Immune-regulatory genes in amoebic gill disease: potential for immunomodulation

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B.App.Sc. (Hons.)

Submitted in fulfilment

of the requirements for the Degree of

Doctor of Philosophy

University of Tasmania, Launceston, Tasmania

May, 2005

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Abstract

Amoebic Gill Disease (AGD) is the most significant health problem affecting Atlantic salmon, *Salmo salar*, culture in Tasmania, Australia. Caused by the ectoparasite *Neoparamoeba* sp., AGD is an emerging disease in several other countries (USA, Ireland, France, Spain, Chile, New Zealand) and affects several other cultured fish species (rainbow trout, *Oncorhynchus mykiss*, coho salmon, *Oncorhynchus kisutch*, turbot, *Scophthalmus maximus*). AGD and the cost of treatment, freshwater bathing, have negatively impacted on the production of Atlantic salmon in Tasmania. Consequently, alternative AGD management strategies are required. Thus an understanding of the disease processes associated with AGD pathology and the immune response to AGD is essential to the development of alternative AGD management strategies. Therefore, the immune response to AGD was partially characterised using molecular techniques, and the potential use of immunostimulants to reduce the impact of AGD on the Tasmanian salmonid industry was assessed.

The transcriptional profile of selected immune-regulatory genes in the gill, liver and anterior kidney of experimentally infected Atlantic salmon and rainbow trout was assessed using semi-quantitative RT-PCR and quantitative real-time RT-PCR. Atlantic salmon interleukin-1β (IL-1β) was shown to be up-regulated in the gills at 14 d post-inoculation (p.i.). In rainbow trout the expression of IL-1β and inducible nitric oxide synthase (iNOS) mRNA was significantly up-regulated in the gills at both 7 and 14 d p.i., while interleukin-8 was significantly up-regulated in the liver

of AGD-affected trout at 7 d p.i. IL-1β mRNA transcripts were localised by *in situ* hybridisation to pavement epithelial cells lining the primary and secondary lamellae of AGD-affected and control Atlantic salmon gills. These data demonstrated the involvement of the immune response to AGD at the molecular level and indicated the significance of the response at the site of infection.

Intraperitoneal administration of immunostimulatory CpG oligodeoxynucleotides increased the resistance of Atlantic salmon to AGD. However, oral administration of β-glucans, a well-known and potentially more cost-effective immunostimulant, was unable to enhance resistance to AGD. Nonetheless, a group of Atlantic salmon were able to survive an experimental AGD challenge. These findings allowed a preliminary AGD infection model to be proposed, and further our knowledge of the fish immune response to parasitic infection. The implications of this research show the potential for an effective immunological-based AGD management strategy such as use of a vaccine, immunostimulants or a selective breeding program.

Acknowledgements

There are many people to thank for their contributions, without whom this thesis would not have been possible. Firstly, I thank my supervisor, Dr Barbara Nowak, for her advice, friendly encouragement and patience throughout this project. Secondly, I owe thanks to my associate supervisor, Dr Richard Morrison, for his invaluable advice, practical assistance and for useful discussions.

Thanks also to members of the AGD research group, Dr Phil Crosbie, Dr Mark Adams, Kally Gross and Benita Vincent, for being great company both on-topic and especially off-topic. I am also grateful for the efforts of Dr Chris Prideaux, Dr Tim Doran, Darren Schafer, Terry Wise and Tony Pye at the CSIRO Australian Animal Health Laboratories (AAHL) during collaborative work. At the School of Aquaculture (UTAS) Mick Attard, Helen Statham and Don Ward were of great assistance, whilst special thanks go to Annabel Tyson and Janette Daniel for their administrative support.

Finally, I express my sincere thanks to my family for their endless support and to my partner Ebony for her love, support and patience.

This study was financially supported by the Tasmanian Aquaculture and Fisheries Institute and the Aquafin Cooperative Research Centre (CRC).

List of Abbreviations

AGD	amoebic gill disease
ANOVA	analysis of variance
APP	acute phase protein
APR	acute phase reaction
BCIP	5-bromo-4-chloro-indolyl-phosphatase
bp	base pair
CMC	carboxymethyl cellulose
COX-2	cyclo-oxygenase-2
CPE	cytopathogenic effect
CpG	cytidine-phosphodiester-guanosine
cRNA	complementary ribonucleic acid
Ct	cycle threshold
CVS	central venous sinus
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
ECP	extracellular product
EDTA	ethylenediaminetetraacetic acid
FBS	foetal bovine serum
GALT	gut-associated lymphoid tissue
H&E	hematoxylin-eosin stain
I.L.U	interlamellar units
i.p.	intraperitoneal
IgM	immunoglobulin M
IL-1R	type I interleukin-1 receptor
IL-1RII	type II interleukin-1 receptor
IL-1β	interleukin-1β
IL-1β1	interleukin-1 beta isoform 1
IL-1β2	interleukin-1 beta isoform 2
IL-6	interleukin-6
IL-8	interleukin-8
iNOS	inducible nitric oxide synthase
ISH	*in situ* hybridisation
LPS	lipopolysaccharide
MGB	minor groove binder
MGB-DQ	minor groove binder-dark quencher
MH	major histocompatibility

MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NBT	nitro-blue tetrazolium
NO	nitric oxide
nt	nucleotide
NTE	sodium chloride-tris-EDTA
O.D.	optical density
ODN	oligodeoxynucleotide
P/S	penicillin/streptomycin
PCR	polymerase chain reaction
PFA-PBS	paraformaldehyde-phosphate buffered saline
PMA	phorbal myristate acetate
qPCR	quantitative polymerase chain reaction
qRT-PCR	quantitative reverse transcriptase-polymerase chain reaction
QTL	quantitative trait loci
RGE	salmon gill epithelium cell line
rIL-1β	recombinant interleukin-1 beta
RNA	ribonucleic acid
RNI	reactive nitrogen intermediates
RP	relative potency
RPS	relative percent survival
rRNA	ribosomal ribonucleic acid
RT	room temperature
RT-PCR	reverse transcriptase-polymerase chain reaction
RTS-11	rainbow trout macrophage cell line
S.E.	standard error
SAA	serum amyloid A
SAP	serum amyloid P
SSC	sodium chloride-sodium citrate
SSU	Small subunit
TBST	tris-buffered saline-tween-20
TCR	T-cell receptor
TGF-β1	transforming growth factor
TNF-α1	tumour necrosis factor alpha isoform 1
TNF-α2	tumour necrosis factor alpha isoform 2
TNF-α3	tumour necrosis factor alpha isoform 3
UV	ultraviolet

CONTENTS

Chapter 1

General Introduction

General Introduction

The costs of the existing method of controlling amoebic gill disease (AGD) continue to hinder the sea-cage culture of Atlantic salmon, *Salmo salar*, in Tasmania, Australia. Consequently alternative strategies are required, and an understanding of the disease processes associated with AGD pathology and immune responses to AGD is essential to the development of cost-effective AGD management strategies.

1.1. Amoebic gill disease (AGD)

Amoebic gill disease (AGD) is an ectoparasitic disease of marine fish caused by the protozoan amoeba *Neoparamoeba* sp. Clinical signs of AGD include lethargy, loss of appetite, obvious respiratory distress, loss of equilibrium, and eventual mortality if untreated. Gills of fish infected with *Neoparamoeba* sp. develop macroscopic multifocal pale mucoid patches that increase in size and frequency with the progression of AGD [1-7]. Microscopic examination of paraffin-embedded gill sections from AGD-affected fish reveals macroscopically visible mucoid patches as areas of epithelial cell hyperplasia. These hyperplastic lesions are numerous in the dorsal portions of the gill arch [3] and have an increased abundance of mucous cells and lesion-associated mucus [7, 8]. Amoebic trophozoites are commonly found closely associated with AGD-induced hyperplastic lesions [2-7, 9]. The degree of histopathology associated with hyperplastic AGD lesions ranges from relatively minor epithelial cell hyperplasia

affecting a few lamellae to lesions consisting of multiple fused lamellae and even fused filaments [2-7]. Hyperplastic lesions have a spongiotic appearance and consist primarily of hypertrophic undifferentiated epithelial cells with minor oedema [3, 7]. Extensive epithelial cell hyperplasia often causes the formation of interlamellar spaces 'crypts' that frequently contain amoebae associated with leucocytes [1, 3]. These leucocytes are presumably part of the host response to infection, and scavenge degraded amoebae. As hyperplastic lesions develop, their morphology changes to that of a mature lesion, often developing a layer of stratified epithelial cells that line the lesion surface [5]. Several studies have characterised the sequential pathology associated with AGD-affected Atlantic salmon during both laboratory-induced and field infections [2, 5, 7].

AGD is generally associated with salmonids, most notably Atlantic salmon and rainbow trout, *Oncorhynchus mykiss*. However, AGD outbreaks have been reported in several marine fish species throughout the world. Cases of AGD have been reported in Atlantic salmon from Tasmania, Australia [1], Chile [10], Ireland [11, 12], France [13] and Spain [12]. Similarly, AGD outbreaks have occurred in rainbow trout in Tasmania, Australia [1], coho salmon, *Oncorhynchus kisutch,* in Washington state and California, USA [14], chinook salmon, *Oncorhynchus tshawytscha*, in New Zealand [15], sea-caged brown trout, *Salmo trutta*, in France [15], turbot, *Scophthalmus maximus*, in Spain [9, 16], and European seabass, *Dicentrarchus labrax*, and sharpsnout seabream, *Diplodus puntazzo*, cultured in the Mediterranean Sea [17, 18].

Although outbreaks of AGD have been reported worldwide, the impact of AGD on marine aquaculture has arguably been greatest on the culture of Atlantic salmon in Tasmania, Australia. AGD was first described in Atlantic salmon in Tasmania by Munday [19] soon after the establishment of Atlantic salmon sea-cage culture. Initially, AGD caused substantial mortalities that were greatly reduced following the introduction of freshwater bathing as a treatment [20]. However, it was soon apparent that freshwater bathing had high labour costs, and that subsequent recovery from AGD was merely temporary and dependent on environmental conditions. For reasons not yet fully understood, Tasmanian salmon culturists have been forced to progressively increase the frequency of freshwater bathing, further increasing the costs of production [21].

The fundamental reason for the high impact of AGD in Tasmania is most likely a combination of local environmental conditions, the most important being high water temperatures in summer. Water temperatures in the Tasmanian summer may approach 20°C, and combined with the appropriate salinity sea water (≥32‰), create conditions conducive to AGD outbreaks [5, 10, 22, 23]. Likewise, AGD outbreaks in turbot have occurred at seawater temperatures of 14.0 to 18.8°C [16]. The influence of both water temperature and salinity is further highlighted by evidence that manipulation of these two factors can increase or decrease the severity of AGD during laboratory-based experimental infection. By maintaining the water temperature at 16°C and a salinity of 35‰ during a laboratory-based infection, a relatively reliable disease progression and outcome that is dependent on the initial inoculation concentration of *Neoparamoeba* sp. is possible [24].

Interestingly, cases of AGD have not been reported from Iceland, Scotland or Norway, presumably due to the lower water temperatures in these countries.

Recently, a detailed morphological and molecular study of 18 *Neoparamoeba* strains/clones isolated from Tasmanian AGD-affected Atlantic salmon, Tasmanian marine sediments in the vicinity of sea-cages, and net material from the sea-cages was performed [25]. This study confirmed the presence of *Neoparamoeba* sp. on AGD-affected salmon [15] and in environmental samples [26, 27]. Importantly, using SSU rRNA gene sequences as molecular markers, Dykova *et al.* [25] identified a new species and named it *Neoparamoeba branchiphila.* Using *N. branchiphila*-specific polymerase chain reaction (PCR) primers, this species was identified in cells isolated from the gills of AGD-affected Atlantic salmon and in marine sediment surrounding sea cages. Likewise, strains/clones isolated from AGD-affected Atlantic salmon and both sediment and sea-cage net environmental samples were identified as *Neoparamoeba pemaquidensis.* While the contribution of *Neoparamoeba aestuarina* to AGD has yet to be disproved, the discovery and isolation of *N. branchiphila* on AGD-affected Atlantic salmon adds further confusion as to the involvement of the three *Neoparamoeba* spp. in AGD. However, of nine strains/clones isolated from AGD-affected Atlantic salmon and identified by Dykova *et al.* [25], seven of these were identified as *N. pemaquidensis*, thus supporting the initial claim that *N. pemaquidensis* is the causative agent of AGD [14].

1.2. Immune response to AGD

Teleost fish are the earliest evolutionary class of vertebrates to possess the majority of immune capabilities observed in higher vertebrates, such as antigen specificity and memory formation [28]. Furthermore, fish are able to mount an immune response to a variety of bacterial, viral, and parasitic pathogens using similar immune mechanisms to those of higher vertebrates such as mammals. Although fish are capable of mounting a response to AGD, as evidenced from the formation of hyperplastic epithelial gill lesions, the mechanisms involved in this response have yet to be elucidated. Exposure to *Neoparamoeba* sp. initiates the proliferation of epithelial cells, presumably in an attempt to prevent further parasitic infestation or damage caused by the parasite, and can therefore be considered the primary immune response.

Several studies of the immune response to AGD in Atlantic salmon have been performed. The major aim of these studies has been to find evidence of a protective acquired antibody response in the hope of producing an effective AGD vaccine. Serum anti-*N. pemaquidensis* antibodies have been identified in cultured Atlantic salmon [29] using a rabbit polyclonal antibody raised against a cultured Tasmanian strain of *N. pemaquidensis* [30]. Similarly, Atlantic salmon produced *Neoparamoeba* sp. specific antibodies when administered a crude preparation of live or sonicated *Neoparamoeba* sp. via intraperitoneal (i.p.) injection [31]. These antibodies did not correlate with AGD resistance after cohabitation with AGD-affected fish and did not confer resistance against AGD. Similarly, protection from

AGD, as determined by the numbers of gill lesions after cohabitation with AGD-affected fish, was not observed in Atlantic salmon administered crude preparations of either live or sonicated *Neoparamoeba* sp. by a variety of routes [32]. Furthermore, Atlantic salmon naturally infected with *Neoparamoeba* sp. or i.p.-injected with crude preparations of *Neoparamoeba* sp. do not necessarily develop serum anti-*Neoparamoeba* sp. antibodies [31].

While antibody-mediated protection is questionable, evidence of resistance to AGD following exposure to *Neoparamoeba* sp. has been reported [13, 33, 34]. Likewise, anecdotal evidence of resistance to AGD in a small number of Atlantic salmon has been mentioned by Tasmanian salmon farmers and research technicians at the School of Aquaculture, University of Tasmania. Therefore, due to a lack of evidence for antibody-mediated resistance, it has been suggested that resistance is due to the stimulation and involvement of the innate immune system [13]. Contradictory to previous studies [13, 33], recent work by Gross *et al.* [35] found that previously infected Atlantic salmon were not protected from reinfection upon re-exposure to *Neoparamoeba* sp. Interestingly, serum anti-*Neoparamoeba* sp. antibodies have recently been detected in Atlantic salmon cohabiting a tank with AGD-affected fish, and these Atlantic salmon are apparently resistant to AGD (Vincent 2005, personal communication). Therefore, whether or not Atlantic salmon are able to produce protective antibodies against AGD is still contentious.

1.3. Immune-regulatory genes

Research to date has provided relatively little information concerning the immune response to AGD. Therefore, further research of the immune response to AGD is required, especially if an immunological approach is taken during development of an AGD management strategy. However, the characterisation of the fish immune response to infection in most species has been hindered by a lack of specific cell markers, including antibodies raised against components of the fish immune system. Conversely, recent molecular studies, including work to characterise the genome of several fish species [36, 37], have elucidated many important immune-regulatory gene sequences. These gene sequences may be used to produce recombinant proteins to which specific antibodies may be raised. Alternatively, the characterisation of the immune response to disease is possible by studying the involvement and regulation of these genes during infection in fish.

Molecular studies have recently been used to characterise the involvement of known immune-regulatory genes during several fish diseases [38-50]. The most commonly assessed genes during infection are those encoding cytokines. Cytokines are proteins that act as soluble mediators and regulators of immune responses. Knowledge of cytokines is greatest in mammals, although orthologues of mammalian cytokine genes are being identified in fish at an increasing rate [51-54]. Similarly, genes encoding cytokine receptors, acute phase proteins [55] and other important components of the fish immune system are also being identified more frequently, and are important to understanding the fish immune response to

pathogens and vaccines. The following are brief descriptions of the immune-regulatory genes studied in this thesis.

Tumour necrosis factor α (TNF-α) is a pro-inflammatory cytokine with a range of biological effects, and is beneficial to the host when expressed at relatively low levels and when tightly regulated [56]. Furthermore, TNF-α is an early response gene and is rapidly transcribed into mRNA in immune cells when stimulated with bacteria and bacterial products, viruses, parasites, nitric oxide and a variety of other stimuli [56]. Stimulated production of TNF-α initiates a cytokine cascade involving the transcription and translation of multiple cytokines and a host of other biological responses [57]. Uncontrolled expression of TNF-α has been associated with various diseases in mammals [58]. The TNF-α gene has been cloned from several fish species [59-64], including two isoforms from rainbow trout [65] and three isoforms from carp, *Cyprinus carpio* [66]. Increased mRNA expression of TNF-α has been demonstrated in both species infected with parasites [40, 42, 44, 49].

Both interleukin-1β (IL-1β) and interleukin-8 (IL-8) are part of the cytokine cascade initiated by TNF-α and are also pro-inflammatory cytokines. IL-1β is a widely studied pleiotropic cytokine that has fundamental roles in innate and acquired immunity [67]. Similar to TNF-α, uncontrolled expression of IL-1β has been established in several pathogenic conditions in mammals [67]. The IL-1β gene has been cloned and sequenced in several fish species [68-71]. In rainbow trout two isoforms of IL-β have been cloned [68, 72]. Both the type I interleukin-1β

receptor (IL-1R) and type II interleukin-1β receptor (IL-1RII) have been cloned in salmonids [73, 74]. IL-1β mRNA expression is induced by lipopolysaccharide (LPS) and recombinant trout IL-1β (rIL-1β) [68, 75]. Increased mRNA expression of IL-1β has been demonstrated during bacterial infection of zebrafish, *Danio rerio*, [38] and parasitic infection of carp [40]. Similarly, increased mRNA expression of IL-1β has been demonstrated during bacterial, viral and parasitic infection of rainbow trout [42, 43, 50, 76]. IL-8 is a pro-inflammatory cytokine that is also a well-known chemokine. Chemokines are a superfamily of small secreted cytokines that direct the migration of immune cells to sites of infection [77]. Production of IL-8 is stimulated by LPS and cytokines such as TNF and IL-1 [78]. Likewise, stimulation of a rainbow trout macrophage cell line (RTS-11) with LPS caused an up-regulation of IL-8 [79]. Viral and parasitic infections of rainbow trout have also been shown to increase IL-8 mRNA expression [42, 50]

Transforming growth factor β1 (TGF-β1) is a cytokine that also has pleiotropic biological effects. Although not a pro-inflammatory cytokine like IL-1β, TNF-α and IL-8, TGF-β1 is important to the immune response and is able to increase or decrease cellular functions of certain immune cells and influence the expression of other immune-regulatory cytokines [80]. The TGF-β1 gene has been sequenced in seabream, *Sparus aurata*, [81], hybrid striped bass, *Morone saxatilis* × *M. Chrysops*, [82], carp [83] and rainbow trout [84]. Bacterial, viral and parasitic infections of rainbow trout have also been demonstrated to increase TGF-β1 mRNA expression [44, 48-50].

Although not cytokines, cyclo-oxygenase-2 (COX-2) and iNOS are immune-regulatory substances that are induced by TNF-α, IL-1β and IL-8. COX-2 expression leads to the production of inflammatory mediators known as prostaglandins (PGs) that have been reported to modulate both immune and inflammatory responses in fish [85, 86]. In rainbow trout, recombinant IL-1β (rIL-1β) [75] and bacterial infection have both been shown to induce mRNA expression of COX-2 in anterior kidney cells [48, 87]. Likewise, increased COX-2 mRNA expression has been demonstrated in rainbow trout during parasitic infection [44, 49]. The importance of nitric oxide (NO) and various reactive nitrogen intermediates (RNI) to the fish immune response to various pathogens is well established [88-91]. The production of NO from L-arginine is catalysed by inducible nitric oxide synthase (iNOS). The mRNA expression of iNOS is induced in rainbow trout by stimulation with LPS and bacteria [92, 93], viruses [50] and parasites [41, 44, 94]. In mammals, iNOS-mediated production of NO is also an important component of the immune response to parasitic infection, but may be destructive to the host when produced in excess or during chronic infection [95]. Likewise, NO production in response to *T. borreli* infection was found to be detrimental to the infected carp, as carp treated with the iNOS inhibitor aminoguanidine had enhanced survival compared with infected control carp [96].

Acute phase proteins (APPs) are a group of plasma proteins that have an increased (positive APP) or decreased (negative APP) synthesis during a physiological response to injury, trauma or infection. This response is known as the acute phase reaction (APR), and has been reviewed in fish by Bayne and Gerwick [55]. The

APR is induced by cytokines such as TNF, IL-1 and IL-6 predominantly during bacterial, viral or parasitic infection [97]. Fish APPs, as in mammals, are primarily produced by parenchymal cells in the liver [55, 97]. However, mRNA expression of APPs has also been shown in the anterior kidney and spleen of Atlantic salmon [45]. Similarly, APP mRNA expression was identified in the gill, liver and anterior kidney of both uninfected and AGD-affected Atlantic salmon (chapter three). Serum amyloid A (SAA) and the pentraxin serum amyloid P (SAP) are two major acute phase proteins in salmonids that have been claimed to have critical roles in the immune response to infection [98, 99]. In carp, an increased mRNA expression of SAA was demonstrated during parasitic infection with the blood flagellate, *T. borreli* [40].

The major histocompatibility complex (MHC) class II β-chain and T-cell receptor (TCR) β-chain are cellular markers of important immune cells. MHC class II genes encode cell-surface molecules capable of binding and presenting short peptides to T-cells via the T-cell receptor. The transcriptional profiles of these cell markers within tissues were quantitated to identify possible AGD-induced changes in the immune cell populations. Increased MHC II mRNA expression was shown in rainbow trout infected with the parasitic ciliate *Ichthyophthirius multifiliis* [41]. No parasite-related mRNA expression changes were observed in rainbow trout infected with the *Gyrodactylus derjavini* [44]. However, decreased MHC II mRNA expression has been shown in carp infected with the parasite *T. borreli* [40].

1.4. Potential AGD management strategies

Freshwater bathing is the only commercially-effective treatment of AGD-affected salmonids, and was first described by Foster and Percival [20]. Freshwater bathing has remained the preferred treatment for AGD in sea-cage cultured Atlantic salmon in Tasmania. However, freshwater bathing does not completely eradicate *Neoparamoeba* sp. trophozoites from the gills of treated fish, implying that an outbreak of AGD may recur [100]. Many different antimicrobial, disinfectant and mucolytic agents have been trialled as treatments against AGD, most of which have been less effective than conventional freshwater bathing [101-104]. Evidence of resistance to AGD, and the possibility that resistance is either antibody-mediated or induced by stimulation of the innate immune response, has led researchers to suggest that an effective AGD management strategy incorporating vaccination, immunostimulation or selective breeding be developed.

Initial experiments using crude vaccine preparations and passive immunisation with sheep anti-*Neoparamoeba* sp. antibodies proved unsuccessful [31, 32]. However, recently a more comprehensive approach has been taken towards developing an AGD vaccine. Research aimed at identifying target antigens for use in an AGD vaccine is currently being undertaken. It has been suggested that surface carbohydrate moieties, probably galactose or N-acetylgalactosamine, are responsible for the adherence of the amoeba to the gill epithelia and are likely antigen candidates for a vaccine [15, 105]. Although the presence and involvement of extracellular products (ECPs) has yet to be demonstrated in AGD, ECPs released

by *Neoparamoeba* sp. provide a possible explanation for a putative cytopathic effect (CPE) of *Neoparamoeba* sp. on an Atlantic salmon gill epithelium cell line (RGE-2) [106]. It is unlikely that this CPE was caused by a virus, as *Neoparamoeba* sp. lysates have since been screened on several occasions and found free of viruses (Gemma Clark, unpublished data). If present, ECPs might also be potential vaccine antigen candidates for use in the development of an AGD management strategy.

Immunological research has primarily focused on aspects of acquired immunity to AGD, with the aim of developing an AGD vaccine. Immunostimulants are chemicals, drugs, stressors or actions that enhance the innate defence mechanisms or the acquired immune response [107]. Interest in the use of immunostimulants in aquaculture is growing, as both consumer and environmental concerns about the use of more traditional chemotherapeutic treatments are increasing. A large range of substances are considered as immunostimulants in vertebrates [108]. Whether these substances stimulate the innate or acquired immune system depends on the characteristics of the immunostimulant. Fish rely more heavily on innate immune responses than mammals, primarily because of the constant presence of pathogens in the water environment. Innate defences in fish include barriers such as skin and scales, lytic enzymes of mucus and sera and functions of leucocytes such as phagocytosis and the production of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI). Therefore the majority of immunostimulants used in fish predominantly stimulate the innate immune system. Many

immunostimulants and aspects of their application to aquaculture are thoroughly discussed in reviews by Anderson [107] and Sakai [109].

Therefore, stimulation of the innate immune system might be beneficial to an AGD management strategy. Levamisole, a known immunostimulant of the innate immune system of fish, has been shown to increase innate immune responses of Atlantic salmon and enhance resistance to AGD when added to a freshwater bath used to treat salmon for AGD [104]. However, no such protection against AGD was conferred when levamisole, or β-glucans, another well known immunostimulant in fish, were orally administered [104]. More recently, AGD-affected Atlantic salmon were found to have suppressed innate immune responses of anterior kidney leucocytes compared with naïve fish [35]. Therefore, it is plausible that immunostimulation of AGD-affected Atlantic salmon might restore innate immune responses suppressed by AGD and prove beneficial in an AGD management strategy. Furthermore, immunostimulants may also act as adjuvants when administered with any potential AGD vaccine.

Prompted by anecdotal evidence of AGD resistance in a small population of Atlantic salmon, research is currently being performed to identify possible hereditable traits associated with AGD resistance. Of particular interest are major histocompatibility (MH) gene polymorphisms potentially associated with AGD resistance which provide quantitative trait loci (QTL). This interest is based on evidence of an association between (MH) gene polymorphisms and resistance to other infections of Atlantic salmon [110]. If discovered, such traits would be useful

for QTL analysis and for the development of a selective breeding program for Atlantic salmon.

1.5. Thesis aims and objectives

This thesis presents the findings of research aimed at further characterising the immune response to AGD using molecular techniques and assessing the potential use of immunostimulants to reduce the impact of AGD on the Tasmanian salmonid industry.

More specifically, the following two questions were addressed:

- Does AGD induce differential immune-regulatory gene mRNA expression?
- Do immunostimulants enhance resistance to AGD?

On a fundamental level it is hoped that these findings will further our knowledge of the fish immune response to parasitic infection, while on an applied level they may be useful to the development of alternative AGD management strategies that reduce the need to freshwater bathe fish.

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Chapter 2

The expression of immune-regulatory genes in rainbow trout, *Oncorhynchus mykiss*, during amoebic gill disease (AGD)

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Fish and Shellfish Immunology 2006, 20, 346-364

Abstract

Amoebic gill disease (AGD) is an ectoparasitic disease caused by infection with the protozoan *Neoparamoeba* sp. and is characterised by epithelial hyperplasia that manifests as gill lesions. In order to examine the nature of the immune response to AGD, the expression of a range of immune-regulatory genes was examined in naïve uninfected rainbow trout, *Oncorhynchus mykiss*, and naïve rainbow trout subjected to a laboratory-induced AGD infection. The immune-regulatory genes examined were interleukin-1 beta isoform 1 (IL-1β1), tumor necrosis factor alpha isoforms 1 and 2 (TNF-α1, TNF-α2), interleukin-8 (IL-8), transforming growth factor beta isoform 1 (TGF-β1), inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), major histocompatibility complex beta chain (MHC-II β-chain) and T-cell receptor beta chain (TCR β-chain). Immune-regulatory genes that were up/down-regulated in AGD-infected trout compared with uninfected controls at 0, 7, and 14 d post-inoculation (p.i.) in gill, liver and anterior kidney tissue were initially identified by means of semi-quantitative RT-PCR. Up/down-regulated immune-regulatory genes were subsequently quantitated and validated by real-time RT-PCR (qRT-PCR). The extent of AGD-associated pathology was consistent amongst all AGD-infected trout at 7 d p.i. and increased considerably by 14 d p.i. At both 7 and 14 d p.i. IL-1β1 and iNOS gene expression was significantly up-regulated in the gills, and IL-8 was significantly up-regulated in the liver of AGD-infected trout at 7 d p.i. These data demonstrate the involvement of the immune response to AGD at the molecular level, and indicate the importance of

this response at the site of infection and the possible involvement of a systemic immune response.

1. Introduction

Amoebic gill disease (AGD) is an ectoparasitic infection of fish gills due to infestation of the gills with the protozoan *Neoparamoeba* sp. Clinical signs of AGD include lethargy, flared opercula, obvious respiratory distress, loss of equilibrium, and mortality if left untreated [1]. Strongly associated with these clinical signs is the presence of grossly visible pale multifocal gill lesions that, when examined histologically, are characterised by epithelial hyperplasia, lamellar fusion and the formation of interlamellar crypts [2, 3]. *Neoparamoeba* spp. have been isolated from several cultured marine fish species during AGD outbreaks throughout many different geographical locations worldwide [4]. However, AGD and the ensuing cost of the current treatment, freshwater bathing, have negatively impacted on the production of Atlantic salmon, *Salmo salar*, in Tasmania, Australia, more so than anywhere else in the world.

Little is known about the involvement of the immune response in AGD infection. Previous investigations have reported the detection of serum anti-*Neoparamoeba* sp. antibodies in farmed Atlantic salmon [5]. However, Atlantic salmon infected with *Neoparamoeba* sp. do not necessarily develop serum anti-*Neoparamoeba* sp. antibodies [5], and previously infected fish are not protected from reinfection upon subsequent re-exposure to *Neoparamoeba* sp. [6]. Histological changes associated

with infection of the gills with *Neoparamoeba* sp. during AGD are relatively well characterised [3, 7-10]. Besides the obvious epithelial hyperplasia that presents as grossly visible pale multifocal gill lesions, there is the less apparent infiltration of leucocytes into the gill lesions and oedema associated with a local inflammatory response. This migration of leucocytes is highly dependent on the stage of infection and age of the lesion [3, 7, 8, 10, 11]. Lesion-associated leucocytes are predominantly found in the central venous sinus (CVS), where they supposedly extravasate into the lesions and are often found in close association with amoebae in interlamellar crypts [10, 11]. These leucocytes appear to participate in lesion repair [8-11]. However, their role in other immune responses to AGD is unknown. Although knowledge of the host immune response in AGD is limited, it is well known that various cytokines and other immune-related factors co-ordinate the immune response to various pathogens.

The identification and characterisation of numerous fish cytokine and immune-regulatory genes in recent years has allowed the study of these genes during disease processes. Thus, the number of studies on the expression of immune-regulatory genes in fish infected with various bacterial, viral and parasitic pathogens, and their involvement in specific disease processes, is rapidly increasing [12-21]. Of particular relevance to the present study are investigations into the expression of rainbow trout, *Oncorhynchus mykiss*, immune-regulatory genes during infection with the ectoparasites *Ichthyophthirius multifiliis* [18, 19] and *Gyrodactylus derjavini* [20, 21]. Infection with the parasitic ciliate *I. multifiliis* resulted in an increased expression of IL-1β, TNF-α and IL-8 at the site of infection, the skin. IL-

1β and IL-8 were also up-regulated in the anterior kidney and spleen, but to a lesser extent [19]. However, the greatest increase in expression was observed in the skin at 4 d post-infection (p.i.), where IL-1β expression was up-regulated relative to controls by 17.8 fold [19]. The immunological importance of the site of infection was also highlighted by Sigh *et al.* [18], who showed that genes encoding complement factor C3, MHC-II, immunoglobulin M (IgM) and iNOS were up-regulated in the skin of trout, *O. mykiss*, infected with *I. multifiliis*. Similarly, IL-1β isoforms 1 and 2, the type II IL-1 receptor (IL-RII, 'decoy receptor'), TNF-α1 and iNOS gene expression were shown to change in skin tissue during parasitic infection of rainbow trout with the monogenean *G. derjavini* [21].

In the present study we examined the expression of selected rainbow trout cytokine and immune-regulatory genes in gill, liver and anterior kidney tissue during infection with the parasitic amoeba *Neoparamoeba* sp. to gain further knowledge of the involvement of AGD-related local and systemic immune responses in this salmonid species.

2. Materials and methods

2.1. Fish

Rainbow trout, *O. mykiss*, weighing approximately 200-250 g, were randomly divided into two groups and each group maintained in a separate 4000 L re-circulating Rathburn tank with biofilter. Sea water was UV-irradiated and 1 μm

filtered before addition to the tanks and maintained at 35‰ salinity at a temperature of 16 ± 0.5°C.

2.2. Amoebae isolation and infection

Amoebae were harvested from the gills of Atlantic salmon according to the procedure described by Morrison *et al.* [22] and used to infect rainbow trout in one of the Rathburn tanks. Isolated amoebae were identified as *Neoparamoeba* sp. by both polymerase chain reaction (PCR) [23] and immunocytochemical staining [11], and then added to the recirculating water supply at a rate of 450 amoebae L^{-1} d^{-1} over three consecutive days.

2.3. Tissue sampling

Gill, liver, and anterior kidney samples were collected from 18 fish in each of the control (AGD-uninfected) and AGD-infected groups at 0, 7, and 14 d post-inoculation (p.i.) with *Neoparamoeba* sp.. These sampling times were chosen as they represented three clearly distinct stages of clinical AGD pathology during the laboratory-based AGD infection. In brief, fish were euthanased with 5 g L^{-1} Aqui-S (Aqui-S NZ Ltd, Lower Hutt, New Zealand) before tissue samples were rapidly dissected and immediately placed in an appropriate volume of RNAlater stabilisation reagent (Qiagen, Clifton Hill, Victoria, Australia). RNAlater-stabilised tissue was placed at 4°C overnight before storage at -20°C until RNA extraction.

For histological analysis the second left gill arch was dissected and placed in seawater Davidson's fixative and routinely processed for histology (5 μm, H & E).

2.4. RNA extraction and cDNA synthesis

Tissues from 18 uninfected and 18 AGD-affected trout per sample time point were individually weighed and combined equally to form three pooled samples (each pooled from six trout) per treatment group. Total RNA was extracted from the pooled RNAlater stabilised tissue samples using an RNeasy RNA extraction kit (Qiagen), a Dounce homogeniser, and QIAshredders (Qiagen) according to manufacturer's instructions with on-column DNAse digestion (Qiagen). Total RNA was eluted in 30 μL RNAse-free water, quantified by spectrophotometry, and an aliquot run on a 1% agarose gel and post-stained with ethidium bromide to verify integrity of the total RNA. All extracted RNA samples had an A_{260}/A_{280} ratio in water of 1.8-2.0 and had well defined 28S and 18S rRNA bands, no sign of degradation, and a 28S band approximately twice as intense as the 18S band as measured by gel densitometry software (Silk Scientific, Utah, USA). Immediately after RNA extraction, 5 μg total RNA was reverse transcribed into cDNA using a SuperScript First-Strand cDNA Synthesis System (Invitrogen, Mount Waverly, Victoria, Australia) following the manufacturer's instructions using oligo $dT_{(12\text{-}18)}$ priming followed by RNAse H digestion. To determine if contaminating genomic DNA was present after RNA extraction and DNAse treatment, controls lacking reverse transcriptase were included at each time point. These samples were then used in both standard PCR and quantitative real-time PCR.

2.5. Standard PCR

Polymerase chain reaction (PCR) was performed using primers for the constitutively expressed housekeeping gene β-actin as both a positive control and for sample normalisation. To make the semi-quantitative PCR analysis as quantitative as possible, the cycle number for each gene examined was kept as low as practically possible to remain within the linear range of PCR amplification whilst still allowing endpoint gel densitometric analysis. The β-actin PCR products obtained from 28 cycles of amplification were used to adjust the initial cDNA dilution, and the subsequent amount used in each PCR. The target genes, primer sequences and predicted amplicon sizes are listed in Table 1, and the cycling conditions for each target gene are indicated in Table 2. PCR reactions were performed on an Eppendorf Mastercyler Gradient thermocycler (Eppendorf, North Ryde, NSW, Australia) using 25 μL reaction volumes containing 12.5 μL PCR Master Mix (Promega, Annandale, NSW, Australia), 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM), 5.5 μL nuclease-free water and adjusted cDNA template in 5 μL. Control reactions without cDNA template (NTC) were performed to ensure that products were not a result of DNA contamination or due to primer-dimer effects. PCR products (10 μL) were visualised on a 2% agarose gel post-stained with ethidium bromide, and semi-quantitative analysis of mRNA amounts were assessed via gel densitometric analysis using a UVP Gel imaging system (Ultra-Violet Products, California, USA) and gel densitometry software (Silk Scientific). Target gene expression was reported relative to β-actin expression after

subtraction of the background pixel intensity. Mean values (+ standard error) from three samples (each sample pooled from six fish) of the target gene expression, relative to β-actin expression, are presented. Statistical differences in gene expression between AGD-infected and uninfected control samples at each sampling time were assessed using a Student's two-tailed heteroscedastic *t*-test, irrespective of the normality of the distribution [24]. A *P* value of less than or equal to 0.05 was considered statistically significant.

Table 1:

Amplicon sizes and gene-specific primers used to amplify rainbow trout immune-regulatory genes

Gene target	Amplicon size (bp)	Primer Designation	Primer Sequence (5'-3')
β-actin	543	Actin-F	ATCGTGGGGCGCCCCAGGCACC
		Actin-R	CTCCTTAATGTCACGCACGATTTC
iNOS	746	iNOS-F4	CATACGCCCCCAACAAACCAGTGC
		iNOS-R5	CCTCGCCTTCTCATCTCCAGTGTC
IL-1β1	873	IL1B-F10	GGATTCACAAGAACTAAGGAC
		IL1B-R3	CTTAGTTGTGGCGCTGGATG
TNF-α1	181	TNF1-F1	CAAGAGTTTGAACCTTGTTCAA
		TNF1-R1	GCTGCTGCCGCACATAGAC
TNF-α2	208	TNF2-F3	CAAGAGTTTGAACCTCATTCAG
		TNF2-R5	GCTGCTGCCGCACATAAAG
IL-8	226	IL8-F3	GGATGTCAGCCAGCCTTGTC
		IL8-R3	TCCAGACAAATCTCCTGACCG
TGF-β1	365	TGFb-F5	GAAGAAACGACAAACCACTAC
		TGFb-R8	GACATGTGCAGTAATTCTAGC
COX-2	382	COX2-F	CTTACTCACTACAAAGGG
		COX2-R	CTGGTCCTTTCATGAAGT
MHC-II β chain	336	ONMY DB001-F	ATGTCGATGCCAATTGCCTTCTA
		ONMY DB318-R	TGTCTTGTCCAGTATGGCGCT
TCR-β chain	412	TCRbeta-F	CTCCGCTAAGGAGTGTGAAGATAG
		TCRbeta-R	CAGGCCATAGAAGGTACTCTTAGC

Table 2.

Summary of cycling conditions used in polymerase chain reaction amplifications.

Target	Cycling protocol			
	Denature	Anneal	Extend	No. of cycles
β-actin	95°C/ 3 min	-	-	1
	95°C/ 35 s	55°C/ 35 s	72°C/ 1 min	28
	-	-	72°C/ 6 min	1
iNOS	95°C/ 3 min	-	-	1
	95°C/ 1 min	56°C/ 1 min	72°C/ 1 min 30s	35
	-	-	72°C/ 6 min	1
IL-1β1	95°C/ 3 min	-	-	1
	95°C/ 45 s	58°C/ 45 s	72°C/ 1 min	28
	-	-	72°C/ 6 min	1
TNF-α1	95°C/ 3 min	-	-	1
	95°C/ 30 s	55°C/ 45 s	72°C/ 45 s	40
	-	-	72°C/ 6 min	1
TNF-α2	95°C/ 3 min	-	-	1
	95°C/ 30 s	58°C/ 45 s	72°C/ 20 s	35
	-	-	72°C/ 5 min	1
IL-8	95°C/ 3 min	-	-	1
	95°C/ 30 s	58°C/ 45 s	72°C/ 20 s	35
	-	-	72°C/ 5 min	1
TGF-β1	95°C/ 3 min	-	-	1
	95°C/ 30 s	58°C/ 45 s	72°C/ 45 s	30
	-	-	72°C/ 6 min	1
COX-2	95°C/ 3 min	-	-	1
	95°C/ 30 s	50°C/ 45 s	72°C/ 45 s	30
	-	-	72°C/ 6 min	1
MHC-II β chain	95°C/ 3 min	-	-	1
	95°C/ 30 s	56°C/ 45 s	72°C/ 45 s	25
	-	-	72°C/ 6 min	1
TCR-β chain	95°C/ 3 min	-	-	1
	95°C/ 30 s	56°C/ 45 s	72°C/ 45 s	25
	-	-	72°C/ 6 min	1

2.6. Real-time PCR conditions

Genes that were found to be up-regulated during semi-quantitative PCR analysis were validated by quantitative real-time PCR (qPCR), using the QuantiTect probe gene expression assay system (Qiagen). In brief, the QuantiTect probe system incorporates a minor groove binder (MGB), Eclipse dark quencher, and modified bases known as Superbases that together enable the use of primers and probe at predefined sequences, such as exon/exon boundaries, to avoid amplification of genomic DNA sequences. In a nonhybridised state in solution, the QuantiProbe has a random coil structure so that the fluorescence of the 3' reporter fluorophore is suppressed by the 5' nonfluorescent quencher (Eclipse Dark Quencher). When the probe hybridises to a complementary target at the annealing temperature (56°C), the quencher and the reporter are separated and fluorescence is emitted. Following hybridisation, the MGB folds into the minor groove of the probe-target duplex and stabilises it, preventing hydrolysis of the QuantiProbe by the 5'→ 3' exonuclease activity of *Taq* DNA polymerase during the 76°C extension phase of PCR. QuantiProbes and primers were designed using the Qiagen Quantitect Custom Assay design software in the custom mode that allowed the development of assays with primers that crossed exon/exon boundaries. This ensured that no genomic DNA was co-amplified with the reverse transcriptase–generated cDNA and that assays had equal PCR efficiencies. The following dye combination for probe generation was used for detection and data normalisation: 6-carboxyfluorescein (FAM) (3' reporter) and MGB-DQ (minor groove binder-dark quencher) for genes of interest and for normalisation control genes. The following probe and primer

sequences were used: for β-actin, sense primer 5'-TCTCTGGAGAAGAGCTAC-3', antisense primer
5'-CAAGACTCCATACCGAGGAA-3', and probe (MGB-DQ)
5'-ACGGACAGGT*CAT*CAC-3' (FAM); for IL-1β1, sense primer
5'-TATCCCATCACCCCATCA -3', antisense primer
5'-CCAACACTATATGT*T*CTTCCAC-3', and probe (MGB-DQ)
5'-CAACCTCATCATCGCCA-3' (FAM); for iNOS, sense primer
5'-TCAGAACCTCCTCCACAA-3', antisense primer
5'-GTGTACTCCTGAGAGTCCTTT-3', and probe (MGB-DQ)
5'-GCACCGACAGCGTCTA-3' (FAM). The symbol * denotes modified bases known as Superbases, which are analogues of the corresponding naturally occurring bases that form strong bonds with their unmodified complementary bases in the target sequence.

Reactions were assembled according to manufacturer's instructions with individual 50 µL reactions consisting of 25 µL 2x QuantiTect Probe PCR Master Mix (including ROX passive reference dye), 2.5 µL 20x Primer Mix, 2.5 µL 20x QuantiProbe Solution, 10 µL RNase-free water and 10 µL of appropriately diluted sample cDNA. The dilution chosen for the cDNA samples was determined from the results of qPCR using the cDNA dilution series also used to assess the qPCR amplification efficiencies of the gene-specific qPCR primers. The qPCR reactions were performed using three uninfected and three AGD-affected samples (each pooled from six fish) from each sampling time and were assayed in duplicate. All reactions were performed using an ABI Prism 7700 Sequence Detector (ABI,

Scoresby, Victoria, Australia) with the following cycle parameters: one cycle of 95°C for 15 min to initiate activity of the HotStarTaq DNA polymerase, followed by 45 cycles of 94°C for 15 s, annealing at 56°C for 30 s, and extension at 76°C for 30 s. Data were collected only during the annealing phase, and raw data were analysed using the ABI Prism Sequence Detection 1.9.1 software (ABI). As an additional control to designing primers that cross an exon/exon boundary, mock reverse-transcription reactions, without the reverse transcriptase, were also performed on a few selected samples. Real-time PCR performed on these samples showed no evidence of any amplified product, thus demonstrating that the reactions were free of contaminating DNA. Agarose gel electrophoresis resulted in a single product of the desired length (β-actin, 113 bp; IL-1β1, 159 bp; iNOS, 93 bp). Additionally, amplicons were sequenced to confirm specificity of amplification.

2.7. DNA sequencing

Amplicons generated from qPCR were electrophoresed on a 3% agarose gel, visualised by ethidium bromide staining, and the products were excised and gel-purified using a spin column and MiniElute PCR purification kit (Qiagen) according to manufacturer's instructions. Purified products were either sequenced directly or cloned into the pGEM-T Easy vector system (Promega). Sequencing reactions were performed with a CEQ Dye Terminator Cycle Quick Start Kit (Beckman Coulter, Fullerton, California, USA), using either the forward or reverse primers used to generate the amplicon, or plasmid DNA purified with a QIAprep Spin Miniprep kit (Qiagen) and an M13 reverse primer. Sequencing was performed

on a Beckman Coulter CEQ8000 automated sequencer (Beckman Coulter) according to manufacturer's instructions.

2.8. Real-time PCR data analysis

The threshold cycle (Ct) of each sample was determined manually for each product and the mean Ct of the replicates calculated. β-actin was used as a housekeeping gene to normalise the gene expression in the samples. For accurate relative gene expression quantitation PCR amplification efficiencies (E) of the target assays should be similar to that of the reference assay (β-actin) [25]. A difference of 0.2 in PCR amplification efficiency between the target and reference gene will result in a more than 10-fold difference in the final result after 25 cycles unless an algorithm is used that corrects for minor differences in E [26]. PCR efficiencies were determined from serial dilutions of β-actin, IL-1β1 and iNOS cDNA and the resulting plots of Ct versus the logarithm of the dilution of the cDNA, using the equation $E = 10^{(-1/slope)}$ [27]. PCR efficiencies were 1.92 for β-actin, 1.95 for IL-1β1, and 1.95 for iNOS. Fold changes in the gene expression of the AGD-infected samples compared with the uninfected control samples at each sampling time point were calculated according to the following equation, using the REST-XL version 2 software [25, 27].

$$\text{Ratio} = (E_{\text{target}})^{\Delta Ct[\text{target(control - AGD-infected)}]} / (E_{\beta\text{-actin}})^{\Delta Ct[\beta\text{-actin(control - AGD-infected)}]}$$

The REST-XL software takes into account minor differences in PCR efficiencies (E) when calculating the fold change in expression, and determines whether the expression of a target gene relative to a reference gene is significantly different between an experimental group and a control group, using a pair-wise fixed reallocation randomisation test [25]. Using the REST-XL software we also validated the use of β-actin as a reference gene for accurate normalisation in experiments comparing relative gene expression in gill tissue from AGD-infected and uninfected rainbow trout, as no difference was evident in the mean β-actin Ct deviation in the control group compared with the AGD-infected group [ΔCt β-actin $_{(\text{mean control - mean AGD-infected})} = 0$]. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Infection

Gross examination of infected fish gills at 7 d p.i. revealed characteristic pale multifocal mucoid lesions associated with AGD (Fig. 1). The distribution and size of the lesions was consistent amongst the sampled gill arches of all fish at 7 d p.i. By 14 d p.i. the distribution and size of gill lesions of infected fish had significantly increased, whilst the degree of gross pathology remained similar amongst all the infected fish at 14 d p.i. (Fig. 1). As expected, gross examination of the uninfected fish at each sample time point showed no signs of AGD or other infections. Gross pathological changes seen in infected fish were confirmed to be the result of AGD

when gill sections were studied histologically. Amoebae were visible in gill sections from infected fish at both 7 d p.i. and 14 d p.i. and were found associated with the lesions (Fig. 2). Lesions at 14 d p.i. consisted of greater numbers of fused lamellae than those at 7 d p.i. and had a thicker layer of hyperplastic epithelial cells towards the lesion surface (Fig. 2). Furthermore, leucocytes were observed in interlamellar crypts as well as within the CVS at 14 d p.i. in AGD-affected trout (Fig. 2). No signs of AGD or any other pathological changes were visible in gill sections from the uninfected fish at 0, 7 and 14 d p.i. No mortalities in the AGD-affected or uninfected control fish were observed over the duration of the experiment.

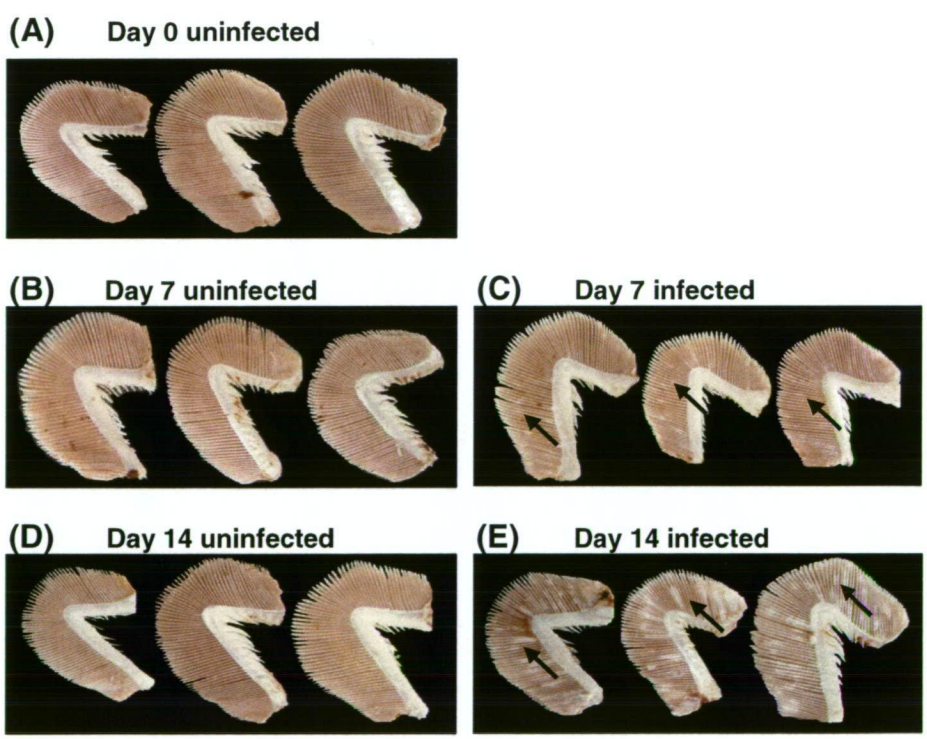


Figure 1.
Gross (macroscopic) images of *Neoparamoeba* sp.-infected and uninfected (control) rainbow trout gills at 0, 7 and 14 d p.i. with *Neoparamoeba* sp. Characteristic pale multifocal mucoid lesions (arrows) associated with AGD are evident in gill images of infected fish at 7 and 14 d p.i.

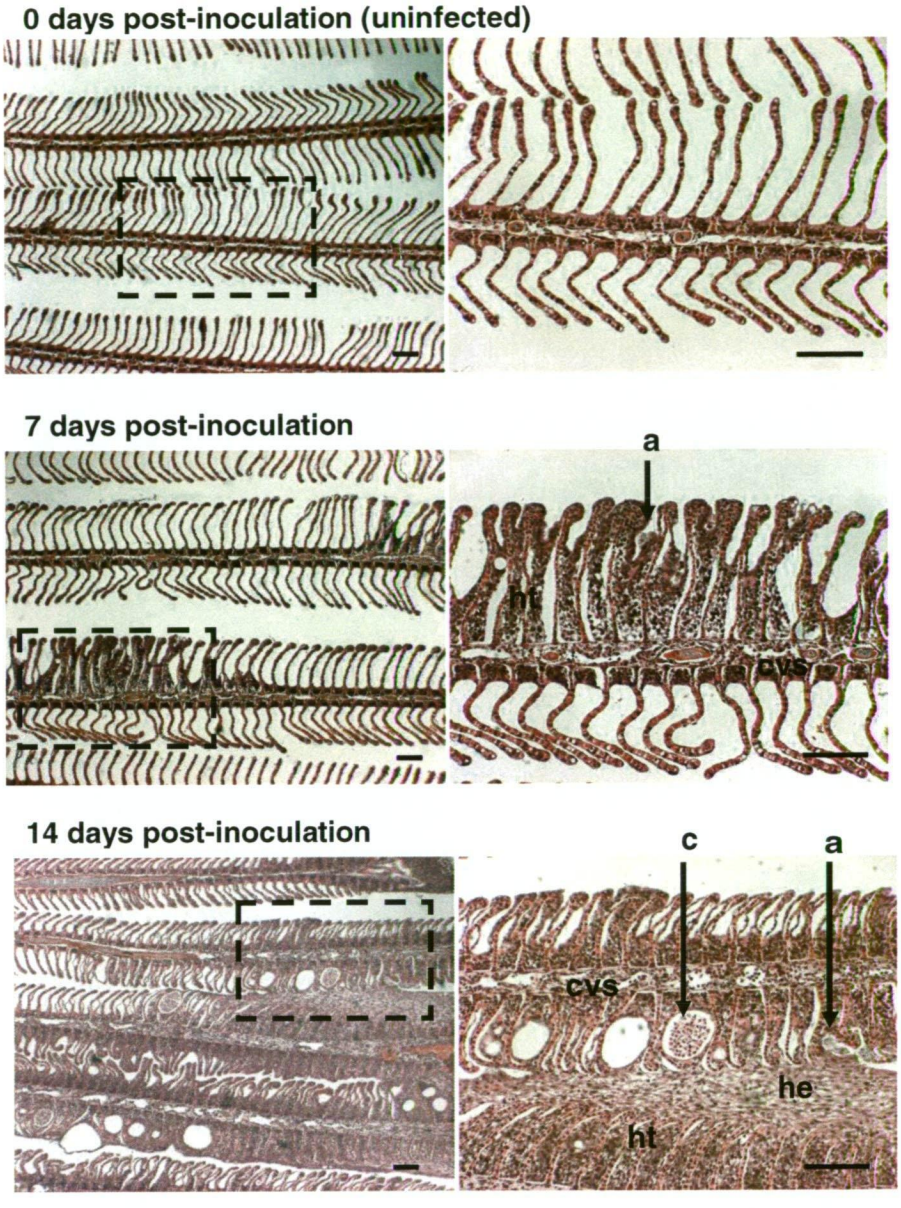


Figure 2.

Histopathology of *Neoparamoeba* sp.-infected and uninfected (control) rainbow trout gills at 0 (uninfected), 7 and 14 d p.i. with *Neoparamoeba* sp. AGD lesions consisted of hyperplastic tissue (ht) and hyperplastic epithelia (he) that fused lamellae and formed a stratified layer of epithelial tissue at the lesion surface. Amoebae (a) can be seen in close association with the lesion surface and are seen in conjunction with leucocytes within interlamellar crypts (c). Leucocytes are also present within the central venous sinus (CVS) (bars = 100 µm).

3.2. Gill gene expression

All of the studied immune-regulatory genes were constitutively expressed in the gills of uninfected fish at each sample time point (Fig. 3). However, the expression of the TNF-α1 gene was only detectable using a nested PCR (data not shown) and was therefore excluded from further analyses due to the unreliability of semi-quantitation after nested PCR. Standard PCR product intensities of the studied genes were reasonably high relative to β-actin, indicating that these genes had moderately abundant mRNA transcript levels in the gill when compared with the liver or anterior kidney. Expression of the studied genes in uninfected trout remained constant, as no significant change ($P > 0.05$) in expression of any of the genes was found at any sample time. AGD infection did significantly ($P < 0.05$) up-regulate the expression of IL-1β1 and iNOS genes at both 7 and 14 d p.i. compared with the uninfected controls (Fig. 3A and B). Gill IL-1β1 gene expression at 7 d p.i. was significantly ($P < 0.05$) up-regulated by 2.8 times relative to controls (Fig. 3A) and increased to 5.2 times the control fish at 14 d p.i. (Fig. 3A). Similarly, iNOS expression was 3.4 times that of the control fish at 7 d p.i. (Fig. 3B) and 7.6 times that of the control fish at 14 d p.i. (Fig. 3B).

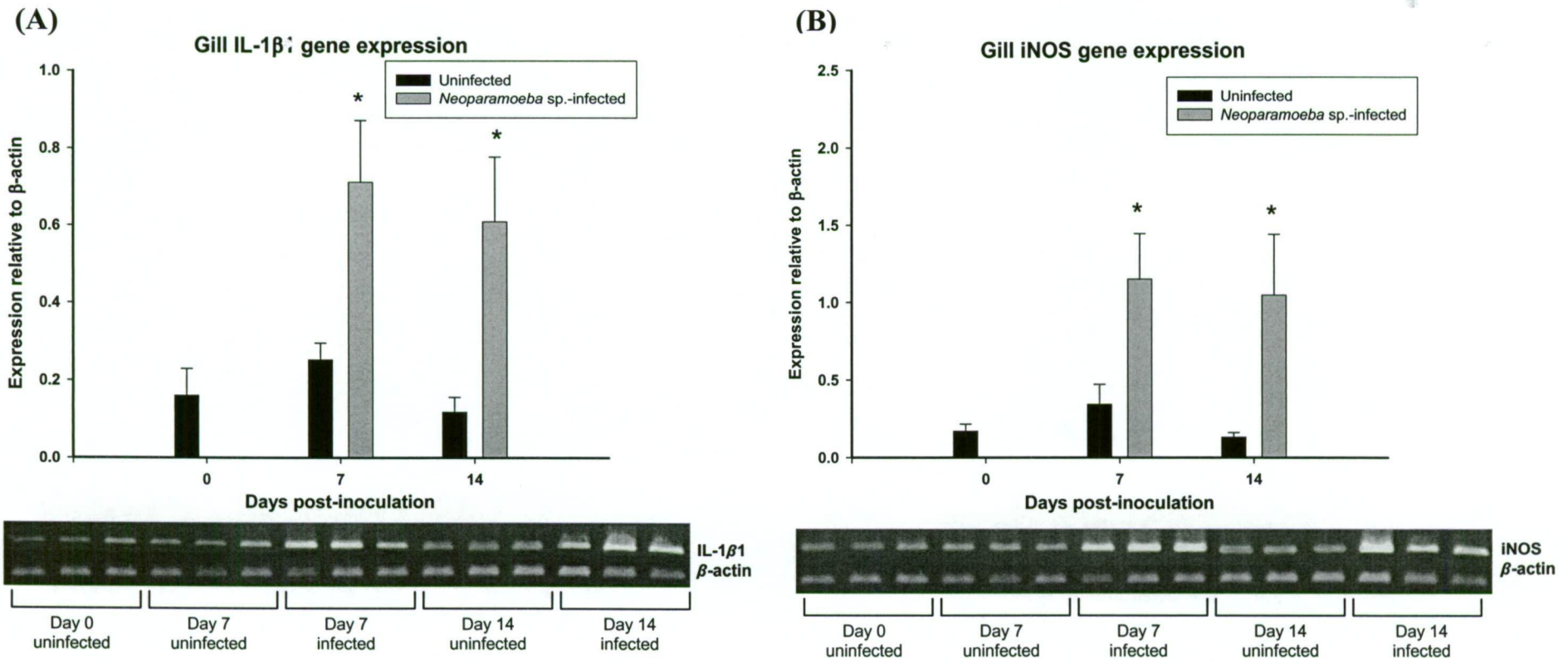
3.3. Liver gene expression

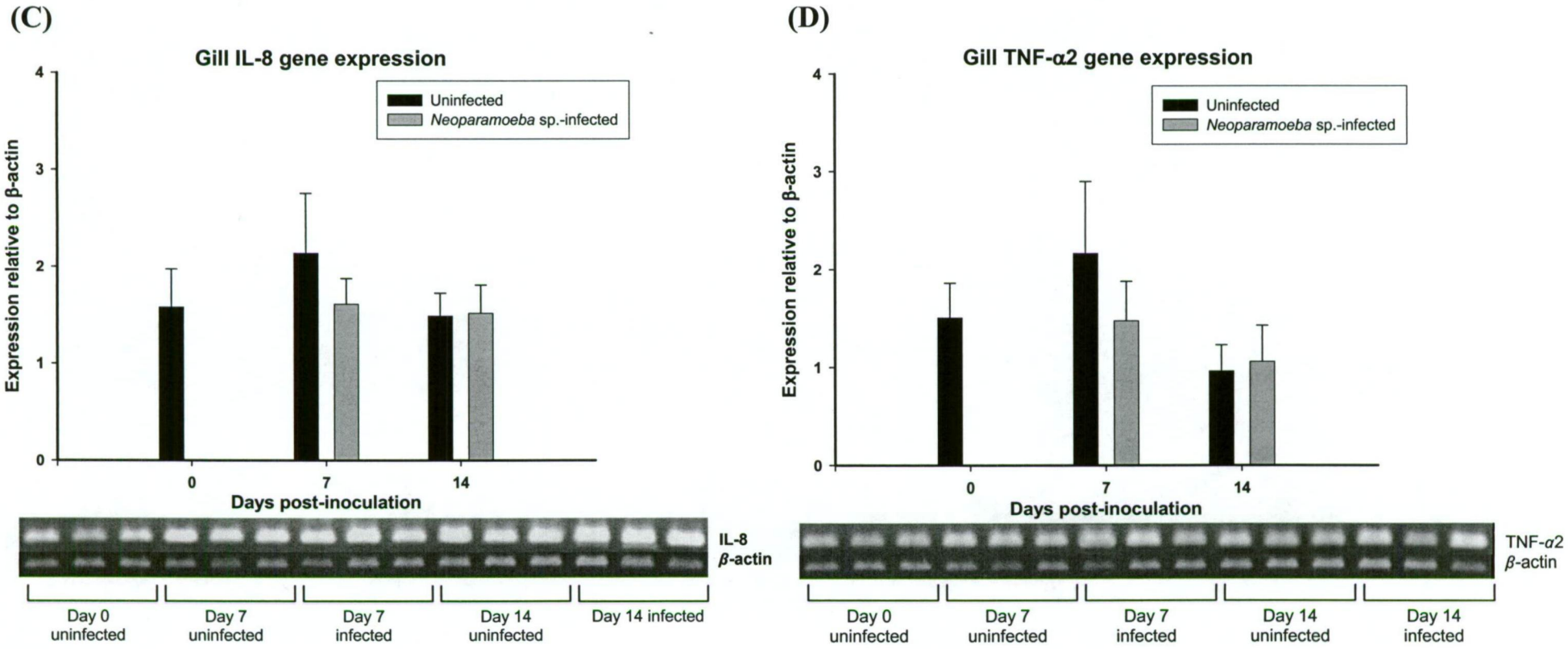
Constitutive expression of the studied immune-regulatory genes, with the exception of TNF-α1 (data not shown), was observed in the liver. At 7 d p.i. an AGD-induced up-regulation of IL-1β1 and iNOS expression was evident, but was not statistically

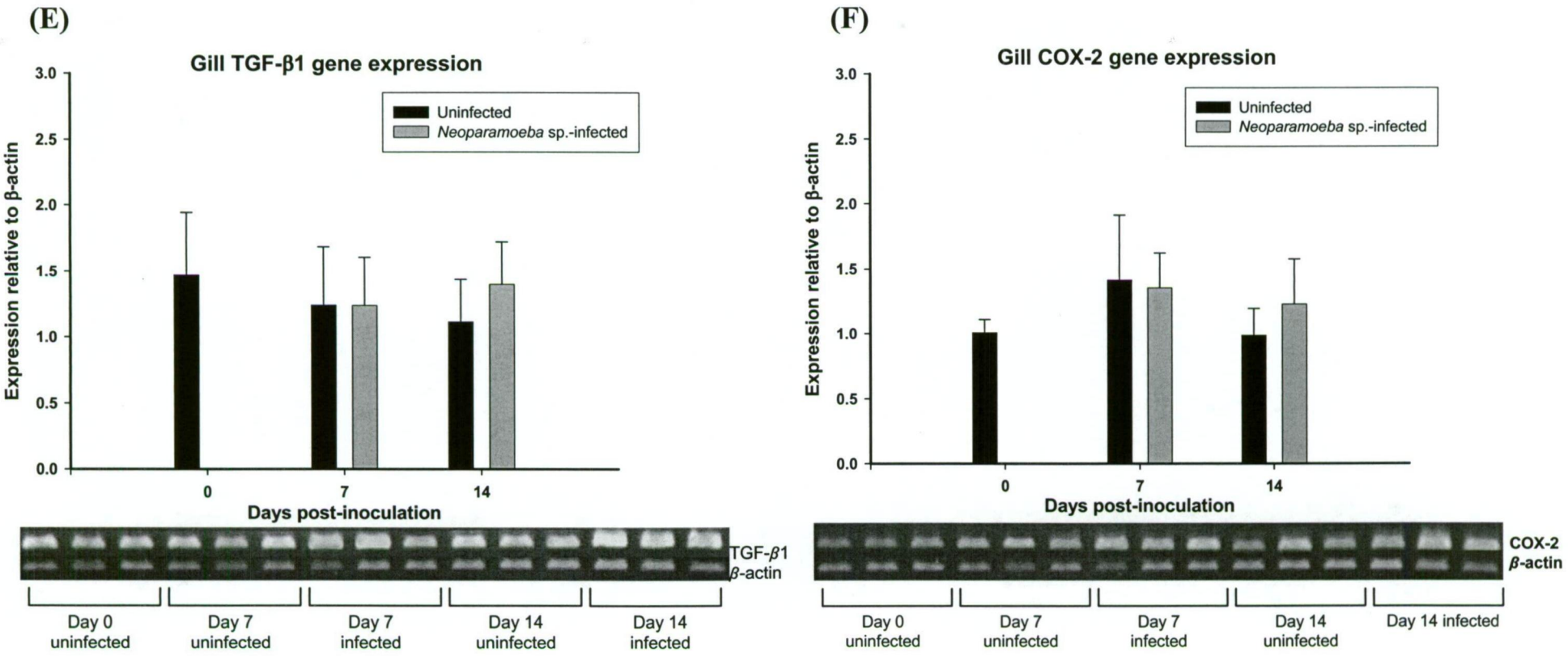
significant and was no different to the control level of expression at 14 d p.i. (Fig. 4A and B). Expression of IL-8 significantly increased ($P < 0.05$) by 4.1 times at 7 d p.i. (Fig. 4C) relative to controls, and like the increase in IL-1β1 and iNOS was no different to the control level of expression at 14 d p.i.

3.4. Anterior kidney gene expression

As was the case in the gill and liver, constitutive expression of the studied immune-regulatory genes, with the exception of TNF-α1 (data not shown), was observed. However, expression of the iNOS gene was undetectable in the anterior kidney at 0, 7 and 14 d p.i. The overall mRNA transcript abundance of the studied genes in the anterior kidney was greater than in the liver and comparable to the levels observed in the gill (Fig. 5). Unlike the gill and liver tissue, no AGD-induced up-regulation of the studied genes was found in the anterior kidney. Although relatively variable and hence not statistically significant ($P > 0.05$), semi-quantitative analysis showed that all of the studied genes, with the exception of IL-1β1, were seemingly down-regulated at 7 d p.i. (Fig. 5). This non-significant ($P > 0.05$) down-regulation was not found at 14 d p.i. when measuring any of the genes other than TNF-α2 (Fig. 5).


(A)
Gill IL-1β1 gene expression
Uninfected
Neoparamoeba sp.-infected
Expression relative to β-actin
1.0
0.8
0.6
0.4
0.2
0.0
*
*
0
7
14
Days post-inoculation
IL-1β1
β-actin
Day 0 uninfected
Day 7 uninfected
Day 7 infected
Day 14 uninfected
Day 14 infected
(B)
Gill iNOS gene expression
Uninfected
Neoparamoeba sp.-infected
Expression relative to β-actin
2.5
2.0
1.5
1.0
0.5
0.0
*
*
0
7
14
Days post-inoculation
iNOS
β-actin
Day 0 uninfected
Day 7 uninfected
Day 7 infected
Day 14 uninfected
Day 14 infected


(C)
Gill IL-8 gene expression
Uninfected
Neoparamoeba sp.-infected
Expression relative to β-actin
0
1
2
3
4
0
7
14
Days post-inoculation
IL-8
β-actin
Day 0 uninfected
Day 7 uninfected
Day 7 infected
Day 14 uninfected
Day 14 infected
(D)
Gill TNF-α2 gene expression
Uninfected
Neoparamoeba sp.-infected
Expression relative to β-actin
0
1
2
3
4
0
7
14
Days post-inoculation
TNF-α2
β-actin
Day 0 uninfected
Day 7 uninfected
Day 7 infected
Day 14 uninfected
Day 14 infected


(E)
Gill TGF-β1 gene expression
Uninfected
Neoparamoeba sp.-infected
Expression relative to β-actin
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
7
14
Days post-inoculation
TGF-β1
β-actin
Day 0 uninfected
Day 7 uninfected
Day 7 infected
Day 14 uninfected
Day 14 infected
(F)
Gill COX-2 gene expression
Uninfected
Neoparamoeba sp.-infected
Expression relative to β-actin
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
7
14
Days post-inoculation
COX-2
β-actin
Day 0 uninfected
Day 7 uninfected
Day 7 infected
Day 14 uninfected
Day 14 infected

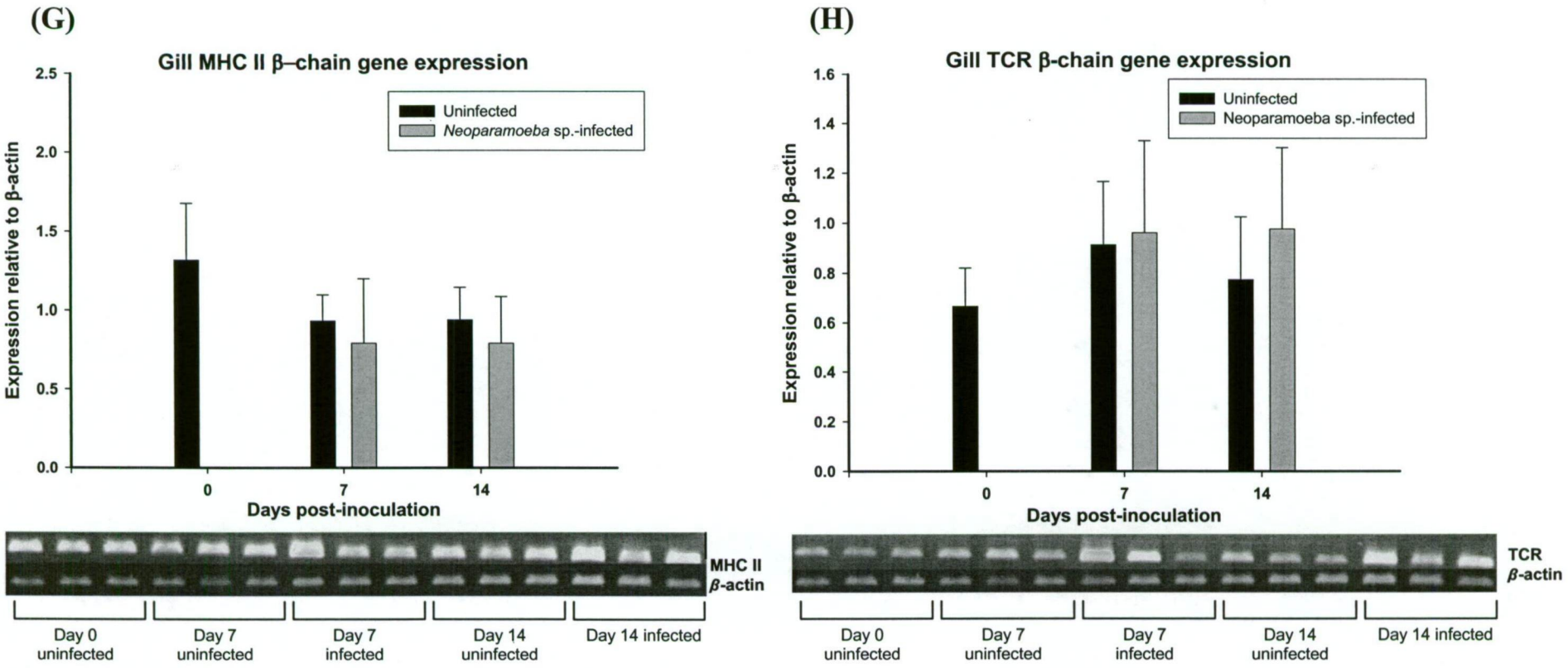
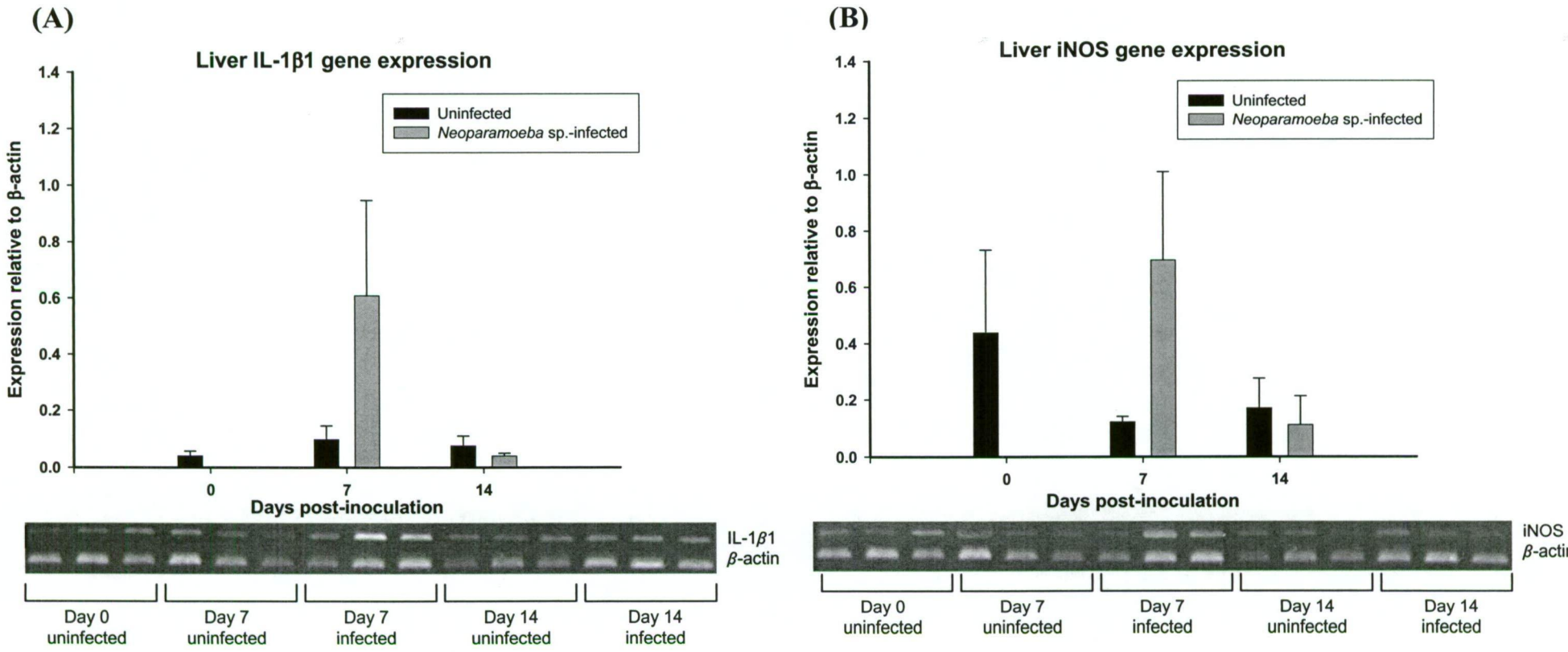
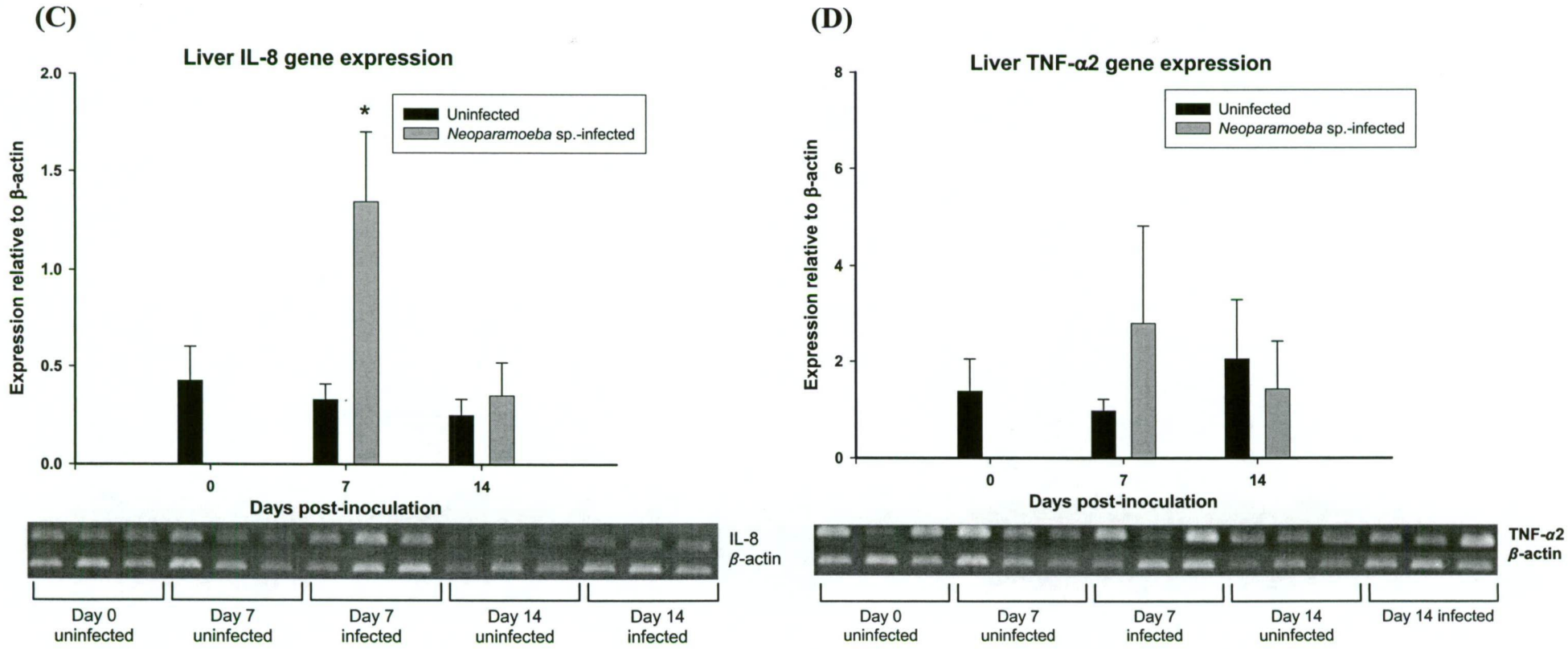


Figure 3.

RT-PCR amplification of immune-regulatory gene expression in gill samples from *Neoparamoeba* sp.-infected and uninfected (control) rainbow trout at 0, 7 and 14 d p.i. (A-H) Products were resolved and visualised on an ethidium bromide-stained gel. Each lane shows amplified products from gill samples pooled from six rainbow trout. Specific expression of immune-regulatory genes relative to β-actin was analysed using densitometry. Bars represent mean values (+ S.E.) of three samples, each pooled from six individual fish. * Denotes statistically significant up-regulation in target gene expression relative to the uninfected control at the same time p.i. ($P < 0.05$).


(A)
Liver IL-1β1 gene expression
Uninfected
Neoparamoeba sp.-infected
Expression relative to β-actin
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
7
14
Days post-inoculation
IL-1β1
β-actin
Day 0 uninfected
Day 7 uninfected
Day 7 infected
Day 14 uninfected
Day 14 infected
(B)
Liver iNOS gene expression
Uninfected
Neoparamoeba sp.-infected
Expression relative to β-actin
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
7
14
Days post-inoculation
iNOS
β-actin
Day 0 uninfected
Day 7 uninfected
Day 7 infected
Day 14 uninfected
Day 14 infected


(C)
Liver IL-8 gene expression
Expression relative to β-actin
2.0
1.5
1.0
0.5
0.0
*
Uninfected
Neoparamoeba sp.-infected
0
7
14
Days post-inoculation
IL-8
β-actin
Day 0 uninfected
Day 7 uninfected
Day 7 infected
Day 14 uninfected
Day 14 infected
(D)
Liver TNF-α2 gene expression
Expression relative to β-actin
8
6
4
2
0
Uninfected
Neoparamoeba sp.-infected
0
7
14
Days post-inoculation
TNF-α2
β-actin
Day 0 uninfected
Day 7 uninfected
Day 7 infected
Day 14 uninfected
Day 14 infected

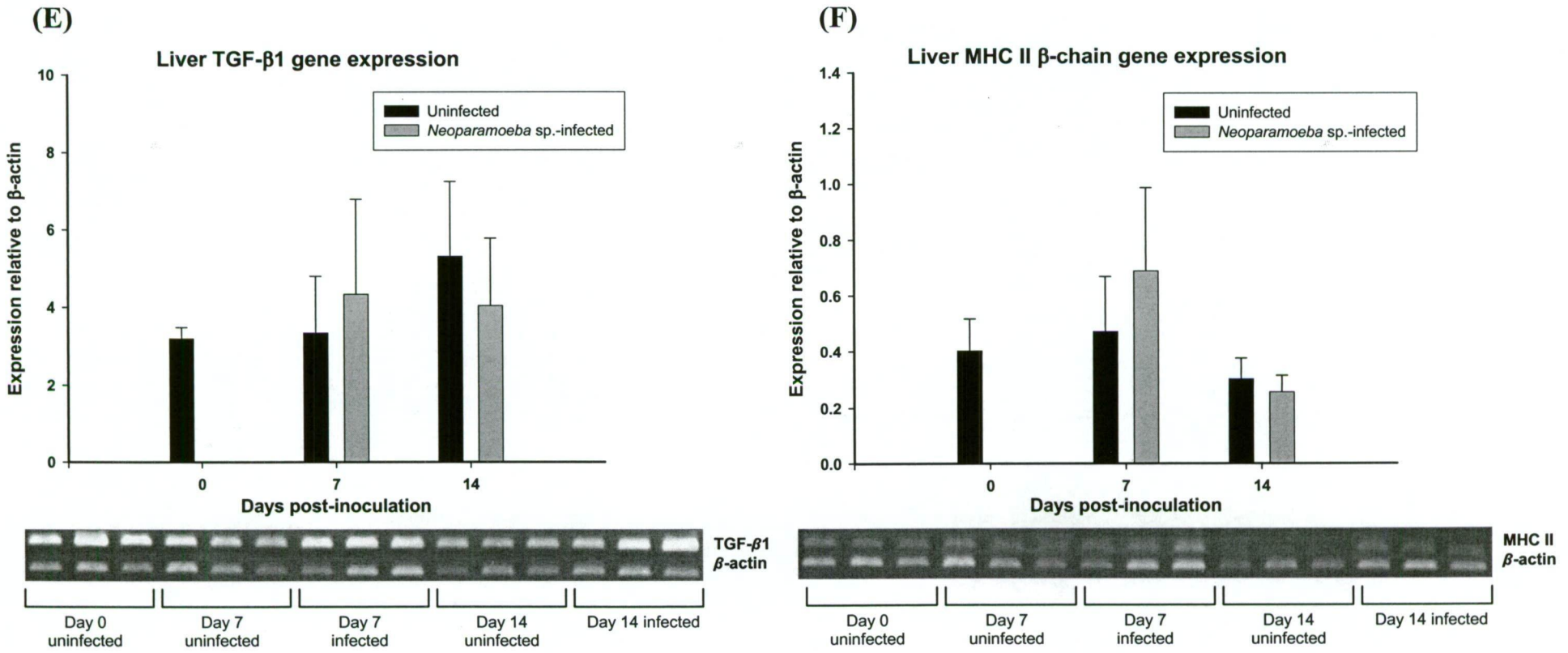
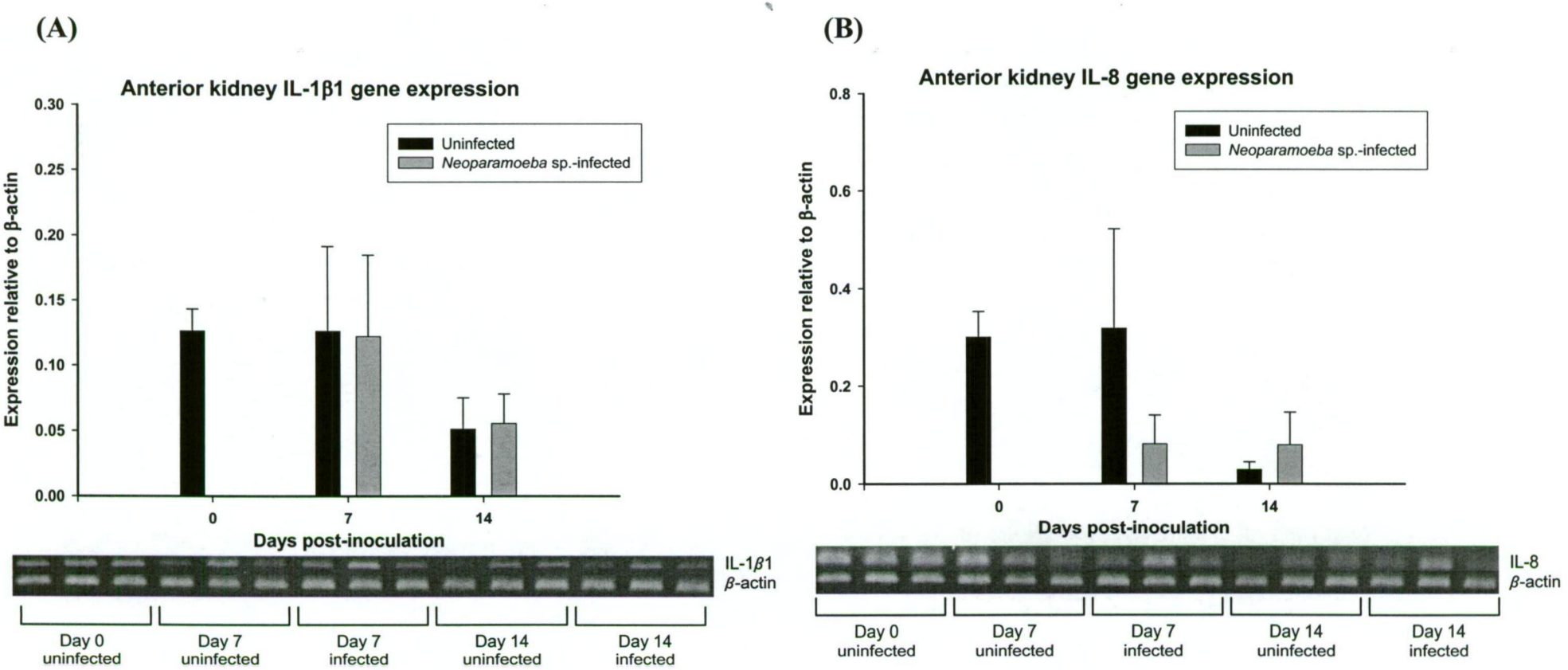
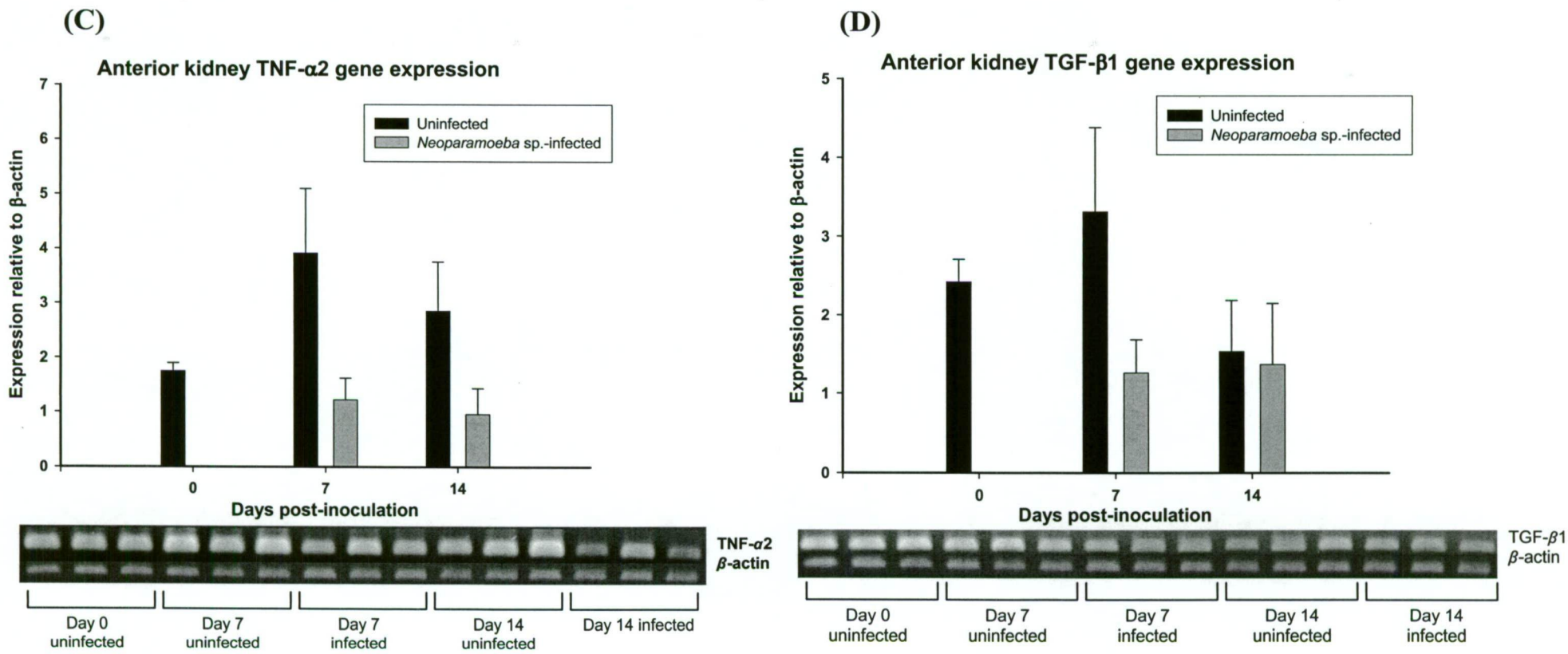
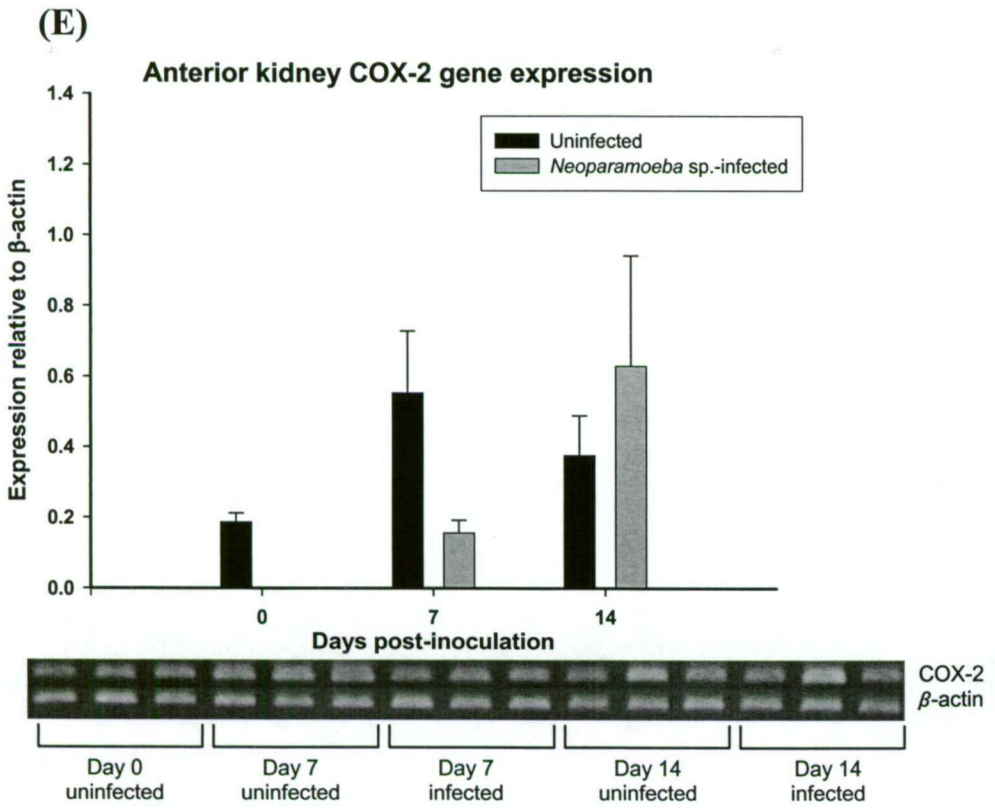


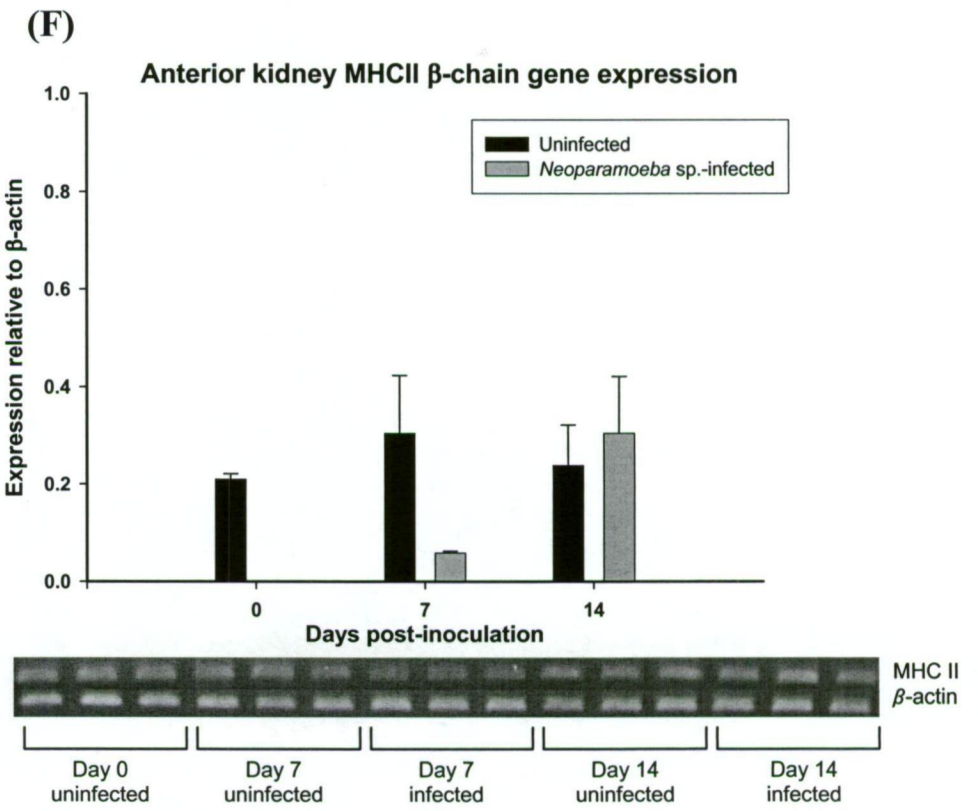
Figure 4.

RT-PCR amplification of immune-regulatory gene expression in liver samples from *Neoparamoeba* sp.-infected and uninfected (control) rainbow trout at 0, 7 and 14 d p.i. (A-F) Products were resolved and visualised on an ethidium bromide-stained gel. Each lane shows amplified products from liver samples pooled from six rainbow trout. Specific expression of immune-regulatory genes relative to β-actin was analysed using densitometry. Bars represent mean values (+ S.E.) of three samples, each pooled from six individual fish. * Denotes statistically significant up-regulation in target gene expression relative to the uninfected control at the same time p.i. ($P < 0.05$).


(A)
Anterior kidney IL-1β1 gene expression
Expression relative to β-actin
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Uninfected
Neoparamoeba sp.-infected
0
7
14
Days post-inoculation
IL-1β1
β-actin
Day 0 uninfected
Day 7 uninfected
Day 7 infected
Day 14 uninfected
Day 14 infected
(B)
Anterior kidney IL-8 gene expression
Expression relative to β-actin
0.8
0.6
0.4
0.2
0.0
Uninfected
Neoparamoeba sp.-infected
0
7
14
Days post-inoculation
IL-8
β-actin
Day 0 uninfected
Day 7 uninfected
Day 7 infected
Day 14 uninfected
Day 14 infected


(C)
Anterior kidney TNF-α2 gene expression
Expression relative to β-actin
Uninfected
Neoparamoeba sp.-infected
Days post-inoculation
TNF-α2
β-actin
Day 0 uninfected
Day 7 uninfected
Day 7 infected
Day 14 uninfected
Day 14 infected
(D)
Anterior kidney TGF-β1 gene expression
Expression relative to β-actin
Uninfected
Neoparamoeba sp.-infected
Days post-inoculation
TGF-β1
β-actin
Day 0 uninfected
Day 7 uninfected
Day 7 infected
Day 14 uninfected
Day 14 infected


(E)
Anterior kidney COX-2 gene expression
Uninfected
Neoparamoeba sp.-infected
Expression relative to β-actin
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
7
14
Days post-inoculation
COX-2
β-actin
Day 0 uninfected
Day 7 uninfected
Day 7 infected
Day 14 uninfected
Day 14 infected


(F)
Anterior kidney MHCII β-chain gene expression
Uninfected
Neoparamoeba sp.-infected
Expression relative to β-actin
1.0
0.8
0.6
0.4
0.2
0.0
0
7
14
Days post-inoculation
MHC II
β-actin
Day 0 uninfected
Day 7 uninfected
Day 7 infected
Day 14 uninfected
Day 14 infected

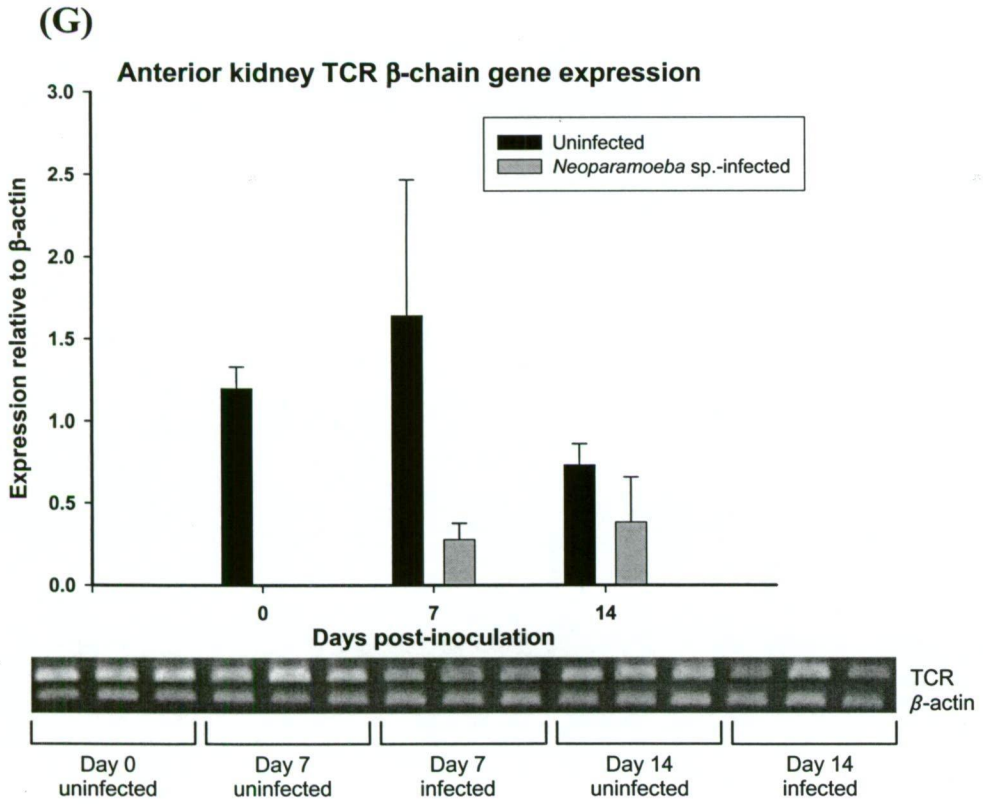


Figure 5.

RT-PCR amplification of immune-regulatory gene expression in anterior kidney samples from *Neoparamoeba* sp.-infected and uninfected (control) rainbow trout at 0, 7 and 14 d p.i. (A-G) Products were resolved and visualised on an ethidium bromide-stained gel. Each lane shows amplified products from anterior kidney samples pooled from six rainbow trout. Specific expression of immune-regulatory genes relative to β-actin was analysed using densitometry. Bars represent mean values (+ S.E.) of the three samples, each pooled from six individual fish.

3.5. Real-time PCR analysis of up-regulated gene transcripts in gill tissue

Real-time PCR analysis confirmed the semi-quantitative PCR results and showed that both iNOS and IL-1β1 gene expression was significantly up-regulated ($P < 0.05$) in the gills of AGD-infected fish relative to the uninfected control fish at 7 and 14 d p.i. (Fig. 6). The expression of β-actin in all samples remained constant at each of the sampling times, and no significant ($P > 0.05$) AGD-induced effect on β-actin gene expression was observed (Fig. 6). At 7 d p.i. expression of the iNOS gene was 4.8 times that of the control fish, and had increased to 6.8 times that of the uninfected control fish at 14 d p.i. Similarly, IL-1β1 gene expression at 7 d p.i. increased from 3.3 times to 7.6 times that of the control fish at 14 d p.i. Sequencing of the amplified products confirmed the specificity of the assays.

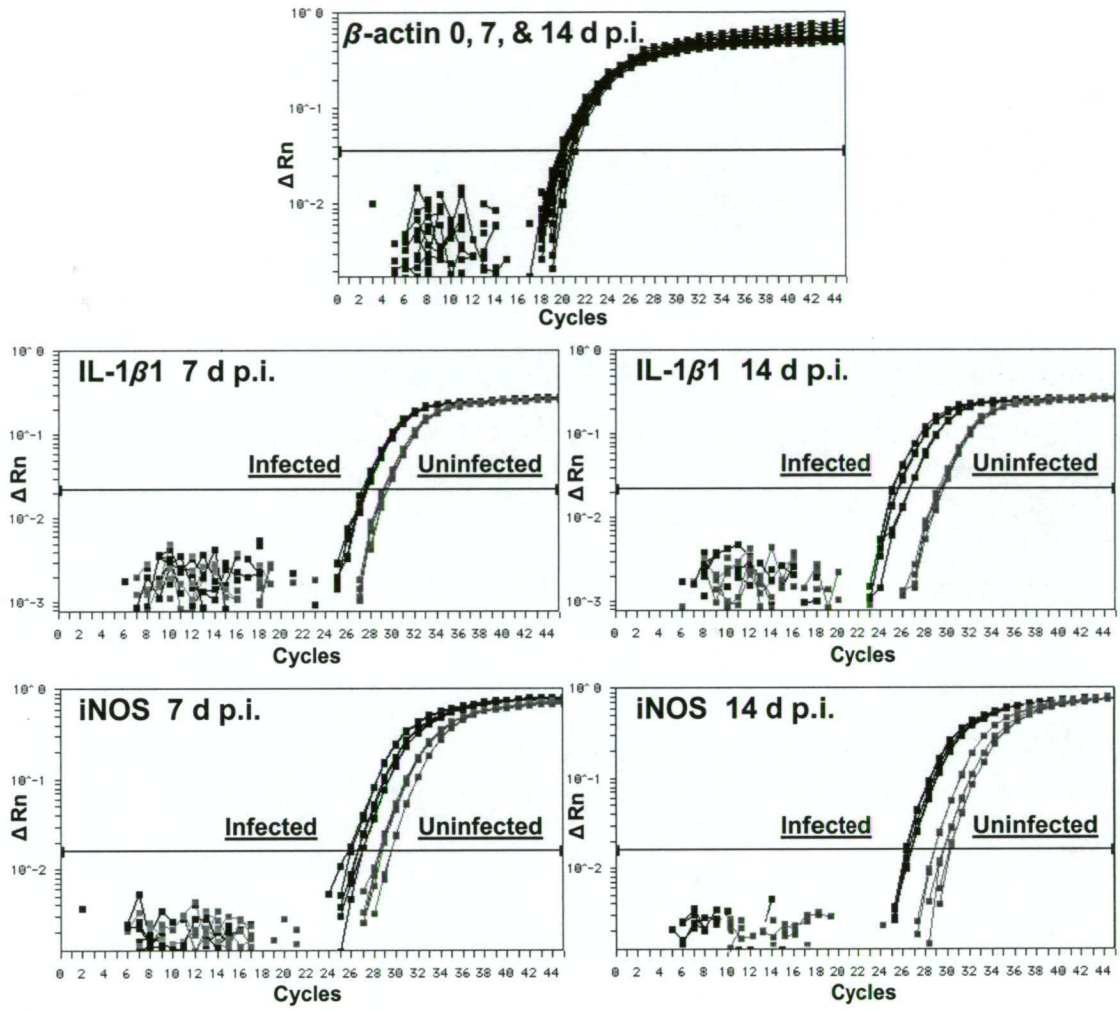


Figure 6.

Real-time PCR amplification plots of β-actin (normalising gene), IL-1β1, and iNOS gene expression in gill samples from *Neoparamoeba* sp.-infected and uninfected (control) rainbow trout at 0, 7, and 14 d p.i. Amplification plots for 0 d p.i. are shown for β-actin only. Real-time PCR was performed on an ABI 7700 sequence detector using QuantiTect probe chemistry. The cycle numbers are plotted against the change in fluorescence relative to a passive reference dye (ROX)(ΔRn). Both iNOS and IL-1β1 gene expression was significantly ($P < 0.05$) up-regulated at 7 and 14 d p.i. compared with the uninfected controls.

4. Discussion

The involvement and importance of immune-regulatory genes in fish diseases has been demonstrated by the growing number of fish disease studies profiling immune-regulatory genes [12-21]. In the present study we demonstrate that AGD in rainbow trout induces the up-regulation of iNOS and IL-1β1 mRNA expression in the gills, and up-regulation of the chemokine, IL-8, in the liver.

In both mammals and fish, iNOS and nitric oxide (NO) are well known immune-regulatory factors important in the defence against various pathogens. The importance of NO production during the immune response has been demonstrated during several pathogen infections in fish [28, 29]. Parasitic infection with the ciliate *I. multifiliis* induced the gene expression of iNOS in the skin of rainbow trout [18]. Likewise, studies of the monogenean ectoparasite *G. derjavini* found that iNOS and IL-1β were up-regulated in the skin of infected rainbow trout and implicated these genes as key mediators of anti-gyrodactylid responses [20, 21]. Constitutive gene expression of iNOS in the present study was easily detected in the gill tissue of uninfected and infected rainbow trout, but was less easily detected in the liver and was absent in the anterior kidney. The lack of detectable iNOS gene expression in the anterior kidney at 0, 7 and 14 d p.i. supports a similar finding by Campos-Perez *et al.* [13] that showed a lack of iNOS gene expression in un-challenged rainbow trout and a transient increase in expression after injection challenge with *Renibacterium salmoninarum* that quickly disappeared 3-5 d post-challenge. Constitutive expression of iNOS gene expression was reportedly absent

in isolated anterior kidney cells from uninfected rainbow trout but detectable in anterior kidney cells isolated from trout challenged with *Aeromonas salmonicida* for 48 h [30]. However, the duration of *A. salmonicida*-induced iNOS expression was unknown, as cells were isolated from fish challenged only for 48 h and no longer. Pathogen–induced iNOS gene expression was also transient in rainbow trout anterior kidney macrophages infected with *R. salmoninarum*. During this study, anterior kidney macrophages infected with *R. salmoninarum* had an increased iNOS gene expression at 2 h post–infection which then returned to undetectable levels at 24 h post-infection [14].

IL-1β is an important inflammatory mediator that is involved in the immune response to many pathogens [31]. IL-1β gene expression in rainbow trout skin infected with the ectoparasitic ciliate, *I. multifiliis*, is increased up to 17.8 times compared with uninfected controls [19]. In addition to the increased IL-1β gene expression at the site of infection, the skin, an increase in this transcript and several other transcripts was also observed in the anterior kidney and spleen of *I. multifiliis*-infected trout late during the infection [19]. However, it is noted by the authors that this apparent systemic IL-1β response may have been the result of non-specific changes induced by the moribund state of the fish during the latter stages of infection [19]. Two genes encoding different IL-1β isoforms, IL-1β1 and IL-1β2, exist in rainbow trout and the expression of both is induced in anterior kidney cells stimulated with LPS [32]. Knowledge of the differential function and expression of both isoforms during disease processes is currently limited. However, an earlier up-regulation of IL-1β1 in the spleen and anterior kidney of *I. multifiliis*-

infected trout, followed by a slightly delayed increased expression of IL-1β2, has been demonstrated [19]. Similarly, a faster down-regulation of IL-1β1 gene expression relative to IL-1β2 has been shown in the skin of *G. derjavini*-infected rainbow trout [20].

Two isoforms of TNF-α also exist in rainbow trout and, as is the case of IL-1β, the functional roles of the different isoforms are still poorly understood. However, both TNF-α1 and TNF-α2 can increase phagocytosis of rainbow trout anterior kidney leucocytes [33], and an increased expression of both isoforms is stimulated by LPS [34]. Previous studies of TNF-α have demonstrated a lack of constitutive expression in various tissues of Japanese flounder, *Paralichthys olivaceus*, [35] or isolated cells from carp, *Cyprinus carpio*, and rainbow trout [34, 36]. However, constitutive TNF-α expression was shown in the gill and anterior kidney from unstimulated trout, *O. mykiss*, [37]. Constitutive TNF-α expression has also been reported from gilthead seabream, *Sparus aurata* [38]. Differential expression of TNF-α isoforms occurs in anterior kidney leucocytes from trout and carp after *in vitro* stimulation with LPS or *T. borreli* respectively [33, 34, 36]. In both of these studies TNF-α2 was the predominantly up-regulated TNF-α isoform upon stimulation. Similarly, constitutive and differential expression of TNF-α isoforms was demonstrated during IHNV, *I. multifiliis* and *G. derjavini* infections of rainbow trout [15, 19, 21]. These infections induced an increase in the expression of the TNF-α1 isoform more so than the TNF-α2 isoform. In contrast, rainbow trout infected with the parasite, *Tetracapsuloides brysalmonae*, the causative agent of proliferative kidney disease (PKD), displayed an increased expression of TNF-

α2 relative to TNF-α1 [16]. This study by Holland *et al.* [16] reflects the findings of the present study that showed TNF-α2 was the predominant TNF-α isoform during AGD in rainbow trout. Thus, the differential expression of TNF-α isoforms is apparently dependent on the specific pathogen or stimulus, the tissue sampled and the species of fish studied.

In the current study an increase in iNOS and IL-1β1 gene expression in the gills of AGD-infected trout was closely associated with an increase in the number and size of AGD lesions. However, the lack of a significant increase in IL-8 transcript level in the gills of infected fish indicated that IL-8 dependent chemotactic migration of cells into the gills at the sample times was unlikely. Furthermore, the lack of a significant increase of MHC-II and TCR transcript levels in the gills of infected fish implied that there was no influx of MHC-II expressing cells and T cells into the gills. Histological examination of the AGD gill lesions confirmed the lack of any great leucocyte infiltration into the infected gills (Fig. 2). Interestingly, the significant increase in liver IL-8 gene expression and a trend showing an apparent up-regulation of iNOS and IL-1β1 gene expression at 7 d p.i. suggests the involvement of a systemic response to AGD infection. Likewise, the apparent, though non-significant, down-regulation of immune-regulatory gene expression levels (IL-1β1 excluded) at 7 d p.i. in the anterior kidney might reflect the possible migration of leucocytes from this organ and thus also suggest the involvement of a systemic response. Thus, further research is needed to investigate the involvement of this potential systemic response of fish to AGD.

Based on the current results and extensive studies of AGD histopathology [3, 7-10] we propose a preliminary model of infection and host response whereby the association of *Neoparamoeba* sp. with the gill tissue, whether attached to the gill epithelium or trapped in mucus surrounding the gill epithelium, evokes an inappropriate gill response. This response is characterised by the up-regulation of iNOS and the pro-inflammatory cytokine IL-1β1 in the gill tissue, which in turn initiates epithelial hyperplasia resulting in gill lesions. A lack of *in situ* hybridisation studies on AGD means that the cellular sources of these mRNA transcripts are currently unknown. However, the most likely sources of IL-1β expression in teleosts are thought to be macrophages, epithelial cells and fibroblasts [39, 40]. The excess mucus associated with AGD lesions may be the result of IL-1β, which is known to increase production and alter the composition of mucus in various mammalian epithelial cells [41, 42]. IL-1β is also able to activate transcription factors such as NF-κB, which has been shown to be important for iNOS transcription in fish [43]. Therefore, the proposed *Neoparamoeba* sp.-induced IL-1β activation and subsequent increase in iNOS expression and subsequent production of reactive nitrogen intermediates (RNIs) may damage the pathogen, while the continuous stimulation of this defence response may inadvertently harm the host and further the pathology associated with AGD [44]. Thus, more studies into the role of iNOS gene expression and regulation, protein expression, NO production and RNIs during infection with AGD are required. Interestingly, an apparent down-regulation in the expression level of all the studied genes in the present study, with the exception of IL-1β1, was seen in the anterior kidney at 7 and 14 d p.i. This supports a previous study showing decreased

phagocytic and respiratory burst activity in the adherent cell population isolated from the anterior kidney of AGD-infected Atlantic salmon [6]. This finding is not surprising, as parasite-induced immune suppression is a common occurrence during parasitic infections and helps the pathogen to survive host responses [45]. However, as mentioned above, the apparent down-regulation of immune-regulatory gene expression in the anterior kidney of AGD-affected Atlantic salmon might also be attributed to the possible AGD-induced migration of leucocytes from the anterior kidney.

These findings illustrate the involvement and importance of the immune-regulatory genes iNOS and IL-1β, and for the first time provide data of the host response to AGD at a molecular level. Using these data we propose an AGD infection model whereby *Neoparamoeba* sp.-induced mRNA expression of IL-1β and iNOS in gills evokes an inappropriate immune response resulting in extensive gill lesions, and leads to eventual mortality. The preliminary nature of this model, and the many unknowns involving the role of the immune response to AGD, means that further elucidation of this model is currently needed. At a molecular level, future studies using *in situ* hybridisation are required to identify the cellular origin of important mRNA transcripts, whilst on a protein level further studies are needed to examine the protein expression resulting from immune-regulatory genes.

Acknowledgements

This work formed part of a project of Aquafin CRC, the Australian Government's CRCs Programme, and received funds from the Fisheries R & D Corporation and other CRC participants. The authors thank Dr Chris Prideaux, Dr Tim Doran and others at the CSIRO Australian Animal Health Laboratories (AAHL) for assistance with qRT-PCR. Thanks also go to Dr Mark Adams and Ms Helen Statham for assistance with gill photography and histological processing.

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Chapter 3

Quantitation of immune-regulatory gene expression and cellular localisation of interleukin-1β mRNA in Atlantic salmon, *Salmo salar* L., affected by amoebic gill disease (AGD)

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(submitted)

Abstract

The characterisation of selected immune-regulatory genes during amoebic gill disease (AGD) in Atlantic salmon, *Salmo salar* L., was performed using semi-quantitative RT-PCR, quantitative real-time RT-PCR (qRT-PCR), and *in situ* hybridisation (ISH). The immune-regulatory genes of interest were interleukin-1β (IL-1β), inducible nitric oxide synthase (iNOS), serum amyloid A (SAA), and serum amyloid P-like pentraxin (SAP). Atlantic salmon were inoculated with the ectoparasite *Neoparamoeba* sp., the causative agent of AGD, and gill, liver and anterior kidney tissue sampled at 0, 7 and 14 d post-inoculation (p.i.). Semi-quantitative RT-PCR was performed on the tissue samples to identify up/down-regulated mRNA expression relative to uninfected control fish and normalised to the housekeeping gene, β-actin. Interleukin-1β (IL-1β) mRNA was shown to be up-regulated in the gills by semi-quantitative RT-PCR and then accurately quantitated using probe-based qRT-PCR. The cellular localisation of the IL-1β mRNA expression in the gills of uninfected and infected fish was then determined by ISH using an IL-1β-specific biotinylated cRNA probe. Expression of IL-1β mRNA was localised to filament and lamellar epithelium pavement cells in gills of uninfected and infected Atlantic salmon. These data demonstrate the importance of IL-1β at the site of infection, the gills, of Atlantic salmon during AGD. This work confirms previous studies demonstrating the importance of IL-1β in the regulation of the fish immune response to parasitic infection and shows the cellular localisation of fish IL-1β mRNA expression during infection.

Keywords: Gene expression; *In situ* hybridisation; Real-time PCR; Interleukin-1β; AGD; Atlantic salmon; *Salmo salar*; Gill

1. Introduction

Amoebic gill disease (AGD) is an ectoparasitic disease of marine fish caused by infection with the protozoan *Neoparamoeba* sp. AGD-affected fish develop multifocal gill lesions as a result of epithelial cell hyperplasia induced by *Neoparamoeba* sp. [1-3]. AGD predominantly affects the culture of Atlantic salmon in Tasmania, Australia and the current treatment consists of freshwater bathing to limit the progression of the disease. Untreated fish display obvious respiratory distress, lethargy, loss of equilibrium, and mortality. Unlike infection with the model parasites *Ichthyophthirius multifiliis* [4] and *Gyrodactylus derjavini* [5], little is known about the involvement of the immune response to infection with *Neoparamoeba* sp. and AGD. In general, studies to investigate the immune-regulatory processes during infection in fish have been hindered by a lack of species-specific antibodies against important proteins of the immune response. However, antiserum specific to seabream, *Sparus aurata*, interleukin-1β (IL-1β) was used in a recent study to investigate the production of IL-1β and to identify leucocytes expressing this cytokine during vibriosis [6]. In contrast, the genes encoding immune-relevant proteins in many different fish species are being discovered at an increasing rate. Therefore the study of the expression of these genes during parasitic diseases has allowed the partial characterisation of immune processes during infection in fish, albeit at a molecular level. Gene expression

studies in rainbow trout, *Oncorhynchus mykiss*, during infection with the model parasites *I. multifiliis* and *G. derjavini* demonstrated the parasite-induced regulation of several pro-inflammatory cytokine and immune-regulatory genes [7-10]. Likewise, gene expression studies in carp, *Cyprinus carpio*, infected with *Trypanosoma borreli*, and rainbow trout infected with *Tetracapsuloides bryosalmonae*, have also furthered the characterisation of the immune response to fish parasites [11, 12].

The majority of studies investigating gene expression in fish isolate RNA from selected tissue, which is then used in either Northern blots or reverse transcriptase PCR using gene-specific probes or primers. These studies demonstrate the presence, and in some cases the abundance, of the specific mRNA of interest in a tissue, but do not show the cellular localisation of the specific mRNA. Therefore, *in situ* hybridisation (ISH) studies are needed to identify the cell types within a particular tissue that express the mRNA of interest to further our knowledge of the immune mechanisms during infection in fish. Recent results from our laboratory have shown an up-regulation of IL-1β and inducible nitric oxide synthase (iNOS) gene expression in the gills of rainbow trout infected with *Neoparamoeba* sp. [13]. Based on these results, IL-1β, iNOS and the acute phase proteins (APPs), serum amyloid A (SAA) and serum amyloid P-like pentraxin (SAP), gene expression was investigated in the gill, liver, and anterior kidney of AGD-affected Atlantic salmon. The type and quantity of immune-regulatory molecules, the timing and location of their release, and co-ordinated expression with other immune-regulatory signals all contribute to the nature of the immune response and disease outcome. Therefore, to

investigate the immune mechanisms involved in AGD, we used semi-quantitative RT-PCR, quantitative real-time RT-PCR (qRT-PCR) and ISH to quantitate and locate specific immune-regulatory gene responses in AGD-affected Atlantic salmon.

2. Materials and methods

2.1. Fish

Seawater-acclimatised Atlantic salmon, *S. salar*, weighing approximately 100 g, were maintained in two 4000 L recirculating Rathburn tanks. Sea water was UV-irradiated and 1 μm filtered before being added to the tanks. Sea water was then maintained at 35‰ salinity at a temperature of 16 ± 0.5°C, and nitrogenous waste was controlled by biofiltration.

2.2. Amoebae isolation and infection

Neoparamoeba sp. were isolated from the gills of AGD-affected Atlantic salmon, as described by Morrison *et al.* [14] and were confirmed to be *Neoparamoeba* spp. using polymerase chain reaction (PCR) [15] and immunocytochemical staining [16]. Atlantic salmon in one of the Rathburn tanks were inoculated by adding 450 amoebae L^{-1} d^{-1} to the sea water over 3 consecutive days.

2.3. Tissue sampling

At 0, 7, and 14 d post-inoculation (p.i.) with *Neoparamoeba* sp., 18 fish from the control (AGD-unaffected) and AGD-affected tanks were euthanised with 5 g L^{-1} Aqui-S® (Aqui-S NZ Ltd, Lower Hutt, New Zealand). Gill, liver and anterior kidney samples were collected and immediately placed in RNA*later*™ (Qiagen, Clifton Hill, Victoria, Australia) to preserve RNA integrity. Samples collected in RNA*later* (Qiagen) were held at 4°C overnight before storage at -20°C until RNA extraction. For histological analysis the second left gill arch was dissected and fixed in seawater Davidson's fixative overnight and routinely processed for histology (5 μm, H & E).

2.4. RNA extraction and cDNA synthesis

Tissues from 18 uninfected and 18 AGD-affected salmon per sample time point were individually weighed and combined equally to form three pooled samples (each pooled from six salmon) per treatment group. Total RNA was extracted from the pooled RNA*later*-stabilised tissue samples using an RNeasy® RNA extraction kit (Qiagen), a Dounce homogeniser (Wheaton Scientific, Millville, New Jersey, USA) and QIAshredders™ (Qiagen), following the manufacturer's instructions. Total RNA was DNAse treated with on-column DNAse digestion (Qiagen) and eluted in 30 μL RNAse-free water. Spectrophotometry was used to quantitate the RNA and to verify the integrity of the total RNA. All RNA samples had an A_{260}/A_{280} ratio in water of 1.8-2.0 and well defined 28S and 18S rRNA bands with

no sign of degradation when run on a 1% agarose gel and stained with ethidium bromide. Analysis of the total RNA on the gel using densitometry software (Silk Scientific, Utah, USA) showed that the 28S rRNA band was approximately twice as intense as the 18S rRNA band. Immediately following RNA extraction and quantitation, 5 µg total RNA was reverse transcribed into cDNA using oligo dT $_{(12\text{-}18)}$ priming (SuperScript™ First-Strand cDNA Synthesis System, Invitrogen, Mount Waverly, Victoria, Australia). Samples were RNAse H digested to remove the RNA template strand from the resulting cDNA. In addition to DNAse digestion of the isolated total RNA, controls lacking reverse transcriptase were performed on samples from each sampling time to determine if contaminating genomic DNA was present after RNA extraction. These samples were then used in both standard and quantitative real-time PCR.

2.5. Semi-quantitative PCR

Polymerase chain reaction (PCR) was performed on an Eppendorf Mastercycler® Gradient thermocycler (Eppendorf, North Ryde, NSW, Australia). PCR reactions were performed using a 25 µL total reaction volume containing 12.5 µL PCR Master Mix (Promega, Annandale, NSW, Australia), 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), 5.5 µL nuclease-free water and adjusted cDNA template in 5 µL water. The target genes, primer sequences and predicted amplicon sizes are listed in Table 1 and the PCR cycling conditions for each target gene are shown in Table 2. Control reactions without cDNA template (NTC) were performed to check that amplified products were not a result of DNA

contamination or due to primer-dimer effects. PCR products were visualised on a 2% agarose gel post-stained with ethidium bromide and analysed using a UVP Gel imaging system (Ultra-Violet Products, California, USA) and gel densitometry software (Silk Scientific). Semi-quantitation involved subtracting the background pixel intensity from the target gene pixel density and reporting this normalised expression relative to the housekeeping gene, β-actin. At each sample time, 18 AGD-affected fish and 18 uninfected control fish were assessed by semi-quantitative PCR from three pooled samples per treatment group (each pool from six fish). Data were presented as the mean (+ standard error) of target gene expression relative to β-actin from the three pooled samples and differences in gene expression between AGD-affected and uninfected control samples at each sampling time were assessed using a two-tailed Student's *t*-test. Differences in gene expression were considered statistically significant if $P < 0.05$.

2.6. Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was used to validate and accurately quantitate the relative mRNA expression of up/down-regulated genes identified by semi-quantitative PCR. Real-time PCR was performed using the QuantiTect® Probe Gene Expression Assay System (Qiagen) and an ABI Prism® 7700 Sequence Detector (ABI, Scoresby, Victoria, Australia). The QuantiTect probe system utilises a minor groove binder (MGB), Eclipse™ Dark Quencher, and modified bases known as Superbases. These components of the QuantiTect system allowed the use of primers and probes at exon/exon boundaries to exclude the co-

amplification of genomic DNA, whilst maintaining optimum PCR efficiency. QuantiProbes and primers (Table 1) were designed using the Qiagen QuantiProbe design software in the custom mode. Reactions were assembled according to manufacturer's instructions and performed using a 50 µL total reaction volume. ABI Prism 7700 cycling conditions are shown in Table 2, and raw data were analysed using the ABI Prism Sequence Detection 1.9.1 software (ABI). In addition to designing primers that crossed an exon/exon boundary, mock reverse-transcription reactions without reverse-transcriptase were also performed. Samples subjected to these mock reactions showed no sign of any amplified product after qPCR. Primers were shown to amplify products of the predicted size (Table 1) when run on a 3% agarose gel and stained with ethidium bromide. The specificity of the reactions was further assessed by gel-purifying the amplified products and either direct sequencing or sequencing the amplicons after cloning into the pGEM®-T Easy Vector System (Promega). Sequencing reactions were performed using a CEQ™ DTCS Quick Start kit (Beckman Coulter, Fullerton, California, USA) and sequenced on a Beckman Coulter CEQ8000 automated sequencer (Beckman Coulter).

2.7. Real-time PCR data analysis

The threshold cycle (Ct) and baseline for the analysis of each target gene in the samples was determined manually and the mean Ct of the replicates calculated. Samples were normalised to β-actin when comparing relative gene expression in gill tissue from AGD-affected and uninfected Atlantic salmon. The housekeeping

gene β-actin was considered a suitable gene for normalisation, as its level of expression was independent of AGD [ΔCt β-actin $_{(\text{mean uninfected control - mean AGD-affected})} = 0$]. For accurate relative gene expression quantitation, PCR amplification efficiencies (E) of the target assays should be similar to that of the reference assay (β-actin) [17]. A difference of 0.2 in PCR amplification efficiency between the target and reference gene will result in more than a 10-fold difference in the final result after 25 cycles unless an algorithm is used that corrects for minor differences in E [18]. Real-time PCR performed on serial dilutions of β-actin, IL-1β, and iNOS cDNA and the resulting plots of Ct versus the logarithm of the dilution of the DNA were used to calculate PCR efficiencies. PCR efficiencies (E), where $E = 10^{(-1/\text{slope})}$, were 1.94 for β-actin, 1.88 for IL-1β, and 1.94 for iNOS. Fold changes in the gene expression of the AGD-affected samples compared with the uninfected control samples at each sampling time point were calculated by REST-XL© version 2 software [17] using the following equation

$$\text{Ratio} = (E_{\text{target}})^{\Delta Ct[\text{target(control - AGD-affected)}]} / (E_{\beta\text{-actin}})^{\Delta Ct[\beta\text{-actin(control - AGD-affected)}]}$$

The REST-XL software uses a pair-wise fixed reallocation randomisation test [17] when calculating the fold change in expression, and determines whether the expression of a target gene relative to a reference gene is significantly different ($P < 0.05$) between an experimental group and a control group.

Table 1.

Amplicon sizes and gene-specific primers and probes used to amplify Atlantic salmon, *S. salar*, immune-regulatory genes in semi-quantitative and quantitative real-time PCR. Included are the gene-specific primers and T7 RNA polymerase promoter adapter primer used to generate the PCR template for *in vitro* transcription and the production of sense and antisense *in situ* hybridisation IL-1β cRNA probes.

Gene target	Amplicon size (bp)	Primer Designation	Sequence (5'-3')
PCR			
β-actin	289	Beta actin-F	TCGCTGGAGATGACGC
		Beta actin-R	TCCCTGTTGGCTTTGG
IL-1β	353	IL-1F	AGGGAGGCAGCGGCTACCACAA
		IL-1R	GGGGGCTGCCTTCTGACACAT
SAA	193	SAA-F	AGCTGCTCGAGGTGCTAAAG
		SAA-R	ATGTCCTCGACCACTGGAAC
SAP-like pentraxin	183	SAP-F	GTCTCAGAGCCCATTTCTGC
		SAP-R	TGGCAAACTGATGAAATCCA
qPCR			
β-actin	113	Qactin-F	TCTCTGGAGAAGAGCTAC
		Qactin-R	CAAGACTCCATACCGAGGAA
		Qactin probe	ACGGACAGG**T*CAT*CAC**
IL-1β	159	QIL-1F	TATCCCATCACCCCATCA
		QIL-1R	CCAACACTATATG**T*T***CTTCCAC
		QIL-1 probe	CAACCTCATCATCGCCA
iNOS	93	QiNOS-F	TCAGAACCTCCTCCACAA
		QiNOS-R	GTGTACTCCTGAGAGTCCTTT
		QiNOS probe	GCACCGACAGCGTCTA
ISH probe PCR			
IL-1β (Antisense)	304 (242 nt probe + 62 nt T7-adapter)	HybIL-1F	GATGAGTGAGGCTATGGA
		Lign'Scribe T7 adapter primer 1	GCTTCCGGCTCGTATGTTGTGTGG
IL-1β (Sense)	304 (242 nt probe + 62 nt T7-adapter)	HybIL-1R	TCTGCTGGCTGATGGA
		Lign'Scribe T7 adapter primer 1	GCTTCCGGCTCGTATGTTGTGTGG

* denotes modified bases

Table 2.

Summary of cycling conditions used in semi-quantitative PCR, quantitative real-time PCR, and standard PCR used to generate the PCR template for *in vitro* transcription and the production of sense and antisense *in situ* hybridisation IL-1β cRNA probes.

Target	Cycling protocol			
	Denature	Anneal	Extend	No. of cycles
PCR				
β-actin	95°C/ 3 min	-	-	1
	95°C/ 30 s	56°C/ 30 s	72°C/ 30 s	25
	-	-	72°C/ 6 min	1
IL-1β	95°C/ 3 min	-	-	1
	95°C/ 25 s	56°C/ 30 s	72°C/ 30 s	30
	-	-	72°C/ 6 min	1
SAA	95°C/ 3 min	-	-	1
	95°C/ 25 s	58°C/ 30 s	72°C/ 30 s	30
	-	-	72°C/ 6 min	1
SAP-like pentraxin	95°C/ 3 min	-	-	1
	95°C/ 25 s	56°C/ 30 s	72°C/ 30 s	30
	-	-	72°C/ 6 min	1
qPCR				
β-actin	95°C/ 15 min	-	-	1
	94°C/ 15 s	56°C/ 30 s	76°C/ 30 s	45
IL-1β	95°C/ 15 min	-	-	1
	94°C/ 15 s	56°C/ 30 s	76°C/ 30 s	45
iNOS	95°C/ 15 min	-	-	1
	94°C/ 15 s	56°C/ 30 s	76°C/ 30 s	45
ISH Probe PCR				
IL-1β (Antisense)	95°C/ 3 min	-	-	1
	94°C/ 25 s	58°C/ 25 s	72°C/ 45 s	30
	-	-	72°C/ 3 min	1
IL-1β (Sense)	95°C/ 3 min	-	-	1
	94°C/ 25 s	58°C/ 25 s	72°C/ 45 s	30
	-	-	72°C/ 3 min	1

2.8. Synthesis of cRNA probes for in situ hybridisation

Antisense and sense (control) IL-1β specific cRNA probes were prepared using a Lign'Scribe™ No-cloning promoter addition kit (Ambion, Austin, Texas, USA) according to manufacturer's instructions. Briefly, a PCR-generated 242 nt IL-1β cDNA template was ligated to a 62 nt T7 RNA polymerase promoter adapter (Table 1). The ligated template cDNAs were then directionally amplified with a T7 promoter adapter primer and a gene specific primer by PCR to produce both antisense and sense cDNA templates for *in vitro* transcription. A Maxiscript® *in vitro* transcription kit (Ambion) was then used to synthesize the 242 nt antisense and sense cRNA probes which were then biotinylated using a BrightStar® Psoralen-Biotin nonisotopic labelling kit (Ambion).

2.9. In situ hybridisation

Excised gill tissue was fixed for 24 h at 4°C in freshly prepared 4% paraformaldehyde-phosphate buffered saline pH 7.4 (PFA-PBS). Tissues were dehydrated through an ethanol series, cleared in xylene and embedded in paraffin wax. Paraffin sections (8 µm) were mounted on Polysine™ slides (Menzel-Gläser, Braunschweig, Germany) and dried overnight at room temperature (RT). Sections were then deparaffinised with xylene, rehydrated through graded ethanol (100%-50%) to PBS pH 7.4, and incubated for 10 min at RT in proteinase K (2 µg mL^{-1} in PBS pH 7.4). Slides were then rinsed in PBS pH 7.4, incubated for 10 min at RT in glycine (0.2% w/v in PBS pH 7.4) and incubated for a further 10 min at RT in 4×

saline sodium citrate (SSC). Probes were heated to 80°C for 5 min, diluted to 400 ng mL^{-1} in pre-heated (50°C) hybridisation buffer (50% deionised formamide, 0.6 M NaCl, 10 mM Tris, 1.7 mM EDTA, 1× Denhardt's solution, 10% dextran sulfate, 250 μg mL^{-1} tRNA, and 250 μg mL^{-1} sheared cod DNA), and added to the sections. Sections were incubated in a humidified chamber for 16 h at 50°C and then washed (2× SSC, 50% deionised formamide) for 30 min at 50°C. Non-specific and partially hybridised probe was then digested with RNAse A (20 μg mL^{-1}) for 30 min at 37°C, washed in NTE buffer (0.5 M NaCl, 10 mM Tris, 1 mM EDTA) for a further 10 min at 37°C, and washed three times for 20 min in 0.1× SSC at 50°C.

For detection of hybridised biotinylated-cRNA probes, sections were blocked with 2.5% casein in Tris-buffered saline and 0.1% Tween 20 pH 7.4 (TBST) for 45 min at 37°C. Sections were then incubated for 30 min at RT with streptavidin-alkaline phosphatase (Vector laboratories, Burlingame, California, USA) diluted 1:1000 in 2.5% casein-TBST. After three washes in Tris pH 9.5, substrate consisting of nitroblue tetrazolium (NBT) and 5-bromo, 4-chloro, 3-indolylphosphate (BCIP) was added to the sections. Colour was allowed to develop overnight at RT in the dark. Colour development was stopped by rinsing the slides in distilled water for 1 min, in PBS for 1 min, and again in distilled water for 1 min. Sections were quickly dehydrated in 70% ethanol (10 s), 95% ethanol (10 s), cleared in xylene (15 s) and mounted in VectaMount™ (Vector laboratories).

3. Results

3.1. Infection

An examination of the gills of fish sampled at 7 d p.i. revealed typical AGD-associated gross pathology in the *Neoparamoeba* sp.-infected fish. Numerous small multifocal pale mucoid lesions were consistently present on all of the gills from *Neoparamoeba* sp.-infected fish sampled at 7 d p.i. (Fig. 1). By 14 d p.i. gross examination of the gills of infected fish showed that there was a clear and consistent increase in AGD-associated pathology compared with that at 7 d p.i. as measured by the number and size of mucoid lesions (Fig. 1). Importantly, no signs of AGD were visible upon gross examination of the gills of the uninfected control fish at 0, 7 and 14 d p.i. Histological examination of the AGD-associated pathology of infected fish at both 7 and 14 d p.i. confirmed that the grossly visible pale mucoid lesions were areas of hyperplastic epithelia (Fig. 2.). At 14 d p.i. lesions were not only more numerous than at 7 d p.i. but consisted of a greater number of fused lamellae and a greater degree of epithelial cell hyperplasia (Fig. 2). Furthermore, amoebae were often found associated with the hyperplastic lesion surface (Fig. 2). No histological signs of AGD or any other pathological changes were found in the uninfected fish sampled at 0, 7 and 14 d p.i. No mortalities in the *Neoparamoeba* sp.-infected or the uninfected control fish occurred during the experiment.

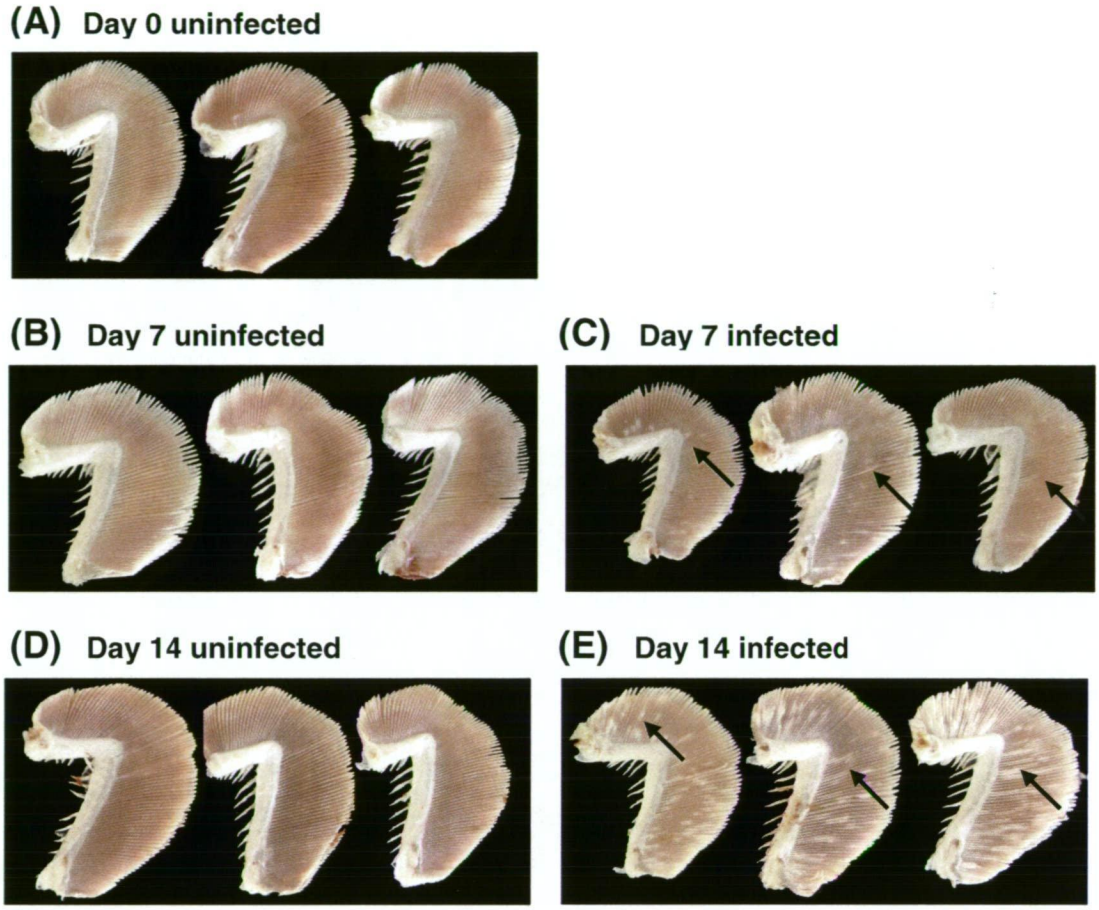

Figure 1.

Gross (macroscopic) images of *Neoparamoeba* sp.-infected and uninfected (control) Atlantic salmon, *S. salar*, gills fixed in seawater Davidson's fixative at 0, 7 and 14 d p.i. with *Neoparamoeba* sp. Characteristic pale multifocal mucoid lesions (arrows) associated with AGD are evident in gill images of infected fish at 7 and 14 d p.i.

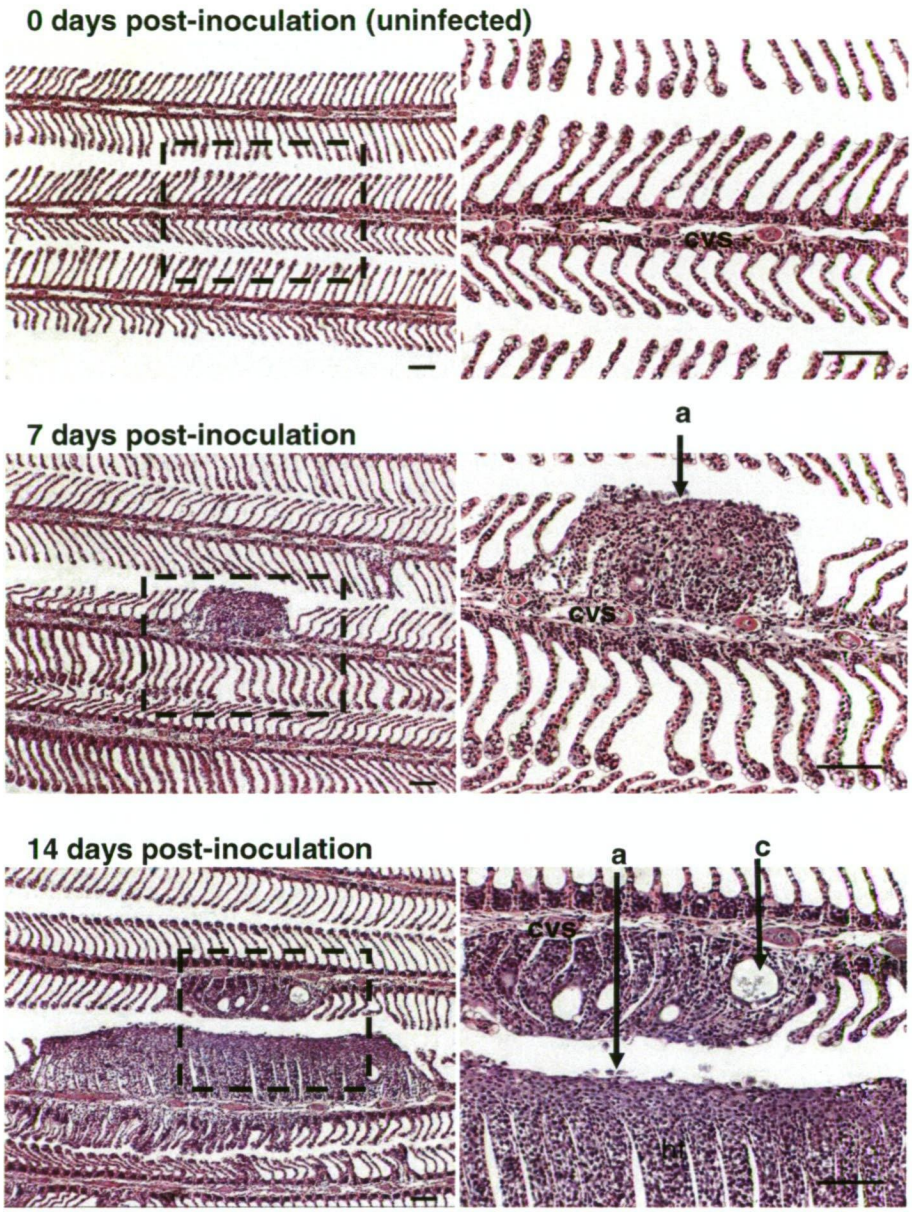

Figure 2.

Histopathology of *Neoparamoeba* sp.-infected and uninfected (control) Atlantic salmon, *S. salar*, gills at 0 (uninfected), 7 and 14 d p.i. with *Neoparamoeba* sp. Highlighted boxes within images on the left of the figure are shown at greater magnification directly adjacent the corresponding box. AGD lesions consist of hyperplastic tissue (ht) that fuse lamellae and form a stratified layer of epithelial tissue at the lesion surface. Amoebae (a) can be seen in close association with the lesion surface and are often seen in conjunction with leucocytes within interlamellar crypts (c). Central venous sinus (CVS) (bars = 100 μm).

3.2. Semi-quantitative RT-PCR gene expression

The immune-regulatory genes IL-1β, SAA, and SAP-like pentraxin were constitutively expressed in the gill, liver and anterior kidney of uninfected fish at 0, 7 and 14 d p.i. (Fig. 3, 4 & 5). The IL-1β-specific mRNA transcript abundance, relative to β-actin, was approximately equal in the three tissues sampled from the uninfected fish. However, SAA and SAP-like pentraxin mRNAs were more abundant in the primary APP organ, the liver. Gill IL-1β mRNA transcript expression was found to be significantly ($P < 0.05$) up-regulated in AGD-affected Atlantic salmon. This AGD-induced up-regulation was found in the gill tissue at 14 d p.i. and was 5.0 times the IL-1β-specific mRNA transcript level observed in the uninfected control fish at this sampling time (Fig. 3A). No other statistically significant AGD-induced up/down regulation of IL-1β, SAA, or SAP-like pentraxin gene expression was found in the gill, liver or anterior kidney at 0, 7 or 14 d p.i. ($P > 0.05$).

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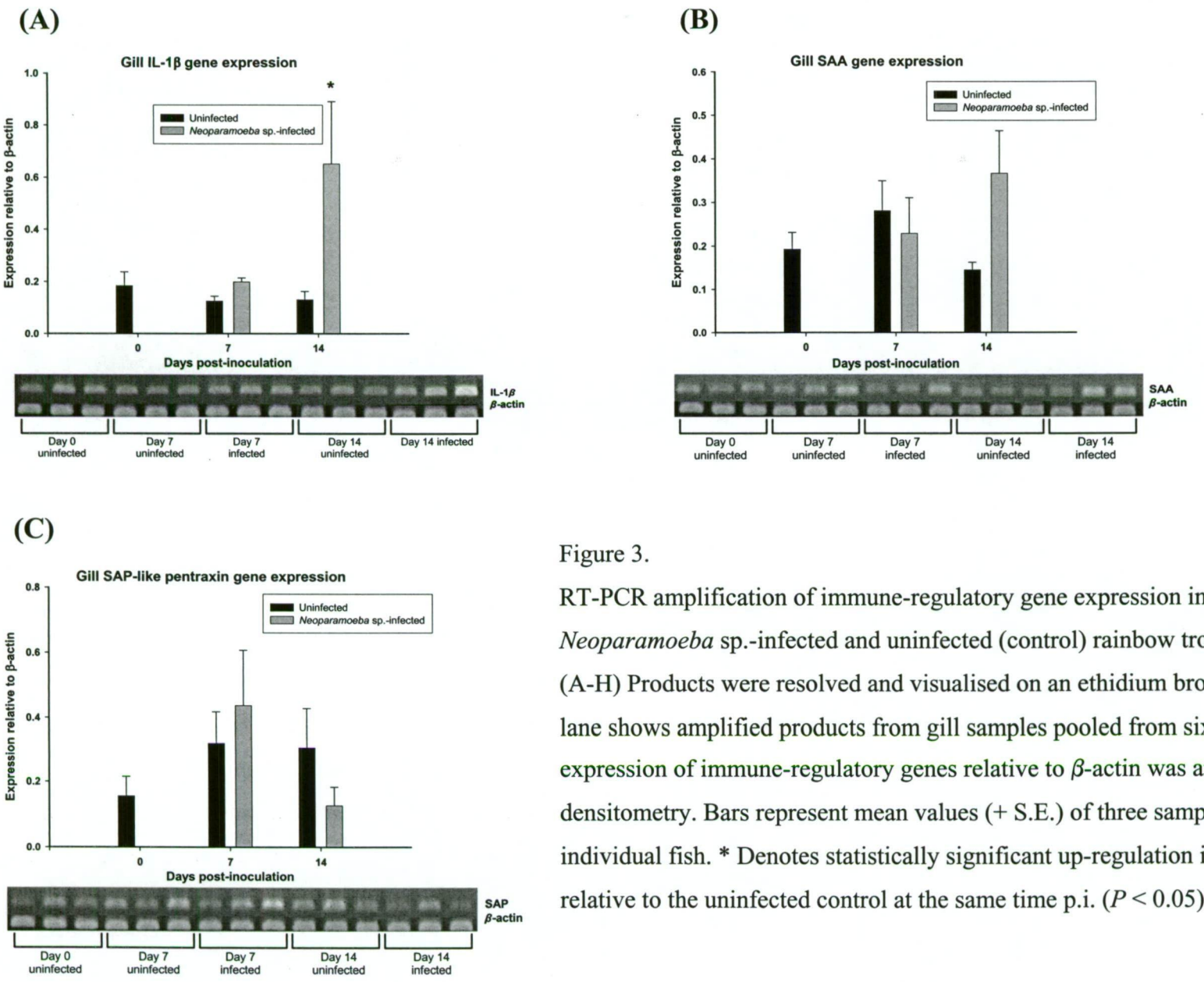

Figure 3.

RT-PCR amplification of immune-regulatory gene expression in gill samples from *Neoparamoeba* sp.-infected and uninfected (control) rainbow trout at 0, 7 and 14 d p.i. (A-H) Products were resolved and visualised on an ethidium bromide-stained gel. Each lane shows amplified products from gill samples pooled from six rainbow trout. Specific expression of immune-regulatory genes relative to β-actin was analysed using densitometry. Bars represent mean values (+ S.E.) of three samples, each pooled from six individual fish. * Denotes statistically significant up-regulation in target gene expression relative to the uninfected control at the same time p.i. ($P < 0.05$).

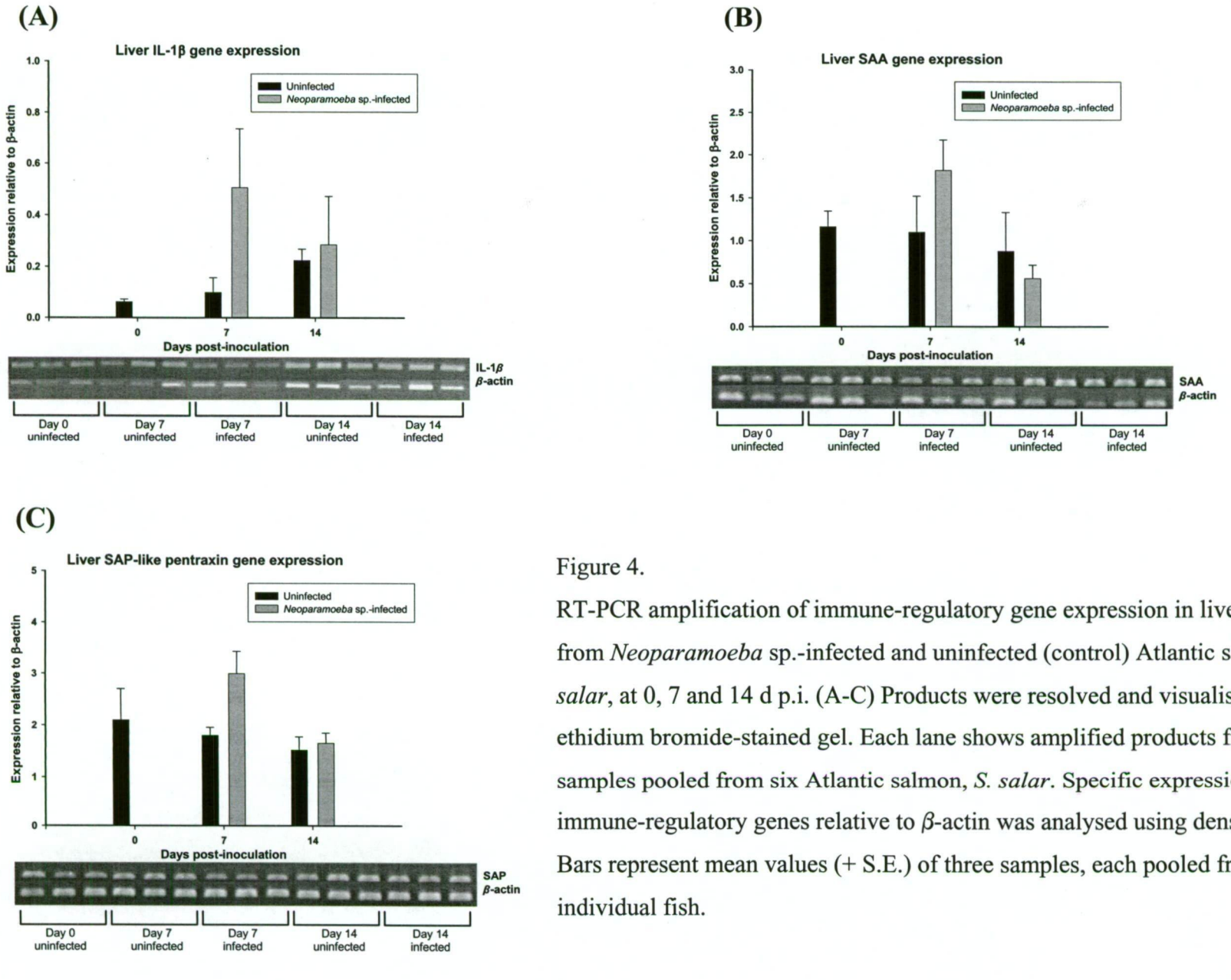

Figure 4.

RT-PCR amplification of immune-regulatory gene expression in liver samples from *Neoparamoeba* sp.-infected and uninfected (control) Atlantic salmon, *S. salar*, at 0, 7 and 14 d p.i. (A-C) Products were resolved and visualised on an ethidium bromide-stained gel. Each lane shows amplified products from liver samples pooled from six Atlantic salmon, *S. salar*. Specific expression of immune-regulatory genes relative to *β*-actin was analysed using densitometry. Bars represent mean values (+ S.E.) of three samples, each pooled from six individual fish.

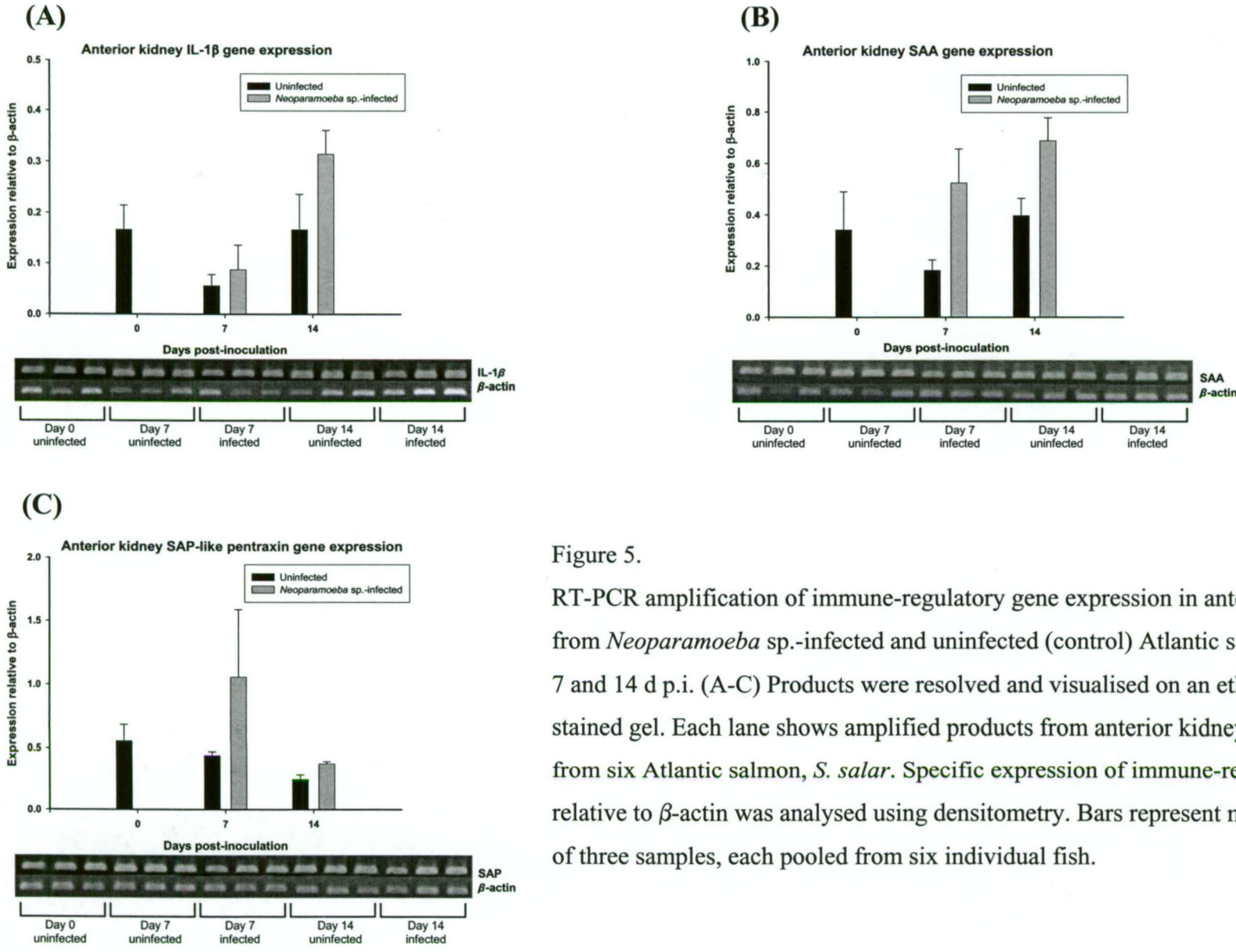

Figure 5.

RT-PCR amplification of immune-regulatory gene expression in anterior kidney samples from *Neoparamoeba* sp.-infected and uninfected (control) Atlantic salmon, *S. salar*, at 0, 7 and 14 d p.i. (A-C) Products were resolved and visualised on an ethidium bromide-stained gel. Each lane shows amplified products from anterior kidney samples pooled from six Atlantic salmon, *S. salar*. Specific expression of immune-regulatory genes relative to β-actin was analysed using densitometry. Bars represent mean values (+ S.E.) of three samples, each pooled from six individual fish.

3.3. Real-time RT-PCR analysis of gill IL-1β gene expression

Accurate quantitation of IL-1β and iNOS mRNA in the gills of AGD-affected Atlantic salmon relative to uninfected control fish was performed using QuantiTect probe real-time chemistry (Qiagen). Real-time PCR analysis confirmed the semi-quantitative PCR result and showed that IL-1β mRNA expression was significantly up-regulated ($P < 0.05$) in the gills of AGD-affected fish at 14 d p.i. relative to uninfected control fish (Fig. 6). AGD-affected fish at 14 d p.i. had 3.7 times the IL-1β mRNA level of the uninfected control fish, whilst no statistically significant AGD-induced up/down-regulation of gill iNOS gene expression was found at 7 or 14 d p.i. β-actin was shown to be a valid housekeeping gene for relative gill gene quantitation during AGD studies as no AGD-induced effect on its mRNA expression was observed at 0, 7 or 14 d p.i. (Fig. 6).

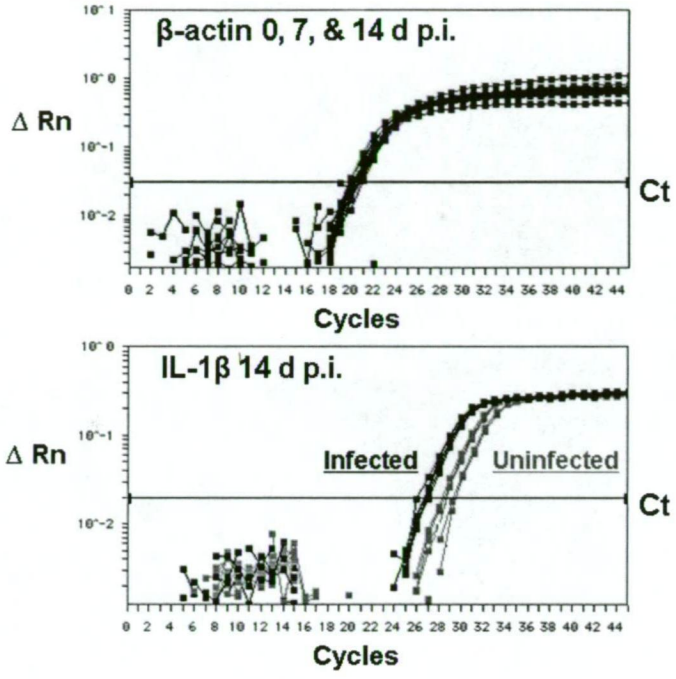

Figure 6.

qPCR amplification plots of β-actin (normalising gene) and IL-1β mRNA expression in gill samples from *Neoparamoeba* sp.-infected and uninfected (control) Atlantic salmon, *S. salar*, at 14 d p.i. The β-actin amplification plot includes mRNA expression at 0 and 7 d p.i. qPCR was performed on an ABI 7700 sequence detector using QuantiTect probe chemistry. The PCR cycle numbers are plotted against the change in fluorescence relative to a passive reference dye (ROX)(ΔRn), and the horizontal line represents the threshold cycle (Ct). IL-1β gene expression was significantly ($P < 0.05$) up-regulated at 14 d p.i. compared with the uninfected controls.

3.4. In situ hybridisation

Gill tissue sections subjected to *in situ* hybridisation using the 242 nt biotinylated IL-1β cRNA antisense probe had an intense signal in what were identified as filament and lamellar pavement epithelium cells by their distribution and morphology (Fig. 7A and B). However, without simultaneous staining using cell-specific markers during ISH it was unclear whether other cells of the filament, such as chloride cells or those found just beneath the filament epithelium pavement cells, were also IL-1β mRNA-positive (Fig. 7A and B). The specificity of the IL-1β antisense probe was proven as no signal was found in serial sections probed with the IL-1β control sense probe (Fig. 7C). Gill sections from AGD-affected fish showed that IL-1β mRNA was found in the squamous epithelial cells lining relatively well-developed hyperplastic lesions but not in the undifferentiated epithelial cells that formed the majority of the AGD-lesion (Fig. 7D).

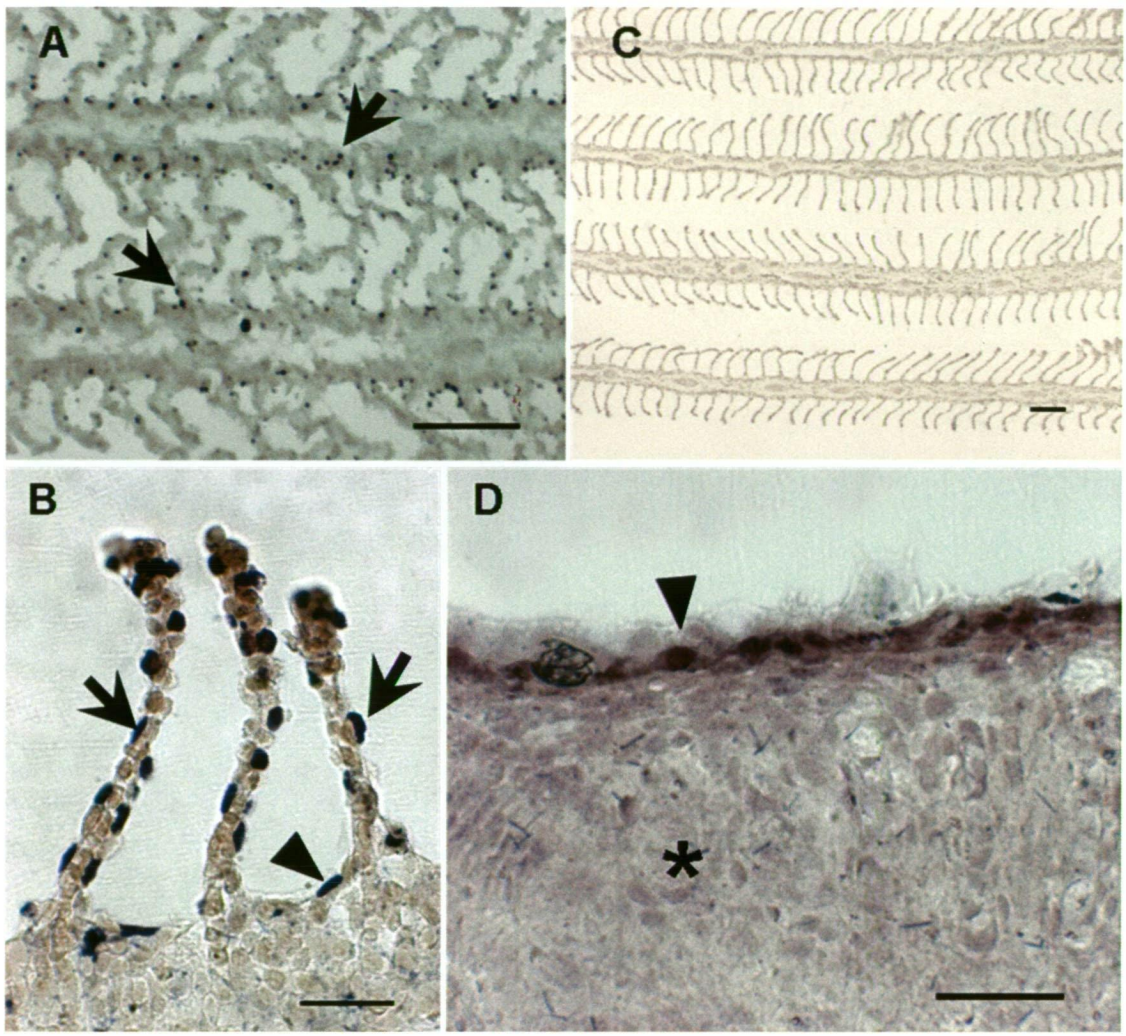

Figure 7.

Uninfected control (A-C) and AGD-affected (D) Atlantic salmon, *S. salar*, gill sections following *in situ* hybridisation using IL-1β sense and antisense biotinylated cRNA probes. (A) IL-1β mRNA positive cells were distributed on both the filaments and lamellae following incubation with the IL-1β antisense probe (arrows, bar = 100 μm). (B) IL-β mRNA positive cells were identified as filament (arrowhead) and lamellar (arrows) epithelium pavement cells (bar = 25 μm) (C) Gill section incubated with the IL-1β sense (control) probe (bar = 100 μm). (D) IL-1β mRNA positive cells in an AGD-affected gill section were localised to the hyperplastic squamous epithelium (arrowhead) lining the surface of the AGD lesion (*) (bar = 25 μm).

4. Discussion

This study supported our previously reported up-regulation of IL-1β mRNA expression in the gills of rainbow trout infected with the causative agent of AGD, the ectoparasite *Neoparamoeba* sp. [13]. However, unlike the previous study which showed both IL-1β and iNOS mRNA expression was up-regulated in AGD-affected rainbow trout, the present study found that only IL-1β was significantly up-regulated in AGD-affected Atlantic salmon. The lack of concomitant mRNA expression of these two genes was possibly the result of different infection dynamics in the two studies. Both gross and histopathological examination of AGD-affected Atlantic salmon in the present study revealed that the Atlantic salmon had less AGD-related pathology at 7 d p.i. compared with the same sampling time in our previous study [13]. Supporting this was the finding that the increased IL-1β mRNA expression was only observed at 14 d p.i. in the present study when the AGD pathology was similar to that at 14 d p.i. in rainbow trout in the previous study [13]. Furthermore, as a result of sampling at 0, 7 and 14 d p.i. we may have failed to detect a possible transient change in iNOS mRNA expression outside the sampling times. It is also possible that the lack of iNOS mRNA up-regulation in the present study was a true Atlantic salmon-specific response to *Neoparamoeba* sp. infection. Nevertheless, the mRNA expression of IL-1β and its regulation in the gills of AGD-affected rainbow trout and Atlantic salmon clearly indicates its importance in the host response to this parasitic disease.

IL-1β is a pleiotropic inflammatory cytokine that mediates the immune response to a variety of pathogens [19]. Recent gene expression studies have reported the pathogen-induced up-regulation of IL-1β during bacterial, viral and parasitic fish diseases [7, 10, 11, 20-24]. Rainbow trout infected with the monogenean parasite *G. derjavini* exhibited an increased IL-1β1 and IL-1β2 isoform-specific mRNA expression in the skin [10]. Likewise, the ectoparasitic ciliate *I. multifiliis* induced an up-regulation of IL-1β1 and IL-1β2 mRNA in the skin of rainbow trout compared with uninfected control fish [7]. These two studies highlight the importance of IL-1β at the site of infection in rainbow trout. Similarly, increased IL-1β mRNA expression was demonstrated in the anterior kidney, liver and spleen of carp infected with the parasitic blood flagellate *T. borreli* [11]. However, unlike systemic infection with *T. borreli*, infection with *Neoparamoeba* sp. and subsequent AGD pathology has been identified only in the host gill. Interestingly, we previously showed an apparent AGD-induced up-regulation of interleukin-8 (IL-8) mRNA expression in the liver of rainbow trout at 7 d p.i. with *Neoparamoeba* sp. [13]. Evidence of systemic regulation of mRNA expression in response to an ectoparasitic infection has been shown during *I. multifiliis* infection in rainbow trout [7, 8]. Therefore, having previously reported an up-regulation of IL-8 mRNA expression in the liver of AGD-affected rainbow trout and hence the possible involvement of a systemic AGD response, we chose to quantitate the mRNA expression of SAA and SAP-like pentraxin.

SAA and SAP are APPs predominantly produced in the liver during an acute phase response (APR). Described as a physiological response to injury, trauma or

infection, the APR in vertebrates is characterised by an increase or decrease in certain plasma proteins [25]. Many of these proteins, including SAA and SAP, are integral components of the innate immune response [26]. Triggered by pro-inflammatory mediators of infection such as IL-1β, tumour necrosis factor α (TNF-α) and interleukin-6 (IL-6), the gene expression and plasma levels of SAA and SAP may increase dramatically during an APR [27]. Carp infected with the parasite *T. borreli*, had increased SAA mRNA expression in the anterior kidney, spleen and liver 4–7 d p.i. [11]. Therefore, in the present study we assumed that a systemic response to *Neoparamoeba* sp. infection might invoke an APR and manifest as an up-regulation of hepatic SAA or SAP-like pentraxin mRNA expression. However, no significant changes in SAA and SAP-like pentraxin mRNA expression were detected at either 7 or 14 d p.i. in the liver, anterior kidney or gill of AGD-affected Atlantic salmon in the present study. Therefore, based on the present findings from gill, liver and anterior kidney tissue sampled at 7 and 14 d p.i., AGD does not invoke a hepatic acute phase immune response in Atlantic salmon, *S. salar*, at the molecular level.

Investigations of the fish immune response to infection have been hindered by a lack of recombinant proteins of the immune system and specific antibodies raised against these important molecules. Hence, the majority of studies investigating specific components of the fish immune response, such as cytokines and other immune relevant molecules, have focused on the mRNA expression of the genes encoding these immune molecules. These gene expression studies so far have been limited to measuring the presence or absence and/or the abundance of specific

mRNA transcripts in cells or tissues. The cellular localisation of specific immune-regulatory mRNA expression is also important during these gene expression studies. However, studies investigating disease-related gene expression in fish tissues have ignored information concerning the cellular origin of the mRNA expression within the tissue. Therefore, having previously identified the up-regulation of IL-1β mRNA expression in the gill tissue of AGD-affected rainbow trout, *O. mykiss*, [13] and Atlantic salmon in the present study, we used ISH to locate the cellular origin of IL-1β mRNA within the gill.

ISH performed on gill sections showed that filament and lamellar epithelium pavement cells were IL-1β mRNA-positive. In mammals, IL-1β is produced by a great variety of cell types and tissues, including monocytes, macrophages, lymphocytes, fibroblasts and epithelial cells [19]. Likewise, fish anterior kidney leucocytes are known to produce IL-1β in response to stimulation with mitogens [28], cytokines [29, 30] and infection [20, 24]. During bacterial infection with *Vibrio anguillarum*, seabream proIL-1β was shown to accumulate in blood acidophilic granulocytes [6]. IL-1 is also thought to be produced in rainbow trout epidermal cells, as cross-reactivity to anti-human IL-1α antiserum was shown in the epidermis of trout infected with *G. derjavini* [31]. Similarly, an IL-1 like factor has been shown in the epidermis of carp [32]. In humans, IL-1 is part of an important immune secretory response of epithelial cells after contact with certain pathogens [33]. Cell damage caused by the amoebic pathogen *Entamoeba histolytica* invokes human epithelial cells to secrete IL-1α that initiates a cytokine cascade as part of the host immune response [34]. In fish, IL-1 secreted by skin

epidermal cells during parasitic infection with *G. derjavini* is crucial for the initiation of the anti-*G. derjavini* response [5]. More recently, studies of IL-1β mRNA expression in the skin of rainbow trout during *G. derjavini* infection support the importance of IL-1β during monogenean infections of fish skin as proposed by Buchmann [5], and provide greater detail of IL-1β involvement in gyrodactylid infections [9, 10].

The results of this study support our previous finding that IL-1β mRNA expression is up-regulated in AGD-affected rainbow trout. However, unlike our previous study, AGD-affected Atlantic salmon showed no evidence of a systemic immune response. These findings, combined with the cellular localisation of IL-1β mRNA in the gill epithelium, stress the importance of the host response at the site of infection. Therefore the results of this study not only increase our understanding of the immune response to *Neoparamoeba* sp.-infection and AGD, but also further our knowledge of the cytokine response during the immune response to parasitic infection in fish.

Acknowledgements

This work formed part of a project of Aquafin CRC, the Australian Government's CRCs Programme, and received funds from the Fisheries R & D Corporation and other CRC participants. The authors thank Professor Chris Secombes (Scottish Fish Immunology Research Centre, University of Aberdeen, Aberdeen, UK) for advice concerning the Atlantic salmon, *S. salar*, IL-1β sequence. Thanks also go to Dr

Chris Prideaux, Dr Tim Doran and others at the CSIRO Australian Animal Health Laboratories (AAHL) for assistance with qRT-PCR.

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Chapter 4

Immunostimulatory CpG oligodeoxynucleotides increase resistance against amoebic gill disease in Atlantic salmon, *Salmo salar* L.

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Journal of Fish Diseases 2003, 26, 367-371
(Short communication)

Amoebic gill disease (AGD) severely affects sea-cage cultured populations of Atlantic salmon, *Salmo salar* L., in Tasmania (Munday, Foster, Roubal & Lester 1990). Caused by the pathogen, *Neoparamoeba pemaquidensis* (Page) (Kent, Sawyer & Hedrick 1988; Howard & Carson 1993), AGD has been an enduring problem in Tasmanian salmonid culture. Cases of AGD are not limited to Tasmania, as the disease has been diagnosed in cultured marine fish throughout the world (Nowak, Carson, Powell & Dyková 2002). Freshwater bathing is currently the preferred form of treatment and control for AGD occurring in Atlantic salmon, *Salmo salar*, in Australia and turbot, *Scophthalmus maximus* L., culture in Spain (Nowak *et al.* 2002). The effectiveness of freshwater bathing as a method for treating AGD is limited as *N. pemaquidensis* is not completely eradicated from the gills of the fish (Parsons, Nowak, Fisk & Powell 2001). In addition, the need for a perennial supply of fresh water and the labour costs required to freshwater bathe fish in the sea-cages mean that an alternative AGD management strategy may prove more cost effective. Vaccination against AGD would be an ideal management strategy, but so far trials have been unsuccessful (Zilberg & Munday 2001). However, this does not mean that all immunological aspects of AGD treatment and control are futile. Manipulation of the innate immune response through the administration of immunostimulants may be an effective strategy for the control and treatment of AGD.

Oligodeoxynucleotides (ODNs) containing cytidine-phosphodiester-guanosine (CpG) motifs are potent immunostimulants that may be beneficial in controlling and treating AGD. Bacterial DNA, unlike vertebrate DNA, contains frequent

unmethylated CpG motifs that have the ability to activate mammalian immune cells, causing them either to proliferate or produce cytokines (Krieg, Yi, Matson, Waldschmidt, Bishop, Teasdale, Koretzky & Klinman 1995; Klinman, Yi, Beaucage, Conover & Krieg 1996). Similarly, *in vitro* studies in fish have shown immune cell recognition of CpG-ODNs, subsequent immune cell proliferation, and cytokine production (Jørgensen, Johansen, Stenersen & Sommer 2001a; Jørgensen, Zou, Johansen & Secombes 2001b; Tassakka & Sakai 2002). However, there have been no studies of the *in vivo* ability of CpG-ODNs to enhance resistance to disease in fish. Therefore, in this paper we report that intraperitoneal (i.p.) administration of CpG-ODNs is able to increase resistance to AGD in Atlantic salmon.

Atlantic salmon smolts, weighing approximately 80 g, were acclimatised to three 1000 L recirculated-seawater tanks over 14 d before treatment. Sea water was maintained at 17°C at 37‰ salinity and the fish fed once daily to satiation with a commercial dry feed (50/14 pellets, Pivot Pty Ltd, Hobart, Australia). Groups of Atlantic salmon were anaesthetised with 50 ppm Aqui-S® (Aqui-S NZ Ltd, Lower Hutt, New Zealand) and the following treatments administered by an i.p. injection of 100 μL $fish^{-1}$: (1) untreated, (2) phosphate buffered saline (PBS), (3) non-CpG 1720 in PBS (50 μg $fish^{-1}$), (4) CpG-1668 in PBS (50 μg $fish^{-1}$). Oligodeoxynucleotides were purchased from Sigma Genosys (Castle Hill, NSW, Australia) and were phosphorothioated to increase their resistance to nuclease degradation. Sequences of ODN are: CpG-ODN 1668, TCC ATG ACG TTC CTG ATG CT and non-CpG ODN 1720, TCC ATG AGC TTC CTG ATG CT. Oligodeoxynucleotide sequences and dosages were selected based on their

effective use in Atlantic salmon, rainbow trout, *Oncorhynchus mykiss* (Walbaum), and murine studies (Jørgensen *et al.* 2001a, b;Weighardt, Feterowski, Veit, Rump, Wagner & Holzmann 2000). Each replicate tank contained seven fish from each of the four treatment groups.

Fish were challenged 6 d post-injection (p.i.) by the addition of *Neoparamoeba* spp. to the recirculating water supply at a rate of 2460 amoebae L^{-1}. Amoebae were harvested and isolated from gills of fish with clinical AGD by a method modified from Howard & Carson (1993). Fish were killed with 0.5% Aqui-S® (v v^{-1}), gill arches were excised, and the mucus was scraped off the gills. Enumeration and viability of the isolated amoebae was assessed using trypan blue. A 100 µL sample of the gill isolate was mixed with an equal volume of 0.5% (w v^{-1}) trypan blue in sea water and viable amoebae counted using a haemocytometer at 100× magnification. A 30 µL sample of the gill isolate was smeared onto a clean glass slide and used to assess the percentage of *Neoparamoeba* spp. in the gill isolate by an immunocytochemical method modified from Zilberg & Munday (2000). Briefly, the gill isolate smears were air-dried overnight and then heat-fixed. Slides were initially quenched of endogenous peroxidase for 10 min in 3.5% hydrogen peroxide (H_2O_2), washed in PBS, stained using rabbit anti-*Neoparamoeba* spp. antiserum (strain PA-027, Howard & Carson 1994) diluted 1:500 in PBS, 0.1% bovine serum albumin (BSA), and incubated for 30 min at 37°C. A peroxidase-based Vectastain ABC kit (Vector laboratories, Sydney, Australia) was used to complete the immunocytochemical procedure, following the manufacturer's instructions, and the slides counterstained with hematoxylin and eosin (H & E). The number of

immunostained *Neoparamoeba* spp. was counted and divided by the total number stained with H & E or the immunostain in 10 random fields of view at 100× magnification. After inoculation with the gill isolate, fish were collected when moribund and killed with 0.5% Aqui-S® (v v^{-1}). A gill smear was taken from the third left gill arch for immunocytochemical detection of *Neoparamoeba* spp. as previously described. The gills were then fixed in seawater Davidson's fixative, and the second left gill arch routinely processed for histology (5 μm, H & E). The percentage of *Neoparamoeba* spp. in the gill isolate used to inoculate the fish was calculated to be 99% as determined using the previously described immunocytochemical method. Survival of the CpG-ODN 1668 treated fish ($n = 21$) was significantly ($P < 0.05$) improved compared with the untreated control fish ($n = 21$) as determined by a log rank test ($P = 0.010$, Fig. 1). Survival of both the non-CpG ODN 1720 treated fish ($n = 21$) and the PBS treated fish ($n = 21$) was not significantly different from the untreated control fish (Fig. 1).

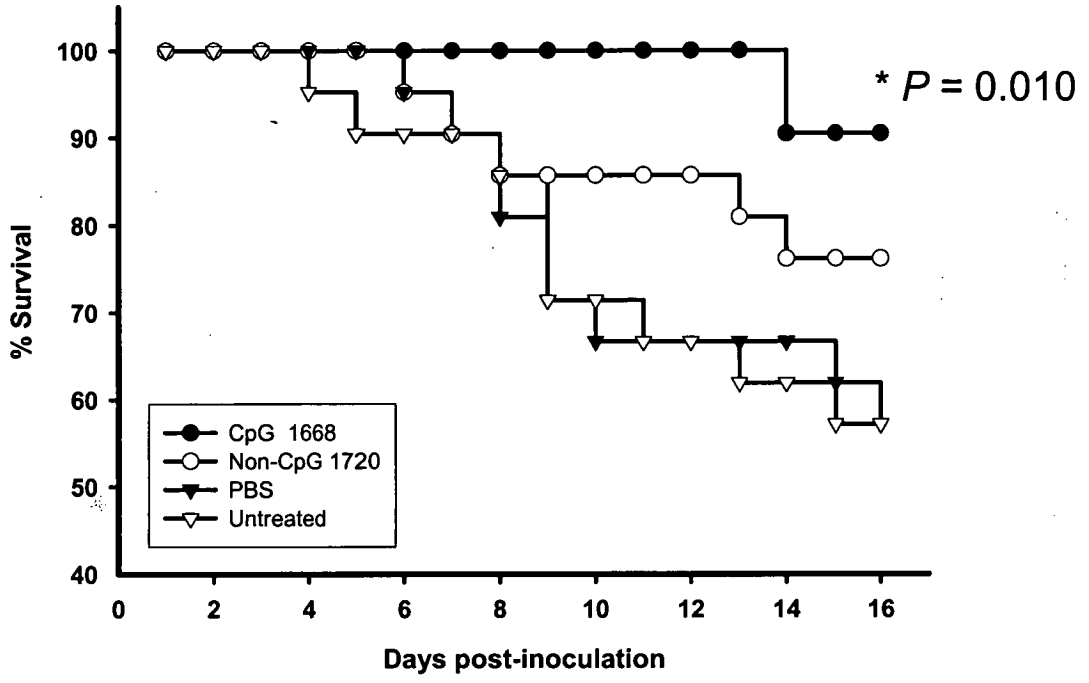

Fig. 1. Improved survival of CpG-ODN 1668 treated Atlantic salmon against amoebic gill disease (AGD). Treatments were i.p. administered 6 d before inoculation with 2460 amoebae L^{-1}. Survival of each group of fish ($n = 21$) was monitored over 16 d and statistical significance ($P < 0.05$), compared with the untreated group, determined using the log rank test.

At the end of the challenge period (16 d), the presence of pale mucoid patches on the gills and immunocytochemical analysis of gill smears, and gill histology showed that all the fish were infected with *Neoparamoeba* spp. and were consequently diagnosed with AGD. Histological examination of the fish treated with CpG-ODN 1668 revealed a more pronounced level of localised inflammation associated with AGD lesions when compared with the other groups of fish (Fig. 2). This type of inflammatory response was characterised by substantial leucocyte infiltration and hyperplastic proliferation of gill epithelia. The majority of the accumulated leucocytes were identified as neutrophils or macrophages based on

their morphology and were observed accumulating along the central venous sinus, and within and surrounding the hyperplastic tissue. In many instances these leucocytes could be seen in close association with the amoebae. Additionally, large numbers of interlamellar cysts were observed within AGD lesions of the CpG-ODN 1668 group of fish (Fig. 3).

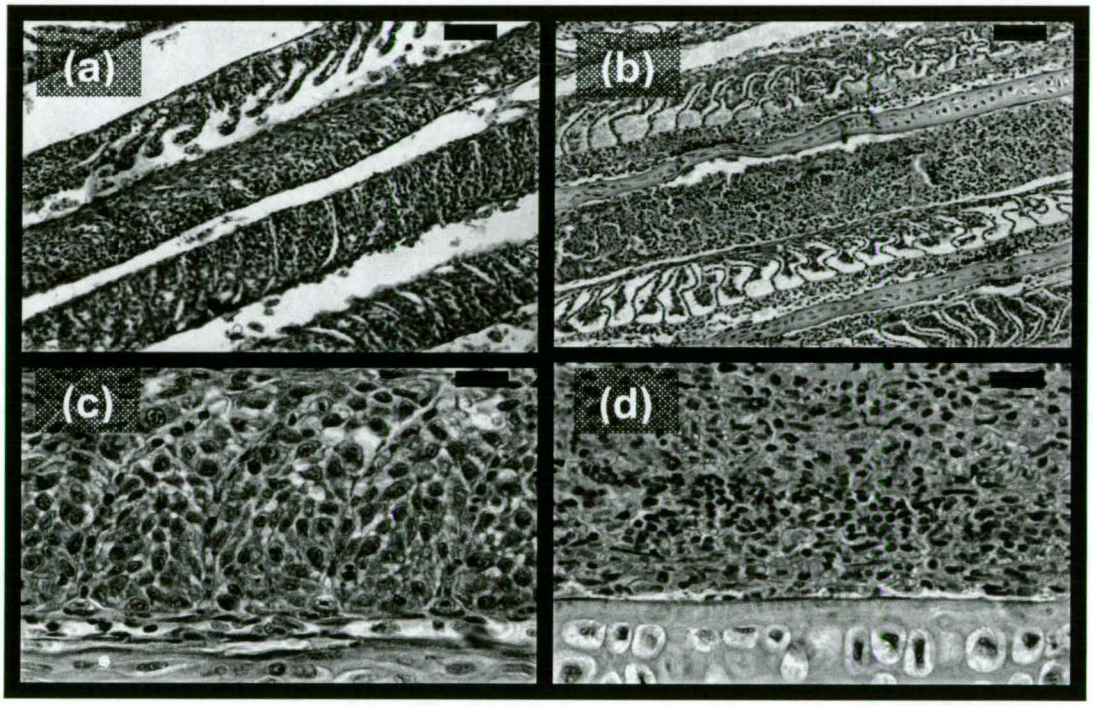

Fig. 2. Host responses to amoebic gill disease (AGD) in untreated (a, c) and CpG-ODN 1668 treated (b, d) Atlantic salmon (bars = 100 μm for a, b and 25 μm for c, d).

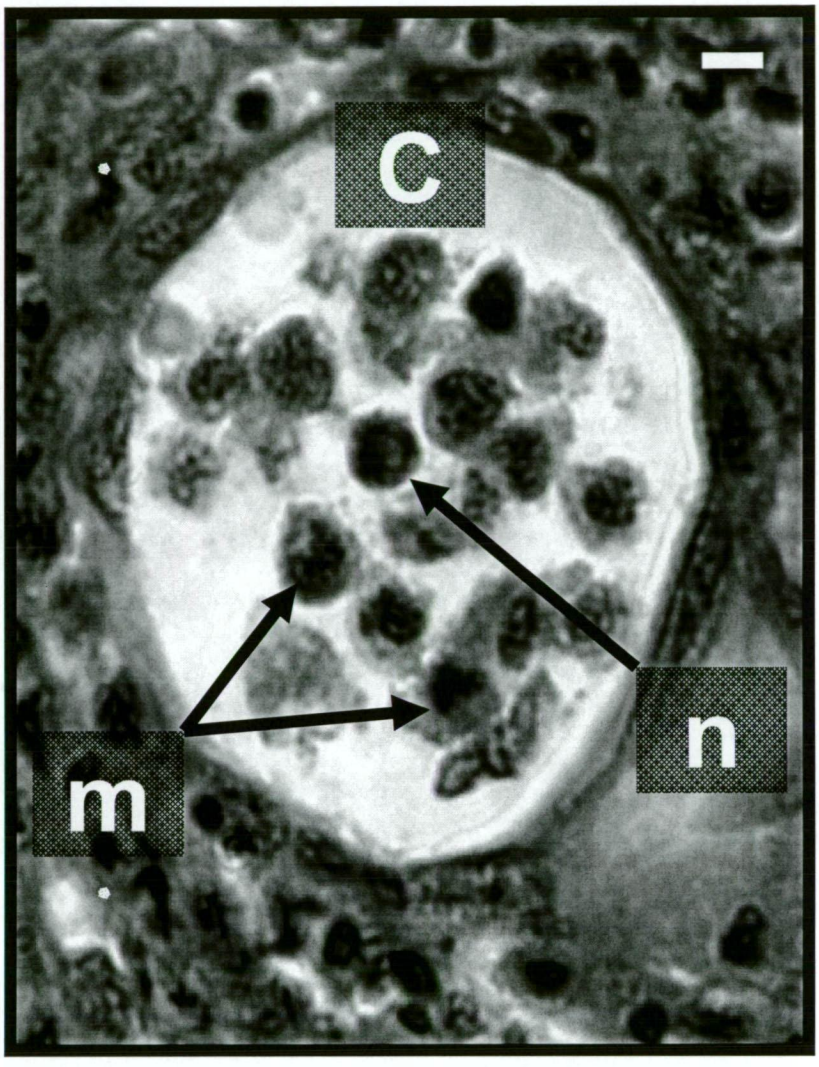

Fig. 3. Cells resembling macrophages (m) and neutrophils (n) within a cyst (C) of a CpG-ODN 1668 treated Atlantic salmon (bar = 10 μm)

Various conserved microbial products associated with bacterial and fungal cell walls are recognised by pattern recognition receptors (PRRs) of the innate immune system in both mammals and fish (Medzhitov & Janeway 1997). More recently, several short bacterial DNA sequences have been shown to activate the innate immune system. Recognition of DNA by the innate immune system and its level of activation depend upon the presence, frequency and position of unmethylated CpG motifs (Krieg *et al.* 1995). Both bacterial and synthetic CpG-ODNs are able to

stimulate the secretion of various cytokines, B-cell proliferation, and immunoglobulin production in mammals (Krieg 2002). The DNA from protozoan parasites also contains unmethylated CpG dinucleotides and is mitogenic for bovine B lymphocytes, stimulates macrophage expression of interleukin-12 (IL-12), tumor necrosis factor alpha (TNF-α), and nitric oxide (Shoda, Kegerreis, Suarez, Roditi, Corral, Bertot, Norimine & Brown 2001). Similarly, synthetic CpG-ODNs are able to stimulate Atlantic salmon leucocytes to produce interferon (IFN)-like cytokines (Jørgensen *et al.* 2001a). Rainbow trout head kidney leucocytes are also activated by CpG-ODNs to produce IFN-like cytokines and interleukin-1β (IL-β) (Jørgensen *et al.* 2001b). Several studies have shown the ability of CpG-ODNs to protect mice against infectious challenge with a variety of intracellular pathogens (Krieg, Love-Homan, Yi & Harty 1998; Elkins, Rhinehart-Jones, Stibitz, Conover & Klinman 1999; Klinman, Verthelyi, Takeshita & Ishii 1999). Immunostimulatory CpG-ODNs have also increased resistance against polymicrobial sepsis in mice via enhanced neutrophil responses (Weighardt *et al.* 2000). Neutrophils were found to have an up-regulation of phagocytic receptors, an increased phagocytic activity and an elevated production of reactive oxygen metabolites (Weighardt *et al.* 2000). Similarly, Tassakka & Sakai (2002) reported the stimulation of cellular immune functions in carp, *Cyprinus carpio* L., injected with CpG-ODN that demonstrated an enhanced phagocytic ability and reactive oxygen species (ROS) production by head kidney phagocytes. It is interesting to note that in the present study the survival of the non CpG-ODN treatment group appeared to be slightly improved in comparison with the untreated and PBS groups of fish (Fig. 1). This apparent enhanced survival was not as pronounced as in the

CpG-ODN 1668 treatment group and was not statistically significant ($P > 0.05$) from the untreated fish. Although the non-CpG-ODN 1720 did not contain any CpG motifs, it did have a phosphorothioated backbone. ODNs with phosphorothioated backbones and lacking any CpG motifs have been shown to elicit at least some amount of immune stimulation in mice (Monteith, Henry, Howard, Flournoy, Levin, Bennett & Crooke 1997). In the present study, histopathological observations from the CpG-ODN 1668 treatment group indicate that gill leucocytes are involved in an innate immune response that is enhanced by the CpG-ODN. It has been suggested by Adams & Nowak (2001) that cyst formation, and a subsequent leucocyte infiltration into the cysts, is an integral process in the destruction and clearance of amoebae from affected gill tissue.

The above-mentioned studies show that CpG-ODNs are potent activators of the innate immune response in vertebrates. Therefore, we suggest that the increased resistance to AGD in the present study is possibly the result of a CpG-ODN enhanced innate effector cell response. This study is the first to demonstrate the ability of CpG-ODNs to enhance resistance to disease in fish, and highlights their possible use as a disease control treatment or vaccine adjuvant.

Acknowledgements

The authors would like to thank Dr Jorunn Jørgensen for advice

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Chapter 5

The effect of β-glucan administration on macrophage respiratory burst activity and Atlantic salmon, (*Salmo salar* L.) challenged with amoebic gill disease (AGD) - evidence of inherent resistance

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Journal of Fish Diseases 2005, 28, 347-356

Abstract

Previous studies have demonstrated that β-glucans stimulate Atlantic salmon (*Salmo salar* L.) head kidney macrophages both *in vitro* and *in vivo* and increase protection against various pathogens. Based on our previous work that showed potent immunostimulatory CpG motif containing oligodeoxynucleotides increased resistance to amoebic gill disease (AGD), the present study investigated the immunostimulatory effects of three commercial β-glucan-containing feeds and their ability to increase resistance to AGD. All three commercial β-glucans were able to stimulate the respiratory burst activity of Atlantic salmon head kidney macrophages *in vitro*, albeit at different times and in different concentrations. However, dietary incorporation of the β-glucans was unable to stimulate the *in vivo* respiratory burst activity of head kidney macrophages, or serum lysozyme production, and did not increase resistance against AGD. However, this trial showed for the first time that a small subpopulation of Atlantic salmon subjected to a severe AGD infection was able to resist becoming heavily infected, and furthermore to survive the challenge.

Introduction

Amoebic gill disease (AGD) is a parasitic infection of fish gills that is the result of infestation of the gills with the protozoan *Neoparamoeba* sp. Clinical signs of AGD manifest as lethargy, flared opercula, obvious respiratory distress and loss of

equilibrium. The majority of cases, if left untreated, culminate in mortality (Munday, Foster, Roubal & Lester, 1990). These clinical signs are strongly associated with the presence of grossly visible pale multifocal gill lesions. Histologically, gill lesions are characterised by epithelial hyperplasia, lamellar fusion and the formation of interlamellar crypts (Roubal, Lester & Foster, 1989; Adams & Nowak, 2001). Less obvious is the infiltration of leucocytes and oedema associated with a local inflammatory response.

Neoparamoeba spp. have been isolated from several marine fish species during AGD outbreaks throughout many different geographical locations worldwide (Munday, Zilberg & Findlay, 2001). Within the Tasmanian salmonid industry mortality associated with AGD, and the labour and fresh water supply costs of the current preferred form of treatment, freshwater bathing, have dramatically hindered the sea-cage culture of Atlantic salmon (*Salmo salar* L.). Therefore, alternative AGD management strategies that reduce the need to freshwater bathe the fish are required. A variety of antimicrobial, disinfectant and mucolytic agents such as narasin (Cameron, 1992), levamisole (Zilberg, Findlay, Girling & Munday, 2000), chlorine dioxide and chloramine-T (Powell & Clark, 2003; Powell & Harris, 2004) have been trialled as AGD treatments, but with limited or no success. In contrast, investigations of the host immune response to AGD as a possible preventative strategy have highlighted the potential of both immunomodulation and vaccination as a method of controlling AGD. Although limited, host pathogen interaction studies involving the role of the host immune response to AGD are elucidating important immunological aspects of the pathology of AGD. Anecdotal evidence by

Tasmanian salmon farmers and research technicians at the School of Aquaculture, University of Tasmania suggesting that certain individual Atlantic salmon are more resistant to AGD than the majority of the Atlantic salmon population, has fuelled hope that resistance of Atlantic salmon to AGD can be boosted by either selective breeding, vaccination or immunostimulation.

Although initial experiments by Zilberg & Munday (2001) using crude vaccination preparations were unsuccessful, a more comprehensive approach has now been adopted to elucidate protective antigens and potential vaccination strategies. Additionally, research is currently being undertaken into possible genetic aspects of resistance to AGD, to evaluate the viability of a selective breeding program and investigate the role that the acquired immune response might play in a vaccination strategy. Evidence that innate immunity might play a part in protective immune responses to AGD has been suggested by Findlay & Munday (1998), Zilberg *et al.*, (2000) and Bridle *et al.* (2003).

Many substances containing conserved structures found in a variety of microbial pathogens are able to stimulate innate immunity and increase the resistance of animals to a diverse range of pathogens. Glucans with β-1,3 and β-1,6 glycosidic linkages (β-glucans) are fundamental structural components of yeast and fungal cell walls that have been proven to increase host resistance to certain pathogens. Both intraperitoneal (i.p.) and oral administration of β-glucans are able to stimulate innate immune responses in fish (Brattgjerd, Evensen & Lauve, 1994; Dalmo & Seljelid, 1995; Jørgensen & Robertsen, 1995). Increased lysozyme and

complement activity, phagocytosis and respiratory burst are immuno-enhancing properties of β-glucan administration. The immunostimulatory ability of β-glucans in fish and mammals is thought to occur by its binding to specific receptors for β-glucans predominantly found on macrophages/monocytes (Dowling, Wadman, Collins, Gans, Newton & Harris, 1992; Engstad & Robertsen, 1993). Evidence of successful oral β-glucan-enhanced microbial disease resistance, and our previous work showing that immunostimulatory synthetic CpG-containing oligodeoxynucleotides (CpG-ODN) were able to increase resistance of Atlantic salmon to AGD, therefore prompted us to investigate whether oral administration of β-glucans was able to increase resistance to AGD in Atlantic salmon.

Materials and Methods

Fish

Atlantic salmon (*Salmo salar* L.) weighing approximately 150-200 g were maintained in a 3000 L re-circulating tank with biofilter. Sea water was UV-irradiated and 1 μm filtered before addition to the tanks and maintained at 35‰ salinity at a temperature of 16 ± 0.5°C.

Isolation of head kidney macrophages

Anterior kidney macrophages were isolated using the method described by Secombes (1990). Briefly, anterior kidneys were aseptically removed from the salmon and disrupted by passing through a 100 μm mesh using Leibovitz L-15 medium (Invitrogen, Mulgrave, Australia) containing heparin (10 U mL^{-1}) (Sigma-Aldrich, Castle Hill, Australia), 1% penicillin/streptomycin (P/S) (Sigma-Aldrich) and 2% fetal bovine serum (FBS) (Invitrogen). The resulting tissue suspension was layered on a 34/51% Percoll (Sigma-Aldrich) density gradient and centrifuged at 400 × *g* for 30 min at 4°C. The interphase was collected and the cells washed twice at 400 × *g* for 5 min in L-15 containing 0.1% FBS. The cells were resuspended in a small volume, counted, and resuspended to 1×10^7 cells mL^{-1} before 100 μL aliquots were dispensed into 96 well microtiter plates. After 3 h, the non-adherent cells were removed by gently washing with L-15 medium and left overnight before washing the adherent cells again. Finally, cell monolayers were maintained in L-15 (1% P/S, 5% FBS) at 18°C.

β-glucan preparations and macrophage stimulation

Three commercial β-glucan products identified as A, B and C were prepared in L-15 medium and added at 0, 0.1, 1 and 10 μg mL^{-1} to the macrophage monolayers for either 3 or 7 d at 18°C before analysis of respiratory burst and lysozyme activity.

Respiratory burst activity assay

Macrophage respiratory burst activity was assessed after incubation of cells with phorbol myristate acetate (PMA) (Sigma-Aldrich) and nitroblue tetrazolium (NBT) (Sigma-Aldrich) (Secombes, 1990). Briefly, after incubation with the β-glucans, the cultures were stimulated with PMA (1 μg mL^{-1}) in the presence of NBT (1 mg mL^{-1}) for 1 h and the optical density measured at 620 nm. The number of macrophages in control wells, incubated with L-15, was determined by adding lysis buffer (0.1 M citric acid, 1% Tween 20, 0.05% crystal violet) for 2 min, then counting released nuclei using a haemocytometer. The results were expressed as O.D. at 620 nm per 10^5 cells.

Lysozyme activity

Culture supernatant or serum (20 μL $well^{-1}$) was placed in triplicate in a 96-well microplate and 180 μL of a 0.75 mg mL^{-1} *Micrococcus lysodeikticus* (Sigma-Aldrich) suspended in 50 mM sodium phosphate buffer pH 6.2 was added. After shaking the plate, the absorbance at 450 nm was recorded at 15 s intervals for 10 min and the rate of change of absorbance calculated. Lysozyme activities were converted to lysozyme concentration using hen egg white lysozyme (Sigma-Aldrich) as a standard.

In vivo experimental design

Atlantic salmon weighing approximately 150-200 g were transferred to 12 circular tanks (300 L) connected to a 3000 L holding tank and gradually acclimatised to sea water over two weeks. Sea water was maintained at 35‰ salinity, a temperature of 16 ± 0.5°C, and was UV-irradiated and 1 µm filtered before addition to the tanks. Water quality was monitored every second day and total ammonia and nitrite concentrations were maintained at ≤0.5 mg L^{-1} and ≤0.2 mg L^{-1}, respectively. Fish were held for a further two weeks to ensure they were healthy and free of gross signs of disease before starting the trial. Four treatments were assigned to the tanks, with each treatment consisting of triplicate tanks (20 fish $tank^{-1}$). Treatments consisted of feeding the fish one of three commercial β-glucan-containing diets and a control diet.

β-glucan feed incorporation

The commercial β-glucan products, A (1%), B (1%), and C (0.3%) were incorporated into a commercial Atlantic salmon feed according to the manufacturer's recommended rate of incorporation. The control diet had no β-glucan added. Briefly, an Atlantic salmon HP kernel diet supplied by Skretting (Hobart, Australia) was hammer-milled to produce a dry feed mix. The experimental 4 mm pellet diets were produced by adding fish oil, water, carboxymethyl cellulose (CMC) (Sigma-Aldrich) and the β-glucan product to the feed mix before pelleting. Inclusion rates of these standard ingredients were

14.30%, 8.00%, and 0.84% respectively, and were calculated allowing a 50% water loss after drying the pellets.

Feeding regime

Feed intake varied over the trial, and fish were fed once per day to satiation. As a result feed intake never exceeded 1% body weight per day. Fish were fed their respective experimental diets for 1 week before the challenge, followed by 3 weeks of the control diet. This 1:3 feeding regime was maintained over the duration of the trial.

Sampling

Four fish were sampled at 0 d before they had been fed the experimental diets, followed by four fish from each treatment group at 3 and 7 d post-initial feeding of the experimental diets, and anterior kidney respiratory burst activities measured as previously described. Fish were anaesthetised with 50 mg L^{-1} Aqui-S in sea water (Aqui-S NZ Ltd, Lower Hutt, New Zealand) and bled from the caudal vein before being euthanased in an overdose of Aqui-S (5 g L^{-1}) and the anterior kidney sampled. Blood collected for serum was allowed to clot for 2 h at room temperature, incubated overnight at 4°C, and after centrifugation the serum was removed and stored at -20°C until required.

Challenge

Fish were challenged at the end of the first 7 d of feeding with the experimental β-glucan feeds by the addition of *Neoparamoeba* spp. to the recirculating water supply at a rate of 1.15×10^3 amoebae L^{-1}. Amoebae were harvested from the gills of fish with clinical AGD according to the procedure described by Morrison *et al.* (2004) and 384 amoebae L^{-1} d^{-1} were added to each of the 12 tanks over 3 d. Harvested amoebae were identified as *Neoparamoeba* spp. by morphological inspection using light microscopy, immunocytochemical staining with an anti-*Neoparamoeba* spp. antiserum as described by Bridle *et al.* (2003) and polymerase chain reaction (PCR) using *Neoparamoeba* spp. 18S ribosomal DNA primers (Wong, Carson & Elliott, 2004). Fish were collected and recorded when moribund and euthanased with 5 g L^{-1} Aqui-S before the gills were excised and fixed in seawater Davidson's fixative. Moribund fish were considered as mortalities for the calculations of percent survival and relative percent survival.

Gill pathology

Seawater Davidson's fixed gill hemibranchs were photographed with an Olympus Camedia C5050 digital camera after 1-2 h in fixative, allowing the amount of gross gill pathology to be visually assessed before the gill hemibranchs were processed for routine histology (5 μm, H & E).

Data analysis

Data were analysed using SPSS version 10. One-way analysis of variance (ANOVA) and Student's *t*-tests (Bonferroni corrected) were used to analyse the respiratory burst data, and Kaplan-Meier survival curves were compared using the log rank test. Relative percent survival (RPS) was evaluated according to Amend (1981) and calculated as: RPS = [1 – (% β-glucan diet mortality/ % control mortality) × 100]. Results of analyses were considered statistically significant if $P < 0.05$.

Results

In vitro macrophage stimulation

To determine whether each of the three commercial β-glucan products was able to modulate the respiratory burst activity of anterior kidney macrophages, the macrophages were cultured for 3 and 7 d in the presence 0.1-10 μg mL^{-1} of the β-glucan products. After incubating the 3 d macrophage cultures with PMA (1 μg mL^{-1}) for 1 h at 18°C, intracellular O_2^- production significantly increased relative to the control (0 μg mL^{-1}) in the macrophages incubated with β-glucan A at 1 and 10 μg mL^{-1} ($P < 0.05$) (Fig. 1). Macrophages incubated for 7 d with both of these concentrations of β-glucan A were still significantly increased when compared with the control. However, β-glucan C at 10 μg mL^{-1} and the β-glucan B at 0.1 and 1 μg

mL^{-1} were also significantly greater than the control (Fig. 1). Intracellular O_2^- production in each of the treatments that were statistically increased compared with the control after 7 d incubation was also greater than the production after 3 d β-glucan incubation (Fig. 1). No significant differences were found between the lysozyme activities of supernatants from macrophages incubated with 0.1-10 μg mL^{-1} of each β-glucan diet for 3 and 7d when compared with the control (data not shown).

***In vivo* macrophage stimulation**

Anterior kidney macrophages isolated from fish sampled at 0, 3, and 7 d post initial feeding of the experimental β-glucan feeds were neither directly stimulated (NBT alone) or primed (NBT + PMA) relative to the control diet (Fig 2). The concentration of lysozyme in the serum of fish sampled at 7 d post-inoculation (p.i.) and the surviving fish at 72 d p.i. was not significantly affected by any of the glucan diets (data not shown).

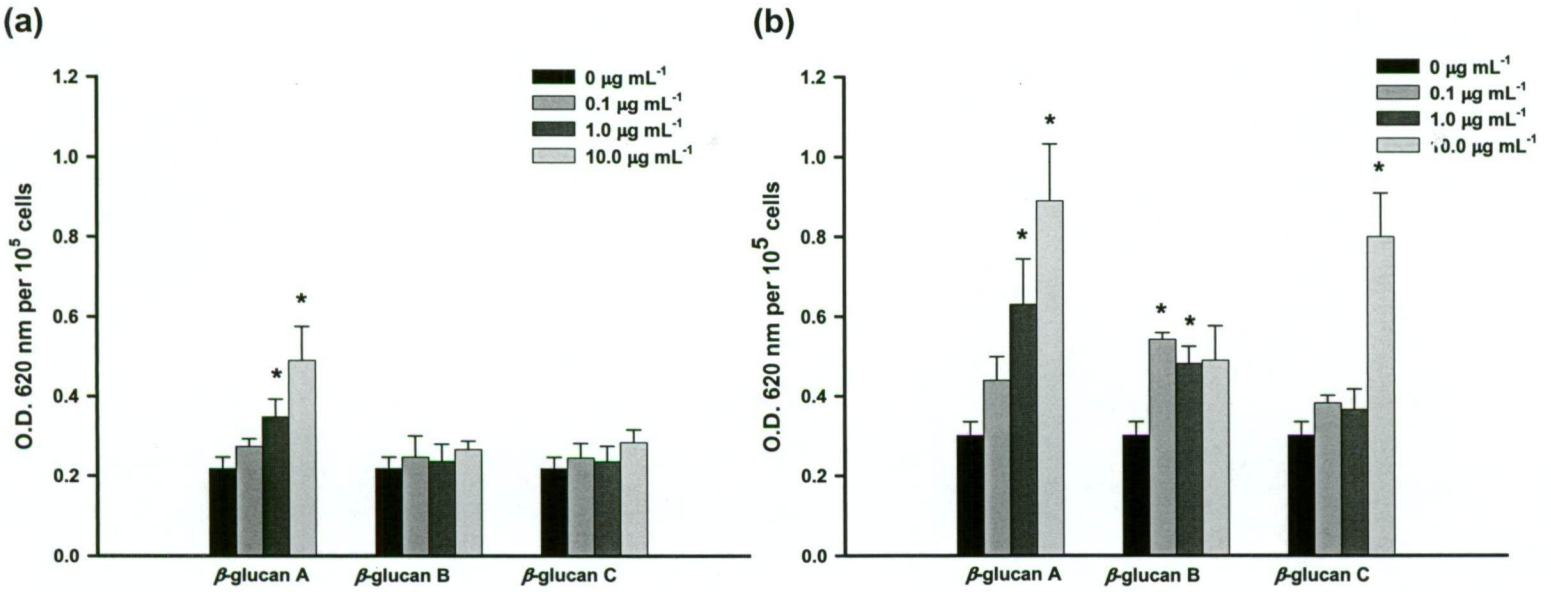

Figure 1 Respiratory burst activity of Atlantic salmon head kidney macrophages upon PMA-stimulation after incubation with different concentrations of three commercial β- glucans for 3 (a) and 7 (b) d. Macrophages were isolated over a Percoll density gradient and monolayers established in 96-well tissue culture plates. After incubation cultures were stimulated with PMA (1 µg mL^{-1}) in the presence of NBT (1 mg mL^{-1}). * indicates a significant difference between glucan treatments and macrophages incubated with 0 µg mL^{-1} ($P < 0.05$). Data are means ± S.E. of eight wells from four fish.

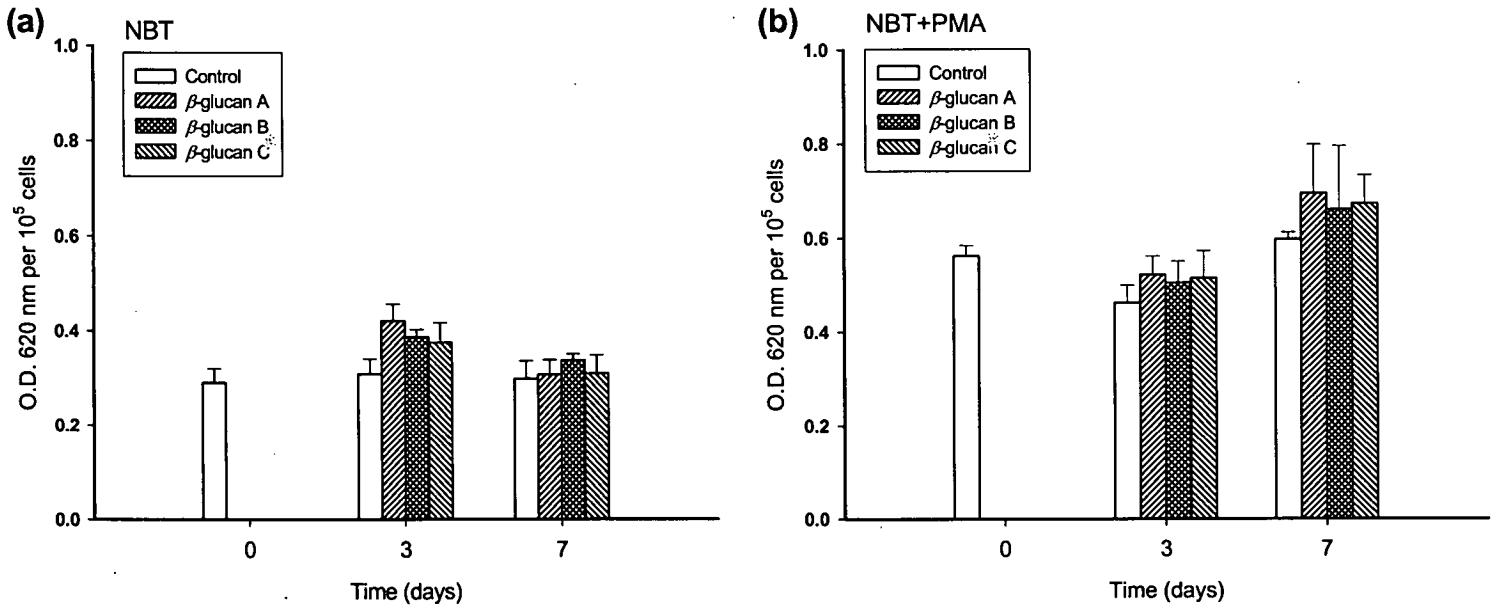

Figure 2 *In vivo* respiratory burst activity of Atlantic salmon head kidney macrophages isolated at 0, 3 and 7 d post-initial feeding. Atlantic salmon were held in sea water at 16°C and fed one of three commercial β-glucan diets or a control diet (no β-glucan) over 7 d. At each sampling time fish were euthanased, the head kidney sterilely dissected, and the macrophages isolated over a 34/51% Percoll density gradient. Cell monolayers were established in 96-well tissue culture plates and incubated with NBT (1 mg mL^{-1}) alone (a), or NBT (1 mg mL^{-1}) and PMA (1 μg mL^{-1}) (b) for 60 min. No statistically significant differences ($P > 0.05$) were found between the different β-glucan diets including the control diet at each sampling time using ANOVA. Data are means + S.E. of eight wells from four fish.

Challenge

Moribund fish were evident 27 d p.i. and showed obvious clinical signs of AGD infection before death. Excised gills from the moribund fish had gross lesions affecting a large proportion of gill surface area. None of the experimental diets, β-glucan A (1%), β-glucan B (1%), or β-glucan C (0.3%), were able to increase the survival of fish that had been infected with 1.15×10^3 amoebae L^{-1} (Fig. 3). RPS values of β-glucan A, β-glucan B, and β-glucan C were 14.5, -10, and 6.4, respectively (Table 1). During the last three weeks of the experiment the rate of moribund fish due to AGD began to plateau, leaving a group of surviving fish exhibiting very few or no mortalities independent of which diet they were fed. To show that the surviving fish were not a result of the absence of the causative agent, the tanks were left empty at the end of the experiment for 24 h before six Atlantic salmon were added to one of the three tanks previously used per diet. Two weeks later these fish were sampled and histological signs of AGD identified on each of the fish confirming that the tanks still contained viable *Neoparamoeba* sp. and were able to infect fish and cause AGD.

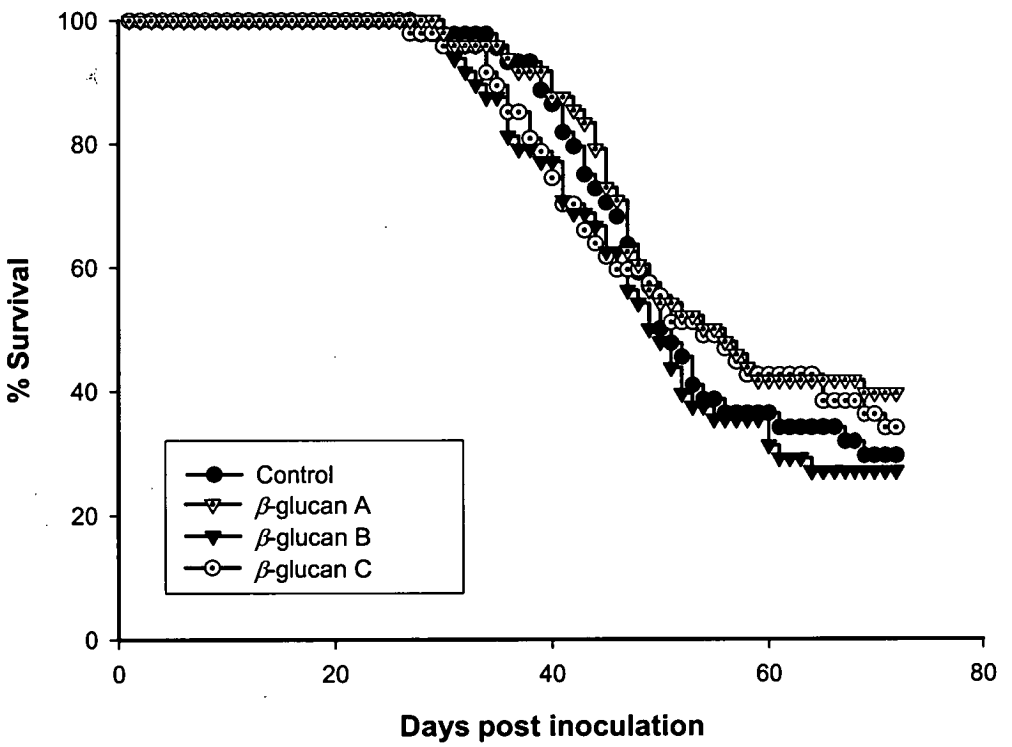

Figure 3 No statistically significant differences were found between the mean survival of triplicate groups of fish fed the four experimental diets and challenged by the addition of 1.15×10^3 amoebae L^{-1}. Experimental diets containing either, β-glucan A (1%), β-glucan B (0.3%), β-glucan C (1%) or a control diet (0%), were fed to the fish for 1 week before the challenge followed by 3 weeks of the control diet. This 1:3 feeding regime was maintained over the duration of the trial. Survival of each group of fish was monitored over 72 d and Kaplan-Meier survival curves statistically compared using the log rank test ($P < 0.05$).

Table 1 Specific mortality, relative potency (RP), and relative percent survival (RPS) of Atlantic salmon fed experimental diets containing either, β-glucan A (1%), β-glucan B (0.3%), β-glucan C (1%), or a control diet (0%), for 1 week before being challenged by the addition of 1.15×10^3 amoebae L^{-1}. Mortalities in each group of fish were monitored for 72 d p.i.

Diet	Total Number of Fish	Number Specific Mortality	Specific Mortality (%)	RP	RPS
β-glucan A	48	28	58.3	1.2	14.5
β-glucan B	48	36	75	0.9	-10
β-glucan C	47	30	63.8	1.1	6.4
Control	44	30	68.2	1.0	

Gill Pathology

Gross pathology associated with the moribund fish was substantial, with the majority of the gill filaments affected by advanced lesions (Fig. 4). This significant level of pathology was even more apparent, histologically with lesions displaying characteristics of a very aggressive infection (Fig. 4). In contrast, the majority of surviving fish at 72 d p.i. were shown on both gross and histological examination to have very few lesions. More importantly, a large number of these lesions showed signs of lesion repair and recovery, indicating that although being colonised by amoebae the fish were able to actively resist the infection (Fig. 4). No gross or histopathological differences were found between groups of survivors fed the different glucan diets or the control diet, indicating that this apparent resistance was inherent in this surviving subpopulation of fish (Table 2).

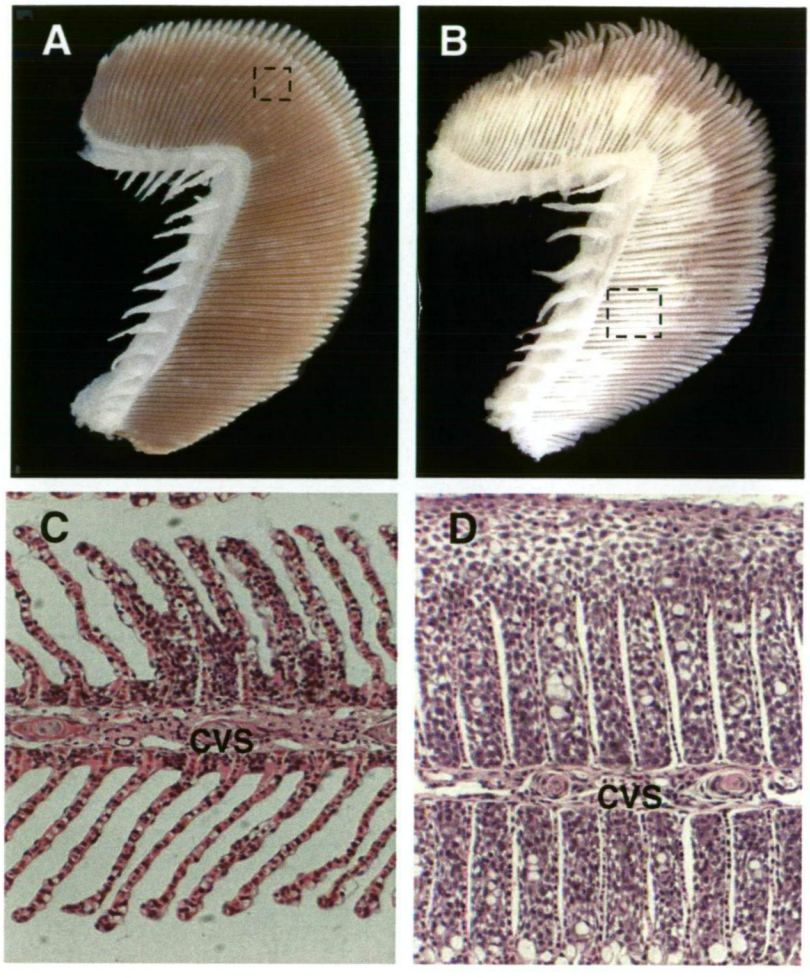

Figure 4 Representative examples of the gills from surviving (A & C) and moribund (B & D) fish challenged with AGD. Surviving fish at 72 d p.i. had very few lesions on both gross (A) and histological (C) examination when compared with moribund fish. The surviving fish had relatively small focal lesions affecting few lamellae scattered over the gill arch (A - pale patch within the broken square). Interestingly a large number of these lesions showed signs of possible lesion repair (C). In comparison moribund fish exhibited extensive epithelial hyperplasia as shown by the pale coloured areas affecting the majority of the gill arch (B – total area within the broken square). Histologically, the lesions of moribund fish were areas of extensive epithelial hyperplasia resulting in total lamellar fusion (D). The central venous sinus in C & D is denoted by CVS.

Table 2 Percentage AGD lesion affected filaments, average lesion size, and percentage AGD lesions with amoebae of the surviving fish 72 d p.i. Fish were euthanased, the gill arches excised and fixed in seawater Davidson's and routinely processed for histology (5 µm, H & E). Average lesion size was expressed as interlamellar units per filament (I.L.U./filament), and was calculated from the number of lamellae affected with epithelial hyperplasia divided by the number of lesion affected filaments. Data are represented as means (± S.E.).

Diet	AGD lesion affected filaments (%)	Average lesion size (I.L.U./filament)	AGD lesions with amoebae (%)
β-glucan A	29.2 ± 7.2	8.2 ± 1.1	25.3 ± 6.6
β-glucan B	26.1 ± 8.4	6.2 ± 1.3	18.6 ± 7.1
β-glucan C	20.6 ± 4.6	10.0 ± 1.4	23.5 ± 5.1
Control	19.5 ± 4.4	10.2 ± 4.8	10.2 ± 4.8

Discussion

It is well established that β-glucans are recognised by receptors expressed on fish monocytes/macrophages and are able to stimulate the innate immune system of fish. Increased production of both nitrogen and oxygen intermediates by phagocytes is indicative of stimulation of the innate immune system both in mammals and fish. Therefore, the results from the present study demonstrate the immuno-enhancing ability of three commercial β-glucan preparations by their ability to prime Atlantic salmon anterior kidney macrophages *in vitro* resulting in

an increased O_2^- production. However, while these preparations were able to stimulate anterior kidney macrophages *in vitro*, albeit at different concentrations and incubation times, no such stimulation was evident from *in vivo* stimulated macrophages after dietary intake of the preparations for either 3 or 7 d.

Previous studies have reported enhanced respiratory burst activity of snapper (*Pagrus auratus*) anterior kidney macrophages both *in vitro* (Cook, Hayball, Hutchinson, Nowak & Hayball, 2001) and after dietary administration of 0.1% v v^{-1} EcoActiva paste (Bio-Resources Division of Carlton and United Breweries, Melbourne, Australia) (Cook, Hayball, Hutchinson, Nowak & Hayball, 2003). A large amount of variation exists among the concentrations and incubation times that various β-glucan products require to elicit enhanced macrophage respiratory burst activities *in vitro*. This is further highlighted by species-specific responses. For instance, snapper macrophages responded to 0.001-0.1% v v^{-1} EcoActiva paste after both 1 and 3 h incubation followed by PMA stimulation (Cook *et al.*, 2001). Atlantic salmon macrophages incubated with MacroGard (Biotec Pharmacon ASA, Tromsø, Norway) for 4 d with 1 μg mL^{-1}, and 7 d with 0.1 and 1 μg mL^{-1}, and then stimulated with PMA had significantly enhanced respiratory burst activities when compared with the control (0 μg mL^{-1}) ($P < 0.05$). However, the same study demonstrated that macrophages incubated with 10 μg mL^{-1} MacroGard for 4 and 7 d and then stimulated with PMA had an O_2^- production no different to the control and that 50 μg mL^{-1} was inhibitory (Jørgensen & Robertsen, 1995). Results of a study by Castro *et al.*, (1999) demonstrate that at high concentrations (25–500 μg mL^{-1}) β-glucans can directly stimulate O_2^- production in both turbot (*Psetta*

maxima) and gilthead seabream (*Sparus auratus*) anterior kidney macrophages. However, the same study also found that macrophages incubated at similarly high concentrations of β-glucans and then stimulated with PMA had greatly reduced respiratory burst activity when compared with the macrophages incubated with β-glucans and NBT only. Inhibition of 'priming' following incubation with high concentrations of β-glucans led Castro *et al.*, (1999) to suggest that high concentration of β-glucans can excessively stimulate fish macrophages and after time exhaust the cells.

While enhanced innate immune responses in fish macrophages incubated *in vitro* with β-glucan based products have been clearly demonstrated, the effectiveness of these products when administered *in vivo* is dependant on several important factors. Firstly, β-glucans containing β-1,3 and β-1,6 glycosidic linkages possess a potent stimulatory ability that is affected by the degree of branching of these β-1,3 and β-1,6 polymer chains. Uptake via the digestive tract and presentation to the gut associated lymphoid tissue (GALT) in mammals is closely associated with particle size (Tabata & Ikada, 1988). Particles of approximately 1 μm diameter that do not form aggregates *en route* through the digestive system are absorbed by the GALT more efficiently than poorly processed β-glucan particles that readily form aggregates when exposed to water (Hunter, Gault & Berner, 2002). Therefore, differences in the dose-response and timing of respiratory burst activation between the different commercial β-glucan preparations are possibly explained by different manufacturing processes.

Dietary administration of commercial β-glucans enhances macrophage respiratory burst activity in turbot (Toranzo, Devesa, Romalde, Lamas, Riaza, Leiro & Barja, 1995), African catfish (*Clarias gariepinus*) (Yoshida, Kruger & Inglis, 1995), snapper (*P. auratus*) (Cook *et al.*, 2001; 2003), and rainbow trout (*Oncorhynchus mykiss*) (Siwicki, Anderson & Rumsey, 1994). However, dietary administration of the three β-glucan products used in the present study did not prime the respiratory burst activity of Atlantic salmon macrophages. The differences in immunostimulatory abilities reported in the previously mentioned β-glucan studies and the present study are most likely explained by differences in experimental factors such as the species-specific immune system responses, water temperatures, diet and β-glucan formulations and feed intakes. Administration of β-glucans either by intraperitoneal (i.p.) injection or within the diet has been demonstrated not only to stimulate fish macrophages but also to increase resistance to a variety of diseases, presumably as a result of enhanced innate immune responses (Robertsen, Rorstad, Engstad & Raa, 1990; Siwicki *et al.*, 1994).

Evidence that innate immunity might play a part in protective immune responses to AGD has been suggested by Findlay & Munday (1998). Freshwater bath treatments with the addition of levamisole, a known immunostimulant of the innate immune system, have also been shown to reduce mortality due to AGD in laboratory-based infections (Zilberg *et al.*, 2000). More recently, we have shown that i.p. administration of a potent immunostimulatory CpG-ODN is able to increase resistance to AGD in Atlantic salmon (Bridle *et al.*, 2003). Therefore, it is not surprising that the lack of *in vivo* macrophage stimulation has translated into a lack

of improved resistance to AGD in the present study. As respiratory burst activation was used as a measure of innate immune system activation it is plausible that cells other than anterior kidney macrophages may have responded to the dietary β-glucans, or that other innate immune responses were enhanced. It is also possible that the β-glucan feed inclusion rates used in the present study were sub optimal, and given greater access to resources could have been further optimised in additional dose-response feed trials.

Although none of the β-glucan diets were shown to increase survival to AGD the challenge did identify a population of fish from each treatment group that survived 72 d of an extremely aggressive AGD infection. More importantly, this population showed relatively minor gill pathology and even signs of possible lesion repair. This supports the findings of Findlay *et al.* (1995) and Findlay & Munday (1998), who demonstrated resistance in Atlantic salmon previously exposed to AGD and a reduction in the number of AGD lesions in this resistant group over time. However, these studies contradict a more recent study of AGD resistance in Atlantic salmon by Gross *et al.* (2004) that found no resistance to AGD was conferred by prior AGD infection followed by bathing and subsequent re-infection. This contradiction between the studies is possibly explained by the different experiment regimes and the vastly different dynamics and duration of infections as evident from the survival/mortality figures of the studies. Although the challenge used in our study was highly aggressive, and fish in a farm situation are unlikely to be exposed to such an aggressive infection, it nonetheless demonstrates varying levels of inherent resistance to AGD within Atlantic salmon populations and further strengthens the

potential for a selective breeding program. Whether this inherent resistance is due to an immunological trait that may be strengthened by immunomodulation is yet to be fully determined.

In conclusion, although we were able to demonstrate an enhanced *in vitro* macrophage respiratory burst activity with each of the commercial β-glucan products, albeit with varying time/dose responses, we were unable to demonstrate improved survival to AGD in fish treated with dietary β-glucans. However, this trial showed for the first time that a subpopulation of Atlantic salmon subjected to a highly aggressive AGD infection was able to resist becoming heavily infected and to survive the challenge. Therefore the results of this experiment warrant further investigations into this inherent AGD resistance and into determining the involvement of the immune response in developing resistance to AGD.

Acknowledgements

This work formed part of a project of Aquafin CRC, the Australian Government's CRCs Programme, and received funds from the Fisheries R & D Corporation and other CRC participants.

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Chapter 6

General Discussion

General discussion

Amoebic gill disease (AGD) continues to impact negatively on the culture of Atlantic salmon, *Salmo salar*, in Tasmania, Australia. Partial control of the disease has been achieved by regularly bathing fish in fresh water when pale mucoid patches are observed on their gills. However, for unknown reasons the frequency of baths required to maintain fish with as few patches as possible is increasing. The constant burden of labour and freshwater supply costs associated with the freshwater bathing continues to hinder the sea-cage culture of Atlantic salmon in Tasmania. Consequently, alternative AGD management strategies are required. Thus an understanding of the disease processes associated with AGD pathology and the immune responses to AGD is essential to the development of AGD management strategies.

Presented in this thesis are the findings of research aimed at further characterising the immune response to AGD using molecular techniques, and assessing the potential use of immunostimulants to reduce the impact of AGD on the Tasmanian salmonid industry.

6.1. Fish species

Rainbow trout, *Oncorhynchus mykiss*, were chosen for AGD gene expression studies as more immune-regulatory genes have been identified and characterised in this species than in any other fish species that are susceptible to *Neoparamoeba* sp.

infection. Although fewer immune-regulatory genes have been identified and characterised in Atlantic salmon, AGD has been extensively studied in this species. In addition, molecular studies have shown that many immune-regulatory genes identified in fish share a relatively high degree of nucleic acid sequence similarity with other fish species [1]. Even greater similarities exist between Atlantic salmon and rainbow trout, as these two species belong to the family Salmonidae. Furthermore, both species are believed to have evolved from ancestors with tetraploid genomes, explaining the existence of multiple isoforms of various genes in both species [2]. Similar immune-regulatory gene expression profiles have been identified during bacterial infection in both species [3, 4].

Although AGD has been extensively reported in Atlantic salmon and rainbow trout, there are few comparative descriptions of AGD-associated pathology of these species. Munday *et al.* [5] reported more diffuse mucoid branchitis in AGD-affected rainbow trout when compared with AGD-induced branchitis in Atlantic salmon. Similarly, lesions containing many mononuclear cells were reported along the primary lamellae, especially in the basal interlamellar tissues, and were more obvious in rainbow trout than in Atlantic salmon [5]. Similar histopathological changes have been reported in non-salmonid fish infected with *Neoparamoeba* sp. In AGD-affected turbot, *Scophthalmus maximus*, amoebic trophozoites were found to accumulate in the gill interlamellar spaces and were accompanied by hyperplasia and hypertrophy of the gill epithelium and fused lamellae [6, 7]. However, individual virulence properties and the pathological consequences of different *Neoparamoeba* spp. have yet to be characterised. Therefore, it is not known what

impact possible differences in the proportions of the *Neoparamoeba* spp. might have on comparisons of AGD pathology between different infections. Thus, it should be noted that comparisons between different AGD infections, whether in the same or different species, may be confounded by the possibility that different proportions of *Neoparamoeba* spp. are responsible for the resulting AGD pathology. The possibility that different proportions of *Neoparamoeba* spp. were in the inocula used to infect the Atlantic salmon and rainbow trout in the experimental infections reported in chapters two and three seems irrelevant, as the qualitative AGD-pathology and immune-regulatory gene expression between these species were similar. Thus, important similarities exist between the immune-regulatory genes, disease-induced differential expression of these genes and the described AGD pathology of Atlantic salmon and rainbow trout. Therefore it can be confidently stated that patterns of immune-regulatory gene mRNA expression identified in rainbow trout correspond to similar molecular responses in Atlantic salmon, and *vice versa*. However, as discussed in chapter three, it is important to make comparisons between specific gene mRNA expression patterns in fish with similar AGD pathology. Whether similar patterns of immune-regulatory gene mRNA expression occur during AGD in more evolutionary divergent species such as turbot is more uncertain. Thus the possibility exists that potential AGD management strategies proposed as a result of the research performed on salmonids affected by AGD, especially those of an immunological nature, may prove inappropriate and ineffective in non-salmonid species.

6.2. Immune-regulatory gene mRNA expression

Several approaches may be taken when trying to characterise the transcriptional profile of genes during infection. Recent molecular approaches such as subtractive hybridisation [8], differential display [9], serial analysis of gene expression (SAGE) [10] and cDNA microarrays [11] have allowed the collection of large amounts of data concerning the differential gene mRNA expression during infection. In fish, cDNA chips for microarray studies are increasingly being developed, but rely heavily on expressed sequence tag (EST) sequences of mostly uncharacterised fish genes [12-15]. Furthermore, differential gene mRNA expression data generated from these studies requires verification using quantitative real-time PCR (qRT-PCR). An alternative approach, and the approach taken in this thesis, involves assessing the differential mRNA expression of genes with proven importance to the immune response during infection.

Therefore, semi-quantitative RT-PCR and quantitative real-time RT-PCR were used to quantitate the mRNA expression in AGD-affected and uninfected control rainbow trout and Atlantic salmon. The immune-regulatory genes studied in this thesis were chosen based on the availability of gene sequences in rainbow trout and Atlantic salmon, the molecular and functional characteristics of the genes during infection, and those genes of known importance in infections of mammals. Immune-regulatory gene mRNAs were quantitated in the gill, liver and anterior kidney tissues of AGD-affected and uninfected control fish. The gills were chosen as they are the site of *Neoparamoeba* sp.-infection and changes within gill tissue

represent the local immune response to AGD. The anterior kidney is an important haematopoietic organ in fish, and the liver is the primary source of acute phase proteins (APPs). Thus, changes within these two organs represent the systemic response to AGD. Tissues were sampled at 7 and 14 d post-inoculation (p.i.) during the experiments described in chapters two and three. These sampling times were chosen as AGD-affected fish had uniform, yet distinctly different, AGD-associated pathology at each sampling time. Future studies might benefit from a greater sampling frequency, thus allowing a more detailed assessment of the transcriptional profile and the possibility of capturing transient mRNA expression of immune-regulatory genes in AGD-affected fish during the course of infection.

Although the degree of AGD-associated pathology was approximately equal amongst fish at each sampling time, the distribution of lesions on the gill was less uniform. In experiments described here, samples were consistently excised from approximately the same location on the gills of AGD-affected and uninfected control fish. Assuming the transcriptional response of the immune-regulatory genes was localised to gill lesions, then a potential source of variation between gill samples of individuals may have been introduced during sampling of the gills of AGD-affected fish. Furthermore, individual variation in tissue mRNA expression, which may have been introduced by subtle differences in AGD pathology between individuals, possibly contributed to the relatively large variation among pools. As the among pool variation was consistent between control and AGD-affected fish, and three pools of six fish were assayed, it appears that the among pool variation was heavily influenced by individual variation. For future studies several

alternative approaches are possible. The quantitation of mRNA expression in individual fish would assess the contribution of individual variation to the among pool variation in the present study and help identify the most appropriate pool size for future studies. It might also prove useful to study the transcriptional profile of gill lesions with varying degrees of histopathology. To do so, the whole gill arch might be excised and fixed in an RNA preservation reagent, the RNA extracted from micro-dissected lesions and quantitated using qRT-PCR. This might not be feasible in the case of very small AGD lesions that affect only a few secondary gill lamellae. Another alternative might be a semi-quantitative *in situ* hybridisation (ISH) approach, which would also demonstrate the cellular localisation of the studied mRNA transcript [16].

The cellular localisation of the studied mRNA transcripts using an ISH approach, and hence the ability to assess mRNA expression on a per cell basis within tissues, eliminates the need to normalise the mRNA expression to that of a housekeeping gene. This would overcome another potential problem associated with quantitating mRNA transcripts from hyperplastic tissue. If the immune-regulatory gene mRNA is preferentially expressed by cells other than the abundant epithelial cells within AGD lesions, then during normalisation to the housekeeping gene the ratio of the immune-regulatory gene mRNA expression to the housekeeping gene mRNA expression will potentially be at a lower ratio in the AGD-affected fish compared with uninfected fish. Thus it is possible that during the experiments performed in chapters two and three this effect masked the true differential expression of immune-regulatory gene mRNA transcripts.

6.3. AGD infection model

The major limitation to the study of the immune response in fish is a lack of species-specific antibodies raised against important components of the fish immune system. However, recent molecular studies are overcoming limitations associated with the more conventional proteomic approach to research of the immune response to infection and disease in fish. Therefore, information on gene sequences from the numerous EST libraries and the completed genome sequencing of fugu, *Takifugu rubripes*, [17] and zebrafish, *Danio rerio*, [18] is important to the continued study of the immune system of fish at a molecular level.

Importantly, care must be taken when making assumptions about the implications of altered gene mRNA expression during research of the immune response to infection. While the transcriptional profile of certain genes may alter, this does not necessarily correlate with a similar alteration in the translational profile and production of protein. This has been highlighted in a study of seabream, *Sparus aurata*, anterior kidney and peripheral blood leucocytes during bacterial infection with *Vibrio anguillarum* [19]. During this study *V. anguillarum* up-regulated interleukin-1β (IL-1β) mRNA expression and subsequent IL-1β pro-peptide (proIL-1β) within the cell but did not increase the release of the mature form of the IL-1β polypeptide [19]. For this reason, molecular studies of mRNA expression should preferably be supported by similar proteomic studies to generate a more complete understanding of the immune-response. Paradoxically, molecular studies may also

help to achieve this aim, as DNA sequences can be used to produce recombinant proteins to which antibodies are raised and used in proteomic studies.

Immune responses to both *Ichthyophthirius multifiliis* and *Gyrodactylus derjavini* have been extensively studied at a protein level, and more recently at a molecular level, thus allowing the development of disease models for both these parasitic diseases [20, 21]. Similarly, research is elucidating immune processes involved in carp, *Cyprinus carpio*, infected with the parasitic blood flagellate, *Trypanoplasma borreli*, [22-25]. One of the objectives of this thesis was to characterise the immune response of Atlantic salmon to AGD at a molecular level and therefore initiate the development of an AGD infection model (Fig. 1). Findings from research reported in this thesis imply that as in the well-studied parasitic infections caused by *I. multifiliis*, and *G. derjavini*, IL-1β is also important during AGD. The increased gill IL-1β mRNA expression of AGD-affected fish described in chapters two and three suggests the importance of this cytokine at the site of infection.

6.3.1. Involvement of interleukin-1β (IL-1β)

Epithelial cell hyperplasia and proliferation of mucous cells has been documented in the epidermis of tilapia, *Oreochromis mossambicus*, that were administered murine IL-1β [26]. In addition, cross-species reactivity of anti-human IL-1α antiserum showed the presence of IL-1 in the epidermis of rainbow trout infected with the monogenean ectoparasite *G. derjavini* [27]. Likewise, IL-1β mRNA expression was induced in the skin of rainbow trout infected with the ectoparasites

I. multifiliis and *G. derjavini* [28, 29]. The IL-1β protein expression was not determined in the gills of fish during experiments two and three. However, by using ISH, AGD-affected and control Atlantic salmon were shown to express IL-1β mRNA in both filament and lamellae epithelium pavement cells. The increased gill IL-1β mRNA expression of AGD-affected fish in experiments two and three was attributed to increased IL-1β mRNA expression of gill epithelial cells. Thus it was hypothesised that infection with *Neoparamoeba* sp. induces the increased expression of IL-1β that initiates epithelial cell and mucous cell hyperplasia, resulting in the characteristic gill lesions associated with AGD histopathology. Furthermore, IL-1β may have several other biological consequences that contribute to AGD pathology. In mammals, IL-1β not only increases mucus production but can also alter the composition of mucus secreted by epithelial cells [30, 31]. Thus IL-1β might alter the composition of the mucus on the gills of AGD-affected fish. The pleiotropic nature of IL-1β [32] means that the synthesis or release of a variety of other cytokines, growth factors and immune-regulatory substances is possibly initiated by IL-1β during AGD. For example, *in vitro* administration of recombinant rainbow trout IL-1β (rIL1-β) has been demonstrated to increase mRNA expression of IL-1β, COX-2, MHC II β-chain, and proliferation of a murine cell line [33]. When administered *in vivo*, rainbow trout rIL-1β was shown to increase systemic expression of IL-1β, COX-2, lysozyme, enhance effector cell responses and augment resistance to *Aeromonas salmonicida* [34]. Therefore, future research might investigate the effects of recombinant Atlantic salmon or rainbow trout IL-1β on similar effector cell and cytokine responses, as well as investigating its effects on cell populations in the gill. Similar experiments might

also be performed using *Neoparamoeba* sp. lysates, sonicates and conditioned culture media added *in vitro* to a gill epithelium cell line, gill explants, or administered *in vivo*.

Several aspects of the research within this thesis, especially the role of acute phase proteins (APPs), highlight the hypothetical nature of the proposed AGD infection model (Fig. 1). Although the liver is considered to be the primary source of APPs in salmonids, a recent study by Tsoi *et al.* [4] showed for the first time that differential expression of mRNA transcripts of several APPs were also present in the spleen and anterior kidney of bacterially-infected Atlantic salmon. Similarly, constitutive expression of serum amyloid A (SAA) and serum amyloid P (SAP)-like pentraxin mRNA was found in the gills and anterior kidney of Atlantic salmon during experiments detailed in chapter three. However, as no differential mRNA expression of these genes was observed in the gills, anterior kidney or liver of AGD-affected Atlantic salmon it is unclear whether the gills participate in an acute phase reaction (APR) to other infections or stimuli (chapter three).

The production of APPs in a tissue that is not the site of infection during an APR is evidence of a systemic response to the infection. It is well known that in mammals APPs are produced by hepatocytes of the liver in response to extremely low molar concentrations of cytokines such as IL-1β, interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) [35]. These cytokines are released into the circulatory system at the site of infection, where they travel to the liver and induce an APR. Likewise, the transcription of SAA in Atlantic salmon hepatocytes was reportedly enhanced

by recombinant human IL-1β, TNF-α and IL-6 [36]. Therefore, the lack of differential expression of SAA and SAP-like pentraxin mRNA in AGD-affected Atlantic salmon suggests the lack of a systemic response. Furthermore, it implies that the increased mRNA expression of IL-1β in the gills of AGD-affected fish is not translated into proIL-1β, or that the proIL-1β is not processed into the mature peptide and released into the circulatory system. Alternatively, the concentration of IL-1β released into the circulatory system may not be sufficient to stimulate cells in an endocrine manner or that it is bound by IL-1β receptors *en route* to the liver, thus reducing its circulatory concentration. However, the latter does not explain the lack of differential expression of SAA and SAP-like pentraxin mRNA in the gills of AGD-affected Atlantic salmon. If the gill tissue is able to produce these two APPs, a lack of autocrine/paracrine stimulation of the gill by IL-1β released from gill epithelial cells supports the previous suggestion that the increased gill IL-1β mRNA in AGD-affected fish is not released as the mature peptide from the epithelial cells expressing the mRNA transcript. If this is true, then the proposed hypothesis that the epithelial cell hyperplasia is a result of *Neoparamoeba* sp.-induced IL-1β expression is doubtful. However, it is also possible that due to the constant stimulation of the gills by waterborne pathogens, the concentration of stimulants such as IL-1β needed to initiate certain responses of the gills such as the production of APPs is much greater, and possibly receptor-mediated.

Although studies of ligands of the immune system are important to the characterisation of the immune response to infection, so is an understanding of the expression of their corresponding receptors. Receptor expression studies not only

identify the cells/tissues capable of responding to the ligands but also provide information on the potential regulation of the biological response to the functional ligands. This is especially important in relation to IL-1β, as the IL-1 type II receptor binds IL-1β but has no signal transducing function, thus effectively down-regulating the biological functions of IL-1β by sequestering secreted IL-1β [37]. In fish infected with *I. multifiliis*, the IL-1 type II receptor was up-regulated and was suggested to regulate the expression of IL-1β at the site of infection [29]. Therefore, future research to elucidate the specific role of IL-1β and other identified genes of importance would benefit from the inclusion and study of their corresponding receptors.

6.3.2. Involvement of iNOS and IL-8

The mRNA expression of iNOS and IL-8 was also shown to be induced in AGD-affected fish. Whether the increased mRNA expression of iNOS is due to a direct pathogen interaction with the gill tissue or due to IL-1β is unknown. However, the induction of iNOS mRNA expression in the gills and the production of nitric oxide (NO) and reactive nitrogen intermediates (RNIs) is a plausible defence mechanism against the parasite, as occurs in other fish [38, 39]. However, these defence mechanisms may be detrimental to both the parasite and the host under certain conditions [40], as shown during the immune response to parasitic infection in carp [23]. Thus NO or RNIs produced during the host response to *Neoparamoeba* sp.-infection might inadvertently increase the histopathology associated with AGD.

The exact nature of the role of increased IL-8 mRNA expression in the liver of AGD-affected rainbow trout is unclear. Although IL-8 is a well-known chemokine, no evidence of altered cell populations in the liver of AGD-affected fish has previously been shown [41, 42]. Nor was there an increase in MHC II β-chain and TCR β-chain mRNA expression in any of the tissues studied in this thesis. Furthermore, there was no increase in IL-8 mRNA expression at the site of infection, the gill. Thus it is unlikely that the increased liver IL-8 mRNA expression was a systemic response to cytokines such as IL-1β released from the gills, as mRNA expression of the APPs, SAA and SAP-like pentraxin in AGD-affected Atlantic salmon remained unaltered.

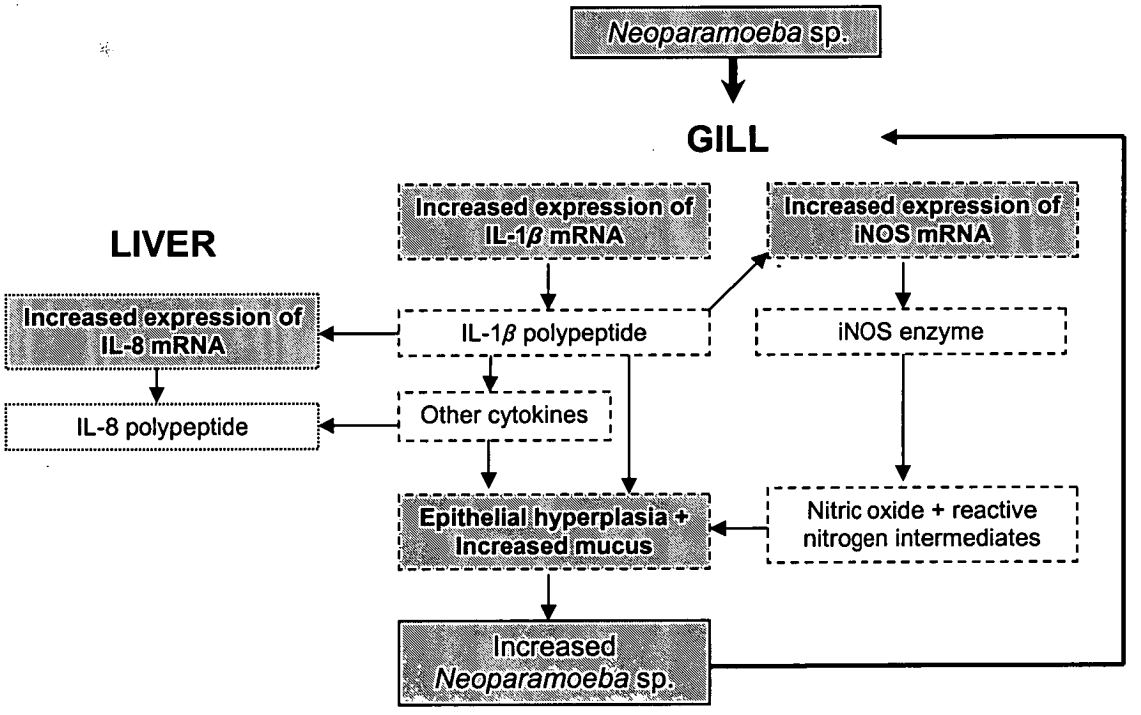

Fig. 1. Hypothetical AGD infection model incorporating immune-regulatory gene mRNA transcripts found to be differentially expressed in AGD-affected salmonids and their proposed effects on the immune response and pathophysiology of AGD-affected fish. Abbreviations: IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; IL-8, interleukin-8.

Therefore, it is proposed that *Neoparamoeba* sp.-induced mRNA expression of IL-1β and iNOS in the gills of AGD-affected fish evokes an inappropriate local response, resulting in extensive gill lesions, increased mucus secretion and eventual mortality. However, although an attempt to present the findings of this thesis in terms of a hypothetical AGD infection model has been made (Fig. 1), it is clear from the many unknowns of the response to AGD that further research is required to establish this AGD infection model. Although the differential mRNA expression of several immune-regulatory genes during AGD has been demonstrated in this thesis, it is important that this research be continued. Future research would benefit from the use of recombinant immune-regulatory proteins and antibodies raised against these recombinant proteins to further elucidate the AGD-induced immune response.

6.4. Immunostimulants vs Immunosuppressants

Whether the hyperplastic lesions formed in response to *Neoparamoeba* sp. are a primary immune response to the parasite e.g. 'walling-off the parasite', or a secondary physiological response to a pathological condition induced by the parasite, is unknown. Both can be considered defence responses, and it has been assumed throughout this thesis that the hyperplastic epithelial lesions were a primary immune response either to the parasite itself or to a possible secreted irritant. Alternatively, the hyperplastic epithelial lesions may form to prevent osmoregulatory dysfunction, perhaps as a result of parasite-induced leaky epithelial cell gap junctions. The implications of both hypotheses are fundamentally

important to the approaches taken to develop AGD management strategies. In both cases, the resulting epithelial cell hyperplasia appears to be excessive and eventually deleterious to the host. Administration of the immunosuppressant cortisol is known to suppress epithelial cell hyperplasia in coho salmon, *Oncorhynchus kisutch*, [43]. This raises the question of whether administration of an immunostimulant or immunosuppressant would be a more appropriate strategy. If as hypothesised, IL-1β is responsible for eliciting the hyperplastic response, would administering an immunostimulant known to enhance the release of IL-1β be beneficial or harmful to the host? The enhanced resistance to AGD conferred to Atlantic salmon after i.p.-injection of a synthetic CpG oligodeoxynucleotide appears to confirm that at least one immunostimulant is beneficial to the host (chapter four).

6.5. CpG oligodeoxynucleotides

DNA containing unmethylated cytidine-phosphodiester-guanosine (CpG) dinucleotides is abundant in bacterial genomes but is far less common in vertebrate genomes. Recognition by the immune system and subsequent immunostimulatory properties of synthetic CpG oligodeoxynucleotides (CpG-ODNs) depends not only on the CpG dinucleotides but also on the sequence of nucleotides that flank them (CpG motif) and the chemical nature of the phosphodiester linkages joining these nucleotides (backbone). In mammals, CpG-ODNs have been reported to stimulate lymphocytes, monocytes and dendritic cells to proliferate, mature and secrete a range of cytokines and immune-regulatory substances [44-46]. Similarly, CpG-

ODNs containing CpG motifs with known stimulatory abilities in humans and mice have also enhanced a variety of immune responses in fish [47]. Recently, the stimulatory abilities of a panel of different CpG-ODNs were tested in Atlantic salmon [48]. Those CpG-ODNs with the greatest ability to stimulate Atlantic salmon leucocytes had not been identified at the time the decision was made to trial a CpG-ODN *in vivo* as an immunostimulant in AGD-affected Atlantic salmon (chapter four). Therefore, a CpG-ODN was chosen that had been shown to enhance resistance to polymicrobial sepsis in mice [49] and that was able to stimulate Atlantic salmon leucocytes *in vitro* [50]. CpG-ODNs have since been shown to enhance resistance of olive flounder, *Paralichthys olivaceous*, and Atlantic salmon to bacterial [51] and viral diseases [48], respectively.

Intraperitoneal injection (i.p.) of Atlantic salmon with a CpG-ODN was able to enhance resistance to AGD. The enhanced resistance to AGD by CpG administration implied that Atlantic salmon were able to mount an immune response to AGD. Evidence of the involvement of the immune response suggests that an immunological management strategy to AGD is possible. Thus further studies are needed to elucidate the involvement of the immune response, and to ascertain whether CpGs enhance the resistance of fish to AGD by stimulating the innate immune system as suggested in chapter four or whether the acquired immune system is also involved. Due to the short-term protection conferred by CpG-ODNs when administered as an immunostimulant [52], and the cost of synthesising CpG-ODNs, it is unlikely that CpG-ODNs would be administered at the same concentration (50 μg $fish^{-1}$) or via i.p.-injection as described in chapter

four. However, an adjuvant effect of CpG-ODNs has been shown in fish [53], suggesting that CpG motifs might prove beneficial as an adjuvant if incorporated into potential DNA vaccines against AGD. Therefore, although CPG-ODNs are useful when characterising the immune response to AGD, a cheaper and more cost-effective substance is needed for use as a potential commercial immunostimulant.

6.6. β-glucans

Glucans with β-1,3 and β-1,6 glycosidic linkages (β-glucans) are fundamental structural components of bacteria, fungi and plants and have been shown to stimulate the immune system and enhance resistance to disease. Most β-glucans are commonly extracted from yeast cell walls, are known to bind with receptors expressed on monocytes/macrophages [54, 55] and are well-known stimulators of the innate immune system of both mammals [56] and fish [57-59]. Both i.p.-injection and oral administration of β-glucans is able to stimulate immune responses and increase resistance to disease in fish [60-62].

Administration of β-glucans was considered more appropriate for commercial immunostimulation than CpG-ODNs, due to the reduced cost of purchase and ease of incorporating β-glucans into fish feed. In a previous study by Zilberg *et al.* [63] the oral administration of β-glucans against AGD was trialled without success. However, the decision to re-assess the use of β-glucans was made after encouraging findings from the use of CpG-ODNs (chapter four), and after trialling the ability of four commercial β-glucan preparations to stimulate the respiratory

burst activity of Atlantic salmon anterior kidney macrophages *in vitro* (chapter five). Although β-glucans were shown to increase the respiratory burst activity of Atlantic salmon anterior kidney macrophages *in vitro*, their incorporation into the diet of Atlantic salmon did not increase the respiratory burst of anterior kidney macrophages *ex vivo* or enhance resistance to AGD. A sub-optimal dosage of the β-glucans and the feeding regime of the β-glucan diets used during this study were suggested as the most probable reason for the lack of *in vivo* immunostimulation and resistance to AGD. However, alternative explanations including the effect of β-glucan particle size, the formation of β-glucan aggregates in water and β-glucan uptake and presentation to the immune system are discussed in chapter five. Therefore, further research incorporating different doses and feeding regimes is needed to assess the usefulness of β-glucans as immunostimulants during AGD.

Although none of the β-glucan diets were shown to increase survival to AGD, the challenge did identify a population of Atlantic salmon that showed resistance to AGD independently of the β-glucan diets. Furthermore, the resistant fish had relatively minor gill pathology and possible signs of lesion repair. Fish immune to the ectoparasite *I. multifiliis* have anti- *I. multifiliis* antibodies in their serum and mucus, and these antibodies have been shown to immobilise theronts and trophozoites *in vitro* [20]. However, the presence of gill lesions and amoebae on the AGD-resistant fish meant that they were unable to prevent the colonisation of their gills by *Neoparamoeba* sp. and subsequent gill histopathology. Therefore, the relatively minor gill pathology and possible signs of lesion repair suggest that the gill pathology and colonisation by *Neoparamoeba* sp. was actively minimised or in

a state of constant flux. Thus, the hyperplastic response to *Neoparamoeba* sp. might be elicited by IL-1β and be initially beneficial, whilst the continued response is detrimental to the host. Therefore, the administration of immunostimulants such as CpG-ODNs, capable of eliciting the production of IL-1β, might also be initially beneficial to the host. The deleterious effect of the continued expression of IL-1β might then be secondary to an enhanced resistance conferred by the immunostimulant, which possibly acts as an adjuvant, and induces an effective antibody-mediated response. Future studies that utilise the administration of an immunostimulant to fish challenged with AGD should also assess the anti-*Neoparamoeba* sp. antibody response in these fish. Furthermore, the administration of an immunosuppressant such as cortisol might help elucidate the mechanisms of the host response to AGD.

The presence of AGD lesions on the gills of AGD-resistant fish not only has implications as to the mechanisms of the immune response to AGD, but is also important to the way AGD-resistance is assessed in fish. This is especially significant during the selection of quantitative trait loci (QTL) used as markers to genetically identify AGD-resistant fish for selective breeding. Thus QTL should select for resistance based on survival rather than the degree of gill histopathology associated with AGD. Furthermore, these findings support research into the development of a vaccine that is not necessarily based on preventing attachment of the *Neoparamoeba* sp. to the gill. Evidence of inherent resistance of fish to AGD highlights the importance of further research to characterise the protective immune mechanisms responsible for the resistance to AGD.

6.7. Conclusions

It has been demonstrated in this thesis that the transcription of several immune-regulatory genes was increased in the gill and liver tissue of AGD-affected fish. The transcription of the pro-inflammatory cytokine gene IL-1β was shown in the filament and lamellae pavement cells of gills and appeared to be an important mediator of AGD histopathology. Furthermore, evidence of inherent resistance to AGD was found in a relatively small population of fish, and administration of an immunostimulant was shown to enhance resistance to AGD. These findings increase our understanding of the immune response to AGD and further our knowledge of fish immuno-parasitology. It is envisaged that these findings will contribute to future research aimed at developing immunological-based alternative AGD management strategies such as a potential vaccine, use of immunostimulants or a selective breeding program.

6.8. References

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