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Effects of single and repeated infections with *Neoparamoeba perurans* on antibody levels and immune gene expression in Atlantic salmon (*Salmo salar*)

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ABSTRACT

Amoebic gill disease (AGD) is the main health problem for the salmon industry in Tasmania, Australia and is now reported in most salmon producing countries. Antibody and gene expression responses to the pathogen, *Neoparamoeba perurans*, have been studied independently following primary exposure; however, the effects of sequential reinfection, which can often occur during net-pen culture of salmon, remain unclear. The association between the transcription of immunoglobulin (Ig) and their systemic and mucosal antibody levels in regards to AGD is unknown. Herein, we assessed the antibody responses as well as Ig transcription in the gills of Atlantic salmon infected only once and also sequentially with *N. perurans*. After four successive AGD challenges, no significant differences in plasma or skin mucus levels of IgM were observed between AGD-naïve and challenged fish. However, IgM gene expression in gill lesions of AGD-affected fish increased up to 31 d after infection, while no changes in IgT, TCR and CD8 transcription were observed. Changes at IgM transcription level did not match the lack of antibody response in mucus, which is possibly explained by weak correlations existing between protein and mRNA abundances in cells and tissues. In the second experiment, which investigated Ig responses to AGD at the transcriptional as well as antibody production level in salmon after a single infection, the levels of serum or skin mucus IgM antibody were not affected and no changes in the IgM or IgT transcription were induced.

1. Introduction

Amoebic gill disease is an important disease affecting salmonids worldwide [1–4], and is the main condition threatening the marine grow-out phase of Atlantic salmon culture in Australia. The causative agent is *Neoparamoeba perurans* [5], a ubiquitous amphizoic marine amoeba. The main signs of the disease are lethargy and respiratory distress and outbreaks can lead to high mortalities [6]. The characteristic disease presentation is restricted to the gills, where excessive mucus production and white raised lesions can be observed. Histologically, these lesions are characterised by hyperplasia of epithelial cells, with extensive lamellar fusion, formation of large vesicles, and loss of normal gill structure with limited response of immune related cells [6–8]. The only commercial treatment is freshwater bathing of the affected fish [6], which requires significant infrastructure and is labour intensive. Due to the high costs associated with this disease and subsequent treatment strategies, significant efforts have been directed at the prevention of AGD through the use of immunostimulatory diets [9–11], selective breeding programs [12,13] and vaccine development [14–17]. Furthermore, numerous studies have focused on attempting to understand the innate and specific immune processes to the disease (for a review see Ref. [18]).

Studies focussing on antibody responses against *N. perurans* in Atlantic salmon have measured the presence of immunoglobulin M (IgM) [19–22] which is known to occur in systemic and mucosal responses [23]. There is no clear evidence that these antibody responses are protective and they have typically been measured only after a single AGD infection. However, during commercial culture of Atlantic salmon and following three rounds of natural AGD infection and freshwater bath treatments, the percentage of seropositive

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fish was shown to increase in AGD-affected fish, but the magnitude of the antibody response was not documented [20]. A new class of Ig, known as IgT, was described in rainbow trout (*Oncorhynchus mykiss*) in 2005 [24]. IgT has been shown to play an important role in mucosal responses in the gut [25], the skin [26] and the gill [27,28] of this species. Unfortunately, reagents required to measure the IgT levels in Atlantic salmon are not readily available and, in our experience, the antibodies raised against rainbow trout IgT [25,28] do not react against this molecule in Atlantic salmon.

Since measuring direct antibody responses in mucosal surfaces of Atlantic salmon have proven difficult, some characterization of the host immune response against the parasite has been achieved using gene expression [29–32]. Most gene expression studies have focused on a single challenge with AGD, and therefore there is no information available on the immune response at transcriptome level in fish after multiple rounds of infection. Until recently, the majority of the transcriptional responses to AGD was measured in lesions and characterised by a general down-regulation of immune-related genes, including various genes associated with MHC-I and MHC-II antigen presentation as well as IgM and IgT heavy chain transcripts [31], and with the exception of an up-regulation of IL-1β [29,32]. Recently, researchers in our group were able to demonstrate an increase in gill gene expression of different immune cell markers, namely MHC-I, IgM, IgT, MHC-II, TCR, CD4 and CD8, which varied according to lesion severity in AGD-affected fish up to 10 d post infection [33]. This has shown that *N. perurans* does indeed induce a classical inflammatory response in AGD-affected Atlantic salmon providing evidence of the infiltration and involvement of immune cells in and surrounding AGD lesions.

The aim of this study was to assess the systemic and mucosal antibody responses and both IgM and IgT gene expression in the gills of fish that had been sequentially exposed to AGD (i.e. four consecutive challenges), as this experimental model resembles more closely the progression of the infection under commercial conditions. Since previous evidence has shown that the levels of seroprevalence in Atlantic salmon increase with successive infections and freshwater bathing cycles in a commercial setting [20], we aimed to determine if this increase in seroprevalence correlates with an actual increase in the levels of antibodies in the serum and mucus of AGD-affected fish, and with their transcription levels. Additionally, in a second experiment, we investigated the effect that a single AGD infection has on the same parameters, transcription level and antibody production level, measured on fish at the same time.

2. Materials and methods

2.1. Effects of repeated AGD exposure on antibody levels and transcription of immune genes

2.1.1. Fish

Atlantic salmon ($n = 100$) with average body weight of 162.75 g (SD 35.86 g) were obtained from a commercial farm and held at the Aquaculture Centre, University of Tasmania. Animals were held for 10 d prior to the experiment and for the duration of the trial in four individual but adjacent seawater recirculation systems consisting each of a 1000 L glass fronted tank and biofilter unit, and maintained at 15 ± 1 °C. Two tanks were assigned for infection and two tanks were used as negative controls. Fish were fed a commercial diet equivalent to 1.5% of their body weight as a daily ration twice a day during acclimation and experimental period. This project was approved under UTAS Animal ethics committee approval number A0009717.

Fish from the 2 infection tanks were initially exposed to 150 amoebae/L, and re-exposed to the parasite at the same density 5, 8 and 14 weeks later, to emulate a recurrent infection. Between challenges, at 21, 42 and 84 d after the primary infection, fish from both infection and control tanks were freshwater (<5 ppt salinity) bathed for approximately 3–5.5 h to limit the disease progress as per industry practice. Infections were initiated using *N. perurans* harvested from an ongoing infection tank located at University of Tasmania, following procedures described previously [34]. In brief, amoebae were left to attach to Petri dishes, washed and incubated at 18 °C overnight, covered in seawater with antibiotics (ampicillin at 9.6 μg/mL and oxolinic acid at 20 μg/mL), harvested and counted using a haemocytometer, placed into 1 L sterile seawater and added to the infection tanks.

2.1.2. Sampling procedures

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

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

Fish were bled from the caudal vein, and blood was aliquoted into heparinised tubes for collection of whole blood and plasma or into clean tubes for sera. Whole blood was centrifuged at 1000 × *g* for 10 min, plasma retrieved and frozen at −80 °C.

After mucus and blood collection, fish were killed by an overdose of anaesthetic. Perfusion of the organs was performed with heparinised 0.9% physiological saline (Baxter, Deerfield, IL, USA) via puncture of the bulbous arteriosus as previously specified [35] to remove any remaining blood, until the gills were white. The gill basket was carefully removed and four hemibranchs were placed into 20 mL mucus buffer for 3–4 h until sampling was finished. They were centrifuged on the same day at 15,000 × *g* for 1 h, supernatant collected and frozen at −80 °C until used. Another two hemibranchs were placed into 25 mL of nucleic acid preservation solution (NAPS, 4 M ammonium sulphate, 25 mM sodium citrate, 10 mM EDTA; pH 5.5) as described elsewhere [36]. All the reagents were kept on ice during collection. Gill arches in NAPS were stored for 24 h at 4 °C and then transferred to −20 °C.

2.2. Effects of a single AGD challenge on antibody responses and transcription of immune genes

2.2.1. Fish

Atlantic salmon ($n = 380$) with average body weight 150.74 g (S.D. 34.11) were obtained from a commercial hatchery and maintained at the Aquaculture Centre at the University of Tasmania. Fish were kept in a recirculating system, comprised of four 250 L fibre glass tanks ($n = 25$ fish per tank), in 20 ppt salinity water and 14 °C for a week; and then acclimated to full strength salinity and 16 °C over two weeks. Fish were maintained for 10 weeks before challenge, under a continuous 24 h light regime, and water quality was checked daily, with temperature ranging 16 ± 0.5 °C over the study. During acclimation and challenge, fish were fed three times daily to satiation with a commercial diet. The feed consumption level varied between 1.1 and 1.25% of body weight per day before and during challenge, and it dropped during the last week of challenge to levels below 1% of body weight per day [11].