish after challenge, as well as in those fish sampled at the end of the experiment which were positive to *Y. ruckeri* infection (Pearson's  $r=0.389$ ,  $P=0.447$ ). Similarly, average AGD lesion size did not correlate with bacterial  $C_t$  values in samples obtained from AGD-affected fish at the end of the experiment (Pearson's  $r=-0.081$ ,  $P=0.878$ ).

Table 5.4 *Yersinia ruckeri* infection status ((+) = positive) in serum of moribund Atlantic salmon by real-time qPCR, before and after the second challenge with *Neoparamoeba perurans*. Salmon were subjected to different vaccination treatments with a recombinant protein before AGD challenge.

| Vaccination treatment | AGD Status     | Days post-challenge with <i>N. perurans</i> | <i>Y. ruckeri</i> status |
|-----------------------|----------------|---|--------------------------|
| RP                    | Pre-challenge  | -   | (+)                      |
| RP                    | Pre-challenge  | -   | (+)                      |
| RP                    | Pre-challenge  | -   | (+)                      |
| RP                    | Pre-challenge  | -   | (+)                      |
| BF                    | Pre-challenge  | -   | (+)                      |
| mRP                   | Pre-challenge  | -   | (+)                      |
| mADJ                  | Pre-challenge  | -   | (+)                      |
| mADJ                  | Pre-challenge  | -   | (+)                      |
| mBF                   | Pre-challenge  | -   | (+)                      |
| mBF                   | Pre-challenge  | -   | (+)                      |
| mBF                   | Pre-challenge  | -   | (+)                      |
| mBF                   | Pre-challenge  | -   | (+)                      |
| mBF                   | Pre-challenge  | -   | (+)                      |
| mRP                   | Post-challenge | 30  | (+)                      |
| RP                    | Post-challenge | 32  | (+)                      |
| BF                    | Post-challenge | 36  | (+)                      |
| ADJ                   | Post-challenge | 37  | (+)                      |
| ADJ                   | Post-challenge | 37  | (+)                      |
| RP                    | Post-challenge | 43  | (+)                      |
| RP                    | Post-challenge | 47  | (+)                      |
| ADJ                   | Post-challenge | 47  | (+)                      |
| RP                    | Post-challenge | 48  | (+)                      |
| RP                    | Post-challenge | 49  | (+)                      |
| RP                    | Post-challenge | 49  | (+)                      |
| BF                    | Post-challenge | 50  | (+)                      |
| mADJ                  | Post-challenge | 56  | (+)                      |

Table 5.5 Number (n) of surviving Atlantic salmon and their *Yersinia ruckeri* infection status ((+) = positive or (-) = negative) detected by real-time qPCR in serum, at the end of the second challenge with *Neoparamoeba perurans* 58 d post-infection. Salmon were subjected to different vaccination treatments with a recombinant protein before AGD challenge. A negative AGD challenge control was located in a different uninfected tank.

| Vaccination treatment | AGD status | <i>Y. ruckeri</i> status (n) |          |
|-----------------------|------------|------------------------------|----------|
|                       |            | (+)                          | (-)      |
| RP                    | Challenged | 2                            | 1        |
| ADJ                   |            | 1                            | 1        |
| BF                    |            | 1                            | 2        |
| mRP                   |            | 4                            | -        |
| mADJ                  |            | -                            | 2        |
| mBF                   |            | -                            | 2        |
| <b>Total</b>          |            | <b>8</b>                     | <b>8</b> |
| RP                    |            | Negative challenge control   | -        |
| ADJ                   | 1          |                              | -        |
| BF                    | 2          |                              | 1        |
| mRP                   | 1          |                              | 1        |
| mADJ                  | 2          |                              | -        |
| mBF                   | 1          |                              | 1        |
| UU                    | 1          |                              | 1        |
| <b>Total</b>          | <b>8</b>   |                              | <b>6</b> |

## 5.5 DISCUSSION

We have tested a recombinant protein (*r22C03*) identified from *N. perurans* as a potential candidate vaccine against AGD. Recombinant proteins are a popular method used for vaccines against parasites, since they are easy to identify and produce [134]. In this study *r22C03*, an MBP-like factor of *N. perurans*, was identified from a cDNA library and was considered as a potential vaccine candidate based on the role of analogous proteins in other amoebae. An MBP-like protein has been identified in *Acanthamoeba* spp., an amoeba affecting mucosal surfaces of mammals, as a key mediator of host-parasite interactions and a virulence factor (as reviewed by Ref. [213]). Polyclonal antibodies developed against this protein, were capable of inhibiting the adhesion of the amoeba to mucosal surfaces, preventing cytopathic effects in epithelial cell cultures [215] and later, an oral vaccine consisting of purified recombinant protein provided significant protection against the disease [228].

In the present study, two different vaccination strategies with *r22C03* were evaluated, one involving an initial and booster i.p. immunisation (RP) and the other including an initial parenteral immunisation followed by dip (mRP). A significant antibody (IgM) response to *r22C03* was induced in serum with both vaccination strategies, beginning at nine weeks after the initial immunisation in the mRP group and observed at five and twelve weeks in the RP, before the amoebae challenge. However, no significant mucus antibody response was measured for any of the treatment groups. This is in partial agreement with our previous study, where *r22C03* induced systemic and mucosal IgM responses in Atlantic salmon, and these antibodies were capable of binding the surface of fixed *N. perurans* [226], suggesting their potential neutralising activity against the parasite. The dose used in the injected group in the present study (250 µg) was slightly higher than that used previously (200 µg) [226] in i.p. immunised fish (RP group). Additionally, the average serum but not the average mucus antibody response, was considerably higher than that observed earlier, indicating a possible effect of the dose on the level of response; however further research is required to clarify this effect. Nine weeks after the initial immunisation, the RP group did not show any difference in the average antibody levels compared to

the control groups, but this could have been due to high variability within the group, since only two of the fish presented a considerably higher antibody level.

In addition to the serum and mucus responses, *r22C03* also generated significant antibody responses measured in the explants obtained from gill and skin of Atlantic salmon from the mRP group. This differed slightly from previous studies, which showed the ability of soluble antigens injected via i.p. to generate IgM responses in mucosal tissues [152, 170], indicating the systemically simulated B-cells could migrate to mucosal surfaces and produce antibodies locally. In our study, only the group injected and then stimulated in the mucosal surface by dip showed responses in the explanted skin and gills. Similarly to serum responses, this could be due to the variability in the antibody responses within the RP group.

Mucus antibody levels, measured before and after the challenge, were in general low or near zero with a few exceptions for both vaccination regimes. However, we have been previously shown that i.p. injection of antigens can induce a certain level of mucosal response in Atlantic salmon [152, 226] which we anticipated to observe in the present experiment. Additionally, the second vaccination protocol (i.p. injection followed by dip-immersion) was tested, as immersion can facilitate uptake by the skin, the gills and the gut (i.e. drinking), triggering local stimulation. Local stimulation of gills with IgM production on site following immersion has been previously documented [171, 194, 201]. Future studies could test the use of adjuvants specifically designed to elicit mucosal responses, such as bacterial binding proteins from the cholera toxin or the *E. coli* heat-labile enterotoxin [229]. These adjuvants have been commonly delivered orally in fish [230, 231], and even though there is evidence that mucosal stimulation in one site (i.e. intestinal mucosa) can generate antibody responses at other mucosal sites (i.e. gill and skin) [200, 230], this is not always the case [152, 170]. Alternatively, other methods attempting to improve vaccine uptake by immersion delivery such as hyperosmotic infiltration, the use of ultrasound and even prolonged immersion could be tested [232], and their effect on the mucosal antibody levels studied in the future in regards to AGD vaccines.

The length of time during which we monitored the immune responses - up to 29 weeks after initial immunisation and 58 d after the second exposure to *N. perurans* -

could reflect the situation in a commercial grow-out setting. To our knowledge, no other experiment has measured the mucosal immune responses in salmonids for such an extended period of time after immunisation [115, 149, 170, 175, 180, 202]. Recent studies in parasites affecting mucosal surfaces of salmonids [97, 118], have indicated that IgT and not particularly IgM, is responsible for the Ig responses in mucosal surfaces in rainbow trout. Efforts in our laboratory have been unsuccessful in characterising Atlantic salmon IgT using the available mAbs raised against this molecule [97, 149], therefore we have been unable to monitor IgT responses in the present study. This could partially explain the low levels of antibody (IgM) response observed in our fish after the extended period. However, it is also known that levels of antibodies in mucosal surfaces are lower than those observed in serum of fish [97, 130], which could be the case in this study.

Following the principles established by Amend [138] to test for the potency of fish vaccines, indicating that the infection or mortality rate on the vaccinated groups should be  $\leq 24\%$  and that of controls groups should be  $\geq 60\%$ , neither of our vaccine strategies provided effective protection against the amoebae. Throughout the challenge, survival curves for both vaccinated groups did not differ from the controls, and neither did the percentage of affected filaments and the size of lesion in AGD-affected gill areas at the end of the experiment. Using these particular doses and immunisation schedules, this candidate vaccine does not represent a commercially viable treatment option for AGD. In the future, parasite vaccines could be directed more towards control rather than eradication of diseases, due to the low efficacy rates obtained in the past [134].

There are a number of potential causes for the lack of protection observed in the candidate vaccine in the present study. Firstly, the role of antibodies in the protection against to AGD is yet to be fully understood (as reviewed by Ref. [103]). Some authors have postulated that the amoebae might be capable of internalising the antibodies once they have attached to their surface, and either degrade them or release them into the seawater at a later stage [221]. Additionally, antibodies might lose their ability to bind antigens when exposed to extreme salinity conditions [223], as those expected in seawater surrounding the mucosal surfaces. Since neither of

these scenarios was tested in our experiment, they could potentially represent causes of failure of the vaccine. Secondly, the production process of recombinant proteins could lead to loss in their functionality, due to the loss of crucial protein structures [233]. Considering this issue, some vaccine attempts are heading back to use of the whole parasite approach with refinements [233]; however, these approaches have already been tested for AGD using different antigens and administrations methods, with unsuccessful outcomes [135, 136]. In addition, the protein used in the current study has been shown to induce antibody responses against *N. perurans* antigens and these antibodies were able to bind the surface of the amoebae [226], and therefore it is very unlikely that loss of crucial structures could be affecting its functionality.

Finally, there was the issue of a bacterial co-infection. An initial diagnosis of overgrowth of normal microflora characterised by the presence of *Vibrio* sp. in the external surfaces of affected fish, lead to the treatment of the concurrent infection with oxytetracycline, a common antibiotic used in aquaculture [234]. Time and budget constraints prohibited the restocking of animals to reinitiate the experiment, and therefore a decision was made to reinitiate the challenge, using the existing animal stock. Serum analysed at a later stage for the presence of potential pathogens indicated that of all of the moribund and a large proportion of survivor fish during and at the end of the amoebae challenge were positive to the presence of *Y. ruckeri*, with a broad range of *Ct* values observed in the positive samples. Even though PCR represents a very reliable pathogen identification method, the positive identification of the pathogen does not mean that it is viable and an active infection is occurring [235]. However, the external signs observed in the fish correlated to those described for the condition in smolts [16], and the moderate-*Ct* value obtained from a moribund fish, indicated a possible infection in course. Concurrent outbreaks of yersiniosis and AGD have been observed in the past during similar trials, with high numbers of fish succumbing to the bacteria [51].

In retrospect, the treatment of fish with oxytetracycline might not have been the best option for our experiment. Previous research into the effect of antibiotic treatment on *Y. ruckeri*, have shown that doses of 30 mg per kg of fish/d for 10 d failed to reduce the clinical infection level with the bacteria, and infection became persistent after the

treatment [236]. It is noteworthy that this dose was more than 100 x lower than the one used in the present study. However our study and the above mentioned, failed to measure the tissue or plasma concentration of the drug, and therefore no specific conclusions can be made on the effectiveness of the treatment. The reduction on the mortality rates after the antibiotic treatment could have been favoured by the simultaneous reduction in water salinity and water temperature. When treating *Y. ruckeri in vitro*, it has been demonstrated that the minimum inhibitory concentration of oxytetracycline is higher than those required for other antibiotics (such as oxolinic acid and sulphonamids); the effect of the drug is only bacteriostatic if removed before the bacterial cell killing and continuous exposure can generate resistance to the compound [234]. These characteristics of the antibiotic could have led to a persistent infection with the pathogen. Unfortunately, we did not achieve the correct identification of *Y. ruckeri* until after the treatment had been carried out, and it is likely that an earlier identification would have led to the use of a more appropriate antimicrobial treatment.

The concurrent infections in the present study might represent a more realistic disease outbreak scenario, similar to those observed in commercial salmon farming, where both pathogens might affect the fish simultaneously during the seawater grow-out phase. However, regarding the experimental testing of the vaccine, the results obtained need to be treated with caution as it is unknown how the concurrent *Y. ruckeri* infection may have affected the severity of AGD and/or response to the vaccine. In future experiments, testing the fish for the presence of the bacteria before experimental challenges should be considered of importance.

In conclusion, we have demonstrated that *r22C03* is capable of inducing antibody response in serum, mucus and gill and skin explants. However, these antibodies did not confer protection to *N. perurans* infection, and did not have any effect on the average extension of the lesions in the gills of affected fish. A co-infection with *Y. ruckeri* possibly represented a situation more comparable to infections observed in commercial setting, but most likely interfered with the survival during the parasite challenge, and could have had an effect on the ability of the fish to respond to the

amoebic infection. In the future, the use of fish that are free of other pathogens should be considered a high priority.

## **5.6 ACKNOWLEDGEMENTS**

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## CHAPTER 6.

### DIFFERENTIALLY EXPRESSED PROTEINS IN GILL AND SKIN MUCUS OF ATLANTIC SALMON (*Salmo salar*) AFFECTED BY AMOEBIC GILL DISEASE

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#### **KEYWORDS**

Amoebic gill disease; *Salmo salar*; gill mucus; skin mucus; proteomics.

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## 6.1 ABSTRACT

The external surfaces of fish, such as gill and skin, are covered by mucus, which forms a thin interface between the organism and water. Amoebic gill disease (AGD) is a parasitic condition caused by *Neoparamoeba perurans* that affects salmonids worldwide. This disease induces excessive mucus production in the gills. The host immune response to AGD is not fully understood, and research tools such as genomics and proteomics could be useful in providing further insight. Gill and skin mucus samples were obtained from Atlantic salmon (*Salmo salar*) which were infected with *N. perurans* on four successive occasions. NanoLC tandem mass spectrometry (MS/MS) was used to identify proteins in gill and skin mucus of Atlantic salmon affected by AGD. A total of 186 and 322 non-redundant proteins were identified in gill and skin mucus respectively, based on stringent filtration criteria, and statistics demonstrated that 52 gill and 42 skin mucus proteins were differentially expressed in mucus samples from AGD-affected fish. By generating protein-protein interaction networks, some of these proteins formed part of cell to cell signalling and inflammation pathways, such as C-reactive protein, apolipoprotein 1, granulins, cathepsin, angiogenin-1. In addition to proteins that were entirely novel in the context in the host response to *N. perurans*, our results have confirmed the presence of protein markers in mucus that have been previously predicted on the basis of modified mRNA expression, such as anterior gradient-2 protein, annexin A-1 and complement C3 factor. This first proteomic analysis of AGD-affected salmon provides new information on the effect of AGD on protein composition of gill and skin mucus. Future research should focus on better understanding of the role these components play in the response against infection with *N. perurans*.

## 6.2 INTRODUCTION

The external surfaces of fish, such as gill and skin, are covered by mucus, which forms a thin interface between the organism and the water [121]. The majority of mucus present in these surfaces is produced by goblet cells, but other epithelial cells may contribute different substances [120, 121]. Mucus is a poorly understood matrix that has been associated with a variety of functions, including gas and ion exchange, excretion, locomotion and defence, and is mainly composed of water and

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glycoproteins, specially mucins (for a complete review on fish mucus functions and composition see Ref. [120]). Several other components, including immune factors, have been also described in mucus of fish. Antimicrobial peptides, lectins, proteases, lysozyme, complement and immunoglobulins have been identified in the skin mucus of several fish species [125, 126, 183, 220, 237, 238], and their presence and abundance can be affected by the disease status of the fish [125, 126, 238].

Amoebic gill disease (AGD) is a parasitic condition that affects salmonids worldwide [22, 34, 37, 41]. The disease, which occurs during the marine grow-out phase and can be fatal if untreated [20], is caused by *Neoparamoeba perurans*, a free-living and opportunistically parasitic amoeba [46]. The presence of the parasite induces excessive mucus production in the gills [239], which could be explained by the recruitment of mucous cells to the gill surface [55, 240]. White raised patches can be seen macroscopically on the surface of gills [54], and histologically, severe changes in the gill structure can be observed including lamellar fusion and epithelial hyperplasia [55], as well as sloughing of the chloride cells from the lesions [55].

While a limited knowledge of the immune response to AGD has been gained through gene expression studies [107-110, 112], antibody response [113-116] and histopathology [55, 57], the host response to AGD is still not fully understood. *N. perurans* infection induces expression of pro-inflammatory cytokines such as interleukin-1 $\beta$  [107, 109, 110, 241] in areas of the gill affected by the parasite [108]. Besides this particular gene, early studies presented evidence that lesions were characterised by a general down-regulation of immune related genes mainly in late infection stages [109]; however a recent gene expression study demonstrated that the disease does in fact cause a classic inflammatory response with cellular infiltration 10 d post-infection, since T and B-cells markers were found up-regulated in lesion areas [112]. Additionally, microarray and immunohistochemical analyses showed that an Atlantic salmon (*Salmo salar*) anterior gradient-2 (asAG-2) gene and protein are highly expressed in AGD lesions [108, 242, 243]. Although gene expression studies are useful to study the cellular response to AGD, they are of limited use for detection of marker proteins in mucus itself. Hence, there is a knowledge gap regarding the relative protein abundance in mucus.

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Proteomics has the potential to provide further insight into the protein response in mucosal surfaces of fish affected by AGD. So far it is only known that the protein concentration increases in the gill mucus of fish affected by *N. perurans* [122]. However, a comprehensive comparison of naïve versus AGD-affected mucus has yet to be carried out. As opposed to genomics, proteomics can provide information of post-transcriptional and post-translational regulation, and therefore contribute data about an organism's physiological state, which could have been missed by the transcriptome [124]. 2-D gel electrophoresis has been used successfully in the past to study fish mucus proteomes [125, 126], however it does not have the capacity to detect the full spectrum of proteins present in a sample, which is necessary for comprehensive analysis of biological material [127]. Gel-free approaches are becoming more popular [128], complementing and sometimes challenging 2-D gel systems, due to the reduction in their costs and moreover, because they allow a deeper proteome coverage, particularly for high throughput proteomics research [124, 129].

In this study we have used nanoLC-MS/MS to identify proteins in gill and skin mucus of Atlantic salmon affected by AGD and compared them to mucus samples from control fish, not affected by AGD. The samples were obtained from fish exposed multiple times to *N. perurans* (i.e. four consecutive challenges), based on previous studies of mucus composition where fish were subjected to repeated infections with other skin parasites [126]. It was thought that changes in the protein profile of mucus would be more substantial following multiple challenges. Statistical analyses were used to identify proteins that were differentially expressed in each sample of mucus from fish affected and not affected by AGD. These proteins were mapped onto protein interaction network, and these results associated AGD with an effect on cell to cell signalling and inflammation pathways.

This array of proteins identified in mucus included some proteins that were previously associated with AGD at the gene expression level, and others that can be considered novel in the host response to this infection. We have shown the feasibility of gel-free approaches to study fish mucus during infection, in particular in diseases like AGD which have a direct effect on an external mucosal surface. This

information provides a baseline for further proteomics studies, for example, subsequent validation studies on the effect of AGD on protein composition of gill and skin mucus. This data could also help to better understand the role of these components in the mucus, and how they might participate in the response against infection with *N. perurans*.

## **6.3 MATERIALS AND METHODS**

### **6.3.1 Fish and experimental procedures**

Atlantic salmon (n=100) with average body weight of 162.75 g (SD 35.86 g) and average fork length of 245.83 mm (SD 14.43) were obtained from a commercial farm and held at the Aquaculture Centre, University of Tasmania, as part of a larger study. Animals were acclimated for 10 d and held during the experiment at approximately 15.0°C and pH 7.0 in a seawater recirculation system consisting of 4 x 1,000 L tanks, where half the tanks were assigned for infection and the other half were used as non-infection controls.

Fish in AGD infection tanks were exposed to 150 amoeba/L, and re-exposed to the parasite at the same density 5, 8 and 14 weeks later. For the challenges, *N. perurans* were harvested from salmon held in an ongoing infection tank located at University of Tasmania, following the procedure previously described elsewhere [151]. After isolation, amoebae were incubated at 18°C overnight in seawater with antibiotics (ampicillin at 9.6 µg/mL and oxolinic acid at 20 µg/mL) to prevent spread of bacteria into the infection tanks. Amoebae were counted using a haemocytometer, then placed into 1 L sterile seawater and added to the infection tanks. Between infections, fish in all 4 tanks were freshwater bathed for about 5.5 h (3 ppt) to limit the disease progress, which is a practice commonly used by the industry as a control measure [20]. All these procedures were performed under the animal ethics committee guidelines from the University of Tasmania, approval number A0009717.

### **6.3.2 Sampling procedures**

Gill and skin mucus were collected from all the surviving fish at the end of the experiment, 18 weeks after the original infection. Fish were killed before sampling using 1 mL clove oil diluted in 10 L of freshwater. Skin mucus was scraped from the

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sides of the fish and transferred into microcentrifuge tubes containing 1 mL “mucus extraction” buffer (2 mM PMSF, 10 mM EDTA, 0.02% sodium azide in 0.85% saline with 10  $\mu$ L anti-protease cocktail (Sigma Aldrich, Castle Hill, NSW, Australia) per tube.

To remove any remaining blood from the organs, perfusion of the organs via puncture of the bulbous arteriosus was performed with 0.9% physiological saline (Baxter, Deerfield, IL, USA) containing 1 IU of heparin/mL as previously specified [152], until the gills were white. The gill basket was carefully removed from the fish and four hemibranchs (i.e. one half of the gill basket) were placed into 20 mL mucus extraction buffer. On the same day, all mucus samples were centrifuged at 15,000  $\times$  g for 1 h, the supernatant collected and frozen at -80°C until used.

**6.3.3 Mucus preparation for proteomics**

Samples from 5 AGD-affected fish and 5 control fish were randomly selected from both replicate tanks for this study and they were processed as described by Ref. [126] with slight modifications. Mucus was mixed 1:4 with 100 mM  $\text{NH}_4\text{HCO}_3$  pH 7.5, centrifuged, mixed with a final concentration of 1 mM PMSF and 50 mM EDTA and frozen at -80°C. Thawed mucus samples were dialysed against 10 mM Tris-HCl pH 8.0, centrifuged and supernatants were freeze-dried. Lyophilised mucus was resuspended in sterile distilled water, and protein samples for nanoLC-MS/MS analysis were processed as previously described [244]. In brief, proteins were sequentially reduced and alkylated under nitrogen by incubation in 10 mM dithiothreitol and 50 mM iodoacetamide. Proteins were co-precipitated with 1  $\mu$ g of trypsin (Promega, Madison, WI, USA) at -20°C in methanol, washed with chilled methanol, dried and reconstituted in 100 mM ammonium bicarbonate. Trypsin digestion was allowed to proceed at 37°C for 5 h, with an extra 1  $\mu$ g of trypsin added after 3 h. Digests were terminated by freezing at -80°C.

Prior to digestion, proteins were analysed by SDS-PAGE to assess consistency among samples. Mucus samples were resolved through 4–12% acrylamide Bis-Tris NuPAGE® Novex® Mini Gels (Life Technologies, Carlsbad, CA, USA), using approximately 6  $\mu$ g of protein per lane and proteins were visualised by silver staining. Protein concentrations were measured by Qubit® 2.0 Fluorometer (Life

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Technologies) and adjusted to 1 mg/ml. Protein bands of interest were excised with a clean scalpel blade, destained and subjected to in-gel trypsin digestion with 250 ng of proteomics-grade trypsin using standard methods [245].

**6.3.4 nanoLiquid Chromatography-LTQ-Orbitrap Tandem mass spectrometry.**

Peptide samples were analysed by nanoLC-MS/MS using an LTQ-Orbitrap XL (ThermoFisher Scientific, Waltham, MA, USA). Aliquots of tryptic peptides were loaded at 0.05 ml/min onto a C18 capillary trapping column (Peptide CapTrap, Michrom BioResources, Auburn, CA, USA) controlled by an Alliance 2690 Separations Module (Waters). Peptides were then separated using a Surveyor MS Pump plus (ThermoFisher Scientific) on an analytical nanoHPLC column packed with 5 µm C18 ProteoPep II media (PicoFrit Column, 15 µm i.d. pulled tip, 10 cm, New Objective) as previously described [246].

The LTQ-Orbitrap XL was controlled using Xcalibur 2.0 software (ThermoFisher Scientific) and operated in data-dependent acquisition mode where survey scans were acquired in the Orbitrap using a resolving power of 60,000 (at 400 m/z). MS/MS spectra were concurrently acquired in the LTQ mass analyser on the eight most intense ions from the FT survey scan. Charge state filtering, where unassigned and singly-charged precursor ions were not selected for fragmentation, and dynamic exclusion (repeat count 1, repeat duration 30 s, exclusion list size 500) were used. Fragmentation conditions in the LTQ were: 35% normalised collision energy, activation q of 0.25, 30 ms activation time and minimum ion selection intensity of 500 counts.

**6.3.5 Database searching and criteria for protein identification**

Centroid mode spectra acquired were converted from .RAW files into .mzXML peak list files using the msConvert command (Proteowizard). The extracted MS/MS data were searched against the Clupeocephala protein database of 227,414 entries downloaded from the National Centre for Biotechnology Information on 23/05/2013. This taxonomic group included the subset of 15,810 entries belonging to Atlantic salmon database. Semi-tryptic searches using parent ion and fragment ion mass tolerances of 10 ppm and 0.5 Da, respectively, were performed using X!Tandem

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running in the Computational Proteomics Analysis System (CPAS), an open-source bioinformatics resource for analysing large proteomics datasets [247].

S-carboxamidomethylation of cysteine residues was specified as a fixed modification and oxidation of methionine was specified as a variable modification. The Peptide Prophet and Protein Prophet algorithms were applied to the X!Tandem search results to assign probabilities to peptide and protein matches, respectively [248, 249]. Peptide-spectrum matches were accepted if the peptide was assigned a probability >0.95 by the Peptide Prophet algorithm. Protein identifications were accepted if the protein contained two or more unique peptide sequences and the protein was assigned a probability >0.95 by the Protein Prophet algorithm. For all samples these filtration criteria constrained the protein false discovery rate to <1%.

**6.3.6 Statistical analyses**

For the statistical analysis of LTQ-Orbitrap mass spectrometry data, relative protein abundance was based on MS/MS spectral counts (SpC), a sampling output commonly used for label-free protein quantitation [250] and normalisation based on the SpC totals recorded for each sample [251]. Changes in the expression level of proteins in the mucus of control and AGD-affected fish were estimated using fold-differences between the mean normalised SpCs and statistical significance ( $P < 0.05$ ) was assigned using the beta-binomial test implemented in R [252]. To adjust for multiple comparisons and provide an estimate of the false discovery rate (FDR),  $q$ -values for each protein were calculated using qvalue online software. FDR of  $X\%$  means that among all individual features that meet the criteria for statistical significance,  $X\%$  of these are truly null on average [253, 254].

Principal component analysis (PCA) was carried out on the dataset containing proteins that were reliably detected ( $\geq 2$  peptides) and in a minimum of 5 samples. This analysis was performed using The Unscrambler® X version 10.3 (Camo Software, Oslo, Norway), with the resulting components plotted as a grid to illustrate the PCA transformation.

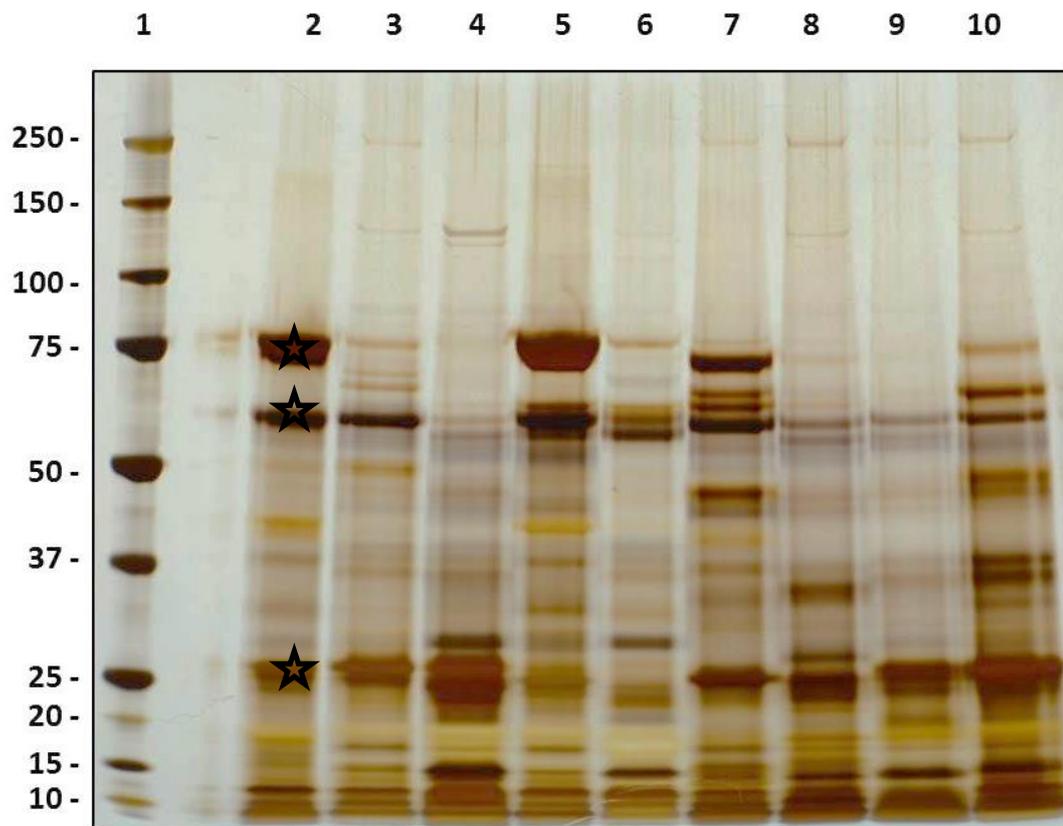
Proteins that were differentially expressed in AGD-affected fish were used to develop a protein interaction network, using Ingenuity Pathway analysis

(www.ingenuity.com). This network was developed using a list of mammalian orthologues of these proteins, generated through blast searches on NCBI database.

## **6.4 RESULTS AND DISCUSSION**

We have used proteomics analysis by nanoLC-MS/MS to characterise changes in the proteome of gill and skin mucus in Atlantic salmon affected by AGD. To the best of our knowledge, this is the first proteomic study to examine the changes in protein abundance in gill mucus for this species and one of a few existing on skin mucus [125, 126, 128]. Mucus provides an informative biological matrix for study of AGD-affected salmon, since the majority of proteins produced locally in the gill would be present. To evaluate the broad pattern of proteins we used SDS-PAGE in the protein extractions. The protein content of gill mucus samples obtained from diseased and control fish, ranged between 1.0 and 1.3 mg/mL. While we obtained relatively consistent amounts of protein between mucus samples, there was marked heterogeneity in the protein bands (Figure 6.1); with little consistency between AGD-affected and AGD-naïve mucus proteins in this analysis.

A total of 508 non-redundant protein groups were identified on the basis of two or more distinct matching peptide sequences, similar to the number of mucus proteins identified in a previous gel-free proteomics analysis [128], of which 186 were found in mucus from gill and 322 in mucus sampled from skin. Of these non-redundant protein groups, 15% of gill and 21% of skin mucus proteins were specifically identified only in mucus samples from AGD-affected fish. Serum-derived proteins were identified in the mucus. These included serum albumin and serotransferrin, that have been previously detected in proteomics analyses of mucus [126, 128], as well as haemoglobin, which has also been detected in mucus of teleosts in occult amounts a short time after handling stress [255].

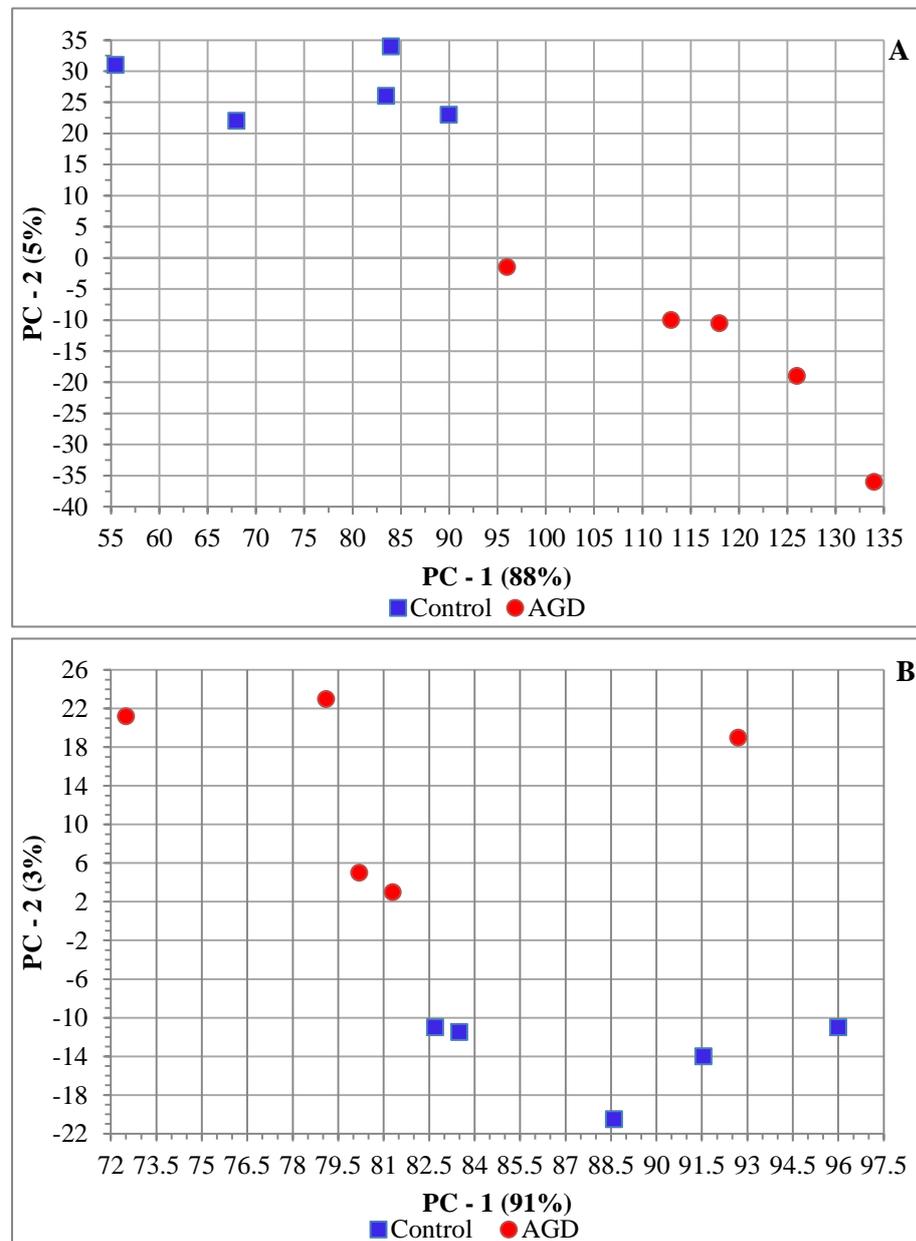


**Figure 6.1** Protein extractions from Atlantic salmon (*Salmo salar*) gill mucus resolved by Bis-Tris 4-12% NuPAGE® Novex® Mini gel and silver stained. Each lane contains a similar amount of protein yield (~ 6 µg per lane), after dialysis and lyophilisation. Lane 1 MWM, lanes 2-6: gill mucus samples from AGD-naïve fish, lanes 7-10: gill mucus from AGD-affected fish. Stars (☆) indicate bands that were excised and subjected to in-gel digestion for identification by nanoLC-MS/MS.

In particular, serum albumin can account for a high proportion of the total protein in mucus [256] and in our samples SpC values for isoforms 1 and 2 of serum albumin accounted for 25-50% of the valid MS/MS spectra acquired (data not shown). However, the protein band detected at the approximate molecular weight of albumin (68 kDa) by SDS-PAGE, varied in abundance from most intensity-stained protein (Figure 6.1, lanes 2 and 5) to undetected (Figure 6.1, lanes 4 and 9). To investigate further, this ~70 kDa band and two other common protein bands were excised and in-gel digested with trypsin (Figure 6.1). Analysis of the protein digests by nanoLC-MS/MS unequivocally identified albumin peptides in all three samples. This is consistent with low mass variants of albumin previously detected by proteomic analysis of salmon serum [257] and accounts for the broadly similar SpC values obtained between samples.

PCA of normalised spectral counts were used to evaluate the global relationships between biological replicates and AGD status. The PCA results showed that for both gill and skin mucus samples, AGD-naïve fish clustered together, as did the mucus samples from AGD-affected fish, based on the effect of PC2 for skin and both PC1 and PC2 for gill (Figure 6.2). However, PC2 accounted for a small percentage of the variation, 5% for gill and 3% for skin mucus, while PC1 accounted for 88% and 91% in gill and skin mucus samples, respectively (Figure 6.2).

Using normalised spectral counts and beta-binomial distribution test, we identified proteins differentially abundant in mucus of AGD-affected and control fish. Fifty-two gill and 42 skin mucus proteins were significantly and differentially abundant between AGD-affected and control fish (beta binomial,  $P < 0.05$ ). Q-values were below 8% for the gill proteins and below 20% for the skin proteins that were differentially expressed (Table 6.1 and Supplementary Table 6.2, respectively). Some of the protein changes were found in both skin and gill mucus while others were specific to each mucus type.



**Figure 6.2** Principal component analysis of the full set of proteins identified at a high confidence level ( $\geq 2$  peptides) in the biological replicates ( $n=5$ ) of gill (A) and skin (B) mucus of Atlantic salmon. The blue dots denote the AGD naïve fish and the red dots represent AGD-affected fish.

**Table 6.1** Proteins significantly and differentially abundant in gill mucus of AGD-affected Atlantic salmon. Proteins were identified by nanoLC-MS/MS. Proteins with  $P < 0.05$  and fold change  $> 2.0$  are in bold letters. SpC C, Spectral count control group; SpC D, spectral count diseased (AGD) group; FC, Fold change.

| #  | Description  | Accession number  | SpC C | SpC D | FC    | P-value | Q-value |
|----|--|---|-------|-------|-------|---------|---------|
| 1  | Angiogenin-1 precursor ( <i>Salmo salar</i> )  | NP_001134561.1  | 2.6   | 29.8  | 11.6  | 0.016   | 0.04    |
| 2  | Death-associated protein 1 ( <i>Salmo salar</i> )  | NP_001133551.1  | 0.63  | 3.45  | 5.465 | 0.043   | 0.071   |
| 3  | Enolase 3, (beta, muscle) ( <i>Danio rerio</i> ), Enolase ( <i>Sparus aurata</i> ), alpha-2 enolase-1 ( <i>Salmo trutta</i> ), enolase 3-2 ( <i>Salmo salar</i> ), Enolase 3-1 ( <i>Salmo salar</i> ), Beta-enolase ( <i>Osmerus mordax</i> )  | NP_999888.1, AAO92646.1, AAI07495.1, AAG16311.1, CAF89801.1, AAH92869.2, NP_001133193.1, NP_001135172.1, ACO09283.1   | 0     | 4.8   | >4.8  | 0.011   | 0.032   |
| 4  | Hemoglobin subunit beta ( <i>Salmo salar</i> , <i>Oncorhynchus mykiss</i> , <i>Oncorhynchus. masou formosanus</i> , <i>Oncorhynchus. nerka</i> )   | HBB_SALSA, ACO07576.1, ACO07923.1, ACN12702.1, 1009195A, ABY21329.1, ACI68214.1, ACO07819.1, HBB4_ONCMY, ACO08038.1, ACI66980.1, ACO08017.1, CAA65948.1, ACI69922.1, ACH70759.1, ACO07581.1, ACI68603.1, ACO07741.1, ACI66413.1, AAR19289.1, ACN12210.1, ACI66559.1, ACN12791.1, ACN12547.1, HBB1_ONCMY, ACM08711.1, ACI68343.1, CAA65954.1 | 7.2   | 34    | 4.7   | <0.001  | 0.009   |
| 5  | Hemagglutinin/amebocyte aggregation factor precursor ( <i>Salmo salar</i> )  | ACI69514.1, ACI68653.1, ACI69319.1, NP_001134776.1  | 2.5   | 11.4  | 4.6   | 0.002   | 0.017   |
| 6  | Neural precursor cell expressed, developmentally down-regulated 8, or NEDD8 precursor ( <i>Danio rerio</i> , <i>Esox lucius</i> , <i>Anoplopoma fimbria</i> , <i>Salmo salar</i> , <i>Osmerus mordax</i> , <i>Oncorhynchus mykiss</i> ), novel ubiquitin-like protein ( <i>Danio rerio</i> ) | NP_001002557.1, ACO13376.1, ACQ58998.1, CAF97715.1, ACI66066.1, XP_001331626.1, NP_958478.1, ACO09617.1, ACI66839.1, ACI69149.1, CAQ14646.1, ACO07821.1, CAF89342.1, ACI68249.1, ACI67176.1, CAG05755.1   | 0     | 4     | >4.0  | 0.012   | 0.032   |
| 7  | D-dopachrome decarboxylase ( <i>Salmo salar</i> , <i>Osmerus mordax</i> )  | ACM08272.1, ACO09793.1, ACM09324.1, ACM08253.1, ACI69753.1  | 0.6   | 2.5   | 3.9   | 0.033   | 0.064   |
| 8  | Nattectin precursor ( <i>Salmo salar</i> )   | ACI69483.1, ACI67625.1, ACI68243.1  | 6     | 20.7  | 3.5   | 0.017   | 0.041   |
| 9  | Caspase 6 precursor ( <i>Oncorhynchus mykiss</i> ), caspase 6B and 6A ( <i>Salmo salar</i> )   | AAAY28971.1, NP_001117743.1, AAY28974.1   | 6.8   | 22.2  | 3.2   | 0.005   | 0.029   |
| 10 | Complement C3-1 (s), Complement C3 ( <i>Salmo marmoratus</i> )   | AAB05029.1, ACF75925.1  | 5.3   | 16    | 3     | 0.006   | 0.032   |
| 11 | Apolipoprotein A-I-1 and A-I-2 precursor ( <i>Oncorhynchus mykiss</i> , <i>Salmo trutta</i> , <i>Salmo salar</i> )   | ACI69572.1, NP_001134612.1  | 5.7   | 14.5  | 2.5   | 0.026   | 0.054   |
| 12 | Lumican ( <i>Salmo salar</i> )   | NP_001133534.1  | 0     | 2.4   | >2.4  | 0.01    | 0.032   |
| 13 | Transgelin ( <i>Salmo salar</i> , <i>Esox lucius</i> , <i>Osmerus mordax</i> ), smooth muscle cell-specific protein SM22 alpha ( <i>Epinephelus coioides</i> )   | ACM09025.1, ACN12512.1, ACO13748.1, ACI67196.1, ACO14008.1, ACO09284.1, ACI66772.1, NP_001134980.1, ABW04145.1  | 0     | 2.4   | >2.4  | 0.012   | 0.032   |
| 14 | Annexin A1 ( <i>Salmo salar</i> , <i>Osmerus mordax</i> ), Annexin max1 and max 3 ( <i>Oryzias latipes</i> )   | NP_001134743.1, NP_001098295.1, ACO09900.1  | 0     | 2.3   | >2.3  | 0.009   | 0.032   |
| 15 | Plastin-2 ( <i>Salmo salar</i> , <i>Danio rerio</i> ), plastin 1 ( <i>Danio rerio</i> ), lymphocyte cytosolic protein 1 precursor ( <i>Oncorhynchus mykiss</i> )   | ACI70203.1, NP_571395.1, CAM82803.1, ACM09836.1, CAF95455.1, CAN88059.1, CAG02957.1, NP_956175.1, CAF91288.1, ACM09824.1  | 0     | 2.1   | >2.1  | 0.012   | 0.032   |

## Differentially expressed proteins in mucus of AGD-affected Atlantic salmon

| #  | Description  | Accession number   | SpC<br>C | SpC<br>D | FC              | P-<br>value      | Q-<br>value  |
|----|--|--|----------|----------|-----------------|------------------|--------------|
| 16 | Anterior gradient protein 2 homologue precursor ( <i>Salmo salar</i> , <i>Esox lucius</i> )  | ACI66035.1, ABB96968.1, ABB96969.1, ACI69433.1, ACM09796.1, ACI67616.1, ACO13414.1   | 0        | 1.8      | >1.8            | 0.013            | 0.032        |
| 17 | PolyrC-binding protein 2 ( <i>Salmo salar</i> , <i>Danio rerio</i> , <i>Osmerus mordax</i> )   | ACI34263.1, NP_001133381.1, NP_957486.1, ACO09656.1  | 0        | 1.7      | >1.7            | 0.012            | 0.032        |
| 18 | Ecto-ADP-ribosyltransferase 4 precursor ( <i>Salmo salar</i> )   | AAF89686.1AF170069_1, ACI69791.1, NP_570987.1, NP_001133774.1<br>ACN11170.1, ACL99859.1  | 0.6      | 0        | <-0.6           | 0.047            | 0.071        |
| 19 | Ester hydrolase C11orf54 homologue ( <i>Salmo salar</i> )  | NP_001134380.1, ACI67203.1   | 0.6      | 0        | <-0.6           | 0.047            | 0.071        |
| 20 | FucU homologue ( <i>Salmo salar</i> )  | NP_001133117.1   | 0.7      | 0        | <-0.7           | 0.043            | 0.071        |
| 21 | Myosin binding protein H-like ( <i>Salmo salar</i> )   | ACN12357.1   | 0.7      | 0        | <-0.7           | 0.043            | 0.071        |
| 22 | Phospholipase B-like 1 ( <i>Salmo salar</i> )  | ACN10983.1, ACN60282.1   | 0.7      | 0        | <-0.7           | 0.043            | 0.071        |
| 23 | Integrin beta-1 precursor ( <i>Salmo salar</i> )   | ACN11424.1, ACN58712.1   | 0.9      | 0        | <-0.9           | 0.007            | 0.032        |
| 24 | Inorganic pyrophosphatase ( <i>Salmo salar</i> , <i>Oncorhynchus mykiss</i> , <i>Anoplopoma fimbria</i> , <i>Danio rerio</i> , <i>Osmerus mordax</i> )   | ACI66684.1, ACI69926.1, ACO08390.1, ACQ57839.1, ACI67039.1, CAX14788.1,<br>ACI67097.1, NP_001017833.1, ACO08739.1, ACI68982.1, ACO08872.1    | 0.9      | 0        | <-0.9           | 0.008            | 0.032        |
| 25 | S100-A11 ( <i>Salmo salar</i> )  | ACI69172.1   | 0.9      | 0        | <-0.9           | 0.008            | 0.032        |
| 26 | Catalase ( <i>Danio rerio</i> , <i>Salmo salar</i> , <i>Ctenopharyngodon idella</i> )  | NP_001135055.1   | 1        | 0        | <-1.0           | 0.046            | 0.071        |
| 27 | FK506 binding protein 2, 13kDa ( <i>Danio rerio</i> , <i>Osmerus mordax</i> , <i>Oncorhynchus mykiss</i> , <i>Salmo salar</i> ), Zgc:101826 protein ( <i>Danio rerio</i> )   | NP_001004677.1, ACO10067.1, ACO08817.1, ACI67147.1, ACI67860.1,<br>ACI68911.1, ACI66312.1, ACI70071.1, AAI55100.1                            | 1        | 0        | <-1.0           | 0.009            | 0.032        |
| 28 | Cleavage and polyadenylation specificity factor subunit 5 ( <i>Esox lucius</i> , <i>Salmo salar</i> , <i>Danio rerio</i> , <i>Oncorhynchus mykiss</i> )  | ACI66857.1, ACI68640.1, ACN12429.1, ACI68292.1, ACI66239.1, ACI69177.1   | 1.1      | 0        | <-1.1           | 0.047            | 0.071        |
| 29 | Calcium/calmodulin-dependent protein kinase type II delta chain ( <i>Salmo salar</i> )   | ACN58731.1   | 1.2      | 0        | <-1.2           | 0.009            | 0.032        |
| 30 | N-acetylglucosamine-6-sulfatase precursor ( <i>Salmo salar</i> )   | ACO14322.1, CAF99562.1, ACM08683.1, ACM08212.1, AAI65427.1,<br>ACN12771.1, CAN88764.1, ACM09751.1, ACO08099.1, XP_001921533.1,<br>ACI68139.1 | 1.2      | 0        | <-1.2           | 0.045            | 0.071        |
| 31 | Nuclear transport factor 2 ( <i>Salmo salar</i> , <i>Oncorhynchus mykiss</i> , <i>Esox lucius</i> )  | ACN12528.1, ACI68533.1, ACI66047.1, ACO08669.1, ACO14237.1, ACI66665.1,<br>ACO13744.1  | 1.2      | 0        | <-1.2           | 0.005            | 0.029        |
| 32 | Lipocalin precursor ( <i>Salmo salar</i> )   | ACI68588.1, ACI68110.1, ACI69560.1   | 23.9     | 20.7     | -1.2            | 0.035            | 0.067        |
| 33 | Lysosomal protective protein ( <i>Salmo salar</i> )  | NP_001133654.1   | 1.4      | 0        | <-1.4           | 0.002            | 0.016        |
| 34 | Ferritin, heavy subunit ( <i>Salmo salar</i> , <i>Osmerus mordax</i> , <i>Dicentrarchus labrax</i> , <i>Oncorhynchus mykiss</i> , <i>O. masou formosanus</i> , <i>O. nerka</i> , <i>Chionodraco rastrospinosus</i> ) | P85837.1, ACO09727.1, CAF92096.1, ACN80998.1, ACN12571.1, ACO07472.1,<br>CAL92185.1, P85838.1, ABY21333.1, AAK08117.1, AF338763_1            | 1.6      | 0        | <-1.6           | 0.005            | 0.029        |
| 35 | Cathepsin S precursor ( <i>Salmo salar</i> )   | ACN12760.1   | 1.8      | 0        | <-1.8           | 0.01             | 0.032        |
| 36 | <b>Aldehyde dehydrogenase family 7 member A1 homologue (<i>Salmo salar</i>)</b>  | <b>NP_001133255.1</b>  | <b>2</b> | <b>0</b> | <b>&lt;-2.0</b> | <b>&lt;0.001</b> | <b>0.009</b> |

## Differentially expressed proteins in mucus of AGD-affected Atlantic salmon

| #  | Description  | Accession number  | SpC<br>C | SpC<br>D | FC    | P-<br>value | Q-<br>value |
|----|--|---|----------|----------|-------|-------------|-------------|
| 37 | Arhgdia protein ( <i>Danio rerio</i> ), Rho GDP dissociation inhibitor (GDI) alpha ( <i>Danio rerio</i> , <i>Salmo salar</i> )   | AAI64027.1, AAH63968.1, ACI33476.1, CAG06134.1  | 2.8      | 1.4      | -2    | 0.038       | 0.07        |
| 38 | Myeloperoxidase precursor ( <i>Salmo salar</i> )   | ACN60208.1  | 29       | 13.5     | -2.2  | 0.002       | 0.017       |
| 39 | Fibronectin 1b ( <i>Danio rerio</i> ), Fibronectin ( <i>Salmo salar</i> )  | CAM13026.2, CAG10108.1, CAQ13985.1, CAF99793.1, NP_571595.1, AAI62574.1, AAP06798.1, ACN60244.1, NP_001013279.1, AAU14809.1, AAP80678.1     | 2.5      | 0.7      | -3.4  | 0.032       | 0.063       |
| 40 | Cystatin precursor ( <i>Salmo salar</i> )  | ACI68361.1, NP_001134331.1  | 1.3      | 0.4      | -3.7  | 0.042       | 0.071       |
| 41 | Gelsolin precursor ( <i>Salmo salar</i> ), scinderin-like $\alpha$ /gelsolin protein ( <i>Epinephelus coioides</i> )   | ACL98119.1, ACN60223.1  | 2.8      | 0.8      | -3.7  | 0.019       | 0.043       |
| 42 | Beta-hexosaminidase beta chain ( <i>Salmo salar</i> )  | NP_001135106.1  | 3.8      | 0        | <-3.8 | <0.001      | 0.009       |
| 43 | CREG1 precursor ( <i>Osmerus mordax</i> ), CREG1 protein ( <i>Salmo salar</i> )  | ACO08939.1, NP_001133995.1  | 1.6      | 0.4      | -4    | 0.02        | 0.045       |
| 44 | C-reactive protein precursor ( <i>Salmo salar</i> )  | ACN12329.1  | 2.1      | 0.4      | -5.5  | 0.006       | 0.032       |
| 45 | SH3 domain-binding glutamic acid-rich-like protein ( <i>Anoplopoma fimbria</i> , <i>Salmo salar</i> , <i>Osmerus mordax</i> , <i>Oncorhynchus mykiss</i> )   | ACQ58643.1, ACN12504.1, ACO10145.1, ACO08489.1  | 4.3      | 0.8      | -5.5  | <0.001      | 0.009       |
| 46 | Malate dehydrogenase, mitochondrial precursor ( <i>Salmo salar</i> , <i>Osmerus mordax</i> , <i>Danio rerio</i> , <i>Sphyaena idiaestes</i> )  | NP_001135174.1, ACO10024.1, ACI67899.1, ACH70948.1, CAG12894.1, NP_998296.1, ACI68220.1, AAK69767.1, NP_001133198.1, ACI66609.1, ACI66741.1 | 5.7      | 0        | <-5.7 | <0.001      | 0.009       |
| 47 | AMBP protein precursor ( <i>Salmo salar</i> )  | ACI68724.1  | 4.67     | 0.7      | -6.6  | <0.001      | 0.009       |
| 48 | Ganglioside GM2 activator, Ganglioside GM2 activator precursor ( <i>Salmo salar</i> )  | NP_001133828.1, ACN11254.1  | 2.6      | 0.4      | -6.7  | 0.002       | 0.017       |
| 49 | Gamma-interferon-inducible lysosomal thiol reductase precursor ( <i>Salmo salar</i> )  | ACN12705.1, ACI69367.1, ACI66390.1, ACI67174.1  | 4.5      | 0.7      | -6.8  | <0.001      | 0.009       |
| 50 | Cathepsin Z precursor ( <i>Osmerus mordax</i> , <i>Danio rerio</i> , <i>Salmo salar</i> , <i>Oncorhynchus mykiss</i> , <i>Cyprinus carpio</i> , <i>Fundulus heteroclitus</i> ), Cathepsin Y ( <i>Oncorhynchus mykiss</i> ), Cathepsin ( <i>Siniperca chuatsi</i> ) | ACO09238.1, NP_001117967.1, ACO07857.1, ACN60340.1, AAY79283.1, AAX51298.1, AAO64476.1, NP_001006043.1, ACN60319.1                          | 4.8      | 0.5      | -9    | 0.026       | 0.054       |
| 51 | Carbonic anhydrase ( <i>Anoplopoma fimbria</i> , <i>Oncorhynchus mykiss</i> , <i>Salmo salar</i> )   | ACQ58008.1, AAR99329.1, NP_001117693.1, ACN10477.1, NP_001117692.1  | 7.2      | 0.7      | -10.2 | 0.01        | 0.032       |
| 52 | Granulins precursor ( <i>Salmo salar</i> )   | ACN10922.1  | 3.1      | 0.2      | -17.6 | 0.007       | 0.032       |

Supplementary Table 6.2 Proteins significantly and differentially abundant in skin mucus of AGD-affected Atlantic salmon. Proteins were identified by nanoLC-MS/MS. Proteins with  $P < 0.05$  and fold change  $> 2.0$  are in bold letters. SpC C, Spectral count control group; SpC D, spectral count diseased (AGD) group; FC, Fold change.

| #  | Description   | Accession numbers  | SpC C | SpC D | FC   | P-value | Q-value |
|----|---|--|-------|-------|------|---------|---------|
| 1  | <b>S100-A16 (<i>Salmo salar</i>)</b>  | NP_001134817.1   | 0.4   | 3.5   | 9.7  | 0.001   | 0.019   |
| 2  | <b>Cystathionine gamma-lyase inhibitor (<i>Oncorhynchus mykiss</i>), Cystathionine gamma-lyase (<i>Salmo salar</i>)</b>   | NP_001118157.1, ACN10804.1   | 0.4   | 2     | 5    | 0.015   | 0.11    |
| 3  | <b>Coactosin-like 1 (<i>Ictalurus punctatus</i>, <i>Salmo salar</i>)</b>  | ABC75560.1, ACN12230.1, CAF98914.1   | 0.4   | 1.9   | 4.7  | 0.027   | 0.148   |
| 4  | <b>Major vault protein (<i>Salmo salar</i>)</b>   | ACN10921.1   | 1.2   | 5.7   | 4.7  | 0.001   | 0.021   |
| 5  | <b>Eukaryotic translation initiation factor 4H (<i>Salmo salar</i>, <i>Danio rerio</i>, <i>Osmerus mordax</i>)</b>  | NP_001133347.1, NP_991258.1, ACO10072.1, ACN10439.1  | 0.6   | 2.7   | 4.6  | 0.011   | 0.103   |
| 6  | <b>Ribosomal protein S7 (<i>Solea senegalensis</i>, <i>Epinephelus coioides</i>, <i>Danio rerio</i>, <i>Takifugu rubripes</i>), 40S ribosomal protein S7 (<i>Oncorhynchus mykiss</i>, <i>Danio rerio</i>, <i>Salmo salar</i>, <i>Fugu rubripes</i>, <i>Perca flavescens</i>, <i>Oncorhynchus mykiss</i>, <i>Ictalurus punctatus</i>)</b>                                    | BAF45895.1, ACH73065.1, CAG01472.1, NP_957046.1, CAA64412.1, ACO08212.1, ACI66988.1, ACI66768.1, ABU54857.1, ACN12304.1, ACI66314.1, NP_001117902.1, ACI67293.1  | 0.5   | 2.1   | 4    | 0.034   | 0.168   |
| 7  | <b>Ribosomal protein L4 (<i>Danio rerio</i>, <i>Oncorhynchus mykiss</i>, <i>Salmo salar</i>), 60S ribosomal protein L4-A (<i>Salmo salar</i>, <i>Osmerus mordax</i>)</b>  | CAK04710.1, CAC43331.1, ACM09838.1, CAC44155.1, ACH70798.1, ACM08761.1, ACO09148.1, CAN88105.1   | 0.7   | 2.7   | 3.8  | 0.037   | 0.172   |
| 8  | <b>SH3 domain-binding glutamic acid-rich-like protein 3 (<i>Salmo salar</i>)</b>  | ACI66430.1   | 0     | 3.1   | >3.1 | 0.001   | 0.024   |
| 9  | <b>Actinin alpha 4 (<i>Danio rerio</i>)</b>   | NP_955880.1  | 1.3   | 3.9   | 3    | 0.007   | 0.088   |
| 10 | <b>Fructose-bisphosphate aldolase A (<i>Osmerus mordax</i>, <i>Esox lucius</i>, <i>Salmo salar</i>, <i>Oryzias latipes</i>, <i>Epinephelus coioides</i>, <i>Danio rerio</i>), aldolase A (<i>Danio rerio</i>), aldolase (<i>Ictalurus punctatus</i>)</b>  | ACO09344.1, ACO14552.1, NP_001133180.1, AAN04476.1, NP_001133181.1, AAO25766.1, BAD17895.1, ACL98138.1, NP_998380.1, AAQ94593.1, ACN10700.1  | 1.7   | 4.9   | 3    | 0.018   | 0.11    |
| 11 | <b>Alanyl-tRNA synthetase, cytoplasmic (<i>Salmo salar</i>)</b>   | NP_001133550.1   | 2     | 5.4   | 2.7  | 0.017   | 0.11    |
| 12 | <b>Proteasome subunit beta type 1-A (<i>Salmo salar</i>, <i>Oncorhynchus mykiss</i>, <i>Carassius auratus</i>, <i>Danio rerio</i>, <i>Osmerus mordax</i>, <i>Gillichthys mirabilis</i>, <i>Anoplopoma fimbria</i>, <i>Esox lucius</i>) 20S proteasome beta subunit (<i>Pagrus major</i>, <i>Cirrhinus molitorella</i>), proteasome beta-subunit C5 (<i>Danio rerio</i>)</b> | ACO07867.1, AAZ73764.1, ACO09437.1, ACI68289.1, AAG13340.1, AF266220_1, ACO08500.1, ACO08383.1, ACQ58517.1, CAG11005.1, AAT68124.1, XP_001921270.1, ACQ58613.1, ACO13854.1, ACI67092.1, ACO07486.1, AAP20145.1, ACO07501.1 | 1.3   | 3.5   | 2.7  | 0.034   | 0.168   |
| 13 | <b>Glutathione transferase omega-1 (<i>Salmo salar</i>, <i>Oncorhynchus mykiss</i>)</b>   | ACN10920.1   | 0     | 2.5   | >2.5 | <0.001  | 0.006   |
| 14 | <b>Eukaryotic translation initiation factor 5A-1 (<i>Salmo salar</i>, <i>Esox lucius</i>)</b>   | ACI34012.1, ACO14228.1, ACM08851.1, ACM08204.1   | 1.3   | 3.3   | 2.5  | 0.038   | 0.172   |
| 15 | <b>Cystatin precursor (<i>Salmo salar</i>)</b>  | ACI66857.1, ACI68640.1, ACN12429.1, ACI68292.1, ACI66239.1, ACI69177.1   | 0     | 2.4   | >2.4 | <0.001  | 0.006   |

## Differentially expressed proteins in mucus of AGD-affected Atlantic salmon

| #  | Description   | Accession numbers   | SpC<br>C | SpC<br>D | FC   | P-<br>value | Q-<br>value |
|----|---|---|----------|----------|------|-------------|-------------|
| 16 | Betaine-homocysteine methyltransferase ( <i>Salmo salar</i> )   | NP_001133157.1  | 1.2      | 2.9      | 2.4  | 0.018       | 0.11        |
| 17 | Glutamate dehydrogenase 1, mitochondrial precursor ( <i>Salmo salar</i> )   | ACN10920.1  | 0        | 2.3      | >2.3 | <0.001      | 0.006       |
| 18 | Keratin 12 ( <i>Oncorhynchus mykiss</i> )   | CAD20811.1  | 5.3      | 11.3     | 2.2  | 0.001       | 0.02        |
| 19 | Keratin type I ( <i>Epinephelus coioides</i> , <i>Sparus aurata</i> , <i>Oncorhynchus mykiss</i> )  | ACL98136.1, CAG13267.1, ACN62548.1, NP_001117826.1  | 0        | 2.1      | >2.1 | 0.011       | 0.102       |
| 20 | Aminopeptidase B ( <i>Salmo salar</i> , <i>Osmerus mordax</i> ), Arginyl aminopeptidase ( <i>Danio rerio</i> )  | ACN10761.1, NP_001002741.1, ACO08981.1  | 0        | 1.8      | >1.8 | 0.014       | 0.11        |
| 21 | Barrier-to-autointegration factor ( <i>Oncorhynchus mykiss</i> , <i>Salmo salar</i> , <i>Esox lucius</i> , <i>Osmerus mordax</i> )  | ACO07632.1, ACI69910.1, ACO13844.1, ACM08201.1, ACI66267.1, ACO08277.1, ACO09060.1  | 0        | 1.8      | >1.8 | 0.001       | 0.028       |
| 22 | Cathepsin H precursor ( <i>Salmo salar</i> ), Cathepsin H precursor ( <i>Salmo salar</i> )  | ACI66855.1, ACI66895.1  | 0        | 1.6      | >1.6 | 0.009       | 0.093       |
| 23 | Deoxyribonuclease gamma precursor ( <i>Salmo salar</i> )  | ACI70073.1, ACI69566.1  | 18.8     | 29.5     | 1.6  | 0.029       | 0.15        |
| 24 | Acidic leucine-rich nuclear phosphoprotein 32 family member A ( <i>Danio rerio</i> ) and family member B ( <i>Salmo salar</i> , <i>Danio rerio</i> )  | ACN11434.1, ACM08449.1, AAI65448.1, ACN12741.1, CAG01930.1, NP_997768.3   | 0        | 1.5      | >1.5 | 0.002       | 0.035       |
| 25 | Anterior gradient-2-like protein 1 ( <i>Salmo salar</i> ), Anterior gradient protein 2 homologue precursor ( <i>Salmo salar</i> , <i>Esox lucius</i> ), Anterior gradient homologue 2 ( <i>Xenopus laevis</i> , <i>Danio rerio</i> )  | ABB96968.1, ACI69433.1, ABB96969.1, ACM09796.1, ACI67616.1, ACO13414.1, AAI52145.1, CAM56358.1  | 6.9      | 10.5     | 1.5  | 0.036       | 0.172       |
| 26 | Guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1 ( <i>Danio rerio</i> , <i>Salmo salar</i> , <i>Osmerus mordax</i> , <i>Esox lucius</i> , <i>Oreochromis niloticus</i> , <i>Anoplopoma fimbria</i> , <i>Danio rerio</i> ), receptor for activated protein kinase c ( <i>Pagrus major</i> , <i>Dicentrarchus labrax</i> , <i>Oncorhynchus mykiss</i> , <i>Sander vitreus</i> , <i>Paralichthys olivaceus</i> , <i>Oreochromis mossambicus</i> , <i>Platichthys flesus</i> ), activated protein kinase C ( <i>Epinephelus akaara</i> ) | NP_571519.1, ACN11311.1, CAG01204.1, ACO09909.1, AAP20196.1, ACO14497.1, ABI26262.1, NP_001118140.1, ABX90099.1, AAT35603.1, ACQ58047.1, ACN12499.1, AAQ91574.1, AAP40018.1, CAE53390.1 | 0        | 1.4      | >1.4 | 0.009       | 0.091       |
| 27 | FAM139A ( <i>Salmo salar</i> )  | ACN60260.1  | 0        | 1.3      | >1.3 | 0.019       | 0.11        |
| 28 | Tumor-associated calcium signal transducer 2 precursor ( <i>Salmo salar</i> )   | ACI69332.1, NP_001134932.1, ACI67643.1, ACI68675.1  | 0        | 1.3      | >1.3 | 0.004       | 0.057       |
| 29 | APEX nuclease 1 ( <i>Salmo salar</i> )  | NP_001135227.1  | 0        | 1.2      | >1.2 | 0.008       | 0.091       |
| 30 | Cleavage and polyadenylation specificity factor subunit 5 ( <i>Salmo salar</i> , <i>Esox lucius</i> , <i>Danio rerio</i> , <i>Oncorhynchus mykiss</i> )   | ACO14322.1, CAF99562.1, ACM08683.1, ACM08212.1, AAI65427.1, ACN12771.1, CAN88764.1, ACM09751.1, ACO08099.1, XP_001921533.1, ACI68139.1  | 0        | 1        | >1.0 | 0.019       | 0.11        |

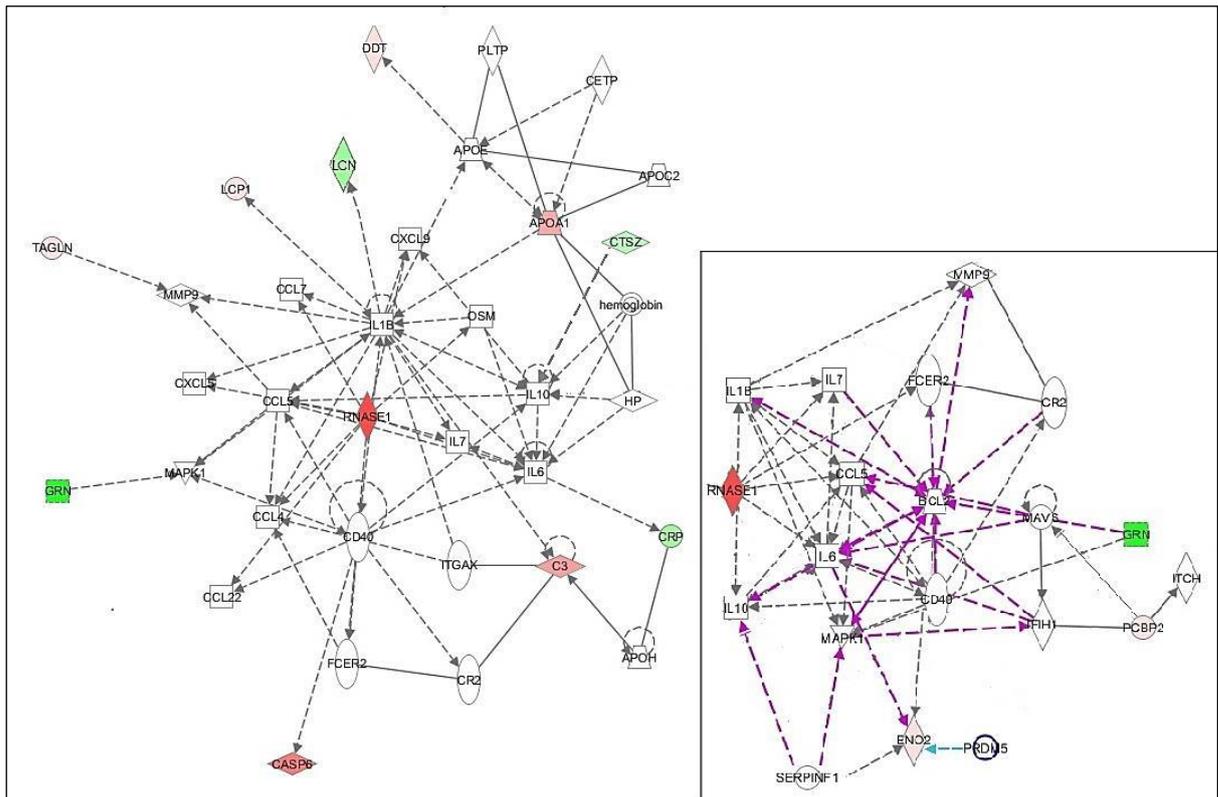
## Differentially expressed proteins in mucus of AGD-affected Atlantic salmon

| #  | Description  | Accession numbers  | SpC<br>C    | SpC<br>D    | FC          | P-<br>value      | Q-<br>value  |
|----|--|--|-------------|-------------|-------------|------------------|--------------|
| 31 | Actin-related protein 3 ( <i>Salmo salar</i> , <i>Esox lucius</i> )  | ACI69786.1, ACO13358.1   | 0           | 0.8         | >0.8        | 0.019            | 0.11         |
| 32 | PREDICTED: similar to nonmuscle myosin heavy chain ( <i>Danio rerio</i> )  | XP_001920004.1, CAG10783.1, XP_683046.3, CAE30366.1, CAF92169.1  | 0           | 0.7         | >0.7        | 0.018            | 0.11         |
| 33 | Myosin-9 ( <i>Salmo salar</i> )  | ACN60211.1, CAF91216.1, CAG06107.1, XP_001920024.1   | 0           | 0.6         | >0.6        | 0.019            | 0.11         |
| 34 | Serum albumin 1 and 2 precursor ( <i>Salmo salar</i> )   | spQ03156, ALBU2_SALSA, spP21848, ALBU1_SALSA   | 140.6       | 109.4       | -1.3        | 0.005            | 0.074        |
| 35 | Myeloperoxidase precursor ( <i>Salmo salar</i> )   | ACN60208.1   | 15.8        | 9.8         | -1.6        | 0.015            | 0.11         |
| 36 | Unnamed protein product ( <i>Danio rerio</i> )   | CAR80295.1   | 30          | 19.2        | -1.6        | 0.02             | 0.11         |
| 37 | Hemoglobin subunit alpha ( <i>Salmo salar</i> , <i>Misgurnus anguillicaudatus</i> )  | ACN12417.1, ACN12305.1, ACN12747.1, ACO08777.1, ACQ59075.1, ACO08865.1, BAC20295.1, ACO13595.1, ACO07584.1, AAB24975.1, ACO07564.1, 1708181A, ACO07614.1, ACO13254.1, ACN12759.1, BAC20294.1, ACQ58404.1, ACO08140.1, ACQ57918.1, AAK12633.1, ACO14036.1, ACO14182.1, ABJ98630.1, ACN12658.1, CAA65949.1, AAM93258.1, ACN12527.1, ACN12414.1, ACO08002.1, ACO07580.1, CAA65946.1, ACI68793.1, ACN12530.1 | 23.5        | 13.9        | -1.7        | 0.002            | 0.035        |
| 38 | Complement C3 group ( <i>Oncorhynchus mykiss</i> , <i>Salmo marmoratus</i> )   | AAB05029.1, ACF75925.1   | 13.6        | 7           | -1.9        | 0.003            | 0.046        |
| 39 | <b>Carbonic anhydrase II</b> ( <i>Oncorhynchus mykiss</i> ), <b>carbonic anhydrase</b> ( <i>Salmo salar</i> )                    | <b>NP_001117693.1, ACN10477.1</b>  | <b>5.7</b>  | <b>1.8</b>  | <b>-3.1</b> | <b>0.015</b>     | <b>0.11</b>  |
| 40 | <b>Hemoglobin subunit beta</b> ( <i>Salmo salar</i> , <i>Oncorhynchus mykiss</i> , <i>O.masou formosanus</i> , <i>O. nerka</i> ) | <b>ACI68214.1, ACM08711.1, ACI68343.1, ACI69922.1, ACH70759.1, ACI68603.1, ACI66413.1, ACN12210.1, ACN12791.1, ACN12547.1, ACN12702.1, ACI66980.1, ACO07576.1, ACO07923.1, 1009195A, ABY21329.1, ACO08038.1, ACO08017.1</b>  | <b>44.6</b> | <b>12.1</b> | <b>-3.7</b> | <b>0.007</b>     | <b>0.084</b> |
| 41 | <b>Tubulin beta-2C chain</b> ( <i>Salmo salar</i> )  | <b>NP_001134313.1, NP_001133265.1, ABQ59661.1</b>  | <b>1.9</b>  | <b>0.4</b>  | <b>-5.1</b> | <b>0.029</b>     | <b>0.15</b>  |
| 42 | <b>Complement component C9</b> ( <i>Oncorhynchus mykiss</i> )  | <b>NP_001117898.1, CAA29037.1</b>  | <b>3.9</b>  | <b>0.4</b>  | <b>-9.9</b> | <b>&lt;0.001</b> | <b>0.014</b> |

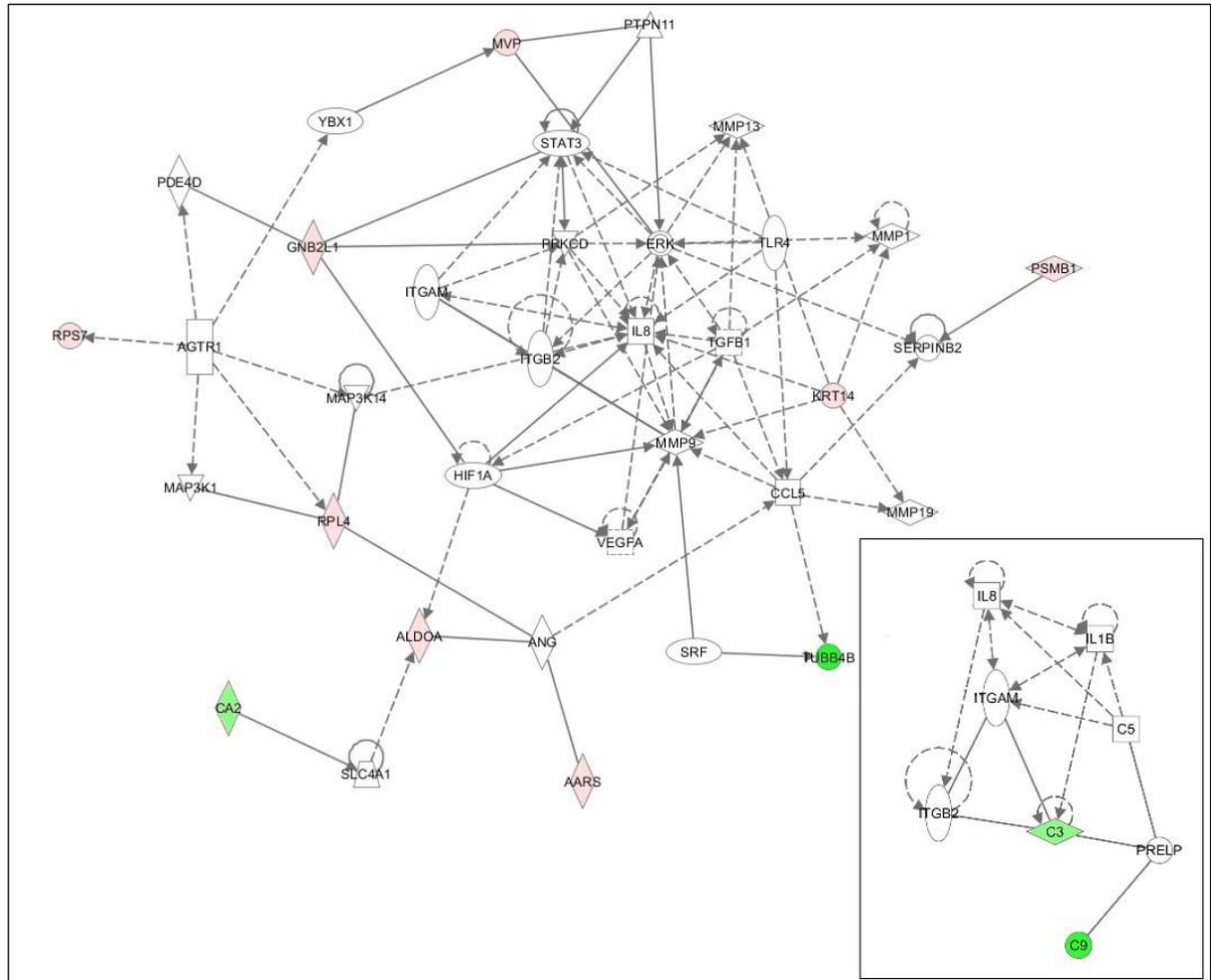
**Differentially expressed proteins in mucus of AGD-affected Atlantic salmon**

To better understand the relationships within the cohort of differentially abundant proteins, Ingenuity Pathway Analysis was used to map interaction networks for gill and skin mucus proteins that were altered between control and AGD-affected fish (Supplementary Figure 6.3 for gill and Supplementary Figure 6.4 for skin protein). In addition to the proteins mapped onto the pathway, there were a number of proteins differentially modulated in gill mucus of AGD-affected salmon, but not represented in the interaction networks. Among these, S-100, FK506, annexin and AG-2 have been previously described as regulated in AGD-affected gill [108]. Atlantic salmon AG-2 protein is detected predominantly in epithelia-rich gill and intestine of Atlantic salmon and it has been previously found to be up-regulated in gill lesions of AGD-affected fish using microarray analyses [108]. Immunohistochemical studies of AGD lesions have demonstrated that AG-2 is highly expressed in cells within the lesions [242, 243]. Our results are in agreement with this previous findings, since the high levels of expression of this protein in the mucus samples analysed, could be explained by the increase number of mucous cells present in AGD lesions [55, 240].

Annexin A1 was detected at elevated levels in the gill mucus of AGD-affected fish (Table 6.1). Gene expression of this protein was up-regulated in AGD-affected Atlantic salmon, both in lesion and lesion-free sites [109]. Annexin A1 is a calcium dependant phospholipid binding protein [258], that has certain anti-inflammatory effects and its expression is increased by endogenous glucocorticoids [259]. Annexin A1 can regulate the activities of innate immune cells, in particular the generation of pro-inflammatory mediators, ensuring that a sufficient level of activation is reached but not exceeded in neutrophils and macrophages [259].



**Supplementary Figure 6.3** Ingenuity pathway of three proteins networks identified during the experiment in gill mucus of Atlantic salmon affected by AGD, one in main image and two in the inset (one in grey, one in purple). Each gene involved in the pathway is denoted by their ENTREZ gene symbol or in some cases full gene name. The proteins indicated in coloured circles showed statistically significant ( $P < 0.05$ ) differential expression by beta-binomial distribution analysis in R (red denotes over expressed, while green denotes under expressed). Figures in white indicate other proteins involved in the pathway. Solid arrows indicate direct protein interactions and dashed arrows indicate indirect protein interactions. Pathway analysis was done based on the mammalian orthologues of the proteins identified.



**Supplementary Figure 6.4** Two ingenuity pathways of proteins identified during the experiment in skin mucus of Atlantic salmon affected by AGD. Each gene involved in the pathway is denoted by their ENTREZ gene symbol or in some cases full gene name. The proteins indicated in coloured circles showed statistically significant ( $P < 0.05$ ) differential expression by beta-binomial distribution analysis in R (red denotes over expressed, while green denotes under expressed). Figures in white indicate other proteins involved in the pathway. Solid arrows indicate direct protein interactions and dashed arrows indicate indirect protein interactions. Pathway analysis was done based on the mammalian orthologues of the proteins identified.

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Gill mucus proteins generated 3 noteworthy networks, including “Cell to Cell Signalling and Haematological System Function”, “Infectious Disease” and “Cell Death and Survival” (Supplementary Figure 6.3). Proteins belonging to the networks showed a high level of interaction with cytokines, in particular the pro-inflammatory molecules IL-1 $\beta$  and IL-6. Gene expression of IL-1 $\beta$  has been previously shown to be up-regulated in Atlantic salmon affected by AGD [107, 109, 110, 241]. However, our data are the first to provide evidence at the protein level for IL-1 $\beta$  associated response. The elevated levels of C3 that we detected in gill mucus may therefore be the result of increased IL-1 $\beta$  signalling. Complement is an integral part of the immune system of vertebrates [260]. C3 and C9 both form part of the classical and alternative complement pathways, and C9 also forms part of the membrane attack complex. Complement factor C3 has been previously identified in the mucus of Atlantic salmon infected with sea lice (*Lepeophtheirus salmonis*) [126] and up-regulated in AGD lesions [108]. Additionally, it has been recently found to be associated with IgT in the host-response to a skin parasite [261].

Also identified by network analysis in association with IL- $\beta$ 1 was lipocalin precursor (LCN) (Supplementary Figure 6.3), which was only slightly down-expressed in the analysis. LCN gene expression was down-regulated in Atlantic salmon cell cultures when exposed to rIL- $\beta$ 1 [262], and this relationship could explain its low expression in the present study. It is not clear what the exact effect of this protein could have on the pathology of AGD, but it is likely related with the production of sterols.

In the present study, the expression of C reactive protein (CRP) was diminished in gill mucus, perhaps showing characteristics of a negative acute phase protein response. CRP form part of a group of evolutionary conserved proteins, the pentraxins, which also includes CRP homologues and serum amyloid protein [81]. CRP is an acute-phase serum protein and a mediator of innate immunity; both pro-inflammatory and anti-inflammatory activities have been described for CRP. As a pro-inflammatory protein, CRP increases the release of IL-6, IL-1, IL-8 and TNF- $\alpha$ , enhances phagocytosis and activates complement (as reviewed by Ref. [263]). In teleosts, serum acute phase proteins have shown different patterns of production depending on the kind of inflammatory stimuli: in some cases serum levels have

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decreased (negative acute phase protein) [264-266] and in other instances serum CRP has increased (positive acute phase protein) [267-269]. The expression of CRP in mucus has been described before, with only a slight increase in skin mucus of tilapia (*Tilapia mossambica*) very shortly after inflammation and necrosis had been induced by injury [270], and in this case it was present in very low quantities.

Natlectin precursor was increased in abundance in the AGD gill mucus. Natlectin is a C-type lectin which was identified in the venom of the Brazilian venomous fish *Thalassophryne nattereri* [271]. Natlectin promotes T-cell differentiation with production of Th1 cytokines with potent pro-inflammatory properties [272], such as the recruitment of neutrophils and IL-1 $\beta$  production in endothelial cell membranes. Its high expression in the gill mucus of AGD-affected fish could be associated with the high levels of expression of IL-1 $\beta$  described in AGD lesions [107, 109, 110, 241].

Transgelin (TAGLN) is an actin binding protein that was overexpressed in the gill mucus from AGD-affected fish. In the protein network described herein, TGLN was related to the matrix metalloprotease-9 (MMP-9) protein (Supplementary Figure 6.3), which is in agreement with previous findings describing the regulation of MMP-9 by TGLN [273]. However, TAGLN has been described as a target of transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) signalling in the human lung, where their interaction generates pulmonary fibrosis with epithelial hyperplasia, with TAGLN representing a key regulator of epithelial cell migration [274]. The gene expression of TGF- $\beta$ 1 in AGD-affected gill has been shown to remain unaffected despite infection [110]. However gill samples in that study, although from AGD-positive fish, were not specifically obtained from gill lesions. As the gene expression of TGF- $\beta$ 1 in actual lesions it is not known, then it is possible that TAGLN could have a similar role related to hyperplasia in the pathogenesis of AGD.

Apolipoprotein A-I (apoA-I) was another immune related protein with increased expression in gill mucus of AGD-affected fish. While the major role of apoA-I is the transport of high density lipoprotein particles, apolipoproteins have demonstrated potential antimicrobial activity in carp (*Cyprinus carpio* L.) epidermis and mucus [275]. Furthermore, apolipoproteins were differentially expressed in skin mucus of Atlantic salmon infected with sea lice [126].

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Myeloperoxidase (MPO) is a reactive intermediate species and one of the antimicrobial systems used by neutrophils (as reviewed by Ref. [276]); and it was significantly down-expressed in the gill mucus of AGD-affected fish. During neutrophil respiratory burst, MPO is released into the phagosome or to the outside of the cell and, in the presence of a halide, catalyses the formation of hypochlorous acid from hydrogen peroxide, which is able to kill microorganisms [276]. There is some evidence suggesting that this MPO-H<sub>2</sub>O<sub>2</sub>-halide system has amoebicidal activity in *Naegleria fowleri* infections in humans [277]. TNF- $\alpha$  has been shown to be regulator of MPO, reducing its mRNA expression levels *in vitro* [278, 279]. Our data showing reduced MPO levels are therefore consistent with evidence from gene expression studies that demonstrated TNF- $\alpha$  signalling in specific AGD lesions [112, 280].

Carbonic anhydrase (CA2) was significantly reduced in the gill mucus of AGD-affected Atlantic salmon (Table 6.1). Carbonic anhydrase has been previously reported from rainbow trout gill epithelia, probably associated with facilitating the diffusion of CO<sub>2</sub> from blood to water [281]. In humans, deficiencies in carbonic anhydrase are associated with metabolic acidosis due to renal tubular acidosis, resulting from either failure to recover sufficient alkaline ions or insufficient secretions of hydrogen protons [282]. This could potentially explain the extracellular acidosis reported in AGD-affected salmon [60].

In skin mucus, proteins like AG-2, MPO and carbonic anhydrase showed similar expression levels to those observed in gill mucus (Table 6.1 and Supplementary Table 6.2). The most significant protein-protein interaction network in skin mucus was related to “Inflammatory Response, Cell Death and Survival, Nutritional Disease”, but proteins were also identified as belonging to a network related to “Cellular Movement, Hematological System Development and Function, Hypersensitivity Response” (Supplementary Figure 6.4, insert). Various proteins were up-regulated in the first network, such as Alanyl-tRNA synthetase, which forms part of the Aminoacyl-tRNA synthetases (ARSs). In addition to protein synthesis, the ARSs are involved in multiple cellular processes including, rRNA synthesis, apoptosis, angiogenesis, and inflammation (as reviewed by Ref. [283]).

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Another protein with increased expression in skin mucus from AGD-affected fish was major vault protein (MVP). MVP is the main component of ribonucleoprotein organelles located in a variety of tissues and eukaryotic organisms [284-286] and has been associated with disease resistance in human epithelial cells. This protein can enhance apoptosis in macrophages and the synthesis of TNF- $\alpha$  [287], which may be one of the roles it played in the gills, as TNF- $\alpha$  gene expression has been shown to be up-regulated in AGD gill lesions [112, 280].

Complement factors C3 and C9 were also identified in the skin mucus of the AGD-affected fish, but at a lower expression level than in healthy fish. Levels of C3 and C9 differed between skin and gill mucus and were located in a second protein-protein interaction network (Supplementary Figure 6.4). Complement factors have been identified in skin mucus of salmon affected by sea lice [126, 128], however these studies did not show clearly whether the presence of the parasite induces an increase or decrease in the expression levels of these proteins.

In summary, we have identified a series of proteins expressed in the mucus of AGD-affected Atlantic salmon. Some of these proteins were related to inflammation and IL-1 $\beta$  expression, which is up-regulated in this disease [107, 109, 110, 241]. Other proteins, such as asAG-2 have already been shown in cells of the gills of salmon infected by *N. perurans* and their mRNA expression levels have been characterised [242, 243].

One limitation of this study is the contamination of mucus samples by high-abundance serum proteins. At present, the source of these serum proteins in mucus is not fully understood, but it has been suggested it could be due to local secretion by the epidermis or leakage from the plasma into the mucus, and in the case of haemoglobin, by release from red blood cells into the surrounding tissues and infiltration into the mucus [126, 255]. Similarly, it is possible that cytokines present in the mucus might have been originated from inflammatory responses in the skin, which could have induced a significant up-regulation of transcription of pro-inflammatory cytokines, as has been observed in rainbow trout infected by Ich [288]. In particular, serum albumin isoforms accounted for a high proportion of the total protein, according to both SpC data and targeted nanoLC-MS/MS analysis of high-

**Differentially expressed proteins in mucus of AGD-affected Atlantic salmon**

abundance protein bands detected by SDS-PAGE. Considering that utmost care was taken to minimise serum contamination of mucus samples, immunoaffinity-based depletion of albumin could be a practical solution to increase detection of lower abundance mucus proteins [256]. Notwithstanding, the cohort of differentially expressed proteins in gill and skin mucus of Atlantic salmon affected by AGD obtained in this study is a reference point on how this disease affects mucus protein composition. Subsequent studies could potentially investigate the proteins present in mucus after a single-infection, and compare them with results from available gene expression studies. Alternatively, future research could also target the gill itself and this information could be used to correlate with the data obtained from mucus analyses, thus providing deeper insight on the role of these identified components, and how they participate in the response against infection with *N. perurans*.

**6.5 ACKNOWLEDGEMENTS**

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**CHAPTER 7.**

**GENERAL  
DISCUSSION**

This research focused mainly on developing a vaccine against *Neoparamoeba perurans*, the causative agent of AGD. Since the information available on mucosal responses against this particular pathogen was limited, the host immune responses and their effects on a potential vaccine candidate against AGD were investigated.

The relationship between the immunoglobulin (Ig) gene expression and the antibody response at systemic and mucosal levels, in animals affected by repeated AGD infections and in fish fed diets containing immunostimulants after a primary infection with *N. perurans*, was considered in Chapter 2. Additionally, this project investigated the best delivery route for a hapten-antigen in order to generate systemic and mucosal responses in Atlantic salmon (Chapter 3). The results from this experiment were used to further assess the production of systemic and mucosal Ig responses against a recombinant protein designed from a putative attachment factor of *N. perurans* (Chapter 4); and a challenge with *N. perurans* was conducted to investigate the level of protection provided by the responses against the recombinant protein (Chapter 5). However, the vaccine candidate did not show a suitable level of protection against AGD; therefore it is possible that the antibody responses obtained were not adequate to control AGD, the fish were over-challenged or, alternatively, the presence of a co-infection could have had an impact on the survival of the experimental fish (see section 7.3). Following from the unsuccessful challenge, the mucus proteome of AGD-affected salmon was analysed by nanoLC-MS/MS, to investigate a possible effect of the disease on the mucosal host-response (Chapter 6).

## **7.1 INTERPRETING THE ANTIBODY RESPONSES TO DIFFERENT ANTIGENS AND *N. PERURANS* IN ATLANTIC SALMON**

An experiment with a larger dose of amoebae produced a larger percentage of seropositive fish, in comparison with a repetitive infection experiment conducted with lower doses of amoebae (Chapter 2). Antibody responses against the parasite and against some particular antigens derived from *N. perurans* have been previously documented (Table 7.1). Studies on field infections have demonstrated an incremental increase in the percentage of seropositive fish over time of natural infection [116, 161]. Similarly, experimental infections conducted in the laboratory

have shown that severity of infection and repetitive exposure to amoebae antigens can also induce higher levels of systemic antibodies in AGD-affected fish [115, 135].

The higher percentage of seropositive fish could be also explained by the use of diets including immunostimulants such as  $\beta$ -glucans, which are known to induce an increase in antibody levels in fish [150, 289]. However, this is the first study to assess the antibody levels in AGD-affected Atlantic salmon which have been fed diets containing immunostimulants, and no effects in the systemic and mucosal Ig levels were found in diseased fish when compared to control fish with the doses and formulations used. Further studies are required to fully understand the role immunostimulants play in the Ig responses against this disease.

Systemic antibodies against *N. perurans* antigens increased in fish after repetitive exposure to the recombinant protein, in Chapters 4 and 5. However, further increases in serum antibodies did not develop in fish after the challenge, when they were exposed to whole parasite antigens (Chapter 5). The lack of increase in serum antibody response following infection could suggest that circulating antibodies play only a very small role in resistance or protection against the disease. Findlay et al. [23] also proposed this hypothesis, as they demonstrated that a group fish exposed to *N. perurans* twice showed lower percentage of seropositive results in serum than naïve fish exposed for the first time. Given the apparent lack of efficacy of systemic antibodies, it was important to improve the understanding of the role of mucosal antibodies on AGD.

In contrast to the antibody response at systemic level, very few studies have attempted detection of antibody responses in mucus [23, 115, 117, 135] (Table 7.1), and only one has previously shown the presence of antibodies against antigens of the parasite in this matrix [115]. In the present work, a response against the parasite was observed in mucus and mucosal organs (gill and skin), when fish were immunised with the recombinant protein *r22C03* (Chapters 4 and 5). Antibodies produced by i.p. injection of fish were able to bind the surface of the parasite, as demonstrated by ICC (Chapter 4). However, antibodies produced with a similar immunisation protocol failed to protect Atlantic salmon in a challenge with *N. perurans* (Chapter 5). There are a few points to consider regarding this mucosal antibody response. Firstly, the

levels of antibodies against *r22C03* in mucus were lower than those observed in serum in the initial experiment of administration of the recombinant protein (Chapter 4) and in the challenge experiment (Chapter 5), and therefore the lack of protection could have been due to a lower level of Ig. Secondly, even though the mucosal response against whole *N. perurans* cells was higher than that of serum, the assay to investigate this was performed under low salinity and neutral pH conditions (i.e. in fixed *N. perurans* cells and immersed in PBS, Chapter 4). The antibody functionality can potentially be affected by the presence of seawater. Previous reports have shown a compromised function of Atlantic salmon serum antibodies when tested in high osmolarity [223]; in contrast to both mucosal and serum antibodies from barramundi (*Lates calcarifer*), which are capable of binding antigens at salinities similar to full strength seawater in a modified ELISA [195]. Further research is required to understand if the lack of protection of the antibodies in the present study was due to differences between systemic and mucosal antibodies or because of an inability of the salmon antibody to function in seawater.

**Table 7.1 Systemic and mucosal antibody responses in Atlantic salmon against whole cells of *N. perurans* or against particular antigens of the parasite, following AGD challenge only or vaccination and challenge.**

| Type of infection or immunisation                                | Location     | Antigen used for response assessment | Days to response after infection | % of sero-positives   | Reference  |
|--|--------------|--------------------------------------|----------------------------------|-----------------------|------------|
| Cohabitation (primary)   | Field        | <i>Neoparamoeba</i> spp.             | N/A                              | 25                    | [135]      |
| Cohabitation (repeated)  |              |                                      | N/A                              | 35                    |            |
| Cohabitation   | Field        | Wild-type amoeba                     | 89                               | 75                    | [161]      |
|  |              |                                      | 136                              | 100                   |            |
| Cohabitation   | Field        | <i>N. perurans</i>                   | 224                              | 12.5 <sup>#</sup>     | [116]      |
|  |              |                                      | 280                              | 62.5 <sup>#</sup>     |            |
|  |              |                                      | 364                              | 57.5 <sup>#</sup>     |            |
|  |              |                                      | 420                              | 81.8 <sup>#</sup>     |            |
| Cohabitation (repetitive infection/bath cycles)                  | Field        | <i>N. perurans</i>                   | 210 (100 <sup>&amp;</sup> )      | 77 <sup>#</sup><br>13 | [114]      |
| Cohabitation   | Experimental | <i>Neoparamoeba</i> spp.             | 28<br>56 (2 x 28) <sup>§</sup>   | 100<br>68             | [23]       |
| Cohabitation (moderate infection)                                | Experimental | <i>Neoparamoeba</i> spp.             | 14                               | 18                    | [135]      |
| Cohabitation (severe infection)                                  |              |                                      | 21                               | 48                    |            |
| Cohabitation   | Experimental | Wild-type amoeba                     | 21-168 <sup>§</sup>              | 50                    | [117]      |
| Inoculation (500 cells/L)  | Experimental | Gill-derived amoeba                  | 63                               | 11.7 <sup>#</sup>     | [113]      |
| Inoculation (1,152 cells/L)                                      | Experimental | Wild-type amoeba                     | 72                               | 4.8 <sup>#</sup>      | [113, 146] |
| I.m. injection of DNA followed by inoculation (500 cells/L)      | Experimental | rproteins from DNA library           | 60 (81*)                         | 100                   | [137]      |
| Inoculation (500 cells/L)  |              |                                      | 39                               | N/A                   |            |
| I.p. injection HMWA + FCA followed by inoculation (500 cells/L)  | Experimental | <i>N. perurans</i>                   | 39 (105*)                        | N/A                   | [115]      |
| Inoculation (repetitive infection/bath cycles) (150 cells/L)     | Experimental | <i>N. perurans</i>                   | 126<br>(4 x 21-28)               | 70                    | Chapter 2  |
| Inoculation (500 cells/L)  |              |                                      | 31                               | 88                    |            |
| I.p. injection with r22C03                                       | Experimental | <i>N. perurans</i>                   | 56                               | 100                   | Chapter 4  |
|  |              |                                      | 70                               | 75                    |            |
|  |              |                                      | 84                               | 100                   |            |
| I.p. injection with r22C03 followed by inoculation (500 cells/L) | Experimental | r22C03                               | 58 (204*)                        | 60                    | Chapter 5  |
| Dip with r22C03 followed by inoculation (500 cells/L)            |              |                                      |                                  | 88                    |            |

SYSTEMIC RESPONSES

**Continuation Table 1**

| Type of infection or immunisation | Location   | Antigen used for response assessment | Days to response after infection | % of sero-positives <sup>+</sup> | Reference                     |           |
|-----------------------------------|--|--------------------------------------|----------------------------------|----------------------------------|-------------------------------|-----------|
| Cohabitation                      | Experimental   | <i>Neoparamoeba</i> spp.             | 56 (2 x 28) <sup>§</sup>         | 0 (G)                            | [23]                          |           |
| Cohabitation                      | Experimental   | <i>Neoparamoeba</i> spp.             | 21                               | 0 (G)                            | [135]                         |           |
| Inoculation (500 cells/L)         | Experimental   | Gill-derived amoeba                  | 63                               | 0 (S) <sup>#</sup>               | [117]                         |           |
| Inoculation (500 cells/L)         |  |                                      | 39                               | 0 (S)                            |                               |           |
| MUCOSAL RESPONSES                 | I.p. injection HMWA + FCA followed by inoculation (500 cells/L)  | Experimental                         | <i>N. perurans</i>               | 39 (105*)                        | 60 (S)                        | [115]     |
|                                   | Inoculation (repetitive infection/bath cycles) (150 cells/L)     | Experimental                         | <i>N. perurans</i>               | 126 (4 x 21-28)                  | 4(S), 0(G)                    | Chapter 2 |
|                                   | Inoculation (500 cells/L)  |                                      |                                  | 31                               | 25 (S)                        |           |
|                                   | I.p. injection with r22C03                                       | Experimental                         | <i>N. perurans</i>               | 28<br>56                         | 100 (S)<br>100 (S)            | Chapter 4 |
|                                   | I.p. injection with r22C03 followed by inoculation (500 cells/L) | Experimental                         | r22C03                           | 58 (204*)                        | 88 (S)<br>75 (GE)<br>100 (SE) | Chapter 5 |
|                                   | Dip with r22C03 followed by inoculation (500 cells/L)            |                                      |                                  |                                  | 88(S)<br>100 (GE)<br>100 (SE) |           |

<sup>§</sup> = Fish were infected twice for 28 d, and given a fresh water bath between infections, <sup>§</sup> = Estimated d post-infection, since fish were located in a constant infection tank at UTAS. \*= Numbers between parenthesis represent d post initial immunisation. &= Number of d after last bath treatment. <sup>+</sup> = Letters between parenthesis indicate origin of mucus analysed G=gill, S=skin, GE=gill explant, SE=skin explant. Percentage of seropositives was evaluated by ELISA, unless noted by <sup>#</sup>, where WB was used.

Effects of diverse exposure routes to antigens on antibody response were also tested during this project. Antibody responses were generated in Atlantic salmon serum after immunisation with two different hapten-antigens (6 weeks post immunisation) and in putative local antibody producing cells located in mucosal organs when the antigens were i.p. injected (Chapter 3). However, when only the gills were exposed to the same antigen through a short immersion, no antibody response was generated. This was possibly due to an impairment of the antigen uptake, mainly related to short

exposure time and fish anaesthesia [179]. More research is required to understand the processes involved in localised gill immune responses in salmonids. In contrast, an Ig response in gill was generated in fish that were initially injected with *r22C03* and boosted by a short dip in a solution of the recombinant protein (Chapter 5), indicating a potential role of other mucosal areas such as skin and gut, in antigen uptake during the short immersion. A recent review by Salinas et al. (see Ref. [76]) has indicated that mucosal IgM responses stimulated on one site can also be measured in other mucosal sites. However, it is unclear how these responses integrate, as there are indications in rainbow trout that IgM might be produced by local mucosal B-cells, or alternatively, also transported from serum to mucosal secretions [97]. Further studies that measure IgM, IgT and IgD classes in skin, gills, gut and serum after mucosal immunisation are required to understand the relationships between responses in different mucosal sites [76].

Skin and gill explants were successfully used to evaluate the antibody responses in Atlantic salmon affected or not by AGD (Chapters 3 and 5); these explants have been effectively used in other species to test for the presence of antibody production in specific sites [170, 206, 207, 222]. In the present project, it was demonstrated that they could be used as an alternative approach to assess mucosal responses (Chapter 3 and 5). Although significant antibody responses were not measurable in the mucus, localised responses were still measured using this technique. A significant drawback for this method is the fact that it requires complete perfusion of the fish with a heparinised physiological solution; therefore its use in smaller fish for investigation of AGD or other diseases could be restricted. Although this project has provided greater understanding of mucosal IgM responses in relation to AGD, the role of mucosal antibodies in protecting fish against *N. perurans* still warrants further research.

## **7.2 ARE ANTIBODY RESPONSES THE BEST METHOD TO TEST FOR VACCINE EFFICIENCY AND AGD PROTECTION?**

Traditionally, antibodies have been one of the principal immune responses evaluated when testing vaccine efficacy, only second to the analysis of protection provided by

survival curves. In recent years, implementation of challenges for vaccine testing has raised some concerns, particularly when taking into consideration the welfare of the large amount of animals used in these experiments [139]. Therefore, it has been recommended as good animal welfare practice, to assess the effectiveness of a vaccine by measuring the levels of protective antibodies in animals, instead of using challenge trials [139]. Increased levels of protection are correlated with significantly elevated antibody levels against antigens from bacterial pathogens such as *Aeromonas salmonicida* [290-294] and *Yersinia ruckeri* [295]; against viral antigens from ISAV [296] and IPNV [297] and against parasites such as *Ichthyophthirius multifiliis* (Ich) [298] in salmonids. The use of serologic methods in fish is yet to be completely established, as antibodies in fish do not always correlate strongly with protection, as in the case of *Vibrio salmonicida* or even AGD [113, 299], and reagents to measure these responses are not always available.

As mentioned above, previous studies have shown conflicting results regarding antibody protection against AGD [23, 113, 114, 161, 221]; however these studies mainly focused on serum antibodies and their results were obtained before the causative agent of AGD was properly identified [46]. Only one previous study [115] and Chapters 4 and 5 of this thesis have shown the presence of antibodies in mucus against *N. perurans* antigens. Even though it was demonstrated that mucosal antibodies bound the surface of amoebae (Chapter 4), protection against the disease by antibody response was not observed during the challenge experiment and is yet to be documented.

One of the initial hypotheses of this project was that antibodies produced in gill mucus by immunisation with a recombinant protein could prevent the attachment of the parasite to the epithelial surface, or even reduce the severity of AGD cases. Previous studies have suggested the possibility that early gill mucosal responses could contribute to protection against certain pathogens by reducing the colonisation of these surfaces [198]. Indeed, blocking attachment factors of *Acanthamoeba* spp. affecting mucosal surfaces of mammals have resulted in reduction of their cytopathic effect [215, 228]. However, the nature of the interactions established between *N. perurans* and the epithelial surfaces is yet to be fully understood. Studies of the

histopathology of the disease have pointed to a very close interaction of the amoebae with the respiratory epithelia [55, 57], but the mechanisms by which *N. perurans* causes pathological changes, including oedema and epithelial hyperplasia have not been completely elucidated. There is a possibility that not only the attachment of the amoebic cells to the respiratory epithelium generates the pathology, but also the presence of extracellular products generated by the parasite. There has been indications that cytolytic products generated by *Neoparamoeba* spp. can cause cytopathic effects when cultured with cell monolayers under specific osmolarity conditions [300]. However, these results should be re-visited in the light of the subsequent identification of *N. perurans* as the causative agent. It would be interesting to test the effects of antibodies produced against these putative extracellular products, if they are ever shown to be generated by *N. perurans* and are properly identified.

In addition to the protective nature of the antibodies, the Ig isotype and its presence in mucosal surfaces should also be taken into consideration when studying the responses against AGD. In salmonids, the role of IgM as the main Ig isotype involved in systemic antibody responses has been known for several years [99], but only in later years have other two Ig isotypes, IgD and IgT been described at transcriptional level [101, 301] and even more recently at functional level for IgT [97]. This latter study demonstrated that IgT is the main Ig isotype involved in mucosal responses, even though IgM is also present in mucosal secretions, but the IgM/IgT ratio is smaller than in serum [97]. In addition, levels of both IgM and IgT are lower in mucus than serum, and only 50% of the cells present in the skin mucosal surface in rainbow trout correspond to IgT<sup>+</sup> IgM<sup>-</sup> B-cells [97]. The development of an antibody specific to Atlantic salmon that recognises IgT, in addition to the ones available for rainbow trout, is warranted [97, 149]. This would allow investigation of the mucosal antibody responses *in-situ* in AGD-affected fish, particularly via IHC or ICC as it has been carried out for other parasitic conditions [149]. Even though a recent study has positively identified IgM and IgT responses at the transcriptional level in AGD lesions [112], we suggested that a correlation was not always present between the transcriptional levels of IgM and the level of antibodies in serum and mucus (Chapter 2). Therefore, the main obstacle for carrying out combined research

approaches involving transcription and protein expression studies is the lack of reagents for Atlantic salmon molecules. This approach could further improve our understanding of immune responses against AGD and other pathogens and could help future vaccine development.

Additionally, research on AGD vaccines would greatly benefit by elucidating the type of immune response that would induce antibodies against *N. perurans* as an end product, as it is not known yet which type of Th cell subset is involved in responses against this parasite. In the case of mammals, Th2 cell type responses, which require IL-4 cytokine for development, are specialized in eliciting immune responses against extracellular parasites [95]. Nevertheless, mice affected by amoebic colitis caused by *Entamoeba histolytica* mounted a strong mixed Th2/Th17 mucosal profile during infection. Neutralisation of IL-4 by antibodies lead to clearance of infection, and this was related to a reduction in the inhibition of INF- $\gamma$  production. INF- $\gamma$  cytokine, which is more representative of a Th1 type response, was hence associated with protection against *E. histolytica* colitis [302]. On the other hand, in corneal keratitis caused by *Acanthamoeba* spp. in mammals, hamsters and pigs immunised with *Acanthamoeba* spp. antigens have been shown to develop lymphoproliferative T-cells responses, with a delayed type hypersensitivity and IgG antibodies which are typical of a Th1 response, however these are not protective [90, 303]. Different parasites affecting different mucosal sites can elicit distinct Th subsets responses, therefore further research is warranted in order to elucidate potential Th responses associated with AGD. In addition, for *Acanthamoeba* spp. keratitis, the protection appears to be mediated solely by secretory IgA antibodies [303], as it has been demonstrated that polyclonal antibodies prepared against a ~400 kDa MBP expressed on the surface of the parasite, inhibit the adhesion of the parasite to the host cell and that this protection can be transferred passively with anti-*Acanthamoeba* IgA antibodies [214, 215, 303]. This is the main reason why it was expected that a protective response induced with the vaccine candidate chosen for this project, recombinant MBP-like, homologue of MBP from *Acanthamoeba* spp., would have been associated mostly with mucosal antibody production.

Mucosal components potentially involved in immune responses against *N. perurans* were investigated using proteomics, as an alternative to offset the lack of reagents needed for measuring directly the antibody isotypes in mucus. Various studies have been recently published which have used proteomics to study mucosal responses to infection in fish [125, 126, 128]. We found that AGD does indeed generate changes in the mucus proteome of affected fish when compared to naïve controls (Chapter 6). In addition, an interesting finding of the proteomics analysis in this thesis was the increased expression of complement C3 molecule in the gill mucus of AGD-affected fish. In rainbow trout, Ich infections increased the expression of C3 at the transcriptional level [149]. Even more interestingly, a recent study on rainbow trout affected by Ich, demonstrated that trophonts of the parasite in the skin mucus were covered in C3 in conjunction with IgT, and that IgT and not IgM activates C3, suggesting an important role of both molecules in the elimination of pathogens from mucosal surfaces [261]. This finding reinforces the need for the development of a monoclonal antibody that reacts with IgT of Atlantic salmon.

### **7.3 OTHER CONSIDERATIONS WHEN TESTING VACCINES**

In all forms of intensive culture, infectious disease agents are easily transmitted between individuals [304]. Vaccines have a high value in the aquaculture industry, since they do not only reduce the transmission of diseases among intensive cultured fish, but also secure the food supply for consumers [139]. Since vaccines were introduced for aquaculture species, the most successful ones have been those administered by i.p. injection with an oil adjuvant [133, 304]. Taking this into consideration, we have used a recombinant protein from *N. perurans* combined with and adjuvant oil as a candidate vaccine in this project.

Vaccine antigens administered through a non-natural route of exposure (i.e. injection) may not provide the adequate signals for a suitable response [217], in this case for a pathogen that affects mucosal surfaces. Moreover the immunogenicity of the vaccine might need to be enhanced by the use of specialised adjuvants [232], as inactivated vaccines based on isolated pathogen antigens could be weakly immunogenic [289]. The oil-based adjuvant used in the present study (FCA) is a potent adjuvant that can stimulate both humoral and cell-mediated responses [216,

289]; however it is possible that adjuvants that have been designed specifically to elicit mucosal responses could have induced a better/stronger response in the case of the vaccine tested in this thesis. Mucosal-binding proteins, such as the bacterial binding proteins from the cholera toxin or the *E. coli* heat-labile enterotoxin, are among the most studied mucosal adjuvants in mammals [229]. In fish, these bacterial proteins have induced an increase in the antibody producing cells in mucosal surfaces of spotted sand bass (*Paralabrax maculatofasciatus*) [230] and have raised systemic antibody levels in the tilapia (*Oreochromis mossambicus*) [231] when used as adjuvants in oral immunisations. Another possible approach could be the use of adjuvants that act as depot of vaccines and can induce elevated humoral responses, as has been demonstrated with injected micro- and nanoparticle formulations of the biocompatible polymer poly(lactic-co-glycolic) acid (PLGA) in Atlantic salmon [305]. Both of these methods present promising research opportunities for the developing of a mucosal vaccine against *N. perurans*.

Another consideration for the future research of AGD vaccines would be the use of specific antigens that could trigger different responses against *N. perurans*. The first pathogen-host interaction is the recognition of PAMPs by the PRRs, and some of these PRRs are coupled to the induction of specific adaptive immune responses [82]. For example, there is an association between the antigens recognized by lymphocytes and the PAMPs recognised by PRRs. In the case of T-cell responses this association is represented by dendritic cells, which act as messengers between the innate and the adaptive immune systems presenting antigens to T-cells after they recognise them via their PRRs. For B-cells, this association between antigen and PAMP can be more direct, as for the case of T-cell independent antigens [306]. There is limited knowledge of the parasite PAMPs recognised by PRRs, but molecules like dominant surface glycolipids (recognised by TLR11), structural protein (recognized TLR11) and genomic DNA (recognised by TLR9) have been described in mammals (for a review see Ref. [307]). Regarding fish parasites, the ciliate Ich expresses immobilisation antigens that are GPI-anchored or glycolipid bound proteins, which are potential PAMPs [308]. In addition, other type of PRRs, like C-type lectins, can recognise and bind surface carbohydrates acting as PAMPs. These types of carbohydrate-lectin interactions have been described for Ich [309], but also for

metazoans such as the monogenean ectoparasite *Gyrodactylus derjavivni* [310]. In the case of AGD, previous work has identified immunodominant glycoproteins expressed in the glycocalyx of *N. perurans*, which were heavily glycosylated and presenting rhamnose as their predominant sugar component [115]. These authors proposed that these molecules might interact with rhamnose-binding lectins, a type of PRR present in mucous cells of Atlantic salmon gills. However, when tested as potential vaccine candidates, these molecules failed to protect the fish after a challenge with the amoebae, even though they induced a strong systemic antibody response [115]. Further research into elucidating PRRs relevant to immune responses against AGD and possible their ligands should be considered for future AGD vaccination strategies.

As mentioned previously, testing of vaccines by using disease challenge methods faces increasing scrutiny regarding animal welfare issues and the measurement of protective antibody levels has been indicated as a good alternative [139], which was one of the approaches used in this project (Chapters 3 and 4). However, conducting a challenge trial was the next step to prove the efficacy of the vaccine before commercial trials could have been performed. One of the issues observed during the challenge was the presence of ulcerative skins lesions on the fish. An initial diagnosis of a *Vibrio* spp. opportunistic infection was obtained and all the corrective measures were carried out for this condition, which included freshwater bathing and the inclusion of antibiotics in the feed. However, a *Y. ruckeri* co-infection was detected by qPCR analyses of serum after the end of the challenge. In retrospect, it would have been more adequate to test the experimental fish -pre-emptively and throughout the trial- for the presence of other pathogens. This could have avoided potentially confounding of results in both in mortality levels and antibody response after the challenge (Chapter 5). Yersiniosis is enzootic to Tasmania, and after the initial infection, an asymptomatic carrier state can be establish in recovered fish, and bacteria can be shed from their intestines following periods of stress [16]. Co-infections of *N. perurans* and *Y. ruckeri* have been documented in the past during experimental trials and they have affected the testing of other candidate vaccines for AGD [137]. However, the co-infection also presented an opportunity to study the interactions between *N. perurans* and *Y. ruckeri* infections. This co-infection is

common in the Tasmanian salmon industry, and thus the situation may represent more closely the challenges suffered by the fish under commercial conditions.

The amoebae challenge dose could have also affected the results. Two or more levels of infection could have been tested in order to follow the guidelines established by Amend [138] for potency testing of fish vaccines. However, the level of infection used in the challenge trial (500 cells/L) and in other experiment in the present work (Table 7.2), was developed as a standard infection method for AGD, capable of causing lesions in 30% of gill filaments from individual fish 14 d post inoculation [151]. As the study by Morrison et al. [151] also recognised a linear relationship between the inoculating dose and the severity of AGD, lower doses were also used in the work presented on this thesis (Table 7.2), to allow for multiple infections without compromising the survival of the experimental animals (Chapter 2). Nevertheless, neither of these infective doses represent the level of amoebae obtained from water samples surrounding commercial cages [311], and therefore might not represent the level of infection present in salmon farms. The development of a challenge model more in accordance with commercial conditions should be included in future AGD research.

**Table 7.2** *Neoparamoeba perurans* dose, number and time of infections used in the present project.

| <b>Dose<br/>(amoebae/L)</b> | <b>Number of infections</b> | <b>Infection times<br/>(weeks)</b> | <b>Reference</b>                      |
|-----------------------------|-----------------------------|------------------------------------|---------------------------------------|
| <b>150</b>                  | 4                           | 0, 5, 8, 14                        | experiment 1, Chapter 2;<br>Chapter 6 |
| <b>500</b>                  | 1                           | 0                                  | experiment 2, Chapter 2               |
| <b>500</b>                  | 2*                          | 0, 5                               | Chapter 5                             |

\* Due to the presence of skin lesion treated with antibiotics, the first challenge had to be terminated by bathing the fish in freshwater.

In conclusion, this thesis increased the knowledge on mucosal responses to AGD. For the first time it was shown that antibody (IgM) developed in the mucus after immunisation is capable of binding the surface of the amoeba. However, the protective nature of this antibody isotype still needs to be further investigated, and the study of IgT responses in AGD-affected fish still remains as an interesting research avenue. Therefore the development of a specific antibody that can measure the IgT responses in Atlantic salmon should be prioritised. Proteomics was used for the first time in the study of mucosal immune responses during AGD, and the results showed that the disease can significantly alter the mucus proteome of affected fish. Finally, the administration route, amoebae infection level as well as the presence of a co-infection could have affected the results obtained in the challenge trial, but this co-existing *Y. ruckeri* infection might represent more closely the conditions affecting salmon after vaccination on commercial farms in Tasmania.

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## APPENDICES

### APPENDIX 1: OTHER MANUSCRIPTS PUBLISHED DURING PHD

- Nowak, B.F., **Valdenegro, V.**, Crosbie, P.B.B., Bridle, A. Immunity to amoeba. *Develop Comp Immunol.* 2014 43, 257–67.
- Andrews, M., Cobcroft, J.M., Battaglione, S. C., **Valdenegro, V.**, Martin, M.B., Nowak, B. F. Parasitic crustaceans infecting cultured striped trumpeter *Latris lineata*. *Aquaculture.* 2013 416–417, 280-8.
- **Valdenegro-Vega, V.**, Naeem, S., Carson, J., Bowman, J., Tejedor de Real, J.L., Nowak, B.F. Culturable microbiota of ranches southern bluefin tuna (*Thunnus maccoyii* Castelnau). *J App Microbiol.* 2013 115: 923–32.
- Kirchhoff N.T., Leef M.J., **Valdenegro V.**, Hayward C.J., Nowak B.F. Correlation of Humoral Immune Response in Southern Bluefin Tuna, *T. maccoyii*, with Infection Stage of the Blood Fluke, *Cardicola forsteri*. *PLoS ONE.* 2012 7: e45742.
- Hardy-Smith, P., Ellis, D., Humphrey, J., Evans, M., Evans, D., Rough, K., **Valdenegro, V.**, Nowak, B. *In vitro* and *in vivo* efficacy of anthelmintic compounds against blood fluke (*Cardicola forsteri*). *Aquaculture.* 2012 334–337, 39-44.

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**APPENDIX 2: CONFERENCE PROCEEDINGS**

- **Valdenegro-Vega V. A.**, Crosbie, P., Bridle A., Leef, M., Wilson, R., Nowak, B.F. Differentially expressed proteins in gill and skin mucus of Atlantic salmon (*Salmo salar*) affected by amoebic gill disease. Oral presentation. 2014 ASP Conference. ANU, Canberra, Australia; 30 June - 3 July, 2014.
- **Valdenegro-Vega V. A.**, Crosbie, P., Vincent, B., Cook, M., Nowak, B.F. Antibody response against recombinant proteins of *N. perurans* and AGD challenge in Atlantic salmon (*Salmo salar*). Oral presentation. 2<sup>nd</sup> Australasian Aquatic Animal Health Conference. Cairns Pullman Reef, Cairns, Australia; 8 - 12 July, 2013.
- **Valdenegro-Vega V. A.**, Crosbie, P., Vincent, B., Cook, M., Nowak, B.F. Antibody response against recombinant proteins of *N. perurans* and AGD challenge in Atlantic salmon (*Salmo salar*). Oral presentation. 1<sup>st</sup> International Conference on Fish and Shellfish Immunology, Vigo, Spain, 25 - 28 June 2013.
- **Valdenegro-Vega, V.**, Crosbie, P., Cook, M., Nowak, B. Antibody response against recombinant attachment proteins of *N. perurans*: an approach to reduce AGD severity. Oral presentation. 6<sup>th</sup> International Veterinary Vaccines and Diagnostics Conference Cairns Convention Centre, Cairns, Australia; 29 July - 1 August, 2012.
- **Valdenegro-Vega, V.**, Crosbie, P., Cook, M., Nowak, B. Antibody response against recombinant attachment proteins of *N. perurans*: an approach to reduce AGD severity. Oral presentation. 2012 ASP Conference, 2 - 5 July 2012, Launceston, Tasmania.
- **Valdenegro, V.**, Crosbie, P., Vincent, B., Cook, M., Nowak, B. Effect of immunization route on mucosal and systemic immune response in Atlantic salmon (*Salmo salar*). Oral Presentation. 15<sup>th</sup> European Association of Fish pathologists: International conference on Diseases of Fish and Shellfish, 9 - 12 September, 2011, Split, Croatia.
- **Valdenegro, V.**, Crosbie, P., Vincent, B., Cook, M., Nowak, B. Effect of immunization route on mucosal and systemic immune response in Atlantic

salmon (*Salmo salar*). Oral presentation. 1<sup>st</sup> Australasian Scientific Conference on Aquatic Animal Health, 5 - 8 July, 2011, Cairns, Australia.