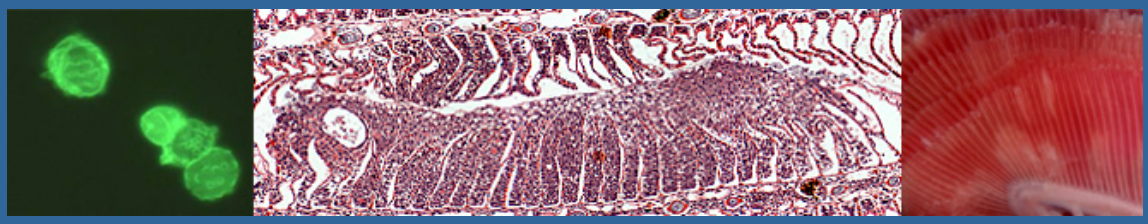

FRDC Salmon Subprogram - Extension funding application – AGD Vaccine Phase III

Philip B.B. Crosbie, Andrew R. Bridle and Barbara F. Nowak

Final report

FRDC PROJECT NUMBER:2008/218

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2008/218 FRDC Salmon Subprogram - Extension funding application – AGD Vaccine Phase III

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OBJECTIVES:

1. Develop techniques to successfully culture virulent *N. perurans*
2. Elucidate the role of saccharide-inhibitable lectins on the ability of the amoeba to attach to salmon tissue (independent of successful *N. perurans* culture)
3. Isolation and sequence identification of the lectin mediating *N. perurans* attachment (progress accelerated by successful *N. perurans* culture) or alternative approach (independent of successful *N. perurans* culture) if the lectin can not be identified
4. Investigate antigenicity and protection against AGD of identified lectin in salmon
5. Maintain and run 4 laboratory tank based trials for Project 2007/234 in 09/10 and 10/11.
6. Provide virulent amoebae for other AGD research

Non-Technical Summary

OUTCOMES ACHIEVED

This project has developed successful culture of virulent *N.perurans*. We have shown that this species of amoeba causes Amoebic Gill Disease in Atlantic salmon. We have developed a method to detect and quantify presence of *N. perurans.* We have run laboratory based challenge trials for project 2007/234 and provided amoebae and amoebae DNA to our collaborators.

We have fulfilled Koch's postulates for *Neoparamoeba perurans* and showed that this species of amoeba causes Amoebic Gill Disease in Atlantic salmon by infecting fish with *N. perurans* after 20 weeks in culture. This was only possible when we developed successful *in vitro* culture methods for this species. This now allows further research into the biology of the amoeba. We determined generation time for cultured *N. perurans* to be 26.66 h.

We have investigated the role of saccharide- inhibitable lectins. The results from the experiments looking at the ability of monosaccharides to inhibit amoebae attachment to the gills of salmon indicated that when amoebae were exposed to galactose at concentrations of 50 and 5 mM there was significantly less pathology seen after 72 h compared to the other galactose concentrations and the positive control.

KEYWORDS:
Atlantic salmon, AGD, *Neoparamoeba perurans,* aquaculture, parasitic disease

Acknowledgments

This study formed part of salmon industry research, and was supported by the Fisheries R&D Corporation. We would like to thank salmon industry for their collaboration and support. We are grateful to Dr Melanie Leef, Dr Stephen Hindrum, Ms Karine Cadoret, Ms Victoria Valdenegro, Dr Benita Vincent, Dr Mark Adams, Dr Richard Morrison, Ms Catarina Norte dos Santos and Ms Jadwiga Sokolowska for their insights and contributions to this project. We would like to thank Prof William A Petri from the University of Virginia for providing monoclonal antibody against the Gal/GalNac surface receptor lectin of *Entamoeba histolytica.*

Background

Histological evidence of AGD pathology clearly shows the association of at least one *Neoparamoeba* sp. with lesions on gill tissue of Atlantic salmon. Until 2007 it was thought that the Neoparamoebae responsible were *N. pemaquidensis* and *N. branchiphila* as these two had previously been cultured from AGD-affected gill material. However the development of species specific oligonucleotide primers and probes by Young et al (2007) resulted in the identification and description of *Neoparamoeba perurans* as the aetiological agent of AGD in Tasmania since the disease was first reported. This species has also been shown to be the aetiological agent in AGD worldwide (Young et al 2008). This new species is morphologically identical to *N. pemaquidensis* and *N. branchiphila* and the three can only be differentiated using molecular tools. At present all efforts to culture Neoparamoebae from the gills of AGD-affected salmon tissue have yielded either *N. pemaquidensis* or *N. branchiphila*, in addition all attempts to induce AGD with these cultured strains have been unsuccessful (Kent et al, 1988, Howard et al, 1993; Findlay, 2001; Morrison et al, 2005, Vincent et al, 2007). All challenge trials are currently initiated using amoebae freshly isolated from AGD-affected salmon gills. Consequently the only supply of infective amoebae is from fish with AGD which necessitates continual maintenance of an infection tank (where salmon are replaced as they succumb to AGD) and a feeder tank (to supply replacement fish). This precludes any research or control approaches requiring large numbers of the amoebae.

Previous studies suggest that the attachment mechanism of *N. perurans* to salmon gill tissue may be lectin mediated and that lectins on the surface of the amoeba bind to carbohydrate residues in the gill tissue of salmon (Vincent 2008 PhD Thesis). Lectins are proteins that bind to carbohydrates, sometimes very specifically, and it is this binding that can facilitate the attachment of parasites to host cells. Vincent (2008) showed that pre-incubation of *N. perurans* with 6 monosaccharides (at relatively high concentrations) or salmon cutaneous mucus significantly inhibited the ability of the amoebae to initiate AGD, but it is not known whether this effect was due to the monosaccharides specifically blocking the carbohydrate recognition domain of the lectin or a concentration effect. This requires further investigation to see if the inhibition is specific to any monosaccharides. Inhibition of attachment by parasitic amoebae to host tissues has been demonstrated in other amoebic attachment models, involving the human pathogen *Entamoeba histolytica*, by blocking the binding sites on the parasite lectin with either saccharides (Boettner et al, 2005), mucins (Chadee et al, 1987) or monoclonal antibodies (Ravdin et al, 1986). This pathogen processes a lectin which binds to galactose (Gal) and N-acetyl-D-galactosamine (GalNac) and is recognised as a major virulence factor as it mediates the amoebae adherence to gut mucins (which contain Gal/GalNAc residues) and targets cells in the human intestine (see review by Petri et al, 2002). Attachment has been inhibited by exposure of *E. histolytica* to Gal or GalNAc or to monoclonal antibodies directed against the lectinand this lectin has now become a target for a vaccine against E. hisolytica (Petri et al, 2006). Vincent (2008) hypothesised that *N. perurans* may express an orthologue of this lectin which may also be inhibited by Gal, GalNAc or other

monosaccharides. Additional studies by Vincent (2008) provide further evidence for this hypothesis by showing that monoclonal and polyclonal antibodies directed against the Gal/GalNAc inhibitable lectin of *E. histolytica* bind to the surface molecules of live *N. perurans*. If specific monosaccharides do inhibit the ability of *N. perurans* to cause AGD then they could provide a tool to identify and sequence the parasite lectin (protein) involved in attachment. The specific monosaccharide could be immobilised on an affinity chromatography column and used as a capture
ligand for the *N. perurans* lectin. Another method to isolate the lectin is byimmunoprecipitation using the monoclonal and /or polyclonal anti-*E. histolytica* lectin antibodies known to have reactivity with surface molecules of *N. perurans*. Once the lectin has been isolated and sequenced it then becomes a vaccine target. This additional work complements the existing AGD Vaccine Phase III (FRDC 2007/234) project and will facilitate use of the challenge facility. Data generated from the first sea trial correlates with that from the challenge system and demonstrates that the system is a valuable tool for screening and testing prospective DNA vaccines.

Need

Amoebic Gill Disease (AGD) significantly contributes to Atlantic salmon production costs in Tasmania. There is an urgent need to successfully culture and preserve *N. perurans* to guarantee future supply of amoebae for research. This will reduce or eliminate the need for an infection tank and reduce the cost of AGD research. The culture of virulent *N.perurans* will facilitate research into its biology by allowing the development of *in vitro* models and improved challenge protocols. We expect that this will provide important insights into the pathogen that will lead to better disease control and/or treatment. The urgent need to successfully culture and preserve *N. perurans* was recognised by the AGD vaccine MAC meeting held on 23 February 2011.

Objectives

1. Develop techniques to successfully culture virulent *N. perurans*
2. Elucidate the role of saccharide-inhibitable lectins on the ability of the amoeba to attach to salmon tissue (independent of successful *N. perurans* culture)
3. Isolation and sequence identification of the lectin mediating *N. perurans* attachment (progress accelerated by successful *N. perurans* culture) or alternative approach (independent of successful *N. perurans* culture) if the lectin can not be identified
4. Investigate antigenicity and protection against AGD of identified lectin in salmon
5. Maintain and run 4 laboratory tank based trials for Project 2007/234 in09/10 and 10/11.
6. Provide virulent amoebae for other AGD research

Methods

Objective 1 Develop techniques to successfully culture virulent *N. perurans*

Several experiments were carried out to achieve this objective. Initially experiments tested culture techniques for *N. perurans* using both solid (agar) and liquid phase culture media. It is generally thought that *N. perurans* on the gills of salmon feed on the gill mucus and when the cells are adhered to a substrate they appear very vacuolated and large in comparison to other species of *Neoparamoeba* which when grown on nutrient poor malt yeast agar (MYA) feed on bacteria and are much smaller and less vacuolated. As previous attempts to culture *N. perurans* isolated from salmon gills directly to agar have failed, the purpose of the first experiment was to attempt to culture the amoeba in liquid phase with a mucus supernatant, various concentrations of ammonia and some antibiotics to control bacterial overgrowth in an effort to encourage the amoeba to feed on bacteria before transfer to MYA plates and subsequent experiments, and to test the impact ammonia may have on growth *in vitro*.

Experiment 1: *N. perurans* culture

N. perurans was isolated from the infection tank and initially inoculated at a concentration of 1000 cells/mL into 14 mL of liquid medium made up of filtered sea water, 1 mL heat sterilised salmon cutaneous mucus supernatant and antibiotics (ampicillin and oxolinic acid at 9.6 µg/mL and 20 µg/mL) with ammonium chloride added at 0, 0.25, 0.5 or 1.0 mg/L. The antibiotics were chosen as they had previously been shown to be non- toxic to *N. perurans* (Crosbie et al, 2007). Media with each ammonium chloride concentration were tested in duplicate polystyrene cell culture flasks and growth was monitored by counting attached cells from 5 fields of view from each flask after observation on an inverted microscope at x100 magnification. In each flask the medium was replaced every 2 days.

Experiment 2: *N. perurans* culture

The culture flasks from Experiment 1 were used in a second experiment where cells were washed and harvested from the flasks and then inoculated dropwise to MYA plates made from 75% or 100% sea water. This method is commonly used for other marine amoebae, including *N. pemaquidensis* and *N. branchiphila*, and the media are often supplemented with heat-killed bacteria. In this experiment heat-killed *S. maltophilia* and *E. coli* (bacteria which have been used in the culture of other *Neoparamoeba* species) were added to plates and amoebae growth evaluated.

Experiment 3: *N. perurans* culture

Amoebae harvested from Experiment 1 were also used in flask cultures to test the impact of the mucus supernatant on growth. Cells (approximately 600 cells/mL) were inoculated to 3 separate culture flasks containing either:

- 14 mL of sea water, antibiotics as described in Experiment 1 and 1 mL of mucus supernatant
- 15 mL of sea water and antibiotics as described in Experiment 1
- 15 mL of sea water only

Media replenishment and cell counts were performed as for Experiment 1.

Experiment 4: *N. perurans* culture

A combination of liquid and solid phase media were used in this experiment namely MYA with sea water overlay. Approximately 3000 *N. perurans* cells in 10 mL of sea water were seeded onto a MYA plate. After 5 days amoebae growth was evident and a small plug of agar was removed and subcultured to a fresh MYA plate. Growth of amoebae was again seen after 5-6 days. A small plug of agar containing a single amoeba was removed and inoculated to a fresh plate and cells were seen on this plate 10 days after the inoculation. From this plate a small sample of cells was taken, DNA extracted using DNAzol direct and a PCR performed using *N. perurans* specific primers to confirm the amoebae identity (Young et al 2007, Crosbie et al 2010).

Experiment 5: *N. perurans* culture and virulence testing

This experiment followed a pilot study that incorporated the use of defined media to increase growth rates. A culture was initiated when approximately 16 *N. perurans* cells were inoculated to a single MYA plate overlaid with sea water. After 5 days when significant growth was visible some of the sea water overlay containing cells was used to inoculate several 25 mL cell culture flasks containing variations of routine cell culture media such as L-15 with sea water and antibiotics, Minimal Essential Medium (MEM) with antibiotics and filtered sea water with no additives. Growth rates were variable and contamination eventually over ran the cultures possibly due to the higher nutrient levels provided by the L-15 and MEM.

The experiment was initiated when amoebae were isolated from AGD-affected Atlantic salmon according to Morrison et al (2004) and then approximately 40 cells were inoculated onto MYA plates made with sea water at 3.5% salinity overlaid with 15 mL of 0.2 µM filtered sea water and incubated at 18°C. Amoebae were subcultured weekly when free-floating cells were removed, inoculated on fresh MYA plates and overlaid with filtered sea water. This procedure resulted in the exclusion of contaminating protozoans and the establishment of non-clonal cultures.

To test the virulence of these amoebae a pilot infection experiment was carried out when amoebae had been in culture for 8 weeks. In this experiment 12 salmon were exposed to cultured cells (approx. 10,000 cells per L) in 3 x 40L tanks for a period of 17 hours after which all fish were transferred to a single 500L system. Individual fish were then sampled at 15, 26 and 38 days post exposure and the trial was terminated after 48 days. Gill samples were taken from all fish and some filaments were used for amoebae retrieval using the amoebae isolation technique of Morrison et al (2004) and others fixed in sea water Davidson's fixative for histology. A control tank housed fish that were treated in the same way but were not exposed to any amoebae.

Experiment 6: Virulence testing of *N. perurans* clonal culture

The failure to introduce the causative agent of AGD into culture in the past has lead to the inability to fulfil Koch's postulates for the disease in salmon and to demonstrate beyond doubt that *N. perurans* is the causative agent and

not other species of *Neoparamoeba*. These postulates are four criteria designed to establish a causal relationship between an organism and a disease. These criteria are:

- The organism must be found in all hosts with the disease
- The organism must be isolated from the host and grown in pure culture
- The disease should be reproduced when the pure cultured organism is introduced into a healthy susceptible host
- The same organism must be re-isolated from the experimentally infected host

As the pilot study (experiment 5) described above was carried out using an amoebae population that was not clonal and therefore not technically 'pure' micromanipulation of single cells was used to establish a clone of *N. perurans*. Individual cells were selected from overlying sea water and placed into individual wells of a 12 well cell plate containing MYA then overlaid with sea water. One clone designated clone 4 was used in the second infection experiment in August 2011. Clone 4 was maintained on several MYA plates as described above and was used to infect a group of 10 salmon in a single 500 L tank at a dose of approximately 5000 cells per L. Two control groups of salmon housed in separate 500 L tanks included those exposed to *N. perurans* freshly isolated from the infection tank and therefore non-cultured (also at 5000 cells per L) and a negative control group not exposed to any amoebae. The positive control group of fish infected with the non-cultured *N. perurans* was included to show that AGD can be established in the experimental population of salmon and the systems used. Prior to infections both the non-cultured, freshly isolated amoebae and clone 4 were identified as *N. perurans* by PCR and viability of both confirmed by a neutral red inclusion dye assay (see Figure 6). Individual fish were sampled 20 and 28 days post exposure for AGD assessment and the experiment terminated 29 days post exposure. Gill samples were taken from all fish for histology and amoebae retrieval as described for Experiment 5.

Experiment 7: Determination of generation times for *N. perurans in vitro*

To determine the generation times of *N. perurans in vitro* three growth trials were carried out on cultured *N. perurans* (clone 4) on MYA plates at 18°C. For each trial 4 replicate MYA plates were seeded with a known number of amoebae which were then counted in 7 fields of view at a magnification of x200 at approximately 24 h periods. The mean count per field of view was calculated from all plates at each time point and growth curves were then constructed. Generation times were determined from the plot of the logarithm of amoebae numbers against time using :

$(\log_{10} N_t - \log_{10} N_0)\text{-}1 \times 0.301t$ where N_t is the number of amoebae after time t in exponential growth phase and N_0 is the number of amoebae at the beginning of the exponential growth phase.

Experiment 8: Effects of some environmental factors on *in vitro* cultures and cryopreservation attempts

8.1 Temperature

The effect of temperature on amoebae growth was examined when *N. perurans* were inoculated to MYA plates then incubated at 20ºC and 25ºC.

Two replicate MYA plates were seeded with a known number of amoebae and were then counted in 7 fields of view at a magnification of x200 at approximately 24 h periods.

8.2 Culture without sea water overlay
As many marine amoebae are amenable to culture on MYA plates without any sea water overlay we also investigated whether vigorously growing plates of *N. perurans* could survive without a sea water overlay with a view to prolong the time between subculture. Overlying sea water was removed from several plates and allowed to partially dry out. The plates were stored at 20ºC then plates were re-hydrated after 7d and 17d.

8.3 Cryopreservation
The cryopreservation method successfully used for cultures of *Neoparamoba pemaquidensis* and *N. branchiphila* was attempted with *N. perurans* (clone 4). This method is used by the American Type Culture Collection for their collection of *N. pemaquidensis*. Cells were harvested from numerous MYA plates enumerated and then equilibrated at room temperature with a 15% solution of dimethyl sulphoxide as a cryoprotectorate in 0.5 mL volumes. Tubes were then placed in a cooling unit allowing cooling at -1ºC per min when placed in a -80ºC freezer. When tubes were cooled to approximately -40ºC (after 1.5 h) the tubes were removed and immediately plunged into liquid nitrogen. One month later 1 tube was removed and rapidly thawed for 2-3 min at 35ºC. Cells were immediately placed onto a MYA plate and overlaid with filtered sea water. A small aliquot was placed in a clean Petri dish for observation.

Quantification of *N. perurans* using qPCR
We developed methods for quantification of *N. perurans* in water samples as described in Bridle et al (2010).

Objective 2 Elucidate the role of saccharide-inhibitable lectins on the ability of the amoeba to attach to salmon tissue.
Initially four experiments were carried out to investigate whether amoebae attachment to the gill tissue of salmon is mediated by lectins on the surface of the amoebae binding to specific saccharide residues in gill tissue. The hypothesis was that the attachment of parasite to host may be inhibited by blocking the binding sites on the lectin with specific saccharides. Therefore *in vivo* attachment experiments were carried out where amoebae were incubated in the laboratory with different saccharides and then the amoebae were used to challenge salmon over a 6 h period, the salmon were then removed to clean sea water tanks with no additional amoebae and gill pathology allowed to develop over 72 h. The rationale is that if there has been any inhibition in amoeba binding to the salmon gills during the 6 h exposure then this would be reflected in less severe gill pathology after 72 h. Four of these experiments were completed in 2008 based on the protocol of Vincent (2008) using 4 different saccharides: mannose, galactose, glucose at final concentrations of 500, 50, 5 and 0.5 mM and n-acetyl-D-galactosamine (GalNAc) at 50, 5, 0.5 and 0.05 mM and an exposure protocol based on Crosby et al (2007). Amoebae were isolated according to Morrison et al

(2004) and each of the saccharides was diluted in phosphate buffered saline (PBS). For each experiment the amoeba suspension was divided into 5 equal parts and then added to the various concentrations of saccharides or PBS only as a positive control and then incubated in 2 mL tubes at 4ºC for 25 min. After incubations amoebae viability was assessed by the ability to attach to the surface of a clean Petri dish using a small aliquot of each amoebae suspension. All the amoebae suspensions were then placed into individual 500 mL bottles with 300 mL of 0.2 µm filtered sea water and immediately used to challenge salmon according to a short-term challenge protocol developed by Crosbie et al (2007). All challenges were for 6 h and occurred in 75 L tanks filled to 30 L for each saccharide at each concentration and used amoebae densities of approximately 5000-6000 cells L^{-1}. There were 2 tanks of 5 fish for each saccharide concentration (treatment group) including control groups challenged with amoeba exposed to PBS only and no amoebae challenge and after the challenge each group of 10 fish was transferred and held in single 350 L tanks for the 72 h pathology development stage after which all fish were euthanased and gills fixed for histology and lesion quantification expressed as the percentage of gill filaments with AGD lesions (i.e. lesions with associated parasome- containing amoebae).

Due to equivocal results a fifth experiment was conducted using mannose only at the same concentrations using the same protocol with the exception that there were 3 replicate tanks used for each challenge with each concentration and 4 salmon per tank.

For all experiments the data were analysed by ANOVA using the Microsoft Excel Program.

Objective 3 Isolation and sequence identification of the lectin mediating *N. perurans* attachment

The logic behind this objective was to use antibodies directed against homologous proteins to the lectin from *N. perurans* and then use these antibodies as tools to isolate the lectin from *N. perurans* which could then be sequenced and used a vaccine target.

Initially antisera to 2 different recombinant proteins were produced in rabbits and partially characterised. A third protein used to immunise Atlantic salmon. The 3 proteins were:

- A peptide derived from cDNA library of *Neoparamoeba perurans* which has significant homology with mannose binding lectins from amoebae (designated 22CO3)
- A peptide derived from cDNA of *Entamoeba histolytica* which represents part of the Gal/GalNac surface receptor lectin (designated 895-998)
- A peptide derived from cDNA of *Entamoeba histolytica* which represents another part of the Gal/GalNac surface receptor lectin (designated 578-1154)

The recombinant proteins were all supplied by collaborators from the CSIRO. Antisera production in rabbits was outsourced to Flinders University as the production facility at the Animal House at UTAS in Hobart was not available at that time. The proteins were prepared in 3 equal doses of 30 µg/mL to enable

a primary and 2 booster immunisations per protein per rabbit and transported to Flinders University. When the antisera were received antisera were initially screened by enzyme- linked immunosorbant assay (ELISA) and Western blot to confirm an immune response from the rabbits to the peptide antigens and the antisera were then screened against a preparation of lysed *N. perurans* to test for reactivity against any proteins. The ELISA was performed by the antisera producers at Flinders University and the Western blots done at UTAS using standard blotting procedures applying A tris buffered saline washes and 1% skim milk in TBS as a blocker.

A small group of Atlantic salmon were also immunised with the 895-998 lectin and the 578-1154 lectin. Total protein yield from all the 2 proteins derived from *Entamoeba histolytica* cDNA were not sufficient to immunise both rabbits and salmon. Six salmon were immunised and boosted with 895-998 and five with 578-1154 at 4 week intervals. Initial immunisations were with the protein antigens emulsified in Freund's complete adjuvant to maximise the likelihood of a response whilst the boosters were emulsified in Freund's incomplete adjuvant. After 6 weeks test bleeds were taken for 2 fish and Western blot analyses done to confirm an immune response from the fish to the protein antigens. Once a response was confirmed the salmon were euthanased and antiserum collected. However when two of the salmon antisera were screened against lysed *N. perurans* prepared as for the screening of the rabbit antisera no reactivity was seen.

The rabbit antisera were then used in immunoprecipitation experiments to isolate the particular protein antigen from *N. perurans* lysates. Immunoprecipitation is a technique employed to purify and isolate particular proteins from cell lysates or other extracts. The technique relies on the ability of an antibody to bind to a specific protein antigen and form a complex which can then be separated from the rest of the lysate material. Antibodies in the antisera described above have been shown to bind to proteins from *N. perurans* lysates, as reported above, and are therefore potentially useful immunoprecipitation tools.

For the immunoprecipitation experiments 13.5 million *N. perurans* were collected over several occasions, washed in PBS then frozen at -80ºC, after thawing the suspension was sonicated (5 x 5 min pulses in an Unisonics bath sonicator) until the cells were completely lysed, then the protein content of the sonicate determined (4.95 mg per mL). A commercial kit (Catch and Release® v2.0 Reversible Immunoprecipitation System, Chemicon) was used for each experiment. Approximately 500 µg of protein (110 µL of the sonicate) was incubated with 10 µL of each rabbit antiserum and pre-bleeds (as negative controls) then applied to capture column with a ligand that binds to rabbit immunoglobulin, extraneous unbound proteins were then washed off and the antibody-antigen complex was eluted and collected. The eluates were then electrophoresed on a polyacrylamide gel and then some gels were silver stained to see eluted proteins and others used for electrotransfer to nitrocellulose membranes for Western blot analyses using standard techniques previously described.

Objective 4 Investigate antigenicity and protection against AGD of identified lectin in salmon

Unfortunately this objective could not be addressed by the project as the lectin isolation and identification experiments were not successful.

Objective 5 Maintain and run 4 laboratory tank based trials for Project 2007/234 in 09/10 and 10/11.

Four vaccine challenge trials were completed in conjunction with collaborators from the CSIRO. All the trials were carried out at UTAS in four replicate 4000 L tanks with recirculating sea water using challenge techniques developed by the project.

The first vaccine efficacy trial started in April 2009 and was completed in June 2009. This trial commenced when 741 Atlantic salmon were transported to the aquaculture centre at Launceston on April 16 after being vaccinated at the hatchery several weeks beforehand. The fish were transferred to 4 x 4000 L tanks (180 fish per tank) and after a 3 week of a tank, temperature and sea water acclimation period challenged with *N. perurans* on 5 occasions for a total dose of 200 cells per L. AGD progression was assessed at 16 and 21 days post challenge (dpc) and when the disease was evident in all tanks after visual examination (presence of gross pathology) the trial was terminated (at 29 dpc). All fish were euthanased and gills removed and placed in sea water Davidson's fixative. Collaborators at the CSIRO removed and photographed the second left gill arch from all salmon to quantify the degree of pathology. Vaccine efficacy was assessed by a reduction in pathology.

The second experimental vaccine trial was performed in September 2009. The trial commenced when 794 Atlantic salmon smolts were transported from the Saltas hatchery at Wyatinah and transferred to 4 x 4000 tanks (described as E1-E4) at the Launceston facility on September 8. After final sea water and temperature acclimation to 35 ppt and 15ºC respectively by September 16 all fish were challenged with *N. perurans* over 5 days with a cumulative dose of 300 amoebae cells per L. However, there were problems with this trial beginning on September 19 (3 days post complete acclimation and 11 days post transfer to tanks) when 9 dead/moribund fish were removed from tank E3 and another 8 dead/moribund fish removed from tank E2 on September 19 - 20. Tanks E1 and E4 had no mortalities at this time. Four fish were taken to DPIPWE laboratory at Mt Pleasant for post mortem examination additionally some blood samples taken for osmolality testing at the University. There were no external or obvious signs of pathology and reports from DPIPWE suggested sea water acclimatisation failure. Osmolalities of > 550 mmol/kg, which exceeded the normal range for salmon (320-350 mmol/kg) were found after analyses of 2 plasma samples from moribund fish were done at UTAS lab. We had been advised by the hatchery that the fish had smolted and that our sea water acclimation regime was appropriate. On September 21 (5 days post acclimation) fish began to present with lateral skin lesions and mortalities found in Tanks E2 and E3. Samples were again submitted for post mortem examination at Mt Pleasant and results showed that the lesions were due to opportunistic mixed bacterial infections. Fish were observed daily and any

with lesions from all tanks were removed and euthanased. By September 25 losses in E2 and E3 had exceeded 20% (the point at which tanks would be terminated as agreed between UTAS and CSIRO) and all remaining fish were removed and euthanased on September 25. By October 13 similar events had occurred in Tanks E1 and E4 and all remaining fish were removed and euthanased. These types of mixed infections can occur when salmon are stressed, however since arrival at the university fish were subjected to limited external stressors. Transfer from the transport tanks to holding tanks occurred directly through a pipe and the fish were not handled or netted, water quality was monitored daily and water exchanged every 2 days. The transfer and husbandry procedures for this trial were the same as those used for the previous 2 trials in October 2008 and April 2009 when no mortalities due to bacterial infections occurred. All fish handling and manipulations including anaesthesia, insertion of passive integrated transponder tags (PITtags) to the peritoneal cavity, primary and booster vaccinations and size grading occurred at the hatchery in a 6 week period before transport to the University.

The situation was extensively discussed at the AGD Management Advisory Committee meeting at Taroona on October 19 with participants from the University, CSIRO and the salmon industry. For future trials it was agreed to confirm smoltification at the hatchery prior to transport. It was also agreed to test the holding systems at the University for any inherent problems with bacterial contamination by acclimating some smolts in the tanks and holding them in sea water for a period. Due to a lack of available smolts only 1 tank could be used for this exercise and E3 was chosen as it housed the most severely infected fish. 120 salmon (100-200g) were transferred to E3 at a salinity of 30ppt and at 15ºC, salinity was increased to 35ppt after 2 days and fish were observed to be feeding well. Water quality was monitored daily and water exchanged every 2-3 days. After 7 days 20 salmon were anaesthetised and examined and all were healthy and free of lesions. After 14 days all fish were feeding well and free of lesions.

The third experimental vaccine trial was performed in April-May 2010 at the Aquaculture centre at Launceston. The trial commenced when 659 Atlantic salmon smolts were transported from the Saltas hatchery at Wyatinah and transferred to tanks E1-E4 at the Launceston facility on April 19. After final sea water and temperature acclimation to 35 ppt and 15ºC respectively all fish were challenged with *N. perurans* over 3 days with a cumulative dose of 300 amoebae cells per L. When it was determined that representative fish from all tanks had clinical signs of AGD the challenge was terminated (20 days after addition of amoebae) and all fish were euthanased and the gills removed and fixed for assessment of the severity of AGD. The gills were dissected and photographed and numbers of AGD lesions per gill filament determined by collaborators from the CSIRO. There was no issue with non AGD mortalities during this trial.

The fourth trial was performed in September-December 2010 and commenced when 460 Atlantic salmon smolts were transported from the hatchery and transferred to experimental tanks (E1-E4) on September 16. After final sea water and temperature acclimation to 35 ppt and 15ºC

respectively by September 29 all fish were challenged with *N. perurans* over 2 days with a cumulative dose of 120 amoebae cells per L. However, on October 3 there were some mortalities in one tank due to skin lesions caused by a bacterial infection. After discussion with collaborators at the CSIRO remedial action was taken and all fish from all tanks were given a freshwater bath. All fish were then held at reduced salinity (20ppt) to allow recovery. By October 25 all fish were again at full sea water and challenged again, however some mortalities again occurred in all tanks and the fish were freshwater bathed a second time and returned to full sea water. At this stage fish loss due to skin lesions across all 4 tanks (E1-E4) were 25%, 19%, 12% and 6% respectively and the decision was made to continue the challenge in E3 and E4 only as losses in E1 and E2 had or were about to exceed the cut off point of 20% mortality. Fish from E1 were euthanased and samples taken by CSIRO to verify the response of the fish to the DNA vaccine. There were no more losses due to skin lesions for the remainder of the trial and the challenge proceeded in E3 and E4 until clinical signs of AGD were seen then the fish in these tanks were euthanased and all gills taken for assessment and quantification of AGD by the CSIRO. There were no more mortalities due to bacterial infection in E2 and the fish were used to supply samples (mucus, serum and gills) for another project.

The fifth and final trial was carried out between March and June 2011. The salmon were transported from the hatchery and transferred to the tanks on March 21 and were sea water acclimated by April 15. The challenge dose was 300 cells per L administered over 2 weeks and after assessing the AGD status the trial was terminated on June 2 when all fish were euthanased. The gills were removed and taken to the CSIRO for AGD assessment. A subsample of fixed gills was processed for routine histology at UTAS and slides sent to CSIRO for AGD assessment. There were no bacterial infections problems in this trial.

Objective 6 Provide virulent amoebae for other AGD research

The infection tank is being maintained and continues to supply amoebae as required and the cultured strain of *N. perurans* is also available as required by collaborators and other researchers.

Amoebae were isolated from gills of moribund fish from the infection tank (Morrison et al 2004) and provided when requested.

Results/Discussion

Objective 1 Develop techniques to successfully culture virulent *N. perurans*

Experiment 1

Amoebae growth followed a similar trajectory for each medium regardless of the concentration of ammonium chloride with numbers peaking 2 days after inoculation indicating daily doubling of numbers before decreasing and stabilising to numbers similar to the inoculation level by day 9 (Figure 1). Both flasks with the medium containing 1.0 mg/mL ammonium chloride were overgrown with contaminating organisms at day 5 post inoculation and were discarded on day 7. Although media were regularly replaced many cells became unattached after 9 days and all flasks contained contaminating organisms such as ciliates and flagellates. The results suggest that ammonium chloride concentration had no impact on growth using this culture method and that the amoebae are difficult to maintain in liquid phase. In particular, it is very difficult to isolate *N. perurans* without contamination by other protozoans.

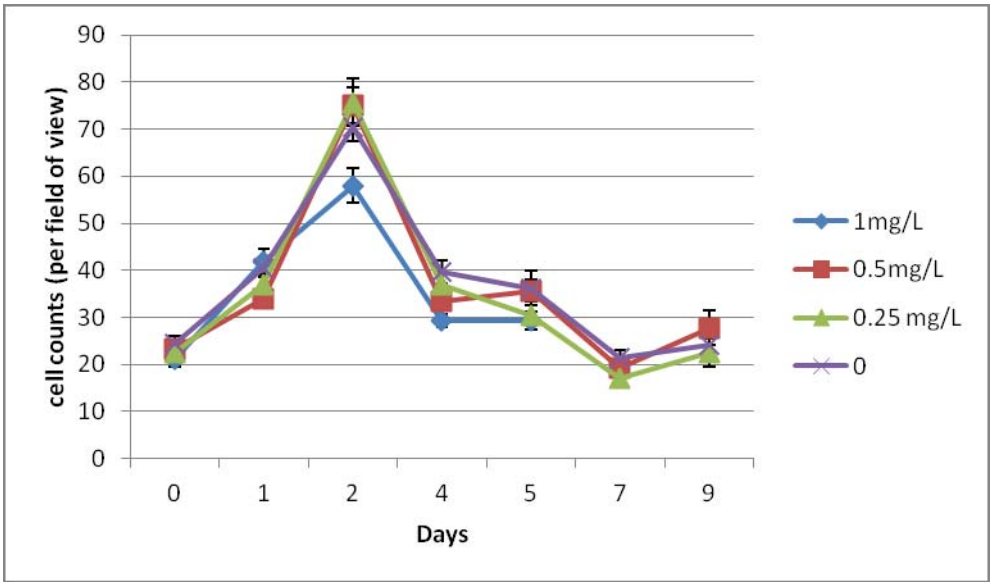

Figure 1. Growth curves for *N. perurans* over 9 days in 15 mL cell culture flasks with sea water media containing Atlantic cutaneous mucus, antibiotics and concentrations of ammonium chloride ranging from 0 to 1mg/L. Cell numbers are means (± S.E) of counts (n=10).

Experiment 2

Amoebae were evident on the 100% sea water agar after 2 days although appeared very sparse. A small number of cells were removed by swab and DNA extracted and PCR performed which confirmed the identity of the cells as *N*. *perurans*. Growth was very slow on MYA plates in comparison to other *N. pemaquidensis* or *N. branchiphila* in the same medium which can produce confluent growth over 5-6 days, especially when plates are seeded with heat-killed or salt tolerant live bacteria. Attempts to subculture *N. perurans* to MYA plates seeded with both heat-killed and live *S. maltophilia* and *E. coli* (bacteria

which have been used in the culture of other *Neoparamoeba* species) were not successful.

Experiment 3

After 5 days there were too few cells to count in media 2 (sea water and antibiotics as in experiment 1) and 3 (sea water only) whereas cell growth in medium with mucus supernatant followed a similar path to that seen in Experiment 1 where the same medium was used (Figure 2). The cell numbers were not as high as in Experiment 1 which may have been due to lower numbers used to inoculate the culture flask. The result indicates that there is some soluble component of the mucus that encourages growth of *N. perurans.* A subsequent growth trial used amoebae isolated from salmon gills and directly inoculated to MYA plates with mucus overlay but was rapidly overgrown with contaminants.

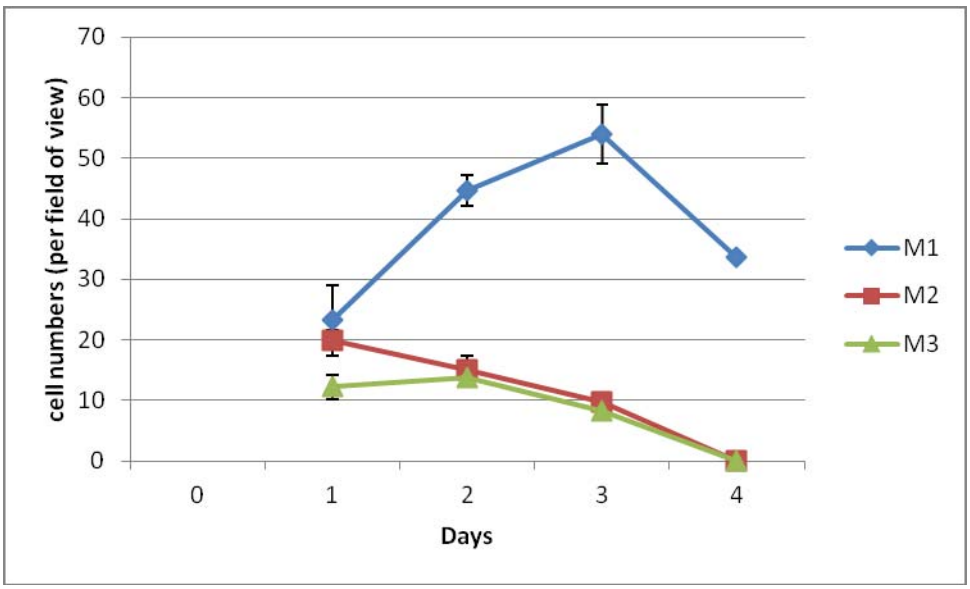

Figure 2. Growth curves for *N. perurans* over 4 days in 15 mL cell culture flasks with media consisting of sea water, antibiotics and mucus supernatant (M1); sea water and antibiotics (M2) and sea water only (M3). Cell numbers are means (± S.E) of counts (n=5).

Experiment 4

The use of MYA plates with a sea water overlay was successful in maintaining *N. perurans* in culture. The PCR showed that the cultured amoeba was N. perurans (Figure 3). However on this occasion contamination of the culture with flagellates, ciliates and other motile protozoans remained a problem and the clonal culture was overgrown 8 weeks after initiation. Contaminants can be controlled to some extent using 70% ethanol as a rapid surface wash as many of the contaminating organisms are in the overlying sea water whilst the majority of amoebae are slightly embedded in the agar.

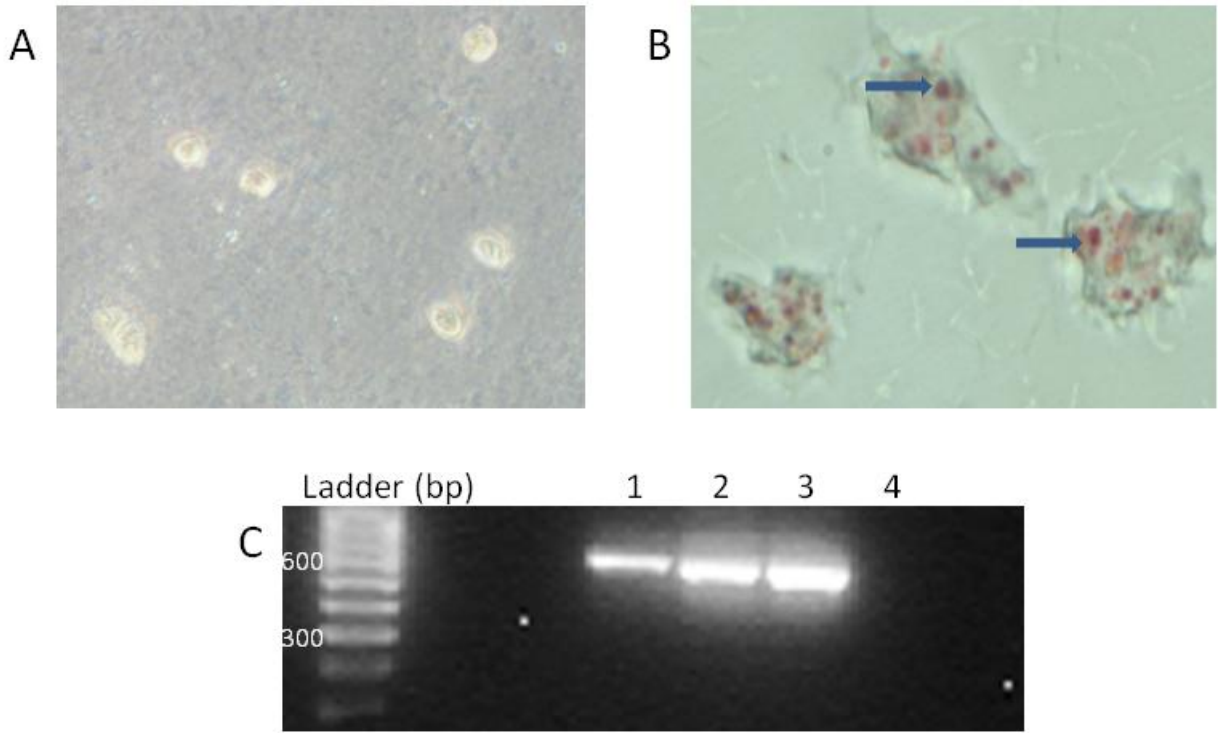

Figure 3. A. *Neoparamoeba perurans* cells in culture on malt yeast agar. B. Cells from the same plate after viability staining with neutral red showing that the dye has been actively taken up and stored in vacuoles (see arrows). C. PCR results confirming the identity of the cells as *N. perurans* (lane 1) with an amplified product consistent with the 636 bp target region of the *N. perurans* 18s rRNA gene, positive controls (lanes 2 & 3), no template negative control (lane 4).

Experiment 5

The weekly subculture of amoebae from MYA plates overlaid with sea water led to the eventual removal of contaminating protozoans such as ciliates and flagellates. This procedure is now commonly used to culture *N. perurans* and the amoeba has now been in continuous culture since February 2011.

The pilot infection experiment using non-clonal *N. perurans* showed that the cultured amoeba could cause AGD. At the conclusion of the experiment all salmon exposed to amoebae had gross signs of AGD and on histological examination displayed typical AGD pathology with lesions and associated amoebae (Figure 4).

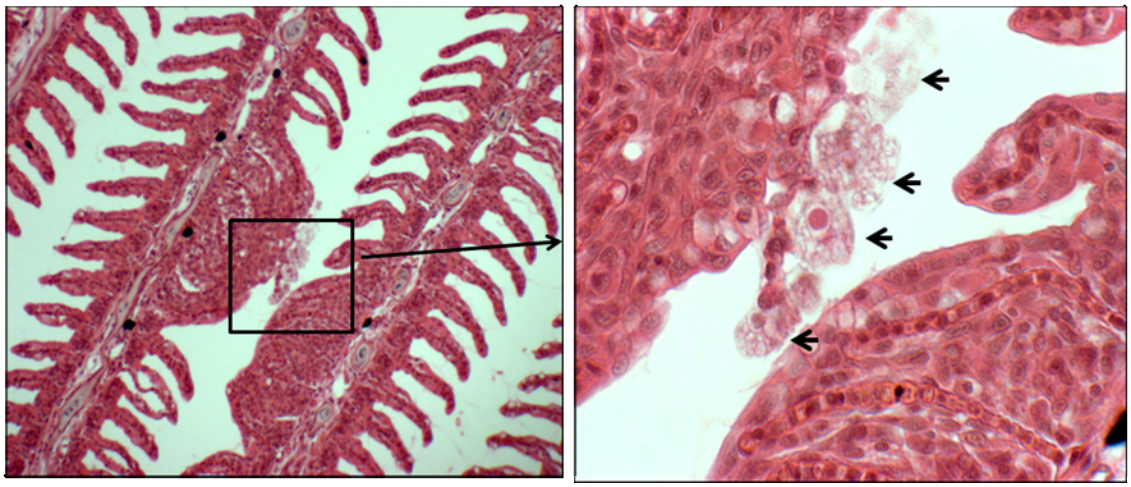

Figure 4. Haematoxylin and eosin-stained section of gill tissue from salmon exposed to cultured *Neoparamoeba perurans* showing typical hyperplastic lesions with associated amoebae (see arrows).

In addition, when species-specific oligonucleotide probes that hybridise to either *N. perurans, N pemaquidensis* or *N. branchiphila* 18s rRNA were applied to fixed sections of gill tissue to identify the amoebae associated with the lesions to species level during *in situ* hybridisation (ISH) experiments (according to Young et al, 2007), the attached amoebae were shown to be *N. perurans* (Figure 5). Amoebae were successfully isolated from all the amoebae-exposed fish on termination of the experiment and these amoebae were identified as *N. perurans* by PCR. Histological examination of sections from the negative control fish showed them all to be free of any AGD lesions.

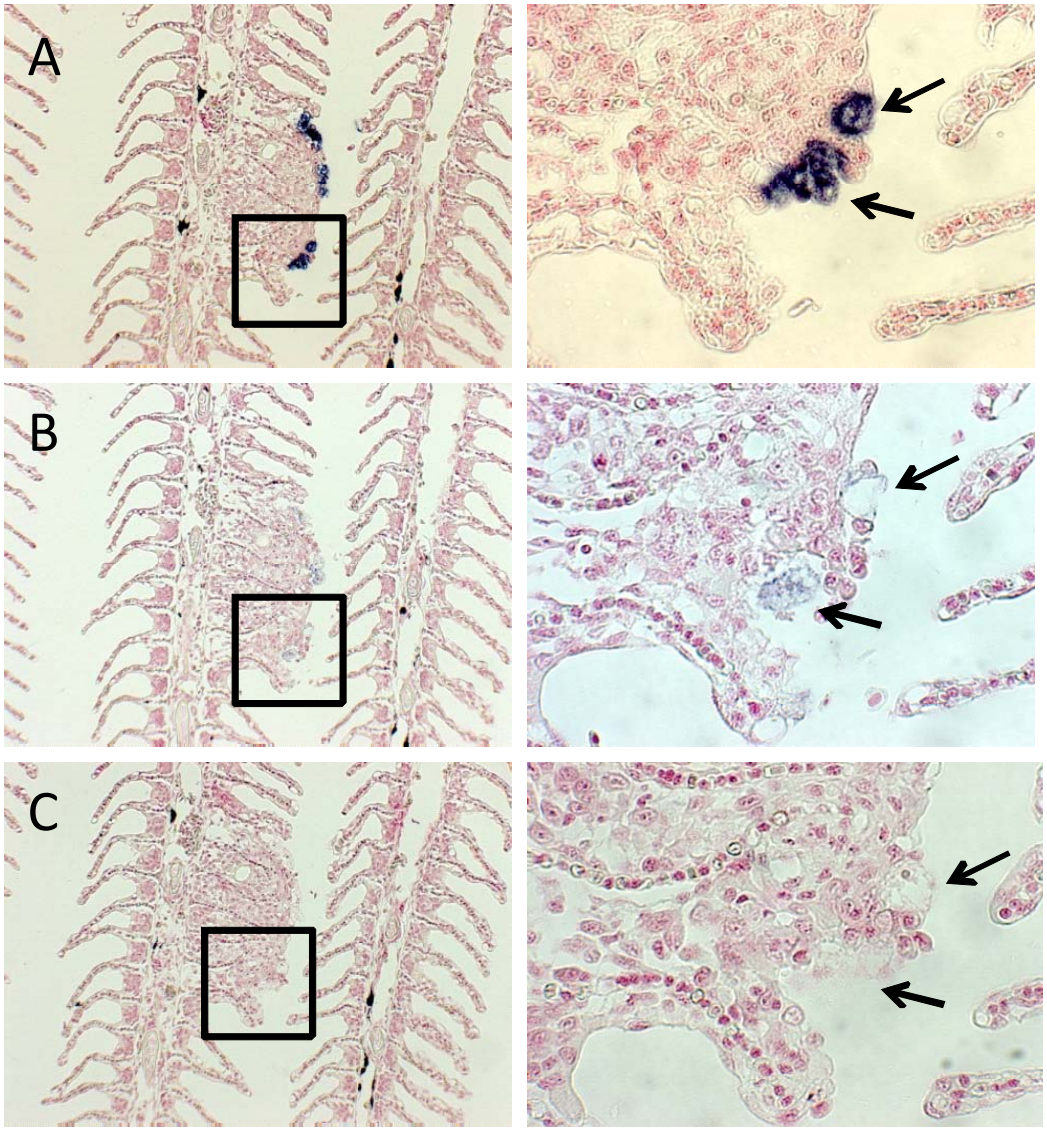

Figure 5. *In situ* hybridisation using species-specific oligonucleotide probes that hydridise to rRNA and differentiate between *Neoparamoeba perurans*, *N. pemaquidensis* and *N. branchiphila*. A. *N. perurans* probe showing positive reactive dark cells (see arrows). B. and C. showing the same section with *N. pemaquidensis* and *N. branchiphila* probes respectively and non-reactive cells (see arrows).

Experiment 6

The infection experiment using the clonal culture (clone 4) resulted in clinical AGD. Amoebae were isolated from salmon gills which had been exposed to clone 4 and freshly isolated *N. perurans* and these were introduced into culture for 1-2 weeks before identities were confirmed as *N. perurans* by PCR. Histological examination revealed typical AGD pathology with associated amoebae in all the fish sampled from the clone 4 infected fish and from those exposed to freshly isolated *N. perurans* (Figure 7). There were no AGD lesions seen on the gill sections from the negative control group that were not exposed to amoebae.

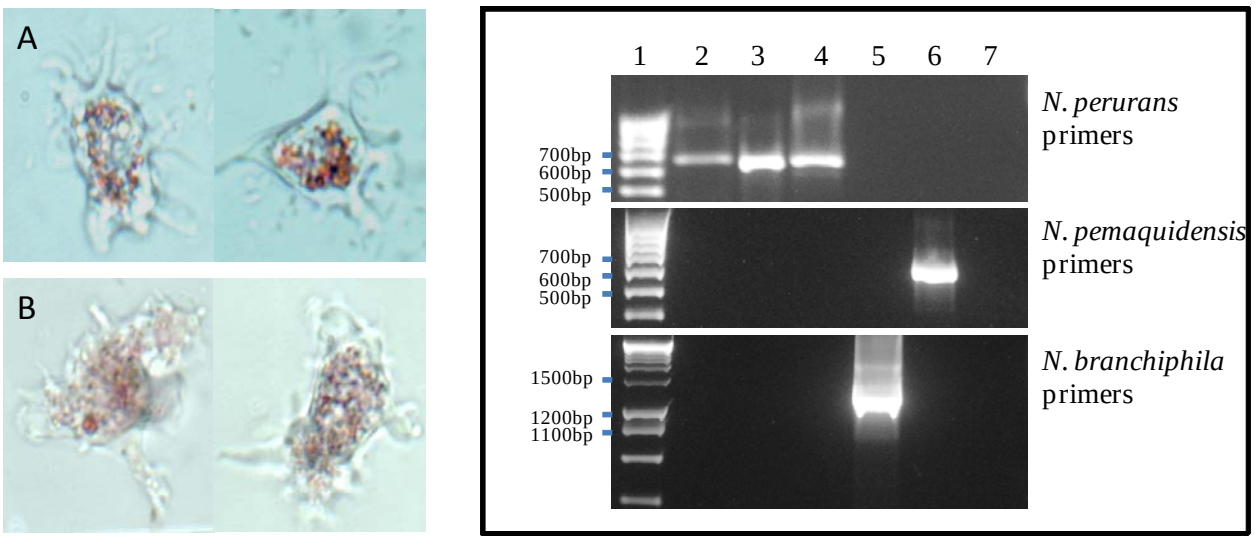

Figure 6. Viability stain with neutral red showing dye uptake by viable amoebae: A. cultured clone 4 and B. freshly isolated, non-cultured amoebae. C. PCR results and identity confirmation as *Neoparamoeba perurans* of clone 4 and freshly isolated amoebae using species specific oligonucleotide primers. DNA templates by lane are: 2 -clone 4, 3- freshly isolated, non-cultured amoebae, 4 -*N. perurans* positive control, 5-*N. pemaquidensis* positive control, 6-*N. branchiphila* positive control and 7-no template negative control

The amoebae associated with lesions in both infections were shown to be *N. perurans* exclusively using ISH (Figures 8 and 9). The culture /challenge experiments have shown that AGD can be induced in salmon by exposure to *N. perurans* that had been in culture for 8 weeks. Additionally a clonal culture clone 4 was capable of inducing AGD after 8 weeks in culture. This clone was established from the initial population of cells that was first isolated and cultured in February 2011 so had effectively been in culture for 20 weeks by the time of the infection trial. Koch's postulates have now been fulfilled for AGD and *N. perurans*.

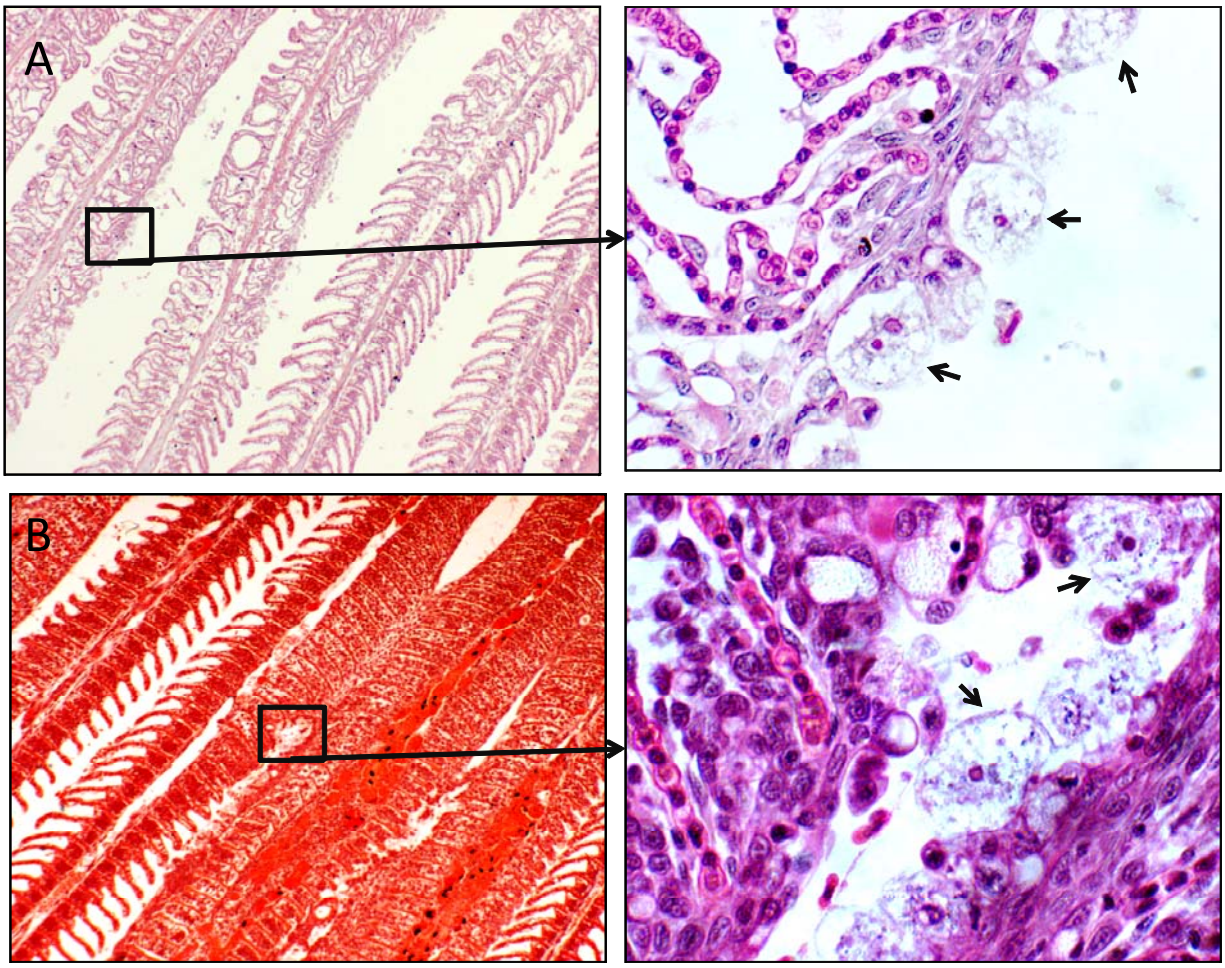

Figure 7. Haematoxylin and eosin-stained section of gill tissue showing typical hyperplastic lesions with associated amoebae (see arrows) after exposure to: A. cultured clone 4, and B. freshly isolated, non-cultured amoebae

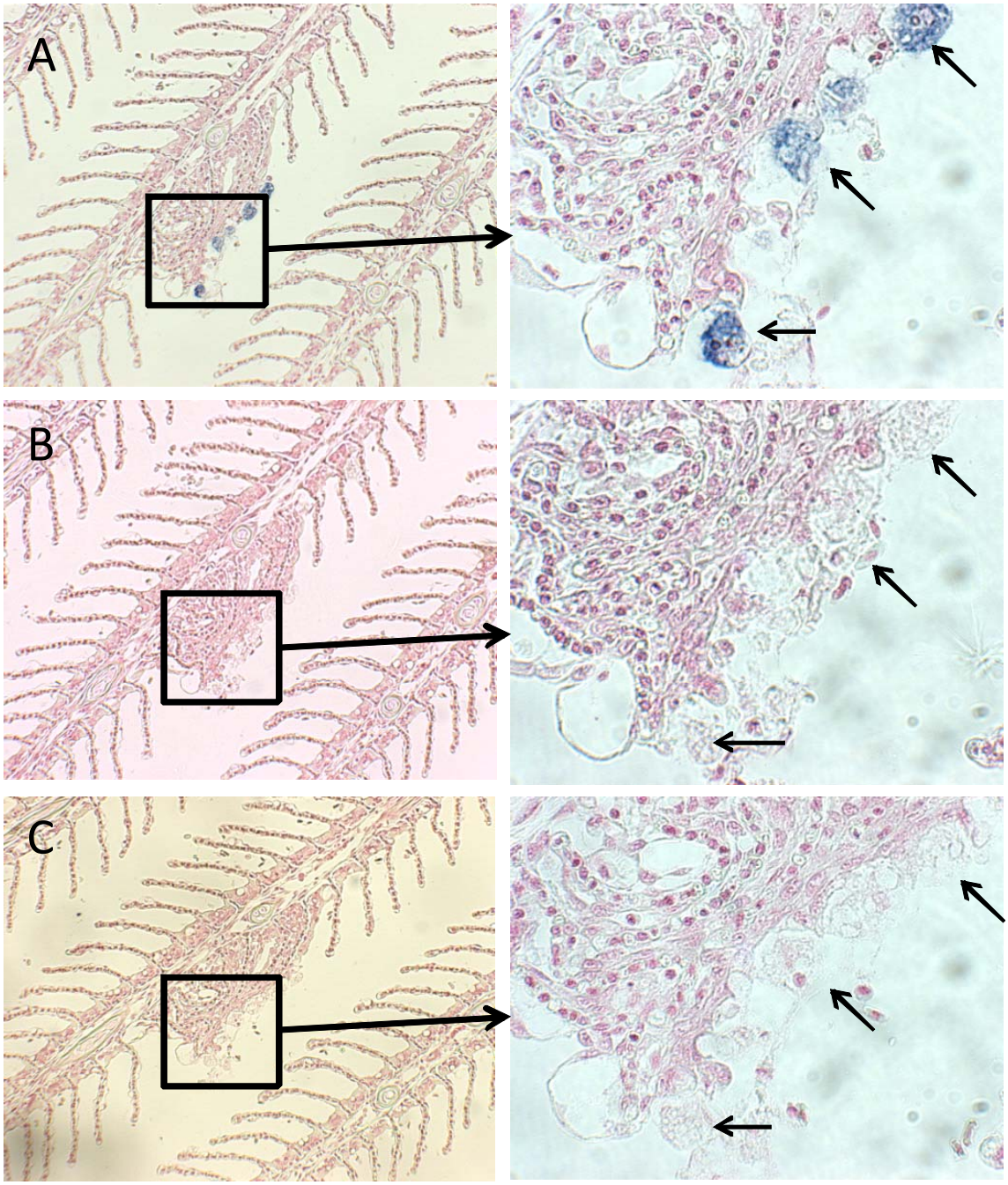

Figure 8. *In situ* hybridisation using species-specific oligonucleotide probes on gill sections from the infection with the freshly isolated *N. perurans*. A. *N. perurans* probe showing positive reactive dark cells (see arrows). B. and C. showing the same section with *N. pemaquidensis* (B) and *N. branchiphila* (C) probes including non-reactive cells (see arrows).

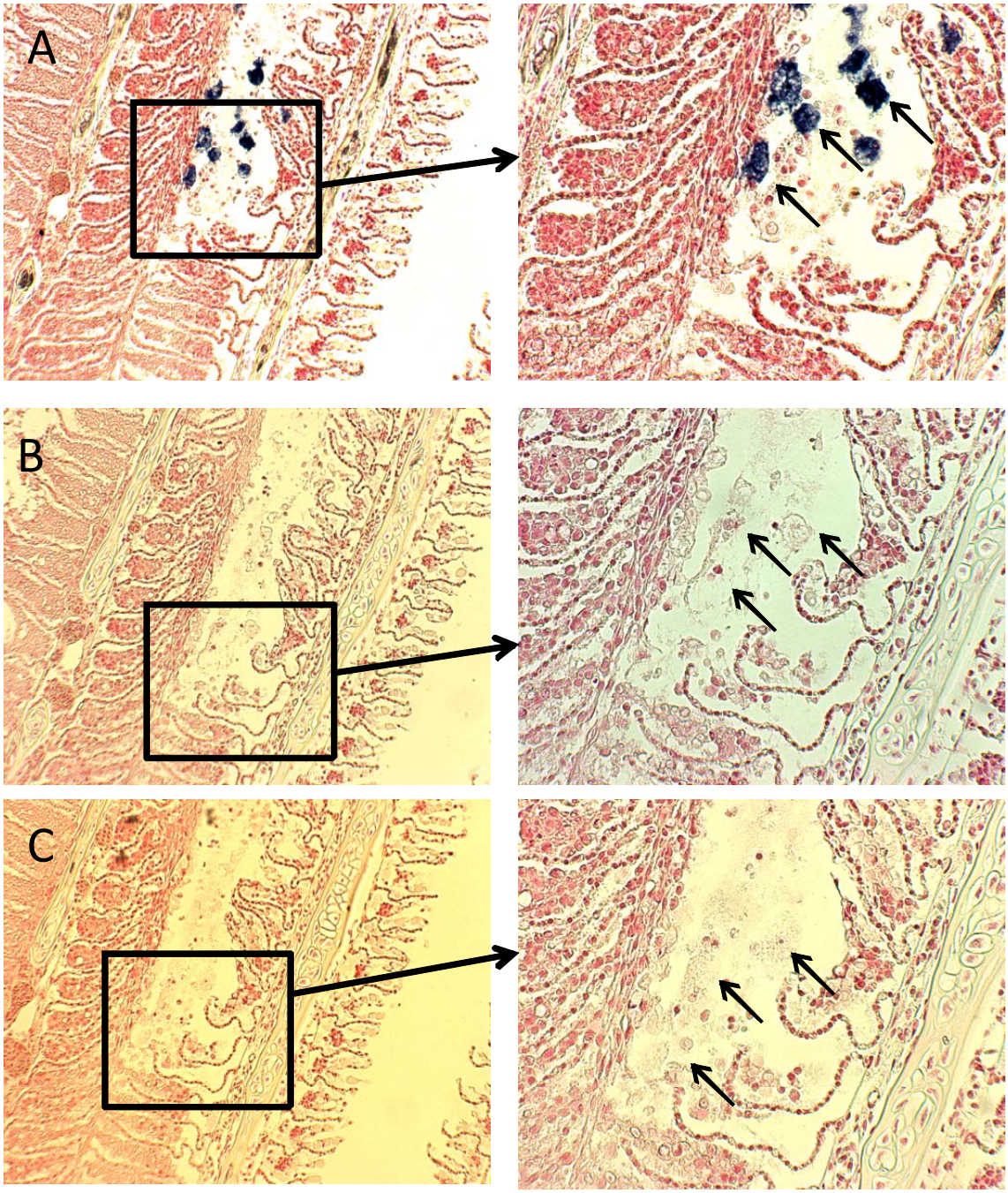

Figure 9. *In situ* hybridisation using species-specific oligonucleotide probes on gill sections from the infection with cultured *N. perurans* (clone 4.) A. *N. perurans* probe showing positive reactive dark cells (see arrows). B. and C. showing the same section with *N. pemaquidensis* and *N. branchiphila* probes respectively and non-reactive cells (see arrows).

Although amoebae belonging to the genus *Neoparamoeba* have long been known to be the causative agent of AGD in marine-cultured Atlantic salmon, this project was the first to successfully culture the amoebae and fulfil Koch's postulates for *N. perurans.* This experiment also demonstrated that the amoeba is free-living but able to infect a host, and therefore can be termed amphizoic (Page 1974). We have previously described the limitations resulting from the lack of success in culturing *N. perurans* (Crosbie et al

2010). The current availability of a virulent cultured clone will provide substantial benefits for AGD researchers and in the future this will remove the need to maintain a perpetual laboratory-based infection. At present vigilant subculturing is still needed to control contaminants and cryopreservation attempts have not as yet been successful (see Experiment 8.3).

Experiment 7

Growth trials with clone 4 to determine generation times showed that exponential growth began within the first 24h and continued for the duration of the trials except during trial 1 when growth began to decline after 144h.

Generation times ranged from 25.41 to 28.69h (Table 1) and were determined from two time points within exponential growth phase. For all trials exponential growth was evident at the first time point and continued for the duration of trials 2 and 3 at 164 and 139h respectively but was declining after 144h during trial 1. For consistency 90-96h was chosen as the second time point within exponential growth phase for generation time calculations for each trial.

Table 1 Generation times (*t gen*) for *N. perurans* grown on MYA plates with a sea water overlay and incubated at 18ºC. *t gen* was calculated using ($\log_{10} N_t$ – $\log_{10} N_0$)-1 x 0.301*t* where N_t is the number of amoebae after time *t* in exponential growth phase and N_0 is the number of amoebae at the beginning of the exponential growth phase.

	Trial 1	Trial 2	Trial 3	Mean (± SE)
t (h)	96	90	91	
Log N_t	1.598	1.495	1.364	
Log N_0	0.591	0.429	0.306	
t gen (h)	28.69	25.41	25.88	26.66 (± 1.02)

Although the literature is scant on *in vitro* growth dynamics for *Neoparamoeba* spp. generation times for *Paramoeba invadens* (a pathogen of sea urchins) of 20.33 h at 15ºC and 19.41h at 20ºC have been reported (Jellet and Scheibling, 1988) and are similar to the mean generation time calculated for *N. perurans* of 26.66 h. Growth rates as determined by generation time are likely to be temperature dependent.

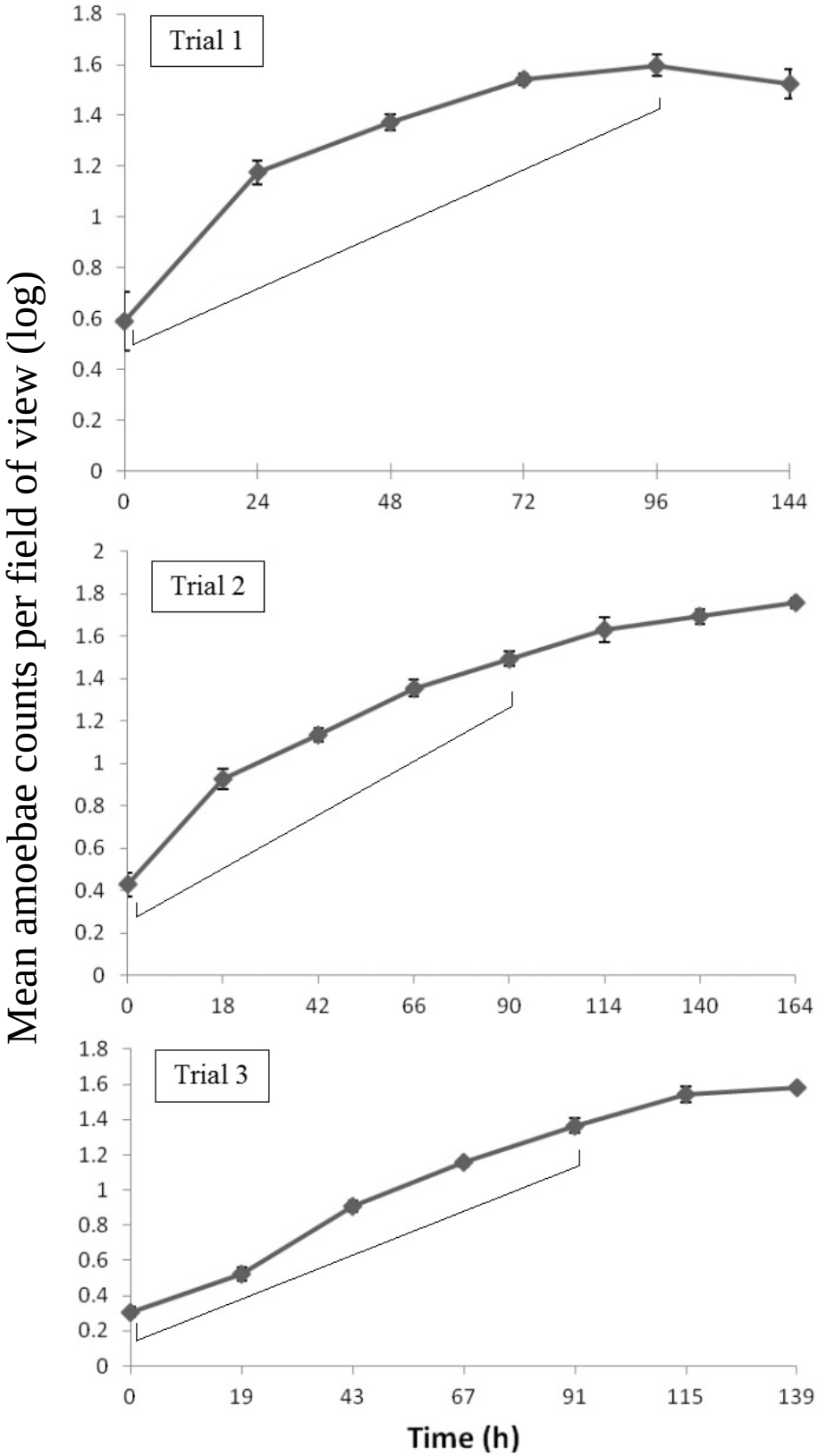

Figure 10. Growth curves for *N. perurans* (clone 4) grown on MYA plates with sea water overlay and incubated at 18ºC during trials. Time points within exponential phases to calculate generation times are indicated by straight lines. All values are means (± SE, n = 4).

Experiment 8

8.1 Temperature

The temperature trial showed that the amoebae did not grow at 25ºC as there were no cells seen on the plates after 24h. The control plates at 20ºC showed that amoebae were growing after 24 h when the experiment was terminated.

8.2 Culture without sea water overlay

Neoparamoeba perurans can be held on culture plates without sea water for up to 17d. In this experiment amoebae survived when overlaying sea water was removed and plates partially dried out, then rehydrated after 7 and 17 d. After rehydration on both occasions amoebae were seen free floating in the sea water overlay 1-2h after rehydration. These cells were subcultured to new MYA plates with a sea water overlay and growth was seen after 2-3 d. This suggests that the cells are capable of surviving without the sea water overlay once they are established in culture and contaminating protozoans are not present.

8.3 Cryopreservation

The cryopreservation experiment using the method of the ATCC was not successful. Thawed cells appeared rounded after 30 min (Figure 11) and showed signs of lysing.

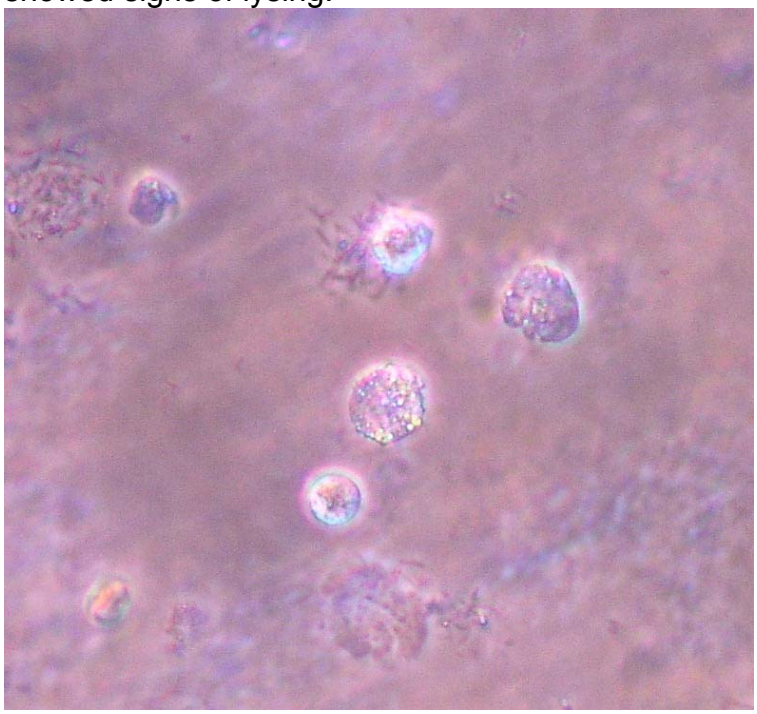

Figure 11. *Neoparamoeba perurans* 30 min post thaw after being frozen using the American Type Culture Collection cryopreservation method (for marine amoebae). Cells are rounded due to the loss of cell membrane integrity and appear more granular and show signs of lysis.

Thawed cells placed onto MYA plates with a sea water overlay did not survive and could not be seen on any plates 3 d post thaw. Although this method has been successfully used for *N. pemaquidensis* and *N. branchiphila* held in the

amoebae library at UTAS unfortunately it does not seem to be effective for *N. perurans*.

Quantitative method to measure *N. perurans* concentrations in water

We developed a qPCR based method to quantify presence of *N. perurans* in water (Bridle et al 2010). This method is used to determine the concentration of the amoebae in water during challenges and has been applied to environmental samples in epidemiological studies. Using this method we were able to detect a single 18S rRNA gene copy and readily detected *N. perurans* with the lowest detection limit for *N. perurans* cells spiked in sea water being one cell (100% detection rate). The method was applied to seawater samples collected from both an experimental AGD infection tank and a variety of environmental sites including those used to culture Atlantic salmon in Tasmania, Australia. Detectable populations were highly abundant from sites in and closely surrounding cage culture of Atlantic salmon (Bridle et al 2010). Furthermore, the method when applied to gill swabs from an on-farm gill pathology assessment demonstrated that non-destructive semi-quantitative analysis of amoebae loads from these fish was possible.

Objective 2 Elucidate the role of saccharide-inhibitable lectins on the ability of the amoeba to attach to salmon tissue

The results from the experiments investigating the ability of monosaccharides to inhibit amoebae attachment to the gills of salmon indicated that when amoebae were exposed to galactose at concentrations of 50 and 5 mM there was significantly less pathology seen after 72 h compared to the other galactose concentrations and the positive control (Figure 12).

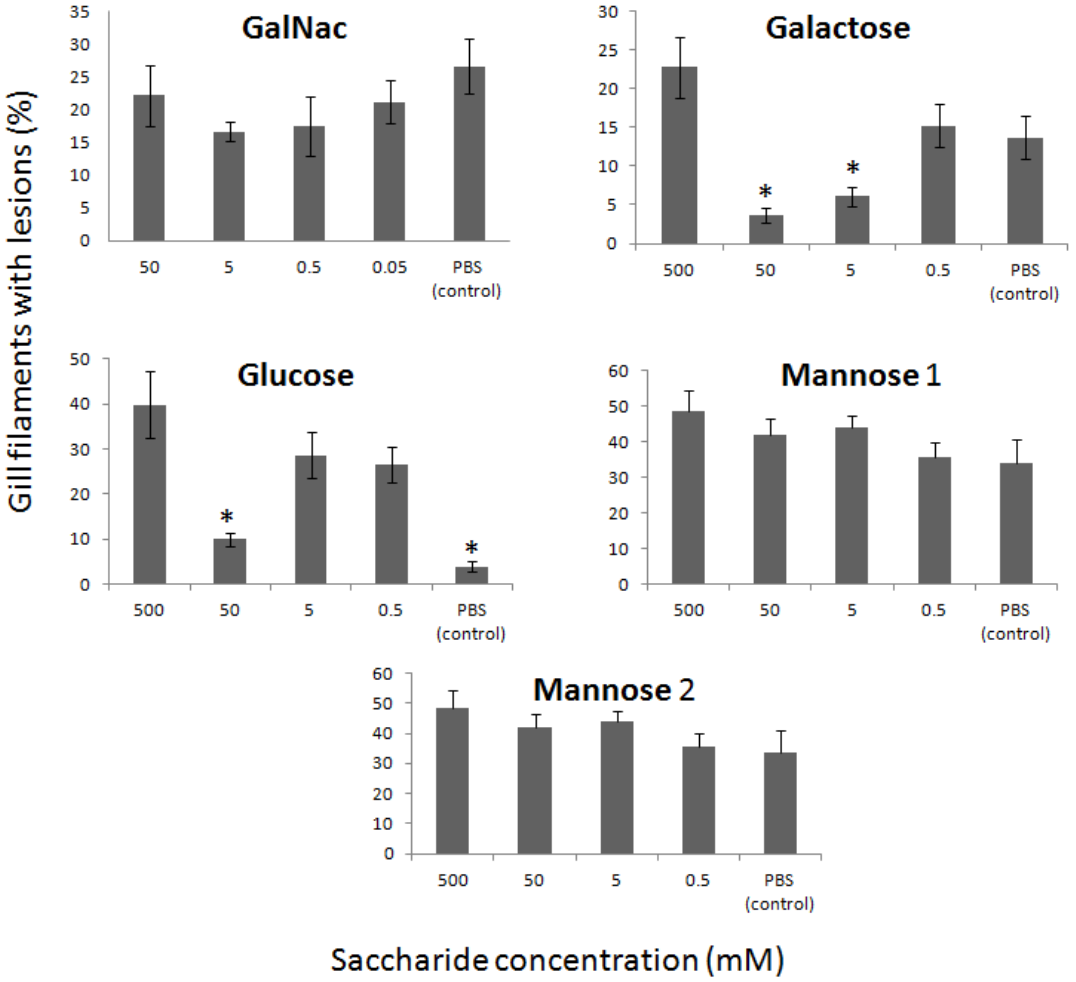

Figure 12. Amoebic gill disease (AGD) pathology quantified by percentage of gills with typical AGD lesions (+ SE) 72 h after challenge with amoebae previously incubated with saccharides : N-acetyl-D-galactosamine (GalNac), galactose, glucose and mannose at 4 concentrations (n=10 except glucose at 500mM where n=7 and mannose 2 where n=11).* indicates significant differences

A similar observation was seen when amoebae were exposed to glucose at 50 mM, however with the positive control group (where amoebae were exposed to the saccharide diluent only; PBS) during the glucose experiment had the lowest measure of pathology, with 3.8% of the gill filaments having AGD lesions, and was significantly lower than pathology quantified from amoebae after exposure to the other concentrations of glucose. The glucose positive control was also substantially lower than the degree of pathology seen for the positive control groups used for the other 4 experiments which ranged from 13.67 to 33.84% and is indicative of a problem with this experiment and makes it difficult to draw any conclusions about the impact of glucose on amoebae attachment. The experiments were to be repeated, but so far only mannose has been repeated and produced similar results.

Objective 3 Isolation and sequence identification of the lectin mediating *N. perurans* attachment.

The antisera produced against recombinant proteins showed reactivity with *N. perurans* antigens (Figure 13) with anti-895-998 antiserum reacting with 2 putative *N. perurans* proteins of around 45 and 50kDa and a smaller one at 6 kDa. Similarly the anti-22CO3 antiserum reacted with possibly the same 50kDa protein and a larger one of around 116 kDa.

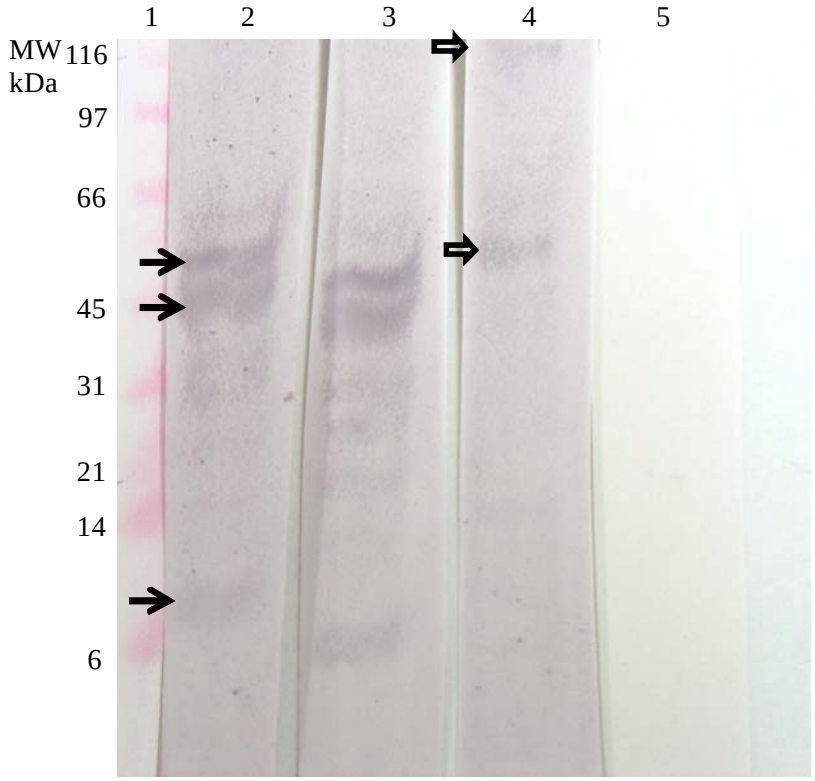

Figure 13. Western blot analyses of rabbit anti-lectin 895-998 (lanes 2 & 3) shows reactivity with putative *N. perurans* proteins of around 6, 45 and 50 kDa (solid arrows) and rabbit anti-lectin 22CO3 (lane 4) shows reactivity with proteins of around 50 and 100 kDa (hollow arrows). Lane 1 is molecular weight markers and lane 5 is a negative control.

The results suggested that the rabbit antisera would be appropriate reagents to use in immunoprecipitation experiments designed to isolate the reactive proteins form *N. perurans* and to then characterise and determine the amino acid sequence.

Immunoprecipitation experiments to isolate the particular protein antigen from *N. perurans* lysates were not successful. Results from the gels suggest that the non-immune rabbit sera (pre-bleeds) react with some *N. perurans* antigens. It was expected that after the immunoprecipitation that the eluates would only contain the antibody-antigen complex, however the silver stained gel clearly shows several protein bands. When the sonicate is incubated with non-immune rabbit serum (i.e. rabbit serum that should have no activity against amoebae antigens) it would be expected that there would be no antibody-antigen complex formation and therefore no amoebae protein bands

evident. However there were protein bands in lanes 4 and 8 (Figure 14) which were incubations of the amoebae sonicate with rabbit sera taken from the rabbits before they were immunised with the recombinant proteins.

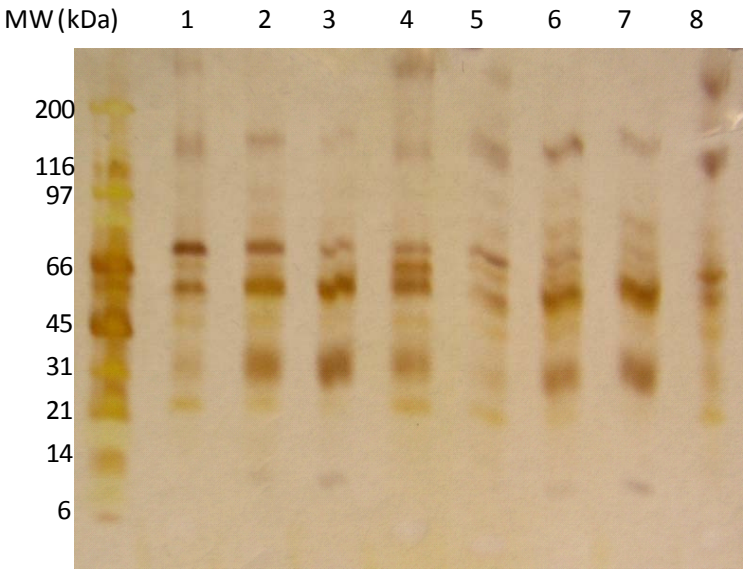

Figure 14 Polyacrylamide gel silver stained to show eluted proteins from a sonicate of *N. perurans* cells after immunoprecipitation. Lanes 1-3 are eluates after incubation of sonicate with antiserum 895-998, lanes 5-7 after incubation with antiserum 22CO3. Lanes 4 and 8 are after incubation with non-immune rabbit serum pre-bleeds.

The Western blot analyses (Figure 15) were performed to show that the antigen of interest (i.e. the antigen to which each antiserum is directed) was present in the eluate after immunoprecipitation. Both antisera reacted with a protein band of approximately 50kDa which was consistent with results shown in figure 13, however they also indicated that those antigens were present in eluates where they should not be present (i.e in lanes 1 and 8 from Figure 15).

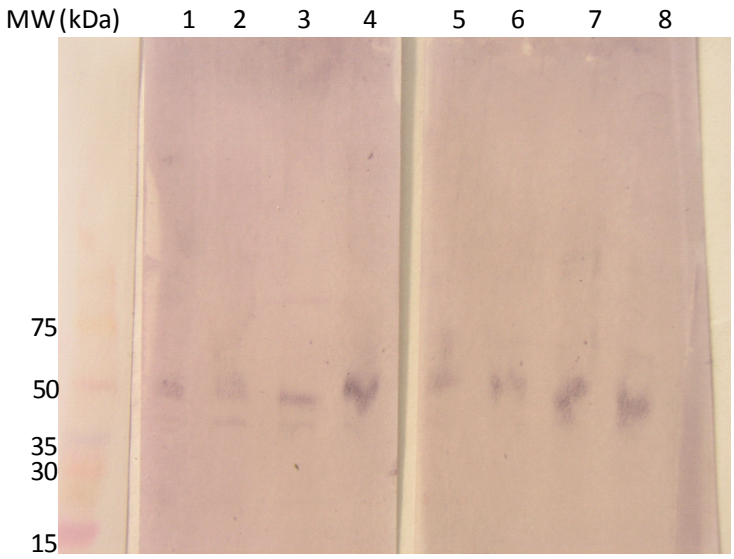

Figure 15. Western blot analyses of eluted proteins from a sonicate of *N. perurans* cells after immunoprecipitation. Lanes 2-4 are eluates after incubation of sonicate with antiserum 22CO3, lanes 5-7 after incubation with antiserum 895-998. Lanes 1 and 8 are after incubation with non-immune rabbit serum

Immunoprecipitation experiments were continued by collaborators at the CSIRO and in addition to the antisera used above (895-998 and 22CO3) a monoclonal antibody designated 3F4 was used. This antibody was provided as a gift by Prof William A Petri from the University of Virginia and was also raised against the Gal/GalNac surface receptor lectin of *Entamoeba histolytica.* The experiments were performed as previously described using both freshly isolated *N. perurans* and cultured *N. pemaquidensis* and products electrophoresed on polyacrylamide gels and stained with coomassie blue to visualise proteins (Figures 16 and 17).

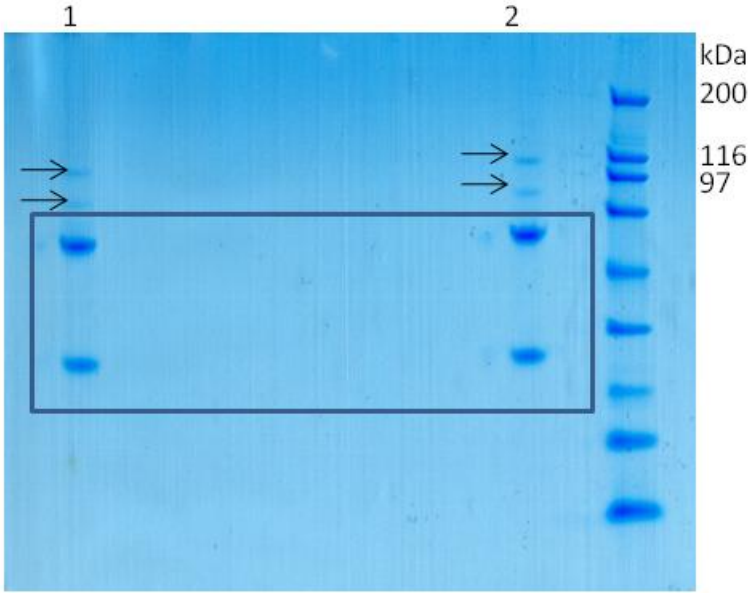

Figure 16. Polyacrylamide gels stained to show eluted proteins from sonicates of *N. perurans* and *N. pemaquidensis* cells after immunoprecipitation. Lane 1 shows eluate after incubation of *N. perurans* sonicate with monoclonal antibody 3F4 and lane 2 is *N. pemaquidensis* sonicate with monoclonal antibody 3F4. Arrows indicate isolated amoebae proteins with a molecular weight (MW) of approximately 100 and 80 kDa. The bands inside the box are the heavy and light chain components of the 3F4 monoclonal antibody.

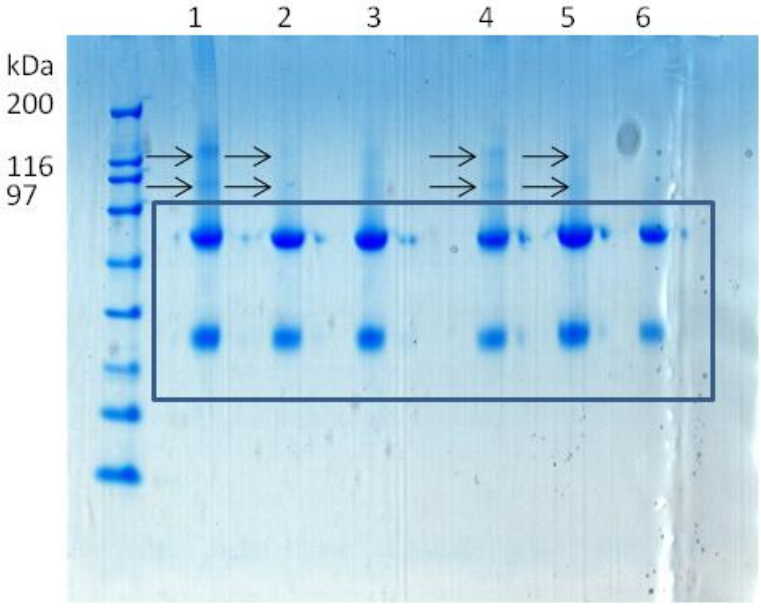

Figure 17. Polyacrylamide gels stained to show eluted proteins from sonicates of *N. perurans* and *N. pemaquidensis* cells after immunoprecipitation. Lanes 1- 3 are eluates after incubation of *N. pemaquidensis* sonicate with antibody 895-998 (lane 2) and 22CO3 (lane 3).

Lanes 4-6 are eluates after incubation of *N. perurans* sonicate with antibody 895-998 (lane 5) and 22CO3 (lane 6). Arrows indicate isolated amoebae proteins with a molecular weight (MW) of approximately 100 and 80 kDa. The bands inside the box are the heavy and light chain components of the 895-998 and 22CO3 antibodies.

All the antibodies used for the immunoprecipitation experiments reacted with proteins of the same size from both *N. perurans* and *N. pemaquidensis* and these proteins that were submitted to the proteomics facility of CSIRO in Brisbane for amino acid sequencing. Unfortunately no meaningful sequences could be identified. There is a potential to continue the immunoprecipitation experiments with cultured *N. perurans* once the cultures can be partially purified.

Objective 4. Investigate antigenicity and protection against AGD of identified lectin in salmon

Unfortunately this objective could not be addressed as the immunoprecipitation experiments were not successful and the putative lectin in *N. perurans* has not been identified.

Objective 5. Maintain and run 4 laboratory tank based trials.

A total of five vaccine and challenge trials were carried out at UTAS during the project using experimental vaccines produced by the CSIRO. The results of those trials are the final report of project 2007/234.

Objective 6. Provide virulent amoebae for other AGD research

The AGD infection is at present still maintained and provides infectious amoebae and the cultured strain of *N. perurans* is also available and has been supplied to other AGD researchers. We have provided positive controls for *N. perurans*, *N. pemaquidensis and N. branchiphila* to laboratories in Ireland, Chile and Japan. We confirmed presence of *N. perurans* in AGD affected fish in Chile and Japan.

We have provided over 20 million gill-isolated ameobae to CSIRO researchers (Dr Matt Cook and Dr Richard Taylor), 2.8 million to Dr Nathan Bott (SARDI) and several millions to Dr Mark Adams (UTAS). Genomic DNA from *N. perurans* was sent to Dr Simon Jones (PBS DFO, Canada), Dr David Graham (Veterinary Sciences Division, Agri-food and Biosciences Institute, Belfast Nth Ireland), Dr Patricio Bustos (ADL Chile) and Prof Kazuo Ogawa, (Japan). Additionally either cultures or DNA of *N. pemaquidensis* and *N. branchiphila* were supplied to Celeste Knowles (PhD student Flinders University and CSIRO), Dr Nathan Bott (SARDI), Dr Simon Jones (PBS DFO, Canada), Dr David Graham (Veterinary Sciences Division, Agri-food and Biosciences Institute, Belfast Nth Ireland), Dr Patricio Bustos (ADL, Chile) and Prof Kazuo Ogawa (Japan).

Benefits and Adoptions

This project directly benefits the industry by development of methods for culture of virulent *N.perurans* and supply of virulent *N.perurans* for other AGD research during the project. Other AGD research projects are already benefiting from the methods developed in this project. In particular cultured *N. perurans* has been supplied to a number of other researchers and the qPCR has been used routinely in challenges. We have used the detection method in industry surveys of net cleaning effluent. Further research is needed before these results can be adopted by the industry.

Further Development

Results of this project have been widely disseminated throughout the salmon industry through industry meetings and email communications. While a culture method for virulent *N. perurans* has been developed (reducing the need for infection tank and thus improving fish welfare) the current technique still requires frequent passaging of the cultures and is laborious and time consuming. Further research should be done to ensure that cultured virulent *N. perurans* remains available. Cryopreservation methods for the virulent pathogen should be developed. Further research should be done on the role of saccharide-inhibitable lectins in the amoebae attachment in AGD.

Planned Outcomes

This project is proactive and directly benefited salmon industry. The planned outcome of the project was the same as that for the AGD Vaccine Phase III project (FRDC 2007/234), that is an efficient commercial vaccination program against AGD for the Tasmanian Atlantic salmon industry with the concomitant economic benefit. More specifically this project will help to identify vaccine target molecules which will contribute to the success of the AGD Vaccine Phase III project through running AGD challenges for vaccinated fish and development of protocols for culture of *N. perurans*.

Outputs of the project include:
1. Protocols for the culture of *N. perurans*
2. A more complete understanding of the binding mechanisms of *N. perurans* to salmon gill tissue

Conclusions

1. We have developed methods for successful culture of *N. perurans* and fulfilled Koch's postulates for this species role in AGD in Atlantic salmon.

2. We have developed a qPCR based method for measuring of *N. perurans* concentrations in water and showed that the concentrations in challenge tanks are lower than nominal concentrations and that the amoeba is present in salmon farming environment.

3. We have provided material for other AGD research. Our international collaborations showed that AGD outbreak in Atlantic salmon in Chile and AGD outbreak in ayu in Japan were caused by *N. perurans.*

4. Exposure to galactose at concentrations of 50 and 5 mM inhibited amoebae attachment and reduced AGD pathology after 72 h.

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Appendix 1

INTELLECTUAL PROPERTY

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

1. Copyright in this report

Appendix 2

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Appendix 3

PUBLICATIONS ARISING FROM THIS PROJECT

Crosbie, P.B.B., Bridle, A.R., Leef, M.J., Nowak, B.F. (2010) Effects of different batches of *Neoparamoeba perurans* and fish stocking densities on the severity of amoebic gill disease in experimental infection of Atlantic salmon, *Salmo salar* L. Aquaculture Research 41, e505-e516.

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