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Comparative susceptibility and host responses of endemic fishes and salmonids to amoebic gill disease in Tasmania

Final Report

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Executive Summary

Report Content

Scientists at the Institute for Marine and Antarctic Studies have completed a two year experimental project on a globally emerging fish disease. Dr Mark Adams and co-investigators Dr Andrew Bridle and Professor Barbara Nowak investigated the comparative susceptibility and host responses of various endemic and salmonid fishes to amoebic gill disease (AGD). The research, conducted at the University of Tasmania's aquaculture research facility in Launceston, found that yellow eye mullet were able to spontaneously resolve pathological signs of AGD under experimental conditions. A tempered disease response to experimental infection with the causative agent *Neoparamoeba perurans* was demonstrated in other native species including Australian salmon, purple wrasse and southern sand flathead.

Negligible differences in immune-regulatory genes were found in Atlantic salmon repeatedly infected with *N.perurans* in comparison to naïve and uninfected fish; a finding that contrasted with concomitant observations at the cellular level. AGD induced tissue remodelling of mucosa associated lymphoid tissues in Atlantic salmon gills. Hybridised Atlantic salmon and brown trout displayed lower levels of disease severity induced by experimental infection supporting recently published field observations (collaboration with Dr Ben Maynard (CSIRO - FRDC project 2011/071).

The disease has affected Tasmanian salmonid aquaculture since its inception three decades ago. AGD has become an increasingly complex management issue as the industry expands and the condition has recently emerged worldwide affecting major producers in the northern hemisphere. It is anticipated that the identification of fish species resistant to or tolerant of AGD will provide insight and linkage toward alternative treatments or prophylaxis for farmed Atlantic salmon.

Background

For three decades AGD has become an increasingly complex management issue for the Tasmanian salmonid industry and the condition is now emerging worldwide affecting major producers throughout the northern hemisphere. During 2010, consultants Panaquatic Health Solutions Pty Ltd undertook a mini-review of research findings for AGD over the preceding 25 years. A key recommendation from this review was that future research be directed toward gaining further fundamental knowledge of host-pathogen interactions particularly in respect to elucidating susceptibility and resistance to AGD. Published and anecdotal evidence to date suggests substantial knowledge gaps in two key areas. Firstly, whether endemic fish (found nearby and within salmon cages) are susceptible to AGD and secondly how does the host response (at the gill level) differ between susceptible and resistant/tolerant salmonids.

Aims/objectives

Broadly, this project had two primary aims;

- To determine susceptibility of selected endemic species with differing life histories to AGD induced under laboratory conditions
- To describe differences in cellular responses and disease progression between and within different salmonid species following infection with *Neoparamoeba perurans*

Methodology

Four endemic fish species were exposed to *N. perurans* under experimental conditions in shared recirculating aquaculture systems with Atlantic salmon *Salmo salar*. The trials for each endemic species were run separately. The four species, with four differing life histories, included an estuarine/marine pelagic carnivore (Australian salmon *Arripus trutta*), an estuarine pelagic detritaphage (yellow eye mullet *Aldrichetta forsteri*), a demersal carnivore (southern sand flathead *Platycephalus bassensis*) and a reef dwelling predator that feeds primarily upon molluscs and crustaceans (purple wrasse *Notolabrus pucicola).* A further challenge trial of extended duration (28 days) was subsequently undertaken using yellow eye mullet cohabited with Atlantic salmon.

Atlantic salmon and rainbow trout were co-habited, acclimated to marine conditions and experimentally challenged with *N.perurans.* Atlantic salmon were challenged a further two times as rainbow trout were withdrawn from the experiment due to marine acclimation difficulties. A group of identically managed unchallenged control fish were then introduced to the system housing the previously infected salmon and all fish were then challenged and sampled.

Atlantic salmon, brown trout and two hybrid crosses (male/female – female/male) of the species were cohabited, acclimated to marine conditions and challenged with *N.perurans* for 18 days. This experiment was collaboratively conducted using fish provided by Dr Ben Maynard (FRDC project 2011/071).

Results/key findings

Experimental challenge with *N.perurans* elicited AGD in all endemic species and Atlantic salmon. Overall the native species tested displayed lower levels of characteristic AGD induced pathology concomitant with lower amoebic loads in comparison to Atlantic salmon. Despite initially showing diagnostic signs of AGD, yellow eye mullet were free of characteristic AGD pathology 28 days post exposure suggesting this species is able to defend and/or tolerate experimental infection by *N. perurans.*

Evidence of infection/disease resolution was observed histologically in fish previously exposed to three rounds of infection with *N.perurans*. Proportionally, Atlantic salmon naïve to infection with *N.perurans* displayed a more severe disease response compared with the re-infected salmon. Negligible differences in immune-regulatory genes were found in Atlantic salmon repeatedly infected with *N.perurans* in comparison to naïve and uninfected fish; a finding that contrasted with concomitant observations at the cellular level. AGD was found to induce tissue remodelling of mucosa associated lymphoid tissues in Atlantic salmon gills. The cohort of rainbow trout to be used as a comparator species cohoused with Atlantic salmon were unable to successfully acclimate to full marine conditions and were withdrawn from the project following the initial amoebae challenge. Results from this study were unable to conclusively delineate whether an adaptive or innate response drives the emergence of AGD resistant sub-populations of Atlantic salmon.

Brown trout and trout-salmon hybrids had a significantly lower level of gill pathology associated with infection by *N.perurans.* The amoebae burden harboured by both Atlantic salmon and brown trout was substantially lower in the hybridised strains. Differences in the cellular responses of hybridised fish in comparison to their pure bred siblings were observed suggesting that hybrid gill surfaces could be inherently unsuitable for colonisation by *N.perurans* and/or assume a refractory or fortified construct once infection commences. Encouragingly, findings from this project provide evidence that an innate capacity for other fish species or strains to resist, defend, or tolerate experimental challenge with *N.perurans*.

Collectively, for Atlantic salmon used throughout the life of this project, seven populations from three cohorts were challenged with one population challenged several times. Each infection elicited responses

demonstrating marked variation in terms of pathological severity and cellular responses for each population both between and within challenges.

Implications for relevant stakeholders

The major beneficiary for this project is the Tasmanian salmonid industry. The project outcomes address fundamental knowledge gaps related to susceptibility and resistance of fish to AGD. Ultimately the conclusions derived from this project have strengthened the fundamental knowledge link requisite for ultimately developing alternative management strategies for AGD. Results from this research have provided industry with new insight into the disease process by detailing aspects of susceptibility and/or resistance based responses to AGD. Knowledge transferred by this project can assist with industry research prioritization. Additionally, given the global emergence of AGD in the northern hemisphere the outcomes of this project can be used by other researchers and salmonid growers internationally.

Recommendations

This project has furthered the collective knowledge of AGD host susceptibility and comparative host responses to AGD. However there are still several knowledge gaps to be addressed before bridging the divide between fundamental information and applied outcomes. Yellow eye mullet and salmon-trout hybrids have shown excellent potential as future comparative research candidates for understanding how fish react and defend against AGD innately. Using these candidates for further comparison based investigation should include research directed toward:

- Characterising the pathobiology and primary interactions of *N.perurans* with gill tissues
- Elucidate functional properties of mucosal constituents responding to infection
- Immuno-pathological studies combining microscopic, genomic and proteomic approaches
- The impact of host age and development upon reactivity to amoebic infection
- Further exploration, diversification and revision of AGD challenge and sampling methodologies

Keywords

Atlantic salmon, amoebic gill disease, AGD, *Neoparamoeba*, yelloweye mullet, sand flathead, purple wrasse, Australian salmon, brown trout, rainbow trout, hybrid vigour.

Introduction

Amoebic gill disease (AGD) has affected the culture of Atlantic salmon during the marine grow-out phase of production in Tasmania since inception (Munday 1986) and is now a disease of global significance. The causative agent of amoebic gill disease is *Neoparamoeba perurans* (Young, Crosbie, Adams, Nowak and Morrison 2007; Crosbie, Bridle, Cadoret and Nowak 2012; Oldham, Rodger and Nowak 2016) which when present upon the gills causes extensive proliferation of epithelial and mucous cells resulting in significant physiological disruption (Adams and Nowak 2003; Munday 1986; Munday, Zilberg and Findlay 2001; Powell, Leef, Roberts and Jonesk 2008; Zilberg and Munday 2000). Affected fish present with signs of lethargy and rapid respiration preceding significant mortality in untreated fish stocks. In Tasmania the only effective and commercially used treatment for AGD is to bathe affected fish in freshwater. Each production pen can require up to 12 baths during the 12 – 18 month grow-out phase which imparts significant infrastructure cost, labour expense and production losses due to reduced growth (adding between $1-$2 per kilo of product). Additionally, but no less importantly, the inherent requirement for a nearby freshwater source presents challenges. Land locked storages and rivers are finite resources which are particularly vulnerable during summer or extended periods of low rainfall. The reliance upon this resource is therefore production limiting, and contributes to limited expansion into open water sites. Almost since its commencement the Tasmanian salmon industry has recognized that freshwater bathing is not sustainable long term. Indeed significant effort and expense has been invested in the identification of a therapeutic agent that rivals freshwater in terms of AGD control.

During 2010, consultants Panaquatic Health Solutions Pty Ltd produced a mini-review of research predominately focussed upon the first15 or so years of AGD research. A key recommendation from this review was that future research effort be directed toward gaining further fundamental knowledge of host-pathogen interactions particularly with respect to elucidating mechanisms of susceptibility and resistance to AGD. Published and anecdotal evidence to date suggested substantial knowledge gaps in two key areas. Firstly, whether endemic fish (found nearby and within salmon cages) are less susceptible to AGD and secondly how does the host response (at the gill level) differ between AGD naive salmonids and those previously exposed to AGD. In this context we proposed firstly to target differences in the host response of fish for evidence of innate defence capacity using a comparative species susceptibility approach and secondly to investigate the host response of repeatedly infected salmonids.

Since the first reported outbreaks of AGD (Munday 1986) and until recently the disease had been primarily of concern to producers of Atlantic salmon in south eastern Tasmania. Globally however, AGD has been reported in other salmonid and non-salmonid species including Chinook salmon, Coho salmon, rainbow trout, brown trout, turbot, sea bass, sea bream, ballan wrasse, olive flounder and ayu (Crosbie, Ogawa, Nakano and Nowak 2010; Dyková, Figueras and Novoa 1995; Kent, Sawyer and Hedrick 1988; Munday *et al.* 2001; Young, Dykova, Snekvik, Nowak and Morrison 2008b; Karlsbakk, Olsen, Einen, Mo, Fiksdal, Aase, Kalgraff, Skår and Hansen 2013; Kim, Cho, Lee, Huh and Kim 2005). Other reports have emerged recently from the northern hemisphere pertaining to AGD occurrence in corkwing wrasse, lump sucker and mackerel. In Tasmania, AGD was detected histologically from one of twelve wild fish opportunistically sampled from a salmon cage during 2005 (Adams, Villavedra and Nowak 2008). Earlier research (albeit of an anecdotal nature) had indicated the presence of both *Neoparamoeba* sp. and AGD-like lesions upon the gills of couta, bastard trumpeter and mullet (Jones 1988). In contrast, during 1999, a histological survey of 325 wild fishes

(comprising 12 species) collected around and within salmon cages failed to identify AGD or the causative agent within those samples (Douglas-Helders, Dawson, Carson and Nowak 2002). The same paper also suggested that AGD could not be experimentally induced in flounder or seahorses**.** Striped trumpeter farmed on a trial basis in the Huon Estuary (within AGD prone leases) during 2008/9 did not present with any mortality attributed to AGD, however these fish were not examined directly for presence of amoebae or signs of disease. There are a number of different factors that may influence the susceptibility of various fishes to AGD, some of which are likely to be interrelated. For example, fish behaviour (swimming speeds, ventilation frequencies and amplitude) may directly influence water flow velocities and patterns through the gills or indirectly through evolutionarily adaptations to gill structure. Inter-fish proximity/density/distribution will affect horizontal transmission dynamics of the pathogen. Mucosal/epithelial properties such as presence/absence/levels of antimicrobial factors, biochemical composition and influences from other microflora/fauna may also play a crucial role in *N. perurans* capacity to colonize the gills. Clearly there is a requirement to systematically examine the question of endemic fish susceptibility to AGD under experimental conditions. This could potentially identify species which are inherently resistant (or tolerant) to infection with *N.perurans*. The use of a controlled experimental environment will eliminate many of the variables described above and importantly provide a negative reference for comparison. The possibility that wild fish may be less susceptible to AGD presents a unique opportunity to compare host tissue reactivity between AGD affected salmon and various endemic species. The provision of such information would additionally prove invaluable from an epidemiological perspective regardless of the observed outcomes. Indeed the aforementioned AGD review noted "Fundamental to understanding the epidemiology of AGD is an understanding of the susceptibility of other common fish species to AGD especially those found around cages".

This project therefore aimed to examine the susceptibility of four Tasmanian endemic species to experimentally induced AGD and to describe their respective response following exposure to *N.perurans*. The four species trialled here in included species with four distinctive life histories, an estuarine/marine pelagic carnivore (Australian salmon *Arripus trutta*), an estuarine pelagic detritaphage (yellow eye mullet *Aldrichetta forsteri*), a demersal carnivore (southern sand flathead *Platycephalus bassensis*) and a reef dwelling predator that feeds primarily upon molluscs and crustaceans (purple wrasse *Notolabrus pucicola)*.

Tank based populations of Atlantic salmon exposed to initial infection by *N.perurans* have consistently resulted in AGD across the entire population. Interestingly it is well documented that a proportion of Atlantic salmon populations (both experimentally and in the field) demonstrate resistance to re-development of AGD following mitigation of the preceding infection (Findlay, Helders, Munday and Gurney 1995; Findlay and Munday 1998b). The basis for resistance is poorly understood as a definitive role for either innate or adaptive immunity has eluded previous attempts to determine their respective roles in previously infected fish. Field based studies lack the essential comparison with control fish groups held under identical conditions. To date there have been no published studies conducted under experimental conditions to that describe cellular responses of naive with previously infected fish. Considering the value of previous pathological studies to subsequent work conducted over the last 15 years, revisiting some of those aspects, in conjunction with gene expression studies could provide valuable insight into the disease process. Here we investigated the responses of Atlantic salmon, rainbow trout and Atlantic salmon-brown trout hybrids to experimentally induced AGD.

Methods

Objective 1: Determine the susceptibility of sea-cage associated endemic fishes to amoebic gill disease in comparison to Atlantic salmon

Experiments 1 – 4: AGD susceptibility trials for four endemic species with differing life histories

Four species of Tasmanian endemic fish including Australian salmon *Arripis trutta*, yellow eye mullet *Aldrichetta forsteri*, purple wrasse *Notolubrus pucicola* and sand flathead *Platycephalus bassensis* were infected experimentally with *Neoparamoeba perurans*. The fish were captured from three locations on the Tamar River by different capture techniques during March, April, May and June 2012 (Table 1).

Table 1. Endemic species ID, weight, length, capture techniques and location collected

Fish Species	Common Name	Mean Weight	CV% Weight	Mean Length	Capture Method	Grid Reference
A.trutta	Australian Salmon	145 g	53.1	208 mm	Hook and Line	41.05.09 & 146.48.20
A.forsteri	Yellow eye Mullet	149 g	13.4	229 mm	Box Net	41.09.56 & 146.51.51
N.pucicola	Purple Wrasse	274 g	28.2	257 mm	Hook and Line	41.05.09 & 146.48.20
P.bassensis	Sand Flathead	124 g	34.3	278 mm	Hook and Line	41.08.54 & 146.51.24

Australian salmon, wrasse and flathead were caught by hook and line using a sabiki rig with barbless hooks. These fish were quickly removed from the rig after capture and placed in a sedative bath (3 $\mu l.l^{-1}$ clove oil). Once sufficient fish were caught they were transferred to a 750 L fish transporter filled with sterilized seawater with sedative (1 $\mu l.l^{-1}$ clove oil). Upon arrival at the Universtiy of Tasmania's northern aquaculture research and teaching facility (based at Launceston) the fish were further sedated in the transport unit until loss of equilibrium. The fish were then transferred to a 2000 L holding system until the commencement of each experiment (seven days). Mullet were caught by a box net from an Atlantic salmon production cage during routine grading.

Atlantic salmon (183g, CV 24.6% Expt 1; 182g, CV 25.1% Expt 2; 260g, CV 14.9% Expt 3; 278g, CV 27% Expt 4) were from a single cohort of smolt originally sourced from Saltas hatcheries during September 2011. These fish were held in freshwater (15°C ± 2°C) until required for each experiment where upon each group (n=60) was acclimated to 35 ‰ seawater over a three week period.

A twin tank (2 x 1000 l) experimental recirculating system (Figure 2) was constructed in duplicate to house and expose fish during each trial. Each system was comprised of 2 x 1000 l conical tanks (one for Atlantic salmon n=20 and one for each native species n=20), a 300 l conical submerged/moving bed biological filter, particulate filtration bed, foam fractionator and UV disinfection. For each trial the water temperature was maintained at 16.5°C (17.5°C for flathead), salinity at 33-35‰, pH 8.2, total ammonia $\leq$1.0 $mg.l^{-1}$. All seawater used during the experiment was chlorinated at 5 $mg.l^{-1}$ overnight and de-chlorinated using sodium thiosulphate. Fish were fed to satiation twice daily using a 3-4mm sinking pellet (Skretting Spectra SS).

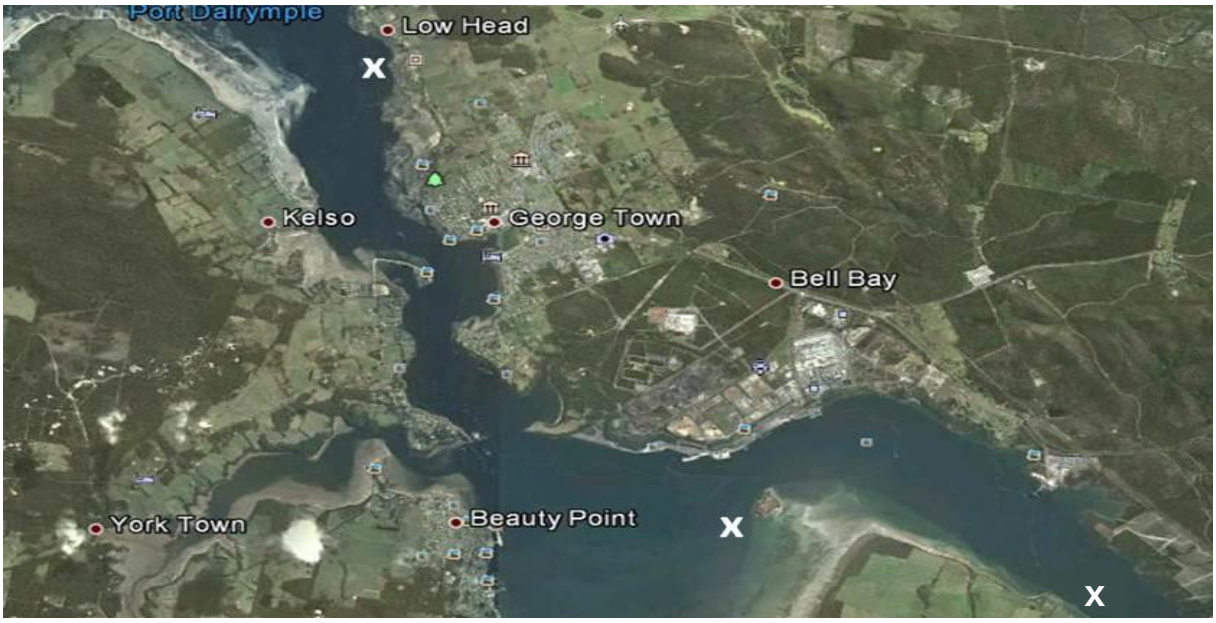


Figure 1. Location of each endemic fish collection on the Tamar River. The white crosses indicate positions for capture of Purple Wrasse (south of Low Head), Sand Flathead (first cross east of Beauty Point), Mullet and Australian Salmon (second cross east of Beauty Point at Van Diemen Aquaculture, both within and next to commercial salmon cages, Rowella).

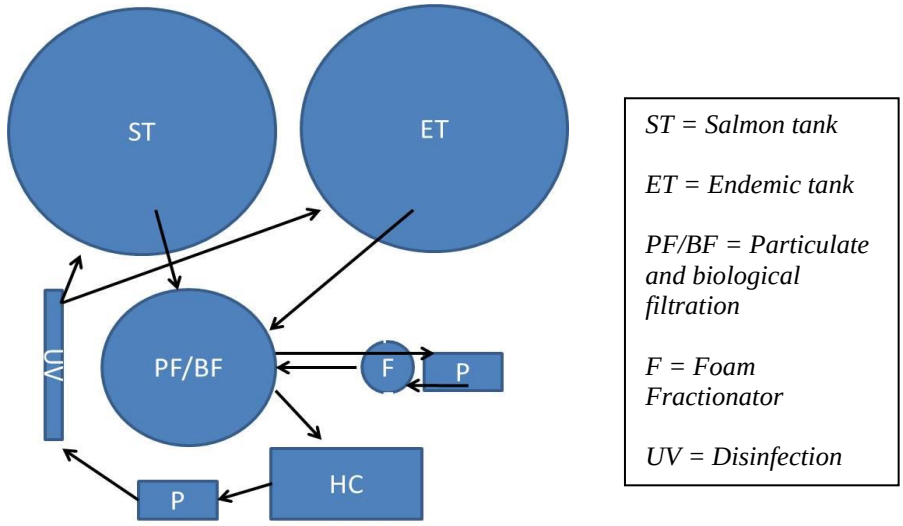


Figure 2. Recirculating system configuration for experiments 1 – 4.

To experimentally induce AGD, *Neoparamoebae perurans* were isolated from the gills of Atlantic salmon sourced from an ongoing infection using methods adapted from Morrison, Crosbie and Nowak (2004). Briefly, moribund or dead fish were removed from an ongoing infection, anaesthetised if moribund until movement ceased (clove oil 30 $\mu l.l^{-1}$) and killed by brain spiking. Excised gill arches were gently agitated with distilled water and the suspension added to an equivalent amount of double strength seawater (70 ppt). The solution was then overlaid in several petri dishes, left for 30-60 min, rinsed and half filled with antibiotic supplemented seawater for 12 -18 hr. Trophozoites were detached with trypsin or distilled water and enumerated with a haemocytometer.

Fish were unfed for 24 h prior to the introduction of *N. perurans* to the experimental system. Immediately preceding exposure fish weights, lengths, condition, gills, blood and mucus samples (gill sub-samples and skin mucus were provided to Dr Ben Maynard CSIRO, FRDC Project # 2011/071) were collected from 20 salmon and 20 endemic fish. Fish (in one system) were subsequently exposed to *N. perurans* for eight hours at concentrations of 1100, 735, 755 and 850 trophozoites.l^{-1} for experiments 1-4 respectively. The other system did not have any amoebae added but was managed identically as follows: Immediately prior to the exposure period, each experimental system's circulation was turned off and the tank volume decreased to 500 l. Aeration was supplied to ensure vigorous water mixing and sufficient oxygenation during this period. Following the exposure period the experimental system's circulation and filtration were resumed. For experiment 1 the final samples were collected seven days after exposure to *N.perurans* and 10 days post exposure for experiments 2-4.

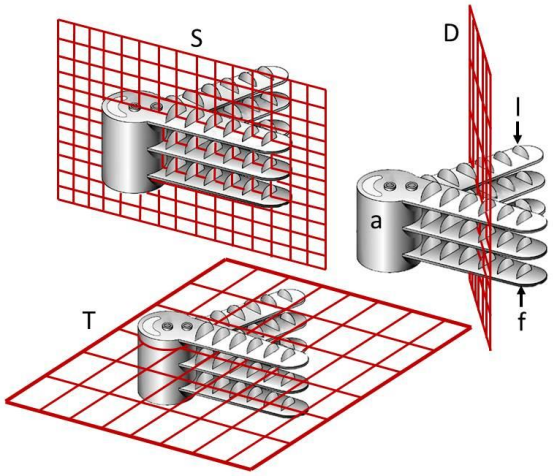


Figure 3. Orientation of gills for histological sectioning in saggital (S), dorsal (D) or transverse (T) planes (a – arch, f – filament, l – lamella). Filament diagram modified from: https://allyouneedisbiology.wordpress.com/tag/suprabranchial-chambers/

To facilitate the collection of samples from each fish, the system pumps were turned off. Clove oil (3µl/l) was then injected into the tank water's aeration stream and allowed to mix (5 min) at which point fish became sedated sufficiently for easy removal from the tank. Fish were then transferred to a 100L tub containing 30 µl.l^{-1} clove oil. Following loss of reactivity by the fish to touch (2-4 mins), the weight and length was measured, blood collected from the caudal vein and the gill basket excised, rinsed and holobranchs allocated to three fixative (SW Davidson's [< 24h since prep], 10% NBF & RNA-Later). The second left and second right anterior hemibranch (fixed in SW Davidson's fixative and NBF respectively) were processed for routine histology. Holobranch sections (4µm) were cut initially in the saggital plane (FIGURE X) and then re-orientated for sectioning in the dorsal plane (Dalum, Austbø, Bjørgen, Skjødt, Hordvik, Hansen, Fjelldal, Press, Griffiths and Koppang 2015) . To determine the infection burden (or relative number of trophozoites) present upon selected holobranchs, (n = 10/group) the first-right holobranchs (fixed in RNA-later) were processed for qPCR (Wright, Nowak, Oppedal, Bridle and Dempster 2015). Each reaction consisted of 5 µL 2X MyTaq HS Mix (Bioline), forward and reverse primers (400 nM each), Taqman probe (100 nM) and 2 µL of DNA template in molecular grade water to a final volume of 10 µL. Cycling conditions consisted of an initial activation of DNA polymerase at 95°C for 3 min, followed by 40 cycles of 5 s at 95°C and 30 s at 55°C. Assay results were quantified by analysis of raw fluorescent unit (rfu) data using the CM3 mechanistic model included in the qPCR package (v. 1.4-0) for R Studio statistical computing software.

Experiment 5 – Comparative sequential pathology of AGD in Atlantic salmon <u>Salmo salar</u> and yellow eye mullet <u>Aldrichetta forsteri</u>

Atlantic salmon (mean initial weight = 565g, CV = 17.4%), naïve to AGD and yellow eye mullet (mean initial weight = 205g, CV = 24.8%) were cohabited in two separate twin tanks (2 x 2000 l) recirculating systems (Figure 4). Mullet were seine-netted from commercially stocked salmon cages (Van Diemen Aquaculture), transferred immediately to a fish transport unit, then transferred and housed as previously described. Atlantic salmon (naïve to AGD) were from the same cohort source as experiments 1 - 4 and were acclimated to seawater as previously described. Water temperature was maintained at 16.5°C, salinity at 35‰, pH 8.2, total ammonia ≤1.0 mg.l^{-1}. All fish were feeding well at the commencement of the experiment. All seawater used during the experiment was chlorinated at 5 mg.l-1 (2-4 h) and de-chlorinated using sodium thiosulphate before addition to experimental tanks. Prior to the introduction of *N. perurans* to one of the systems, the weights, lengths, condition, gills,

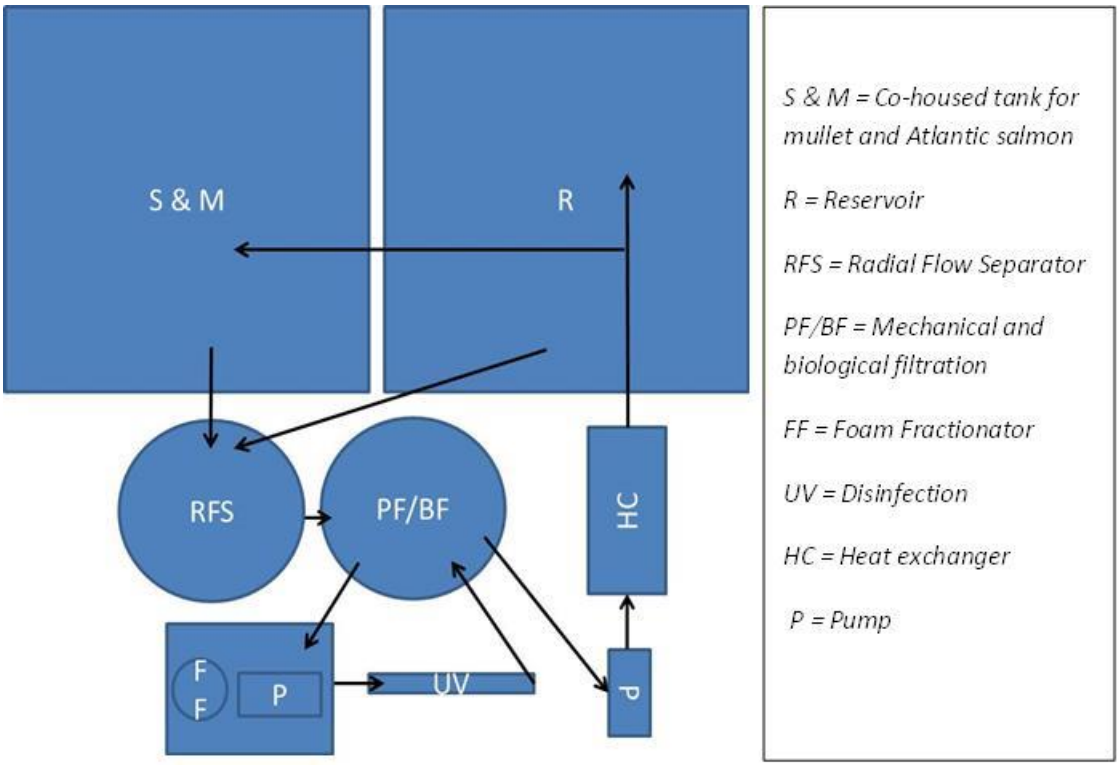


Figure 4. Recirculating system configuration used for housing fish during Experiment 5.

blood and mucus samples (gill sub-samples and skin mucus were provided to Dr Ben Maynard CSIRO, FRDC Project # 2011/071) were collected from 10 salmon and 10 mullet. Fish (in one system) were subsequently exposed to *N. perurans* for eight hours at a concentration of 500 amoebae.l^{-1}. No trophozoites were added to the other system but it was managed identically as follows. Immediately prior to the exposure period, each experimental system's circulation was turned off. Aeration was supplied to ensure vigorous water mixing and sufficient oxygenation during this period (DO_{sat} >80%). Following the exposure period each system's circulation and filtration were resumed. As per Table 2 for each trial samples (as described above) were collected 3, 7, 14, 28 days after exposure. There was an additional collection of samples (not initially planned) on day 21 from the mullet only. After 14 days of exposure, 10 salmon (mean initial weight 205g, naïve to AGD, different cohort) were added to each system to assess the presence/absence and intensity of an ongoing amoebic infection. Sample collection and analysis were undertaken as per experiments 1 – 4.

Table 2 Numbers and days of gill samples collected for Experiment 5.

	Days post exposure						
Species	0	3	7	14	21	28	28*
Salmon -ve	5	10	10	10	0	10	10
Mullet -ve	5	10	10	10	10	10	na
Salmon +ve	5	10	10	10	0	10	10
Mullet +ve	5	10	10	10	10	10	na

* Atlantic salmon naïve to AGD introduced to the population on day 14

Pathological responses for exposed and unexposed fish populations were analysed separately by two-way ANOVA with day (six levels) and species (2 levels) as the main effects. As an interaction was observed ($P < 0.001$), Tukeys post hoc test was used to investigate differences between days and species. Assumption of normality was checked with the Shapiro-Wilk test and the data homogeneity checked by Levene's test. A *p* value of < 0.05 was adopted for the rejection of the null hypothesis and the significance level lowered to 0.01 [19] where assumptions of normality or homogeneity failed. Naïve Atlantic salmon introduced to the challenge system on day 14 were not included in these analyses.

Additional sampling and analysis was undertaken to investigate whether AGD affects the recently described interbranchial lymphoid tissue, a secondary lymphoid structure located between hemibranchs and extending as a diffuse mucosal lymphoid tissue distally along predominatly the trailing edge of filaments (Dalum *et al.* 2015; Haugarvoll, Bjerkås, Nowak, Hordvik and Koppang 2008; Kim *et al.* 2005). The first left holobranch (from Atlantic salmon only) was dissected (post-transfer to 70% EtOH) to excise three anterior and posterior filaments from the dorsal, medial and ventral regions. The excised filaments were processed, embedded and sectioned in the transversal plane (Koppang, Fischer, Moore, Tranulis, Dijkstra, Köllner, Aune, Jirillo and Hordvik 2010). Sections were stained with H&E and images were captured basally and apically from proximal interbranchial lymphoid tissue (PILT) at 1000x magnification. Lymphocyte numbers (lymphocytes.100 μm^2) were quantified manually using image analysis software (ImageJ - Kurt De Vos, University of Sheffield, Academic Neurology http://imagej.nih.gov/ij/plugins/cell-counter.html). The area and length of the ILT were measured from images captured at 100X magnification. PILT length was measured between the basal membrane and the apical extremity of the ridge. Additional sections from fish sampled from exposed and unexposed groups at both 14 and 28 DPE (n=3/group) were immune-histochemically probed for proliferating cell nuclear antigen (PCNA). Sections (5µm) were mounted on poly-L-lysin-coated slides (PolysineTM, Menzel-Gläser, Germany), dewaxed and rehydrated, immersed in citrate buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0), microwaved (12 min), rinsed in phosphate buffered saline (PBS), blocked (5 min – 30% H_2O_2), rinsed, incubated (PCNA 1:700, Sigma-aldrich, NSW, Australia, 30 min), rinsed, incubated with polymer/peroxidase (EnVison™ - Dako corp., Carpinteria, CA, USA, 10 min), rinsed and incubated with DAB to develop the visualizing chromogen (≈5 min). The reaction was stopped with a dH2O rinse and the section was then counterstained with hematoxylin (5 s), rinsed in tap water, dehydrated in in an ethanol series, cleared in xylene and mounted with DPX.

Lymphocyte numbers, ILT area and length were analyzed by three-way analysis of variance (ANOVA) with treatment (2 levels), day (5 levels) and gill site (3 levels) as the main effects. Assumption of normality was checked with the Shapiro-Wilk test and the data homogeneity checked by Levene's test. A *p* value of < 0.05 was adopted for the rejection of the null hypothesis and the

significance level lowered to 0.01 [19] where assumptions of normality or homogeneity failed. Where a 3-way interaction was found the data were analyzed separately for each gill site by 2-way ANOVA. Tukey's honest significant difference (HSD) was used for post-hoc analysis. A regression analysis was used to assess the relationship between AGD severity and the area and length of the ILT of infected fish. All statistical analyses were performed with SPSS ® (IBM SPSS Statistics 20, SPSS Science.

Objective 2: Investigate the comparative host responses of Atlantic salmon and rainbow trout naive and previously exposed to amoebic gill disease

Experiment 6: Comparative pathology of AGD and immune gene expression in Atlantic salmon Salmo salar and rainbow trout Oncorhynchus mykiss following repeated infections with Neoparamoeba perurans.

Fish holding and acclimation pre-challenge:

Atlantic salmon (n= 307, mean weight 55g) and rainbow trout (n=299, mean weight 64g) were collected from a commercial hatchery site (Petuna Seafoods Ltd, Cressy) on March (20th) 2013. After sedated transfer (1 µl.l^{-1} clove oil) to the Aquaculture Centre (University of Tasmania, Newnham) the fish were split and the species mixed then divided evenly between two twin tank (2 x 2000 l) freshwater recirculating systems previously used with a modified filtration and disinfection configuration (

Figure 5). One month period of holding/habituation to the systems then ensued. Fish were fed a 3 mm sinking pellet at 2% BW/day (Skretting Spectra & Spirit Supreme). Water temperature was maintained at 15.5°C, salinity 0‰, pH 6.8-7.2, TA-N ≤1.0 mg.l^{-1}, NO_2 < 2 mg.l^{-1}, NO_3 ≤ 40 mg.l^{-1} and DO_{sat} >85%.

Following habituation the fish were lightly sedated (5µl.ml^{-1} clove oil) and transferred in groups of ten to a 100 l tub filled with water (from the same tank the fish were removed from) and a sufficient amount of anaesthetic to attain a concentration of 30 µl.l^{-1}. Once the fish were unresponsive a small incision was made anteriorly of the right pelvic fin with a sterilized scalpel. A sterile passive integrated transponder (PIT) tag was inserted through the incision which was dressed with a sealant mixed with antibiotic (2% w/w Tricon). Approximately 20 larger rainbow trout were removed from the experiment at this time. The fish were not fed for the following two days and strong feed response was noted thereafter.

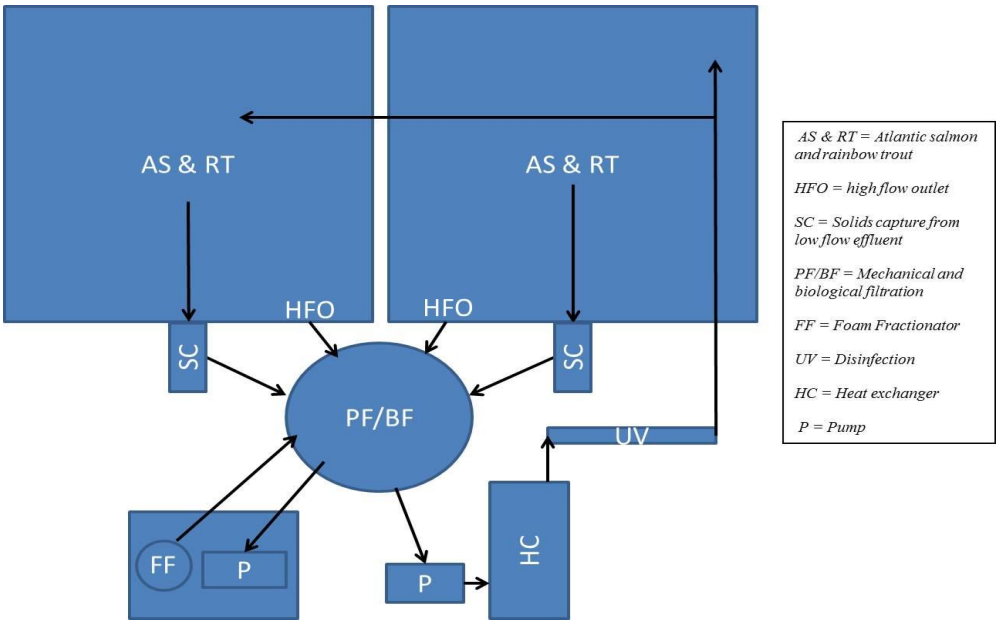


Figure 5. Modified recirculating system configuration used for housing mullet and Atlantic salmon during Experiment 6.

A salinity acclimation period of four weeks was planned prior to the first challenge with *N.perurans* however three significant issues arose that lengthened this phase of the experiment considerably. Firstly as the salinity was increased in each system the healing tag incision sites showed signs of inflammation and ulceration in approximately 15% of both the salmon and trout. To maintain a clean wound site the fish were bathed (15-16°C, pH 7.0, hardness <20 mg.l^{-1} $CaCO_3$, clove oil 1.5 µl.l^{-1}) approximately weekly over a five week period as the salinity was gradually increased. This problem recurred approximately four weeks later in a smaller proportion of fish shortly after an increase in salinity from 25 to 28 ppt. This was eventually mitigated with a combination of freshwater treatments and infeed administered antibiotics (Terramycin). Secondly, feeding behaviour observations were indicating that rainbow trout were consistently holding a higher station within each tank and were becoming noticeably larger than the salmon. A weight check undertaken at the end of May confirmed that rainbow trout had doubled the weight of cohabiting salmon. The populations were therefore segregated and the trout ration restricted to 1% BW/day. Thirdly, simultaneous to increasing system salinities above 28 ppt, a dribbling mortality of rainbow trout began appearing. The salinity was decreased to 25 ppt and the mortality ceased but recommenced as the salinity was re-elevated. Moribund specimens exhibited high serum osmolality values (> 450 mosm.kg^{-1}). Owing to the substantial amount of time taken to prepare fish for amoebic challenge the decision was taken to commence the challenge phase conceding that rainbow trout may need to be withdrawn from the experiment. Subsequently any rainbow trout seen unresponsive to feed input, isolated from other fish and sluggishly responsive to external stimuli were captured and returned to a freshwater holding system. Figure 6 describes the timeline and interventions for the duration of the experiment. At the completion of the acclimation phase, the fish were sedated and then transferred temporarily to a single tank (with oxygenation) and randomly reallocated to either a twin tank 2x1000 l system (Figure 2) or returned to the one of the original systems. Any poorly conditioned or otherwise unhealthy fish were euthanized by anaesthetic overdose (30µl.l^{-1} clove oil > 20 min). Following re-allocation the combined stocking density of salmon and trout in the 4000 l system prior to infection with *N.perurans* was 16.9 kg.m^3. The unexposed group within the 2000 l system had a stocking density of 9.3 kg.m^3. Figure 7 details the challenge timeline post-acclimation.

Table 3. Mean fish weight, length and condition factor (K) and numbers (n) during experiment 6.

Event	**Atlantic salmon**				**Rainbow trout**			
	Weight g	**Length mm**	**K**	**n**	**Weight g**	**Length mm**	**K**	**n**
Hatchery transfer	55	-	-	307	64	-	-	299
PIT Tag insertion	70	183	1.15	297	82	185	1.18	281
First Challenge (unexposed)	181	245	1.28	50	231	267	1.19	40
First Challenge (exposed)	188	242	1.28	181	232	269	1.21	151
Final Sampling (unexposed)	516	330	1.41	21	n/a	-	-	n/a
Final Sampling (exposed)	397	306	1.26	174	n/a	-	-	n/a

First exposure Neoparamoeba perurans

Following the acclimation period, fish in the 4000 l system were exposed to *N.perurans* at 194 trophozoites.l^{-1} under the same exposure conditions described for experiment 5. At three weeks post-exposure, 10 fish Atlantic salmon and rainbow trout were removed by dip-netting, anaesthetised with clove oil (30 µl.l^{-1}) and inspected for gross gill lesions to confirm progression of the infection to a diseased state. One week later all the fish in each tank and system were sedated (4µl.l^{-1} clove oil) and

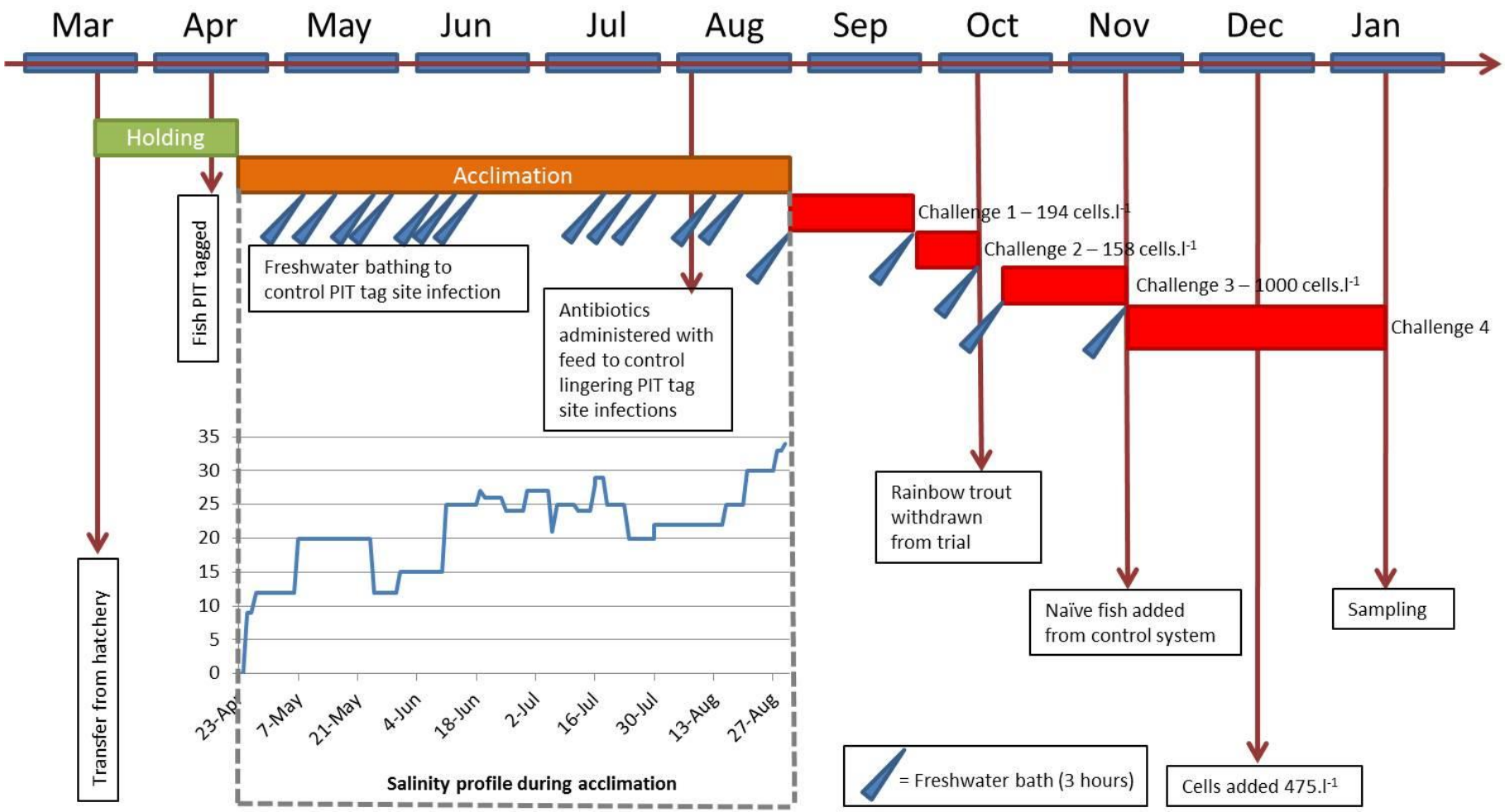


Figure 6. Timeline for Experiment 6

	Aug-30	Sep-26	Oct-14	Oct-23	Nov-17	Dec-17	Jan-14	Jan-14
Gill Health	**Start**	**GC & Bath**	**GC & Bath**	**Bath only**	**GC & Bath**	**GC only**	**GC Repeats**	**GC Naive**
Clear %	100	0	100	na	0.0	70.0	6.9	0
Light %	0	36	0	na	15.4	30.0	39.6	25
Moderate %	0	61	0	na	50.7	0.0	40.3	44
Heavy %	0	3	0	na	33.8	0.0	6.9	31

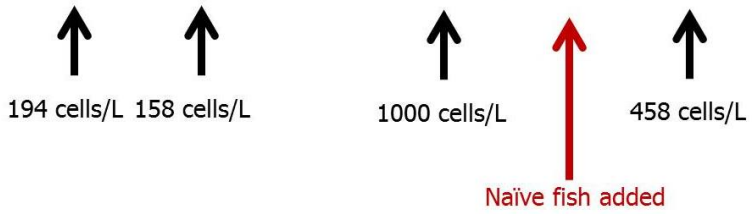


Figure 7 Challenge timeline. GC = Gross gill lesion assessment. Repeats = Fish previously challenged with *N.perurans*. Naïve = Sourced from fish maintained under identical husbandry conditions until challenge.

incrementally (10 fish at a time) transferred to a 100 l tub with a higher anaesthetic concentration (30 ul.l^{-1}) until unresponsive (3-5 minutes). After scanning the fish for the PIT tag code, the gills were inspected and the clinical severity based on the number and coverage of macroscopic lesions was recorded. The severity of disease expression was interpreted macroscopically (Figure X) as being either:

- Clear (no lesions observed)
- Light (one or more small lesions affecting one or more hemibranchs viewable hemibranchs).
- Moderate (multiple lesions affecting all hemibranchs)
- Heavy (lesions affecting all hemibranchs with substantial coverage)

All fish were then bathed in freshwater for three hours as described above. The experimental tanks were simultaneously drained, cleaned and refilled with sterilized seawater (chlorinated with sodium hypochlorite [5 mg.l^{-1} total chlorine] for two hours and de-chlorinated with sodium thiosulphate) adjusted to the correct temperature and salinity. At the completion of the bath, the clove oil concentration was increased to further sedate fish for transferring back to the experimental tanks. The unexposed group were handled and treated in an identical manner.

Second exposure to Neoparamoeba perurans

Immediately following the recovery of fish from bathing (gauged by a suitably strong response to a small feed input) the fish were re-exposed to *N.perurans* at 158 trophozoites.l^{-1} without stopping system flow but removing mechanical filtration and UV disinfection for 24 hr. After 17 days a preliminary macroscopic gill assessment was performed to determine AGD status. All fish were subsequently bathed and returned to experimental tanks and re-bathed one week later once a sufficient number of amoebae were harvested.

Third exposure to Neoparamoeba perurans

Fish were re- exposed to amoebae (1000 trophozoites.l^{-1}) again under the same conditions as the first challenge. Ten days later the rainbow trout were removed from the experiment due to ongoing morbidity. Three weeks post re-exposure another preliminary macroscopic assessment was undertaken and the fish immediately bathed and returned to experimental systems as above. Twenty fish from the unexposed group were transferred to the system previously inoculated with amoebae to serve as a naïve infected comparison.

Final exposure to Neoparamoeba perurans

Fish were returned to the experimental systems without the addition of gill isolated amoebae. This approach has been used previously (Vincent, Morrison and Nowak 2006; Adams, Crosbie and Nowak 2012) albeit assuming that amoebae surviving upon the gills post-treatment would reinitiate disease in a manner partially mimicking a field re-infection (Adams and Nowak 2004b). This approach was pursued for one month, at which point all fish were sedated and inspected for macroscopic gill lesions and then returned to the experimental systems. Additional amoebae (475 trophozoites.l^{-1}) were then introduced to the this system as described above. The sub-sample of fish were checked (n=20) three weeks after the addition of amoebae for gross signs of AGD and all fish were subsequently sampled one week later.

Collection of samples

To facilitate the collection of fish samples, each tank level was dropped by 50% and fish were carefully removed by dip net (n = 10 per collection) and transferred to a 100 l tub containing seawater from the same tank and clove oil (30 µl.l^{-1}). Once the fish were unresponsive to handling their weights, lengths, gill conditions and any anomalous external signs were recorded. Blood was collected laterally from the caudal vein above the anal fin, the gills were excised and divided between solutions of SW Davidson's fixative (all LHS holobranchs), neutral buffered formalin (1^{st} & 2^{nd} RHS holobranchs) and RNA-later (4 M ammonium sulphate, 25 mM sodium citrate, 10 mM EDTA, pH 5.2)(3^{rd} RHS holobranch). Samples collected in RNA-later were temporarily stored at 4^0C before freezing (-20°C).

Selection of fish groups for histopathological and comparative gene expression analysis

Four clinical history patterns represented by seven fish / pattern were identified and selected for sub-sample collection:

1. *Clear* - No macroscopic lesions detected upon final assessment regardless of clinical history
2. *Light* - Severity deemed clinically light by gross gill inspection
3. *Moderate* - Severity deemed clinically moderate by gross gill inspection
4. *Naïve moderate* - Fish previously naïve to infection with AGD severity deemed clinically moderate by gross gill inspection.

Sub-samples were also collected from fish (n = 7) unexposed to amoebae (categorized as *control*). Fish of approximately the same size (~ 409 g, ~ 315 mm) that satisfied the above criteria were selected for further analysis. RNA-later preserved gill holobranchs were photographed anteriorly and posteriorly through a stereomicroscope. Each gill arch was punched twice with a 2 mm biopsy punch. One punch, classified as lesion (-), of gill tissue with a normal appearance was taken from positions with no visible lesion (CROSS REF). The second biopsy punch, classified as lesion (+),was collected

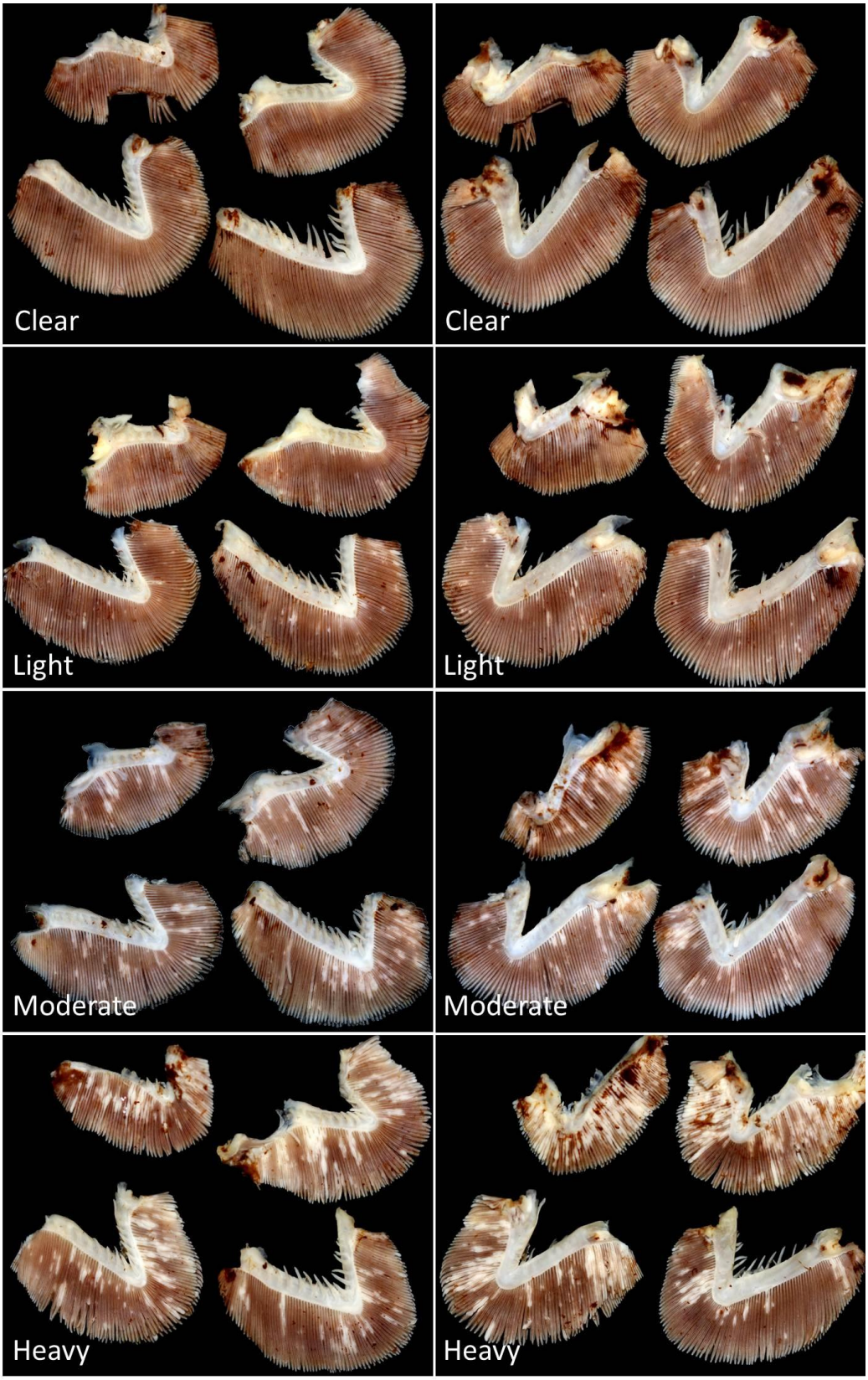


Figure 8. . Representation AGD severity categories (clear, light, moderate, heavy) based on the grossly observable presence of gill lesions and the extent of lesion size/coverage.from lesion affected tissue.

For the categories *clear* and *control* tissue punches were collected from proximally to medially area relative to the gill arch.

RNA extraction and cDNA synthesis

Total RNA was extracted from semi-dried gill tissue punches, using 400 µL of nucleic acid extraction buffer (4M urea, 1% SDS, 0.2 M NaCl, 1 mM sodium citrate pH 8.2) containing 20 U of proteinase K (Bioline), homogenised manually with a micro-tube pestle on ice. Following protein digestion (30 min), centrifugation (4^{0}C, 16,000x g, 3 min) in 7.5 M ammonium acetate (eliminating cellular debris and detergent) a≈nd isopropanol precipitation (room temperature, 16,000 x g, 8 min), RNA was resuspended in 190uL RNAase-free water, heated 55^{0}C resuspended and re-precipitated in 10 µL 7.5M LiCl and 400 µL 100% ethanol and spun (16,000 x g for 10 min). After centrifugation the supernatant was decanted and pellet was resuspended in 180uL RNAase-free water, heated 37^{0}C and resuspended.

DNAse buffer (20uL 10x) was added at 37^{0}C and completely removed after dual treatments with Baseline-Zero DNAse (2 units each, 15 and 30 min respectively, 37^{0}C). After DNAse treatment, RNA was re-precipitated in 10 µL 7.5M LiCl and 400uL 100% ethanol, briefly vortexed, spun at 16,000 x g

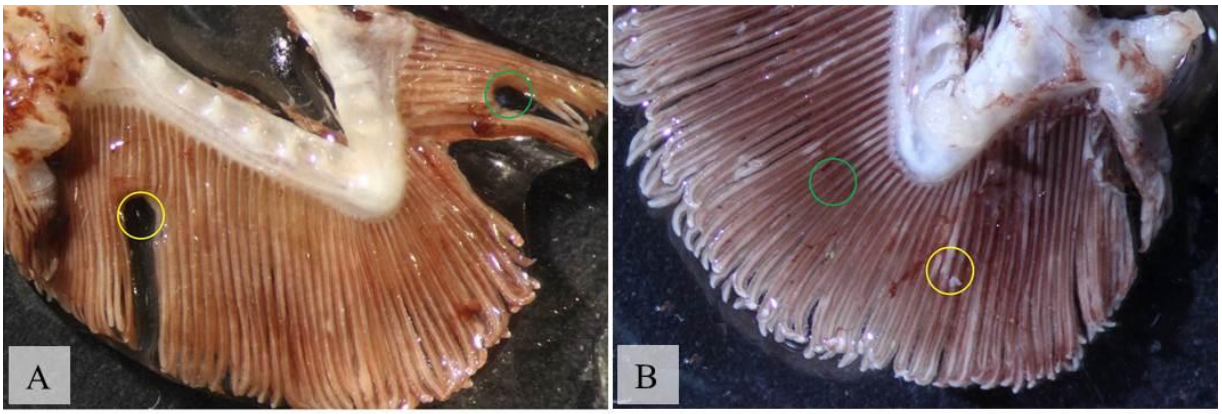


Figure 9. (A) RNA-Later fixed holobranch following removal of tissue by a 2mm biopsy punch. Each punch extracted tissue from 3-4 filaments. (B) Example of normal (green circle) and a lesion (yellow circle) targeted for biopsy.

for 10 min at RT and finally washed 1x with 70% ethanol. The DNA-free total RNA was re-suspended in 15 µL RNAase-free water + dTT, quantified using a Qubit fluorometer (Invitrogen) and an aliquot run on a 1% Agarose gel to verify integrity of the total RNA. All extracted RNA samples showed no sign of degradation and had well-defined 28S and 18S rRNA bands the former displaying twice the intensity of the latter.

After RNA extraction 1µg of total RNA was reverse transcribed into cDNA using a cDNA Synthesis Kit (Bioline) with Oligo $(dT)_{18}$ primer mix as per manufacturer instructions. Controls, lacking reverse transcriptase, were run to confirm that genomic DNA contamination did not occur after RNA extraction and DNAse treatments.

Quantitative RT-PCR and data analysis

A CFX Connect Real-Time PCR detection system (Bio-Rad) was used for quantitative RT-PCR. SYBR green chemistry was used to measure expression of target genes. Each PCR reaction master mix consisted of 2x polymerase/syber mix (SensiFast) and forward and reverse primers (400 nM

each). The mastermix (8 µL) was transferred to each column of the plate and 2 µL cDNA template in molecular grade water was added to produce a final volume of 10 µL. Cycling protocol included an initial activation of DNA polymerase at 95^{0}C for 3 min, followed by 40 cycles of 5 s at 95^{0}C, 10 s at 60^{0}C and 10 s at 72^{0}C. All samples were run in duplicates and at the end of the cycling protocol melt curve analyses were conducted to ensure amplification specificity. Cycle threshold (Ct) values were copied to an excel spread sheet and data imported into qBasePLUS® analysis software (Biogazelle, Zwijnaarde, Belgium). To normalise mRNA expression results, the means of two reference gene elongation factor 1alpha (EF1α) and beta actin (β-actin) were used. One-way analysis of variance assessed differences in immune gene mRNA expression among different groups. Values were considered significantly different where $p < 0.05$.

Gross and Histopathological Assessment

To gauge the relative severity of AGD for each fish after the final challenge, all holobranchs were excised from the left side of the gill basket 24-48 h post-fixation in SW Davidson's fluid, immersed in EtOH (70% v/v) and immediately digitally photographed. Images were processed and analysed as detailed in the figure below.

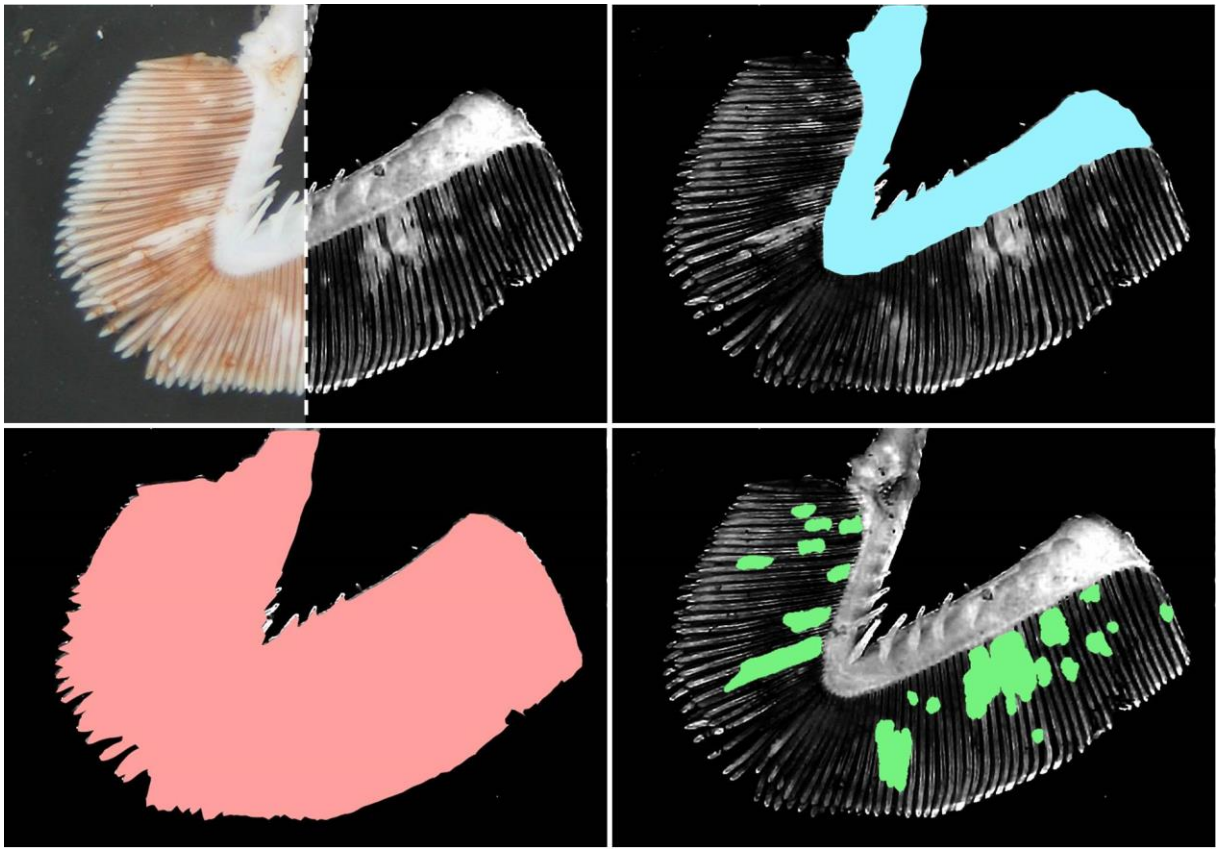


Figure 10 Image processing and analysis to assess the relative severity of AGD for each fish. Top right – The original image is converted to greyscale, sharpened and contrasted. The pixel count from the arch (in blue) is deducted from the viewable holobranch pixel count (bottom right) to give the total filament area; grossly visible lesions are also enumerated and expressed as a percentage of the total filament area. The process is repeated for all LHS anterior and posterior hemibranchs.

Relative severity values based on the proportion of the filament area displaying proliferative changes was analysed by one-way ANOVA and Tukey's multiple comparison test. A Mann-Whitney two-tailed U-test was used to assess differences between anterior and posterior holobranchs for each group. Data were arcsin transformed to ensure variance homogeneity (Levene's test).

The second left gill arch was dissected from the gill basket, arch excised and placed into biopsy pad-lined tissue cassettes and immersed in 80% ethanol until processing. Tissues were dehydrated, infiltrated in turn by xylene and paraffin, placed into wax moulds and sectioned (5µm) initially in the saggital plane and then in the dorsal plane. H&E sections were assessed for the presence and length (interlamellar units - ILU)(Adams and Nowak 2001) of hyperplastic lesions upon each filament and whether lesions were colonised by amoebae and values expressed as a percentage of either the total filament or lesion count. Microscopic observations in the sagittal plane were made qualitatively but systematically from four key branchial regions:

1. Lamellae
2. Basal filamental epithelium
3. Central venous sinus and drainage vessels
4. Connective tissue and vasculature surrounding the cartilage

Each region was examined for micro-structural deviation from normal morphologies expected for these tissues relative to the unexposed group. Differences between groups for histological values were assessed by One-way ANOVA and Tukey's multiple comparison test ($P < 0.05$) where assumptions of variance and normality were met.

Experiment 7: Comparative pathology of experimentally induced AGD in pure and hybrid Atlantic salmon <u>Salmo salar</u> and brown trout <u>Salmo Trutta</u>

On August 7 & 8 2013 crosses of Atlantic salmon (SxS), Atlantic salmon x brown trout (SxT), brown trout x Atlantic salmon (TxS) and brown trout (TxT) were transferred from a commercial hatchery (TASSAL Ltd - Russell Falls) to University of Tasmania (Maynard, Taylor, Kube, Cook and Elliott 2016). The fish were housed in separate 4000L Rathbun tanks (one cross per tank) sharing a common recirculating fresh water source. The fish were provided with continuous light (24:0 L:D) and fed twice daily to satiation for the following eight weeks (holding period). Fish were maintained during this period with an increasing ambient temperature range (10 - 13.5°C), total ammonia-nitrogen (TA-N) < 0.5 mg.l^{-1}, nitrite (NO_2) < 0.5 mg.l^{-1}, nitrate (NO_3) < 50 mg.l^{-}1, pH 6.6-7.2 and dissolved oxygen saturation (DO_{sat}) >80%. After three weeks the fish were tattooed with a sub-cutaneous injection of dye (2% w/v Alcian Blue) at the base of either the left or right pectoral or pelvic fins providing a unique identifier for each group.

Five weeks later, the fish in each tank were lightly sedated (2.5 mg.l^{-1} clove oil) and 10 fish were dip-netted from each tank and transferred to a single 400L tub containing aerated seawater at 35 ppt for 24 hours. The fish were subsequently anaesthetised (30 µl.l^{-1} clove oil) and blood collected from the caudal vein to assess osmoregulatory competence. Simultaneously, three groups of 20 fish from each tank were transferred into one tank of a twin tank (2 x 1000 l) system (figure 2) or into both tanks of a 2 x 2000 l system (

Figure 5). Therefore each tank was allocated 20 fish from each cross for a mixed cross population total of 80 and 160 fish per system at an equivalent initial stocking density (2.5 kg/m^3). The timeline and experimental design is summarized in Figure 11 and Figure 12. During the acclimation phase the water temperature in each system was increased to 16°C, salinity from 22 - 35‰, and pH from 7.6 - 8.2. TA-N was maintained at ≤1.0 mg.l^{-1}, NO_2 ≤ 5 mg.l^{-1}, NO_3 ≤ 80 mg.l^{-1} and DO_{sat} >90%.

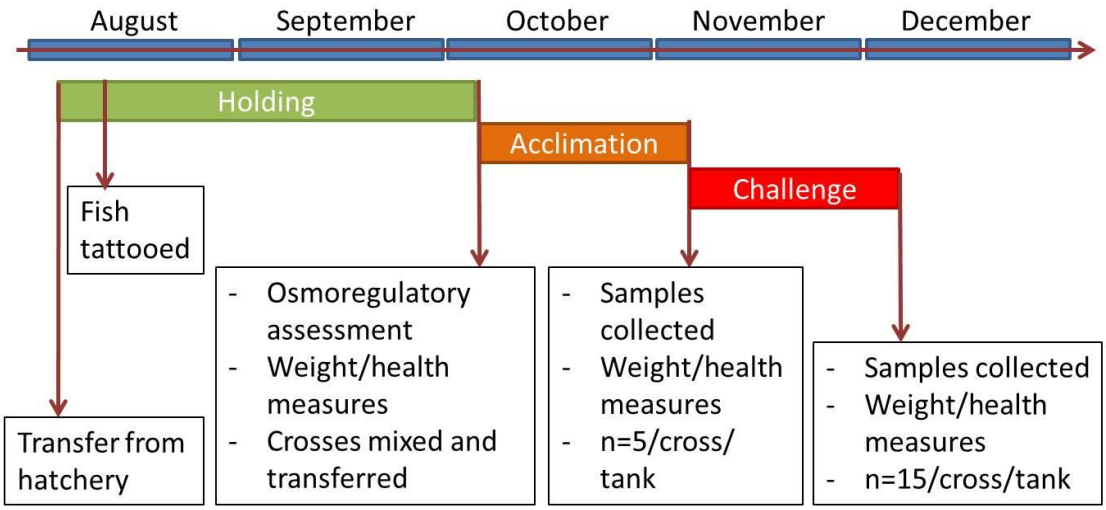


Figure 11. Timeline for experiment 7 inclusive of transfer, acclimation and challenge.

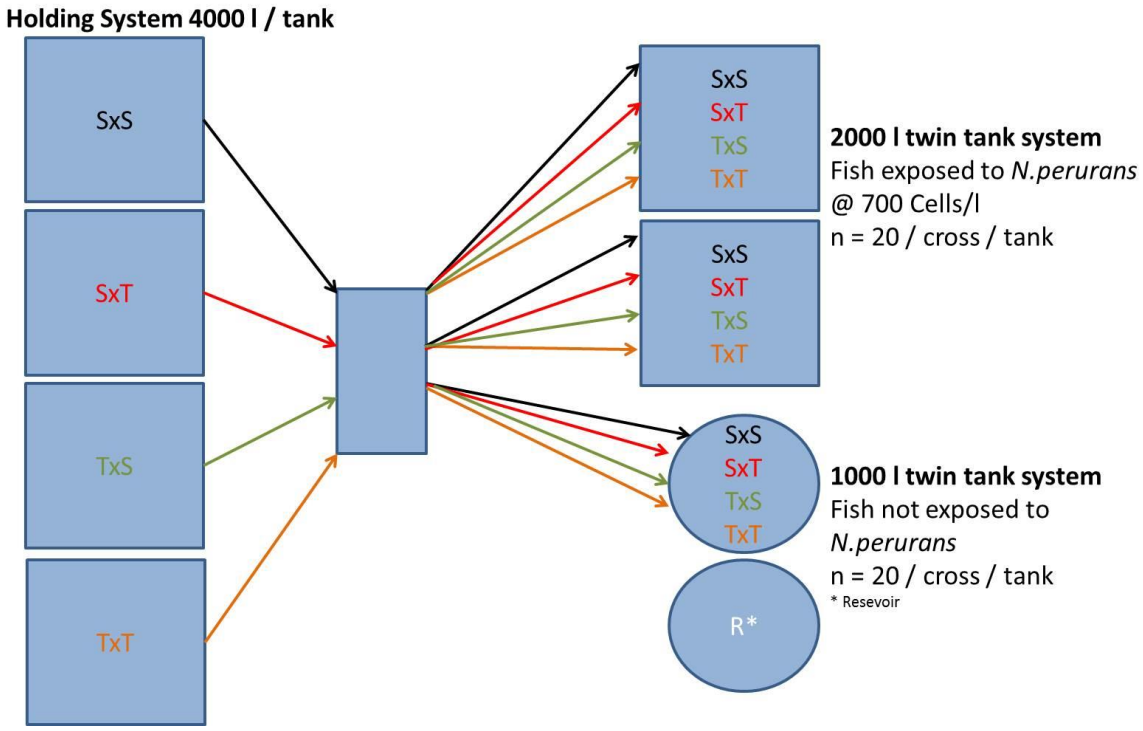


Figure 12. Fish group (cross) allocations during the holding and experimental periods. SxS = Atlantic salmon♂/♀ cross, SxT = Atlantic salmon ♂/ brown trout ♀ cross, TxS = brown trout ♂ / Atlantic salmon ♀ cross, TxT = brown trout ♂/♀ cross

Prior to the introduction of *N. perurans* to the 2x2000 l system, the weights, lengths, condition, gills, blood and mucus samples (gill sub-samples and skin mucus were provided to Dr Ben Maynard CSIRO, FRDC Project # 2011/071) were collected from five fish from each cross from each tank providing a pooled pre-challenge comparison (n = 15 per cross). Fish were subsequently exposed to *N.perurans* for six hours at a concentration of 700 trophozoites.l^{-1}. The other system did not have any trophozoites added but was managed identically as follows. Each experimental system's circulation was turned off before addition of amoebic trophozoites. Aeration was supplied to ensure vigorous water mixing and sufficient aeration to maintain DOsat at >80% during this period. Following the exposure period each system's circulation was resumed and filtration reactivated the next day.

The infection period progressed for 18 days and samples collected (as per pre-exposure) from 15 fish / cross / tank. The fish in each tank were in turn lightly sedated with clove oil to facilitate easy capture by dip net. Groups of approximately ten fish were transferred to a separate 100 l tub containing clove oil (20 µl.l^{-1}) and then removed once equilibrium was lost and the fish easily captured by hand to assess the location of the tattoo determining the species cross for allocation into holding tubs. Four 200 l tubs with aeration and seawater supplied from each experimental system (removed prior to clove oil addition) were used to hold the fish post-sedation until sampled. Tubs were cleaned and refilled between tank groups. Groups of approximately ten fish were then removed by dip-net and transferred to a 100 l tub, anaesthetised, gills scored, lengths /weights/gross anomalies recorded, the gills excised and fixed in SW Davidson's solution. The second left anterior holobranch was dissected from the gill basket and processed routinely to sections (5um). The filaments for each hemibranch were assessed for the presence/absence of hyperplastic lesions and the presence of amoebae facilitating the calculation of the percentage of filaments with hyperplastic lesions, percentage filaments amoebae colonised lesions and the percentage of hyperplastic lesions colonised for each section. Data were analysed by two-way ANOVA (Species x Tank) and data were pooled if no interaction occurred.

Results and Discussion

Objective 1: Determine the susceptibility of sea-cage associated endemic fishes to amoebic gill disease in comparison to Atlantic salmon

Experiment 1: Comparative susceptibility of Australian salmon Arripis trutta and Atlantic salmon Salmo salar to experimentally induced amoebic gill disease

Trichodinids (parasitic ciliates) was present upon the gills of Australian salmon prior to challenge with *Neoparamoeba*, often in large numbers (Figure 13). Although largely there were no aberrant histological signs consistently associated with their presence occasionally trichodinids were entrapped by proliferative tissue. There were no other significant histopathological findings upon the gills of either the Australian salmon or Atlantic salmon prior to exposure to *N. perurans*.

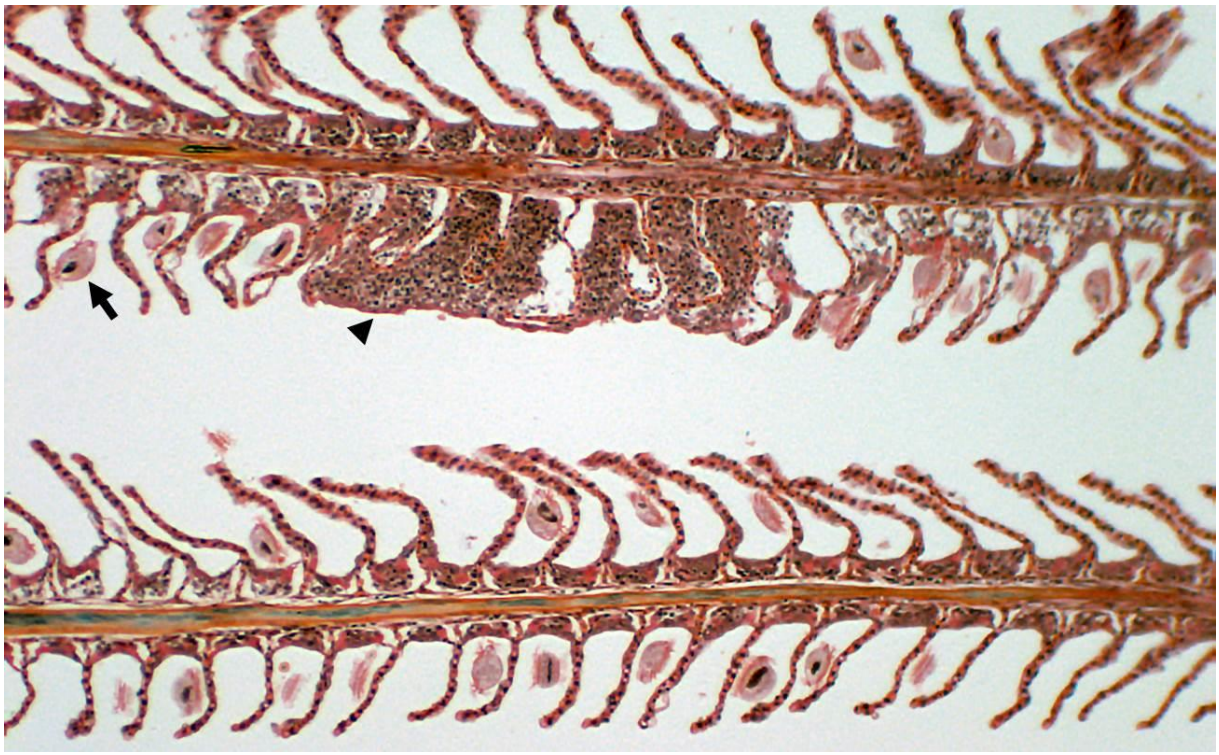


Figure 13. Australian salmon gills not exposed to *N.perurans*. Note the presence ofa trichodinid(arrow) on filaments with both a normal appearance and with a lymphocytic lesion (arrowhead).

Atlantic salmon prior to challenge displayed no remarkable gill pathology aside from two fish with small singular hyperplastic foci fortified apically with a squamous epithelial covering interspersed with mucus cells. In contrast, small raised white spots were grossly observed on the gills of 40% of unexposed Australian salmon sampled at the completion of the experiment. Histologically, these lesions were somewhat variable in cellularity but an often spongiotic epithelial matrix infiltrated with small mononuclear leucocytes (MNL) were common (Figure 13). No amoebae were observed in gill sections from unexposed fish of either species.

Atlantic salmon and Australian salmon exposed to *N. perurans* showed 100% and 95% prevalence respectively of gills with hyperplastic lesions (Table 4). The mean percentage of filaments with lesions higher for Atlantic salmon (47% : 32%) although not statistically significant (P = 0.087). However the percentage of lesions colonised by *N.perurans* was significantly lower (32% - 13%, P = 0.0014) and the abundance of amoebae found associated with lesions was appreciably lower in Australian salmon gills. Amoebic trophozoites were generally found singularly or in small clusters mostly at lesion peripheries. The range of hyperplastic pathologies found in the gills of Australian salmon ranged broadly from no discernible gill lesions to 64% of filaments containing lesions with 35% of those colonised by amoebae.

Table 4. Prevalence of Australian salmon and Atlantic salmon displaying hyperplastic lesions and fish with lesions colonised by amoebae.

	Prevalence of hyperplastic gill lesions			**Prevalence of fish with gill lesions colonised by *N.perurans***		
Species	Day 0 Unexposed	Day 10 Unexposed	Day 10 Exposed	Day 0 Unexposed	Day 10 Unexposed	Day 10 Exposed
Australian Salmon	0	10	95	0	0	65
Atlantic salmon	0	10	100	0	0	90

Table 5. The mean percentage of filaments with hyperplastic lesions and the proportion of lesions colonised by amoebae in Australian salmon and Atlantic salmon

	% Filaments with hyperplastic lesions			**% Hyperplastic lesions +ve for N.perurans**		
Species	Day 0 Unexposed	Day 10 Unexposed	Day 10 Exposed	Day 0 Unexposed	Day 10 Unexposed	Day 10 Exposed
Australian Salmon	0.00	18.40	31.90	0.00	0.00	13.30*
Atlantic salmon	0.00	0.65*	46.90	0.00	0.00	31.60
**Indicates means that are significantly lower at each day between species*						

qPCR results indicated a significantly higher (P<0.05) relative load of *N.perurans* in Atlantic salmon (1118.7 ±SE 158.2 cells.g^{-1} filaments) compared to Australian salmon (196.9 ±SE 91.7 cells.g^{-1} filaments).

Hyperplastic lesions associated with the presence of amoebae in Australian salmon were comprised primarily of epithelial cells often tightly packed apart from some lesion margins (Figure 14). Small MNLs were abundant in these regions and intermittently encountered within the lesion and CVS (central venous sinus). Mixed lymphocytic/spongiotic gill lesions morphologically similar to those found in the unexposed group of Australian salmon were also found upon the gills of fish exposed to *N.perurans.* Atlantic salmon exposed to *N.perurans* displayed hyperplastic reactivity characteristic for AGD. Most fish (95%) displayed hyperplastic lesions comprised of epithelial tissue and infiltrating inflammatory cells (data not shown). *N.perurans* were not observed in association or adhered to normal gill epithelium in either species. Trophozoites of a trichodinid were also found on the majority of Australian salmon in both the exposed (Figure 15) and unexposed groups. No interactions were

detected between the different parasites. Trichodinid ciliates were not detected within gill sections of Atlantic salmon from any of the experimental groups.

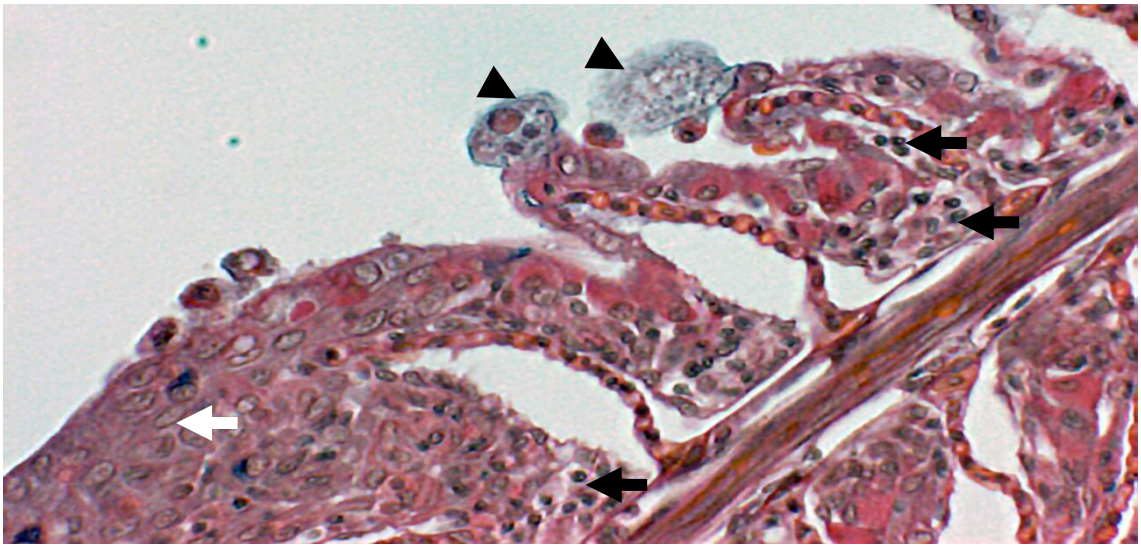


Figure 14 - *N. perurans* trophozoites (arrowheads) at the periphery of hyperplastic gill lesion. Margins are infiltrated with MNLs (black arrows) whilst epithelial cells (white arrow) dominate the bulk of the lesion.

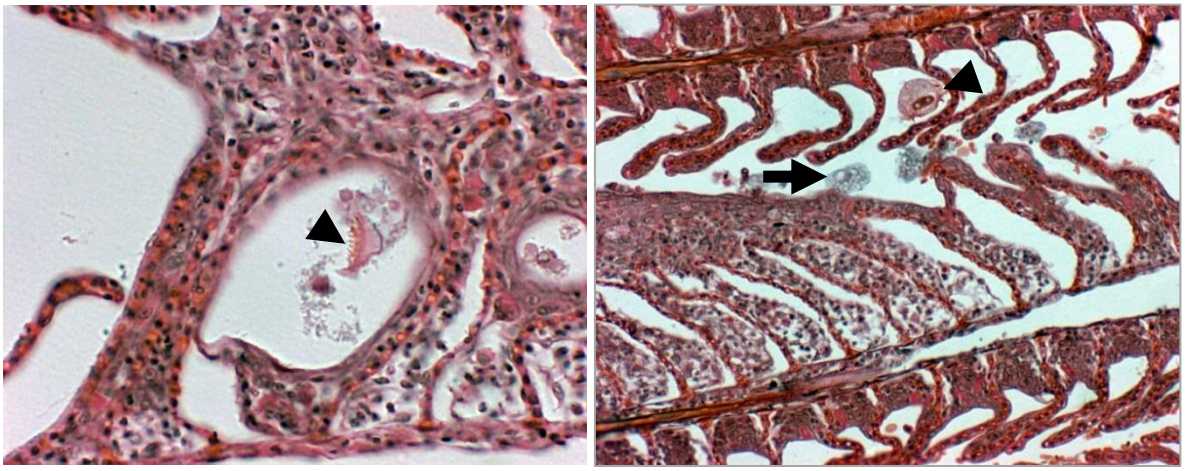


Figure 15 - A fragment of a trichodinid(arrow head) entrapped within an inflamed lamellar lesion from an Australian salmon not exposed to *N.perurans* (image on right). The margin of a gill lesion from an Australian salmon exposed to with amoebae (arrowhead) and a single trichodinid ciliate (arrow) on the adjacent filament.

In dorsally orientated Atlantic salmon gill filaments, AGD induced substantial alterations to the filamental mucosa and distal interbranchial lymphoid tissue (see Dalum *et al.* 2015). Epithelial hyperplasia and intraepithelial oedema was consistently observed adjacent to hyperplastic lamellae and MNL numbers were markedly lower in comparison to unaffected edges (Figure 16). Substantial inflammatory infiltration was observed in lesion adjacent localities of the collateral sinuses adjacent the filament arteries, arterial & cartilaginous connective tissue surrounds and endothelial-basal membrane interface within the CVS (Figure 17). Similar observations were made from dorsally orientated Australian salmon gill filaments although the extent of epithelial hyperplasia was generally lower and intraepithelial oedema was more pronounced in affected filaments (Figure 18 & Figure 19).

The sub-mucosal tissue of the filamental edges were consistent with a diffuse mucosal associated lymphoid tissue.

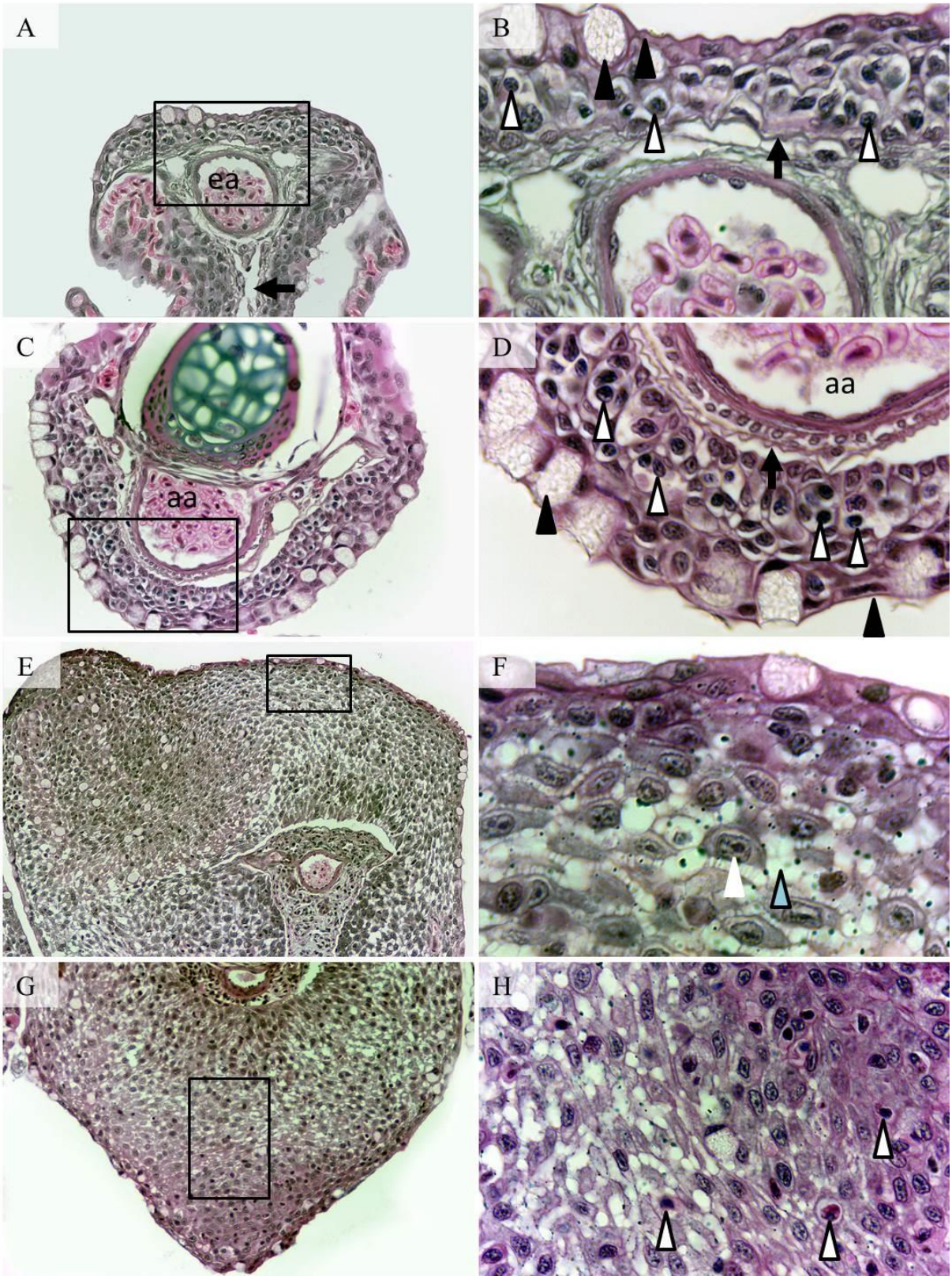


Figure 16. Dorsal aspect of Atlantic salmon gill filaments exposed to *N.perurans* demonstrating AGD induced alterations to mucosal and sub-mucosal tissues. A – Leading edge not colonised by *N.perurans*. B – Higher magnification of A showing the mucosa (black arrowheads) (cont. over page)

and basement membrane (arrow) bordering intraepithelial MNLs (white arrowhead). C – Trailing edge of a filament not colonised by *N.perurans*. D – Higher magnification of C showing a thinner intraepithelial MNL network to that in B. E – Leading edge of an enlarged filament. F – Higher magnification of E showing intraepithelial presence of EMNLs (eosinophilic mononuclear leucoytes) and MNLs (white arrowheads). G – Trailing edge of a hyperplastic filament showing intra-epithelial eodema (arrow) and occasional MNLs (arrowhead).

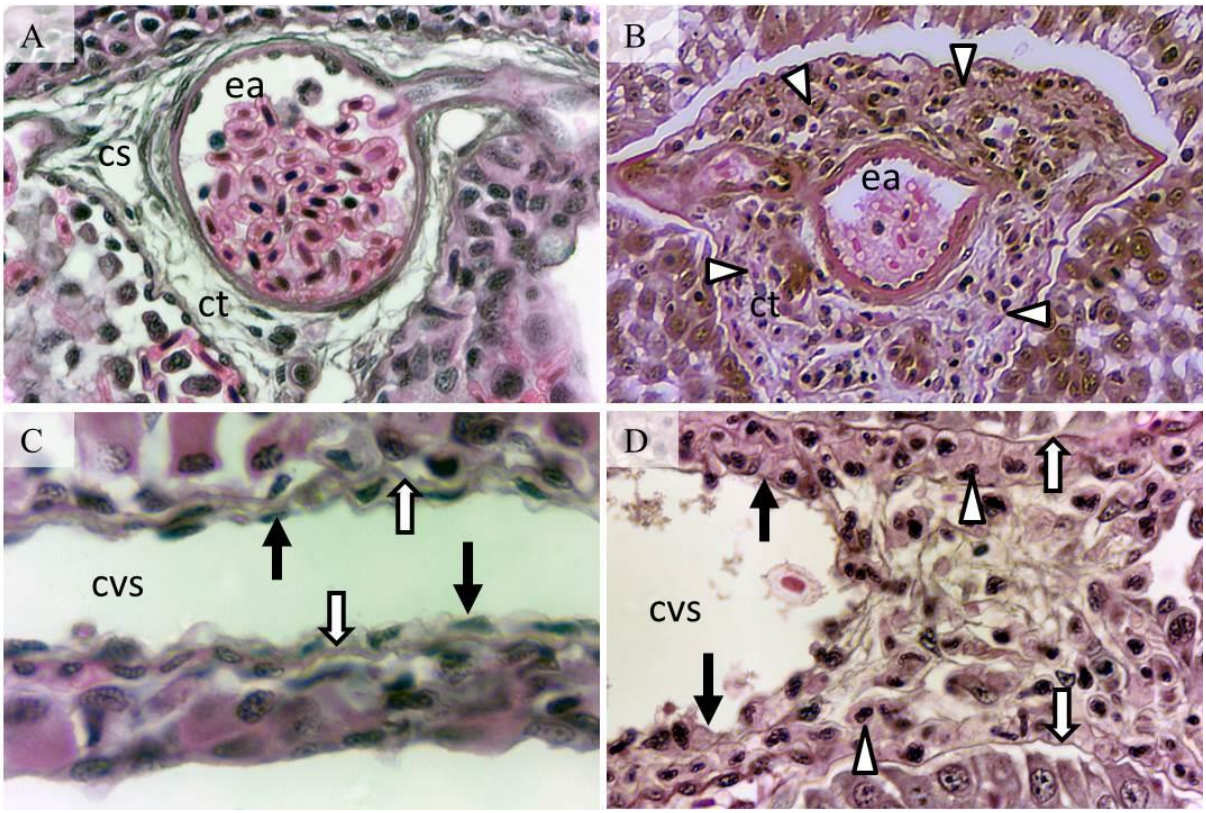
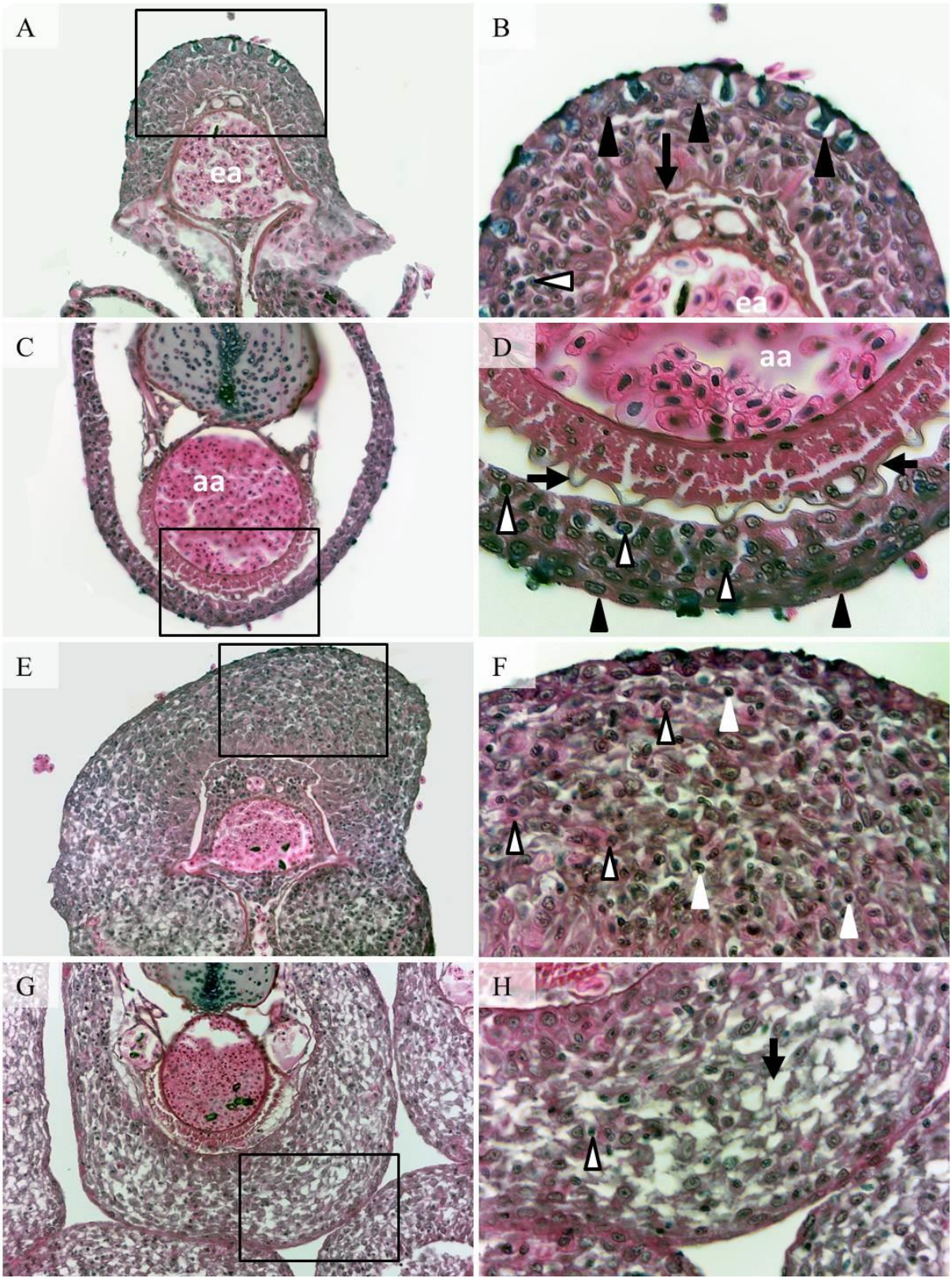


Figure 17. Dorsal aspect of Atlantic salmon gill filaments exposed to *N.perurans* showing AGD induced circulatory inflammation. A – Within an un-parasitised filament the collateral sinuses are visible within loose connective tissue (ct) surrounding the efferent artery (ea) in contrast to the connective tissue congested with an inflammatory infiltrate (arrowheads) in a hyperplastic filament (B). C – Endothelium (black arrows) is adherent to the basal filamental membrane encasing the central venous sinus (CVS) of an un-parasitised filament. D – EMNLs (white arrowheads) and occasional PMNLs infiltrating the endothelium (black arrows) overlaying the basal membrane (white arrows) interface and into the CVS in a parasitised filament.

Figure 18 (next page). Dorsal aspect of Australian salmon gill filaments exposed to *N.perurans* demonstrating AGD induced alterations to mucosal and sub-mucosal tissues. A – Leading edge not colonised by *N.perurans*. B – Higher magnification of A showing the mucosa (black arrowheads) and basement membrane (arrow) bordering intraepithelial MNLs (white arrowhead). C – Trailing edge of a filament not colonised by *N.perurans*. D – Higher magnification of C showing a thinner intraepithelial MNL network to that in B. E – Leading edge of an enlarged filament. F – Higher magnification of E showing intraepithelial presence of EMNLs (bordered arrowheads) and MNLs (white arrowheads). G – Trailing edge of a hyperplastic filament showing intra-epithelial eodema (arrow) and occasional MNLs (arrowhead).



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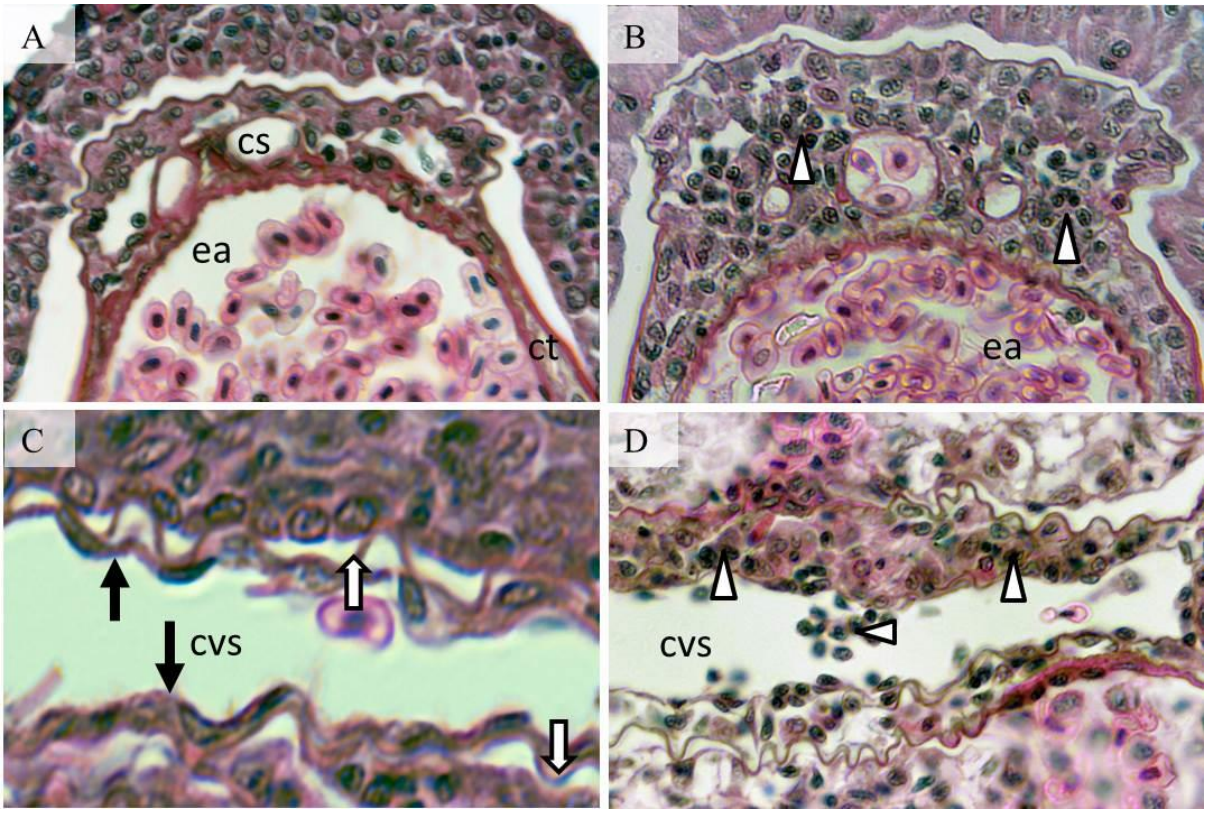

Figure 19. Dorsal aspect of Australian salmon gill filaments exposed to *N.perurans* showing AGD induced circulatory inflammation. A – Within an un-parasitised filament the collateral sinuses are visible within loose connective tissue (ct) surrounding the efferent artery (ea) in contrast to the connective tissue congested with an inflammatory infiltrate (arrowheads) in a hyperplastic filament (B). C – Endothelium (black arrows) is adherent to the basal filamental membrane encasing the central venous sinus (CVS) of an un-parasitised filament. D – EMNLs (white arrowheads) infiltrating the endothelium (black arrows) overlaying the basal membrane (white arrows) interface and into the CVS in a parasitised filament.

Table 6. Prevalence of external lesions upon Atlantic and Australian salmon.

	External lesion location					
Species	Eyes	Dorsal	Flank	Ventral	Fins	Tail
Atlantic salmon	1.7	0.0	11.7	8.3	48.3	30.0
Australian Salmon	3.3	0.0	0.0	0.0	0.0	3.3

indicates there were very few lesions other than those described for the gills present upon Australian salmon. From the 60 fish of this species examined prior to bleeding and gill excision, two fish presented with minor tail erosion and two with slight exopthalmia. Erosion of the right pectoral fin and tails was frequently noted in Atlantic salmon along with 20% of these fish presenting with focal petachia or small ulcers (≈ 5mm diameter).

Experiment 2: Comparative susceptibility of yellow-eye mullet <u>Aldrichetta forsteri</u> and Atlantic salmon <u>Salmo salar</u> to experimentally induced amoebic gill disease

Gross gill examinations were unremarkable for all fish examined prior to challenge with *N.perurans*. At day 10, 65% of mullet unexposed to amoebae had developed focal to multifocal white spots and/or patches. No gross gill lesions were observed in the unexposed group of Atlantic salmon. All mullet and salmon presented with macroscopic gill lesions 10 days after exposure to *N.perurans*. Histopathological observations from the gills of both fish species sampled prior to *N. perurans* exposure were unremarkable aside from 5% incidence of focal lymphoid lamellar fusions typically involving 2-4 lamellae (Figure 20).

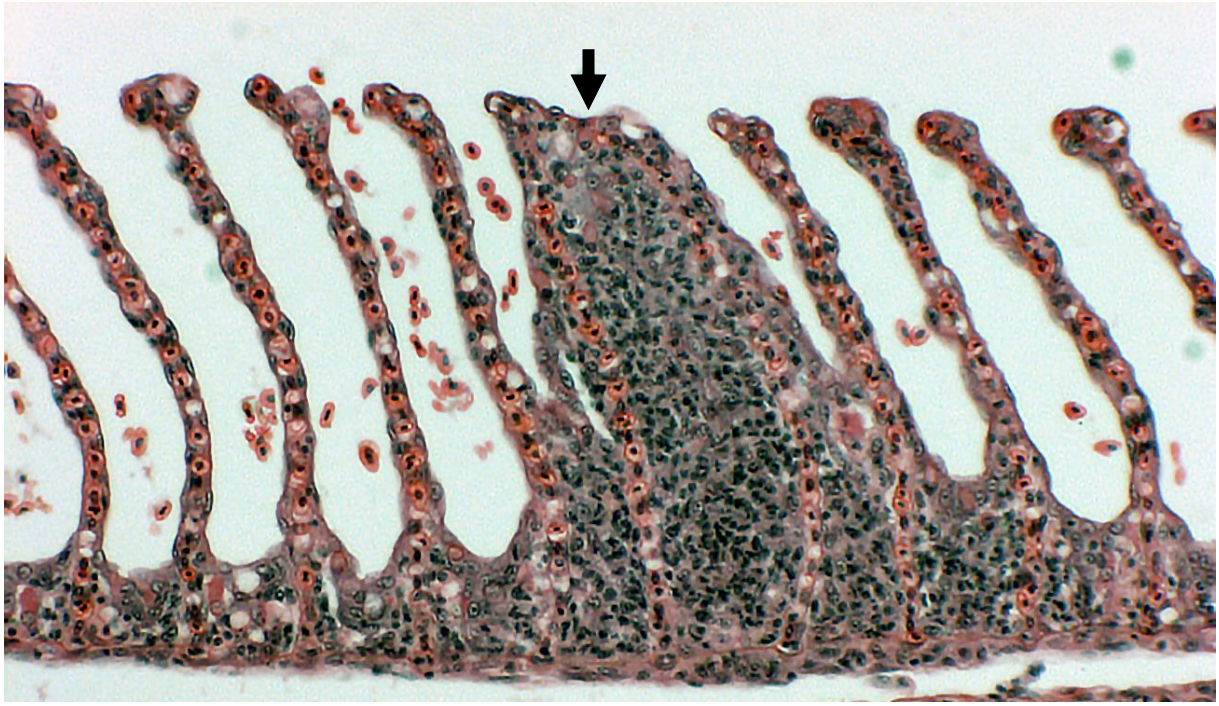

Figure 20. A small lesion (arrow) comprised predominately of small mononuclear cell types in mullet prior to exposure with *N.perurns*.

50% of the mullet unexposed to amoebae and sampled at day 10 displayed gross gill lesions which histologically displayed a proportionally variable mixture of small MNLs and eosinophilic mononuclear leukocytes (EMNCs) encapsulated by epithelium (Figure 21). Erythrocyte congestion within lamellar channels and presence of inflammatory cells in the CVS and apposed sinusoids and vessels was also noted in some cases. There was no obvious aetiological cause associated with the presence of these lesions.

Table 7. Prevalence of mullet and Atlantic salmon displaying hyperplastic lesions and fish with lesions colonised by amoebae.

	Prevalence of fish with hyperplastic lesions			**Prevalence of fish with gill lesions colonised by *N.perurans***		
Species	Day 0 Unexposed	Day 10 Unexposed	Day 10 Exposed	Day 0 Unexposed	Day 10 Unexposed	Day 10 Exposed
Yellow eyed mullet	0	55	100	0	0	30
Atlantic salmon	0	5	100	0	0	100

Table 8. The mean percentage of filaments with hyperplastic lesions and the proportion of lesions colonised by amoebae in mullet and Atlantic salmon

	% Filaments with hyperplastic Lesions			**% Hyperplastic lesions positive for *N.perurans* / gill section**		
Species	*Day 0 Unexposed*	*Day 10 Unexposed*	*Day 10 Exposed*	*Day 0 Unexposed*	*Day 10 Unexposed*	*Day 10 Exposed*
Yellow eyed mullet	*0.00*	*6.47*	*29.40**	*0.00*	*0.00*	*3.00**
Atlantic salmon	*0.00*	*0.48**	*50.60*	*0.00*	*0.00*	*56.20*

**Indicates means that are significantly lower at each day between species*

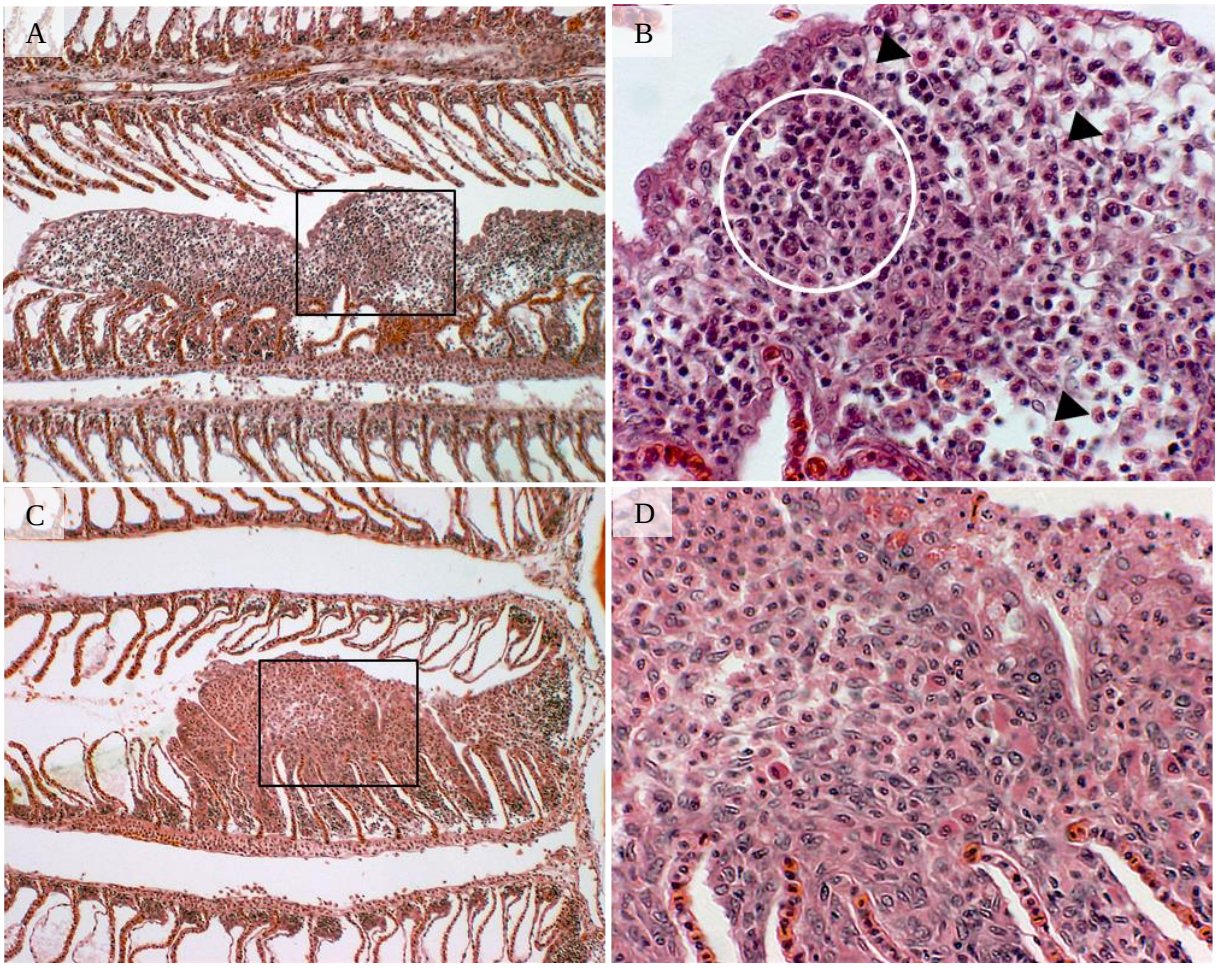

Figure 21. Mullet unexposed to *N.perurans* at day 10 showing lesions with variably proportional MNL/EMNL infiltration. Higher magnification of boxed regions (in A & C) are to the left (B&D) of the corresponding low magnification image.

There was no unusual pathology noted in the gills of Atlantic salmon at day 10 that were from fish inhabiting the inhabiting the same system. All mullet that were exposed to *N.perurans* presented grossly with white spots and patches consistent with signs of AGD as did Atlantic salmon. Hyperplastic lesions of variable size were present in all fish examined histologically although the percentage of filaments containing lesions was significantly lower (P = 0.006) in mullet (29%) than in Atlantic salmon (51%). All Atlantic salmon had a proportion of lesions colonised with amoebae compared to only 30% of mullet. Where amoebae were present upon gill lesions in mullet, their numbers were minimal compared to a substantial abundance noted upon Atlantic salmon gill lesions.

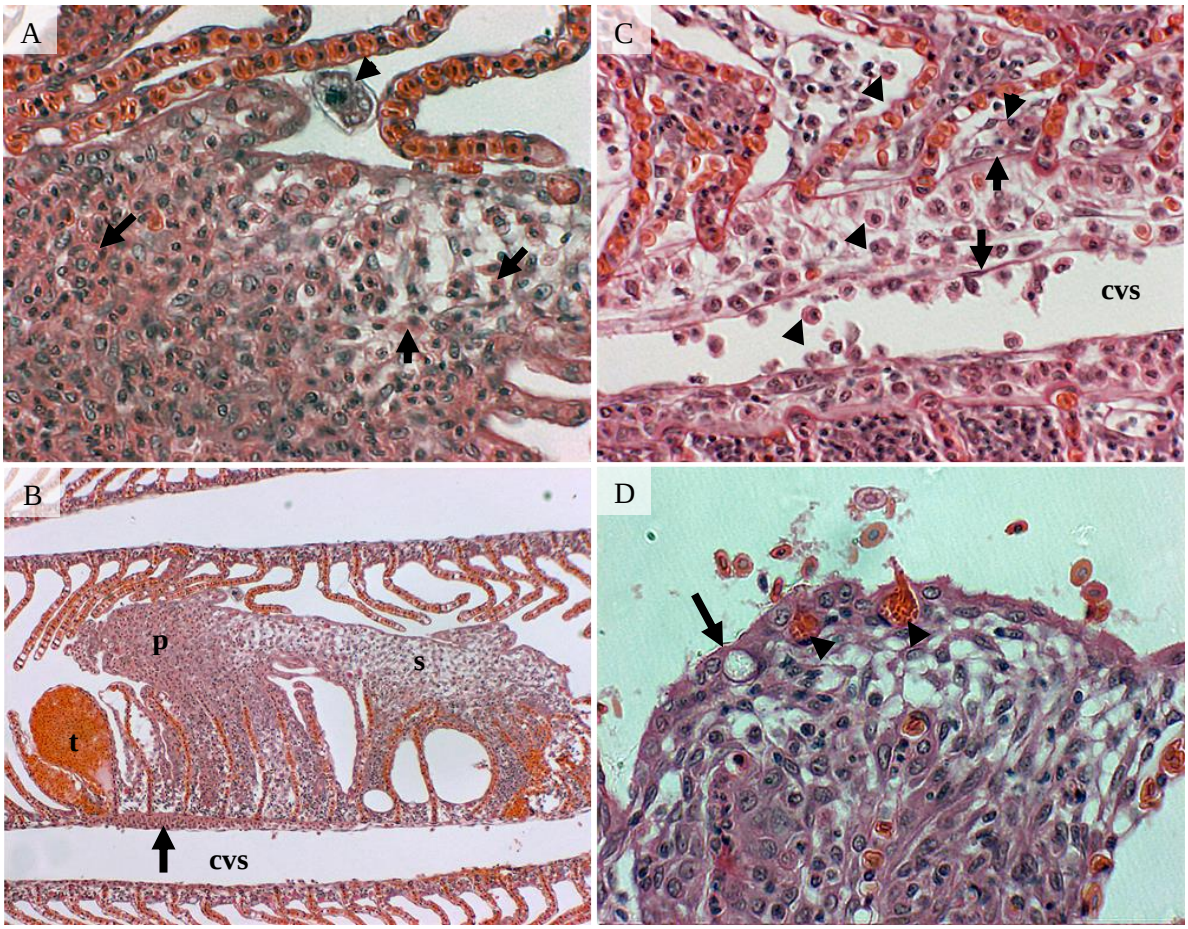

Figure 23. Gill lesions from mullet exposed to N.perurans. A – Infiltration of hyperplastic epithelium by eosinophilic MNLs (arrows) and an associated amoeboid trophozoite (arrowhead); B – Lower magnification of the corresponding lesion (from A) showing a gradient of spongiotic (s) to packed (p) hyperplastic tissue and peripheral lamellar telangiactasia (t). The central venous sinus (cvs) with peripherally adhered EMNLs (arrow) seen more clearly in C ; C – EMNLs (arrowheads) within the CVS infiltrating the basal membrane (up arrow) and endothelial lining (down arrow). The latter was observed tethered to the basal mebrane in unnaffected filaments (data not shown). D – Acidophilic granular mucus cells (arrowheads) and a non-staining mucus cell (arrow) at a lesion surface.

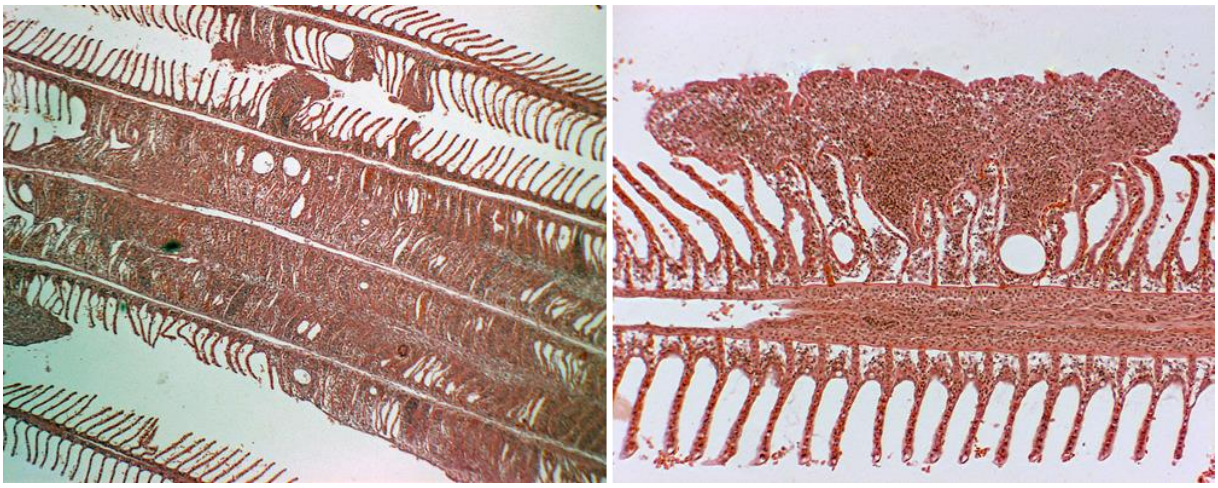

Figure 22. Gill lesions from mullet exposed to *N.perurans*. Hyperplastic lamellar epithelium fusing several filaments and devoid of amoebae (on right). A smaller lesions bearing marked similarity to lesions found in unexposed mullet also uncolonised by amoebae (on left).

Lesions, morphologically comparable although less lymphoid compared to those described for unexposed mullet, were present in the majority of the gill sections analysed (Figure 23). This is reflected in Table 8 where the percentage of lesions colonised by amoebae within the gills of mullet (3%) was significantly lower ($P < 0.0001$) than co-housed Atlantic salmon (56%). Amoebic trophozoites were found isolated singularly or occasional groups of two or three. No particular lesion location was preferentially colonised by amoebae. Trophozoites were often unattached in the interstitial milieu between filaments and sometimes with displayed a marked margin between the cell membrane and endoplasm giving a ballooned appearance (Figure 16A; also see experiments 3 & 5). This was also noted occasionally for amoebae attached to hyperplastic regions in both species. qPCR results indicated no significant difference ($P=0.20$) between the relative load of *N.perurans* in the first holobranch of Atlantic salmon (1487.5 ±SE 1478.0 cells.g^{-1} filaments) compared to mullet (110.8 ±SE 35.9 cells.g^{-1} filaments). Interestingly, small amounts of DNA from *N.perurans* were detected upon two mullet (equivalent of 3&6 cells.g^{-1} filaments) prior to exposure to *N.perurans* although both species were negative in the un-inoculated system at day10.

Gill lesions with a variably dense epithelial scaffold found in the exposed mullet gills were often infiltrated by MNLs/EMNLs. Some lesions (and/or lesion margins) displayed a more spongiotic appearance with limited inflammatory infiltration. Gill lesions were generally larger than lesions described for unexposed mullet. Occasionally lamellar pillar cell channels had ruptured allowing erythrocytes to permeate lesions. The CVS of lesion affected filaments were often congested peripherally with EMNLs adhered to the basal filamental membrane underlying the endothelium of the CVS. Isolated observations of acidophilic granular secretory cells (distinct from the mucus cell population found elsewhere) were made from two fish upon apical lesion regions. Non-staining mucus cells were more numerous upon some lesions relative to unnaffected filaments but did not feature prominently in comparison to those observed in amoebae-exposed Atlantic salmon gills.

In dorsally orientated Atlantic salmon gill filaments, AGD induced substantial alterations consistent with those described for experiment 1. Similar observations were made from dorsally orientated mullet filaments which were also as described for experiment 1(Figure 24).

Table 9. Prevalence of external lesions in Atlantic salmon and mullet.

	External lesion location					
Species	Eyes	Mouth	Flank	Ventral	Fins	Tail
Atlantic salmon	0.0	0.0	20.0	1.0	32.0	43
Yellow eyed mullet	0.0	7.0	4.0	0.0	0.0	2.0

External somatic lesions were observed upon the flanks (mostly petechiae & occasional small ulcers), fins (eroded right pectoral) and tails (minor erosion) of Atlantic salmon. Contrastingly there were very few or no lesions of these types in mullet (Table 9).

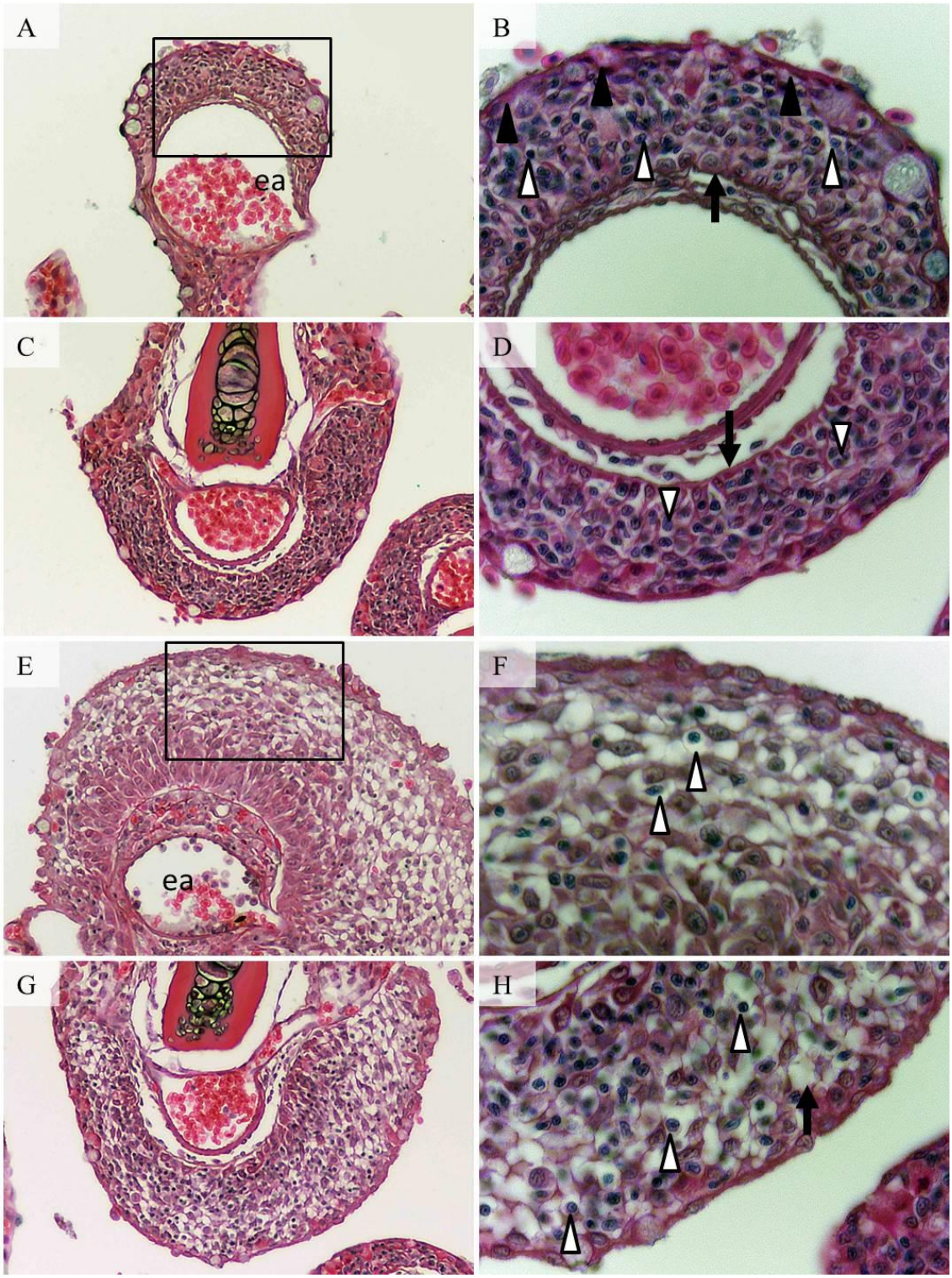

Figure 24. Dorsal aspect of yellow eye mullet gill filaments exposed to *N.perurans* demonstrating AGD induced alterations to mucosal and sub-mucosal tissues of the leading and trailing edges. A – Leading edge not colonised by *N.perurans*. B – Higher magnification of A showing the mucosa (black arrowheads) and basement membrane (arrow) bordering intraepithelial MNLs (white arrowhead). C – Trailing edge not colonised by *N.perurans*. D – Higher magnification of C showing the intraepithelial MNL network. E & G – Leading/trailing edge of AGD affected filaments. F & G – Higher magnification of E & G respectively showing intra-epithelial eodema (arrow) and interspersed MNLs (arrowheads).

Experiment 3: Comparative susceptibility of purple wrasse <u>Notolabrus fucicola</u> and Atlantic salmon <u>Salmo salar</u> to experimentally induced amoebic gill disease

No remarkable pathology was noted grossly upon the gills of salmon or wrasse prior to exposure to *N.perurans*. Somewhat similar to Australian salmon and mullet, unexposed wrasse sampled at day 10 presented histologically with variably spongiotic and sometimes vesicularised hyperplastic lesions infiltrated with small MNL/EMNLs the latter sometimes appearing rounded and granular (**Error! eference source not found.**). Apically located large basophilic mucus cells were also consistently present both upon lesions (**Error! Reference source not found.**) and the leading edge of unaffected ilaments. Atlantic salmon gills also displayed small (1-2 inter-lamellar units - ILU) occasional small lesions dominated by small MNLs with a single-layer squamated epithelium without mucus cells. The

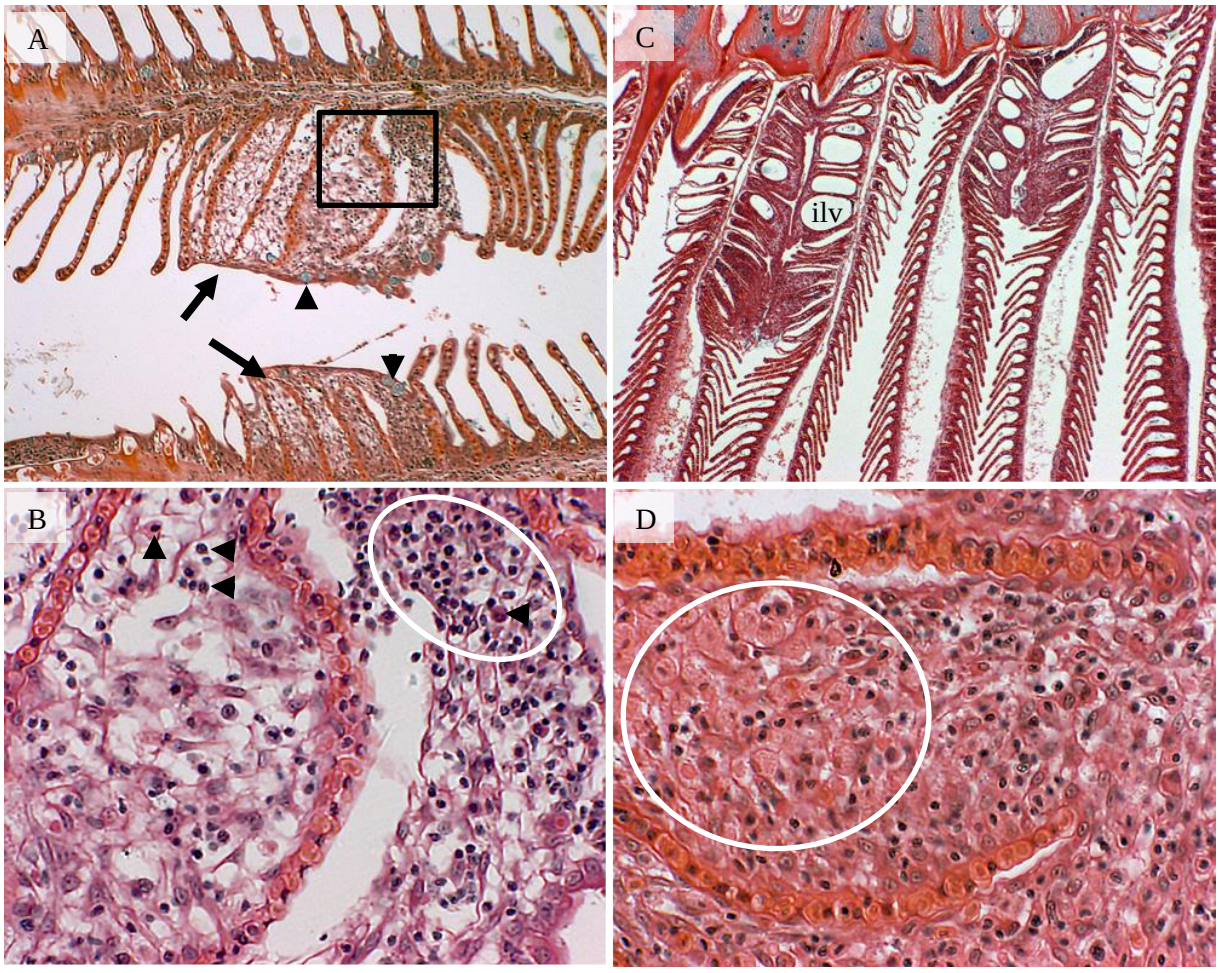

Figure 25. Gill lesions in purple wrasse not exposed to N.perurans at day 10. A – Spongiotic hyperplastic lesion with apical basophilic mucus cells (arrowheads); B – higher magnification of A, spongiotic epithelial tissue infiltrated with occasional EMNLs (arrowheads) and MNLs (majority of cells encircled); C – Vesicularised lesions (ilv = interlamellar vesicle); D – Rounded, granular EMNLs the dominant cell type within a gill lesion (encircled).

Table 10. Prevalence of purple wrasse and Atlantic salmon displaying hyperplastic lesions and fish with lesions colonised by amoebae.

	Prevalence of fish with hyperplastic lesions			**Prevalence of fish with gill lesions colonised by *N.perurans***		
Species	Day 0 Unexposed	Day 10 Unexposed	Day 10 Exposed	Day 0 Unexposed	Day 10 Unexposed	Day 10 Exposed
Purple wrasse	0.0	45.0	100	0.0	0.0	60
Atlantic salmon	0.0	0.00	100	0.0	0.0	100

Table 11. The mean percentage of filaments with hyperplastic lesions and the proportion of lesions colonised by amoebae in purple wrasse and Atlantic salmon

	% Filaments with hyperplastic lesions				**% Hyperplastic lesions positive for *N.perurans* / gill section**		
Species	Day 0 Unexposed	Day 10 Unexposed		Day 10 Exposed	Day 0 Unexposed	Day 10 Unexposed	Day 10 Exposed
Purple wrasse	0.0	6.82	35.50*	0.0	0.0	15.20*	
Atlantic salmon	0.0	0.0	57.20	0.0	0.0	42.00	

**Indicates means that are significantly lower at each day between species*

The percentage of lesions colonised by amoebae was significantly lower for wrasse (15%) than for Atlantic salmon (42%) (Table 11). Amoebae colonising the gill lesions of wrasse were often tenuously or unattached to proliferative lesions and were generally present in low numbers. Pronounced space between the outer cytoplasmic margin and cell membrane of amoebae was frequently observed in comparison to trophozoites colonising the gill lesions of exposed Atlantic salmon. qPCR results indicated significant differences ($P<0.05$) between the relative load of *N.perurans* in the first holobranch of Atlantic salmon (10177 ±SE 3953.9 cells.g^{-1} filaments) and wrasse (228.5 ±SE 72.2 cells.g^{-1} filaments). Interestingly, small amounts of DNA from *N.perurans* was detected upon a single wrasse (equivalent of 3 cells.g^{-1} filaments) prior to exposure to *N.perurans* and upon two salmon (<2 cells.g^{-1} filaments) in the un-inoculated system at day10. Hyperplastic gill lesions in wrasse with associated or attached amoebae were often infiltrated with small MNLs, EMNLs and apically with secreting basophilic mucus cells. Although sometimes similar in cellularity and morphology to lesions described for unexposed wrasse, the presence of sloughing and sloughed epithelium, epithelial hyperplasia, cellular debris and basophilic exudate featured regularly where amoebae presence was noted.

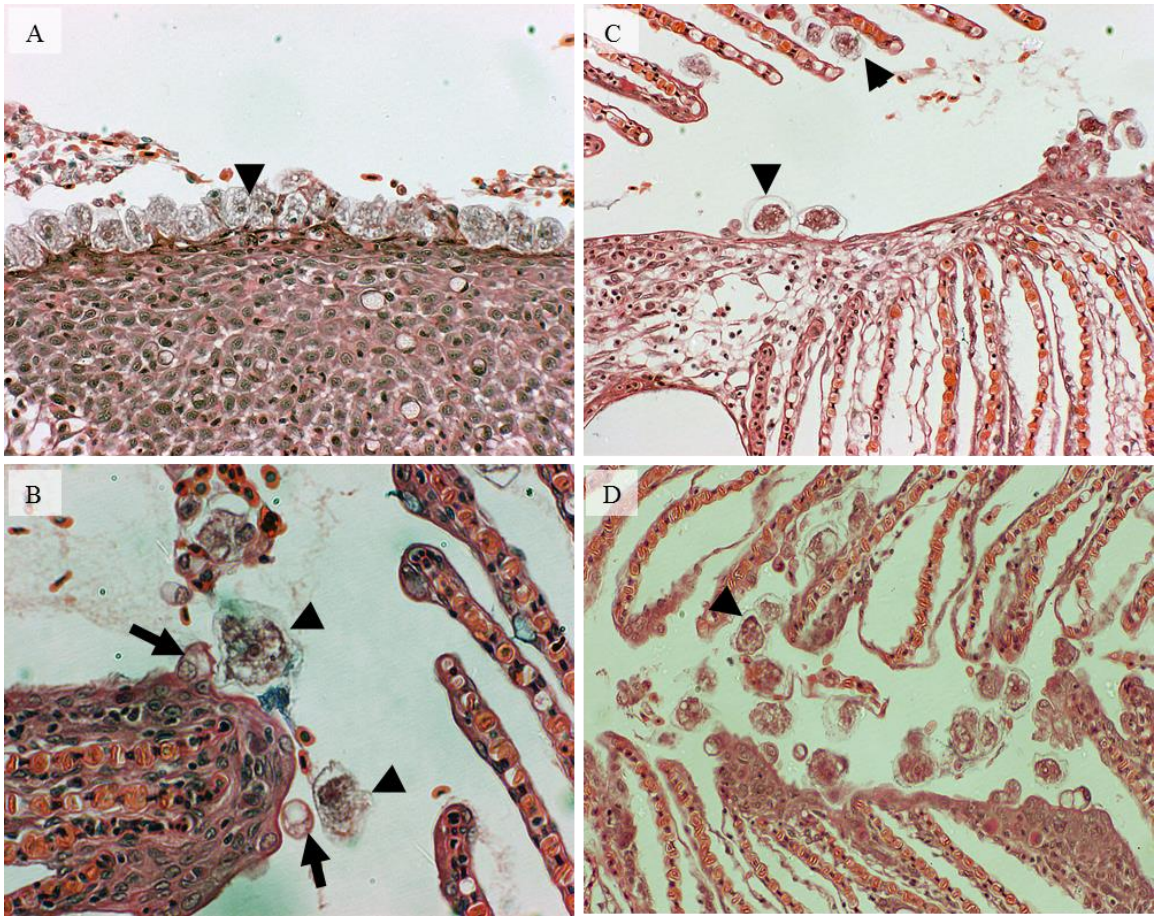

Figure 26. Gill lesions colonised by *N.perurans* (arrowheads). A – A row colonising amoebae attached to a gill lesion from Atlantic salmon; B – Amoebae attached to the surface of a wrasse gill lesion. Note the margin between the membrane and endoplasm; C – Amoebae (arrowhead) and an exfoliating epithelial cells (arrows) at a lesion surface; D – Unattached amoebae (arrowheads) in adjacent to hyperplastic lesions.

In dorsally orientated Atlantic salmon gill filaments, AGD induced substantial alterations consistent with those described for experiment 1. Similar observations were made from dorsally orientated wrasse filaments which were also as described for experiment 1 with substantially prominent intraepithelial oedema of sub-mucosal regions (Figure 27 – next page).

Table 12. Prevalence of external lesions upon Atlantic salmon and purple wrasse.

	External lesion location					
Species	Eyes	Mouth	Flank	Ventral	Fins	Tail
Atlantic salmon	0.0	0.0	9.0	1.5	40.0	32.0
Purple Wrasse	5.0	0.0	0.0	0.0	0.0	0.0

Aside from two observations of exopthalmia in wrasse, there were no remarkable lesions noted grossly. Erosion of the right pectoral fin and tail was commonly encountered in Atlantic salmon along with less frequent observations of petechiae (**Error! Reference source not found.**).

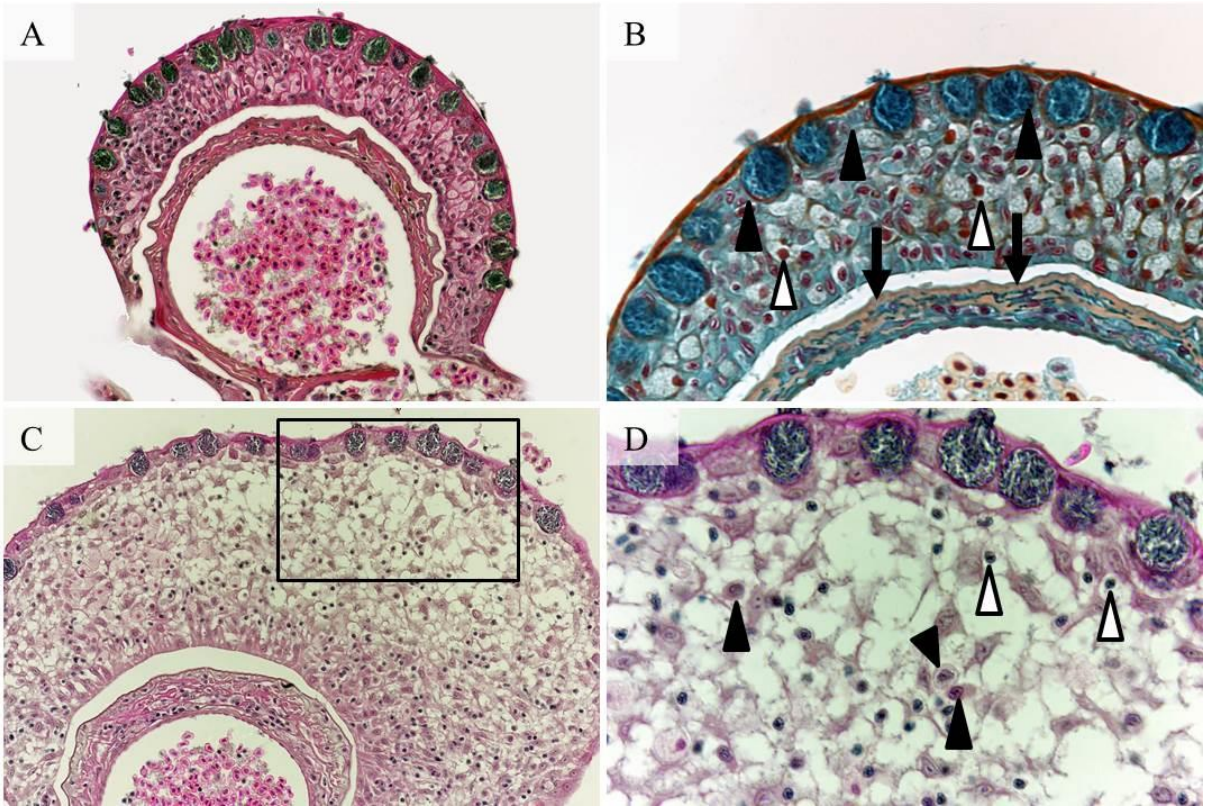

Figure 27. Dorsal aspect of purple wrasse gill filaments exposed to *N.perurans* demonstrating AGD induced alterations to mucosal and sub-mucosal tissues of the leading edge. A – Leading edge not colonised by *N.perurans*. B – Serial section of A showing the mucosa (black arrowheads) and basement membrane (arrow) bordering intraepithelial granular MNLs (white arrowheads) (modified Dane's stain). C – Leading edge of AGD affected filament. D – Higher magnification of C showing extensive intra-epithelial eodema (arrow) and interspersed MNLs (white arrowheads) and occasional EMNLS (black arrowheads).

Experiment 4: Comparative susceptibility of southern sand flathead Platycephalus bassensis and Atlantic salmon Salmo salar to experimentally induced amoebic gill disease

Pre-exposure samples revealed the presence of monogenean dactylogyrid flukes upon the gills of 55% of southern sand flathead. There was little tissue reactivity associated with their presence inclusive of the haptoral anchorage sites (Figure 28). There were no other significant histopathological findings for the gills of either the flathead or Atlantic salmon prior to exposure to *N. perurans*. A similar prevalence (65%) of flathead gills with monogenean parasites was noted in unexposed flathead gills at day 10 and they were not detected in Atlantic salmon gills. There were no other significant findings upon the gills of Atlantic salmon unexposed to *N.perurans* sampled on day 10.

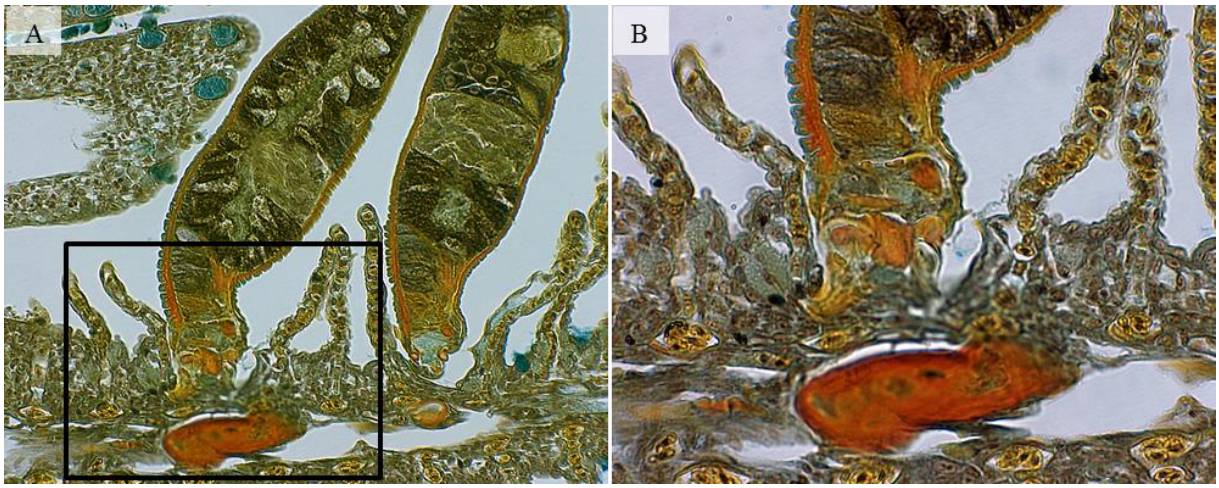

Figure 28. A - Monogenean gill flukes attached to the gills of southern sand flathead prior to the cohort's exposure to *N.perurans;* B – Higher magnification of box in A, no significant host response at the attachment site was a consistent finding for all flathead parasitized by gill flukes (Danes stain).

Hyperplastic lesions were found upon the gills of all Atlantic salmon and 84% of flathead exposed to *N. perurans* (Table 13). The severity of disease expressed as the percentage of lesion affected filaments was significantly lower ($P < 1x10^{-9}$) for flathead (22%) compared to salmon (85%) (Table 14). The percentage of gill lesions colonised by amoebae was likewise significantly lower ($P < 1x10^{-6}$) in flathead compared to salmon. Where present, singular, paired and small groups of up to 10 amoebae were seen associated with gill lesions in flathead, sometimes attached to hyperplastic epithelium but often free amongst sloughed tissue and proteinaceous material between filaments (Figure 29).

Table 13. Prevalence of sand flathead and Atlantic salmon displaying hyperplastic lesions and fish with lesions colonised by amoebae

	Prevalence of fish with hyperplastic lesions			**Prevalence of fish with gill lesions colonised by *N.perurans***		
Species	Day 0 Unexposed	Day 10 Unexposed	Day 10 Exposed	Day 0 Unexposed	Day 10 Unexposed	Day 10 Exposed
Sand flathead	0	0	84	0	0	63
Atlantic salmon	0	0	100	0	0	100

Table 14. The mean percentage of filaments with hyperplastic lesions and the proportion of lesions colonised by amoebae in sand flathead and Atlantic salmon

	% Filaments with hyperplastic Lesions			% Hyperplastic lesions positive for N.perurans / gill section		
Species	Day 0 Unexposed	Day 10 Unexposed	Day 10 Exposed	Day 0 Unexposed	Day 10 Unexposed	Day 10 Exposed
Sand flathead	0.0	0.0	21.5*	0.0	0.0	30.8*
Atlantic salmon	0.0	0.0	94.3	0.0	0.0	88.4

**Indicates means that are significantly lower at each day between species*

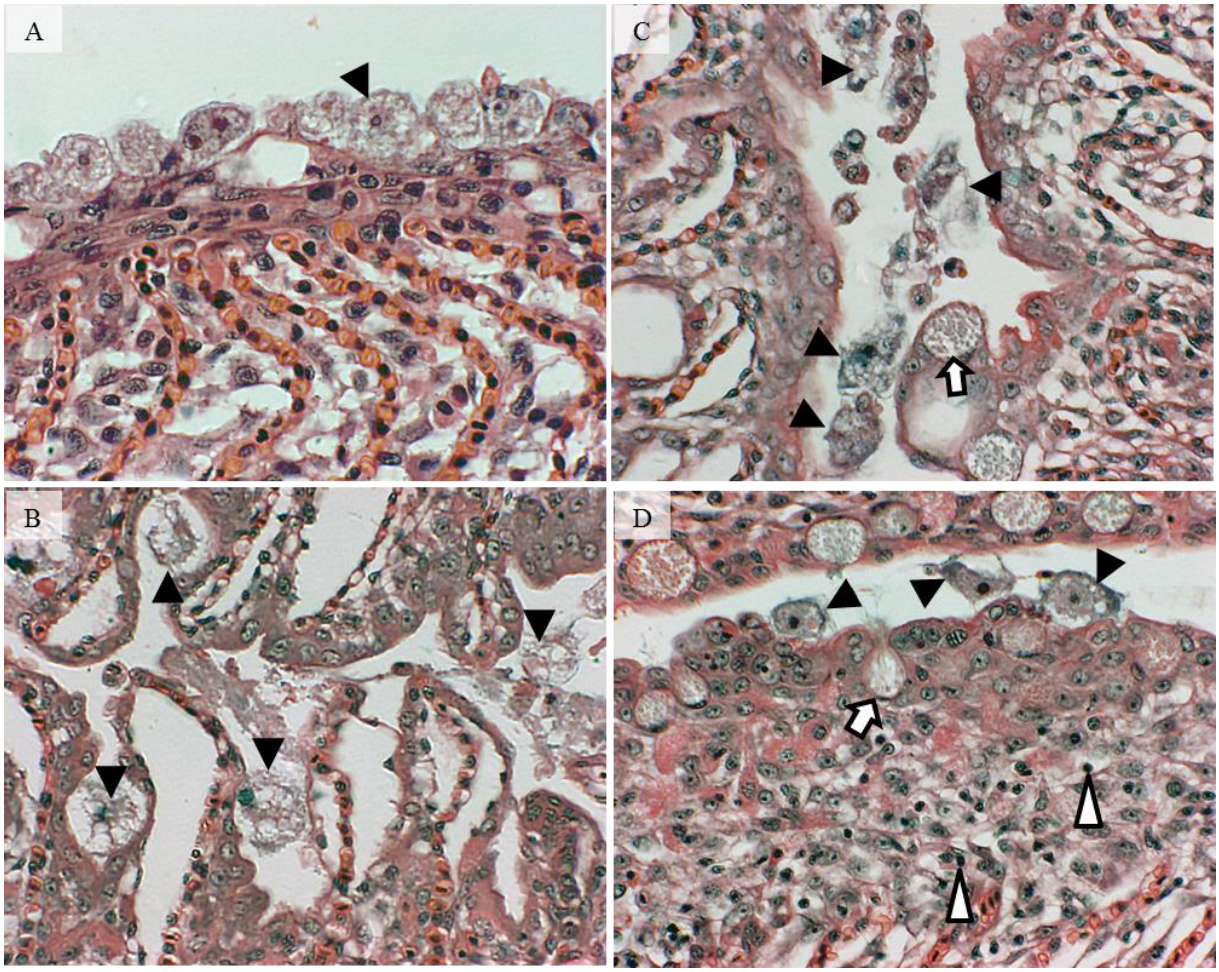

Figure 29. Hyperplastic gill lesions and colonising amoebae in Atlantic salmon (A) and southern sand flathead (B-D). A – A row of amoebae (arrowhead) attached to hyperplastic gill epithelium in Atlantic salmon; B – Degenerating amoebae (arrowheads) partially enclosed by proliferating lamellar epithelium; C – Amoebae seemingly unattached from hyperplastic lesions within the interfilamental mileau along with sloughed epithelium, large basophilic granular mucus cells (+ve for alcian blue – data not shown) were often present at lesion surfaces in flathead gills; D - Amoebae on gill lesion in flathead with foamy cytoplasm and pyknotic nuclei. Several large granular mucus cells are present and a translucent mucus cell is releasing its contents (white arrow). The lesion is infiltrated with occasional small MNLs (white arrowheads).

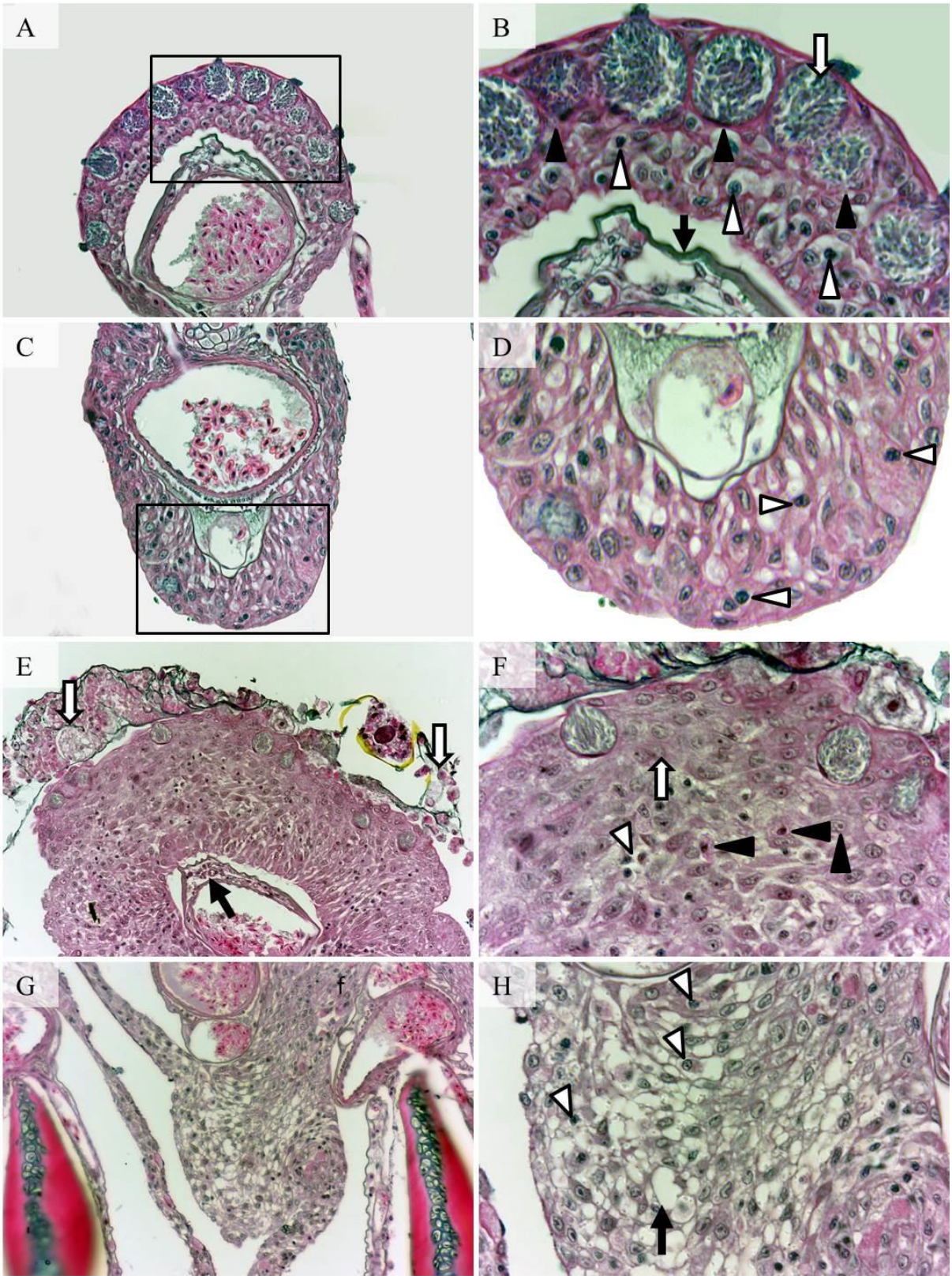

Figure 30. Dorsal aspect of sand flathead gill filaments exposed to *N.perurans* demonstrating AGD induced alterations to mucosal and sub-mucosal tissues of the leading and trailing edges. A – Leading edge not colonised by *N.perurans*. B – Higher magnification of A showing the mucosa (black arrowheads) with large basophilic mucous cells (black arrowheads) and basement (cont. over page)

membrane (arrow) bordering intraepithelial MNLs (white arrowheads). C – Trailing edge not colonised by *N.perurans*. D – Higher magnification of C showing occasional intraepithelial MNLs. E & G – Enlarged leading/trailing edge of AGD affected filaments (note sloughed mucus, amoebae, host cells/cellular debris and fused filaments f). F – Higher magnification of E showing intra-epithelial eodema (arrow) and interspersed MNLs (arrowheads). H – Higher magnification of E showing occasional MNLs within oedematous epithelial hyperplastic tissue.

Amoebae associated with gill lesions in flathead frequently showed disintegration of the cellular membrane and cytoplasmic organelle. Contrastingly, salmon gill lesions were often coated by a single or dual layer of trophozoites with mostly intact trophozoites (Figure 29). qPCR results indicated no significant difference (P=0.23) between the relative load of *N.perurans* in the first holobranch of Atlantic salmon (9633.0 ±SE 6098.3 cells.g^{-1} filaments) compared to flathead (3061.4 ±SE 968.11 cells.g^{-1} filaments). *N.perurans* were detected upon four flathead in low numbers (<6 cells.g^{-1} filaments) prior to exposure to *N.perurans*. Salmon were negative in the un-inoculated system at day10 however, a single flathead was positive (1617 cells.g^{-1} filaments). No histopathological signs of disease were present either grossly or histopathologically upon this fish or any other fish of either species collected on day 10.

Gill lesions in flathead associated with the presence of amoebae were predominantly comprised of epithelial cells. In contrast to other endemic species challenged during experiments 1-3, there was a limited infiltration of lesions and the CVS by MNLs and EMNLs, the latter of which were rarely observed. The apical regions of lesions were often populated with large, often actively secreting, basophilic mucus cells (Figure 29).

In dorsally orientated Atlantic salmon gill filaments, AGD induced substantial alterations consistent with those described for experiment 1. Similar observations were made from dorsally orientated flathead filaments which were also as described for experiment 1 with intraepithelial oedema and epithelial hyperplasia of sub-mucosal regions (Figure 30).

Table 15. Prevalence of external lesions upon Atlantic salmon and southern sand flathead.

	External lesion location						
Species	Eyes	Mouth	Flank	Ventral	Fins	Tail	Scale Loss
Atlantic salmon	3.3	0.0	9.0	1.5	48.0	33.0	0.0
Flathead	1.7	8.3	23.0	5.0	0.0	43.0	6.0

In contrast to other endemic species, lesions of the tail flank and mouth were often noted in flathead sampled on day 10 (Table 15). The conical tank design conflicted with the sedentary behaviour of flathead and was likely the underlying cause for the majority of lesions. These mostly appeared to be the result of secondary inflammation/infection arising as a result of rubbing either the internal standpipe or floor of the tanks. External lesion observed upon salmon were consistent with those previously described for experiment 1-3.

Experiment 5: Comparative sequential pathology of yellow-eye mullet <u>Aldrichetta forsteri</u> and Atlantic salmon <u>Salmo salar</u> during experimental challenge with <u>Neoparamoeba perurans</u>

Pre-challenge gross pathology and histopathology

There was no remarkable gross pathology observed upon the gills of salmon sampled prior to the challenge period. Two mullet presented with small hyperplastic/inflammatory lesions comparable in structure and cellularity to those observed in unchallenged mullet from experiment 4 (day 10).

Mullet and salmon exposed to <u>N.perurans</u> (Days 3 – 28): Histopathology

There were no significant differences between mullet and salmon for the percentage of filaments with hyperplastic gill lesions observed on days 3, 7 and 14 (Figure 31). Both species displayed small hyperplastic foci colonised by one or two visible amoebae (Figure 31) although the prevalence of fish with lesions colonised by amoebae was markedly lower in mullet than for the salmon (Table 16) prior to day 21. By day 14 lesions were generally larger than days 3 and 7 (Figure 32) although amoebae numbers colonising lesions were sparse in mullet, consistent with findings in experiment 2. Similarly, lesion morphologies aligned with those described for experiment 2.

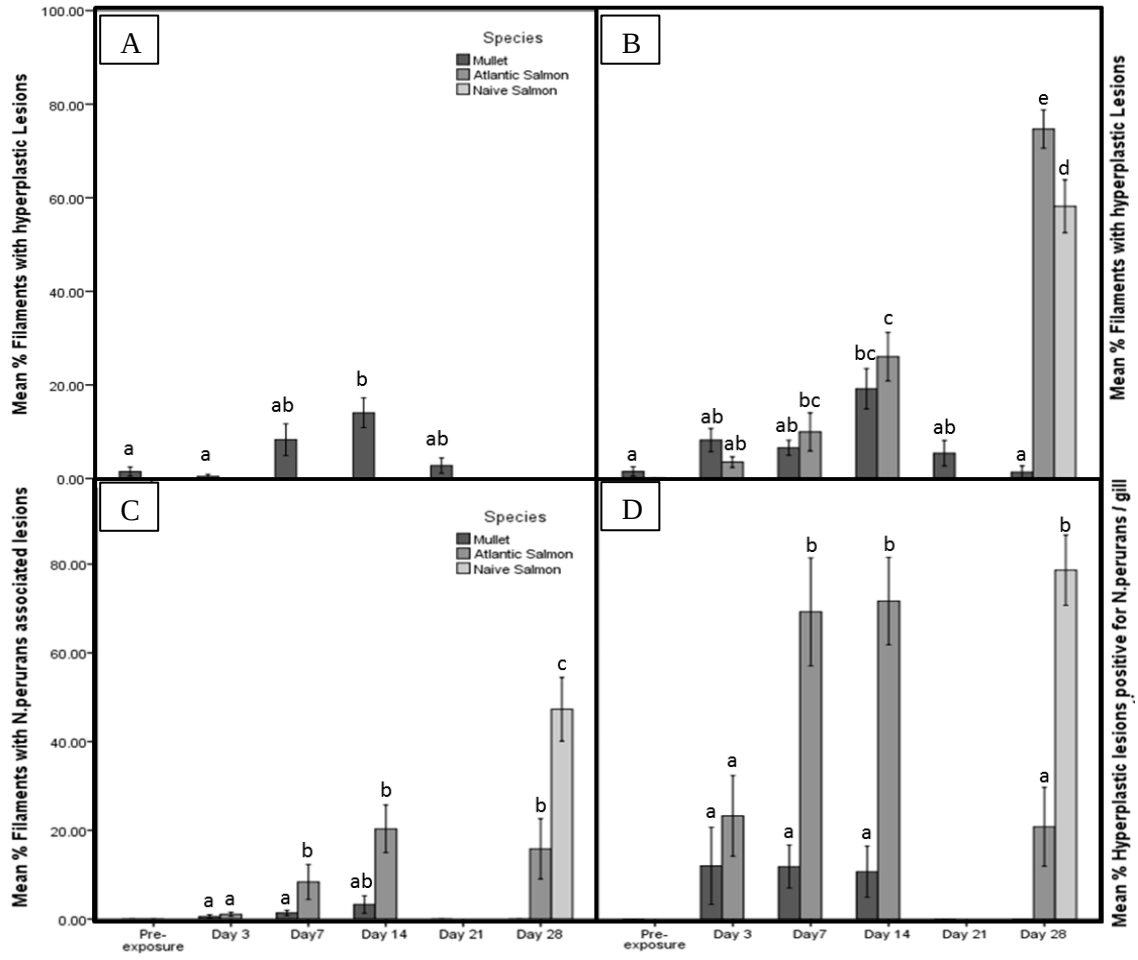

Figure 31. Percentage of filaments affected with hyperplastic lesions (A – Unexposed, B - Exposed). The percentage of filaments affected with lesions colonised by *N.perurans* (C). The percentage of lesions colonised by amoebae per gill section (D). Note – salmon were not sampled on day 2. Salmon naïve to AGD were introduced to the exposed system on day 14. Letters indicate means that were significantly different.

Table 16. Prevalence of fish with hyperplastic lesions colonised/uncolonised by *N.perurans*

	NP*	Salmon**	Mullet**	Salmon	Mullet	Salmon	Mullet	Salmon	Mullet	Salmon	Mullet	Salmon	Mullet	Salmon (N)	Mullet (N)
Day	**-**	**0**	**0**	**3**	**3**	**7**	**7**	**14**	**14**	**21**	**21**	**28**	**28**	**28**	**28**
% Group NP +ve	-	0	0	0	0	0	0	0	0	0	0	0	0	n/a	n/a
% Group Lesion +ve	-	0	20	0	10	0	60	20	90	0	50	0	0	n/a	n/a
% Group NP +ve	+	0	0	50	20	80	50	90	40	n/a	0	80	0	100	n/a
% Group Lesion +ve	+	0	20	60	80	100	80	90	80	n/a	50	100	10	100	n/a

*+ indicates group challenged with *N.perurans (NP),** Data drawn from same sample pool*

No amoebae were detected histologically in mullet sampled at day 21and 28 (Figure 33 & Table 16), the prevalence of mullet with hyperplastic lesions was significantly lower (Table 16) and the mean percentage of lesion affected filaments had also diminished significantly compared to mullet sampled at day 14 (Figure 31). Aside from occasional small hyperplastic/inflammatory lesion foci the gills of mullet sampled on day 28 were comparable to mullet in the unexposed group ().

In contrast to mullet, the percentage of hyperplastic gill lesions from Atlantic salmon was significantly higher by day 28 compared to day 14, all fish were affected with lesions and 80% of fish had lesions colonised by amoebae (Figure 31 & Table 16). Interestingly a significantly lower percentage of lesions were colonised by amoebae on day 28 compared with the previous sampling on day 14 (Figure 31). Lesions from this group were showing signs of resolution indicated by smaller sizes, undulating lesion surfaces and a marked presence of mucus cells apically aligned upon lesions and apposed filaments (Figure 33). Similarly, where lesions were colonised by amoebae their numbers were comparatively low relative to the naïve fish, typically observed singularly or in small groups of 2 – 4 trophozoites. Infective pressure within the system was still clearly apparent as naïve salmon introduced to the system on day 14 had rapidly developed signs of AGD. This was indicated by the prevalence of affected fish, the percentage of lesion affected filaments, the percentage of filaments with colonised lesions and the high proportion of lesions colonised by amoebae (Figure 31, Figure 34 & Table 16). Gill lesions in naïve fish displayed familiar AGD lesion morphologies with marginal oedema, leukocyte infiltration, epithelial sloughing and clusters of colonising amoebae (Figure 34).

Mullet and salmon unexposed to N.perurans (Days 3-28): Histopathology

There were no unusual pathological findings in the gills of Atlantic salmon unexposed to *N.perurans* for the duration of the experiment except for day 14 where two fish presented with multifocal fusion of lamellar pairs (data not shown). The gills of mullet sampled on days 3 – 21 presented with hyperplastic, often spongiotic lesions infiltrated with EMNLs and small MNLs as described for lesions in mullet gills from experiment 2 (Figure 33). An aetiological agent was not identified in association with any gill lesions presenting in either fish species not exposed to *N.perurans*.

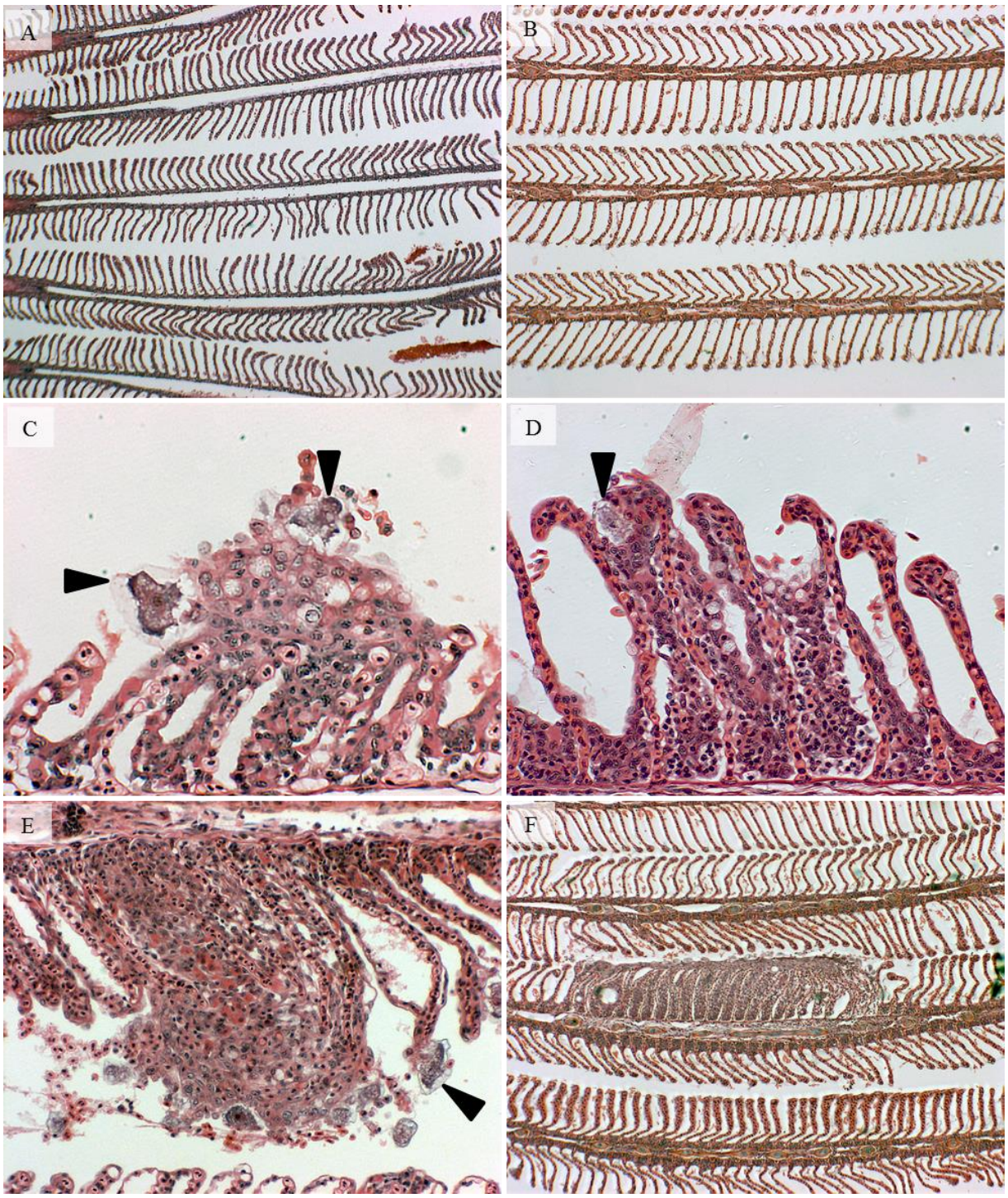

Figure 32. Lesion development in mullet and salmon gills exposed to *N.perurans* at days 0,3 and 7. A- Mullet gills prior to exposure; B – Salmon gills prior to exposure; C – Mullet gill lesion 3 days post-exposure (DPE) showing ballooned amoebae (arrowhead); D – A single trophozoites attached to a lesion in salmon 3 DPE; E – Multiple amoebae attached and associated with a gill lesion from mullet at 7 DPE; F - Salmon gill lesion at 7 DPE.

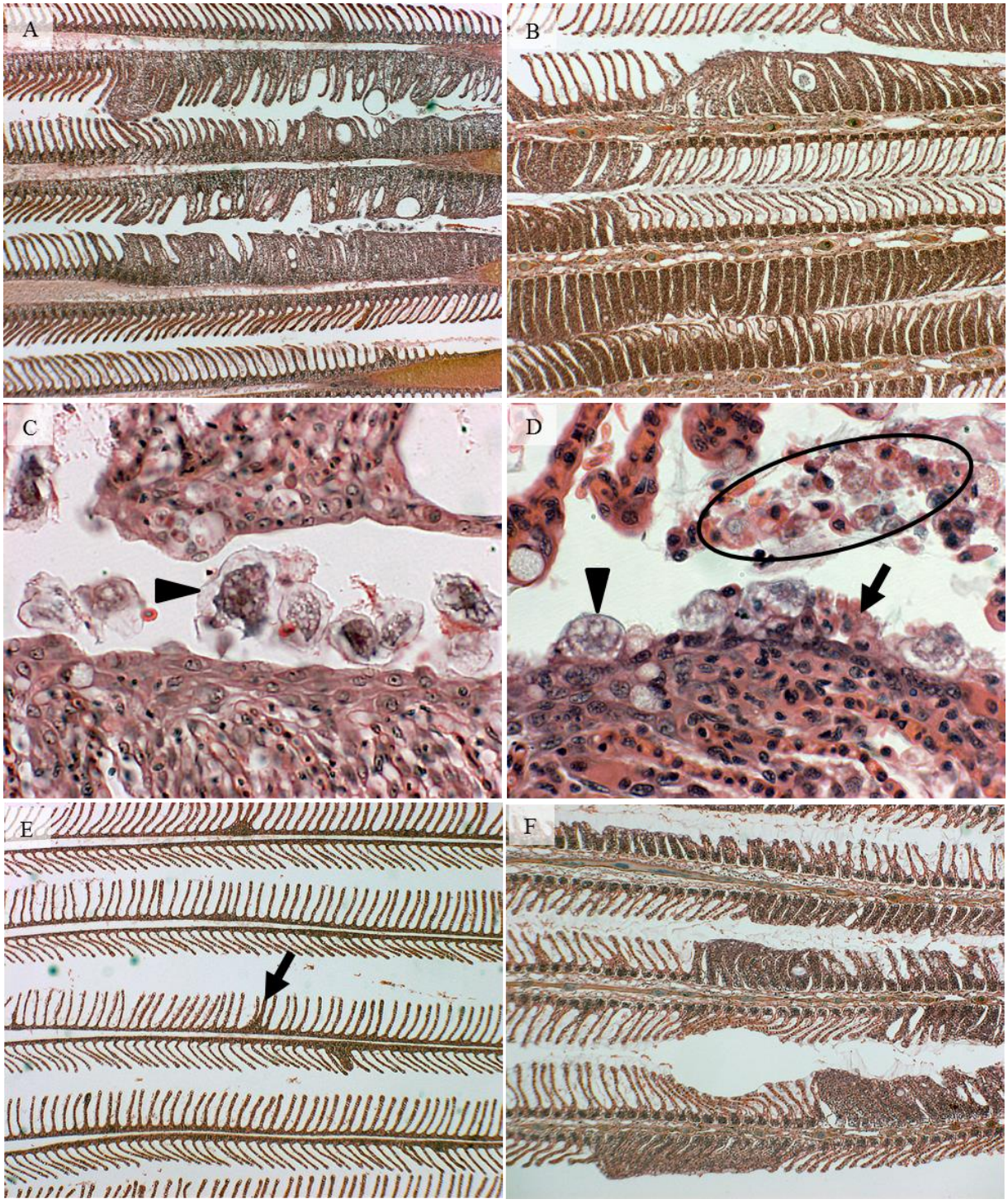

Figure 33. Lesion development in mullet and salmon gills exposed to *N.perurans* at days 14 and 28. A- Mullet gill lesion spread across three filaments at 14 DPE; B – Salmon gill lesion spread across three filaments at 14 DPE.; C – Mullet gill lesion 14 days post-exposure (DPE) showing several ballooned amoebae (arrowhead) unattached to hyperplastic epithelium; D – Trophozoites attached to a lesion in salmon at 14 DPE with sloughing (arrow) and sloughed host cells (circle); E – Mullet gill at 28 DPE, small MNL infiltrated lesions are present without ameobae (arrow); F - Salmon gill lesions present at 28 DPE.

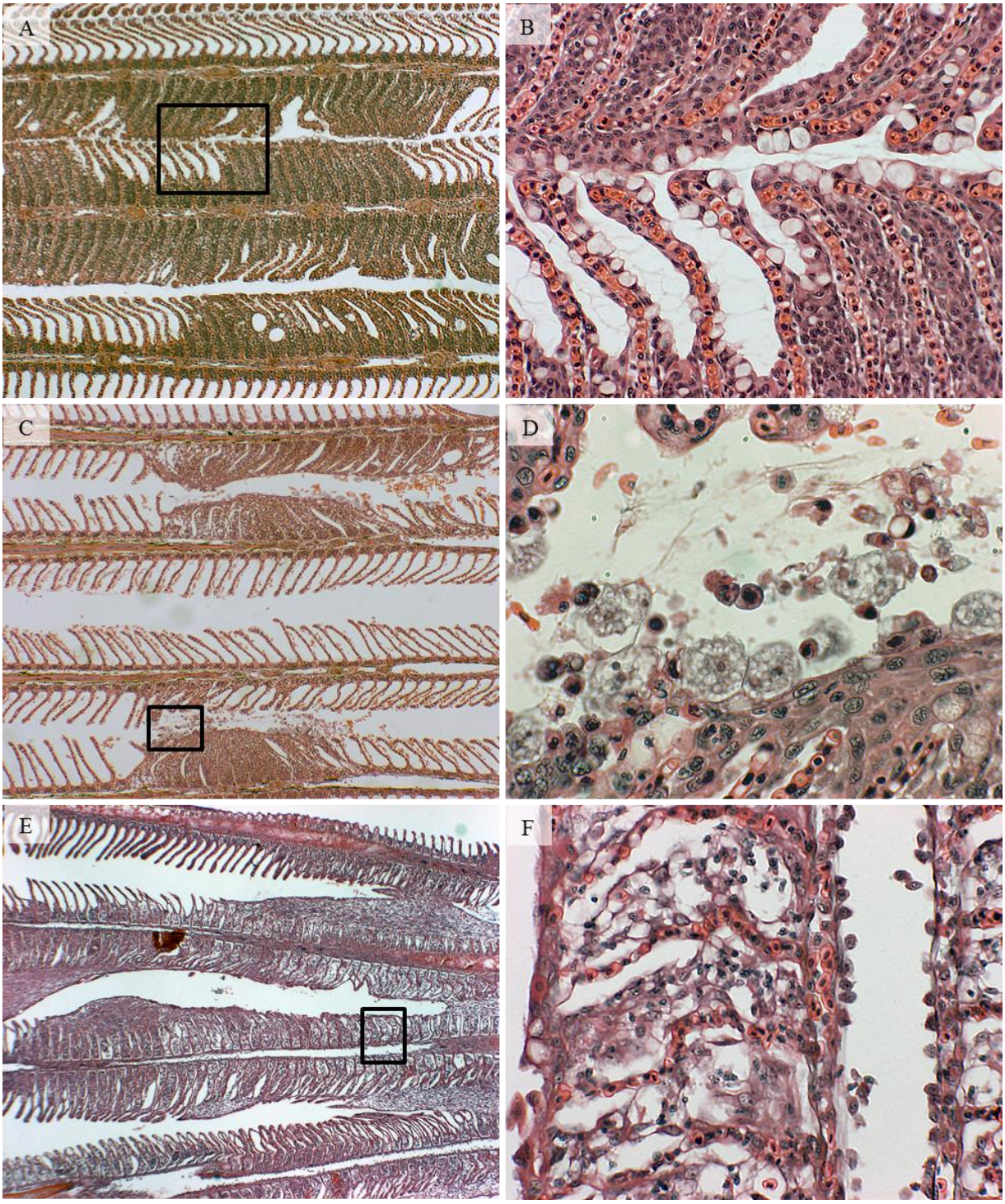

Figure 34. Lesion comparison in salmon gills exposed to *N.perurans* at day 28 and naïve salmon introduced at 14 DPE (A-D). Mullet gill lesions observed in mullet not exposed to amoebae at day 14. A- Salmon gill lesion spread across three filaments 28 DPE without amoebae; B – Higher magnification of A showing high abundance of mucus cells and mucoid material lacking marginal oedema, leukocyte infiltration and sloughing of host cells/cellular material; C – Gill lesions in salmon introduced at 14 DPE and sampled on 28 DPE; D – Higher magnification of C showing several trophozoites attached to a lesion sloughing and sloughed host cells; E – Mullet gill at 14 DPE showing extensive lamellar fusion across three filaments. F – Higher magnification of E showing spongiotic epithelial lamellar fusion and leukocyte infiltration.

Salmon and mullet – All groups (Day 0-28): qPCR

Pre-challenge, a single mullet was positive for the presence of *N.perurans* albeit at a low relative load (3 cells.g^{-1} filaments) (Figure 35). Salmon exposed to *N.perurans* showed an increasing infection prevalence and relative amoebae load from day 3 to day 14 and day 28 (Figure 35). The relative load of amoebae in salmon was significantly higher ($P<0.05$) at day 14 compared days 0,3 and 7 and comparable to day 28. Interestingly the prevalence of amoebic infection in mullet remained high throughout whereas the relative load did not increase significantly though time although a peak trend was evident at day 14 (Figure 35).

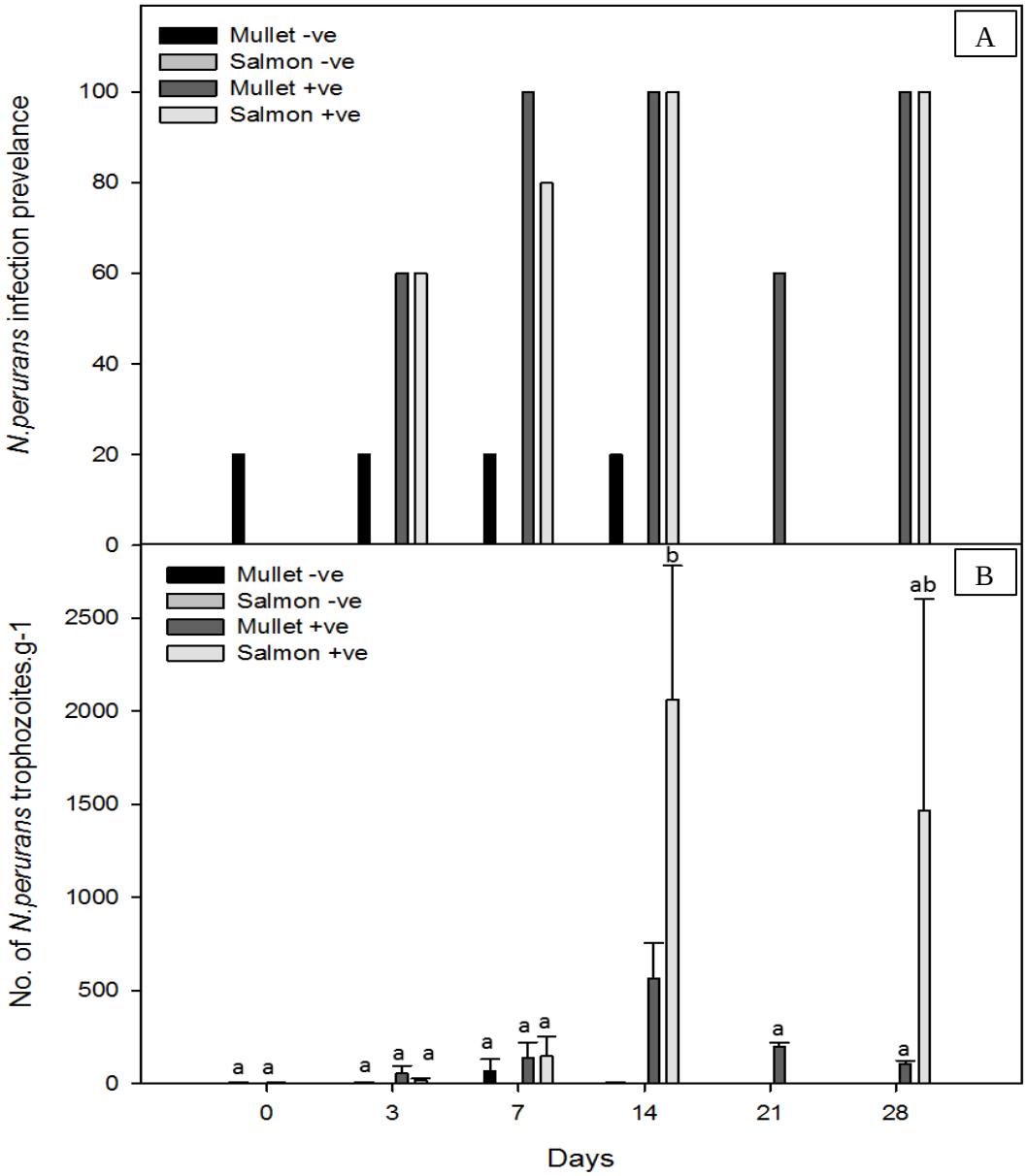

Figure 35. Prevalence of infection with *N.perurans* (A) and the relative trophozoite load upon the first-left holobranch detected and quantified respectively by qPCR (B)(salmon holobranchs not collected on day 21) (n=5/bar).

Morphometry/morphology of inter branchial lymphoid tissue (ILT) in Atlantic Salmon

The proximal ILT (PILT) of salmon contained a network of reticular epithelial cells forming a mesh-like structure enclosing populations of small mononuclear leucocytes resembling lymphocytes consistent with previous anatomical descriptions (Haugarvoll *et al.* 2008; Dalum *et al.* 2015).

Morphometric differences in PILT assemblage were detected in the gills of the fish in the later stages of infection (days 14 and 28). The PILT area (pooled) and length (dorsal) were significantly larger compared to all other groups at all other time points (P< 0.001) (Figure 36). The medial site of infected salmon was significantly smaller than both the dorsal and ventral sites ($P<0.05$ and $P<0.01$, respectively) but not significantly different to the medial and dorsal regions of unexposed salmon (data not shown). There was substantial individual variability in the size of the PILT from infected fish. Regression analysis indicated that AGD severity accounted for 40% of the variation of the area of the PILT ($P< 0.001$) and 27% for length ($P= 0.004$) (Figure 37).

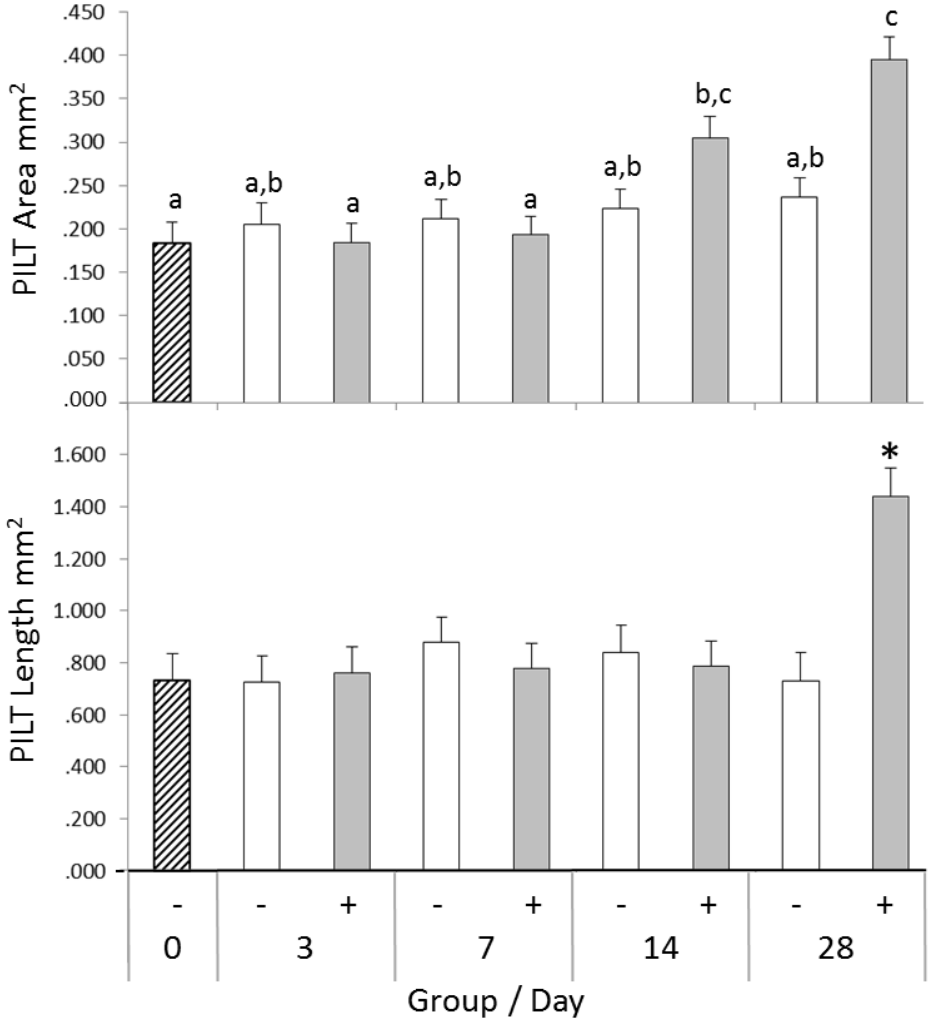

Figure 36. (Top) The mean ± SE area and length (dorsal region) (bottom) of PILT from salmon on days 0, 3, 7, 14 and 28. Different letters or an asterisk indicate values that are significantly different (P< 0.05).

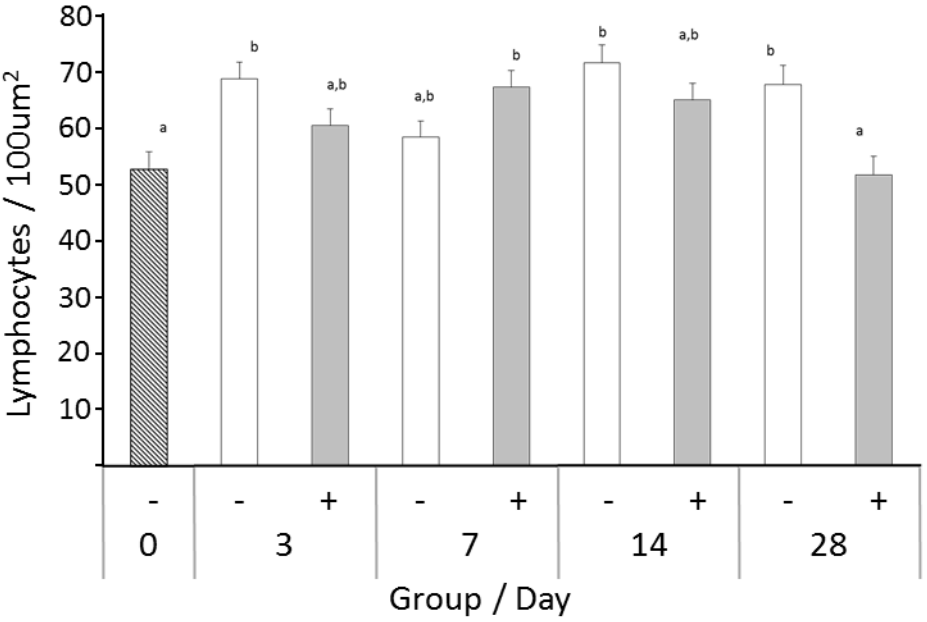

Figure 38. The relative number of lymphocytes in the PILT of fish at 0, 3, 7, 14 and 28 DPE. Values are mean ± SEM. Different letters indicate values that are significantly different ($P < 0.05$) (- Unexposed, + exposed to *N.perurans*).

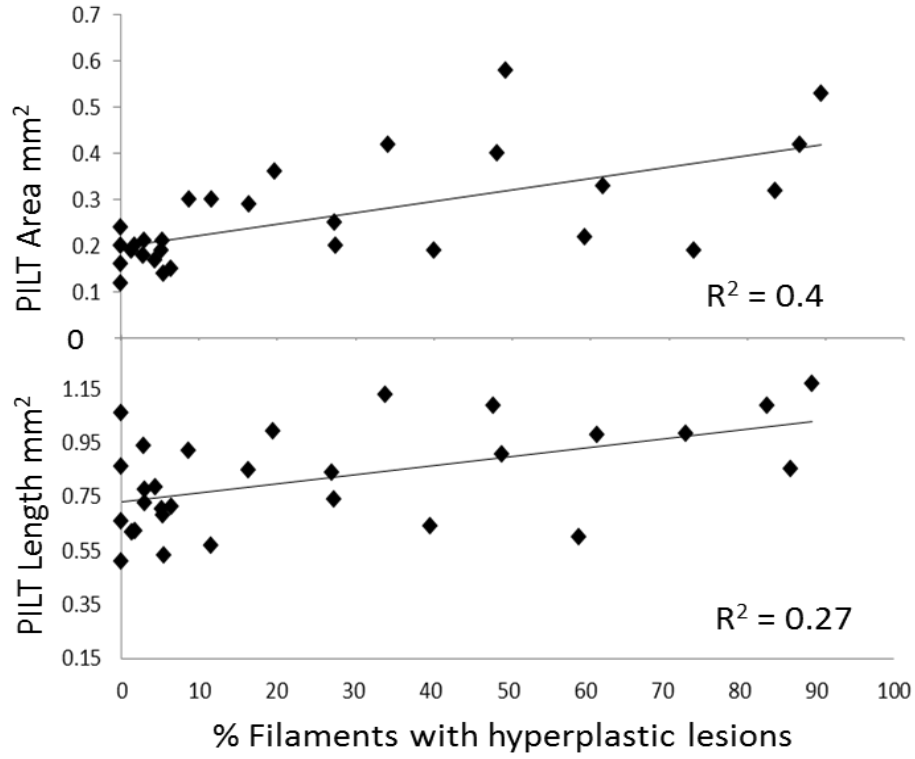

Figure 37. Relationship between the area of the PILT (area (mm2)/log (weight) + 1) ($F = 19.79$, $df = 1,28$, $P < 0.001$) (top) and length of the PILT (length (mm)/log (weight) + 1) and AGD severity expressed as percentage of filaments with hyperplastic lesions ($P < 0.01$) (bottom)

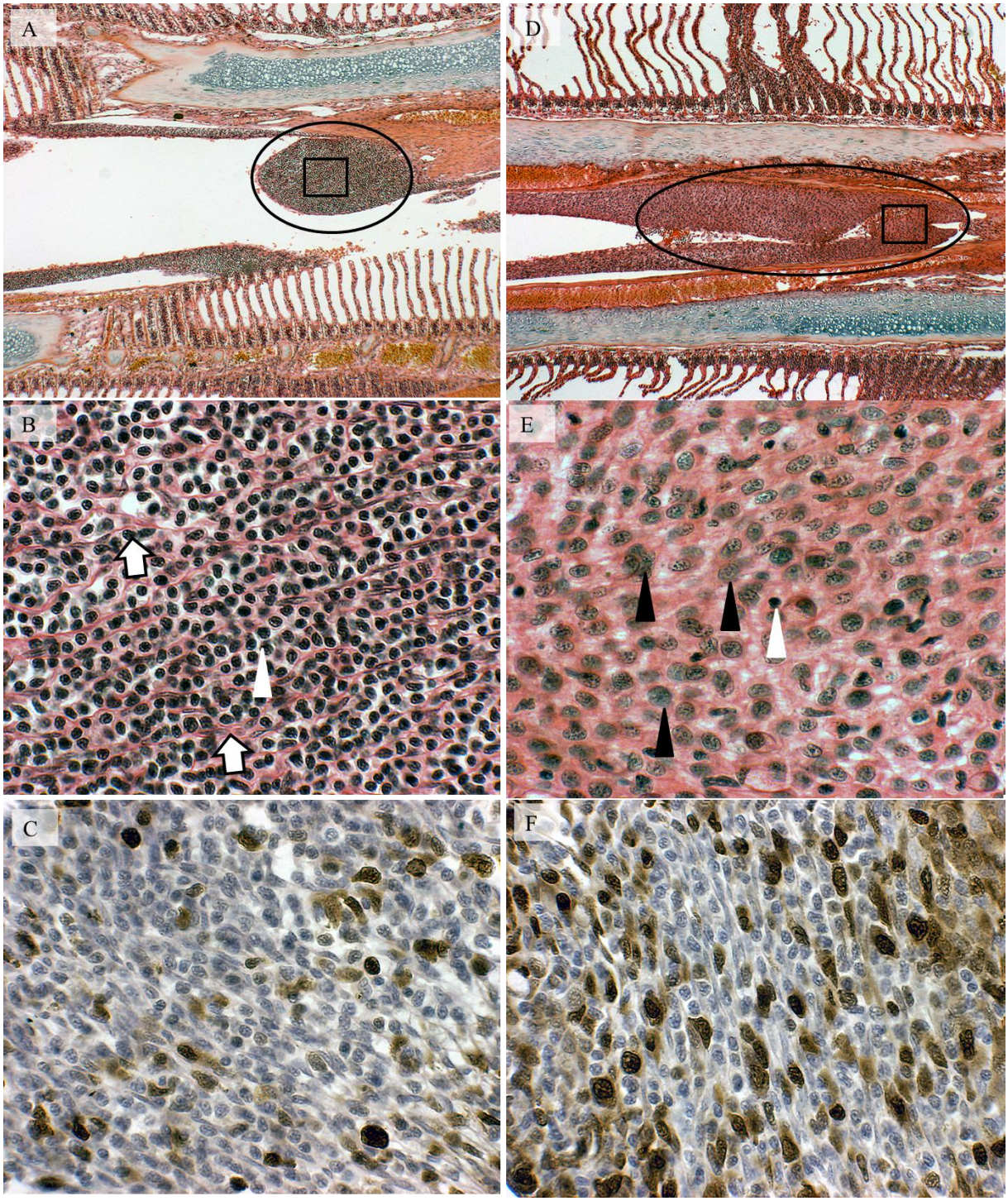

Figure 39. (A) Transversal section of a gill filament of an unexposed fish sampled on day 28 showing PILT (inside circle).(B) Higher magnification of box inset of (A) depicting lymphocytes (arrowhead) interspersed amongst a reticular epithelial mesh (arrows). (C) PCNA +ve (recently divided) cells in PILT unexposed to amoebae on day 28. (D) AGD affected salmon on day 28 with enlarged PILT(inside circle) with hyperplastic lesions evident above and below (arrows). (E) Higher magnification of box inset of (D) showing transformed PILT dominated by undifferentiated epithelial cells (black arrowhead). (F) Partially transformed PILT from AGD affected salmon on day 28. Note contrast with (C) for higher frequency of PCNA positive cells.

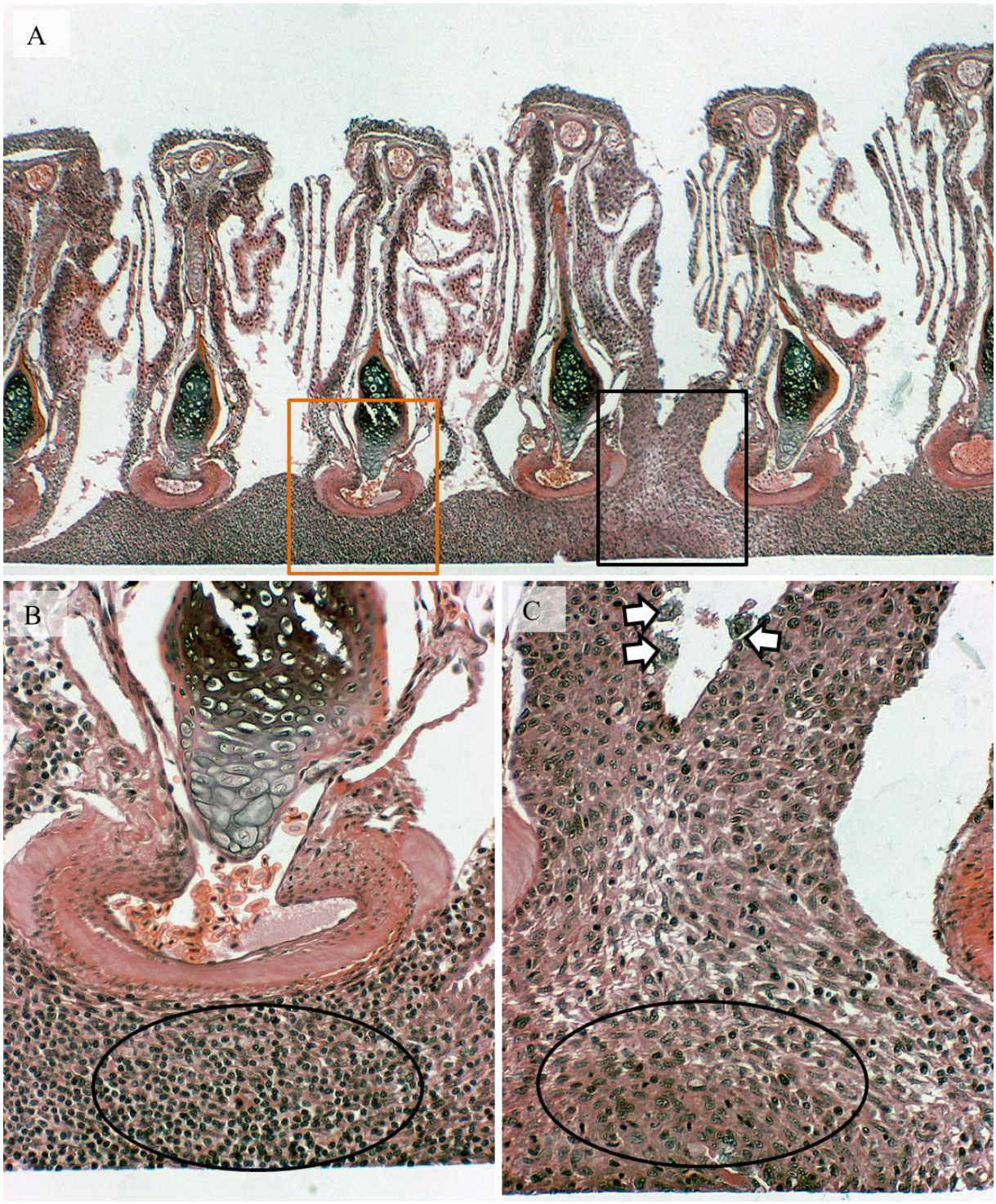

Figure 40. (A) PILT of an AGD affected salmon (day 28) viewed dorsally. PILT adjacent to a normal filament is highlighted (orange box). Hyperplastic epithelium infiltrating a region adjacent to apposed lesions (black box). (B) Higher magnification of orange box showing lymphoid morphology. (C) higher magnification of black box showing epithelial cell dominant tissue in contrast to (B).

In uninfected salmon the PILT area was significantly larger in the ventral holobranchs region than in the medial region ($P< 0.001$) (data not shown). Otherwise, there were no other remarkable findings in terms of morphometric divergence in PILT within the unexposed groups.

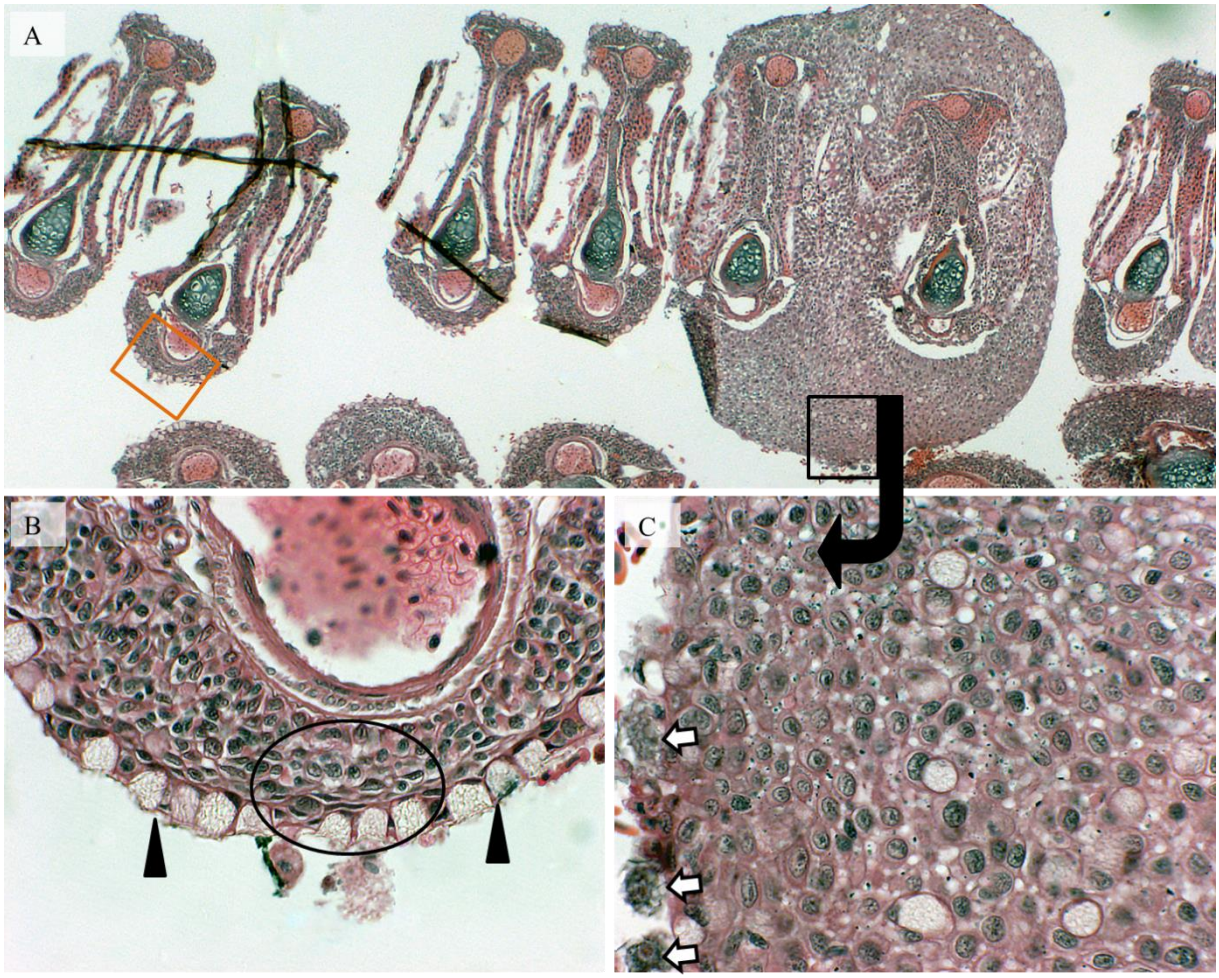

Figure 41. (A) Dorsal view of filaments from an AGD affected salmon (day 28). DILT associated with the trailing edge of a normal filament is highlighted (orange box). Proliferated epithelium is evident between fused filaments transforming this region (black box). (B) Higher magnification of orange box showing normal DILT construct contained between mucosa (arrowheads). (C) Rotated higher magnification of black box showing epithelial cell dominant tissue with attached trophozoites (arrows) and loss of normal mucosal surface in contrast to (B).

PILT lymphocyte numbers in AGD affected salmon was significantly higher on day 7 when compared with day 0 ($P < 0.05$) (Figure 38). Larger PILT size in AGD affected salmon at the individual level were concomitant with remodeling of the reticular epithelial network, particularly when hyperplastic lesions were present nearby or coalescing with PILT (Figure 39, Figure 40). Lymphocytes were less abundant and epithelial cells assumed an undifferentiated morphotype (Figure 39, Figure 40). On day 28, a significantly lower density of lymphocytes was found in the PILT of infected fish compared to day 7 ($P < 0.05$) and compared to unexposed salmon ($P < 0.05$) (Figure 38). Lymphocyte density unexposed salmon PILT was significantly higher on day 3 ($P<0.05$) but did not change significantly for the subsequent duration (Figure 38). There was no significant difference in lymphocyte numbers between gill sites ($P = 0.140$). PCNA positive cells, indicative of recent cell division, were present in both infected and non-infected salmon. PCNA positive cells more numerous in PILT of AGD affected fish from days 14 and 28 and it appeared that epithelial cell nuclei accounted for the majority of signaling (Figure 39).

A similar pattern of tissue remodeling was evident in the distal ILT (DILT)(Figure 41), which is present as a diffuse mucosal lymphoid tissue particularly prominent upon the trailing edge of the filament (Dalum *et al.* 2015). Unaffected filaments, even those immediately juxtaposed to those with lesions, presented a distinct mucosal surface comprised of a single or dual layer of squamous epithelium with mucous cells prominently embedded within. In dorsally sectioned lesions the reticular meshwork normally contained between the mucosa and basal membrane of the trailing filament edge was indistinct and dominated by undifferentiated epithelial cells particualalry when trophozoites had colonized the lesion (Figure 41). Mitotic figures within epithelial nuclei were often encountered along with occasional eosinophilic MNLs and lymphocytes (data not shown). The mucosa was generally devoid of mucous cells particularly when amoebae were located nearby (Figure 41).

Objective 2: Investigate the comparative host responses of Atlantic salmon and rainbow trout naive and previously exposed to amoebic gill disease.

Experiment 6: Comparative pathology of AGD in Atlantic salmon Salmo salar and rainbow trout Oncohynchus mykiss following repeated infections with Neoparamoeba perurans

Histological observations from fish exposed to Neoparamoeba perurans:

Group classification

Generally, the group classification based on the gross gill score reflected both the percentage of gross affected area calculated by the image analysis (FIGURE) and the histological assessment of AGD severity (Table 17). In particular, the correlation analysis between the percentage of gross affected area and the mean lesion size for the second left gill arch showed that there was a positive correlation ($P< 0.01$ and $r^2 = 0.6$).

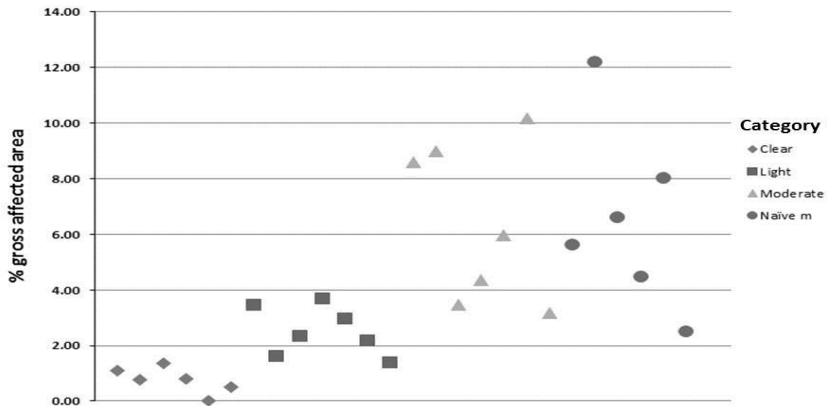

Figure 42. Grossly affected area derived from the left hand holobranchs set (8 x hemibranchs) for each gross category.

Table 17. Histological assessment of AGD severity for each grossly categorised AGD history

Histological parameters	*Clear*	*Light*	*Moderate*	*Moderate**
% of filaments with hyperplastic lesions	35.9	36.6	39.5	32.5
% of filaments with lesions colonised by amoebae	2.4	15	13.7	16.3
% of lesions colonised with amoebae	5.7a	40.9b	36.8abc	52.2c
Mean lesion size (ILU)	7.5a	13.3ab	17.4bc	21.0c

*Naïve infection / no treatment

Interestingly the mean percentage of filaments with hyperplastic lesions was essentially the same across all infected groups. Lesions occupied increasing lengths of filaments as the clinical severity increased as indicated by the mean lesion size. The clear group also showed a five to six fold negative difference between the percentage of filaments with lesions colonised by amoebae and similar differences in the percentage of total lesions harbouring amoebae. There were no significant

differences for amoebic lesion colonisation between the light, moderate and naïve moderate groups. Unexposed fish did not harbour any lesions consistent with infection by *N.perurans*.

Histolopathological response according to gross categorical history

Moderate (Naïve fish infected with N.perurans):

Lesion sizes ranged from small (2 -10 interlamellar units - ILU), medium (10-50 ILUs) to large (>50 ILUs) which occupied more than half the length of an individual filament (mean length = 17.4 ILU; Table 17).

The lamellar and filamental epithelium around small developing lesions was often desquamated with underlying oedema separating the epithelium from their basal membranes (Figure 43). These voids were proportionally expansive as lesions spread and were frequently infiltrated by EMNLs and occasional PMNLs. Chloride cells remained upon the exterior periphery of desquamating epithelium occasionally sloughed from the epithelium along with degenerating epithelial cells & occasional leukocytes.

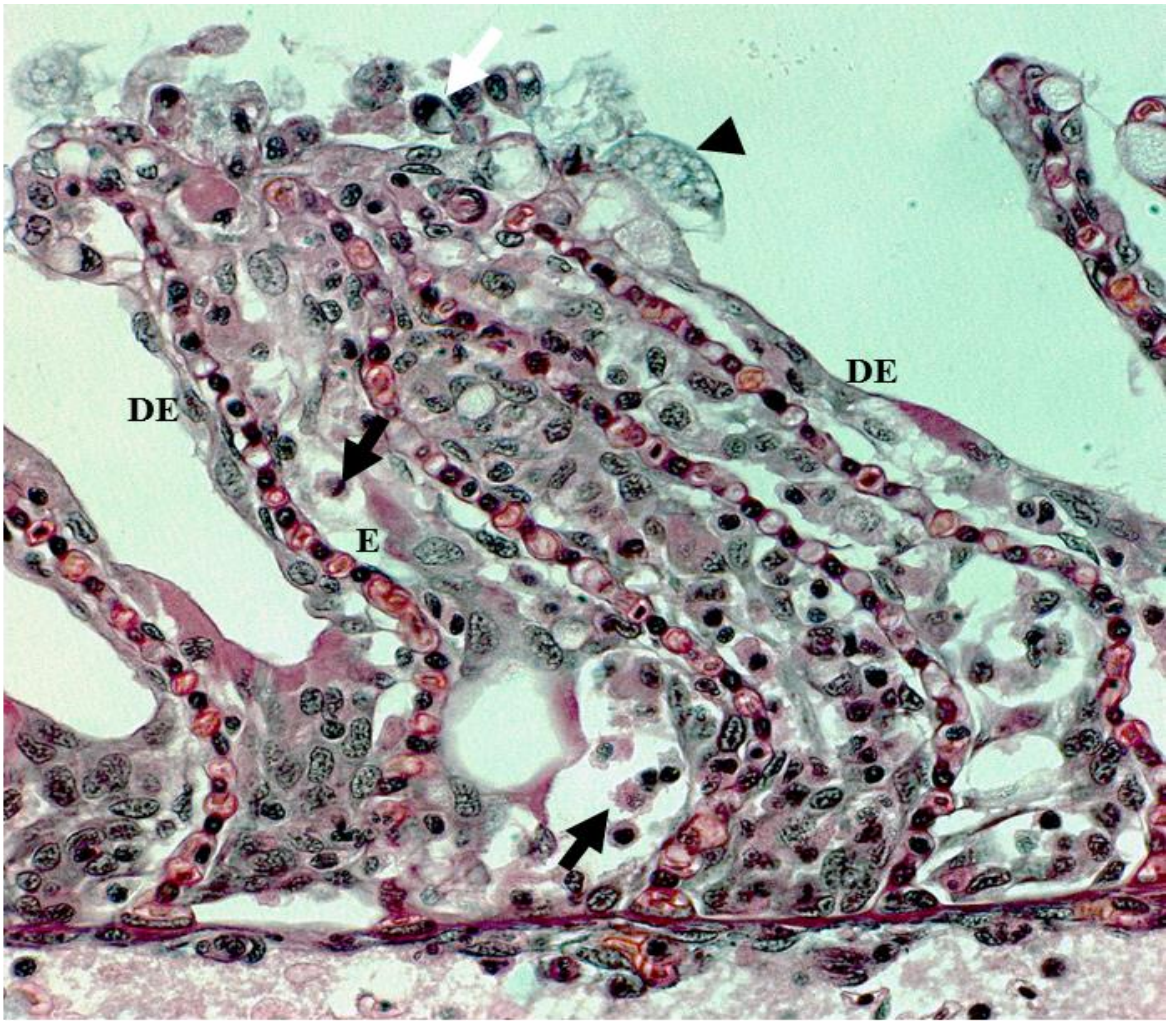

Figure 43. A small developing lesion in gills of Atlantic salmon previously unexposed to *N.perurans*. Amoebae (arrowhead) and sloughed cells (white arrow) evident apically. Desquamating epithelium is seen peripherally (DE) with EMNLs (black arrows) infiltrating large inter-epithelial channels.

Larger lesions had sometimes spread and occasionally fused to apposed filaments. The margins were typically infiltrated by leucocytes as described for smaller developing lesions and amoebae were most frequently observed clustered at these locales. The internal regions were dominated by variably packed epithelial cells (contrast illustrated by figures 5 & 6) sometimes extruding well beyond the lamellae tips where a looser or spongiotic matrix was sometimes observed. Epithelial cells were easily discerned by their large nucleus, stippled chromatin and prominent nucleolus. Internally, lesions were sometimes infiltrated by EMNLs (figure 5) and occasional PMNLs. EMNLs were more readily detectable in spongiotic or loosely packed epithelial matrices (figure 5). The presence of EMNL infiltrated epithelial channels and inter-epithelial compactness varied both between fish, between

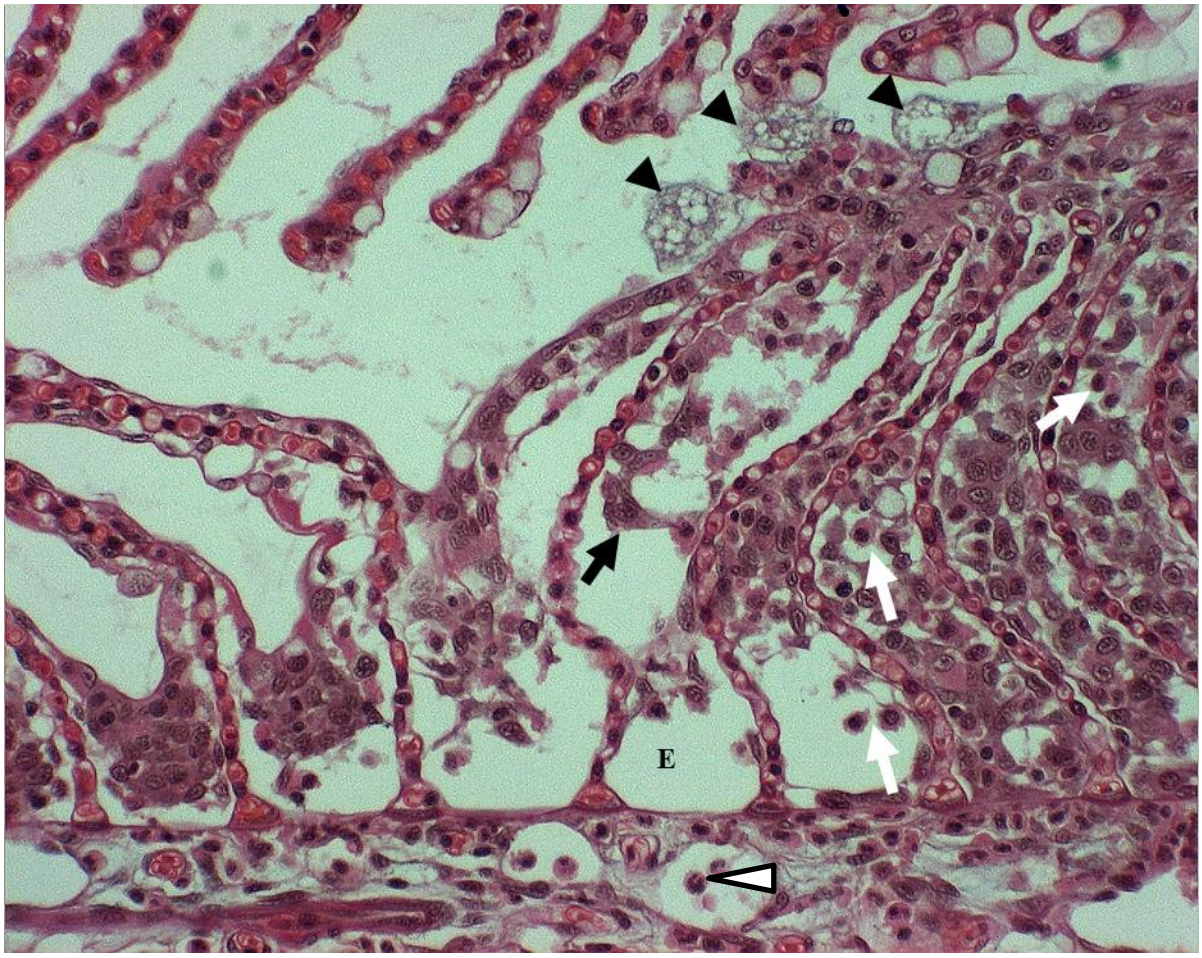

Figure 44. The margin of a larger lesion from an AGD-naïve Atlantic salmon colonised by ameobae (arrowheads). EMNLs (white arrows) and desquamated pavement cells (black arrow) present within areas of oedema (E). PMNLs were more prominent in vessels and sinuses (highlighted arrowhead).

lesions within individual fish and within individual lesions. Interlamellar vesicles of varying sizes (1-5 ILUs) featured regularly in lesions longer than 10 ILUs. These were mostly empty but occasionally contained necrotic amoebae, host cells and proteinaceous material. Mucous cell were sporadic within hyperplastic lesions, both within and upon the apical periphery of lesions but they were more regularly encountered centrally upon larger lesion surfaces where amoebae were rarely seen. Large and actively secreting mucus cells were notable upon non-hyperplastic lamellae of lesion apposed filaments. Chloride cells were rarely observed apically or within larger lesions.

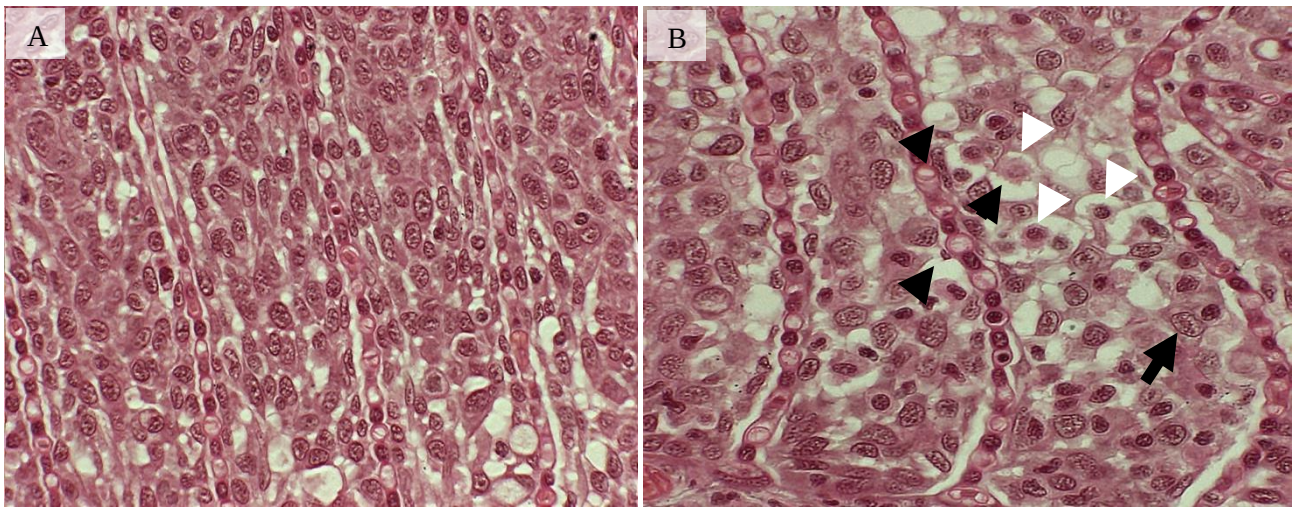

Figure 46. An internal region within a large lesion in gills of Atlantic salmon previously unexposed to *N.perurans*. A – Epithelial cells dominate the inter-lamellar regions – note the contrast in tissue structure and cellularity with B (collected from the same tissue section) where EMNLs (white arrows) occupy inter-epithelial channels (black arrows). Epithelial cells (black arrowheads) are identified by the presence of prominent nucleus and stippled chromatin characteristic of proliferating cells (arrow).

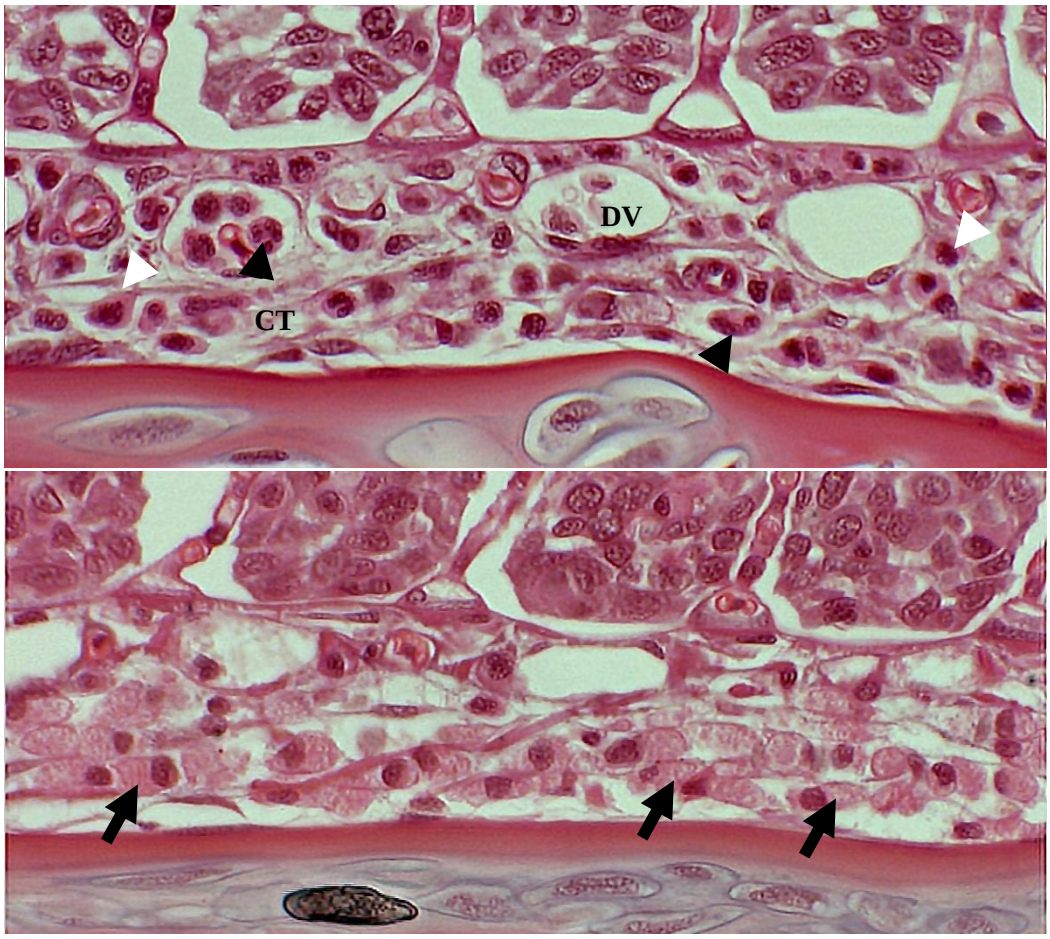

Figure 45. A – The connective tissue surrounding the cartilage adjacent to a hyperplastic lesion in gills of Atlantic salmon previously unexposed to *N.perurans*. PMNLs (black arrowheads) and EMNLs (white arrowheads) present within CVS drainage vessels (DV) and connective tissue (CT). B – Numerous eosinophilic granule cells (arrows) within the connective tissue below a lesion.

The basal filamental epithelium was structurally consistent with control fish in non-hyperplastic regions. Tissue modifications were largely reflective of the inter-lamellar hyperplastic tissues described above at lesion sites. At lesion locations, PMNLs and EMNLs were common (except for a single fish) and more numerous than in unaffected sites; the former were consistently the dominant cell type present. The connective tissue surrounding the cartilage and nutrient supply vasculature had higher numbers of PMNLs and EMNLs at lesion sites (8A) in comparison to unaffected tissues and controls. Elevated numbers of eosinophilic granule cells were observed in one fish juxtaposed to some larger lesions (8B). However, dorsally aligned sections showed a narrow band of eosinophilic granule cells concentrated around the cartilage leading edge (data not shown). A sagittal cut at the appropriate angle could misrepresent actual EGC abundance.

Clear (Fish re-infected with N.perurans):

Small lesions of less than 10 ILUs dominated the gills of fish scored clear of gross lesions at the final sampling point. Colonisation of lesions with amoebae was rare as reflected by Table 17. Small MNLs were numerous within the inter-lamellar tissue and were sometimes mixed with larger EMNLs within inter-epithelial channels. The apical lesion surfaces were consistently lined with large mucus cells (Figure 47).

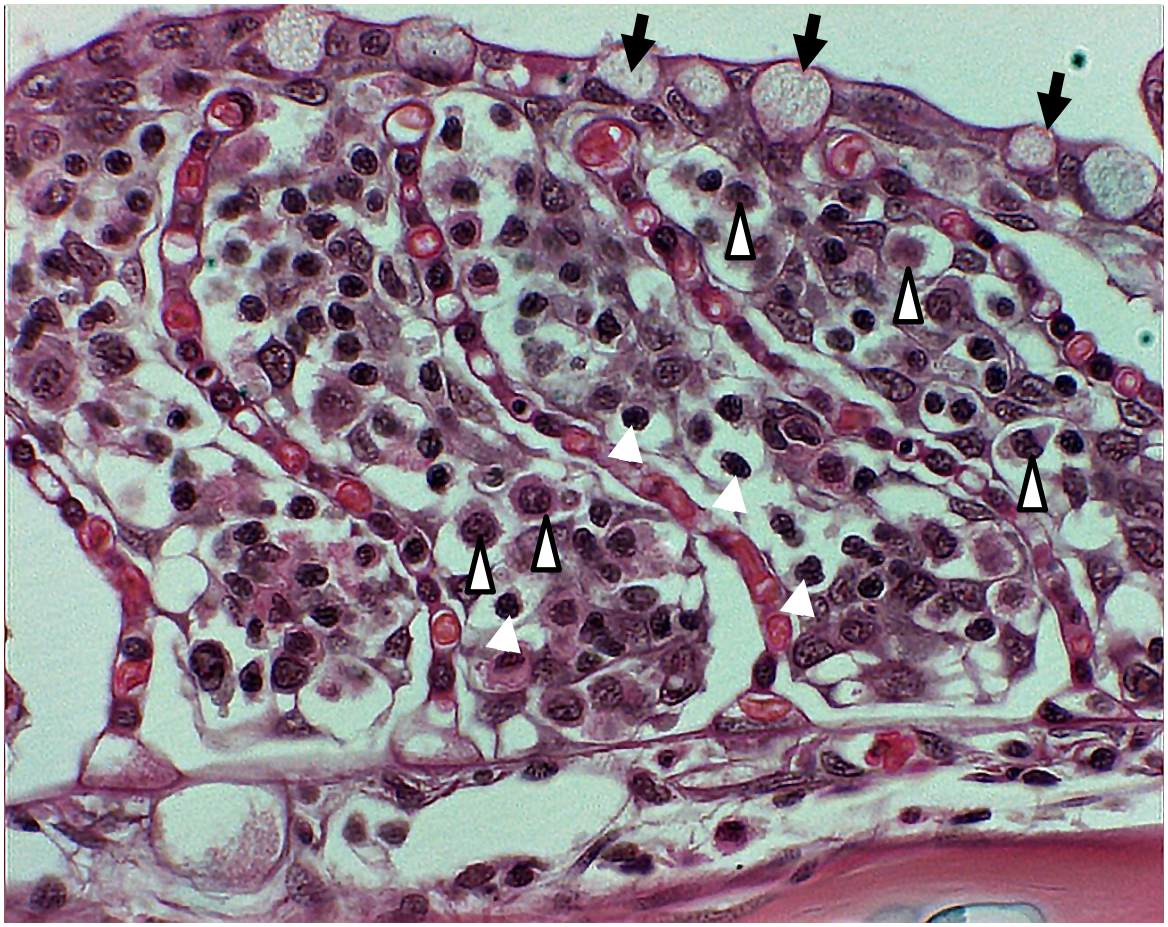

Figure 47. A small lesion from gills previously exposed to N. perurans that presented clear of macroscopic lesions. Many smaller MNLs (white arrowheads) and larger EMNLs (highlighted arrowheads) infiltrating channels between epithelial cells. These lesions were typically rich in large, apically located mucus cells.

Congestion of the CVS and connective tissues with PMNLs, EMNLs, and small MNLs adjacent to lesions did not feature to the extent seen in the other categories. Seemingly higher numbers of eosinophilic granule cells were observed in the connective tissues contiguous to lesion sites in two fish, however it is difficult to ascertain whether these instances may be a section depth and/or filament alignment artefact.

The gills from a single fish contained three larger lesions that were undetected during gross assessment. Interestingly the histological presentation showed that these lesions were lined apically with mucus cells and had very little congestion within the CVS or connective tissues. The lesion surfaces were also undulating and from 39 lesions counted in total upon this fish only three were colonised. It is likely that the larger lesions in this particular fish were receding/resolving and not visually prominent from a macroscopic perspective.

Light (Fish re-infected with N.perurans):

Fish with a light clinical AGD history presented histologically with a similar percentage of hyperplastic gill lesions although they seldom exceeded approximately 10 ILUs and had rarely spread to neighbouring filaments.

In contrast to fish categorized as clear, the percentage of lesions with amoebae was essentially the

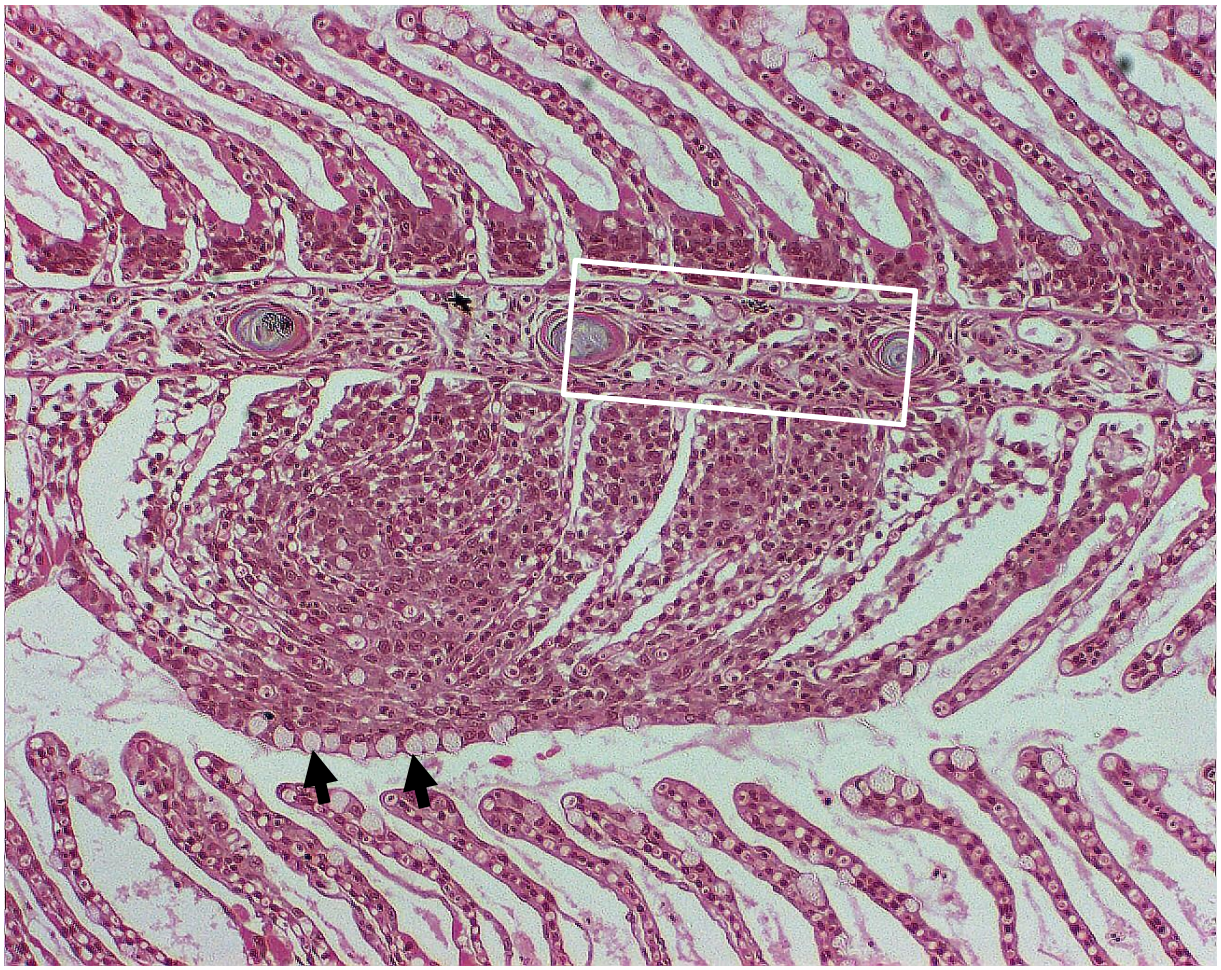

Figure 48. A lesion from a fish with a light clinical AGD history. A congested epithelial matrix with occasional small MNLs and EMNLs infiltrating the lesion. Connective tissue surrounding cartilage and sinusoidal spaces/drainage vessels are infiltrated with various leukocytes (boxed area). Numerous mucus cells line the lesion surface apically (arrows).

same as fish with a moderate clinical history. Consistent with the variation in lesion cellularity seen for moderately affected fish (previously infected) variable numbers of leucocytes were observed within many lesions and within connective tissue abutting the basal filamental membrane (Figure 48). There were no differences in lesion colonisation rates between the light and moderate categories (Table 17), however their abundance on a per colonised lesion basis was low given amoebae were typically seen singularly or occasionally in groups of two or three.

Moderate (Fish re-infected with N.perurans):

Fish that were previously exposed to *N.perurans* infection with a moderate clinical disease history generally exhibited the same infection response patterns described above for naively infected fish with a moderate gross score. Table 17 illustrates a lower proportion of lesions were colonised by amoebae but conversely a higher percentage of lesion affected filaments. A distinct variation in cellularity for some lesions was observed particularly in those of shorter lengths. This was typified by the presence of small MNLs infiltrating inter-epithelial channels within lesions. The same morphotype was seen in connective tissue to corresponding lesions but rarely in the central venous sinus and associated drainage vessels. A dense monolayer of mucus cells were found upon the apical periphery of smaller lesions that were infiltrated by small MNLs. Amoebae were rarely observed upon lesions of this type consistent with findings in fish categorised as clear or light. Small MNL infiltration of lesion affected tissue was not easily discernible in all lesions examined. Typically a mixture of cellularity patterns was evident in the majority of lesions examined with variability existing both between and often

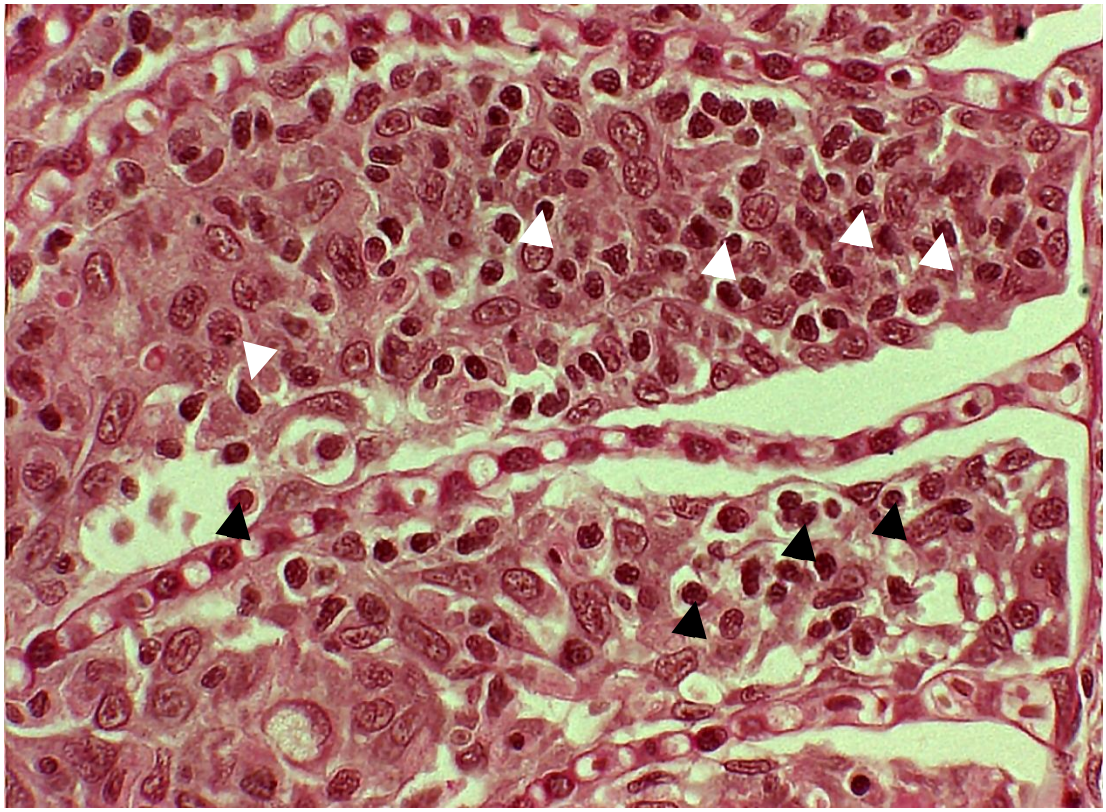

Figure 49. The internal region of a smaller lesion from moderately affected gills of Atlantic salmon previously exposed to *N. perurans*. A number of smaller MNLs within the epithelial matrix either within inter-epithelial channels (black arrowheads) or between epithelial cell junctions (white arrowheads).

within individual lesions. This was particularly apparent in larger lesions where the sectional depth along the filament length varied.

Similar to the moderately affected naïve group, tissue modifications to the basal epithelial regions generally mirrored the sub-apical inter-lamellar hyperplastic tissues. In non-hyperplastic regions the basal filamental epithelium was morphologically consistent with control fish.

Immune gene expression

Gene expression between groups where non-hyperplastic tissue was sub-sampled:

The pattern of TCR-α chain and MHC-IIα mRNA expression in the light and moderate groups were significantly lower relative to unexposed fish (P = 0.0067 & 0.0371; Figure 50A &D) but no differences were found relative to the other infected groups. CD8 and IgM mRNA expression was significantly lower (P = 0.0167 & 0.0328) compared to the unexposed fish with no differences found compared to other infected groups. All groups aside from those classified as light showed significantly lower (0.0085) expression of IgT mRNA. All groups showed significantly lower (P = 0.0096) expression of MHC-1 mRNA relative to unexposed fish. No significant differences were found for CD4 or IL-1β mRNA gene expression.

Gene expression between groups where hyperplastic tissue was sub-sampled:

Quantitative RT-PCR showed that mean mRNA expression of TCR-α chain and CD8-α was significantly lower (P = 0.0006 & 0.0016) in light and moderate groups compared to the unexposed and clear fish (Figure 51 I & J) with the moderate group suggesting a 2-fold down-regulation for the former gene's expression. CD4 mRNA expression was significantly lower (P = 0.0028) in both light and moderate groups relative to the unexposed group (Figure 51K). MHC-IIα mRNA expression was significantly lower (P < 0.0001) in light, moderate and naïve moderate groups (Figure 51L) with the former two showing a two-fold down regulation. The light group's IgM mRNA expression was significantly lower than all other groups (P = 0.0067). IL-1β mRNA expression in the moderate group was the only significantly higher mean (for all genes) relative to the control and clear groups. No significant differences were found between any groups for IgT or MHC-I mRNA gene expression. Table 18 summarizes the post-hoc test outcomes conducted subsequent to a one way analysis of variance. Figure 50A-H and Figure 51 I-P detail the individual responses from fish for each group.

Table 18. Statistical comparison of immune gene expression for salmon exposed to *N.perurans*

	Non-Lesion				Lesion			
Gene	**Clear**	**Light**	**Mod.**	**Naïve Mod**	**Clear**	**Light**	**Mod**	**Naïve Mod**
TCR	NS	L	L	NS	NS	L	L	NS
CD8	NS	NS	L	NS	NS	L	L	NS
CD4	NS	NS	NS	NS	NS	L	L	L
MHC-IIα	NS	L	L	NS	NS	L	L	L
IgT	L	NS	L	L	NS	NS	NS	NS
IgM	NS	NS	L	NS	NS	NS	L	NS
MHC-I	L	L	L	L	NS	NS	NS	NS
IL-1β	NS	NS	NS	NS	NS	NS	H	NS

NS = No significant difference to unexposed fish, L = Significantly lower expression than unexposed fish , H = Significantly higher expression than unexposed fish

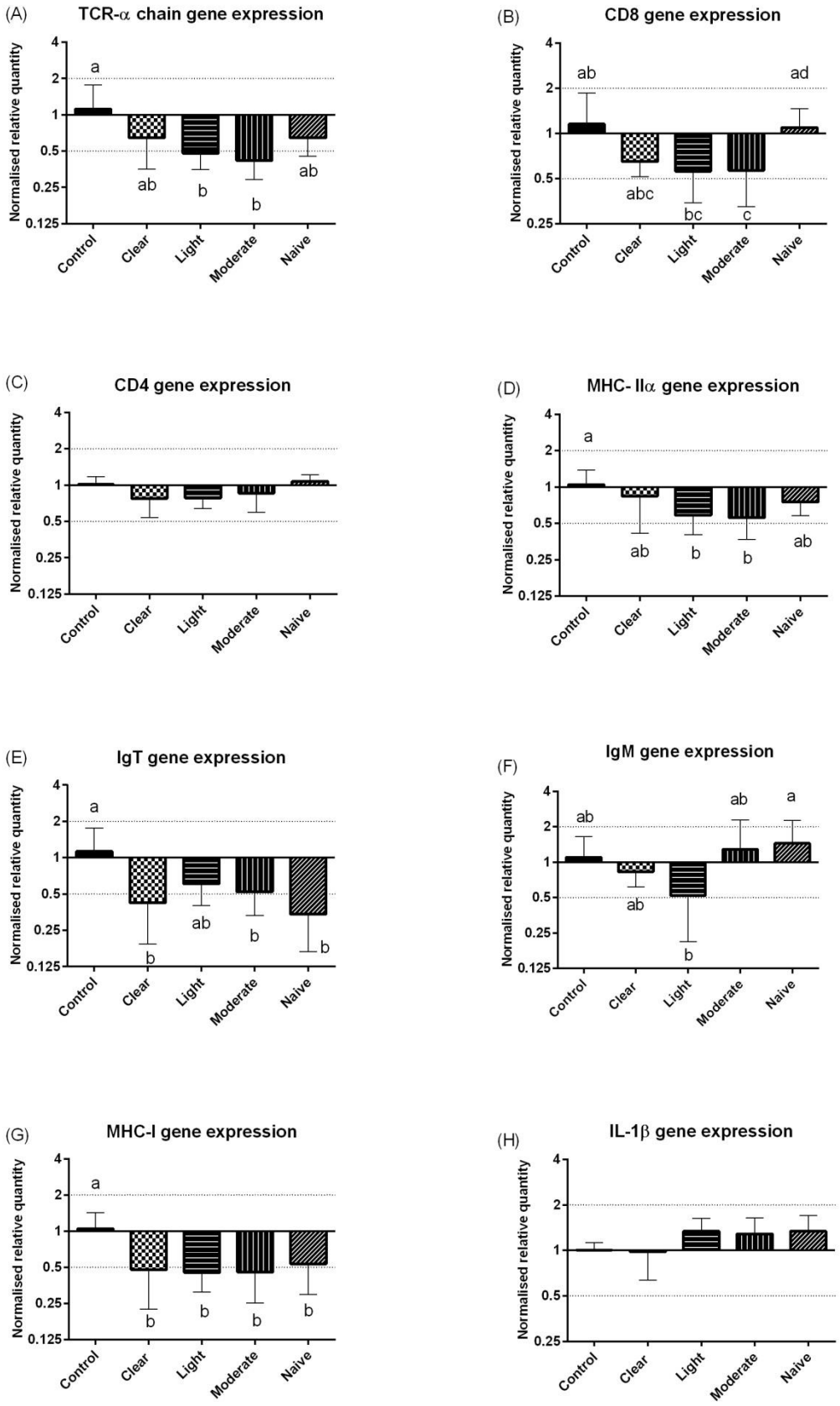

Figure 50 Quantitative RT-PCR analysis of immune related gene expression in gill biopsies (excised next to a lesion site) obtained from Atlantic salmon exposed 4x (clear, light and moderate) to *N. perurans* and FW bathed, 1x (naïve moderate) with no FW bath given; and unexposed fish (control). Products were resolved and visualised on a GelRed-stained gel. The mRNA gene expression is relative to the two reference genes EF1-α and β-actin. Bars represent mean values. Different letters identify groups that are significantly different ($p < 0.05$). Graphs A-H represent close to lesion type biopsies whereas graphs I-P represent lesion type biopsies.

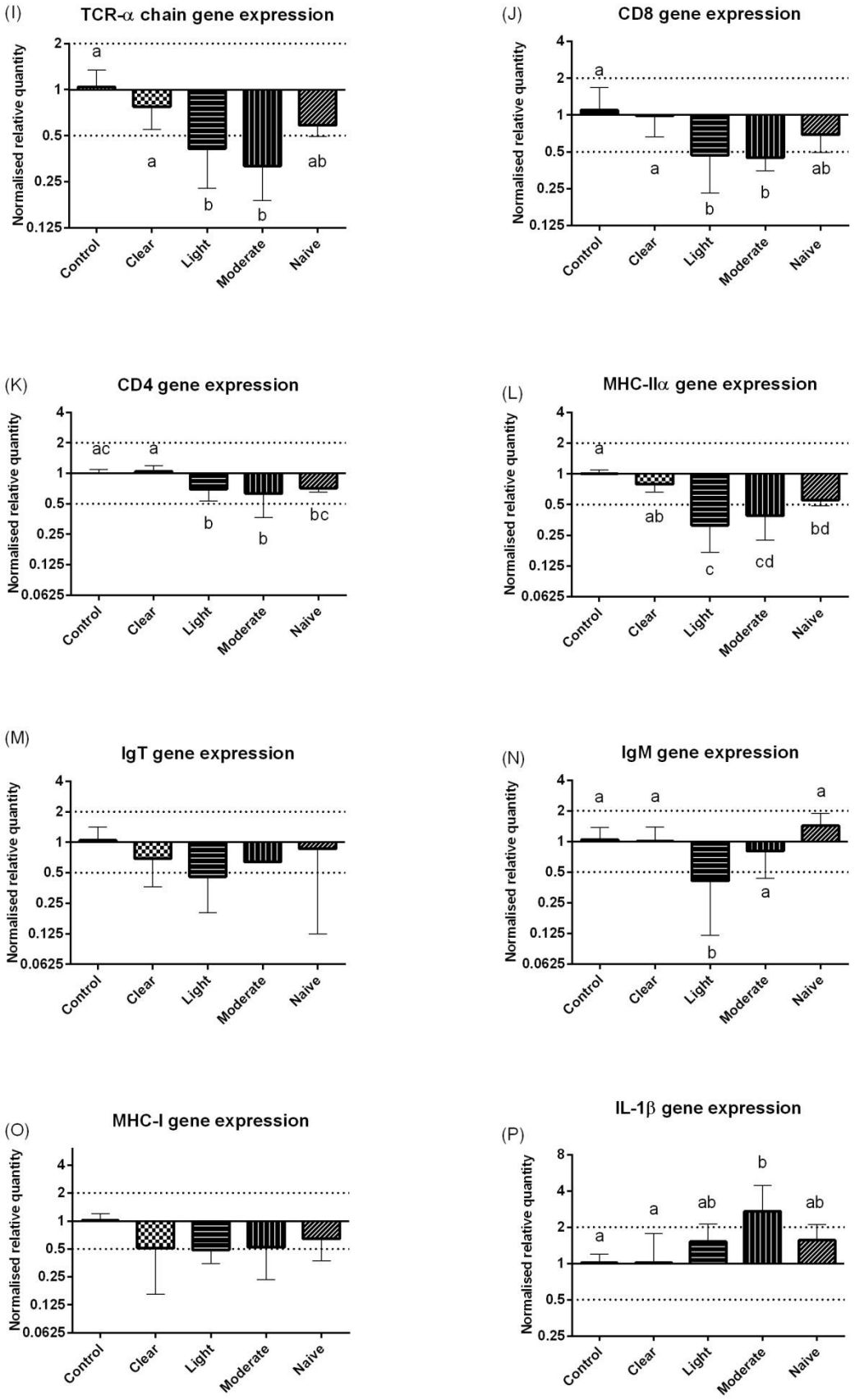

Figure 51. Quantitative RT-PCR analysis of immune related gene expression in gill biopsies (lesion excised) obtained from Atlantic salmon exposed 4x (clear, light and moderate) to *N. perurans* and FW bathed, 1x (naïve moderate) with no FW bath given; and unexposed fish (control). Products were resolved and visualised on a GelRed-stained gel. The mRNA gene expression is relative to the two reference genes EF1-α and β-actin. Bars represent mean values. Different letters identify groups that are significantly different ($p < 0.05$). Graphs A-H represent close to lesion type biopsies whereas graphs I-P represent lesion type biopsies.

Experiment 7: Comparative pathology of experimentally induced AGD in pure and hybrid Atlantic salmon Salmo salar and brown trout Salmo trutta

Gross Scores

Clinical assessment of macroscopic lesions indicated that pure Atlantic salmon strains (SxS) were most affected following challenge with *N.perurans* with a significantly higher score (P<0.001) than hybrid salmon/trout (SxT), trout/salmon (TxS) or pure brown trout (TxT) (Figure 52).

Histopathology

Concurrent with significant differences found for macroscopic lesions SxS had a significantly higher percentage of filaments with hyperplastic lesions (P<0.0001) and percentage of affected filaments colonised by amoebae (P<0.0001) than all other crosses. The percentage of lesions colonised per hemibranch section was highest for SxS but not significantly different to TxT. The latter was not significantly different to either the SxT or TxS groups (Figure 52).

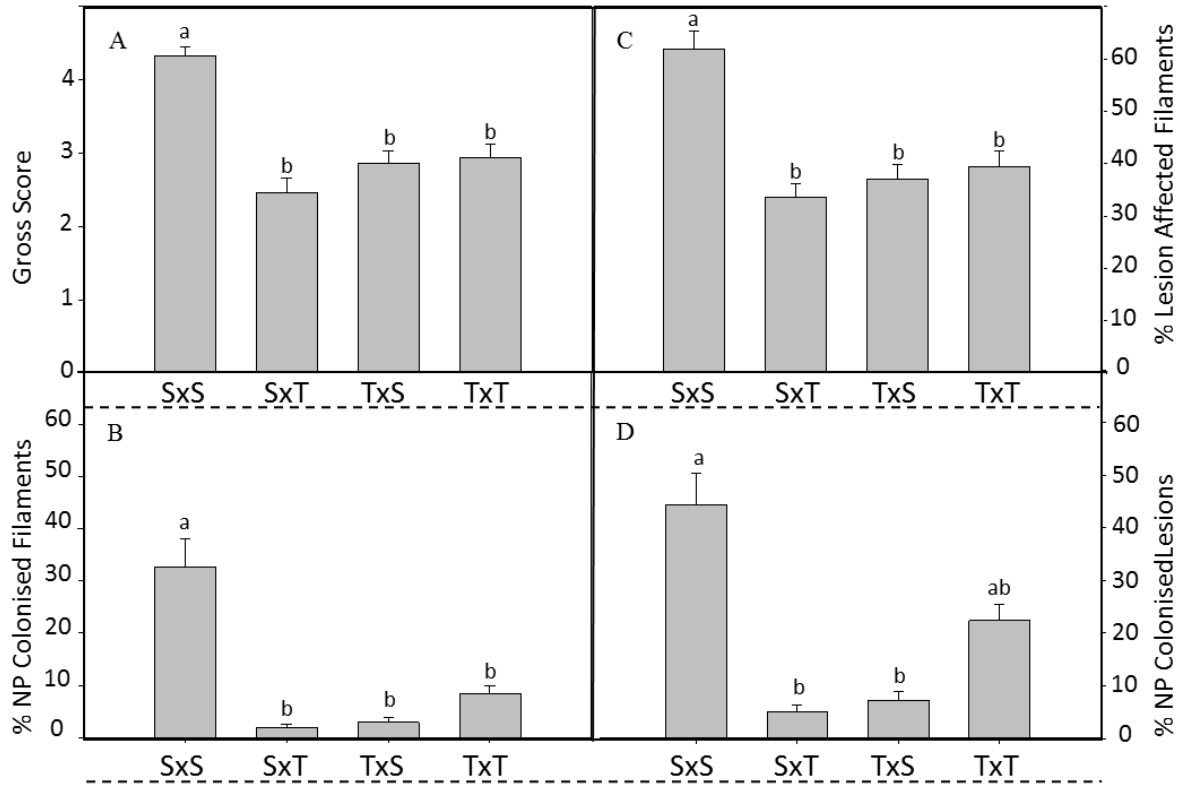

Figure 52. Mean gross scores (A), % lesion affected filaments (B), % *N.perurans* colonised lesion affected filaments (C) and % lesions colonised / hemibranch (D) for SxS, SxT, TxS and TxT crosses.

Figure 52 indicates that the clinical severity of AGD in all crosses was moderate to heavy according to the gross scores and histopathological assessment of the percentage of filaments bearing hyperplastic lesions (amoebae presence +ve and –ve). However, marked differences were apparent for the colonisation of lesions by amoebae as indicated by Figure 52 C & D. Table 19 suggests that amoebae were not detected in high abundance within the gills of hybrid crosses and the SxS groups carried the highest burden of amoebae although variable at the individual SxS level. However, lesions in the SxS group were expansive with two or even three filaments fused resulting in substantial

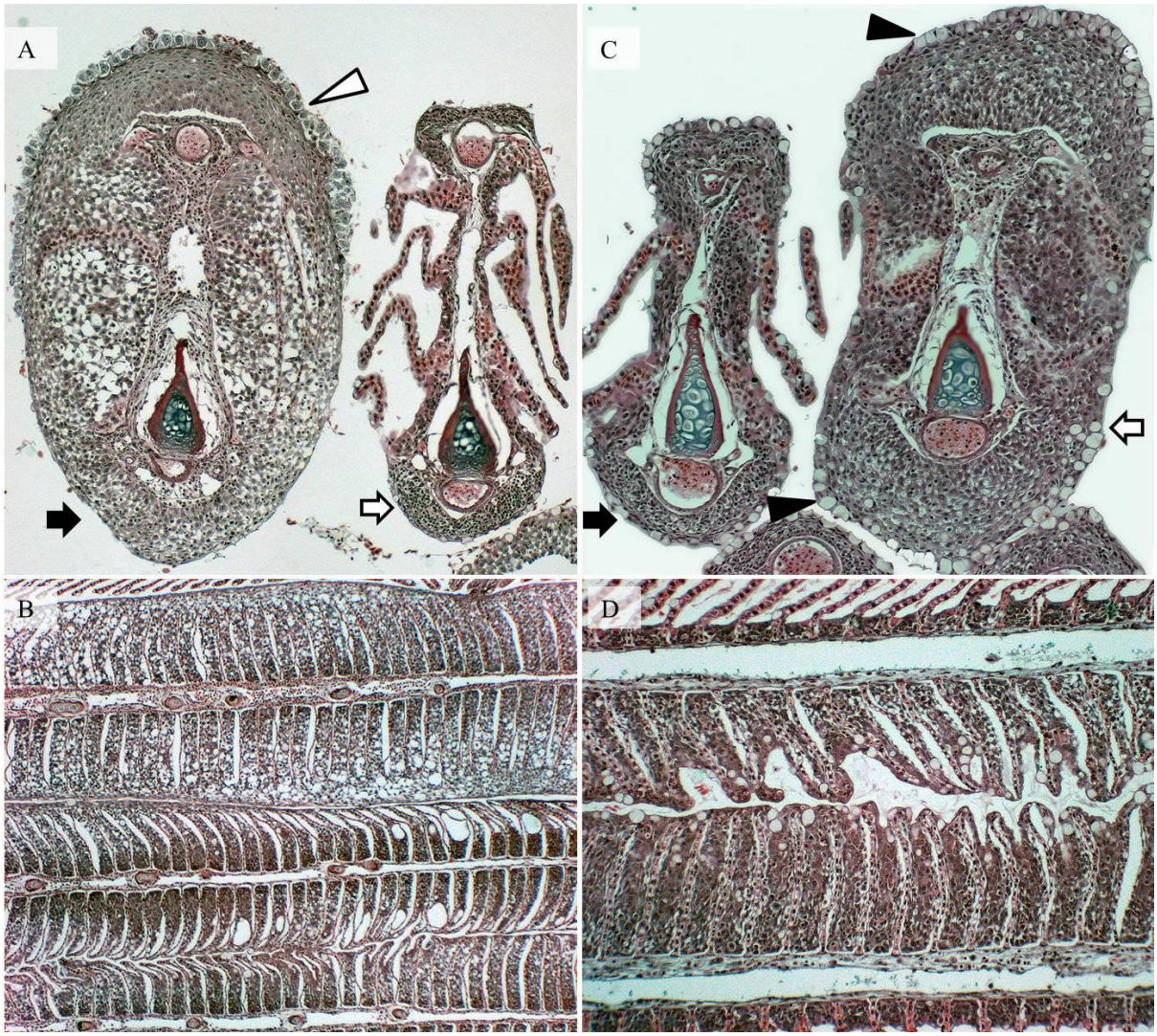

Figure 53. (A) Pure salmon cross (SxS) showing two dorsally orientated filaments. Black arrow indicates a filament colonised by amoebae (arrowhead) and contrasts markedly with normal morphology of the adjacent filament (white arrow). (B) Typical morphology of sagittally orientated lesions found in fish from the SxS group, smooth lesion margin and lack of mucous cells. (C) Trout v salmon cross (TxS) showing two dorsally orientated filaments. White arrow indicates a hyperplastic filament from a fish with no amoebae evident. In contrast to (A) the mucosa is interspersed with mucous cells (black arrowhead) which were prominent in lesions viewed sagittally (D) as well as undulating lesion surfaces.

coverage of affected hemibranchs. Histopathological findings showed a marked inflammatory response in many of the fish from the SxS groups. Oedema and spongiosis was common in hyperplastic lesions with infiltrating leukocytes throughout. and sloughed host cells/cellular debris from lesion surfaces (data not shown). Tissue remodelling of the leading and trailing filamental edges was as previously described for experiment 5. Hybrid crosses and brown trout displayed a tempered response in comparison to the pure salmon strains. The size and coverage of lesions was substantially lower and was reflected by the proportion of filaments bearing lesions. Amoebae were infrequently detected upon lesion surfaces and epithelial and apically aligned mucous cells dominated the cellularity of most lesions. Surface mucus cells featured prominently particularly in lesions with undulating surfaces. The leading and trailing edge of lesion affected filaments in most cases

maintained a morphologically normal mucosa and a variable degree of undifferentiated epithelial cell infiltration of the DILT.

Table 19. Semi-quantitative assessment of amoebae abundance.

	Abundance Category*: % Fish per Cross			
Species	**0**	**1**	**2**	**3**
SxS	0.0	56.7	26.7	16.7
SxT	60.0	40.0	0.0	0.0
TxS	40.0	56.7	3.3	0.0
TxT	10.0	86.7	3.3	0.0

(1) Amoeba(e) generally appear singularly but may be more on larger lesions – generally no more than ten/section
(2) Multiple amoebae, often appearing in clusters peripherally or distributed singularly
(3) Majority of lesion is colonised by amoebae

Heterosis

Estimates of mid-parent heterosis for AGD resistance using the host response parameters of gross score and lesion affected filaments were 27% and 31%, respectively, with best-parent estimates for both parameters of 9% (Table 20). Heterotic estimates for parasite abundance parameters were substantially higher, with mid-parent values of 82% and 88% and best-parent values of 72% and 73% for *Neoparamoeba* colonised filaments and *Neoparamoeba* colonised lesions, respectively (Table 20).

Table 20. Mid-parent and best-parent heterosis for pathological response parameters.

Parameter	**Mid-parent heterosis**	**Best-parent heterosis**
Host response Gross score	27%	9%
Lesion affected filaments	31%	9%
Parasite abundance NP Colonised Filaments	88%	72%
NP Colonised Lesions	82%	73%

Other pathology

Table 21. Prevalence of pure and hybrid fish presenting with external lesions.

	External lesion location						
Species	**Eyes**	**Mouth**	**Dorsal**	**Flank**	**Ventral**	**Fins**	**Tail**
SxS	0.0	0.0	2.0	0.0	0.0	43.0	78.3
SxT	2.0	0.0	0.0	0.0	0.0	48.8	35.5
TxS	6.7	0.0	0.0	0.0	0.0	55.5	3.0
TxT	0.0	0.0	0.0	0.0	0.0	62.0	6.7

The majority of externally observed pathology was found on the fins (generally unilateral presentation) and tails. The SxS and SxT groups appeared most susceptible to tail erosion whereas fin erosion was marginally more prevalent in the TxS and TxT groups. Mean fish weights from transfer until final sampling (approximately 14 weeks) is shown below in Table 22.

Table 22. Mean fish weights August - November.

Transfer	**Pre acclimation**	**Post acclimation**	**Post challenge**
16-Aug	**4-Oct**	**6-Nov**	**25-Nov**
46	102	151	157
45	88	122	126
38	70	113	106
27	44	89	103

Discussion

Four endemic fish species with distinctive life histories were challenged with *N.perurans*. An estuarine/marine pelagic carnivore (Australian salmon), an estuarine pelagic detritaphage (mullet), a demersal carnivore (flathead) and a reef dwelling predator that feeds primarily upon molluscs and crustaceans (wrasse*).* In all species, susceptibility to infection and the development of a pathological response was evident under the experimental conditions. This result supports a growing body of evidence of low host specificity for *N.perurans* given the right conditions in tank or cage based culture/experimental systems. Spontaneous infections of non-salmonid species in such systems resulting in characteristic signs of AGD have been published for turbot *Scopthalmus maximus* ayu *Plecoglossus altivelis*, *Dicentrarchus labrax*, sharpsnout bream *Diplodus puntazzo*, blue warehou *seriolella brama*, ballan Wrasse *Abrus bergylta*, lumpsucker *Cyclopterus lumpus* and corkwing wrasse *Symphodus melops* (Karlsbakk *et al.* 2013; Crosbie *et al.* 2010; Dyková *et al.* 1995; Dyková, Figueras and Peric 2000; Dyková and Novoa 2001; Adams *et al.* 2008). AGD was also induced experimentally in Yellowtail kingfish *Seriola ialandi* and brook char *Salvelinus fontinalis* (unpublished data). Suspected paramoebic infections have also been reported for greenback flounder, big bellied seahorse and gilthead seabream (Douglas-Helders, Dawson and Nowak 2000; Athanassopoulou, Cawthorn and Lytra 2002). Given as many as 23 species of fish from six orders (salmoniformes inclusive) have now been largely confirmed as susceptible to infection then it seems plausible that wild fish could act as a transmission vector for *N.perurans*. During the course of this project (April – October 2012), 20 of the 115 wild fish (collected and assessed by qPCR and histology) were positive for *N.perurans* by qPCR albeit at comparatively low numbers (range 3 – 77 amoebae.g^{-1} gill filaments) to those experimentally exposed during challenge. Given the fish were transferred to an experimental facility and maintained in tanks for up to two weeks before initial gill samples were collected, this finding is not considered definitive without further surveys of wild fish *in situ*. The Tamar estuary has a single commercial salmon site and is only intermittently affected every few years by isolated AGD outbreaks during summer/autumn. Previously a qPCR evaluation of water samples collected from the Tamar estuary (both within farm and 10km distant) did not detect *N.perurans* (Bridle, Crosbie, Cadoret and Nowak 2010). All precautions were taken to eliminate opportunity for amoebic contamination at the tank, sampling and laboratory level but the possibility of such an occurrence cannot be completely dismissed. Hyperplastic lesions without any associated amoebae were found on 33 occasions from this group of experimentally unexposed fish. A simultaneous qPCR positive result was recorded in a single fish so the pathological aetiology remains obscure. Previously it had been found that the gills of one of twelve wild fish opportunistically collected from a production cage in the Huon estuary (Tasmania) was infected with *Paramoeba* sp and had developed early disease signs (Adams *et al.* 2008). In contrast, histopathological evidence of AGD was not found in wild fish (n = 325) captured within and around salmon cages in southern Tasmania despite outbreaks occurring throughout the sampling period (Douglas-Helders *et al.* 2002). A survey of 2348 fish (28 species from 50 trawls) from Scottish coastal waters found a single fish (horse mackerel *Trachurus trachurus*) PCR positive for *N.perurans* (Stagg, Hall, Wallace, Garcia Perez and Collins 2015). Published surveys conducted to date (albeit limited in number) show is no evidence of intra/inter-site issue transfer of *N.perurans* by wild fish. Further investigation of this potential vector is warranted although the environmental ubiquity of *N.perurans* and its horizontal transmission between cages and even sites are likely to be major transmissibility factors (Douglas-Helders, O'Brien, McCorkell, Zilberg, Gross, Carson and Nowak 2003; Wright *et al.* 2015).

Pathological severity of characteristic AGD lesions (experiment 1-4) was significantly lower for endemic fishes compared to simultaneously cohoused Atlantic salmon except Australian salmon. Small MNL infiltrated hyperplastic lesions were encountered upon gills of unexposed endemic fish (except flathead) which may have inflated the severity assessment for the exposed endemic groups. Indeed, the percentage of filaments with lesions colonised by amoebae was significantly lower for all endemic fish and prevalence based on amoebae presence in histological sections ranged from 30-65% compared to 90-100% for Atlantic salmon. The abundance of amoebae in association with lesions in endemic fish was clearly lower and this observation was supported by lower relative numbers of amoebae calculated from qPCR values. Curiously, amoebae observed in mullet, wrasse and flathead displayed cytoplasmic retraction and were often unattached from adjacent lesions. It is unclear whether the abnormal appearance of amoebae observed histologically in the gills of native species was an artefactual observation or indicative of a moribund state. All precautions were undertaken to control for artefacts as standardized conditions were used to capture, anaesthetize, excise, fix and process gills. Native fish and Atlantic salmon gills for each group (pre-exposure, unexposed and exposed) were processed together. Fixation and processing errors are able to adversely affect the normal morphology of amoebae seen in histological sections (pers ob) however host tissue likewise degraded, however this observation was not made here. Retraction of amoebic cytoplasm was previously observed in Atlantic salmon following an on-site in-feed treatment (pers. ob.). Further investigation may entail observation of trophozoites isolated from the gills at the time of sampling in order to assess morphology, growth and/or survival. The challenge conditions used for experiments 1 – 4 could be considered "aggressive" (700-1100 amoebae.l^{-1}) and well above concentrations reported from Tasmanian salmon sea cages to date (Douglas-Helders *et al.* 2003; Bridle *et al.* 2010; Wright *et al.* 2015). Experimental procedures required that native fish were captured, transported and habituated to a tank environment on a modified diet. Even so, the endemic species housed displayed considerable robustness and adaptability with (normally demersal) flathead the only species displaying some degree of difficulty adapting to a sloping tank floor. Given the resistance to (or tolerance of) amoebae demonstrated by endemic fish exposed during these experiments (experiment 5 inclusive), the likelihood of significant pathology developing in the species of Tasmanian endemic fish challenged here is considered unlikely *in situ*.

Collectively the outcomes obtained from experiments 1-4 indicated that yellow eye mullet would be a suitable species to challenge for a longer period during experiment 5. Infection appeared to proceed in mullet in a similar manner to that seen for salmon during the first two weeks as indicated by relative amoebae numbers, infection prevalence (qPCR+) and the extent of pathology. Mullet sampled on days 21 and 28 however showed a marked decline in gill filaments affected harbouring lesions with no signs of disease at the latter time point. Interestingly *N.perurans* were still detected in all fish by qPCR at day 28 at one order of magnitude lower than that the mean *N.perurans* load in salmon gills. The extent of pathology was also significantly higher in salmon at day 28 compared to day 14 although a declining trend for relative numbers of amoebae was noted. AGD naïve smolt from a different cohort, introduced to the system on day 14, provided evidence of infective pressure as indicated by the rapid development of lesions and high abundance of attached trophozoites. Two important observations to note are that mullet were able to resolve infection spontaneously whilst an active infection/disease course was simultaneously developing in both groups of salmon and that the gills of mullet were qPCR positive for *N.perurans* in the absence of gill lesions. It was beyond the scope of this project to investigate further the mechanisms underlying the apparent differential pathological responses observed in endemic fish relative to their salmonid "controls". However the differential in the disease course for mullet and salmon was contrasted markedly and provides a future

avenue for comparative studies targeting innate resistance mechanisms influencing resistance and potentially tolerance to infection with *N.perurans*.

The gills of Atlantic salmon, infected during the course of experiment 5, were subsampled to assess morphometric and morphological attributes of interbranchial lymphoid tissue (ILT) (Haugarvoll *et al.* 2008). The ILTstructure and composition are consistent with mucosa associated lymphoid tissue (MALT), a critical component of the mucosal immune system; the frontline defending pathogen invasion. It is well established that colonisation of the branchial environment by *N.perurans* induces transformation of the lamellar epithelium. Inflammation confluent with epithelial desquamation and hyperplasia fuse the normally demarcated lamellae to form focal or multifocal lesions (Roubal, Lester and Foster 1989; Zilberg and Munday 2000; Adams and Nowak 2004a; Adams and Nowak 2003). Whether structural or cellular transformations affect the mucosa and its associated lymphoid tissue remotely and/or adjacently to lesion affected lamellae required investigation. Results from experiment 5 indicated that *N. perurans* infection was associated with a localised remodeling (size and cellularity) of PILT and DILT in Atlantic salmon challenged during experiment 5. The ILT was longer, concomitant with larger area measurements, particularly as hyperplastic lesions became larger and more numerous throughout the gills later in the experiment. The density of lymphocytes within the ILT changed significantly for both exposed and unexposed groups, however a discernible pattern relating to disease presence/development that could be reasonably argued as biologically significant was not evident. We were unable to determine whether the transition of ILT from that of an organised MALT arrangement to an epithelial cell dominated matrix was due to infiltration of newly divided cells and/or dedifferentiation of reticular epithelial cells to an undifferentiated form in a manner analogous to that found in other animals (Mashanov, Zueva, Rojas-Cartagena and García-Arrarás 2010; Bonventre 2003). The extent of transformation was certainly more pronounced when *N.perurans* trophozoites were more numerous thus increasing the chance of detecting a colonized lesion in a section. Proliferating epithelial cells (PCNA +ve) were more numerous in remodeled ILT but their origin was unknown. The fate of reticular epithelial cells, lymphocytes, other leucocytes and mucus cells seemingly "lost" from remodeled ILT in AGD affected salmon is unclear, although the processes involved could include migration, dedifferentiation, apoptosis and mucosal necrosis/sloughing. ILT remodeling may also contribute to published reports of lesion localised changes in gene expression for various immune and cell cycle responses ((Loo, Sutton and Schuller 2012; Morrison, Cooper, Koop, Rise, Bridle, Adams and Nowak 2006; Morrison, Zou, Secombes, Scapigliati, Adams and Nowak 2007; Wynne, O'Sullivan, Cook, Stone, Nowak, Lovell and Elliott 2008; Young, Cooper, Nowak, Koop and Morrison 2008a)). Pennacchi, Leef, Crosbie, Nowak and Bridle (2014) proposed these differences may be an artifact of cell type given the high ratio of epithelial cells to other morphotypes. Our observations suggest that the "loss" of lymphocytes from lesion affected gill MALT, particularly apparent in colonized lesions, should also be considered for when targeting gill tissue for immune gene expression studies.

Results from the hybrid challenge indicated a significantly higher degree of pathology (both grossly and histologically) in Atlantic salmon compared to brown trout and their respective crosses. Atlantic salmon were scored consistently higher and this was reflected histologically in the percentage of filaments with hyperplastic lesions. Hybrids and brown trout displayed considerable variation in the magnitude of their respective response to amoebic challenge. Histologically, lesions were typically smaller possibly overstating the extent of the severity assessment. Significant differences were noted in the percentage of filaments with lesions that were colonised by amoebae for hybrids and brown trout. The abundance of amoebae where present was also significantly lower compared to Atlantic salmon which may be due in part to the presence of mucus cells which was also observed in Atlantic

salmon. Mucus cell rich apical lesion surfaces have been previously noted as being devoid of colonising amoebae *in situ* (Adams and Nowak 2003; Adams and Nowak 2004b). Relatively higher concentrations of innate defence proteins, glycoproteins and glyconutrients were found in the cutaneous mucus of brown trout compared to other salmonids sampled in a related study (Maynard, unpublished data, FRDC 071/2011). Mechanisms responsible for initial (possibly innate) and ongoing (possibly adaptive) resistance to AGD demonstrated by brown trout and hybrids experimentally and in the field under semi commercial conditions (Maynard *et al.* 2016) also require characterisation. In that context, this experiment has shown that a lab-based challenge approach (perhaps with a tempered exposure regime) would be suitable.

Trends across heterotic estimates for the parameters of gross score and percentage of lesion affected filaments supports field observations by Maynard *et al.* (2016) for fish from the same cohorts. The lower values observed in this experiment may reflect the aggressive nature of the challenge (≈700 amoebae.l^{-1}). Significant heterotic estimates were obtained for the parameters of amoebae-colonised filaments and lesions, suggesting hybrids may have an ability to repel or clear the parasite by advanced stages of first infection. Whether Atlantic salmon x brown trout hybrids can resist *Neoparamoeba* attachment at the initial stages of first infection, or subsequent infections, can only be elucidated through further study. Histopathology has added a unique perspective to this study, and has allowed the partitioning of "host response" and "parasite abundance" parameters to some extent. Estimates of heterosis for AGD resistance using the parasite abundance parameters were substantially higher than the host response parameters, and these values are among the most extreme examples of heterosis reported in livestock literature for pigs (Johnson 1981), cattle (Greiner 2009) and sheep (Leymaster 2002). Molecular techniques could be utilised to further explore the degree to which attachment of *N.perurans* is hindered or controlled to elucidate the relationship between resistance and tolerance to infection.

On a gross categorical basis, results from experiment 6 found that fish, effectively reinfected three times, had a higher proportion of clear and lightly affected individuals and lower proportions of fish moderately and heavily affected by AGD compared to salmon infected only once. This finding is consistent with previous lab and field based studies that had clearly identified the development of resistance following previous exposures (Findlay and Munday 1998a; Vincent *et al.* 2006; Taylor, Kube, Muller and Elliott 2009a; Taylor, Muller, Cook, Kube and Elliott 2009b) with resistance (low gill score measure) now exploited as a selective breeding trait. Interestingly, the percentage of filaments harbouring hyperplastic lesions in clear fish was not significantly different to fish with light or moderate AGD histories. However the lesions found in clear fish (grossly undetected) were substantially smaller, rarely associated with amoebae and often dominated by putative lymphocytes. These lesions differed markedly in cellularity from those described previously that form in response to early stages of infection (Adams and Nowak 2004a; Adams and Nowak 2003; Adams and Nowak 2004b; Crosbie, Adams, Attard and Nowak 2007; Zilberg and Munday 2000). Nowak and Munday (1994) found lymphoid nodules upon Atlantic salmon gills after between two and six weeks following transfer to marine cages prior to the presence of lesions colonised with amoebae five weeks post-transfer. Similarly unpublished observations of lymphoid nodules were made during a sequential field study (Adams and Nowak 2003) from 2 -8 weeks post transfer of smolt to marine cages prior to AGD onset five weeks later. In the present study no substantive lesions in the negative reference group were noted (18 plus weeks at 35 ppt). Although speculative, it is possible the lesions seen here potentially represent the legacy of an amoebic attachment/repulsion sequence. Early descriptions of recovering cases of AGD described the presence of "mononuclear (possibly lymphoid) nodules" along filaments and particularly in the basal interlamaller space (Munday, Foster, Roubal and Lester 1990). Previous

AGD survivorship challenges (field and lab), eluded to surviving fish harbouring little pathology and low amoebae numbers following multiple field infections (Taylor *et al.* 2009b) or single laboratory challenge where lesions were reportedly under repair (Bridle, Carter, Morrison and Nowak 2005); however no detailed histopathological descriptions were given in either study. Further study of fish surviving AGD challenge and clear of gross pathological signs is warranted to elucidate this observation.

Quantitatively there was little difference in the percentage of filaments with hyperplastic lesions and percentage of lesions colonised by amoebae between the groups with light or moderate histories and moderately affected naïve fish; although the latter group had the highest value for each parameter including the size of lesions. Partial ambiguity of quantitative results was reflected somewhat qualitatively in terms of lesion cellularity and structure where some trends were evident. In particular, in fish with moderate histories there were many examples of undulating lesions apically rich in mucous cells with no amoebae present although active lesions with inflammatory margins were also present within individual fish; an observation not so often apparent in fish with moderate pathology infected once only. These inter-gill and sometimes inter-lesion differences may be reflected in the individual variability noted for group and individual mRNA expression of inflammatory and immuneregulatory genes selected for study here. Variability of gene expression between some individuals within AGD affected groups was observed with arguably biologically significant change in certain individuals. However, overall group mean values indicate little transcriptional evidence of appreciable biological differences in inflammatory and immune responses between AGD challenged and unchallenged groups. Where significant differences were identified the change was almost exclusively negative (except IL-1β) and less than two-fold. However, the overall downward trend observed is interesting in the context of AGD related immune gene expression studies to date. Down regulation or dysregulation of immune related genes was reported previously for naive AGD affected fish particularly during later stages (25+ days) (Morrison *et al.* 2007; Young *et al.* 2008a; Wynne, O'Sullivan, Cook, Stone, Nowak, Lovell and Elliott 2008) and it was proposed this may be a function of a parasite-mediated immune evasion capability. Contrastingly an approximately two-fold up-regulation of immune related genes (Ig light chain precursor & MHC2 inv chain-like protein) was found in the gills of fish surviving a natural re-infection with little pathology (100 days) relative to survivors with advanced pathology and a tank based population of naïve fish (Taylor *et al.* 2009b). We found very little difference in immune gene responses between the unexposed group and fish that were grossly clear following 60 days of reinfection aside from modest down regulation of IgT and MHC1. Differences between studies (this study inclusive) are inevitable and it must be recognised that contrasting results between studies could be influenced by methodological divergence. For example, the timing of sample selection, gill subsampling methods (location and quantity of excised samples), sub-sample comparisons (unexposed tissues v lesions v exposed tissues), fish histories, fish source/controls, replication, challenge intensity (initial amoebae concentration), amoebae source (wild type v cultured) and challenge environment (tank v cage) may all contribute to differing infection dynamics or introduce other intrinsic or extrinsic variables resulting in downstream transcriptional and translational outcomes. To some extent, previous studies have not included detailed methodological specifics for one or more of the aforementioned aspects which complicates retrospective interpretation.

In a broader context, this experiment and other studies demonstrate that observational investigation (histology, IHC, ISH, EM) are required concomitantly with gene and protein expression studies to elucidate the underlying explanation for transcriptional and translational variation (Bridle, Morrison, Cunningham and Nowak 2006; Nowak, Cadoret, Feist and Bean 2013; Morrison, Koppang, Hordvik

and Nowak 2006; Morrison and Nowak 2008; Korsten, Ziel-van der Made, van Weerden, van der Kwast, Trapman and Van Duijn 2016). Equally so, visual studies require the fundamental support of transcriptional and translational studies in order to explain the fundamental mechanisms of observational outcomes (Perou, Sorlie, Eisen, van de Rijn, Jeffrey, Rees, Pollack, Ross, Johnsen, Akslen, Fluge, Pergamenschikov, Williams, Zhu, Lonning, Borresen-Dale, Brown and Botstein 2000; Sørlie, Perou, Tibshirani, Aas, Geisler, Johnsen, Hastie, Eisen, van de Rijn, Jeffrey, Thorsen, Quist, Matese, Brown, Botstein, Lønning and Børresen-Dale 2001; Korsten *et al.* 2016). Ultimately results from this and previous studies cannot conclusively delineate whether an adaptive or innate response drives the emergence of AGD resistant sub-populations. Further investigation focussed upon survivors of repeatedly infected fish relative to synchronously uninfected individuals is warranted for immune-prophylactic intervention to be realized.

Six of the seven experiments conducted during the life of this project targeted the innate capacity of fish to defend against AGD in a comparative manner to Atlantis salmon. Differences were observed in susceptibility (as indicated by infection and/or disease prevalence) and host responses (assessed by infection intensity and pathology). Historically, both *in vivo* and *in* situ, Atlantic salmon have shown little innate capability to resist disease development in naïve fish as a result of infection by *N.perurans* without intervention by treatment. This project has demonstrated that under relatively controlled conditions using an aggressive disease challenge approach the endemic and hybrid species studied appear to have an inherent ability to do so. Future focus upon mechanisms that underpin contrasting resistance/tolerance of Atlantic salmon with other species/strains would be required for translation of these findings into prophylactic or therapeutic options. Jointly pursuant should be the elucidation of resistance mechanisms employed by Atlantic salmon surviving beyond initial and repeat development AGD.

Collectively a substantial individual variability in terms of susceptibility to AGD and the nature/ severity of the pathological response between groups and individuals within groups both across and within the experimental themes investigated was evident. Although variations in susceptibility and disease progression for AGD were anticipated given previous reports (Taylor, Wynne, Kube and Elliott 2007; Maynard *et al.* 2016; Taylor *et al.* 2009a; Taylor *et al.* 2009b; Wynne, Cook, Nowak and Elliott 2007; Bridle *et al.* 2005; Nowak, Adams, Vincent, O'Brien and Mitchell 2007; Findlay *et al.* 1995; Findlay and Munday 1998b; Gross, Morrison, Butler and Nowak 2004; Vincent *et al.* 2006), this project provided some additional insight into infection dynamics between *N.perurans* and Atlantic salmon under experimental conditions that warrant further consideration. Interestingly, amoebae loads were not always elevated in fish with relatively extensive pathology (for example wrasse – exp 3, hybrids – exp 7, salmon – exp 5) indicating that, under certain conditions, that fortification of lesions during later stages of disease (Adams and Nowak 2003) may be a refractory host measure. A consistent pathological pattern in naively infected fish emerged in such cases where lesion surfaces were richly populated with mucous cells embedded within a squamous epithelium; essentially mimicking the mucosa normally observable upon the leading and trailing filamental edges. This was also noted in small lesions found upon the gills of reinfected fish with grossly undetectable lesions. These surfaces were rarely colonised by amoebae, indeed where lesions were populated with amoebae, sloughing of epithelial cells/cellular debris indicative of an underlying superficial necrosis and inflammation (epithelial oedema, desquamation,, fenestration & leukocyte infiltration) were prominent and in agreement with previous observational studies (Adams and Nowak 2003; Dyková *et al.* 1995; Kent *et al.* 1988; Lovy, Becker, Speare, Wadowska, Wright and Powell 2007; Roubal *et al.* 1989; Zilberg and Munday 2000; Wiik-Nielsen, Mo, Kolstad, Mohammad, Hytterød and Powell 2016). Alternatively in some fish, gill cellular reactivity associated with amoebae and amoebae loads

were comparatively tempered with no appreciable additive recruitment of mucous cells (particularly Australian salmon & mullet) or a capacity to resolve infection was demonstrated (mullet). Our experiments were unable to detect any evidence of high amoebae loads with no or low level pathology further supporting the contention that attachment is requisite for disease to ensue (Bridle, Davenport, Crosbie, Polinski and Nowak 2015; Adams and Nowak 2004a; Morrison *et al.* 2004; Zilberg, Gross and Munday 2001; Zilberg and Munday 2000; Wiik-Nielsen *et al.* 2016). A somewhat novel observation from experiment 5 suggested that three markedly different infection dynamics occurred simultaneously among co-housed groups (experiment 5). A rampant amoebic infection with rapidly escalating pathology was evident in the co-housed sentinel population that was introduced to the system on day 14). The original cohort of larger salmon (≈ 565g v 205g) infected at the outset of experiment 5, displayed "heavy" pathology with an apparently stabilized amoebae load (albeit high individual variability, day 14 – day 28). Mullet showed little to no remarkable pathology with comparatively low amoebae loads from those sampled later in the experiment (day 21-28). The underlying factors for these clearly demarcated disease response patterns may well be numerous and interrelated (age, size, species, habituation, acclimation, robustness, fitness, stress) and likely to differentially influence an innate capacity to maintain disease in stasis or reduce/resolve disease signs.

Since the initial pathological descriptions of AGD in Tasmania (Munday 1986; Munday *et al.* 1990; Roubal *et al.* 1989) until present, cellular pathological responses to AGD have been described from hemibranch sections orientated in a sagittal viewing plane. Alternative aspects, in the transverse and dorsal planes, have been used more recently for anatomical and functional descriptions of ILT in the gills of Atlantic salmon (Aas, Austbø, König, Syed, Falk, Hordvik and Koppang 2014; Dalum *et al.* 2015; Haugarvoll *et al.* 2008; Koppang *et al.* 2010; Norte dos Santos, Adams, Leef and Nowak 2014). Therefore, to improve our perspective of AGD lesion morphology and cellularity, the alignment of wax embedded holobranchs in the dorsal and transversal planes were similarly undertaken. Transversal sectioning and image analysis indicated dimensional and structural differences occurred in the PILT of AGD affected salmon during experiment 5 (Norte dos Santos *et al.* 2014). Dorsal filamental sections demonstrated the transformational influence of epithelial hyperplasia upon the PILT and DILT of Atlantic salmon adjacent to lesions induced by *N.perurans*. Intraepithelial lymphocytes were reduced in density in the PILT of Atlantic salmon as was the apparently heterogeneous population MNLs in native fish even though epithelial hyperplasia was not consistently prominent. Rather, intraepithelial oedema of the submucosa along the filamental edges adjacent to fused lamellae seemingly enlarged these regions. These apparent reductions of MNL densities may be due to migration to localised sites of infection, as seen in mice GALT (Edelblum, Shen, Weber, Marchiando, Clay, Wang, Prinz, Malissen, Sperling and Turner 2012) using time-lapse confocal microscopy. Dorsal sectioning also provided enhanced perspective of predominately non-lymphoid cellular infiltration of lesion affected gill filaments particularly during the earlier stages of AGD development (1 – 14 days post infection). A consistent finding was inflammatory congestion of connective tissues and collateral vessels surrounding the filament arteries as well as the endothelial-basal membrane interface of the central venous sinus. Inflammatory infiltration of the vascular housing was not as apparent in gills analysed from later time points (experiments 5 & 6). The patho-morphological observations of spatially and temporally heterogeneous tissue constructs described in this study should be taken into consideration for future immune-regulatory studies.

Conclusion

Objective 1: Determine the susceptibility of sea-cage associated endemic fishes to amoebic gill disease in comparison to Atlantic salmon

Amoebic gill disease was experimentally induced in four Tasmanian endemic fish species (Australian salmon, yellow eye mullet, purple wrasse and southern sand flathead) under laboratory conditions. In comparison to Atlantic salmon the disease response was less severe or slower to develop. Yellow eye mullet when infected with *N.perurans* for a longer period were able to resolve the pathological signs contiguous with experimentally induced AGD. The latter result provides a directed opportunity for further research to elucidate the defensive processes/mechanisms engaged by this species.

Objective 2: Investigate the comparative host responses of Atlantic salmon and rainbow trout naive and previously exposed to amoebic gill disease.

Rainbow trout were not able to successfully acclimate to marine conditions suitable for experimental AGD inducement. Histopathological findings demonstrated a differential cellular response to experimental infection with *N.perurans* between Atlantic salmon naïve to infection and salmon previously exposed to three rounds of infection. Contrastingly, negligible transcriptional differences were found for immuno-regulatory genes between fish groups infected once, repeatedly and not infected although individual variability was marked in the infected cohorts. A proportion of the previously challenged population was capable of defending against the progressive development of AGD although whether the mechanism is innate or/and adaptive remains unclear.

Hybridised Atlantic salmon and brown trout showed significantly less severe pathological responses to experimental infection with *N.perurans* than observed in pure salmon and trout crosses. Although the clinical manifestation in all crosses was substantial the amoebic burden was clearly lower for the hybridised fish.

Atlantic salmon were challenged on seven occasions during the course of this project. Overall a substantial variation in disease response and amoebae abundance was observed histologically. Significant pathology was shown to develop with markedly differeing burdens of associated amoebae. Further research capturing underlying mechanisms that affect the host-parasite interaction for this disease warrant further investigation.

Implications

The major beneficiary for this project is the Tasmanian salmonid industry. This project aimed to develop outcomes that address fundamental knowledge gaps related to susceptibility and resistance of fish to AGD. Ultimately the conclusions derived from this project have strengthened the fundamental knowledge link requisite for developing alternative management strategies for AGD. The impact of this research for the salmonid industry in Tasmania can only be estimated in qualitative terms. Results from this research have provided industry with new insight into the disease process by detailing aspects of susceptibility and/or resistance based responses to AGD. Knowledge transferred by this project can assist with future industry research prioritization.

Recommendations

Further development

This project has furthered the collective knowledge of AGD host susceptibility and comparative host responses to AGD. However there are still several knowledge gaps to be addressed before bridging the divide between fundamental information and applied outcomes. The experiments reported here investigated responses measured in days to months and whilst this scale of study is critical for future studies there is presently little information regarding short term host responses to infection with *N.perurans*. Indeed the nature of attachment and/or association itself requires more vigorous investigation.

Yellow eye mullet and salmon-trout hybrids have shown excellent potential as future comparative research candidates for understanding how fish react and defend against AGD. The inherent variation to naïve and repeated infections within Atlantic salmon populations could also be exploited from an immune-pathological perspective.

Using these candidates for further comparison based investigation should include research directed toward:

- Characterising *in vitro* and *in vivo* the pathobiology and primary interactions of *N.perurans* with gills
- Functional properties of mucus, proteins and host cells responding to infection
- Immuno-pathological studies encompassing and combining microscopic, genomic and proteomic approaches
- The impact of host ontogenic development upon reactivity/response to amoebic infection
- Further exploration, refinement and revision of AGD challenge methodologies

It is recommended that these aspects are incorporated to further explore functional differences and disease mechanisms within and between Atlantic salmon populations, yellow eye mullet and salmonid hybrids.

Extension and Adoption

Communication to end-users:

- AGD working group meeting November 2012 IMAS Taroona - Verbal summation
- Visit from Scottish salmon farming representatives, Dr Steve Percival (Technical manager – Huon Aquaculture Company) and Dr Adam Main to experimental facility and research labs hosted by Professor Barbara Nowak – January 2013
- Summary statement issued to TSGA February 2013
- Presentation to TSGA technical committee May 2013
- Presentation to FRDC Second Australasian Scientific Conference on Aquatic Animal Health. July 2013
- Presentation to TSGA technical committee May 2014
- Presentation to the 4th International Meeting of the Gill health Initiative June 2016

Project coverage

Media dissemination:

- Press release issued to AMC and UTas websites - March 2012
- "Fish" Magazine article April 2012
- UTAS "Research to reality" magazine article - December 2012
- Interview broadcast on ABC local TV news, ABC News 24, ABC radio and ABC webpage – February 2013

Project materials developed

Published papers:

Norte dos Santos CC, Adams MB, Leef MJ, Nowak BF. Changes in the interbranchial lymphoid tissue of Atlantic salmon (Salmo salar) affected by amoebic gill disease. Fish Shellfish Immunol. 2014 Dec; 41(2):600-7

Manuscripts in preparation:

Y. Pennacchi, M.B. Adams, A.R. Bridle, B.F. Nowak. Immune gene expression in the gills of Atlantic salmon (Salmo salar L.) following experimental reinfection with Neoparamoeba perurans.

Adams M., Bridle A., & Nowak B. Comparative susceptibility of four Tasmanian endemic fish species and Atlantic salmon Salmo salar to experimentally induced amoebic gills disease.

Adams M., Bridle A., & Nowak B. Comparative histopathology of yellow eye mullet Aldrichetta forsteri and Atlantic salmon Salmo salar during experimental challenge with Neoparamoeba perurans.

Adams M., Maynard B., Cook M. & Taylor R. The susceptibility of pure and hybrid Atlantic salmon and brown trout strains to experimentally induced amoebic gill disease.

Presentations:

Second Australasian Scientific Conference on Aquatic Animal Health. The Pullman Reef Hotel, Cairns, Queensland, Australia

4th International Meeting of the Gill health Initiative June 2016, Stirling Court Hotel, Stirling, Scotland

Appendices

Appendix 1 - List of researchers and project staff

Dr Mark Adams (PI)

Dr Andrew Bridle (CI)

Prof Barbara Nowak (CI)

Ms Catarina Norte dos Santos (PhD candidate)

Ms Ylenia Pennachi (Phd candidate)

Dr Bikram Ghosh (Technical Assistance)

Dr Victoria Valdenegro (Technical assistance)

Ms Karine Cardoret (Laboratory manager – Technical Assistance)

Dr Ben Maynard (Collaborator) CSIRO

Appendix 2 - Bibliography

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