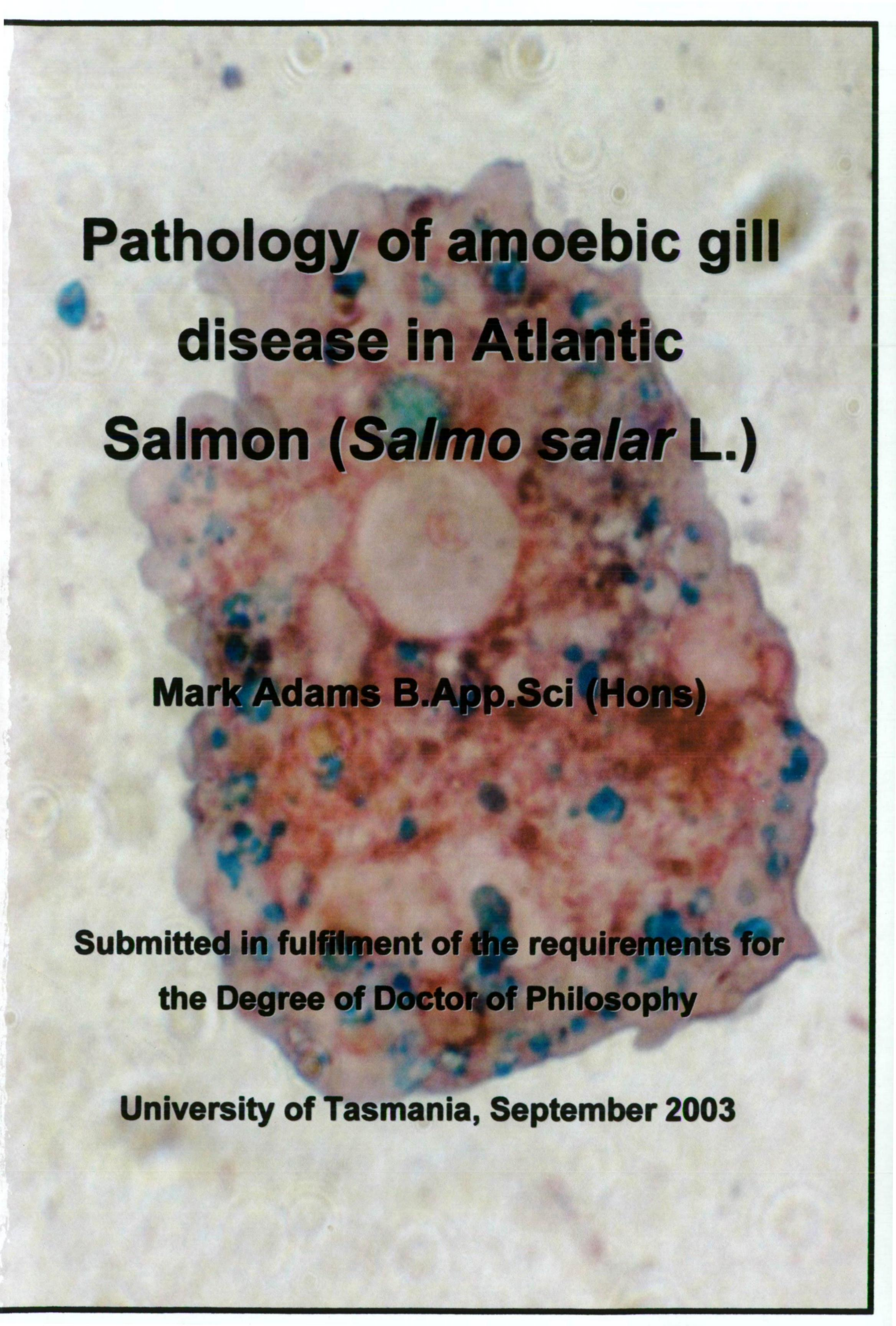

Pathology of amoebic gill disease in Atlantic Salmon (*Salmo salar* L.)

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Submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy

University of Tasmania, September 2003

Declaration

This thesis contains no material which has been accepted for a degree or diploma by the university or any other institution, except by way of background information and duly acknowledged in the thesis and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgements are made in the text of this thesis.

Mark Adams

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Inside cover picture – *Neoparamoeba* sp. isolated from gills of Atlantic salmon & stained with PAS/AB

Abstract

Although AGD has affected the Tasmanian salmonid industry for nearly 20 years, several fundamental questions regarding the pathology of this condition remain unanswered. This thesis elucidates the requirements for AGD outbreaks and how AGD progresses within the commercial culture environment.

Atlantic salmon *Salmo salar* L. gills, affected with amoebic gill disease, were analysed by routine histology to identify lesion morphology and distribution patterns. Interlamellar cyst (or vesicle) function was hypothesised as a host defence mechanism leading to entrapment of trophozoites and clearance from hyperplastic tissues by host cellular processes.

The degree of conformity between clinical signs and histological lesions was investigated in commercially reared Atlantic salmon. Micro-stereoscopic analysis showed that grossly affected tissue regions correspond to areas of hyperplastic lamellae fusion generally in association with attached amoebae. Agreement between gross signs of AGD and histopathological diagnosis, as indicated by Kappa, was moderate to good (0.52 – 0.74). Stage of disease development, lesions derived from other pathogens, assessor interpretation / experience, sampling methods, histological technique and/or experience all featured as potential factors leading to individual case disagreement.

The causal mechanisms for AGD lesion development and the primary role of *Neoparamoeba* sp. were investigated. AGD only occurred when fish were exposed to viable trophozoites. A progressive host response and significant increases ($P<0.001$) in the numbers of attached amoebae was apparent over the 48 h duration. Attachment of Neoparamoeba sp. to damaged gill filaments was significantly lower than upon damaged filaments ($P<0.05$) by 48 h post exposure.

Histopathological observations of AGD from smolts, sampled weekly, following transfer to estuarine/marine sites were investigated. Results suggest that AGD progression was linked to retraction of the estuarine halocline and increases in water temperature. The host response to gill infection with Neoparamoeba sp. is characterized by a focal fortification strategy concurrent with a migration of immuno-regulatory cells to lesion affected regions.

Subsequently, the progression of re-infection (post-treatment) was investigated using a similar sequential investigation. Halocline cessation and increased water temperature appeared to drive the rapid onset of initial infection prior to bathing. Freshwater bathing cleared lesions of attached trophozoites and associated cellular debris. During the post-bath period, non AGD lesions including haemorrhage, necrosis and regenerative hyperplasia were occasionally observed though no evidence of secondary colonization of these lesions by *Neoparamoeba* sp. was noted. We conclude that pathogenesis, during the inter bath period, was identical to initial infection although the source of re-infection remains to be established.

Together, these data have addressed the need for an improved understanding of AGD associated pathology during commercial culture of Atlantic salmon in Tasmania primarily by defining an improved pathological model of AGD. This work forms the basis for not only differential diagnosis per se but also a foundation and/or reference for future research dependent upon histopathological outcomes as an evaluative endpoint.

Acknowledgements

First and foremost I must thank my supervisor, Dr Barbara Nowak, for an immeasurable amount of assistance, guidance, encouragement and opportunity over the last three and a half years. Your enthusiasm, motivation and work ethic as a researcher are inspirational and I am truly grateful for everything. Thankyou so much.

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Also thanks to many people and organizations that have assisted me, technically, financially or otherwise over the duration of my candidature: Tas Uni AGD research groups, Helen Statham, Matt Foale, Greg Kent, Jan Daniel, Annabel Tyson, David Steele, Brian Beam, Ross Daglish, Tassal P/L, Nortas P/L, Aquatas P/L, Dr Iva Dyková, FRDC, EAFP, Dan Fisk, CRC Aquafin, Stuart Fyfe, Cass Wilson, Dion Sydow and anyone I have forgotten to mention.

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CHAPTER 1 - General Introduction

1.1 Salmonid Aquaculture in Tasmania

Commercial culture of Atlantic salmon (*Salmo salar* L.) in Tasmania commenced during 1984/85 with an initial harvest of 53 tonnes during the summer of 1986/87 (Dix 1986; Munday *et. al.* 1990). The production cycle was fairly simplistic, with introduction of spring smolts (September – December) resulting in a large harvest gap each year. To address the gap in production the industry responded with the introduction of photo-manipulated out-of-season smolts and pre-smolts during the 1990's.

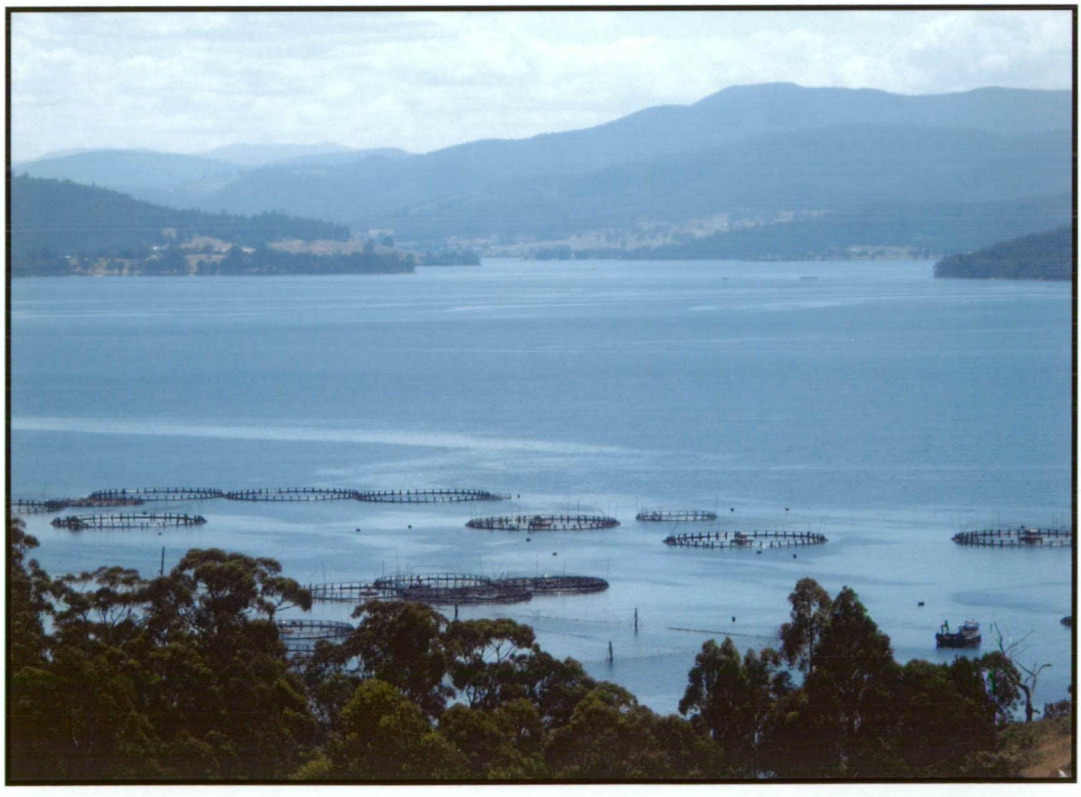

Figure 1. View of an Atlantic salmon production lease in the Huon estuary at Hideaway Bay in southern Tasmania, Australia.

Subsequently, annual production had attained nearly 12000 tonnes by 2000 (O'Sullivan and Roberts 2001).

In terms of production of farmed salmon, Tasmania is a relatively small contributor to the world market accounting for ≈ 1% of global salmon production. However, the Tasmanian product attains premium prices due to excellent growing conditions in pristine waters. Tasmanian produced salmon are free of major diseases commonly encountered overseas thus the use of antibiotics and chemotherapeutics is virtually non-existent.

Although free of exotic bacterial and viral disease, Tasmanian produced salmon are subject to a proliferative gill condition called amoebic gill disease or AGD. The management (monitoring & mitigation) of this condition accounts for 10 – 20% of total production costs and limits potential yields due to site selection limitations. It is imperative that AGD and its associated economic burden is minimized for continued industry development.

1.2 Historical background of AGD in Tasmania

Shortly after commencement of sea-caged Atlantic salmon culture in Tasmanian marine waters, significant mortalities (up to 50%) occurred during the warmer summer months. Clinical signs of disease were symptomatic of respiratory distress including sluggishness and fish swimming with open operculae. A severe mucoid branchialitis was observed upon the gills of afflicted fish (Munday 1986). Although large numbers of an ameboid protozoan were associated with wet mounts of affected tissues, a definitive diagnosis of the causative agent could not be made. A subsequent study, conducted over the spring-summer of 1987/88, attributed infection to the "naked", normally free living *Neoparamoeba* sp. with a morphology closely resembling that of *N. pemaquidensis* (Roubal *et. al.* 1989). After the initial drastic

losses experienced by the industry, an effective mitigation strategy (freshwater bathing) was introduced (Foster & Percival 1988) and mortalities were largely controlled. The practice was hurriedly adopted by all marine farms and is a standard operating procedure remaining in use today albeit at a significant cost to production.

1.3 Global distribution and species affected with AGD in marine aquaculture

Tasmania is the most severely AGD affected salmon producer with regular outbreaks occurring throughout each year particularly during the warmer summer months (Clark & Nowak 1999). However, AGD has been reported in a variety of overseas locations and affecting several species. For Atlantic salmon, outbreaks have been recorded in Ireland, France, Chile and Spain (Rodger & McArdle 1996, Clark & Nowak 1999). Rainbow trout *Oncorhynchus mykiss* (Walbaum) are also affected in Tasmania (Munday *et. al.* 1990) and sporadically in France (Baudin-Laurencin pers. comm. cited by Munday *et. al.* 1990). AGD was diagnosed from marine culture facilities of brown trout in Spain (Baudin-Laurencin pers. comm. cited by Munday *et al* 2001). Kent *et. al.* (1988) described infection attributable to *Neoparamoeba pemaquidensis* in coho salmon *Oncorhynchus kisutch* (Walbaum) farmed in Washington and California, USA. Sporadic occurrences of AGD have been identified in New Zealand in chinook salmon *Oncorhynchus tschawytscha* (Anderson pers. comm. cited by Findlay *et al* 1998). Outbreaks have also been reported in Spain affecting the culture of turbot *Scophthalmus maximus* (L.) (Dyková *et. al.* 1995). Sea bass *Dicentrarchus labrax* (L.) within the Mediterranean have also been periodically afflicted (Dyková *et. al.* 2000).

1.4 The causative agent

1.4.1 Taxonomy

Page (1987) created a new genus within the family Vexilliferidae (Phylum - Rhizopoda; Class – Lobosea; Subclass – Gymnamoebia) and redesignated *Paramoeba pemaquidensis* and *P. aestuarina* as *Neoparamoeba pemaquidensis* and *N. aestuarina*. Some major features defining the classification of the genus include forms being non-scale bearing, containing a nucleus plus parasome(s) (identified as a *Perkinsiella amoebae*-like endo-symbiont closely related to the kinetoplastid *Ichthyobodo* [Dyková *et. al.* 2000; Dyková *et. al.* 2003]) and possessing hexagonal glycostyles. It was several years before this revised classification was adopted within AGD related literature and thus where mentioned in this thesis, *Paramoeba* sp. is equivalent to *Neoparamoeba* sp..38-46,48,51,53-63

1.4.2 Morphology using light microscopy

Neoparamoeba sp. freshly isolated from the gills of infected fish appear in their free floating form as roughly spherical (up to 40 μm in diameter) and possessing multiple digitate pseudopodia (Kent *et. al.* 1988; Munday *et. al.* 1990; Rodger & McArdle 1996; Dyková *et. al.* 1998).

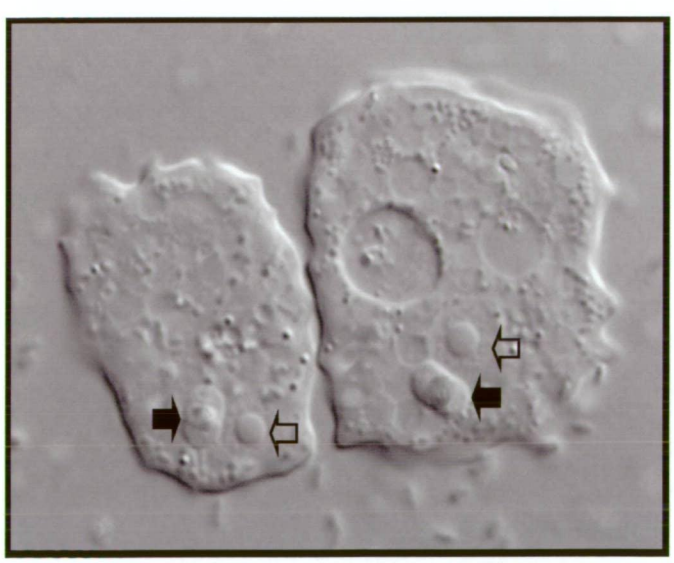

Figure 2. Attached trophozoites of *N. pemaquidensis* viewed using Olympus Nomarski DIC system. Nucleus (unfilled arrow) and "parasome" (filled arrow) are indicated (Plate courtesy of P. Crosbie).

When attached to a substrate *Neoparamoeba* sp. assume a more lobose form and the nucleus and "parasome(s)" are generally visible. In wax and resin sections the same structures are identifiable and trophozoites often appear highly vacuolated (Roubal *et. al.* 1989; Munday 1990; Dyková *et. al.* 1995).

1.4.3 The agent's association with AGD

Kent *et. al.* (1988) implicated *Paramoeba pemaquidensis* as the causative agent infesting the gills of coho salmon in Washington State and California based on observations from wet preparations, culture, histopathology and transmission electron microscopy (TEM). A year later Roubal *et. al.* (1989) attributed AGD in Tasmanian farmed Atlantic salmon to *Paramoeba* sp.. Both studies noted a similar morphological resemblance to that formerly described for *Paramoeba pemaquidensis* (Page 1970; Cann & Page 1982; Page 1983; Bovee & Sawyer 1989). The associated pathological host response was very similar in both cases and the organism was ascribed as the primary agent of the disease. Later Tasmanian studies concurred with these findings (Munday *et al.* 1990; Munday *et. al.* 1993; Nowak & Munday 1994). Outbreaks of AGD in turbot facilities from Spain were initially attributed to infection with an amoebic protozoan (Dyková *et. al.* 1995). The primary agent was later identified as *Paramoeba* sp. although several other strains of amoebae were isolated from clinically diseased and non diseased fish. These were morphologically identified and included species belonging to the genera *Platyamoeba*, *Vannella*, *Flabellula* and *Gruberella* (Dyková *et. al.* 1998; Dyková *et. al.* 1999). The role of these other isolates was not determinable as no direct evidence of pathogenicity was detectable. Leiro *et. al.* (1998) suggested *Platyamoeba* sp. as being the causative agent of AGD in turbot from the same region although the investigation was not sufficiently detailed from a histopathological perspective (Dyková *et. al.* 1999). A comparative study of six

strains from different origins (of the formerly designated *Paramoeba* genus), identified all strains belonging to the genus *Neoparamoeba* (Dyková *et. al.* 2000). It was concluded however, that until a more refined diagnostic approach became available, that the closely related *N. aestuarina* could not be dismissed as a potential agent of AGD (Dyková *et. al.* 2000).

Attempts to infect fish with cultured strains of *Neoparampoeba* sp. have been unsuccessful, thus Koch's postulates remain to be satisfied (Kent *et. al.* 1988; Roubal *et. al.* 1989; Howard 2001; Morrison pers. comm.).

1.4.4 Environmental distribution/reservoirs

Neoparamoeba pemaquidensis was first recovered from marine waters off Maine, USA by Page (1970) and is considered the most common amoeba throughout the marine environment (Cann & Page 1982; Page 1983). In Tasmania, *Neoparamoeba* sp. has been isolated from sediment samples at various locations around Tasmania, both with or without the presence of salmon farms and in marine and estuarine locations (Crosbie *et. al.* 2002). Tan *et. al.* (2002) isolated *Neoparamoeba* sp. from bio-fouling organisms present upon nets from salmon farms affected by AGD in the Huon Estuary. Salmon cage nets treated with a copper-based antifoulant, harboured higher loads of *Neoparamoeba* sp. Wild fishes, sampled from AGD affected salmon farm locations, were not found to be a significant reservoir for *Neoparamoeba* sp. (Douglas-Helders *et al.* 2002). Relative numbers of *Neoparamoeba* sp. were found experimentally to multiply rapidly upon the gills of dead salmon suggesting their presence under culture conditions may be a potential source of infection (Douglas-Helders *et. al.* (2000). Douglas- Helders *et. al.* (2002) found seawater dispersed *Neoparamoeba* sp., derived from the gills of experimentally infected Atlantic salmon, were able to re-infect naïve fish after a non contact period of 14 days. Douglas-

Helders *et. al.* (2003) found densities of *Neoparamoeba* sp. in the water column, at commercial culture sites in the Huon estuary (Southern Tasmania), to be higher in summer. Water samples collected within salmon pens contained amoebae densities that were likewise higher than at reference points 1100 m away (Douglas-Helders *et. al.* 2003).

1.5 Pathology of AGD

1.5.1 Clinical and pathological features

During initial Tasmanian AGD outbreaks in the mid 1980's, clinical signs of disease were associated with respiratory distress, loss of appetite and a severe mucoid branchialitis (Munday *et. al.* 1990; Munday *et. al.* 1993). Outbreaks in the USA, Spain and Ireland were also associated with similar signs (Kent *et. al.* 1988; Rodger & McArdle 1996; Dyková *et. al.* 1998). The introduction of routine farm monitoring and treatment facilitated an earlier response to developing outbreaks, thus perturbations to fish behaviour are presently less frequent. Clinical or gross (macroscopic) signs are largely confined to the gills where focal or multifocal white mucoid patches and profuse mucous production may be seen dependent upon disease severity (Alexander 1991, Clark & Nowak 1999).

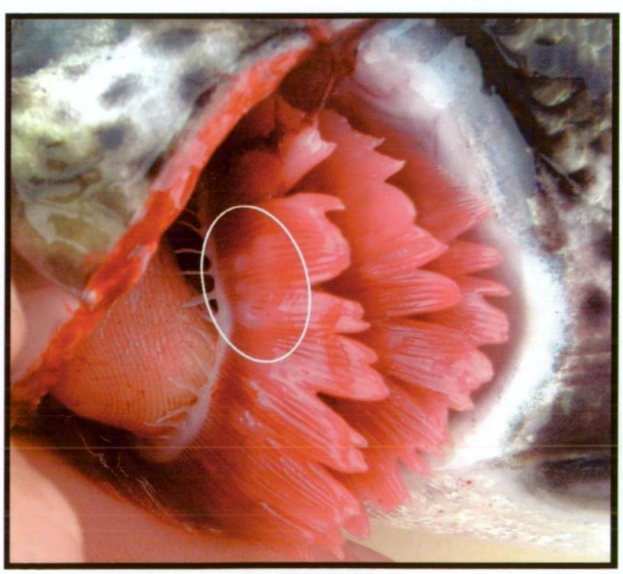

Figure 3. Pale mucoid patches (circled) appearing upon the gills of Atlantic salmon are the gross signs indicative of AGD.

From a field based histological perspective, the prominent feature of AGD is hyperplasia of the lamellar epithelium resulting in fusion of secondary lamellae and formation of interlamellar vesicles (Kent *et. al.* 1988, Roubal *et. al.* 1989; Munday *et. al.* 1990; Dyková *et. al.* 1995). Qualitative observations of reductions to chloride cells and increases to mucous cells are associated with hyperplastic epithelium (Roubal *et. al.* 1989; Munday *et. al.* 1990; Nowak & Munday 1994).

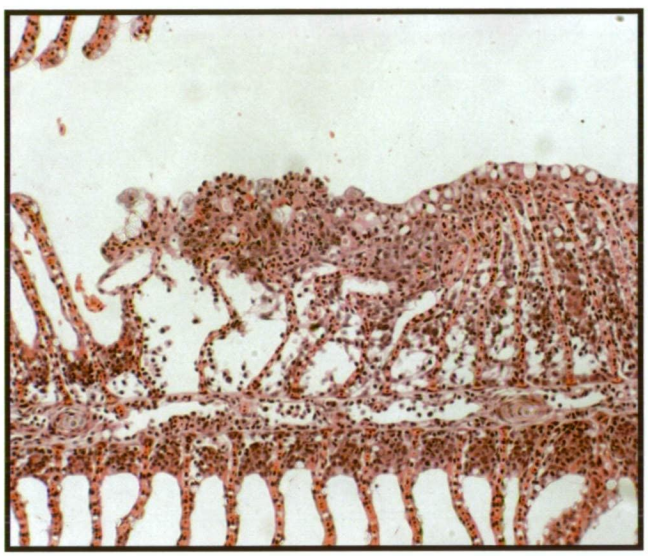

Figure 4. A typical AGD lesion as seen histologically. Note fusion of secondary lamellae.

Leucocytes have been observed within filamental connective tissues, filamental vascular tissues and within areas of hyperplastic epithelium (Roubal *et. al.* 1989; Munday *et. al.* 1990). Amoebae are seen almost exclusively in association with hyperplastic lesions (Munday *et. al.* 1990; Nowak & Munday 1994; Zilberg and Munday 2000).

In contrast to clinical signs of respiratory distress described during earlier, outbreaks of AGD exhibiting mortality (Munday *et. al.* 1990), patho-physiological evidence of oxygen deprivation due to AGD has not been detected although a respiratory acidosis was apparent (Powell *et. al.* 2000; Fisk *et. al.* 2002; Powell &

Nowak 2003). AGD affected fish have been shown to be hypernatremic (Munday *et. al.* 1990), hypertensive (Powell *et. al.* 2002a) and display altered cardiac morphology (Powell *et. al.* 2002c).

1.5.2 Pathogenesis

Although the major features pertaining to histopathological diagnosis are well documented, few authors have attempted to describe temporal aspects associated with AGD in Atlantic salmon. Zilberg & Munday (2000) induced disease under experimental conditions using co-habitation with infected fish and described progression at 1, 2, 4, 7, 14, 28 days post-exposure (DPE). In this study, adherence of amoebae to normal gill epithelium was evident by 2 DPE and small hyperplastic lesions with associated amoebae were evident by day 4 DPE. At 28 DPE, substantial hyperplasia had fused most lamellae (Zilberg & Munday 2000). The severity of infection is influenced by the density of amoebae within the water column (Zilberg *et. al.* 2001; Morrison 2003 unpublished data).

Infection progression was described for an AGD outbreak amongst turbot in a facility from Spain (Dyková *et. al.* 1995). At regions of trophozoite attachment thickening of secondary lamellae due to desquamation, hypertrophy and hyperplasia were observed. Subsequently lamellar fusion and formation of cavities between distally fused secondary lamellae were reported as infection progressed. Trophozoites associated with lesion affected regions were highly vacuolated but little evidence of bacterial ingestion was observed (Dyková *et. al.* 1998). During late stages of infection the relative numbers of attached amoebae had declined markedly (Dyková *et. al.* 1995; Dyková *et. al.* 2001).

Nowak & Munday (1994) presented the only study of AGD development during the initial marine phase of the Tasmanian Atlantic salmon production cycle.

They described the formation of nodules and plaques (small lesions of unknown aetiology showing fusion of 2 – 10 lamellae consisting mainly of mononuclear cells) appearing on salmon gills and suggested such lesions may be predisposing to the onset of AGD possibly suggesting preferential colonization of these regions. It has been suggested that attachment of *Neoparamoeba* sp. to gill epithelium may be influenced by the presence of predisposing lesions. Such lesions may potentially arise from gill insults by other biological agents such as protozoa, bacteria, algae or jellyfish (Kent *et. al.* 1988; Nowak & Munday 1994; Munday & Zilberg 2000; Dyková & Novoa 2001; Handlinger pers. comm. cited by Munday *et. al.* 2001).

1.6 Diagnosis of AGD

On farm diagnosis relies upon the presentation of gross signs of gill infection. Farm technical personnel routinely (approximately monthly depending upon season) monitor the gills of susceptible salmon stocks for the presence of raised, white mucoid spots and/or patches upon the gills. A score is generated for each cage according to

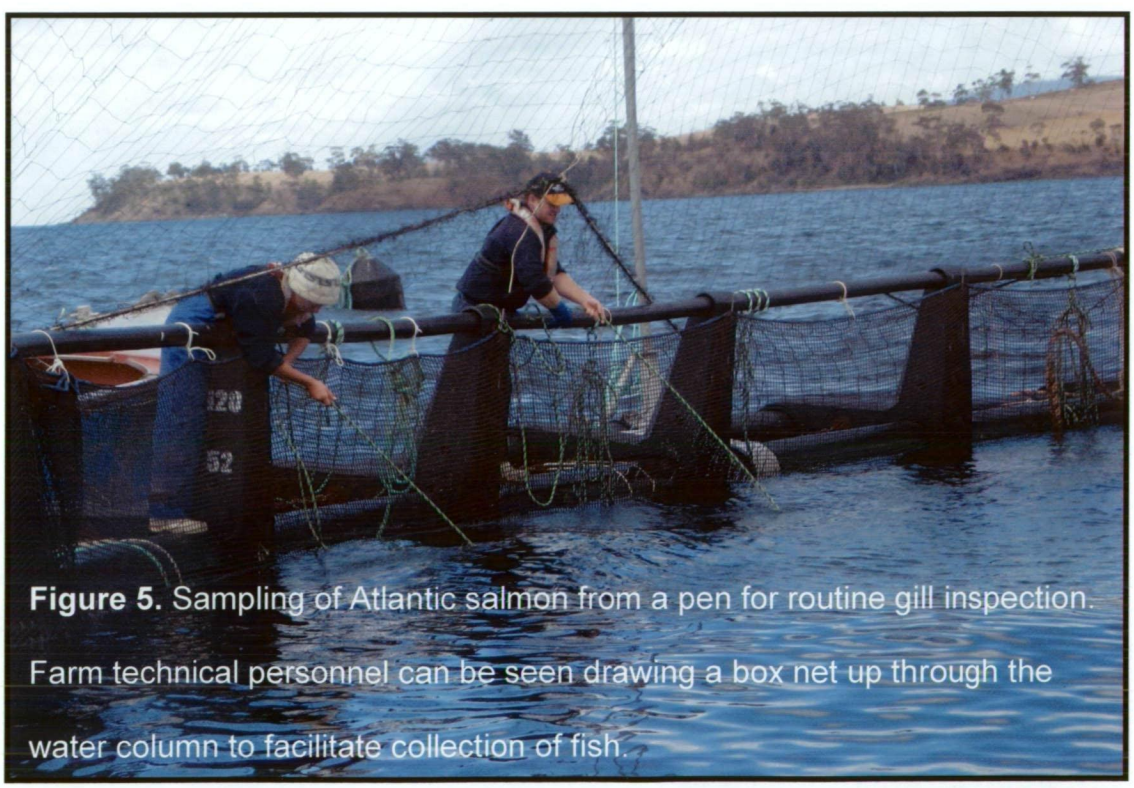

Figure 5. Sampling of Atlantic salmon from a pen for routine gill inspection. Farm technical personnel can be seen drawing a box net up through the water column to facilitate collection of fish.

the prevalence and relative severity of macroscopic (gross) gill change from a sample population ($n \approx 30 - 50$). The score determines the requirement for treating the entire pen population. Farm scoring of affected fish is variable according to the farm methodology and observer experience/interpretation. Gross diagnosis only confirms an altered gill condition but not the presence of the pathogen. Although this method of diagnosis has proven successful in the control of large scale mortalities, Clark & Nowak (1999) suggested this method of diagnosis was unreliable particularly for lighter cases of infection. Where doubt exists regarding diagnosis, samples are collected for microbiological and histopathological examination.

Development of an immuno-fluorescent antibody test (IFAT) (Howard and Carson 1993), using a polyclonal antibody raised against a Tasmanian isolate, provided farms with an alternate non destructive method of detecting the pathogens presence. This test is used to screen gill mucus along with surface tissue scraped from the gills of suspect fish. It was shown to be in excellent agreement with histological AGD diagnosis (Howard & Carson 1993). For diagnostic and research AGD investigation, histopathology is the only true diagnostic method indicating a diseased state and its causative agent. For AGD investigation, histopathological examination is more sensitive than IFAT (Zilberg & Munday 2000) but requires destructive sampling of fish. Gill smears, dried and stained by Quick Dip®, have been shown to be in excellent agreement with IFAT and provide a rapid presumptive diagnostic method for detection of the pathogen (Zilberg *et. al.* 1999). Another presumptive method of diagnosis is the examination of gill mucus for detection of ameboid like cells under a light microscope (Clark *et. al.* 2001).

1.7 Environmental influences and other potential risk factors

During the early years of commercial Atlantic salmon production in Tasmania, AGD outbreaks and associated mortalities were most frequent during late spring to early autumn at full salinity sites (Foster & Percival 1988b; Munday *et. al.* 1990, Munday *et. al.* 1993). Although AGD has eventually become a year round problem, bathing frequency is higher during the summer months and farms located in predominantly estuarine environments can remain free of AGD year round (Clark & Nowak 1999). Salinity and water temperature appear to be the major environmental factors influencing the occurrence of AGD during commercial Atlantic salmon production in Tasmania (Clark & Nowak 1999). Similarly in Ireland, AGD was reported during periods of record sea temperatures at sites experiencing oceanic salinities (Rodger & McArdle 1996).

Other intrinsic and/or extrinsic factors suggested as potential influences upon AGD development include stocking density/biomass, immune status, gill damage (physical [husbandry related] or biological [harmful algae, jelly fish, bacteria) and maturation. However the extent of the role (if any) played by these factors is presently undetermined (Nowak 2001).

1.8 Treatment and Control

As mentioned, the mitigation of AGD under Tasmanian farming conditions is mostly due to the advent of freshwater bathing. Large scale mortalities are generally avoided, although bathing is both expensive (≈ 15% of production costs) and production limiting due to the requirement for an extensive nearby freshwater source. Following gross diagnosis of AGD by routine farm sampling, fish are bathed in freshwater for 3-4 hours (Parsons *et. al.* 2001). The mitigating properties of freshwater bathing

infected fish are not entirely understood. It is suspected that a combination of osmotic challenge to gill associated amoebae, removal of seawater stable gill mucus and dissolution of gill lesions contribute to treatment success (Parsons *et. al.* 2001; Munday *et* al, 2001). When freshwater bathing was initially introduced as a commercial mitigation strategy in the late 1980s, two to three baths provided sufficient alleviation from AGD during the marine production cycle (Foster & Percival 1988a; Clark & Nowak 1999). Presently however, fish may require up to ten baths to successfully circumvent AGD progression for the same period. (Mitchell 2001). Parsons *et. al.* (2001) demonstrated the survival of amoebae within mucous samples following freshwater bathing. In the same study, amoebae were also observed trapped within inter-lamellar vesicles and both instances were suggested as a potential source of recurrent infection. Clark *et. al.* (2003) found gill associated amoebae numbers return to pre-bath levels ten days after treatment. Both *in vitro* and *in vivo*, survival of gill amoebae is lower in soft freshwater (Powell & Clark 2003, Roberts & Powell 2003a). No harmful physiological effects of freshwater bathing were reported during commercial treatment (Powell *et. al.* 2001).

Other methods of AGD mitigation have included freshwater bath supplementation with levamisole (Clark & Nowak 1999; Findlay *et. al.* 2000; Zilberg *et .al.* 2000) seawater addition of hydrogen peroxide or levamisole (Cameron 1993; Munday *et. al.* 2003) or feed additives (glucans) (Zilberg *et. al.* 2000). Experimental results had shown promise (Findlay *et. al.* 2000; Howard & Carson 1993) but when such strategies are transferred to a commercial situation the outcomes have been inconclusive (Cameron 1993, Clark & Nowak 1999, Zilberg *et. al.* 2000). Powell *et. al.* 2002c suggested chloramine-T, used as an additive to experimental freshwater and seawater baths, was more efficacious than freshwater treatment alone although post-

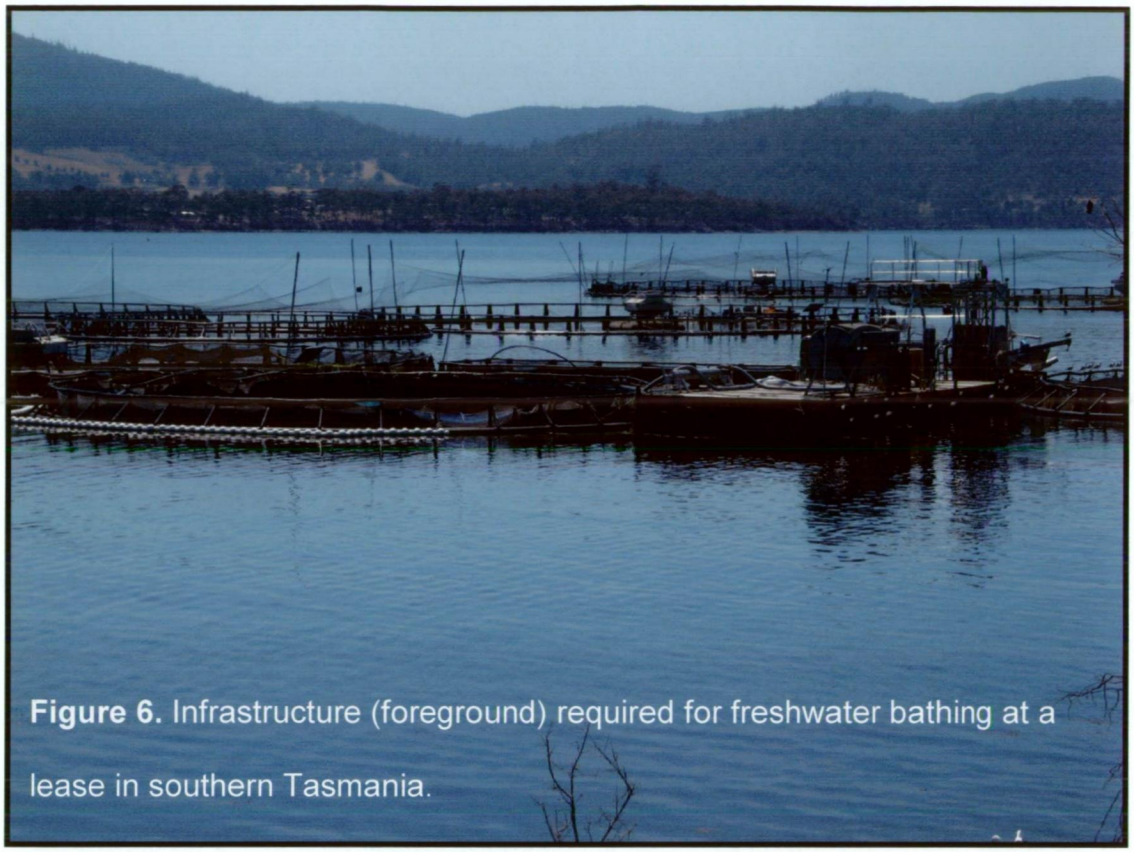

Figure 6. Infrastructure (foreground) required for freshwater bathing at a lease in southern Tasmania.

bath mortality was higher for the former treatments. Subsequent semi commercial trials (Powell *et. al.* 2003) suggested the use of a one hour seawater bath with chloramine-T was as effective in controlling AGD as freshwater bathing although this form of mitigation requires further investigation.

Although a humoral response, in Atlantic salmon and rainbow trout, against antigens from sonicated *Paramoeba* sp. had been described (Bryant *et. al.*1995), protection against infection by passive immunization with the above antigens was unsuccessful (Akhlagi *et. al.* 1996). Similarly, *in vitro* and *in vivo* attempts to hinder the survival and infectiveness of *Paramoeba* sp. using antibodies against the organism also failed (Zilberg & Munday 2001a; Zilberg & Munday 2001b). Findlay *et. al.* (1995, 1998) experimentally described resistance to AGD, attributable to non-specific immunity, in salmon that were bathed twice or held in freshwater for four weeks between an initial and a subsequent exposure to AGD. However, recent experiments

found no evidence of resistance using a similar experimental design and reported depressed phagocytic function of macrophages in re-infected fish (Gross, Morrison, Butler and Nowak 2003). Indeed, under commercial culture conditions, resistance to re-infection has not been documented.

For spring smolts and pre-smolts, re-infection occurs more rapidly after freshwater bath treatment compared to the time taken for initial infection to appear. Additionally, of critical concern to the Tasmanian industry, is the approximate five-fold increase in bathing frequency (Mitchell 2001) during the marine production cycle seen over the last decade.

1.9 Aims and outline of thesis

Although AGD has affected the Tasmanian industry for nearly 20 years (at the time of writing), several fundamental questions regarding the pathology of this condition remain unanswered. This thesis aims to address the more fundamental pathological aspects of AGD in the field, primarily by defining an improved pathological model of AGD. This thesis will elucidate the requirements of AGD development and how AGD development progresses within the commercial culture environment. The approach to solving some of the issues touched upon in this introduction is outlined below.

Chapter 2 describes the analysis of gill lesions from fish showing variable AGD pathology, for distribution, size and gill arch proximity. In this study, lesions were quantitatively analyzed to ascertain whether there is a distinct lesion distribution pattern. An investigation upon lesion structure was also undertaken to further investigate the host response during AGD. The structure of interlamellar cysts, a common feature of AGD lesions, is described and their formation is hypothesized.

Chapter 3, also a field based study, aimed to firstly describe gross pathological gill morphology using micro-stereoscopic and histopathological technique and

secondly investigate the agreement (or disagreement) between clinical and histopathological assessment of AGD at both the sample population and individual levels. The outcome of this study will enable elucidation of factors influencing the diagnostic reliability of clinical assessment as an indicator of amoebic gill disease.

Chapter 4 investigates whether attachment of *Neoparamoeba* sp. is an essential requirement of lesion formation. Also described are the early stages (first 48 hours) of AGD lesion development and the ability of *Neoparamoeba* sp. to colonize pre-existing gill lesions induced by physical trauma. These results in effect serve as an experimental model defining early lesion formation for the following chapters.

In chapter 5, a histological evaluation of the progressive nature of AGD and overall gill health status, under commercial culture conditions, was undertaken after transfer of spring smolts to estuarine/marine conditions. The trial concluded immediately prior to initial AGD treatment. The results were integrated with environmental records for additional consideration of aetiologically and environmentally related aspects of AGD.

Chapter 6 investigates the pathogenesis of re-infection with AGD after initial freshwater treatment. As infection often occurs rapidly following treatment, the plausibility of a modified infection strategy between treatments could then be compared to initial infection of naïve fish, as in chapter 5. The associated environmental observations and general gill health status were also examined for any interrelationships contributing to infection.

Chapter 7 provides a summation of the main results and conclusions integrated within the context of current literary assertions pertaining to many facets of AGD mentioned in the above sections. Consideration is given to future directions in terms

of diagnosis, research methodologies and research strategies that will assist in further developing our understanding of host–pathogen interactions of AGD.

CHAPTER 2 - Distribution and structure of lesions in the gills of Atlantic salmon (*Salmo salar* L.) affected with amoebic gill disease.

M.B. Adams & B.F. Nowak

2.1 Abstract

Atlantic salmon *Salmo salar* L. gills, affected with amoebic gill disease, were analyzed by routine histology to identify lesion morphology and distribution patterns. Numbers of lesions occurring dorsally, medially and ventrally were recorded as was lesion size, proximity to the gill arch and the degree of pathological severity imparted. The mean number of lesions and pathological severity in the dorsal region of the second left gill arch were significantly higher than that found ventrally ($P<0.01$). There were no significant differences in lesion size or proximity of lesions to the gill arch, between gill regions. Serially sectioned lesions revealed interlamellar cysts to be spherical to ovate in shape and fully enclosed within a wall of epithelium. Small to medium size cysts sometimes contained necrotic amoebae. Inflammatory cells, morphologically identified as neutrophils and macrophages were occasionally seen infiltrating medium sized cysts. Larger cysts were mostly clear of any cellular debris.

2.2 Introduction

The most significant health problem affecting the production of Atlantic salmon *Salmo salar* L. in Tasmania is amoebic gill disease (AGD) (Munday, Foster, Roubal

& Lester 1990). This condition is caused by *Neoparamoeba pemaquidensis* (Page, 1970), an amoeba that manifests itself on the gills of not only Atlantic salmon but also rainbow trout *Oncorhynchus mykiss* (Walbaum), coho salmon *Oncorhynchus kisutch* (Walbaum), turbot *Scophthalmus maximus* (L.) and sea bass *Dicentrarchus labrax* (L.) (Kent, Sawyer & Hedrick 1988; Roubal, Lester & Foster 1989; Munday *et al.* 1990; Dyková, Figueras, Novoa & Casal 1995; Speare 1999; Dyková, Figueras & Peric 2000). Outbreaks have been reported in Spain, Ireland, United States, France, Chile and New Zealand (Kent *et al.*, 1988; Dyková *et al.* 1995; Rodger & McArdle 1996; Clark & Nowak 1999).

Severity and duration of an outbreak is primarily a function of increasing salinity and water temperatures (Clark & Nowak, 1999). Outbreaks may also be influenced by other factors such as predisposing nodules or plaques, immune status and stocking densities (Nowak and Munday 1994; Clark and Nowak 1999; Findlay and Munday 1998; Findlay, Zilberg & Munday 2000; Nowak 2001, Zilberg & Munday 2000). Control of AGD is currently achieved by bathing entire pens of afflicted fish in fresh water for 2 – 4 hours (Parsons, Nowak, Fisk & Powell 2001). Consequently, AGD is a substantial economic burden in terms of labour and infrastructure (~14% of total production costs). Additionally, current preventative measures place limitations upon site selection owing to the recurrent need for a nearby, extensive fresh water source. Gross signs of infection are indicated by slightly raised, white mucous patches on the gills. The gill is the only site of infection which presents histologically as hyperplasia generally resulting in fusion of the secondary lamellae (Munday, Lange, Foster, Lester & Handlinger 1993).

Hyperplastic lesions often vary in size and extent. It is presumed that the initial interaction between the amoebae and the gill occurs when amoebae within

surrounding water are passed over the gill during the venting process. Amoebae are often seen adhering or in close proximity to lesions and sometimes entrapped within interlamellar vesicles or "cysts" (Kent *et al.* 1988; Munday *et al.* 1993; Dyková *et al.* 1995; Speare 1999; Parsons *et al.* 2001). It is unclear what the cause or function(s) of these cysts may be although it has been suggested that cysts may protect amoebae from treatment (Parsons *et al.* 2001).

There has been no specific investigation upon morphometric aspects of lesions resulting from AGD. In this study, gill lesions from fish showing variable AGD pathology, were quantitatively analysed for distribution, size and gill arch proximity to ascertain whether there is a lesion distribution pattern inconsistent with that of water flow across the gills. An investigation upon lesion structure was initiated to further characterise the host response to this pathogen by describing the structure of interlamellar cysts.

2.3 Materials and methods

2.3.1 Lesion Distribution

The gill sections selected for this study, previously collected and prepared by Clark and Nowak (1999), were from individual cages on two full salinity farm sites taken in May and December 1996. Each group of fish were independent (May-1995 smolt intake & December – 1996 smolt intake) and their post transfer histories are further described in Table 1.

Table 1. Number of days post-transfer from hatchery to seawater.

Farm Site	Sampling Month	Post-transfer (Days)
1	Dec	42
2	Dec	27
1	May	196
2	May	188

Ten histological slides (second left gill arch), each from a different individual with AGD lesions, were selected from archives representing each cage and month (except Farm 2 [May] where n = 9). The sections were viewed under a light microscope (Olympus, Hamburg, Germany) at 40x – 400x to ascertain the number, size, proximity to the gill arch and location of each lesion. Lesion size and proximity measurements were taken by counting the number of hyperplastic interlamellar units within each lesion. For each fish the total filament length for each region (dorsal, medial & ventral) was recorded to enable the percentage size and proximity to the gill arch of each lesion to be calculated. The overall impact of a lesion, in terms of the space it occupies and the position it occurs along a filament, was considered the primary unit of importance therefore lesion size and proximity were expressed as a function of the length of each filament. The percentage proximity described the distance of each lesion from the gill arch, whereby a lower percentage indicated closer proximity.

Lesions were designated either dorsal, medial or ventral according to their position on the gill. A pathological index was calculated, to permit an analysis of the

pathological severity imparted upon each fish, by multiplying the number of lesions by their average percentage size in each individual fish.

2.3.2 Lesion Morphology

Embedded gill arches (Farm 1 [December]: n=10) were serially sectioned at 5μm. Every second section was attached to a slide, stained with hematoxylin & eosin then viewed under a light microscope at 40x magnification. A CCD video camera (DXC-107P – Sony, Australia), c-mounted to a light microscope and integrated with a PC (Miro Computer Products, Palo Alto, CA), was used to capture images of each lesion section in a sequential manner. Image capture software (Vidcap32 [Microsoft Corporation, Redmond, WA] & Adobe Premiere 5.1 [San Jose, CA]) was used to compile the images into a movie format permitting a transverse, sequential viewing of lesions. Following image capture, each movie was viewed frame by frame to identify the start and endpoint of the cysts found within each lesion. The overall depth of each cyst could then be estimated by the number of frames shown, as each frame represented a 10μm depth increment through the lesion. The movie format for each lesion was also used to determine which frame displayed the maximum width for each cyst. A single frame of each cyst was subsequently captured, from the corresponding slide, for width measurements at a higher magnification (100 – 400x) using image analysis software (Sigma Scan).

An immuno-fluorescent antibody test (Howard & Carson 1993) was performed upon a selection of deparaffinised and rehydrated tissue sections (n=15) with AGD lesions. These sections were randomly chosen from samples used for lesion distribution studies outlined previously.

2.3.3 Statistical Analysis

The results for lesion distribution and associated characteristics were analysed by three way analysis of variance (ANOVA) with farm (fixed, orthogonal, two levels), month (fixed, orthogonal, two levels) and arch location (fixed, orthogonal, three levels) as factors. Tukeys HSD was used for means comparisons where assumptions of normality (Shapiro-Wilk test) and homogeneity (Levene's test) were met. A P value of $P<0.05$ was adopted for rejection of the null hypothesis. SPSS® (version 9.0, SPSS Science) and Sigma Plot (version 4.0, SPSS Science) were used for data analysis and presentation.

2.4 Results

Results from three way ANOVA indicated a significant difference in the number of lesions ($P<0.01$) and pathological severity ($P<0.05$) between arch location and the interaction of farm and month ($P<0.001$). Lesion numbers and pathological severity in the dorsal region of the second left gill arch were significantly higher than the ventral region (Fig. 1). Medial lesion numbers and pathological severity were not significantly different to dorsal or ventral regions although a declining trend was evident across both farms. Lesion numbers on the 2nd left gill arch taken from Farm 2 in December were as much as three times higher than all other groups and the

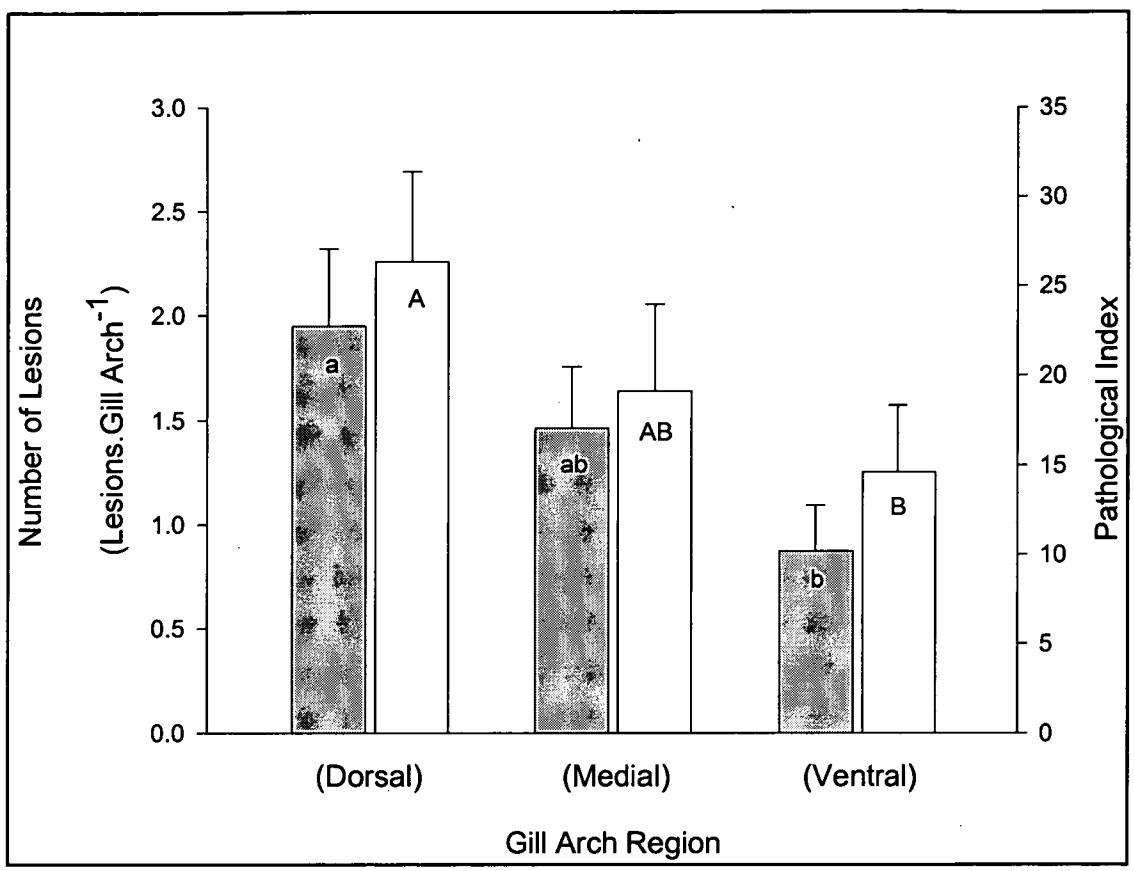

Figure 1. Number of AGD lesions (grey bars) and pathological severity (white bars) for each gill region from 2 farms in December and May (n = 39). Values are means ± SE. Different letters indicate values that are significantly different.

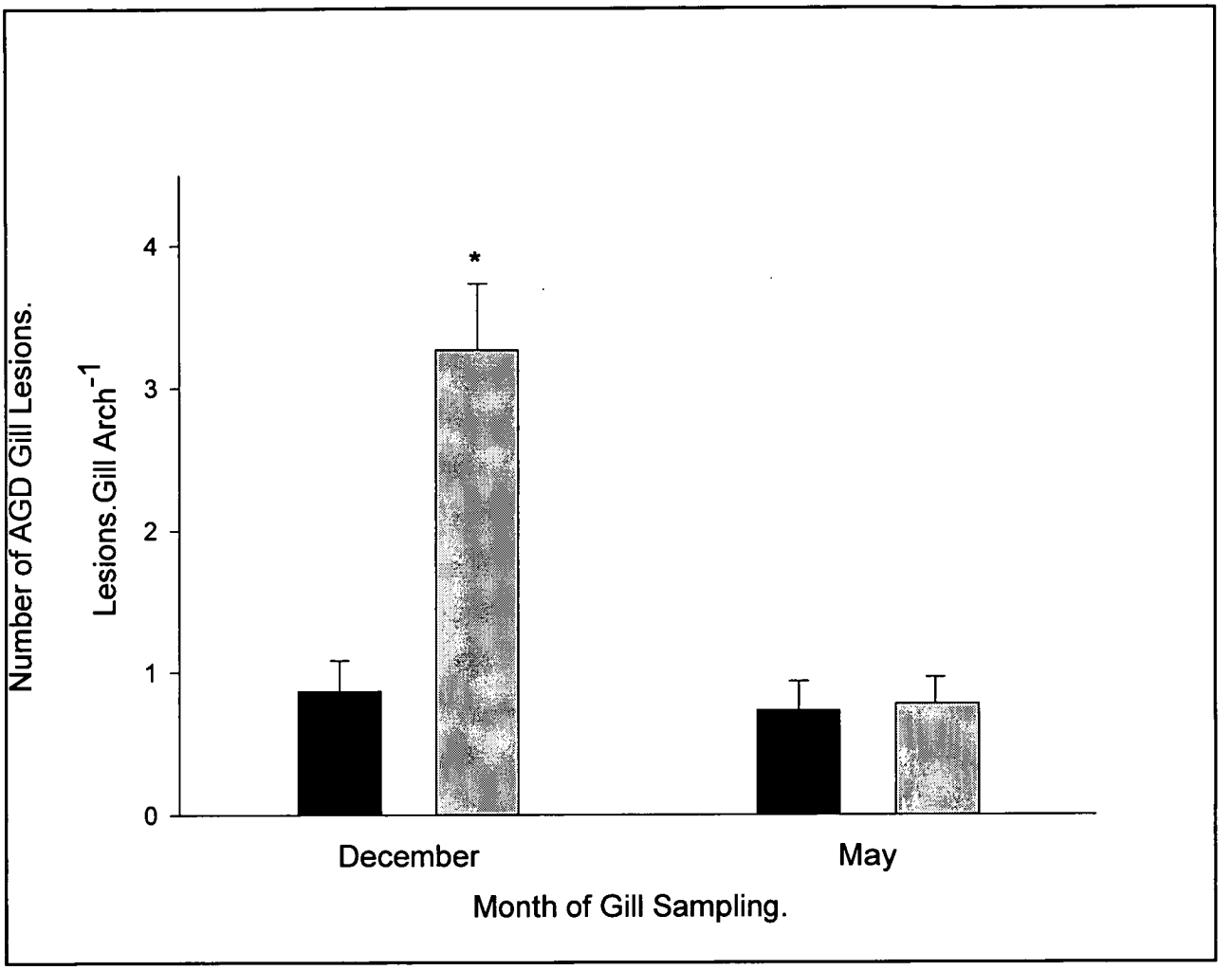

Figure 2. Number of AGD lesions for each farm [farm 1 – black bars & farm 2 – grey bars] and month (n = 39). Values are means ± SE. Different letters indicate values that are significantly different.

differences were significant (P<0.001; Fig. 2). The pathological severity of these lesions also increased for the same farm and month by a similar order of magnitude (P<0.01; data not shown). Percentage size and proximity were found to be significantly (P<0.01) larger and closer to the gill arch in December for Farms 1 & 2 (combined data; Table 2).

Table 2. Percentage size & proximity of AGD lesions. Values are means ± SE. Asterisk depicts those values significantly different for each variable.

Month	December		May	
Percentage Size	*17.44	±1.38	9.59	±1.5
Percentage Proximity	14.83	±1.56	*27.63	±2.93

Pathological observations revealed AGD lesions with variable hyperplasia, lamellar fusion and the presence of interlamellar cysts (Fig 3). A localised inflammatory response was evidenced by the migration of neutrophils, macrophages and lymphocytes along the central venous sinus. These cells were seen adhering and migrating through the endothelia of the central venous sinus into hyperplastic tissues (Fig 4 A,B). In some cases eosinophilic granule cells were seen in close association with the connective tissues of the primary lamellae. Serially sectioned lesions

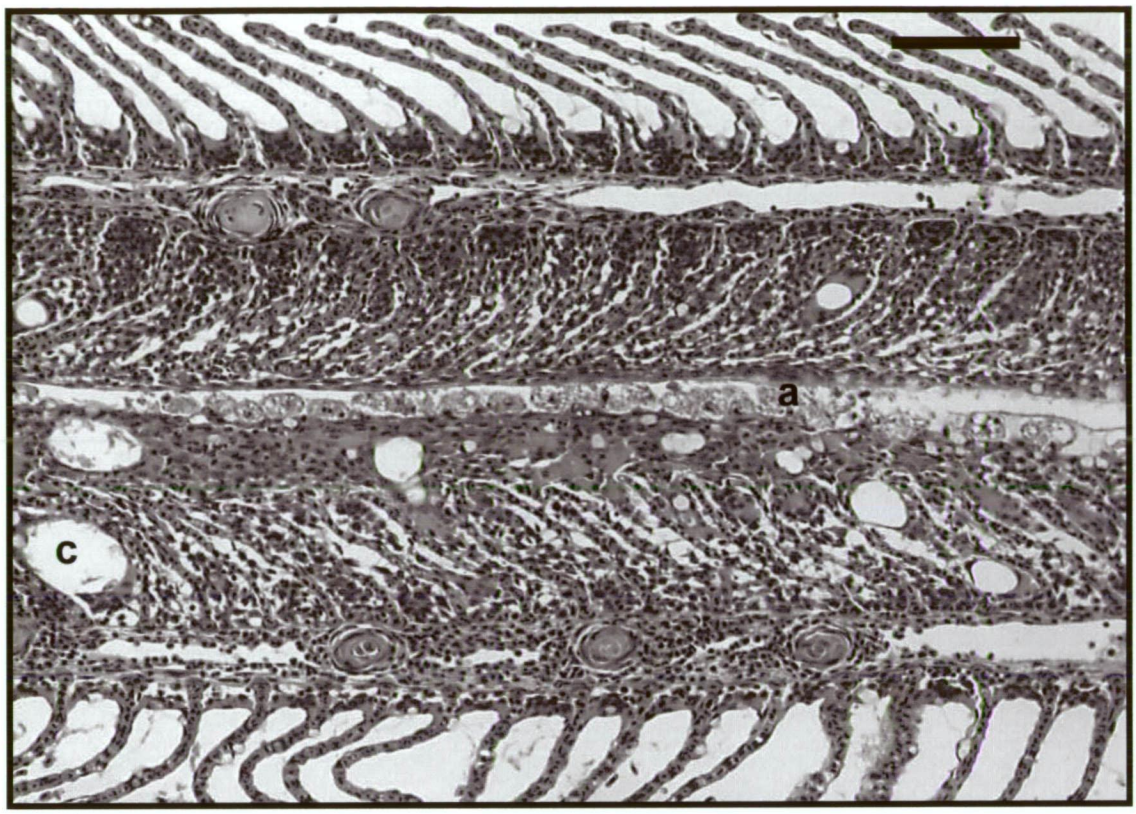

Figure 3. Pathology of amoebic gill disease. Hyperplasia of gill filaments, fusion of secondary lamellae, multiple rows of attached amoebae [a] and the presence of interlamellar cysts [c]. Bar represents 100μm.

enclosed within a wall of epithelial cells. Their sizes ranged from 10 to 140 μm in depth and 16 to 156 μm in width (Fig. 6). Small (20-50 μm) to medium (50-100 μm) sized cysts, sometimes contained necrotic amoebae displaying a swollen and granular appearance (Fig. 5). Inflammatory cells, morphologically identified as neutrophils and macrophages were occasionally seen infiltrating medium sized cysts. Larger cysts (>100 μm diameter) were mostly clear of any cellular debris. Results from the IFAT staining of selected gill sections were unable to positively identify the nature of cellular debris found within the cysts. Positive identification of amoebae is normally associated with a bright green fluorescence as opposed to the background yellow

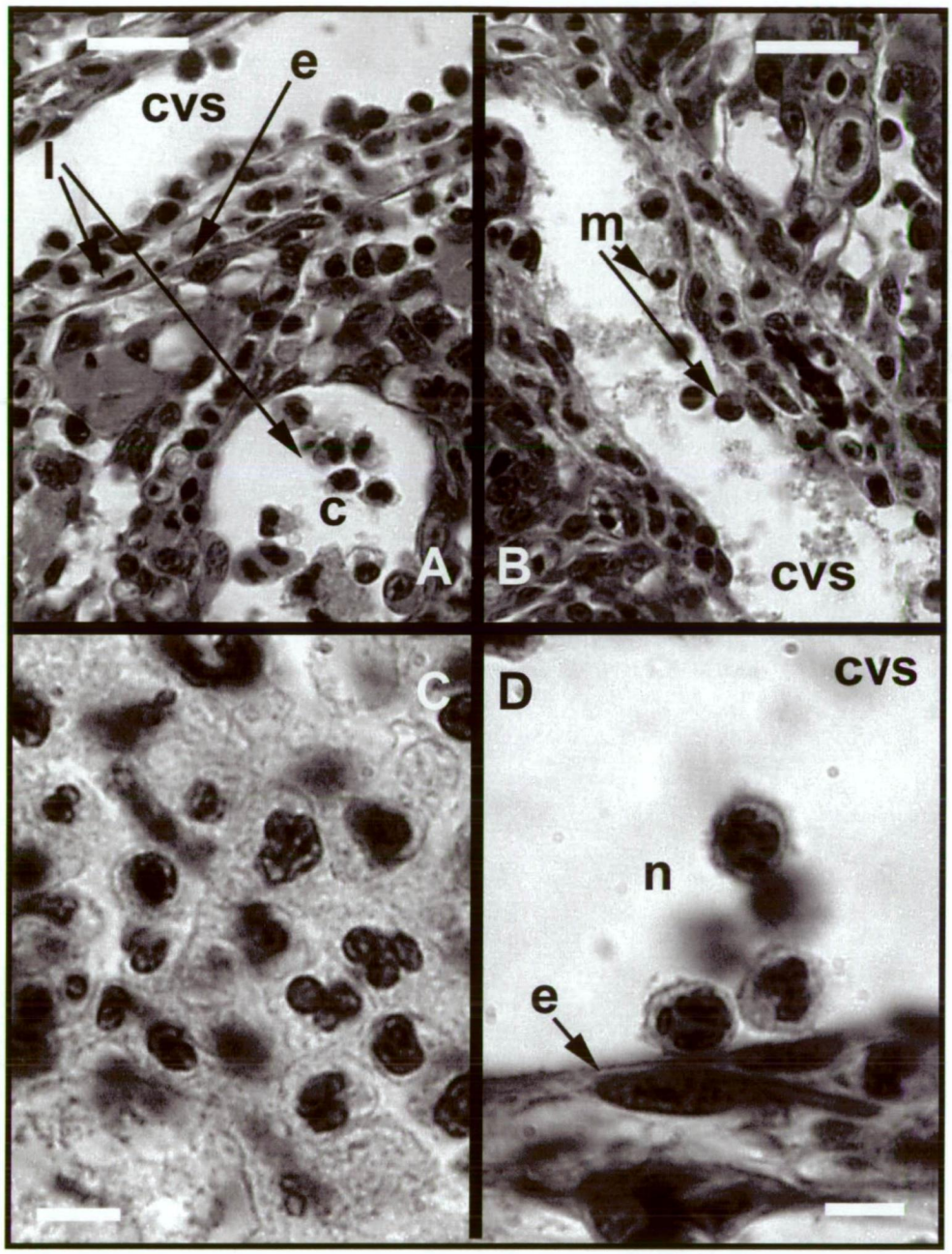

Figure 4. Further host responses to amoebic gill disease. (A) Leucocytes [l] crowding the endothelia [e] of the central venous sinus [cvs] and infiltrating a cyst (c); (B) Cells resembling macrophages [m] prior to migration through the endothelia of the CVS adjacent to a lesion; (C) Neutrophils [n], with swollen cytoplasm, occupying a cyst filled with necrotic debris; (D) Neutrophils in the CVS, adjacent to a lesion, prior to diapedesis. {Bar represents 20μm for (A,B) and 5 μm for (C,D).

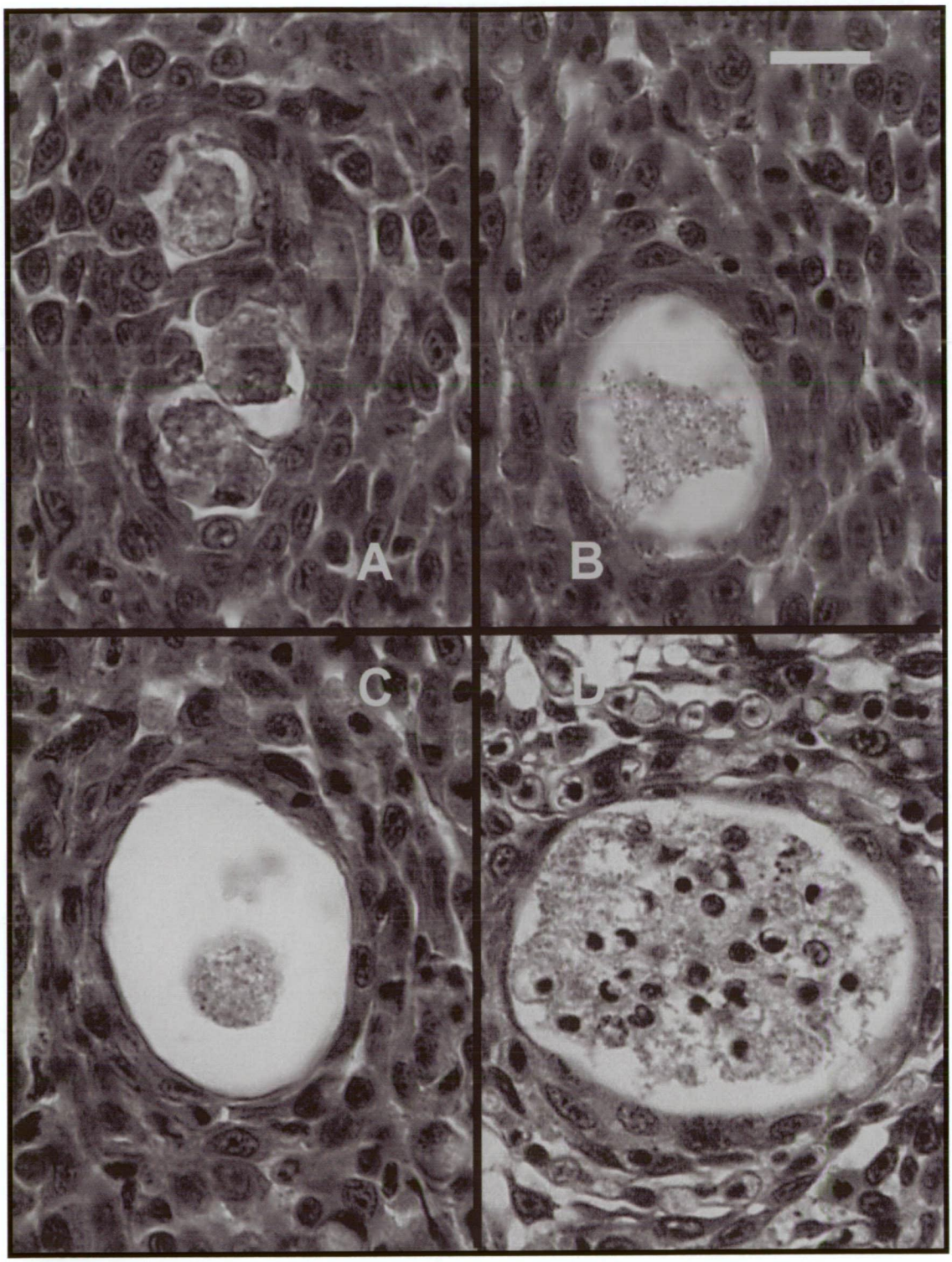

Figure 5. Hypothesized cyst formation and clearance. (A) Entrapment of amoebae by proliferating tissue; (B, C) Re-organization of epithelial cells for amoebic encapsulation; (D) Clearance of amoeba by infiltrating phagocytic cells. Bar represents 20μm.

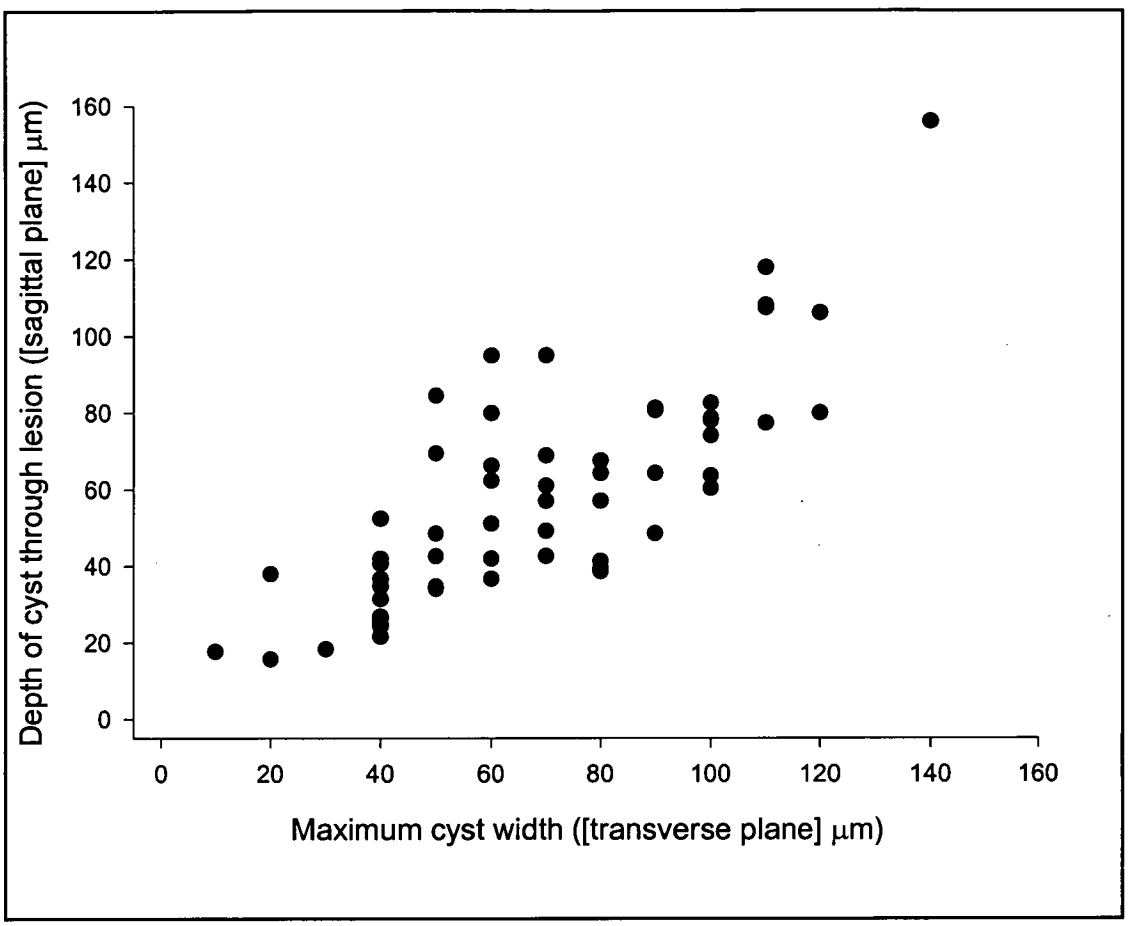

Figure 6. Scatterplot of cyst depth as measured in the sagittal plane (µm) of hyperplastic lesions versus maximum width (µm) of lesion cysts in the transverse plane.

staining exhibited by gill tissue. Only a few cysts from the selected samples contained cellular debris the majority of which were empty or contained trace amounts of debris. In most cases the cellular debris contained within the cysts, from IFAT sections, were necrotic.

2.5 Discussion

Lesion numbers were significantly higher in the dorsal region of the second left gill arch. Knowledge of regional water pattern movements throughout the gills is currently unknown. It is speculated however that water flow patterns may have contributed to the position of lesions on the gill arch. Water flow through the dorsal region of the gills is quite possibly retarded due to the influence of the cranial lobes. This would in effect increase the retention time of water passing the gills in this region, permitting increased contact time between host and pathogen. Ferguson (1989) suggested that "low flow locations" influences the recovery of bacteria from the gills where successful recovery is frequented more so dorsally than ventrally.

The predominantly dorsal appearance of histological lesions, in this study, contrasts with anecdotal evidence indicating a predominantly ventral occurrence of gross gill lesions on site in the field. Similarly, literature findings have failed to conciliate differences between gross and histological gill lesions. Gross gill lesions present macroscopically as raised, white mucous patches on the gill as opposed to histological lesions presenting microscopically as hyperplasia and fusion of the secondary lamellae. Whether or not a gross gill lesion is indicative of a histological lesion is still largely circumspect. Clark and Nowak (1999) described a lack of correlation for light infections when investigating consensus between gross lesion scores (generally scored as 0 – 4 with a high score indicating extensive lesions [Parsons et al 2001]) and lesions presenting histologically. It should be noted however that histology normally uses a single gill arch for diagnosis whereas gross assessment is generally ascertained from all eight gill arches (16 hemibranchs). Light infections rarely see all arches displaying gross lesions both experimentally and in the field (pers. ob.). Zilberg and Munday (2001), using fish infected experimentally with AGD,

found that mucous cell numbers were significantly reduced in hyperplastic tissue as opposed to normal tissue. Nowak & Munday (1994) and Munday et al (1990) conversely described the occurrence of many mucous cells on the surface and within lesions of naturally infected fish. Powell, Parsons & Nowak (2001), found increases in mucous cells on lesion affected filaments from naturally infected fish as did Zilberg & Munday (2000) in experimental trials. It should be noted that different infection regimes (experimental or natural), tissue staining procedures and alterations to lesion morphology over time would substantially influence these findings and hence the apparent differences. A more definitive study, that tracks the gill arch location and numbers of gross lesions followed by histological verification, would be of significant value in further understanding the host response and reconciling discrepancies between gross and histological lesions.

Previous studies on proliferative gill diseases do not quantitatively describe the distribution of lesions throughout the gill arch with one exception. Nowak and Lucas (1997) reported a significantly greater prevalence of fish showing post transfer lesions (non-AGD type) ventrally. The difference between lesion locations of AGD type lesions and post transfer lesions is most probably due to the nature of the irritant.

Fish from farm 2 in December had the highest number of lesions and pathological severity per gill arch. Clark and Nowak (1999) found the corresponding farm and month to have the highest prevalence of AGD amongst the four groups investigated in this study over 1995 -1997. A relationship between AGD prevalence on site and pathogen load upon the fish seems likely, however it is unknown whether a corresponding increase in lesion numbers upon the gills and amoebic loading are related aspects. However, if this assumption were adopted, then it would concur with

anecdotal evidence indicating fish as a primary reservoir for the spread of infection throughout a farm.

AGD tends to abate in its intensity by late autumn (Clark and Nowak 1999) and this is apparently reflected by the percentage size of lesions displayed in Table 2 where lesions were approximately 50% smaller. This result may also be influenced by the reduced prevalence of AGD that affected the 1995 smolt intake. Either scenario would indicate that the incidence of disease is a factor contributing to pathological development and/or recovery.

The closer proximity of lesions to the arch in December fish is most probably related to the size of the lesions. Nowak and Lucas (1997) found that larger post transfer lesions occurred more frequently proximal to the arch. The more aggressive nature of AGD in the summer months may be a factor in the spread of hyperplasia, along and/or between gill filaments, as a response to increased pathogen loads. This is seen in experimental infections (Zilberg and Munday 2001) that typically are far more veracious than a wild infection and are probably due to increased pathogen loads that fish would not normally contend with on the farm. Naturally, larger lesions will therefore occupy more space along the filament.

This study has now characterised interlamellar cysts as being ovate - spherical and fully enclosed by epithelial cells as revealed by serially sectioning lesions. Observations from this study would suggest that the formation of cysts is primarily due to the nature of the proliferative host response. This response most probably begins during the initial phases of tissue proliferation and ultimately results in the complete destruction and clearance of amoeba trapped within proliferating tissue. Parsons et al (2001) found amoebae within cysts and hypothesised that the cysts may be a source of re-infection for recently bathed fish. We suggest that amoebae found

within cysts occur transitionally prior to their ultimate degradation and removal by host cellular processes (Fig 6.). IFAT was unable to verify the nature of the cellular debris found within the cysts. However, the cysts from IFAT sections contained material in an advanced state of necrosis, possibly influencing IFAT sensitivity due to biochemical degradation of epitopes for antiboby binding. The exact mechanisms and sequence for the apparent necrosis and clearance of amoebae requires further investigation.

2.6 Acknowledgments

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CHAPTER 3 - Gross pathology and its relationship to histopathology of amoebic gill disease (AGD) in farmed Atlantic salmon *Salmo salar* L.

M.B. Adams, K. Ellard & B.F. Nowak

3.1 Abstract

Gross pathological assessment of amoebic gill disease (AGD) is the only non destructive, financially viable method for rapid and broad scale disease management of farmed Atlantic salmon (*Salmo salar* L.) in Tasmania. However, given the presumptive nature of this diagnosis, the technique has been considered questionable. We investigated the degree of conformity between clinical signs and histological lesions observed in a commercial setting. Three groups of Atlantic salmon (n = 42, n = 100, n = 100 respectively) were collected from various farm sites in southern Tasmania between December 2001 and April 2003. Micro-stereoscopic analysis showed that grossly affected tissue regions correspond to areas of hyperplastic lamellae fusion generally in association with attached *Neoparamoeba* sp. We also compared agreement between gross signs of AGD and histopathological diagnosis. Kappa analysis indicated moderate to good agreement between methods (k = 0.52 – 0.74). Individual cases of disagreement were further scrutinized and several factors were found to influence the level of agreement between the two methods. Stage of disease development, lesions associated with other pathogens, assessor interpretation / experience, sampling methods, histological technique and/or experience were

potential confounding factors. We concluded that clinical diagnosis is acceptable as a farm monitoring tool only. Removal of grossly affected tissue and subsequent histological examination is recommended to augment diagnostic accuracy.

3.2 Introduction

Amoebic gill disease (AGD) is a significant health problem affecting the commercial culture of Atlantic salmon *Salmo salar* L. in Tasmania and has been described for a variety of cultured fish species worldwide (reviewed by Munday, Zilberg & Findlay 2001; Nowak, Carson, Powell & Dyková 2002). Tasmanian outbreaks of AGD are primarily influenced by salinity and water temperature (Clark & Nowak 1999; Adams & Nowak 2003). Successful mitigation of this condition is provided by freshwater bathing whole pens of afflicted fish (Parsons, Nowak, Fisk & Powell 2001). However, this practice is both expensive and production limiting due to the requirement for an extensive nearby freshwater source.

The primary feature of macroscopically detectable (gross) AGD is the presence of raised, white mucoid spots and/or patches upon the gills (Munday, Foster, Roubal & Lester 1990). In Tasmania, commercial marine salmon growers routinely (approximately monthly depending upon season) monitor the gills of susceptible salmon stocks for such symptoms as an indicator of AGD. The prevalence and relative severity of gross gill change, amongst the sample population, determines the requirement for bathing each pen of fish. However, detection of spots and patches only indicates an altered gill condition, not the identification of the causal agent. Under culture conditions, fish are susceptible to a range of potential toxicants and/or pathogens able to induce a hyperplastic response (Roberts 1989). However gross

monitoring remains the only practical, economically viable tool for the management of AGD on a commercial scale.

Gill patch counts have been used exclusively as a quantitative measure of pathological severity in a number of experimental amoebic infections (Findlay, Helders, Munday & Gurney 1995; Findlay & Munday 1998; Findlay, Zilberg & Munday 2000). Under experimental conditions this method may be appropriate because control over the immediate environment is regulated thus reducing the possibility of other pathogens or toxicants inducing clinical gill lesions. Indeed, comparable patterns of pathological severity were evident when patch counts were used in conjunction with histological lesion counts (Zilberg, Gross & Munday 2001). Occasionally, field studies have relied upon arbitrary gills scores (farm based assessment) as an indicator of infection intensity. Using this method, pathogen presence was confirmed by immunofluorescent antibody testing (IFAT) (Powell, Fisk & Nowak 2000; Fisk, Powell & Nowak 2002).

The exact relationship between the occurrence of gross gill change and histological manifestation of AGD is unclear. For diagnostic and research purposes, histopathology is the only method able to indicate both presence of the pathogen and resultant host response. However, histopathology requires destructive sampling which is potentially limiting to broader scale epidemiological studies (Douglas-Helders, Carson, Howard & Nowak 2001). Histopathologically, affected gills display single or multi focal epithelial hyperplasia and leucocytic infiltration resulting in lamellar fusion (Kent et al. 1988; Munday et al. 1993; Dyková et al. 1995; Parsons, Nowak, Fisk and Powell 2001; Adams & Nowak 2001, 2003). Histopathological assessment of AGD is generally made upon examination of a single hemibranch. Little consideration has been afforded to targeting grossly affected tissue for

histopathological analysis. A preliminary field study indicated that regions of grossly affected tissue manifested histologically as lesions consistent with AGD (Adams, Ellard & Nowak 2002). However, a more comprehensive investigation was warranted in light of previous disparity between gross gill change and histopathological diagnosis (Clark & Nowak 1999; Adams & Nowak 2001, 2004a; Zilberg et al. 2001; Sadler 2002; Adams & Nowak 2003b).

This field based study aims to firstly describe the morphology of gross gill changes using micro-stereoscopic and histopathological technique and secondly investigate the agreement (or disagreement) between clinical and histopathological assessment of AGD at both the sample population and individual levels. The outcome of this study will enable elucidation of factors influencing the diagnostic reliability of gross clinical assessment as an indicator of amoebic gill disease.

3.3 Materials and methods

3.3.1 Sampling regime

Three groups of Atlantic salmon (referred to hereafter as A [n = 42], B [n = 120] & C [n = 100]) were collected from various farm sites in southern Tasmania between December 2001 and April 2003. Figure 1 and table 1 detail sampling locations, times and numbers. Group A salmon, targeted for the presence of macroscopic gill lesions, were collected immediately prior to freshwater bathing and/or during routine monitoring. Group B and group C were collected randomly (n = 10 per group) on a weekly basis prior to initial AGD treatment and between AGD treatments, during consecutive summers respectively.

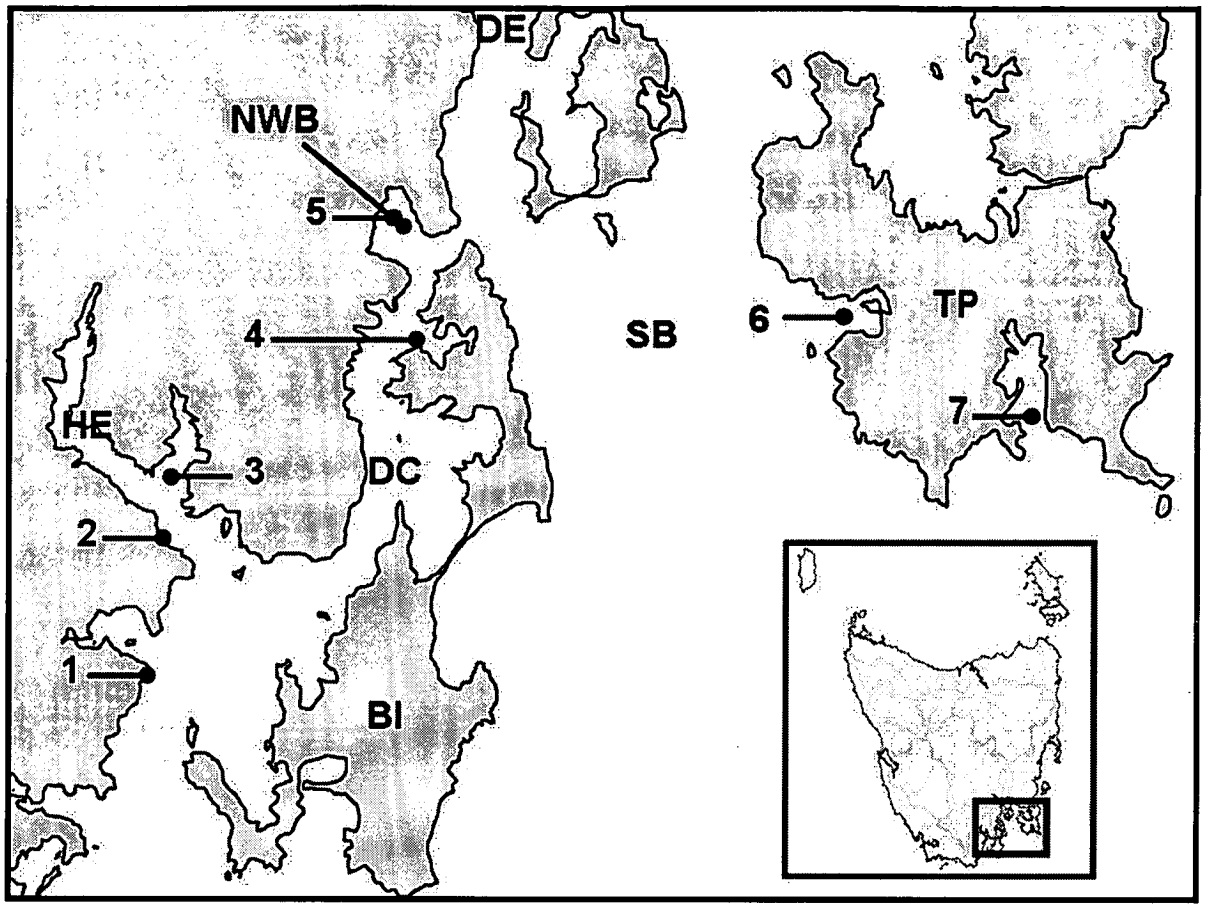


Figure 1. Location of sampling sites (DE) Derwent Estuary (BI) Bruny Island (HE) Huon Estuary (DC) D'Entrecasteaux Channel (SB) Storm Bay (NWB) Northwest bay (TP) Tasman Peninsula. Numbers correspond to sampling sites as per Table 1. Inset - Map of Tasmania showing regional sampling location.

Table 1. Sampling regime.

Site*	Location	n	Group	Collection Date
1	Port Esperance	1	A	Dec-2002
1	Port Esperance	4	A	Mar-2003
2	Hideaway Bay	3	A	Feb-2002
3	Deep Bay	100	C	Dec-Jan 2002/03
3	Deep Bay	120	B	Dec-Feb 2001/02
4	Sykes Cove	5	A	Nov-2002
5	Northwest bay	4	A	Feb-2002
5	Northwest bay	4	A	Oct-2002
5	Northwest bay	3	A	Dec-2002
5	Northwest bay	3	A	Mar-2003
5	Northwest bay	4	A	Apr-2003
6	Nubeena	5	A	May-2002
7	Port Arthur	6	A	Jul-2002
7	Port Arthur	1	A	Dec-2002

*As per Figure 1.

3.3.2 Sampling protocols

Following collection, group A salmon were euthanized in clove oil (0.02% w/v) and the operculum (left or right) was removed. Macroscopic images were taken (Kodak DC4800, New York, USA) of each anterior hemibranch prior to gill excision. Gills were then excised, rinsed gently in 0.22 μm filtered seawater and fixed in seawater

Davidson's fixative for 1 -2 h. Following fixation, macroscopic and micro-stereoscopic (Canon PowerShot G3, New York, USA; Olympus Camedia C5050 & Olympus SZX12, Hamburg, Germany) images were taken of the same hemibranchs and processed for routine histology. Salmon (groups B & C) were collected from each cage by box netting and transferred to an oxygenated holding bin (80L). Each fish was individually removed from the holding bin and terminally euthanized as above. The gills were arbitrarily scored according to lesion severity (Adams & Nowak 2003) thus indicating presence or absence of clinical gill lesions. Gills were then excised, rinsed and fixed as above. For group C, macroscopic gill images for each fish were captured from the second left anterior hemibranch which were subsequently processed for histology. Images were later referenced for comparison with histopathology.

3.3.3 Histopathology

Gills were transferred to 70% ethanol at 24 h post-fixation. For group A, the 2nd left gill arch was dehydrated, embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and eosin (H&E). Group A and C gills were dehydrated and embedded assuring orientation consistent with photographic records. Group A gills were sectioned at 20 μm until protruding tissue was evident within the slice. A single 5 μm section was then selected. Gills were then further sectioned at 20 μm until the entire hemibranch was evident and a second 5 μm section was taken. After staining, all sections were viewed under a light microscope (Olympus BH2, Hamburg, Germany) at 400x magnification and images taken (Leica DC300f, Wetzlar, Germany) of affected tissue.

3.3.4 Clinical-histological agreement

Data from group B and C were analysed for comparison between presence/absence of gross lesions upon any hemibranch with presence/absence of histopathological AGD

lesions upon the hemibranch selected for histology. Group C also compared presence/absence of gross lesions upon the hemibranch selected for histology and presence/absence of histological AGD lesions upon the corresponding hemibranch. The overall prevalence of gross lesions and histological AGD lesions, for each group combination, were compared by calculating kappa (Cichetti & Feinstein 1990) and the relative strength of agreement statistically analysed (table 2).

Table 2. Indicator of agreement strength using kappa value

Value of kappa	Strength of agreement
<0.20	Poor
0.21-0.40	Fair
0.41-0.60	Moderate
0.61-0.80	Good
0.81-1.00	Very good

Group C was further examined on a case by case basis to ascertain the nature of disagreement between gross lesions present/absent on any hemibranch, gross lesions present/absent upon the hemibranch selected for histology and presence/absence of histological AGD lesions upon the corresponding hemibranch. In each case of disagreement the relevant image and histological section were reviewed to ascertain the nature of the disparity.

3.4 Results

3.4.1 Clinical lesion morphology

3.4.1.1 AGD lesions: For AGD affected gills, the pattern of gross lesion presentation ranged from discrete focal spots to extensive regions of coalescing mucoid patches. The detection of gross AGD lesions on the gills of fish (Group A) was dependant upon the stage of development. Smaller lesions were not visible grossly (Fig. 2A), but were discernible micro-stereoscopically (Fig. 2B) and manifested histologically as small focal regions of hyperplastic lamellar fusion (Fig. 2C). Micro-stereoscopic observations of grossly detectable AGD lesions (Fig. 2D) displayed a distinctive protrusion of tissue upon the leading edge of filaments extending deep into the interlamellar regions (Fig 2E). Serial histology indicated that protruding areas of proliferating tissue were comprised mostly of undifferentiated epithelial cells and mucous cells. Deeper sections of corresponding tissue exhibited characteristic AGD lesions. Amoebae (identified morphologically by the presence of the nucleus and endosymbiont) were substantially numerous on the protruding face of most lesions compared to the inter-filamental regions (Fig. 2F). Mucous cells were extremely numerous and hypertrophic when in close proximity to or within a lesion and within the deeper inter-filamental regions. Hyperplastic epithelia heavily populated with mucous cells were rarely colonized by amoebae.

3.4.1.2 Non-AGD Lesions: Grossly detectable lesions not attributable to infection with *Neoparamoeba* sp. were apparent in 11% of Group A cases collected during October 2002 - April 2003. Micro-stereoscopically, proliferation of tissue upon the leading

filamental edge and inter-filamental regions was similar to that observed for clinical AGD (Figure 3A). In most cases of gills with grossly detectable lesions, multiple

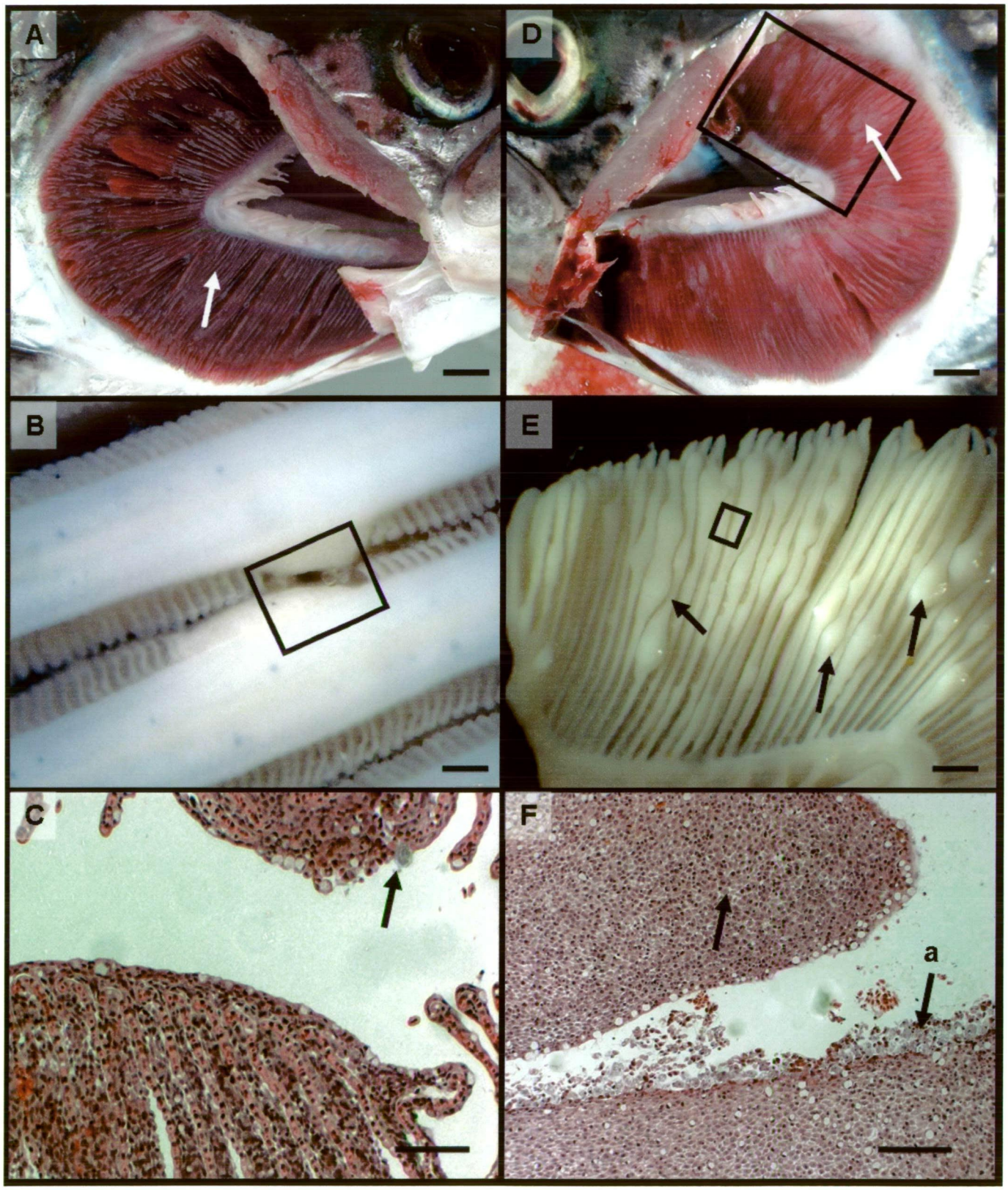
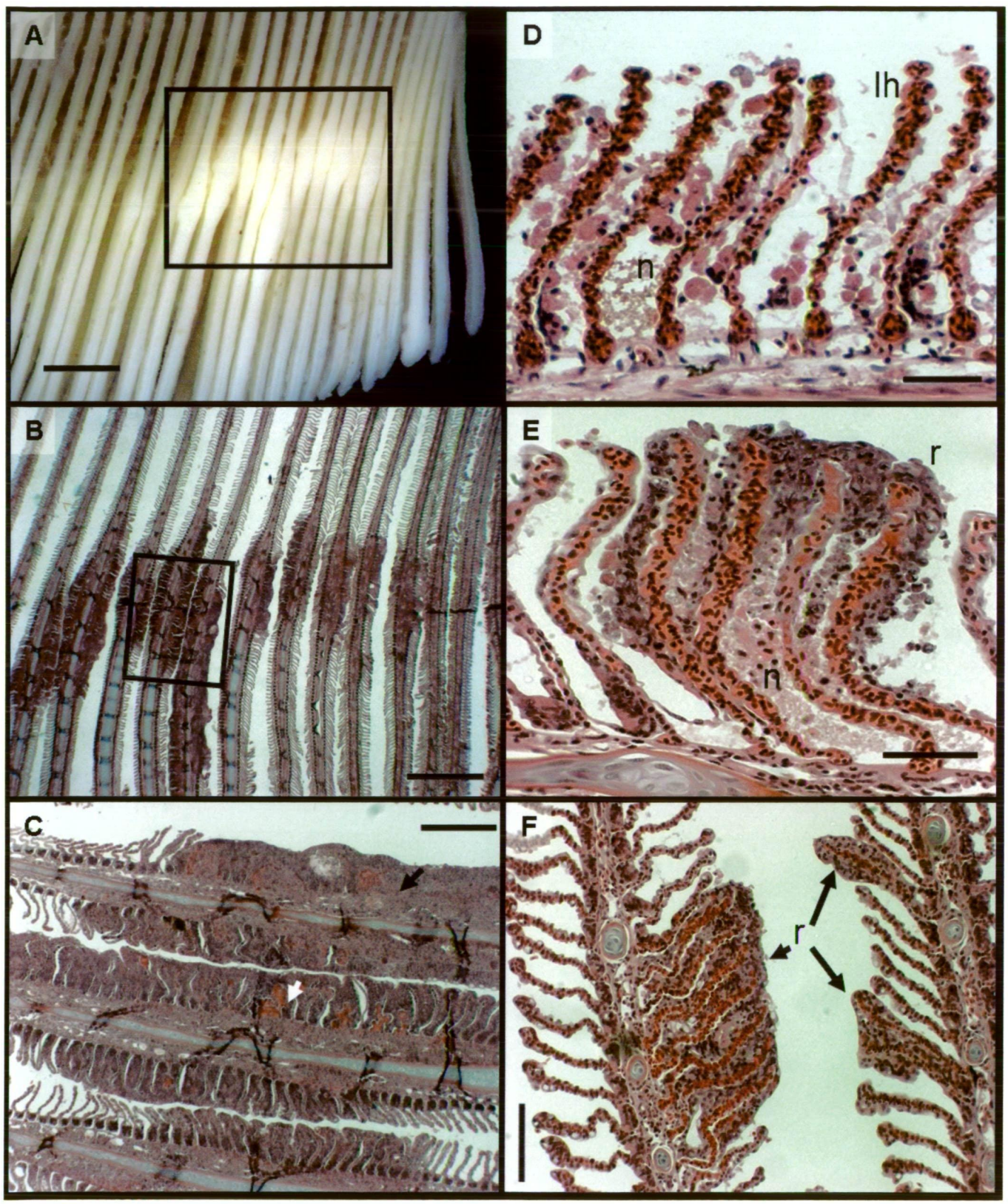


Figure 2 – legend over page.

Figure 2. A – Gross image of hemibranch clear of clinical AGD symptoms. White arrow indicates position of lesion below. B – Micro-stereoscopic view of small lesion not visible macroscopically in A (Bar = 200 µm). C - Corresponding histology from boxed area in B. An amoeba is seen adhered to an early lesion (arrow) (Bar = 100 µm). D - Gross image of hemibranch with advanced clinical AGD symptoms (white arrow). E – Micro-stereoscopic view of boxed area in D. Black arrows indicate sites of filamental tissue proliferation which extend deep into the interlamellar regions (Bar = 2 mm). F - Corresponding histology from protruding filamental tissue indicated by boxed area in E. Tissue is composed mainly of undifferentiated epithilial cells and mucous cells (black arrow). Multiple amoeba (a) are seen adhered to proliferative tissue (arrow) (Bar = 250 µm).

Figure 3. (Next page) A - Micro-stereoscopic view of a gross lesion displaying filamental proliferation (Bar = 1.5 mm). B – Histological section corresponding to boxed area in A showing lamellar fusion across multiple filaments (Bar = 1 mm). C – Higher magnification of boxed area in B. Note hyperplasia and loss of lamellae (black arrow); resolving thrombi (white arrow) (Bar = 250 µm). D, E & F – Re-epithelialization. D – necrosis (n) of lamellar epithelia and hyper-perfused lamellae
(lh) (Bar = 50 µm). E – Partial re-epithelialization (r) commencing distally upon secondary lamellae. Remains of necrotic residues evident between lamellae (n) (Bar = 50 µm). F – Lamellar fusion resulting in formation of small plaques due to re-epithelialization of necrotic damage (Bar = 100 µm).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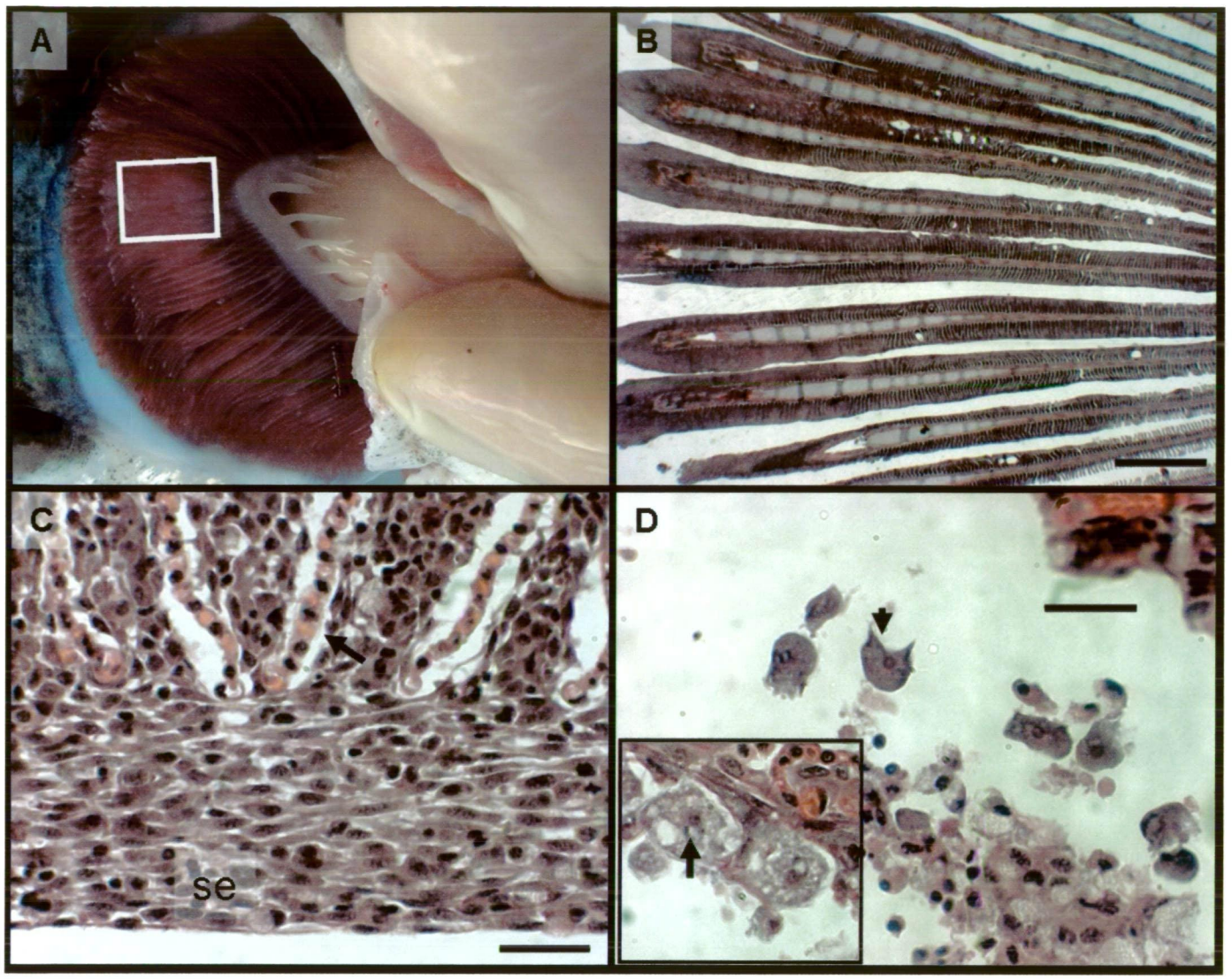

Figure 4. A - Large singular clinical lesions grossly evident upon a hemibranch. B – Severe hyperplasia of filaments corresponding to the boxed region in A, note lamellae and filamental fusion (Bar = 1 mm). C – Higher magnification of surface of hyperplastic lesion in B, note lamellae fusion and extensive stratification of epithelial cells extending well beyond the tips of secondary lamellae (black arrow) (Bar = 25 µm). D – unidentified protozoa (black arrow) amidst a clump of sloughed host cells. Inset provides comparison with *Neoparamoeba* sp. (not present upon pictured fish) showing nucleus and "parasome".

filaments were affected and the lesion was often spread across multiple filaments in a linearly aligned pattern (Figure 3A & 3B). Within these cases, regeneration of epithelium was evident in association with multi-focal regions of necrosis (Fig. 3D, 3E & 3F). Histological examination found no amoebae in association with such lesions thus the causative agent was not evident.

In contrast, a single case of grossly detectable gill change was associated with an unidentified protozoan. The associated gill pathology presented grossly as a single, large multi-filamental mucoid patch (Fig 4A) and micro-stereoscopically as swollen gill filaments (Fig. 4B). Histologically, the macroscopically affected region was markedly hyperplastic with fusion of secondary lamellae along the entire length of afflicted filaments (Fig. 4C). An unidentified protozoan was adhered to the protruding epithelia upon the leading edge of hyperplastic filaments. Cells were more abundant within sloughed tissue debris trapped between filaments (Fig 4D). Compared to cells of *Neoparamoeba* sp. (Fig 4D [inset]) the unidentified cells were smaller, acidophilic, finely vacuolated, lacked a parasome and displayed a limited degree of polymorphism.

3.4.1.3 Other pathology: Distal necrosis of single to multiple filaments was noted in three cases. The gross appearance was quite distinct compared to hyperplastic lesions as affected regions were markedly blanched often with an associated necrotic exudate (Fig. 5A). Coagulative and liquefactive necrosis, oedema and haemorrhage were histologically evident in affected filaments (Fig 5B). Sporadic populations of bacteria were also noted within necrotic tissues.

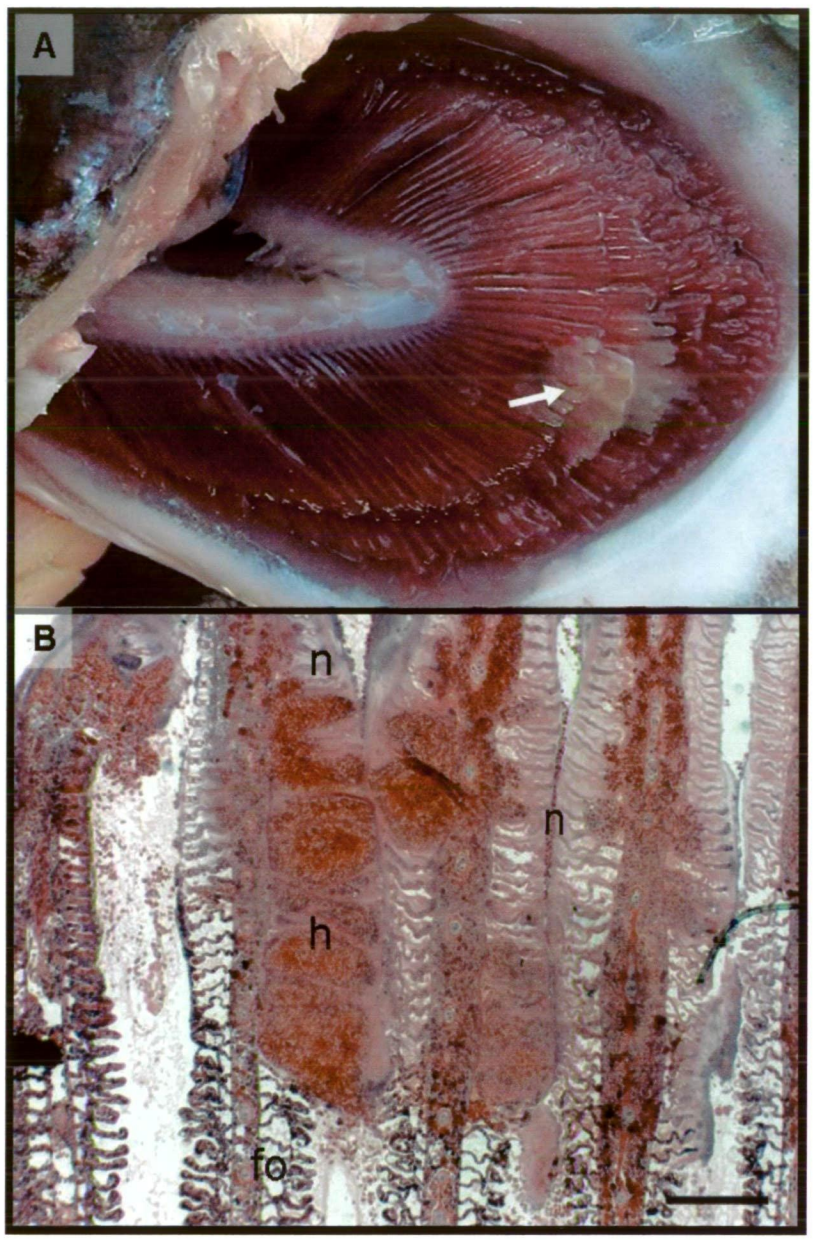

Figure 5. A – Distal lesion grossly evident upon three hemibranchs, note contras in appearance to Figure 2D (white arrow). B – Histology corresponding to gross lesion in A featuring prominent necrosis (n), haemorrhage (h) and filamental oedema (fo) (Bar = 250 µm).

3.4.2 Clinical-Histological Agreement

Agreement between gross and histological diagnosis was good (kappa = 0.74) for group B where comparison was made between clinical presentation of AGD signs upon any arch with the hemibranch selected for histology. Sensitivity and specificity of clinical symptoms were 82% and 91% respectively; positive and negative predictive values were 82% and 92% respectively where AGD prevalence amongst the sample population was 37%.

Agreement statistics were lower for Group C fish. Moderate agreement (kappa = 0.52) was observed between gross presentation of AGD signs upon all arches compared with the AGD diagnosis upon the hemibranch selected for histology. Sensitivity and specificity of clinical signs were 78% and 75% respectively; positive and negative predictive values were both 76% where prevalence was 47%. Agreement was higher when gross signs upon the hemibranch selected for histology were compared to histological diagnosis for the corresponding hemibranch (kappa = 0.60). Sensitivity-specificity was increased to 80%; positive and negative predictive values were 79% and 81% respectively at a prevalence of 47%.

Table 3. Observed reasons for disagreement between clinical signs and histological diagnosis for sample group C.

Gross * (any arch)	Corresponding Hemibranch *		Observed reason for disagreement (n = 25)
	Gross	Histology	
-	-	+	Small lesions (n=5), lesion obscured by arch location (n=1)
-	+	-	Non AGD lesion (n=2)
-	+	+	Lesion not detected - human error (n=2)
+	-	-	No lesions on hemibranch selected for histology (n=4)
+	-	+	Small Lesions on selected arch (n=3)
+	+	-	Lesion was not in section (n=5), Non AGD lesion (n=3)

*Indicates presence or absence of lesions

The level of agreement for Group C fish between presence/absence of gross lesions upon any arch, upon the hemibranch selected for histology and the histological AGD lesions upon the corresponding hemibranch was 74%. Further individual analysis of diagnostic disagreement (26%) identified several sources of potential disparity between analysis of macroscopic signs and histological diagnosis. Lesion size, cause and technical proficiency/methodology featured prominently and results are summarized in table 3.

3.5 Discussion

Micro-stereoscopic observations of gross lesions and subsequent histological examination of the corresponding tissues suggests that white mucoid patches are sites of epithelial hyperplasia resulting in fusion of multiple lamellae. These observations conclusively link macroscopic lesion observation with histological presentation of hyperplastic lesions. Within this study, the majority of such lesions were associated with *Neoparamoeba* sp. However, several factors must be considered before assuming that clinical AGD signs equate to the diagnosis of the disease itself and as such are outlined below.

3.5.1 Disease ontogeny

The developmental stage of disease, whether AGD or some other form of proliferative gill condition, will affect its detection at a macroscopic level. We have indicated that smaller lesions, affecting less than approximately 10 lamellae are clinically undetectable. Gross diagnosis of AGD, in terms of prevalence and pathological severity, has previously proven unreliable for less severe (light) cases (Clark & Nowak 1999; Zilberg *et al.* 2001). Additionally, *Neoparamoeba* sp. have previously

been isolated from turbot (*Scophthalmus maximus* L.) without gross evidence of disease (Dyková & Novoa 2001). Macroscopic lesions do not appear on salmon gills during the first two days of infection even though amoebae can be adhered to gill epithelia (Zilberg & Munday 2000; Adams& Nowak 2004a). The majority of AGD case agreement between clinical and histological lesions seen in this study occurred in moderate to advanced infection. This concurs with previous work where gross diagnosis was proven reliable in heavy cases of AGD (Clark & Nowak 1999).

3.5.2 Alternate causes of gross lesions

Although epithelial hyperplasia due to amoebic infection was the major cause of gill pathology described during this study, some fish displayed gross gill lesions not attributable to amoebic infection. This was evident in 26% of fish from groups A & C, sampled October 2002 – April 2003. Such lesions were mostly associated with fish that were recovering from focal and/or multi focal necrosis. The result of such damage was re-epithelialization of the damaged region, the extent of which influenced its clinical appearance. Indeed, reduction in observed agreement (kappa) between gross diagnosis and histological confirmation of AGD was apparent in groups (B & C) sampled a year apart during summer, from the same site. This result was largely a function of the aforementioned pathology and regenerative response of the affected sample population. Such a mechanism of repair or fortification is a major component of wound healing in fish (Roberts 1989) and is recognizable histologically as a non-AGD proliferative response. The initial cause of the aforementioned pathological response was tentatively assigned to moon jellyfish (*Aurelia aurita*) present during sampling of group C fish (Adams & Nowak 2004b). Similar observations of re-epithelialization have been described in clubbing and necrosis gill syndrome in Tasmania (Clark, Nowak, Handlinger, Munday & Percival 1997).

As previously mentioned, a single anomalous case of dramatic hyperplasia was detected in association with an unidentified protozoan. Unfortunately, identification was not possible as histological detection occurred some weeks after sample collection. As a result a complete diagnostic investigation was not undertaken. Gross signs, similar to that of AGD, were also observed for gill infestation with *Trichodina* sp. during February 2003 in the Huon estuary (Adams & Nowak, unpublished data). The observation of other pathogens being potentially responsible for grossly detectable hyperplastic gill change is the essential consideration and highlights the importance of histological examination for accurate AGD diagnosis.

3.5.3 Interpretation, sampling and histological methodology

A major source of disagreement between gross and histopathological diagnosis was attributed to sampling and histological technique (accounting for 80% of divergent cases in group C). Gross lesions were either not detected or occurred within a hemibranch not selected for histology. Therefore a fundamental sampling requirement should be excision and histological examination of the grossly affected hemibranch as opposed to routine removal of the second left hemibranch.

Where the appropriate hemibranch was histologically examined, the lesion or amoebae were occasionally absent within sectional plane. This problem would be particularly troublesome for inexperienced histologists. Zilberg *et. al.* (2001) described the occurrence of AGD like lesions with no amoebae present at low levels of exposure to the pathogen. Although advanced cases of AGD are more easily observed, we noted a reduced incidence of amoebic attachment to afflicted tissues in the deeper filamental regions where epithelial fortification (Adams & Nowak 2003) against the pathogen was markedly apparent. Similar observations were described for turbot (*Scophthalmus maximus* L.) (Dyková & Novoa 2001) when amoebae were not

always present in high numbers during the final phase of amoebic infection. For the aforementioned problems, serial sectioning is recommended to produce a reliably accurate diagnosis.

In two cases gross lesions were not detected upon examination of all arches but detected upon later image review of the hemibranch designated for histology. This suggests human error was to blame and may have arisen from a multitude of sources including data transfer, insufficient examination or inclement examination conditions (weather, lighting etc).

In this study and as previously reported (Clark & Nowak 1999), disparity predominantly occurred during diagnosis of light levels of infection. Clearly, as AGD progresses, lesions lengthen and spread across filaments (Zilberg & Munday 2000; Adams & Nowak 2003), gross visibility likewise increases as does their appearance upon multiple arches (Adams & Nowak, unpublished data). Therefore it is to be expected that the chance of histological detection and identification of gross lesions would be increased. Careful consideration and execution of sampling, histopathological processing and analysis are a fundamental requirement for inerrant diagnosis.

3.5.4 Conclusion

Gross assessment of AGD as a diagnostic tool provided moderate to good agreement with histological findings. However, estimation of AGD prevalence and/or severity, based on gross assessment alone, may be variably influenced by the stage of disease development, interference with other pathogens and assessor interpretation / experience. As such, AGD diagnosis based solely upon gross assessment is only acceptable as a farm monitoring tool. Research studies, where destructive sampling is not an option, should combine gross signs in conjunction with a test for pathogen

presence or antigen ie. IFAT or dot blot (Howard & Carson1993; Douglas-Helders, Carson, Howard & Nowak 2001). Disparity between gross and histopathological findings was mainly attributable to sampling technique during gill excision. Thus, at a diagnostic level, removal of grossly affected tissue and subsequent histological examination will improve diagnostic accuracy.

3.6 Acknowledgements

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CHAPTER 4 - Experimental amoebic gill disease (AGD) of Atlantic salmon (*Salmo salar* L.): further evidence for the primary pathogenic role of *Neoparamoeba* sp. (Page 1987)

M.B. Adams & B.F. Nowak

4.1 Abstract

Amoebic gill disease (AGD) has been attributed to infection by *Neoparamoeba* sp. The causal mechanisms for AGD lesion development and the primary pathogenic role of *Neoparamoeba* sp. require elucidation. Three groups of Atlantic salmon were exposed to viable gill isolated amoebae, to sonicated amoebae, or to seawater containing viable amoebae without direct contact to gill epithelia. Fish were removed 8 d post exposure and the gills assessed histologically for AGD. AGD occurred only when fish were exposed to viable trophozoites. Consequently, in an accompanying experiment, infection was evaluated histologically at 12 h, 24 h and 48 h post exposure in three groups of salmon, one group being mechanically injured 12 h prior to exposure. A progressive host response and significant increases ($P<0.001$) in the numbers of attached amoebae was apparent over the 48 h duration upon undamaged hemibranchs in both treatment groups. There were no significant differences to mucous cell populations. Attachment of *Neoparamoeba* sp. to damaged gill filaments was significantly reduced ($P<0.05$) by 48 h post exposure. These data further confirm

and describe the primary pathogenic role of *Neoparamoeba* sp. and the early host response in AGD. Presented also is preliminary evidence suggesting lesions resulting from physical gill damage are not preferentially colonized by *Neoparamoeba* sp.

4.2 Introduction

Amoebic gill disease (AGD) imparts a significant economic burden upon the Tasmanian salmon industry and is an emerging disease worldwide (reviewed by Munday, Zilberg & Findlay 2001). AGD is diagnosed grossly by the presence of raised, multi focal white mucoid patches upon the gills. AGD presents histologically as focal and multi-focal hyperplasia of the primary and secondary lamellae and formation of interlamellar vesicles with the presence of *Neoparamoeba* sp. (Roubal, Lester & Foster 1989; Munday, Lange, Foster, Lester & Handlinger 1993, Adams & Nowak 2001).

Although Koch's postulates have not been satisfied for this organism (Howard & Carson 1994), AGD is attributed to infection by *Neoparamoeba* sp. (Kent, Sawyer & Hedrick 1988; Roubal *et al,* 1989; Dyková, Figueras & Novoa 1995; Zilberg, Gross & Munday 2001; Adams & Nowak 2004). Attachment of singular *Neoparamoeba* sp. trophozoites to healthy gill epithelium has been described both experimentally and from the field (Zilberg & Munday 2000; Adams & Nowak 2003).

It has been suggested that *Neoparamoeba* sp. may have a secondary pathogenic role by colonizing pre/co-existing gill lesions (Nowak & Munday 1994; Nowak 2001; Zilberg & Munday 2000; Douglas-Helders, Handlinger, Carson & Nowak 2003). The causal mechanisms for initial AGD lesion formation and subsequent progression require elucidation.

Here we investigate firstly whether attachment of *Neoparamoeba* sp. is an essential requirement of lesion formation. We also describe the early stages (first 48 hours) of AGD lesion development and lastly investigate the ability of *Neoparamoeba* sp. to colonize pre-existing gill lesions induced by physical trauma.

4.3 Materials and Methods

4.3.1 Experiment 1

Atlantic salmon of 120 g (n=36), naïve to AGD, were acclimated to seawater (35 ppt) over 14 d. The fish were divided equally and transferred to four identical experimental recirculating systems each consisting of three 80 l containers (n = 3 fish per container) and an 80 l sump. One of the four systems was fitted with a 20 μm screened, cylindrical pre-pump filter and a post-pump 10, 5 and1 μm filter bank (Cuno® Pacific Pty Ltd, Blacktown, NSW, Australia). The pre-filter housing was vigorously aerated by an external air curtain. All four systems were filled with 1 μm filtered seawater (270 l per system) and a 22% daily exchange of fresh filtered seawater was undertaken for the duration of the experiment. Seawater was maintained at 19.2 °C, pH 8.2, dissolved oxygen 7.6 $mg.l^{-1}$, salinity 35 ppt and total ammonia-nitrogen below 0.2 $mg.l^{-1}$. *Neoparamoeba* sp. were isolated and quantified from the gills of infected salmon as described by Zilberg *et al.* (2001). Briefly, six salmon (approx. 100g) were selected from an ongoing amoebic infection maintained at the University of Tasmania. The fish were euthanized (clove oil 0.02% w/v), gills were excised and mucous scraped from the gills. The mucous was washed twice in sterile filtered seawater by centrifugation. A 100 μl sample of resuspended pellet was diluted with an equal volume of trypan blue (0.25% w/v) in sterile filtered seawater. Viable amoebae

were then counted from this sample using a haemocytometer. A further 30µl sample was dried (37°C) on a glass slide overnight, fixed in 100% methanol and air dried. Slides were then quenched of endogenous peroxidase (3.5% H_2O_2 in 100% methanol), washed in PBS and treated with primary antiserum (rabbit anti *Neoparamoeba* sp.– Howard and Carson 1994, diluted 1:500 in 0.1% BSA) for 30 min at 37°C. The procedure was completed using a peroxidase based kit (Vector laboratories, Sydney, NSW, Australia) following the manufacturers instructions and counterstaining for 20 seconds in haematoxylin. The percentage of *Neoparamoeba* sp. in the isolated gill material was determined from the number of immuno-stained cells divided by the combined number of immuno & haematoxylin stained cells x 100. The isolates were divided equally by volume into three aliquots, each aliquot capable of delivering a final concentration of approximately 2500 cells.l^{-1} when introduced to the appropriate system. The first and second aliquots were added to the sumps of either the modified (contact prevention) system or an unmodified system (positive control). The filter assemblies (as described) were deployed so as to prevent amoebae from leaving the sump or entering the fish containers. The remaining aliquot was sonicated until all cellular material was lysed (Bryant, Lester & Whittington 1995) and added to another unmodified system. No amoebae were added to the remaining system (negative control). The addition of amoebae aliquots was repeated at day 4 post-exposure to supplement potential losses of amoebae due to water exchange. Moribund fish were removed from the system and sampled as described below. At day 8 post exposure, the remaining fish were removed from each system and terminally anaesthetised by overdose with clove oil (0.02% w/v). The gills were grossly examined for AGD, then excised and placed in seawater Davidson's fixative for 12 h. After fixation, the gills were dehydrated in a graded ethanol series, embedded in paraffin, sectioned at 5 µm

and stained with haematoxylin and eosin. Filament numbers on each arch were counted for each section. A filament was only counted when the central venous sinus was visible in at least 2/3 of a filament. For each filament assessed, any lesions were noted and duly recorded.

4.3.2 Experiment 2

Atlantic salmon of 90g (n=27), naïve to AGD, were acclimated to seawater (35ppt) over 14 d. The fish were divided equally and transferred to three identical experimental recirculating systems as described in experiment 1. The systems were filled with 1μm filtered seawater (270 l) and a 50% exchange of fresh filtered seawater was introduced to the system immediately prior to addition of gill isolated amoebae. Seawater was maintained at 16.2 °C, pH 8.2, dissolved oxygen 7.9 $mg.l^{-1}$, salinity 35 ppt and total ammonia-nitrogen below 0.12 $mg.l^{-1}$. Group A were exposed to *Neoparamoeba* sp. (2600 $cells.l^{-1}$) isolated and quantified from the gills of infected salmon as described by Zilberg *et al.* (2001). Group B were exposed to *Neoparamoeba* sp. and had the first and second left anterior hemibranchs abraded with a sterile cotton swab until haemorrhage was evident. Gill isolated amoebae were introduced to the systems 12 h after the gills had been abraded. Salmon in group C (negative control) had their gills abraded as described above but no amoebae were added to the system. Each group was sampled (n=3) at 12, 24 and 48 hours post-exposure. After terminal anaesthesia (clove oil - 0.02% w/v) both the left and right, first and second gill arches were excised, gently rinsed in 0.22 μm filtered seawater and fixed in seawater Davidson's fixative for 1 h. The arches were then transferred to 70% ethanol for 24 h, dehydrated in a graded ethanol series, embedded in paraffin and sectioned at 5 μm. Two sections from each anterior hemibranch were stained using haematoxylin and eosin (H&E). A further two sections were similarly selected and

stained using periodic acid -Schiff-alcian blue technique (AB-PAS [pH 2.5]) as described by Adams & Nowak (2003b). The numbers of attached amoebae per filament were then counted microscopically at 200x magnification on AB-PAS stained sections. Mucous cells were quantified by semi quantitative digital image analysis as described by Adams & Nowak (2003b). General morphological and pathological gill observations were made from H&E stained sections at 20 – 1000x magnification.

Mucous cell populations and amoebae attachment data (un-abraded arches) were analysed by two way analysis of variance (ANOVA) with treatment (fixed, orthogonal, 3 levels) and time (fixed, orthogonal, 3 levels) as factors. Tukeys HSD was used for means comparisons where assumptions of normality (Shapiro-Wilk test) and homogeneity (Levene's test) were met. A paired sample students T test was used to determine differences in amoebae attachment between abraded and un-abraded sides for each treatment. A P value of $P<0.05$ was adopted for rejection of the null hypothesis. SPSS® (version 10.0, SPSS Science) and Sigma Plot (version 6.0, SPSS Science) were used for data analysis and presentation.

4.4 Results

4.4.1 Experiment 1

The experimental period was terminated at day 8 post-exposure however some fish were prematurely sampled due to morbidity or mortality (Table 1). Morbidity / mortality was attributed to severe erosive dermatitis of unknown aetiology (diagnosis provided by Animal Health Laboratory, Department of Primary Industries, Water and Environment, Launceston, Tasmania, Australia). All fish sampled at day 8 post exposure, displayed variable amounts of ulcerative dermatitis along the flanks, fins and mouth. No gill pathology was noted within prematurely sampled fish other than lesions associated with AGD.

Table 1. Sampling details and AGD diagnosis for experiment 1.

System	Mortality / Morbidity	Day Removed	Day 8 Remainder	No. AGD +ve fish *
Positive	3	3,4,6	6	9
Negative	3	5,6,6	6	0
Sonication	2	6,6	7	0
Contact Prevention	5	2,3,4,6,7	4	0

* Diagnosis on histological basis

No AGD gill pathology was apparent either grossly or histologically in either negative control fish (Fig. 1A) or systems where isolates had been sonicated or prevented from fish contact by a filter barrier. There were no other notable signs of gill pathology. All positive control fish (n = 9) were histologically diagnosed with AGD (mean = 35.1% SE ± 5.2 AGD lesion affected filaments). AGD lesions were characteristically hyperplastic with leucocyte infiltration within the central venous sinus and upon lesion surfaces (Fig. 1B). Large numbers of amoebae consistent with the morphology of *Neoparamoeba* sp. (presence of nucleus and endosymbiont) were noted in association with hyperplastic lesions.

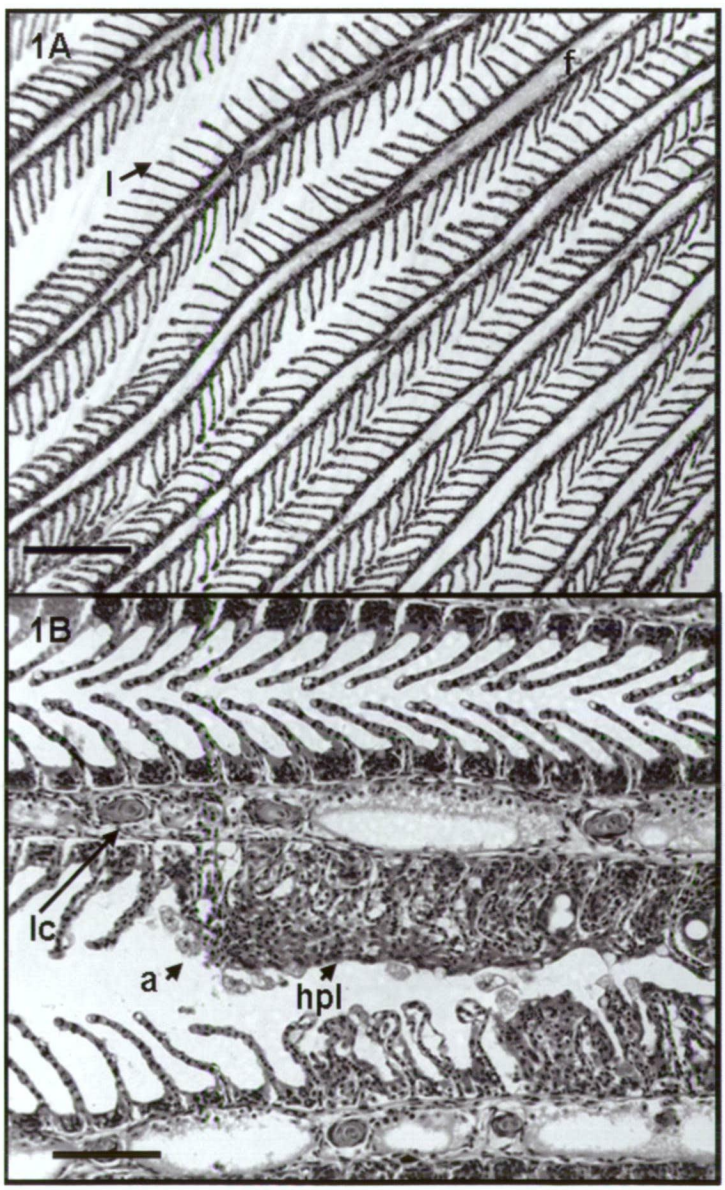

Figure 1. A - Gill filaments (f) from negative control fish showing normal secondary lamellae (l). Picture is representative of all treatments except positive control (Bar = 250µm). B – Hyperplastic AGD lesion (hpl) present upon filaments of a positive control fish. Lesion shows amoebae sp. (a) and leucocytic infiltration (lc) of the central venous sinus (Bar = 100 µm).

4.3.2 Experiment 2

For Group A (infection only), amoebae attachment was observed at 12 h post-exposure and the number of gill attached amoebae had increased significantly ($P<0.0011$) by 48 h post exposure (Fig 2).

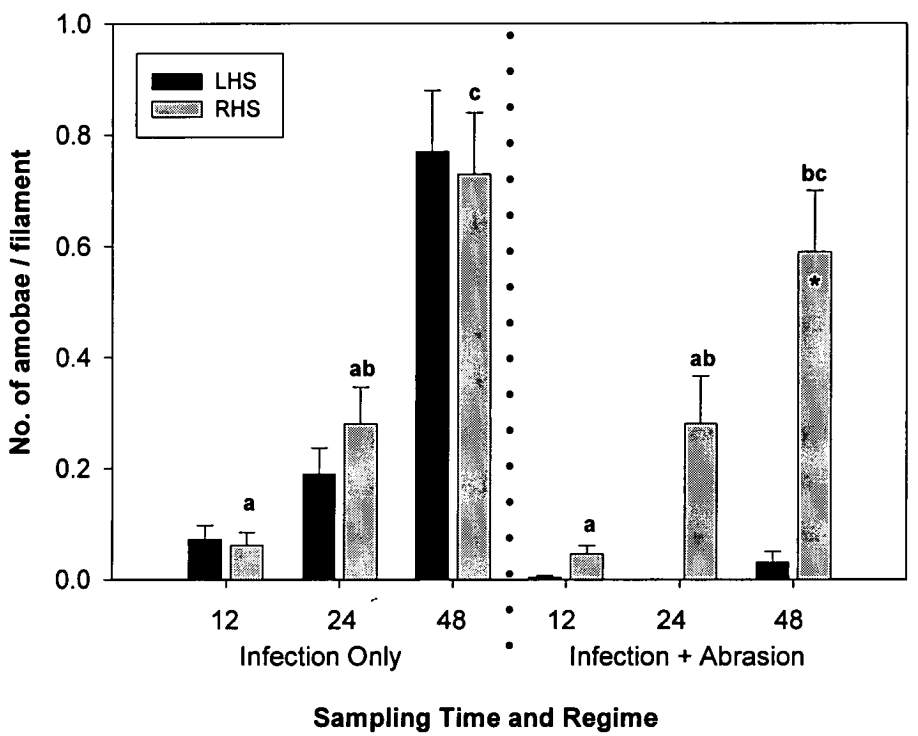

Figure 2. Mean number of amoebae attached per filament over 48 hours for fish infected with *Neoparamoeba* sp. only (Group A) and for fish infected and gill abraded (Group B LHS = abraded hemibranchs, RHS = normal hemibranchs). Values are means ± SE. Different letters indicate significant differences over time. Asterix indicates significant differences between left and right hand side arches.

At 12 h post exposure attachment was by singular amoebae to healthy gill epithelial tissue (Fig. 3A & 3B). Adhered amoebae resembled *Neoparamoeba* sp. as indicated by the presence of a nucleus and endosymbiont (Fig. 3B). Attached amoebae were generally found at the base of secondary lamellae. There were local alterations to host epithelium immediately juxtaposed to amoebae including desquamation and occasional oedema of epithelial cells. Leucocytes were sometimes seen within the central venous sinus (CVS) in regions apposed to amoeba attachment. At 24 h post exposure lamellae fusion in response to attached trophozoites was noted. Localized tissue changes within these early lesions included more pronounced epithelial desquamation and presence of leucocytes within the CVS. By 48 h post-exposure (Fig. 3C & 3D) lamellae fusion affected multiple filaments with many amoebae adhered to these regions. There was also infiltration of leucocytes via the CVS to fused lamellae with macrophages seen apically upon lesions. Lamellae fusion was facilitated by recruitment of undifferentiated epithelial cells. No gross pathology was apparent for Group A during sampling.

Abraded gill filaments (Groups B & C), at 12 h post-exposure, showed oedema, spongiosis, multiple aneurysms, hemorrhaging and leucocyte infiltration (Fig. 3E). At 24 h post-exposure damaged tissue was as described but with increased leucocyte, mucous cell and undifferentiated epithelial cell infiltration/differentiation. By 48 hours (Fig. 3F) oedema had noticeably decreased and lamellae were substantially fused with undifferentiated epithelial cells and hypertrophic mucous cells. For Group B, attachment of amoebae and consequent host response in un-abraded filaments was as described for group A. Significant increases ($P<0.05$) in amoebae attachment was apparent between un-abraded filaments at 12 and 48 h post-exposure. Amoebae attachment was significantly ($P<0.05$) less on abraded filaments

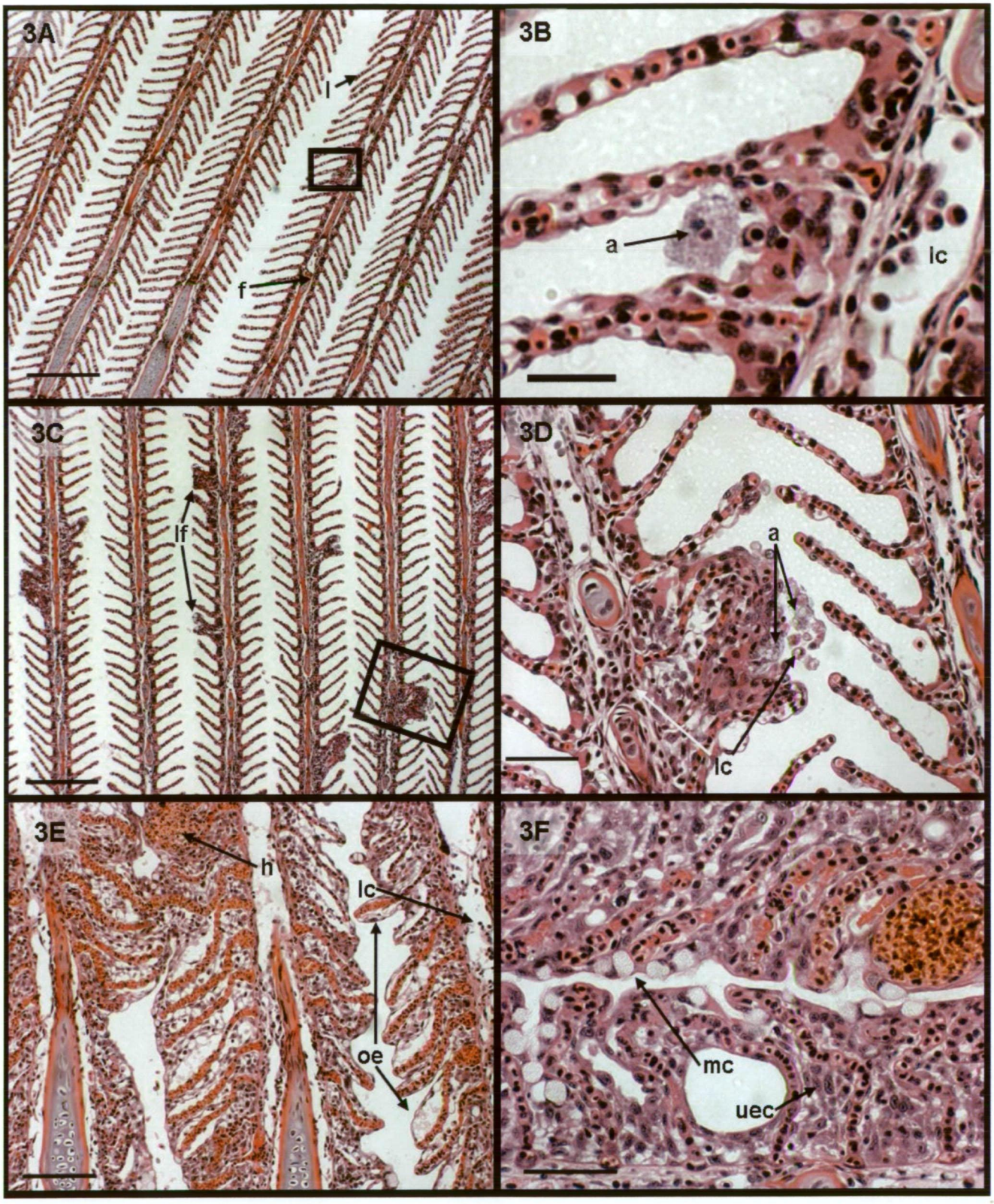

Figure 3 – legend next page.

Figure 3. A – Gill filaments (f) from Atlantic salmon with normal secondary lamellae (l) at 12 h post exposure to *Neoparamoeba* sp. gill isolates. Inset border corresponds to following plate. B – Higher magnification of highlighted area in 3A. Note attachment of *Neoparamoeba* sp. with nucleus and endosymbiont (a) to base of secondary lamellae and discrete epithelial swelling in juxtaposed regions and leucocytic infiltration (lc) within the central venous sinus. C – Gills at 48 h post exposure showing multiple lamellar fusion events (lf). D - Higher magnification of highlighted area in 3C. Note attached amoebae (a) and pronounced leucocytic (lc) infiltration of the central venous sinus and appearance at the lesion surface. E & F – Filaments of fish abraded but not infected at 12 & 48 hr respectively. Note progression of hemorrhagic (h), oedematus (oe) filaments and secondary lamellae with leucocytic infiltration (lc) at 12 h (E) to a fortified arrangement with many mucous cells (mc) and lamellae fused with undifferentiated epithelial cells (uec) at 48 h (F) (Bars = 25 µm for B; 50 µm for D & F; 100 µm for E; 250 µm for A & C).

by 48 h post-exposure (Fig 2.). Group C (negative control) were negative for AGD as no alterations to un-abraded tissue were evident over the duration of the experiment. There were no significant differences between treatments in the number of mucous cells on un-abraded gill filaments. No gross evidence of AGD was apparent upon un-abraded hemibranchs where as abraded hemibranchs displayed swollen, grey/white haemorrhagic filaments.

4.5 Discussion

During experiment 1, it was noted that attachment of viable *Neoparamoeba* trophozoites to gill epithelium was the only treatment leading to subsequent lesion development. This is consistent with other lab and field studies implicating *Neoparamoeba* sp. as the primary pathogen for AGD (Kent *et al.* 1988; Roubal *et al,* 1989; Dykova *et al.* 1995; Zilberg & Munday 2000; Zilberg *et al,* 2001; Adams & Nowak 2003b). Although fish were exposed to amoebic lysate (sonication treatment) and possibly extra-cellular products (ECP) (sonication & contact prevention treatment) a hyperplastic reaction was not evident. The possibility that amoebic toxins may play a role in AGD (Powell, Forster & Nowak 2002) was not perceptible in this study on the basis of lesion inducement. However the likelihood that a more discrete role may be played by amoebic ECPs at the host pathogen interface cannot be excluded. Other pathogenic amoeba species are known to produce ECPs as part of their infective processes. *Acanthamoeba* and *Entamoeba histolytica* produce an assortment of proteases responsible for host cell cytolysis, phagocytosis and apoptosis induction (Niederkorn, Alizadeh, Leher and McCulley 1999; Espinosa-Cantellano & Martinez-Palomo 2000). It is likely that any ECP cytopathic effects (within sonicated and contact prevention treatments) were truncated by dilution. However, the experimental rationale was to examine lesion inducement at an exposure level approximating a commercial culture situation.

Mortality observed during experiment 1, ascribed to a dermal ulcerative infection, did not influence the outcome of the experiment. Mortality was apparent in all treatment groups and there was no histopathological evidence of gill damage other than lesions consistent with AGD.

Recent field observations lead to a formative lesion development model *in situ* (Adams & Nowak 2003b) which described lesion progression from initial trophozoite attachment to advanced hyperplasia. The initial onset and timing of lesion induction therefore required further elucidation. Consequently, results from Experiment 2 described the initial stages of AGD progression following adherence of *Neoparamoeba* sp. to gill epithelia. It was clear that attachment of trophozoites to the lamellar epithelium induced a progressive host response. Initial localized lamella epithelial changes, subsequent lamellar fusion and leucocyte migration were increasingly apparent over time. Observations from the current study concur with lesion formation described in the field (Adams & Nowak 2003). Presence of greater trophozoite numbers in association with developing lesions (in this study) suggests that proliferation occurs after trophozoite attachment. In the field, trophozoites of *Neoparamoeba* sp. are consistently associated with hyperplastic lesions upon the gill filaments (Munday *et al*, 2001). The surfaces of smaller lesions (5-10 fused secondary lamellae) are often blanketed by adhered trophozoites. Upon larger AGD lesions however, trophozoites are often concentrated at lesion margins (Adams & Nowak 2003b). Experimentally, AGD gross gill lesion numbers increase simultaneously with increasing numbers of trophozoites in the water column (Zilberg *et al*, 2001; Morrison 2003 unpublished data). We suggest lesion development is initiated by attachment of *Neoparamoeba* sp. trophozoites. Subsequent lesion progression is then dependent upon proliferation and migration of amoebae along filaments.

In this study, mucous cell populations were unchanged over the initial 48 hours of infection. Previous studies have indicated increases in gill mucous cell populations concurrent with AGD progression over a period of weeks (Nowak & Munday 1994; Zilberg and Munday 2000; Adams & Nowak 2003b; Roberts & Powell

2003). Although no increase in the number of mucous cells was apparent, this outcome does not preclude the possibility that mucus production and/or secretion is altered during initial infection.

Neoparamoeba sp. trophozoites were clearly able to adhere to normal gill epithelium but largely unable to attach to proliferative epithelial tissue generated during repair from epithelial abrasion. This observation suggests that pre-existing lesions may not pre-dispose gill epithelia to colonization by *Neoparamoeba* sp. as previously considered (Nowak & Munday 1994; Zilberg & Munday 2000; Nowak 2001; Douglas-Helders, Handlinger, Carson & Nowak 2003). However, a number of factors may have contributed to the observed lower level of trophozoite adherence to damaged lesions. Gills were abraded 12 h previous to introduction of gill isolated amoebae allowing sufficient time for healing processes to commence. Initial leucocytic infiltration and progressive activation/release of associated non specific immune mediators may have hindered trophozoite attachment. The ensuing repair process of epithelial hyperplasia and stratification accompanied by mucous cell recruitment to these regions may have had a similar inhibitory effect consistent with other ectoparasitic infections (Urawa 1992; Buchmann & Bresciani 1998; Buchmann & Bresciani 1999; Buchmann 1999). Attachment of trophozoites to squamous regions of reparative lesions may also have been hindered by physical dynamics. Normal filament regions possess relatively "sheltered" interstitial spaces between secondary lamellae. Lamellar fusion reduces the sheltered spaces and exposes amoebae to higher water velocities, a factor that may also influence lesion distribution (Adams & Nowak 2001).

In contrast to this study, *Acanthamoeba castellanii* binding expression was increased when normal and damaged rabbit corneal epithelia were damaged and

immediately exposed to trophozoites *in vitro* (Paliakkara, Cao & Panjwani 1998). Conceivably, gill trauma occurring simultaneously with AGD may have produced a differing outcome as could other forms of gill trauma possibly irrespective of timing. Alternatively, it is possible that the experimental duration was not sufficient to fully appraise the long-term effects of gill damage upon the infection's dynamics. Field results however found no interaction between AGD and post-transfer lesions, clubbing and necrosis gill syndrome or coelenterate insult at a histopathological level (Clark, Nowak, Handlinger, Munday & Percival 1997; Adams & Nowak 2003a; Adams & Nowak 2003b).

This study has implicated *Neoparamoeba* sp. as the primary pathogen of AGD in agreement with previous findings by other workers (Kent *et al*, 1988; Roubal *et al*, 1989; Munday, Foster, Roubal & Lester 1990; Dykova *et al*, 1995; Dykova *et al*, 1998; Zilberg & Munday 2000; Adams & Nowak 2003b). However, fulfilment of Koch's postulates for this pathogen has proven difficult. Previous experimental infection attempts with Coho salmon (*Oncorhynchus kisutch*), Atlantic salmon and Rainbow trout (*Oncorhynchus mykiss*) by cultured strains of *Neoparamoeba* sp. have failed to reproduce disease (Kent *et al*, 1988; Findlay 2000; Howard 2001). It is unclear why infectivity is lost following isolation and culture. During culture, amoebae are removed from the nutrient rich environment of the gills and maintained under monoaxenic conditions. It is possible that virulence factors are down regulated as demonstrated for *Vibrio anguillarum*. In that study, significant increases in protease production and membrane protien expression were observed when cultured in artificial media containing salmon mucous (Garcia, Otto, Kjelleberg & Nelson 1997; Denkin & Nelson 1999). Virulence reduction following monoaxenic culture or culture in artificial media has been observed for *Paramoeba invadens* and *Entamoeba*

histolytica. Virulence was restored for these species when passaged through the target host (Jellett and Scheibling 1988; Padilla-Vaca, Ankri, Bracha, Koole & Mirelman 1999). It is possible that involvement of a cryptic pathogen (eg bacterium or virus) is required during infection of salmon with *Neoparamoeba* sp. However there is no evidence indicative of such involvement based on TEM observations of lesions produced by AGD (Roubal *et al*, 1989; Dykova *et al*, 1998). The designation of primary pathogen has been ascribed to organisms such as *Mycobacterium leprae* and *Treponema pallidum*, the agents of leprosy and syphilis in humans, without fulfilment of Koch's postulates (Kreier 2002). *Neoparamoeba* sp. as the agent of AGD, satisfies criteria described by Evans (1976) for acute and chronic diseases of diverse aetiologies which were formulated with an appreciation of limitations to Koch's postulates (Casadevall & Pirofski 1999). Clearly, further research is required to investigate the primary mechanisms for attachment of *Neoparamoeba* sp. to gill epithelia. Elucidation of such mechanisms could provide an understanding of virulence factors required for infection, ultimately assisting with future AGD mitigation strategies of cultured fish species.

4.6 Acknowledgements

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CHAPTER 5 - Amoebic gill disease (AGD): Sequential pathology in cultured Atlantic salmon (*Salmo salar* L.).

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5.1 Abstract

Amoebic gill disease (AGD) affects the marine culture phase of Atlantic salmon (*Salmo salar* L.) in Tasmania. Here, we describe histopathological observations of AGD from smolts, sampled weekly, following transfer to estuarine/marine sites. AGD was initially detected histologically at week 13 post-transfer while gross signs were not observed prior to week 14 post-transfer. Significant increases ($P<0.001$) in the proportion of affected gill filaments occurred at weeks 18 & 19 post-transfer coinciding with the cessation of a halocline and increased water temperature at the cage sites. The progression of AGD histopathology, during the sampling period, was characterized by three phases. (1) Primary attachment/interaction associated with extremely localized host cellular alterations, juxtaposed to amoebae, including epithelial desquamation and oedema. (2) Innate immune response activation and initial focal hyperplasia of undifferentiated epithelial cells. (3) Finally, lesion expansion, squamation-stratification of epithelia at lesion surfaces and variable recruitment of mucous cells to these regions. A pattern of preferential colonization of amoebae at lesion margins was apparent during stage 3 of disease development.

Together, these data suggest that AGD progression was linked to retraction of the estuarine halocline and increases in water temperature. The host response to gill infection with *Neoparamoeba* sp. is characterized by a focal fortification strategy concurrent with a migration of immuno-regulatory cells to lesion affected regions.

5.2 Introduction

Amoebic gill disease (AGD) remains the most significant health problem affecting the production of Atlantic salmon in Tasmania and has had variable impact upon other cultured marine fish species elsewhere (reviewed by Munday, Zilberg & Findlay 2001). Histopathology of AGD, under culture conditions, has previously been described for a number of species including Atlantic salmon (*Salmo salar L.*) (Roubal, Lester & Foster 1989; Adams & Nowak 2001), rainbow trout *Oncorhynchus mykiss* (Walbaum) (Munday, Foster, Roubal & Lester 1990), coho salmon *Oncorhynchus kisutch* (Walbaum) (Kent, Sawyer & Hedrick 1988) and turbot *Scophthalmus maximus* (L.) (Dyková, Figueras & Novoa 1995; Dyková, Figueras, Novoa & Casal 1998). The etiological agent of AGD is *Neoparamoeba* sp., a free living amphizoic amoeba of marine waters. Gross pathology, in infected fish, is characterized by raised, multifocal white mucoid patches upon the gills. AGD presents histologically as epithelial hyperplasia of the primary and secondary lamellae (Roubal *et* al.1989; Munday, Lange, Foster, Lester & Handlinger 1993). Amoebae are often seen adhered to or in close proximity to lesions and sometimes entrapped within interlamellar vesicles or "cysts" (Kent *et al.* 1988; Munday *et al.* 1993; Dyková *et al.* 1995; Parsons, Nowak, Fisk and Powell 2001; Adams & Nowak 2001). Freshwater bathing

of AGD affected fish remains the only effective treatment in managing outbreaks (Munday *et al.* 2001), a practice that is expensive and production limiting.

The major histopathological features of AGD are well documented (Munday *et al.* 2001). However, few authors have attempted to describe the pathogenesis of infection with *Neoparamoeba* sp. upon the gills. At the onset of experimentally induced AGD, trophozoites were seen adhered to normal gill epithelium after one day of co-habitation with infected fish and a progressively worsening condition was described over 28 days of infection (Zilberg & Munday 2000). As Koch's postulates are yet to be fulfilled, some conjecture has arisen regarding the infective process during development of AGD. Nowak & Munday (1994) suggested the formation of nodules and plaques, appearing on salmon gills after transfer to marine conditions, may be predisposing to the onset of AGD. Gill trauma from blooms of potentially harmful algae and jellyfish or other physical insults are considered potential risk factors for AGD development (Nowak 2001, Munday *et al.* 2001). It remains unclear whether *Neoparamoeba* sp.is able to participate as a secondary pathogen. An understanding of the temporal development of AGD under culture conditions is a fundamental prerequisite for future research.

This study histologically evaluated the progressive nature of AGD and overall gill health status after transfer to estuarine/marine conditions until initial AGD treatment. The results have been integrated with environmental records for additional consideration of aetiologically and environmentally related aspects of AGD.

5.3 Materials and methods

5.3.1 Sampling Regime

Commercially reared Atlantic salmon were transferred from two freshwater hatcheries to Pillings Bay in the Huon Estuary, Southern Tasmania in early October 2001 (Fig.1). Fish were initially sampled during transfer to the estuarine site (2 cages, n = 10 / cage) and were sampled identically on a weekly basis thereafter. Immediately following collection of fish at week 12 post-transfer, the cages were towed further toward the mouth of the estuary (Fig. 1). Sampling was completed after 19 weeks immediately preceding initial freshwater bath treatment of fish for AGD.

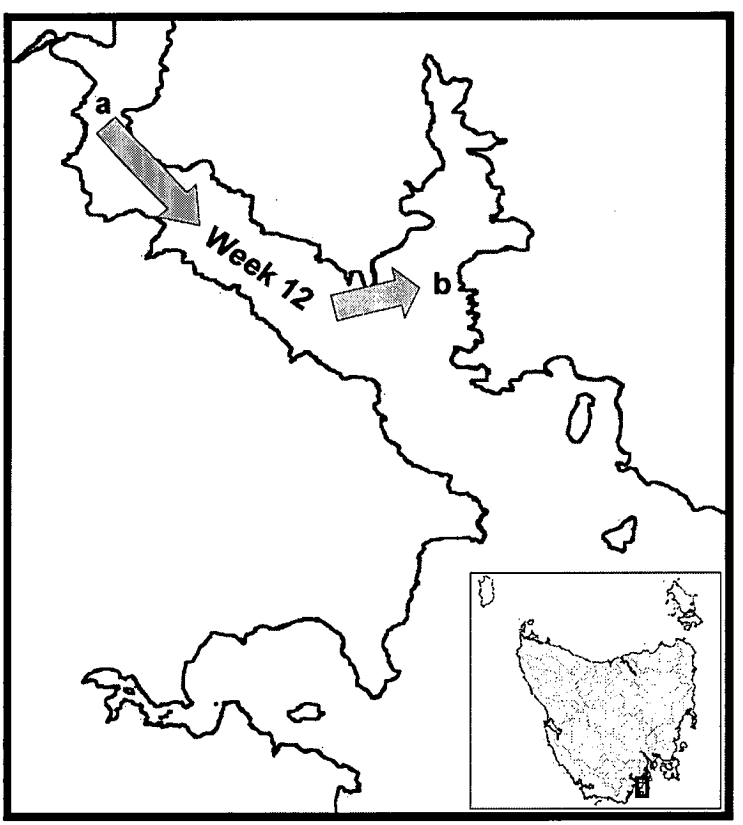


Figure 1. Map of the Huon Estuary. Block arrows indicate the transfer of cages from Pillings Bay (a) to Deep Bay (b) after completion of week 12 sampling. Inset picture – map of Tasmania depicting geographical locality of the Huon Estuary.

Fish were collected from each cage by box netting and transferred to two oxygenated holding bins (80L). Each fish was individually removed from the holding bin and terminally anaesthetized in clove oil (0.02% w/v). The gills were scored for gross signs of AGD (Table 1) and any abnormal observations upon the gills or upon each fish were noted. Fish weights and lengths were recorded and the gills were excised for histology.

Table 1. Scoring scheme for gross signs of AGD.

Infection Level	Score	Number of affected hemibranchs
Clear	0	0
Light	1	1 - 4
Moderate	2	5 - 10
Heavy	3	10 - 16

5.3.2 Histopathology

After excision the gills were placed in Davidson's seawater fixative and after 24 hours were transferred to 70% ethanol. The 2nd left gill arch was removed, dehydrated, embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and eosin (H&E). The sections were viewed under a light microscope (Olympus, Hamburg, Germany) at 400x magnification. Filament numbers on each arch were counted for each section. A filament was only counted when the central venous sinus was visible in at least 2/3 of a filament. For each filament assessed, any lesions were noted and duly recorded.

5.3.3 Histochemistry

The 2nd left gill arches, fixed and processed as above, were stained using periodic acid -Schiff-alcian blue (PAS-AB) technique (pH 2.5) modified from Bancroft & Cook (1994). Briefly, sections were de-waxed and re-hydrated, immersed in alcian blue (AB) (Sigma, Castle Hill, Sydney, Australia)) and microwaved on high (45 sec), left to stand (5 min) and washed in deionized water (DI) (1 min). Sections were then immersed in 1% periodic acid (ICN Biomedicals, Ohio, USA) (10 min), washed (1 min, DI) and immersed in Schiff's reagent (BDH Laboratory Supplies, England), microwaved on high (3 x 15 sec) and left to stand (5 min). Finally sections were washed in running tap water (15 min), counterstained with haematoxylin (15 sec), dehydrated, cleared and mounted.

5.3.4 Immunohistochemistry

Chloride cells, proliferative cells and apoptotic cells were targeted for immunohistochemical identification using methods modified from Ortego, Hawkins, Krol & Walker (1996), Dang, Lock, Flik & Wendelaar Bonga (2000) and Chiu, Ngan, Khoo & Cheung (2001).

Chloride cell staining used gill tissue fixed as previously described. For proliferative and apoptotic cellular staining, the 3rd left gill arch was removed during initial gill excision and fixed in 10% neutral buffered formalin (24 h). Following dehydration and embedding, tissue sections were cut (5 μm), mounted on Vectabond™ (Vector Laboratories, Burlingame, CA, USA) coated slides and processed according to the avidin-biotin-peroxidase (ABC) technique (Kiernan 1999) as follows. To facilitate heat induced epitope retrieval (HIER), sections were dewaxed, rehydrated and placed into citrate buffer solution (pH 6) and microwaved on high (12 min) then allowed to stand (20 min). After a brief rinse in deionized H_2O

(DH_2O), sections were blocked for endogenous peroxidase (3% H_2O_2 – 20 min), washed (PBS – 3 x 1 min) and incubated with normal horse serum (20 min) (Vector Laboratories, Burlingame, CA) to block non specific binding sites. Sections were then blotted dry and incubated in a humid chamber (37.5°C, 1 h) with a mouse monoclonal antibody to Na^+/K^+- ATPase (1:100, IgGά5, Developmental Studies Hybridoma Bank, Department of Biological Sciences, The University of Iowa, USA) or to proliferating cell nuclear antigen (PCNA) (1:700, Clone PC10 mouse IgG_{2a}, Dako Corp., Carpinteria, CA, USA) or M30 Cytodeath (1:100, Clone M30 mouse IgG_{2b}, Roche Diagnostics, Mannheim, Germany). The gills served as internal positive controls for Na^+/K^+- ATPase and PCNA staining. For apoptosis, a section of human breast carcinoma (Dako Corp., Carpinteria, CA, USA) was used as a positive control. Sections were washed then incubated (30 min, 37.5°C) with biotinylated horse anti mouse IgG (ABC kit, Vector Laboratories)(1:500 PCNA, 1:200 Na^+/K^+- ATPase & M30 Cytodeath), washed again, then incubated (30 min, 20°C) with peroxidase conjugated streptavidin (1:200 ABC kit, Vector Laboratories). After a final washing step, slides were flooded with 3-3'-diaminobenzidine (DAB) in peroxide buffer (2 min) (Roche Diagnostics) then rinsed in DH_2O (30 s), counterstained with Mayer's haematoxylin (5 s), rinsed, differentiated in PBS (30 s), dehydrated, cleared and mounted. Omission of the primary antisera served as a negative control.

5.3.5 Digital Image Analysis

Histochemically and immunohistochemically stained sections were analysed and quantified using semi-automated digital image analysis. The methodology was modified from Menter, Hoque, Motiwala, Sahin, Sniege, Liberman & Lippman (2001) and Graham, Bryant, Kirkpatrick & Moltrup (1994).

The rationale for use of digital image analysis in this study was to (1) provide a means of quantification to minimize intra/inter-observer bias or variability, (2) generate a larger less labile data set and (3) perform an efficient and less labour intensive method of data collection.

Grayscale images (150 dots per inch [DPI]) were captured on every second to third filament of each gill section. Images were captured from the proximal half of each correctly orientated filament (as previously described). A Leica DC300F digital camera (Leica Microsystems, Wetzlar, Germany) C-mounted to a light microscope (Olympus, Hamburg, Germany) was used for image capture. Image analysis was performed using Image Tool version 3.0 (University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA). Winbatch (Wilson WindowWare Inc., Seattle, WA, USA) was used to automate image analysis procedures on multiple images.

DAB or AB/PAS positive cells were counted by image thresh-holding. The rationale is as follows: Each pixel within a grayscale image is automatically assigned a particular level of intensity (grey level). Each image is comprised of 256 grey levels where black = 0 and white = 256. A 5 s counterstain (Mayer's haematoxylin) generates nuclei and cytoplasmic staining intensities upwards of approximately 110 - 130 (determined from random images [n=5] for each staining batch). Image tool retained pixels below this threshold and converted the image to binary form. The binary image assigns retained pixels to level 0 (black) and discarded pixels are assigned level 256 (white). Image tool then counts the number of black pixelated objects generated within the binary image.

To ensure accuracy of the automated methodology, a group of images (n=17) were manually counted by eye. These counts were subsequently compared to automated results from the same group of images (data not shown).

As an allometric relationship exists between body mass and gill surface area (Muir 1969; Roubal 1987; Palzenberger and Pohla 1992), data were expressed as the number of positively stained cells / mm of filament / log mass of fish. This calculation allowed for comparisons between groups from sampling commencement to completion where a 10-fold increase in growth had occurred. The log mass division was excluded for chloride cells which were evenly distributed along the basal filamental regions.

5.3.6 Environmental Profiles

Water temperature, salinity and dissolved oxygen results were obtained from farm records. Other records included mortality within selected pens, net changes and periodic algal species observations and were likewise drawn from farm data.

5.3.7 Statistical Analysis

Data were analysed by two way analysis of variance (ANOVA) with pen (fixed, orthogonal, two levels) and sampling time (fixed, orthogonal, 19 levels) as factors. Pen data were pooled where no significant differences were found between pens for each parameter. Tukeys HSD was used for means comparisons where assumptions of normality (Shapiro-Wilk test) and homogeneity (Levene's test) were met. Heterogenous data were transformed (square root) and a P value of $P<0.05$ was adopted for rejection of the null hypothesis. SPSS® (version 10.0, SPSS Science) and Sigma Plot (version 6.0, SPSS Science) were used for data analysis and presentation.

5.4 Results

Aside from an initial mortality spike of nearly 1% during the first sampling week, mortality remained fairly constant at approximately 0.05% to 0.2% of the cage populations throughout the duration of the trial. There were no significant blooms of algal species known to be harmful to salmon for the duration of the trial. Large fluctuations in salinity at 1m prior to the final two sampling weeks were observed (Fig. 2 – next page). A gradual increase in temperature and reductions in dissolved oxygen concentrations were also noted as the transfer period progressed (Fig. 2).

Gross signs of AGD were first apparent from fish sampled after Week 14. Histological evidence of AGD was first noted after week 13 and there were significant increases ($P<0.001$) in the percentage of affected filaments during the final two sampling weeks (Fig. 3). Significant increases ($P<0.001$) in gross pathology were also evident during the final two sampling weeks (Fig. 3).

The combination of histopathological technique and the availability of sequential gill material enabled the elucidation of the progressive nature of lesion development. Initial attachment of trophozoites to secondary lamellae coincided with hypertrophy and/or desquamation of surface epithelial cells within the immediate vicinity of attachment (Fig. 4A*). Thickening of secondary lamellae appeared to begin with hypertrophy and some hyperplasia of epithelial cells as well as oedema of the epithelium (Fig. 4B). These regions progressed to a more pronounced hyperplastic state where fusion of secondary lamellae occurred simultaneously with oedema of the primary filamental epithelium (Fig. 4B & 4C). Zones of filamental oedema were infiltrated by leucocytes emigrating from the central venous sinus (Fig. 4C & 4D). The basal epithelia of the primary filaments were consequently removed to the distal regions of the secondary lamellae. Chloride cells were sloughed off the forming lesion

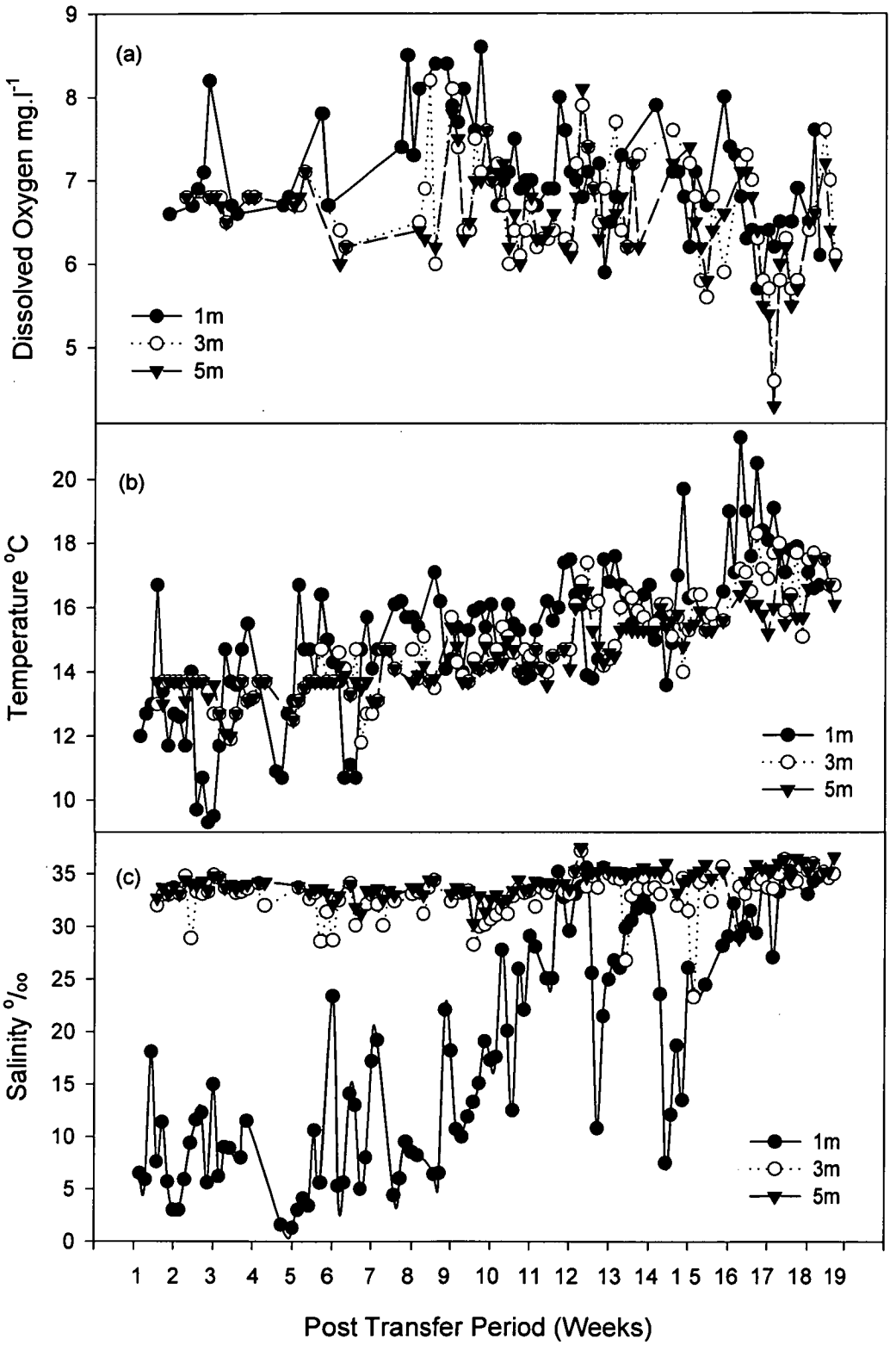


Figure 2. Environmental records including dissolved oxygen (a), temperature (b) and salinity (c) over the duration of the sampling period.

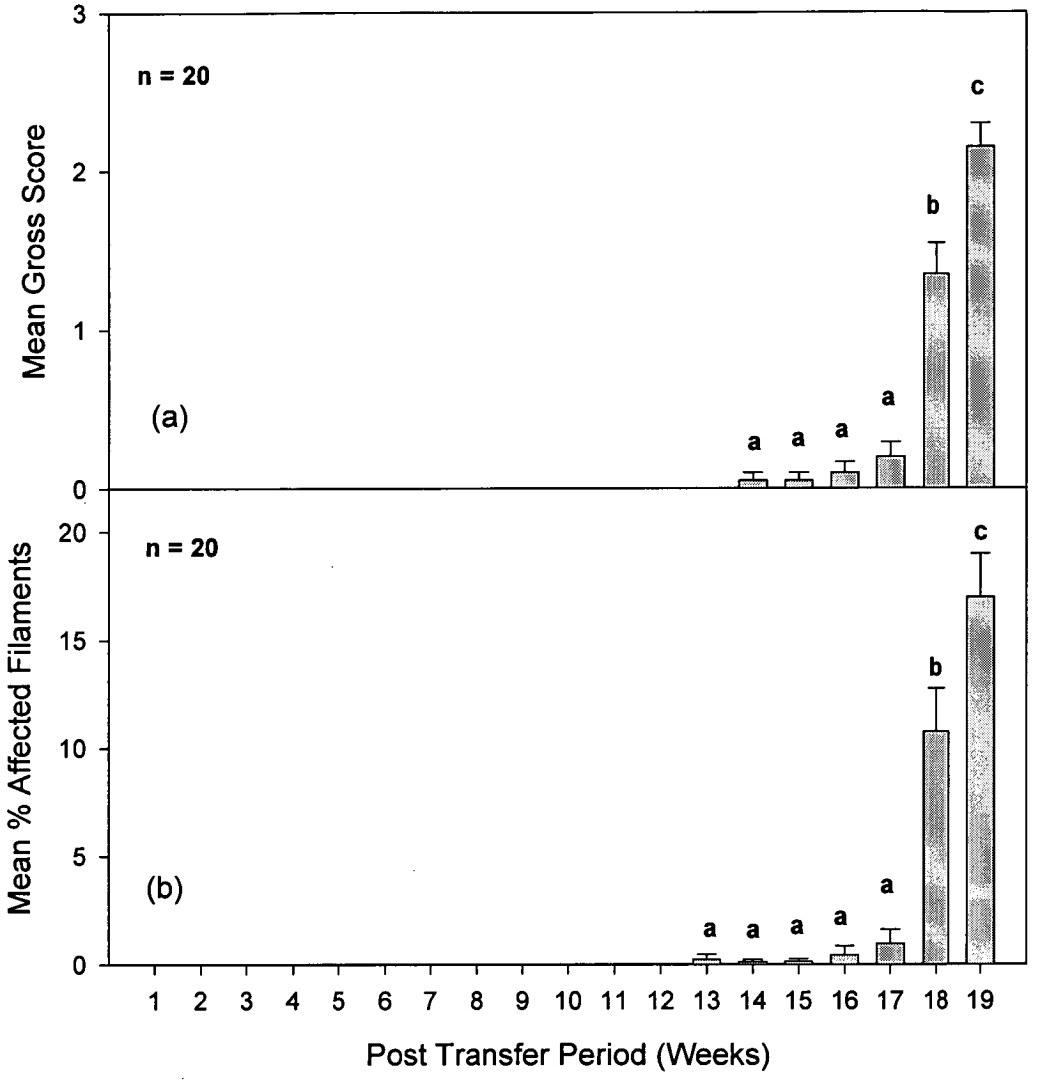
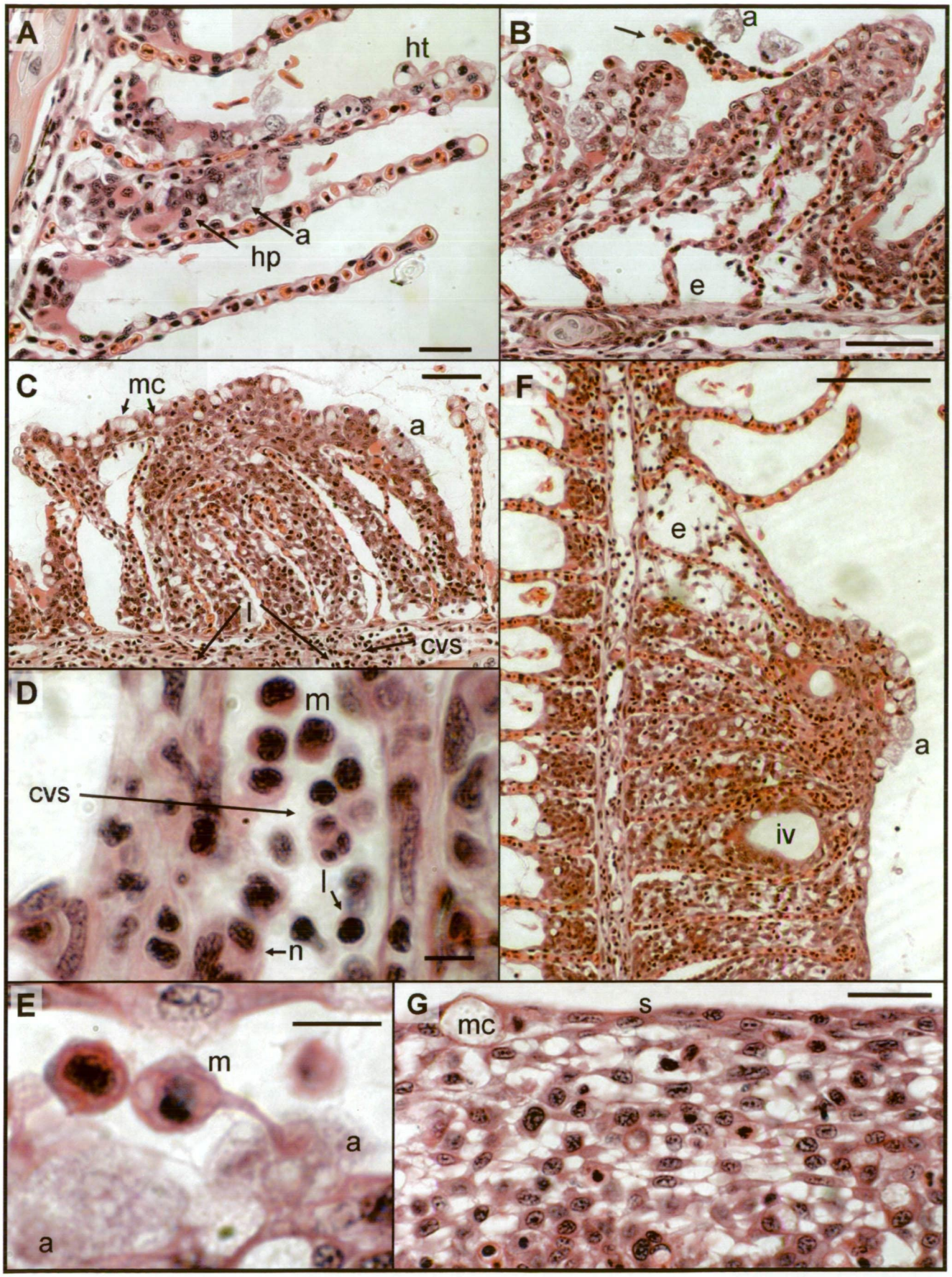


Figure 3. Mean scores for gross pathological assessment of AGD (a) and mean percentage of filaments affected by AGD lesions for each week. Values are means ± SE. Different letters indicate mean values that are significantly different.



* Figure 4 - appears in press as black and white image, figure legend next page.

Figure 4. A – Intitial interaction between a trophozoite (a) and secondary lamellae. Note initial hyperplasia (hp) and hypertrophy (ht) of epithelial cells upon the affected lamellae (bar = 25 μm). B - Progression of hyperplasia and oedema (e) of the basal filamental epithelium. Multiple trophozoites (a) and sloughing of tissue from the forming lesion (arrow) are also shown (bar = 50 μm). C – Small hyperplastic lesion with leucocytes (l) migrating along the central venous sinus (cvs). A single trophozoite (a) and mucous cells (mc) are also evident (bar = 50 μm). D – Various white blood cells within the central venous sinus (cvs) including macrophages (m), neutrophils (n) and lymphocytes (l) (bar = 10 μm). E – Macrophage (m) penetrating a trophozoite (a) with a pseudopod (bar = 10 μm). F – "Mature" AGD lesion displaying oedema (e) and numerous trophozoites at the leading edge. A single interlamellar vesicle (iv) is also present (bar = 100 μm). G – Stratification of epithelial tissue centrally located upon the surface a spongiotic, mature lesion. Note squamous epithelia (s) and absence of trophozoites (bar = 25 μm).

along with a proteinaceous necrotic exudate containing amoebae, epithelial cells and leucocytes as the lesion matured (Fig. 4B & C). At lesion surfaces, epithelial associated amoebae were frequently adhered to by macrophages and in some cases inter-digitating pseudopodia were seen contacting attached trophozoites (Fig. 4E).

Neutrophils were most often associated with the central venous sinus, interlamellar vesicles and endothelia of filamental basal regions. As lesions developed in length along the primary filament, the leading edge of the lesion was preceded by oedema and leucocyte infiltration (Fig. 4F). Large numbers of eosinophilic granule cells/putative mast cells (EGC) were sometimes seen within swollen vasculature surrounding the filamental cartilage within lesion affected regions (data not shown).

Their presence could not be regularly associated with hyperplastic lesions although their absence was conspicuous in unaffected filaments. Larger lesions consisted mainly of undifferentiated epithelial cells (mostly PCNA +ve) and variable inclusions of mucous cells. Trophozoites appeared to colonise the margins of longer lesions. Larger lesions (margins excepted) often displayed a more structured epithelial arrangement where epithelial cells had assumed a stratified, squamous arrangement and were frequently interspersed with mucous cells (Fig. 4G). Recruitment of leucocytes to these regions was negligible and colonization by trophozoites was often non-existent. There were numerous incidents of interlamellar vesicle formation within larger lesions. Spongiosis of hyperplastic tissue was also noted in many lesions.

Image analysis (IA) of histochemically stained gill sections showed significant increases ($P<0.01$) in the number of branchial mucous cells (Fig. 5) following transfer of fish to Deep Bay. There were no significant differences ($P>0.05$) between weeks 1 and 12 (Fig. 5).

At weeks 17-19 mucous cell numbers increased almost threefold to gills of fish sampled at week 12 (Fig. 6A). These quantitative increases were a function of higher mucous cell densities notably at the distal tips of non-hyperplastic secondary lamella (Fig. 6B). Additionally, there were many instances of hypertrophic mucous cells in high densities in the immediate proximity of mature lesions, both on affected filaments and on adjacent non affected filaments. Mucous cells within these regions were most commonly PAS positive - AB negative, indicating the presence of neutral mucins.

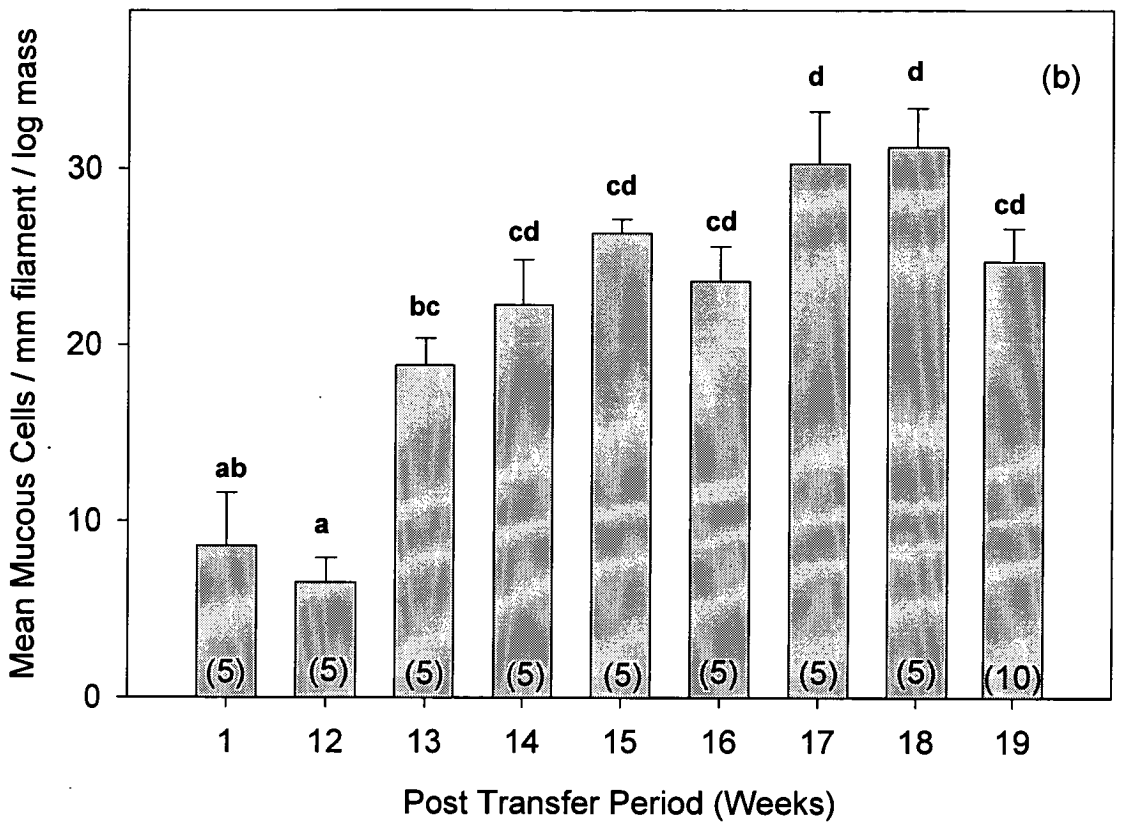


Figure 5. Mean number of mucous cells per mm of filament per log mass. Counts generated using semi-automated digital image analysis. Values are means ± SE. Different letters indicate values that are significantly different.

PCNA positive cells were significantly increased at weeks 17, 18 and 19 compared to week 12 (Fig. 6C) ($P<0.05$, $P<0.01$ and $P<0.01$ respectively [Fig. 7]). It should be noted that due to technical difficulties it was not possible to assess PCNA during all sampling weeks.

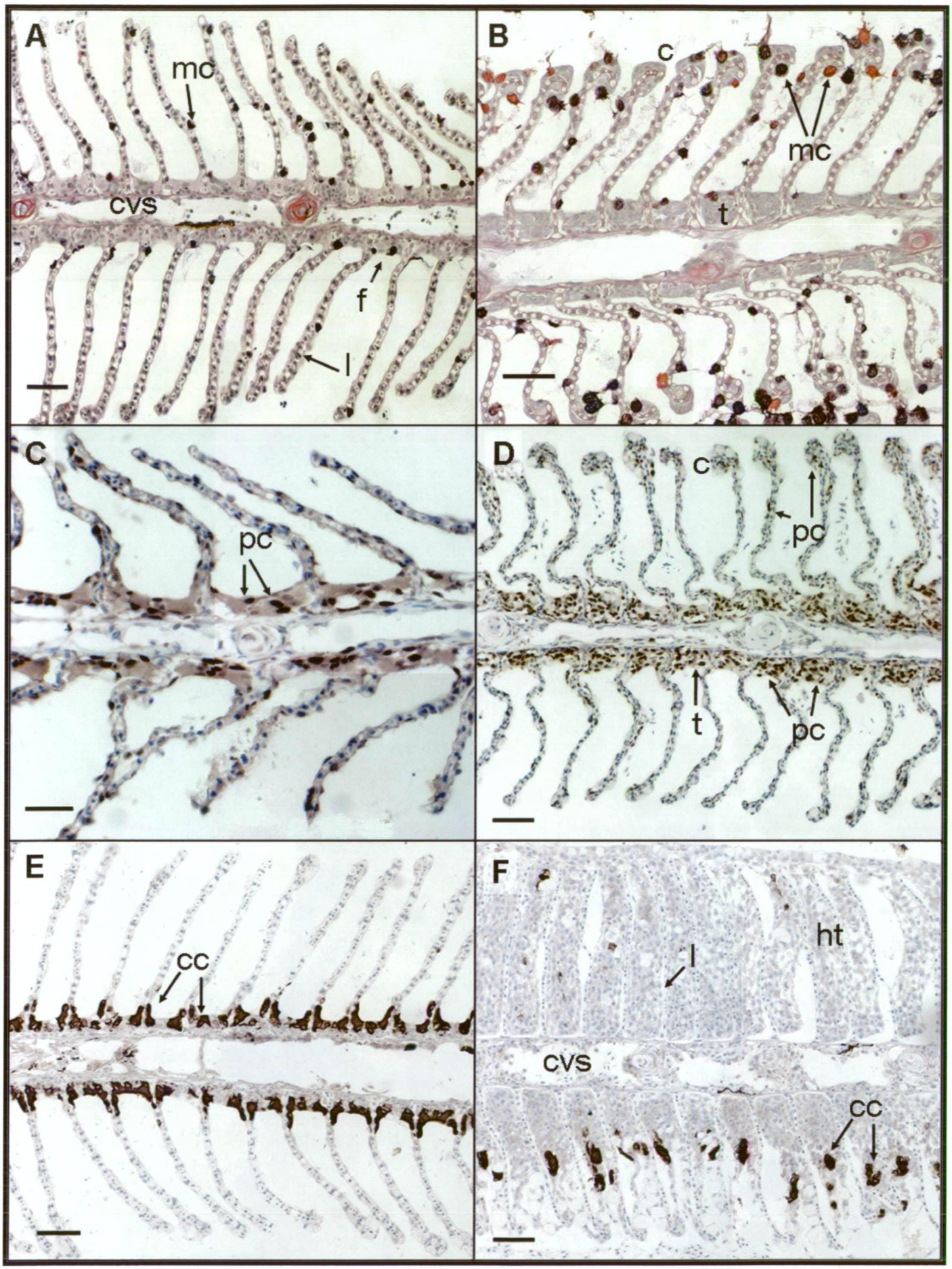

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Figure 6. (A&B) PAS-AB stain for mucous cells. (A) Week 12 sample, note normal structure of filament (f) and lamellae (l) and presence of few mucous cells (mc) (bar = 50 µm). (B) Week 19 filament adjacent to an AGD lesion showing increases in the number of mucous cells (mc), clubbing of distal regions of secondary lamellae (c) and thickening of the basal filamental regions (t) (bar = 50 µm). (C & D) PCNA immuno-stains of gill sections from weeks 12 (C) (bar = 25 µm) and 19 (D) (bar = 50 µm). (C) Note relatively few positive cells (pc) compared to (D) where thickening of the basal epithelium (t) and clubbing of lamellae (c) has occurred in a filament adjacent to an AGD lesion (lesion not shown). (E & F) Na^+/K^+- ATPase immuno-staining for chloride cells. (E) healthy filament from fish with AGD (week 19) with distinguishable chloride cells (cc) (bar = 50 µm). (F) Same section showing reductions in chloride cell presence within hyperplastic tissue (ht) between lamellae (l) (bar = 50 µm).

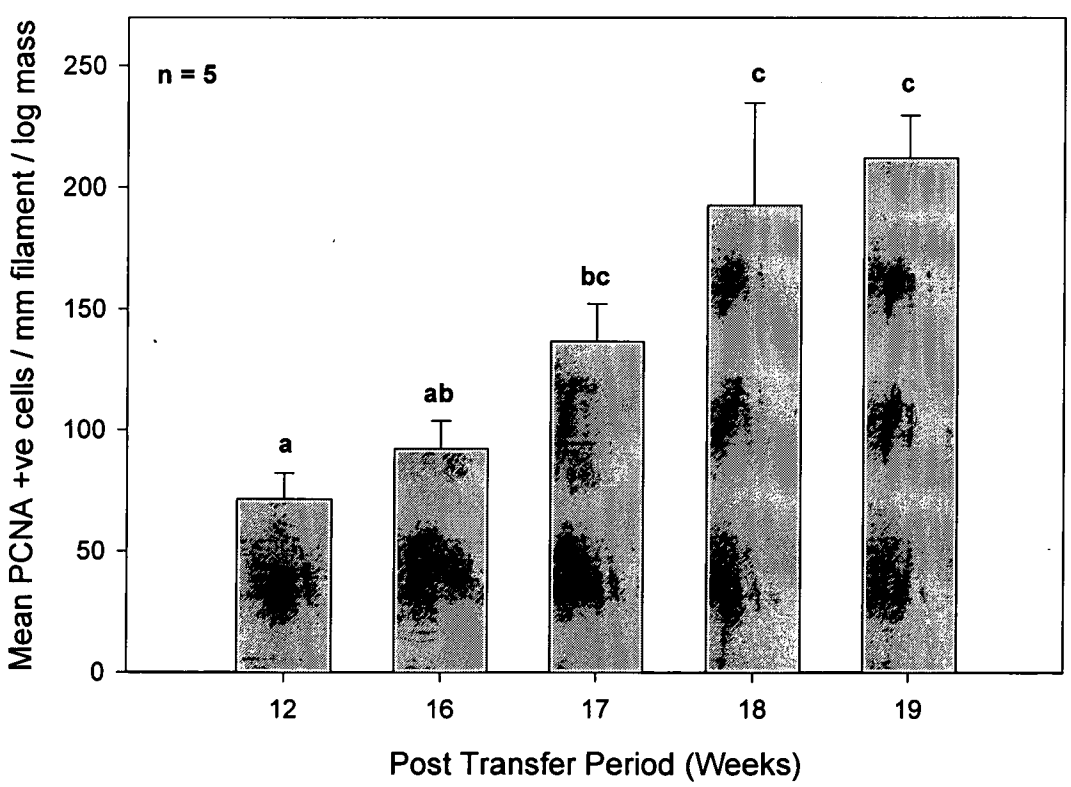

Figure 7. (Previous page) Mean number of PCNA +ve cells per mm of filament per log mass. Counts generated using semi-automated image analysis. Values are means ± SE. Different letters indicate values that are significantly different.

At weeks (17 – 19), PCNA positive cell densities were much higher along the basal filamental regions (Fig. 7). This was particularly apparent upon filaments containing lesions and sometimes those adjacent to hyperplastic regions (Fig.6D). The secondary lamellae of such filaments also displayed higher densities of PCNA positive cells. Within the central venous sinus, adjacent to forming lesions, PCNA positive cells with refractile granules were often noted. Examination of corresponding sections stained with H&E suggested these cells were most probably EGCs. PCNA expression in EGCs was variable while other leucocytes, pillar cells and squamous epithelial cells displayed modest to no staining. In unnaffected filaments, PCNA positive undifferentiated epithelial cells were especially numerous at the filamental leading edge, filamental tips and in basal filamental regions.

There were no significant differences in the number of chloride cells (Na^+/K^+-ATPase positive) during weeks 13 – 19, however, there was an appreciably lower number of chloride cells associated with larger hyperplastic lesions (Fig. 6E & 6F).

Immunostaining of apoptotic cells was negative for all selected samples except control tissue. A piscine positive control was not used for this study. Therefore, the significance of host apoptosis during amoebic infection, if any, could not be fully evaluated.

Nodule/plaque and aneurysm formation was observed during the sampling period with peak activity noted during week 5 and week 9 respectively (data not

shown). The aetiological cause of nodules, plaques and aneurysms could not be determined.

5.5 Discussion

Gross pathology and histopathology indicated a rapid onset of AGD at 18-19 weeks post-transfer to estuarine/marine sites. This coincided with the dissolution of a halocline and increases to water temperature. The halocline's location and zone of influence is dependant upon freshwater input from the Huon River (Butler 2001). Once the halocline retracted to the upper estuary (after cessation of high rainfall), infection was no longer restricted by salinity fluctuations. Additionally, water temperature may also have influenced disease out break. The relationship between salinity and temperature with AGD prevalence is consistent with previous field findings and qualitative risk factor evaluations (Clark & Nowak 1999; Nowak 2001) contributing to disease outbreak.

Primary interactions, defined here as trophozoite attachment, localized epithelial desquamation and oedema of host tissue further confirm the primary pathogenicity of *Neoparamoeba* sp. The same genus (formerly *Paramoeba* sp. [Dyková, Figueras & Peric 2000]) has been identified as the primary agent of AGD in turbot (Dyková *et al.* 1995 & 1998). Attachment of *Neoparamoeba* sp. to normal gill tissue was observed experimentally after one day of co-habitation with infected fish (Zilberg & Munday 2000). A recent experimental infection (Adams & Nowak, unpublished data) confirmed attachment of *Neoparamoeba* sp. to gill epithelium following twelve hours of exposure to gill isolates. The observed patterns of initial alterations to secondary lamellae, following initial attachment, were consistent with the current study.

The cellular response to AGD is similar to that of amoebic infections in higher vertebrates. *Acanthamoeba* sp., agents responsible for *Acanthamoeba* keratitis, initially elicits localized tissue changes including epithelial desquamation and oedema. *Acanthamoeba* also produces an assortment of proteases that assist in the ongoing pathogenesis (host cell cytolysis, phagocytosis and host cell apoptosis induction) of the aforementioned condition (Niederkorn, Alizadeh, Leher and McCulley 1999). *Entamoeba histolytica,* the cause of amoebiasis in humans, promotes similar initial cellular disruptions following attachment and also contain numerous extra-cellular proteases. *E. histolytica* also possess the ability to form amoebapores (channel forming peptides) following attachment that are able to destroy host cells osmotically (Espinosa-Cantellano & Martinez-Palomo 2000). Both of the aforementioned genera bind to host cells by lectin attachment subsequently resulting in cytolysis of the affected cells (Kain & Ravdin 1995; Petri and Shnaar 1995; Cao, Jefferson & Panjwani 1998; Petri, Haque & Mann 2002). To what degree the initial invasive strategy of *Neoparamoeba* sp. relies on the aforementioned adhesion and cytolytic mechanisms is not known and requires further investigation.

A marked innate cellular immunological response was a secondary feature observed histopathologically during lesion formation and along the margin of larger lesions. Macrophages and neutrophils were the most abundant leucocytes identified throughout hyperplastic regions as previously reported (Roubal *et al.* 1989; Munday *et al.* 1993; Dyková *et al.* 1995; Adams & Nowak 2001; Bridle, Butler & Nowak 2003). Specific interactions (adherence) between host immuno-regulatory cells and epithelium associated amoebae have not been previously identified in field infections of AGD. There was no histological evidence that attachment of macrophages to trophozoites was detrimental to the latter. An *in vitro* appraisal would be required to

provide specific observations. Macrophages have shown adherence to *Gyrodactylus derjavini in vitro* resulting in substantial tegument damage (Buchmann and Bresciani 1999). Macrophages are commonly recruited during ectoparasitic insults (Barmanrokh & Woo 2001; Bennett and Bennett 2001; Colquitt, Munday and Daintith 2001). They not only possess phagocytic ability but also contain mediating factors that assist immuno-regulation (Secombes & Fletcher 1992; Buchmann 1999; Koppang, Hordvik, Bjerkas, Torvund, Aune, Thevarajan & Endresen 2003). Neutrophils were also prominent within lesion affected regions. The role of these cells, according to their observed distribution, appears to be confined within the gill as there was little evidence of migration to lesion surfaces as seen with macrophages. Their major function appeared to involve infiltration into interamellar vesicles and surrounding hyperplastic tissues for removal (either directly or adjunctly) of trophozoites entrapped with proliferative tissue. This was consistent with previous observations in field infected fish (Adams & Nowak 2001) and laboratory infection (Bridle *et al.* 2003). Neutrophils are recruited to sites of hyperplasia in bacterial gill disease (BGD) (Speare, Ferguson, Beamish, Yager & Yamishiro 1991a). Eosinophilic granule cells (EGCs) within blood vessels surrounding the filamental cartilage were often noted adjacent to lesions. It is likely that the recruitment and activity of these cells is influenced by the attachment of *Neoparamoeba* sp. to gill tissue as it is for other persistent parasitic insults (Reite 1998; Treasurer and Turnbull 2000; Barmanrokh & Woo 2001; Colquitt *et al.* 2001). The presence of occasional PCNA positive cells suggests EGC mitosis maybe occurring periodically, a process previously detected in rainbow trout gill explants (Flano, Lopez-Fierro, Razquin & Villena 1997). The mechanisms and timing for recruitment, ontogeny, activation and interrelationships of EGCs with other leucocytes requires further understanding.

The occurrence of pronounced filamental and lamellae oedema was a common feature in developing lesions and at the margins of larger lesions. Nodule/plaques did not display peripheral oedema suggesting filamental oedema at lesion peripheries may be specific to hyperplastic lesions induced by amoebic attachment. These events were not considered to be histological artefact and have been mentioned in other superficial infections (Speare, Ferguson, Beamish, Yager & Yamishiro 1991b; Urawa and Yamao 1992; Barmanrokh & Woo 2001; Bruno, Collins, Cunningham & MacKenzie 2001). It was unclear in this study whether oedema formation was a function of internal processes, reduced epithelial integrity or a function of both.

Consistent observations of amoebae colonizing the margins of lesions suggests that progression of lesions along primary filaments most likely results from the migration of proliferating amoebae outward from the point of initial lesion formation. Gill isolated *Neoparamoeba* sp. display typical amoeboid motion (Roubal *et al.* 1989; pers ob). During development of human amoebiasis, *E. histolytica* exploits its motility to advance infection and proliferation amongst surrounding tissues and their densities are generally concentrated at lesion peripheries (Sehgal, Bhattacharya & Bhattacharya 1996; Espinosa-Cantellano & Martinez- Palomo 2000). The active host pathogen interface regions of lesion peripheries may also encourage attachment of nearby mucous associated trophozoites. During amoebiasis infection, areas of superficial epithelial desquamation in the human colon are rendered more susceptible to invasion by *E. histolytica* than unaffected tissues (Espinosa-Cantellano & Martinez- Palomo 2000).

The latter stages of AGD lesion development comprised of substantial epithelial rearrangement including squamation of superficial epithelia and variable recruitment of mucous cells to lesion surfaces. These regions were rarely colonised by

trophozoites. This may be indicative of a "fortification" strategy adopted by teleosts against gill ecto-parasites (Treasurer and Turnbull 2000;Bennet and Bennet 2001; Adams and Nowak 2001) resulting in the internalization/exclusion and destruction/isolation of the pathogen from susceptible tissue. Filament regions with fully fused secondary lamellae also deny trophozoites the opportunity to exploit the relatively "sheltered" interstitial mucous layer between secondary lamellae. Trophozoites would be relatively exposed to greater water velocity possibly facilitating removal from these regions. The squamous morphology of regenerated superficial epithelia may be potentially less suitable for adhesion. The role of mucous cells, recruited to these regions, also warrants careful consideration.

Increases in mucous cell numbers were apparent after the onset of AGD. This corresponds with previous studies indicating similar increases to total mucous cell numbers and the tendency for PAS positive mucin predominance in lesion affected regions (Nowak & Munday 1994; Zilberg & Munday 2000; Roberts & Powell 2003). Increases to total mucous cell numbers have also been observed in BGD (Ferguson, Morrison, Ostland, Lumsden & Byrne 1992). We observed few trophozoites in contact with regions rich in mucous cells, an observation consistent with findings by Urawa (1992) and Buchmann & Bresciani (1998) during *Trichodina truttea* and *Gyrodactylus derjavini* infections respectively. Buchmann & Bresciani (1998) and Buchmann (1999) suggested that inclusion of mucosal immune associated enzymes may influence the course of infection.

Although no overall reductions in chloride cells (Na^+/K^+- ATPase positive) were evident in this study, their numbers were substantially reduced in advanced lesions. Roubal *et al.* (1989), Munday *et al.* (1990), Munday & Nowak (1994) also described similar reductions to chloride cells in hyperplastic AGD lesions. Fish with

severe experimental AGD display elevated blood sodium levels (Munday *et al.* 1990; Findlay 2001) and is likely a result of reductions to this cell type.

Concurrent increases of proliferative (PCNA positive) cells and AGD was reflective of increased cell turnover and recruitment in response to infection with *Neoparamoeba* sp.. Proliferative host cells featured distinctly and numerously upon lesion affected filaments, within hyperplastic epithelia filaments and sometimes within lesion apposed filaments. Cellular proliferation in response to teleostean parasitic infections has not been previously described.

There was no indication of any pathogen or toxicant contributing to AGD development in this study. The percentage of nodule/plaque affected filaments peaked at 5-6 weeks post-transfer (data not shown), a similar timing to that described by Nowak and Munday (1994). However AGD was not apparent for a further six weeks in this study. This may suggest that nodule and plaque formation is a common post transfer event in spring smolts occurring independently of AGD.

To conclude, this study emphasises the importance of salinity and temperature as major factors determining the timing of AGD outbreaks. *Neoparamoeba* sp., the primary pathogen, elicits a tri-phasic host response to infection including localized host tissue responses to adhered trophozoites, non-specific immuno-regulatory cell infiltration and advanced hyperplasia with epithelial fortification. Interactions between AGD and other gill micro-organisms or pre-existing/simultaneous gill disruptions were not evident in this study.

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CHAPTER 6 - Sequential pathology after initial freshwater bath treatment for amoebic gill disease in cultured Atlantic salmon *Salmo salar* L.

M.B. Adams & B.F. Nowak

6.1 Abstract

Freshwater bathing is essential for control of AGD during the marine phase of the Tasmanian Atlantic salmon production cycle, a practice that is costly, production limiting and increasing in frequency. Although the pathogenesis of gill infection with *Neoparamoeba* sp. in naïve Atlantic salmon (*Salmo salar*) is now understood, the progression of re-infection (post-treatment) required elucidation. Here, we describe the weekly histopathological progression of AGD from first to second freshwater bath. Halocline cessation and increased water temperature appeared to drive the rapid onset of initial infection prior to bathing. Freshwater bathing cleared lesions of attached trophozoites and associated cellular debris. Subsequent gill re-infection with *Neoparamoeba* sp. was evident at 2 weeks post-bath and had significantly increased ($P<0.001$), in severity, by 4 weeks post bath. No significant difference in gross pathology was observed until 4 weeks post-bath ($P<0.05$). The re-infective progression of AGD was characterized by localized host tissue responses juxtaposed to adhered trophozoites (epithelial oedema, hypertrophy & hyperplasia), non-specific inflammatory cell infiltration (macrophages, neutrophils & eosinophilic granule cells) and finally advanced hyperplasia with epithelial fortification. During the post-bath

period, non AGD lesions including haemorrhage, necrosis and regenerative hyperplasia were occasionally observed though no evidence of secondary colonization of these lesions by *Neoparamoeba* sp. was noted. We conclude that pathogenesis, during the inter bath period, was identical to initial infection although the source of re-infection remains to be established.

6.2 Introduction

Currently, amoebic gill disease (AGD) impacts heavily upon production costs of commercial Atlantic salmon *Salmo salar* L. culture in Tasmania (Carington Smith & Wadley 2003). AGD is also reported to affect a variety of other marine fish species in other parts of the world (reviewed by Munday 2001; Nowak, Carson, Powell & Dyková 2002).

In Tasmania, AGD is attributed to infection by *Neoparamoeba* sp. (formerly *Paramoeba* sp. [Dyková, Figueras & Peric 2000]), a free living, amphizoic marine amoeba that attaches to the gills of salmon and, left untreated, causes a severe hyperplastic tissue reaction and eventual death (Munday, Foster,Roubal & Lester 1990). AGD outbreaks are primarily influenced by environmental factors, most importantly salinity and temperature (Clark & Nowak 1999; Nowak 2001; Adams & Nowak 2003).

AGD is diagnosed grossly by the presence of raised, white mucoid-like patches upon the gills and excessive mucus production (Munday *et* al. 1990). Histologically, these patches present as focal and multi-focal hyperplasia resulting in variable degrees of lamellar fusion (Adams, Ellard & Nowak 2004). The naïve host response to field infection with *Neoparamoeba* sp. includes localized host tissue

responses to adhered trophozoites, non-specific immuno-regulatory cell infiltration and advanced hyperplasia with epithelial fortification (Adams & Nowak 2001; Adams & Nowak 2003). Other factors have been suggested as possible influences upon AGD outbreaks including pre/co-existing lesions, immune status, stocking densities and other amoebae (Nowak & Munday 1994; Findlay & Munday 1998, Clark & Nowak 1999; Dyková, Figueras & Novoa 1999; Findlay, Zilberg & Munday 2000; Munday *et al.* 2001; Nowak 2001).

The only commercially effective treatment for AGD is freshwater bathing. Following gross diagnosis of AGD by routine farm sampling, fish are bathed in freshwater for 3-4 hours (Parsons, Nowak, Fisk & Powell 2001). The mitigating properties of fresh water bathing are not entirely understood. It is suspected that a combination of osmotic challenge to gill-associated amoebae, removal of seawater stable gill mucus and dissolution of gill lesions all contribute to treatment success (Parsons, Nowak, Fisk & Powell 2000; Munday *et* al, 2001; Clark, Powell & Nowak 2003; Munday & Zilberg 2003; Powell & Clark 2003; Roberts & Powell 2003). Numbers of gill associated amoebae have been shown to return to pre-bath levels within 10 days of bathing (Clark & Powell 2003).

When freshwater bathing was initially introduced as a commercial mitigation strategy in the late 1980s, two to three baths provided sufficient alleviation of AGD symptoms during the marine production cycle (Foster & Percival 1988; Clark & Nowak 1999). Presently however, fish may require up to ten baths to successfully circumvent AGD progression for the same period. For spring smolts and pre-smolts, re-infection occurs more rapidly after treatment compared to the time taken for initial infection to appear (Mitchell 2001). The apparent increase in bathing frequency is a critically important industry concern in terms of production costs.

No information has been forthcoming that sufficiently details the pathogenesis of re-infection with AGD during an entire re-bathing cycle. Consequently, this study describes the development of AGD to determine the plausibility of a modified infection strategy between treatments. Associated environmental observations and general gill health status are also examined for any interrelationships contributing to infection.

6.3 Materials and Methods

6.3.1 Sampling Regime

Commercially reared Atlantic salmon (out of season) smolts were transferred to Deep Bay in the Huon Estuary, Southern Tasmania in June 2002 (see Adams & Nowak 2003a for map reference). Salmon populations from two 80 m pens within this site were selected and monitored approximately fortnightly for gross signs of AGD. On 5th December 2002 the trial pens were deemed by farm management as "heavy" in terms of AGD severity based on gross diagnosis (Table 1.). The pens were subsequently scheduled for initial freshwater bath treatment the following week. Fish were bathed for three hours in oxygenated freshwater sourced from a local dam (total hardness = 165 $mg.l^{-1}$ $CaCO_3$). Stocking densities were 3.34 $kg.m^{-1}$ and 10.72 $kg.m^{-1}$ for pens referred to hereafter as 10 and 14. The population within Pen 14 was subsequently split immediately after bathing reducing the stocking density to 2.14 $kg.m^{-1}$.

Sampling commenced immediately before and after initial bath treatment and then on a weekly basis until a second bath was required to treat re-infection. Pen 10 was re-bathed after week 4 sampling was complete and pen 14 was re-bathed seven

Table 1. Huon Aquaculture Company Pty.Ltd. Gross scoring method for assessment of AGD severity per fish.

Infection Level	*Description*
Clear	Gills are healthy, clean and red
Faint Spots	Small discrete spot, not raised, translucent appearance
Spots	Raised opaque spots upon single filaments, spot display a distinct white colouration on a red gill background
Patches	Raised white patch affecting two or more filaments, excessive mucous production

days later resulting in an extra week of data for this pen. Fish were collected from each pen (n = 10) by box netting and transferred to two oxygenated holding bins (200L). After terminal anaesthetization with 0.2% clove oil, the weight, length, gross gill score (Adams & Nowak 2003) and any anomalous gross observations were recorded for each fish. The gills were then excised, rinsed gently in 0.22 μm filtered seawater and fixed for 1-2 h in seawater Davidson's fixative. Gross gill images for each fish were captured from the second left anterior hemibranch which were subsequently processed for histology.

6.3.2 Histopathology

Gills were dehydrated, embedded in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin (H & E). The sections were viewed under a light microscope

(Olympus, Hamburg, Germany) at 400x magnification. The number of filaments on each arch were counted for each section and any lesions present were recorded. A filament was counted only when the central venous sinus was visible in at least 2/3 of a filament.

6.3.3 Histochemistry

The 2nd left gill arches, fixed and processed as above, were stained using periodic acid -Schiff-alcian blue (PAS-AB) technique (pH 2.5) as described by Adams & Nowak (2003). Briefly, sections were de-waxed and re-hydrated, immersed in alcian blue (AB) (Sigma, Castle Hill, Sydney, Australia) and microwaved on high (45 sec), left to stand (5 min) and washed in deionized water (DI) (1 min). Sections were then immersed in 1% periodic acid (ICN Biomedicals, Irvine, CA, USA) (10 min), washed (1 min, DI) and immersed in Schiff's reagent (BDH Laboratory Supplies, Dorset, UK), microwaved on high (3 x 15 sec) and left to stand (5 min). Finally sections were washed in running tap water (15 min), counterstained with haematoxylin (15 sec), dehydrated, cleared and mounted.

6.3.4 Immunohistochemistry

Chloride cells were immunohistochemically identified using methods described by Adams & Nowak (2003). Following dehydration and embedding, tissue sections were cut (5 μm), mounted on silane (Sigma, Castle Hill, NSW, Australia) coated slides and processed according to the avidin-biotin-peroxidase (ABC) technique (Kiernan 1999) as follows. To facilitate heat induced epitope retrieval (HIER), sections were dewaxed, rehydrated and placed into citrate buffer solution (pH 6) and microwaved on high (12 min) then allowed to stand (20 min). After a brief rinse in deionized H_2O

(DH_2O), sections were blocked for endogenous peroxidase (3% H_2O_2 – 20 min), washed (PBS – 3 x 1 min) and incubated with normal horse serum (20 min) (Vector Laboratories, Burlingame, CA) to block non specific binding sites. Sections were then blotted dry and incubated in a humid chamber (37.5°C, 1 h) with a mouse monoclonal antibody to Na^+/K^+- ATPase (1:200, IgGα5, Developmental Studies Hybridoma Bank, Department of Biological Sciences, The University of Iowa, USA). Sections were washed then incubated (30 min, 37.5°C) with biotinylated horse anti mouse IgG (ABC kit, Vector Laboratories)(1:200), washed again, then incubated (30 min, 20°C) with peroxidase conjugated streptavidin (1:200 ABC kit, Vector Laboratories). After a final washing step, slides were flooded with 3-3'-diaminobenzidine (DAB) in peroxide buffer (2 min) (Roche Diagnostics, Castle Hill, Australia) then rinsed in DH_2O (30 s), counterstained with Mayer's haematoxylin (5 s), rinsed, differentiated in PBS (30 s), dehydrated, cleared and mounted. The gills served as internal positive controls. Omission of the primary antisera served as a negative control.

6.3.5 Digital Image Analysis

Histochemically and immunohistochemically stained sections were analysed and quantified using semi-automated digital image analysis as described by Adams & Nowak (2003). Grayscale images (150 dots per inch [DPI]) were captured on every third filament of each gill section. Images were captured from the proximal half of each correctly orientated filament (as previously described). A Leica DC300F digital camera (Leica Microsystems, Wetzlar, Germany) C-mounted to a light microscope (Olympus, Hamburg, Germany) was used for image capture. Image analysis was performed using Image Tool version 3.0 (University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA). Winbatch (Wilson WindowWare Inc.,

Seattle, WA, USA) was used to automate image analysis procedures on multiple images.

6.3.6 Environmental Profiles

Records for water temperature, salinity, dissolved oxygen, stocking density, mortality, net changes and periodic algal species observations were obtained from farm data.

6.3.7 Statistical Analysis

Re-infection rates for each pen were analyzed by linear regression and the slopes compared using a students T test (dependant variable - percentage of AGD lesion affected filaments; independent variable – number of days post-bath). Week 6 data for pen 14 were subsequently discarded and all remaining data analysed by two way analysis of variance (ANOVA) with sampling time (fixed, orthogonal, 6 levels) and pen (random, orthogonal, two levels) as factors. Pen data were pooled where no significant differences were found between pens for each dependant parameter. Tukeys HSD was used for means comparisons where assumptions of normality (Shapiro-Wilk test) and homogeneity (Levene's test) were met. Heterogenous data were transformed (square root) and a P value of $P<0.05$ was adopted for rejection of the null hypothesis. SPSS® (version 10.0, SPSS Science) and Sigma Plot (version 6.0, SPSS Science) were used for data analysis and presentation.

6.4 Results

The advent of AGD prior to initial bathing (gross score farm data [Fig. 1a]) coincided with stabilization of salinity and a rise in temperature of surface waters (Fig 1b, 1c.).

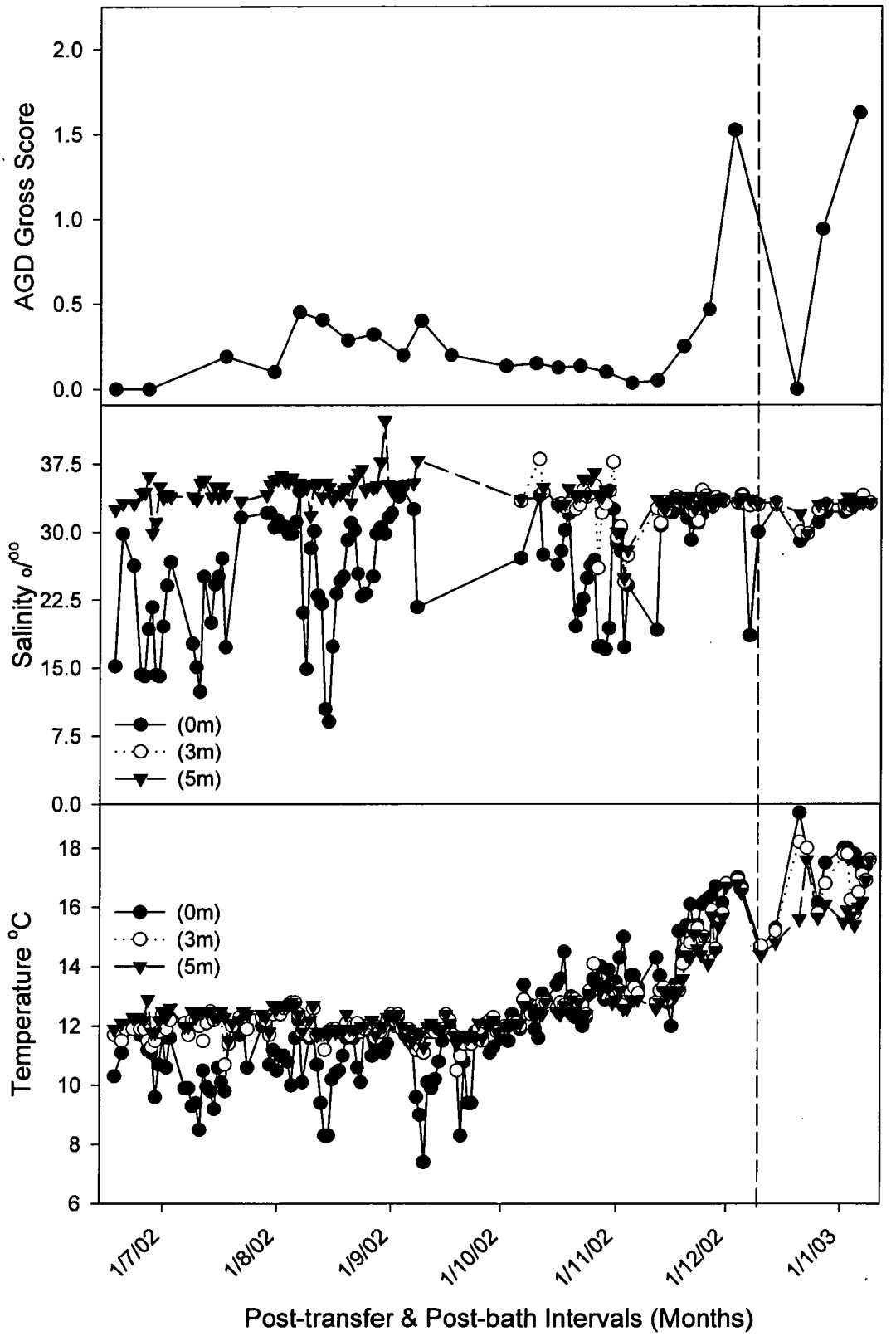
AGD Gross Score
2.0
1.5
1.0
0.5
0.0
Salinity ‰
37.5
30.0
22.5
15.0
7.5
0.0
(0m)
(3m)
(5m)
Temperature °C
18
16
14
12
10
8
6
(0m)
(3m)
(5m)
1/7/02
1/8/02
1/9/02
1/10/02
1/11/02
1/12/02
1/1/03
Post-transfer & Post-bath Intervals (Months)

Figure 1. Combined pen gross score (a), salinity (b) and temperature (c) profiles over the entire post transfer period (data collated from farm records). Dashed line represents timing of initial bath treatment.

The halocline reappeared briefly one to two days pre-bath but had dissipated by three days post-bath (Fig. 1b). Surface water temperatures fluctuated between 16°C & 19°C during the post-bath period (Fig. 1c). Mortality during the post-bath period did not exceed 0.01%.d^{-1}. Mean dissolved oxygen for 1 m – 5 m was 7.9 mg.l^{-1} and ranged from 5.6 mg.l^{-1} – 9.3 mg.l^{-1}. There were no significant blooms of algal species known to be harmful to salmon during AGD re-infection. Moon jellyfish (*Aurelia aurita)* were observed within the immediate site vicinity on numerous occasions from week 2 onwards.

Prior to initial bathing, characteristic epithelial hyperplasia resulting in lamellae fusion was the most prominent histological feature (data not shown). *Neoparamoeba* sp. trophozoites were adhered to these regions. Leucocytes, including macrophages, neutrophils and eosinophilic granule cells (EGC) were apparent within the central venous sinus, within hyperplastic tissue and upon lesion surfaces (macrophages only). Variable lesion sizes/stages were observed starting with fusion of 2 or 3 lamellae and in some cases lamellar fusion of up to 50% of an affected filament. Interlamellar vesicles were often present within lesions.

Initial freshwater bathing reduced the percentage AGD lesion affected filaments by 48% from 8.3% to 4.3% (Fig. 2a). Although this decline was not statistically significant, post-bath lesions were often fragmented and spongiotic. Residual lesion surfaces were also devoid of attached trophozoites, macrophages and tissue debris.

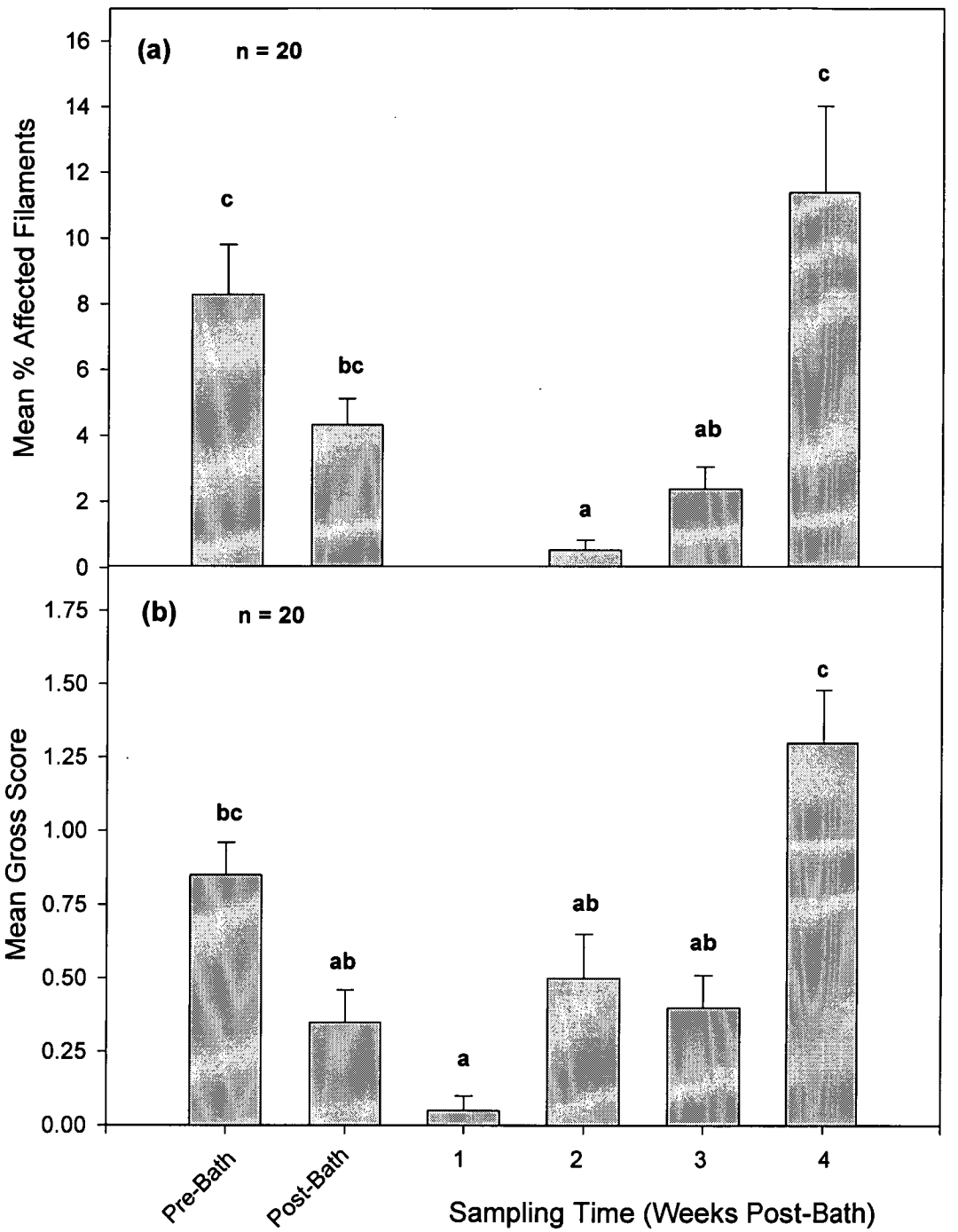

Figure 2. Mean percentage of AGD lesion affected filaments (a) and mean gross scores during sampling (pooled pen data). Values are means ± SE. Different letters indicate mean values that are significantly different.

No histological signs of AGD were apparent at one week post-bath. By two weeks post-bath, light levels of infection were present in 15% of sampled fish. Discrete numbers of focal lesions (2-10 fused lamellae) with adhered amoebae were observed. A similar pattern was observed the following week although lesions were often notably larger. In the final sampling week 75% of fish displayed histological signs of infection. A significant increase in AGD severity ($P<0.001$) was observed with 11.4% of filaments displaying AGD lesions equating to a 4.75 fold increase compared to week 2 (Fig 2a). AGD lesion patterns, during the final week of sampling, were identical to those described prior to bathing.

Generally, gross scores (Fig 2b) displayed a similar pattern to histological data. Significant increases ($P<0.05$) were apparent during the final sampling week compared to all other weeks except pre-bath. No statistical differences in gross scores were apparent from gills collected post bath through to three weeks post-bath. Re-infection progressed as trophozoites attached to normal gill epithelium causing localized epithelial desquamation and oedema in juxtaposed regions (Fig 3A). Where attached trophozoites were observed a hyperplastic reaction was evident and leucocytes were present within the central venous sinus (Fig. 3B). Upon larger lesions, the central surface regions were stratified with squamous epithelial cells and mucous cells. Trophozoite attachment to these regions was generally sparse. However, lesion margins continued to exhibit active hyperplasia with oedema, leucocyte infiltration and concentrated trophozoite attachment (Fig. 3C). The occurrence of inter-lamellar vesicles was also evident for the majority of advanced lesions.

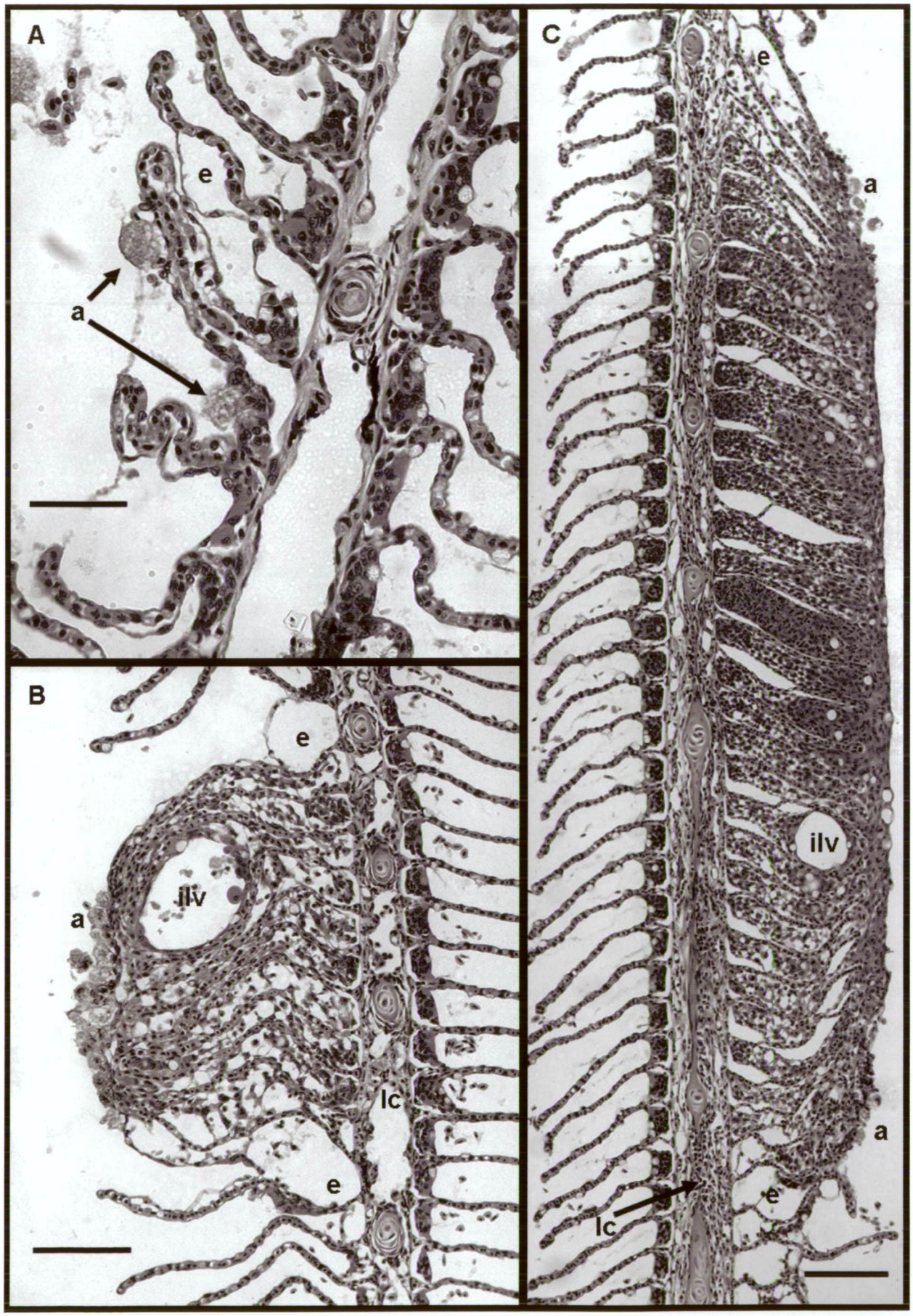

Figure 3 – legend next page.

Figure 3. (Previous page) Progression of AGD lesion formation following re-infection with *Neoparamoeba* sp. A – Adherence of trophozoites (a) to lamellar epithelium and associated localized oedema (e). B – Small lesion with attached trophozoites (a), peripheral oedema (e), inter-lamellar vesicle formation (ilv) and infiltration of leucocytes (lc) within the central venous sinus. C – Advanced lesion showing marginal oedema (e) with nearby trophozoites (a) and prolific leucocytic infiltration (lc). A single interlamellar vesicle (ilv) is present. Note absence of trophozoites from central lesion surface. Bars = 50 µm (A); 100 µm (B); 250µm (C).

Image analysis of histochemically and immuno-histochemically stained sections revealed no significant differences to branchial mucous cell (range = 23.5 – 32.5 cells/mm/logmass) or chloride cell populations (range = 75.3 – 88.2 cells/mm) over the sampling period.

A range of other (sometimes severe) gill lesions were encountered during the course of sampling, particularly from week 2 onwards. Hemorrhaging, detected macroscopically (Fig. 4A) and histologically (Fig.4B & 4C), occurred focally or in narrow, linear demarcated zones along or across many filaments (4A & 4B). This type of lesion was generally associated with substantial epithelial necrosis (Fig. 4C) and in many cases only one side of each filament was injured. Necrotic lamellae were hyper-perfused with erythrocytes (Fig.4C). Telangiectasis (data not shown) was often seen in association with hemorrhaging and necrosis although this feature also presented in the absence of other pathology.

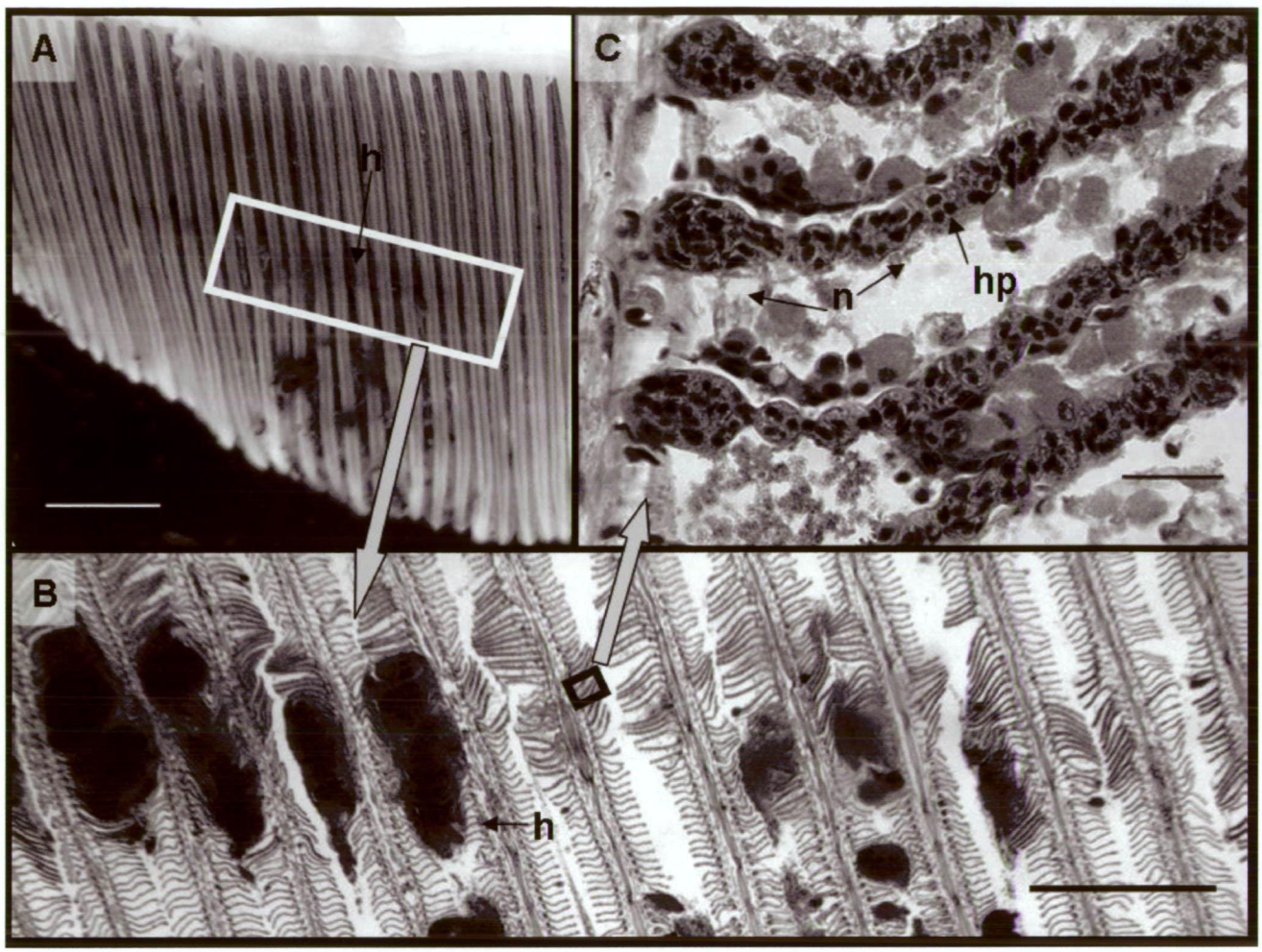

Figure 4. A - Haemorrhage (h) grossly evident upon fixed tissue sample. B – Histological section corresponding to highlighted region in A. Note haemorrhaging (h) and linear alignment of affected tissue across many filaments. C – Higher magnification from highlighted region in B. Note necrosis of lamellar and filamental epithelia and lamellar hyper-perfusion (hp) with erythrocytes. Bars = 0.5cm (A); 1mm (B); 25 µm (C).

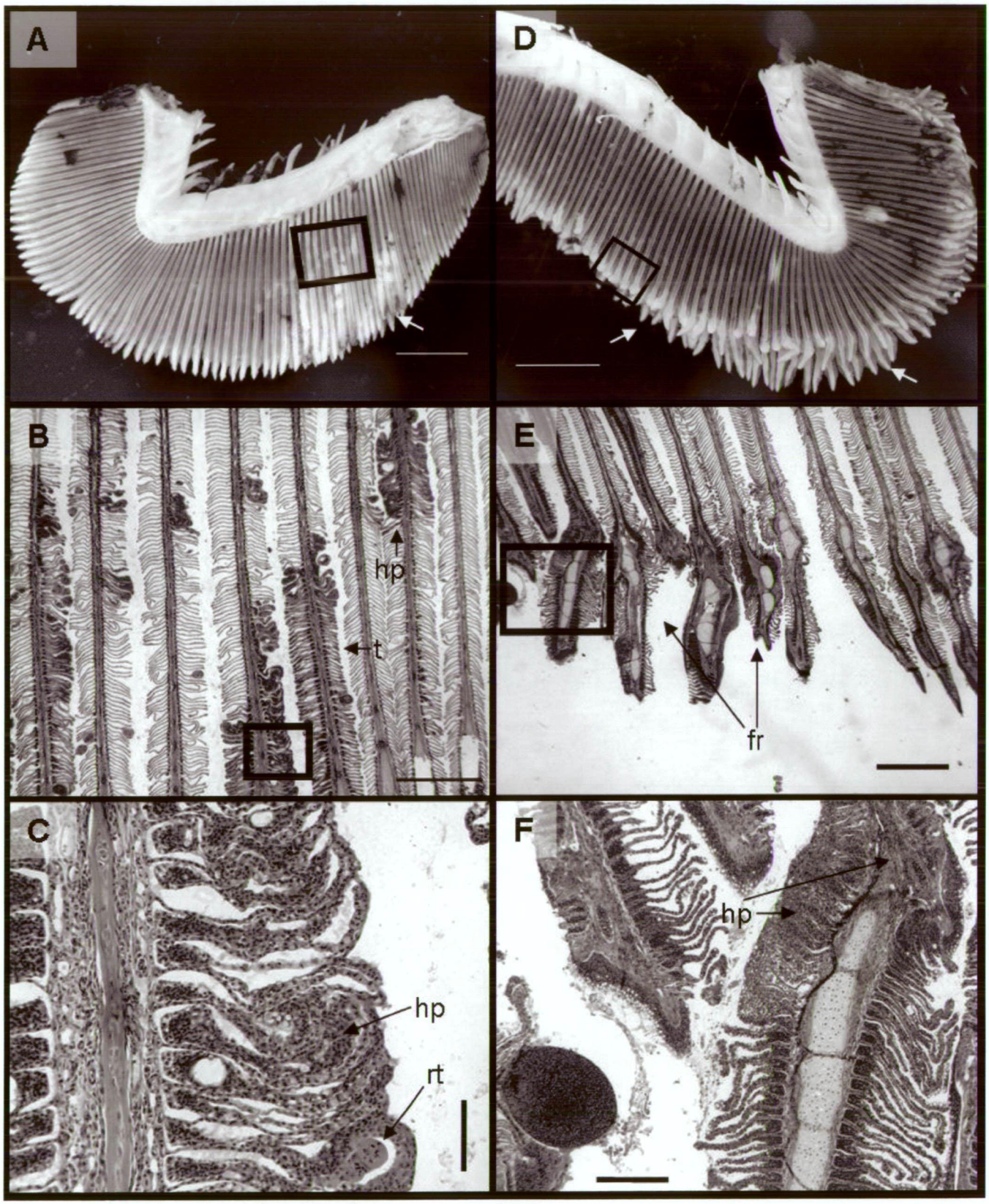

Figure 5 – legend next page.

Figure 5. (Previous page) A - Filamental thickening manifesting grossly as a large diffuse patch in the medial/ventral region of the affected hemibranch (white arrow). B - Histological section corresponding to highlighted region in A. Note regions of lamellar hyperplasia (hp) and lamellar thickening (t) due to epithelial hypertropy. C – Higher magnification of highlighted section in B. Lamellar hyperplasia (hp) and resolving thrombus (rt) are evident. D – Gross view of shortened and clubbed filaments (white arrows). E - Histological section corresponding to highlighted region in D. Note distal filamental restructuring (fr). F - Higher magnification of highlighted section in E. Endothelial and epithelial hyperplasia (hp) are depicted. Bars = 1cm (A&D); 1mm (B&E); 50µm (C); 250 µm (F).

Multi-filamental thickening (Fig 5A) presented histologically as hypertrophy and hyperplasia of the lamellar epithelia (Fig 5B & 5C). Within these regions many resolving thrombi were evident and associated with more pronounced hyperplasia (Fig 5C). In some fish the distal filamental regions were shortened and/or clubbed occasionally inclusive of the entire distal hemibranch region (Fig 5D). These areas were sometimes associated with necrosis and telangiectasis (data not shown). In more pronounced cases, filamental endothelial and epithelial hyperplasia had resulted in significant distal filamental restructuring (Fig 5E & 5F).

Although AGD lesions sometimes co-existed with the aforementioned pathology, there was no evidence of amoebae being directly associated with non-AGD type lesions.

6.5 Discussion

Salinity and temperature were the major factors facilitating the progression of AGD prior to both 1st and 2nd freshwater bath treatments, an observation consistent with previous studies (Rodger & McArdle 1996; Clark & Nowak 1999; Nowak 2001; Adams & Nowak 2003).

The histopathological sequence of host gill tissue response to attachment with *Neoparamoeba* sp, during the inter-bath period, was identical to that described for the infection of naïve salmon prior to initial bathing. The course of infection also progressed over the same period of time (Adams & Nowak 2003). However, the source of re-infection should be given due consideration. It was apparent during this study, immediately post-initial bath, that fresh water bathing had cleared lesions of attached amoebae and associated tissue debris. Total clearance of lesion adhered trophozoites has been previously described 24h post-bath (Clark *et al.* 2003). However, Parsons *et al.* (2001) indicated a 70% reduction of lesion attached amoebae immediately post-bath. Water hardness during bathing, which influences post-treatment survival of amoebae (Powell & Clark 2003; Roberts & Powell 2003), was not given in either study. Treatment efficacy, due to water hardness, may have influenced these outcomes. It is likely that re-infection is derived from a combination of amoebae remaining upon the gills after bathing (Parsons *et al.* 2001; Clark *et al.* 2003; Roberts & Powell 2003) and contact/adherence to gill epithelia with untreated trophozoites present within the water column (Douglas-Helders 2002). However, the latter source is probably the major influence upon re-infection rate. Attachment of waterborne trophozoites to healthy gill epithelia can occur in as little as 12 h - 24 h under experimental conditions with lesion development underway within 2 to 4 days (Zilberg & Munday 2000; Adams & Nowak 2004). Yet experimentally infected

salmon that were bathed and then left in filtered seawater did not display signs of amoebic re-infection for three weeks. The aforementioned fish were not re-exposed to the pathogen (Gross, Butler & Nowak 2003; Adams & Nowak, unpublished data). This suggests that bath tolerant *Neoparamoeba* sp. are unable to attach directly to the gill epithelium and induce lesion formation as rapidly as untreated waterborne trophozoites.

During the current study, there was no evidence of any gill lesions associated with *Neoparamoeba* sp. one week after bathing. Rapid recovery from hyperplastic gill lesions occurs in a few days for channel catfish (*Ictalurus punctatus* L.) and fathead minnows (*Pimepalus promelas* L.) (Yonkos, Fisher, Wright & Kane 2000; Darwish, Griffin, Straus & Mitchell 2002). Re-infection (defined as new lesion formation) did not occur until some time between one and two weeks post-bath. Over a 10 day sampling period, Clark *et al.* 2003 found no significant difference in the number of lesions/filament examined pre-bath through 1, 3, 5, & 10 days post-bath. Whether lesions were post-bath residuals, newly formed or a combination of both was not investigated. It is also possible that re-infection rate may have differed compared to our study. The summer of 1999/2000 (during which time the Clark *et al.* 2003 study took place) was particularly extreme in terms of low rainfall and high temperatures which may have facilitated a faster re-infection rate. Indeed, higher seasonal water temperatures correlate with higher numbers of trophozoites within the water column (Douglas-Helders 2002) and infection severity has been experimentally demonstrated as amoeba concentration dependant (Zilberg, Gross & Munday 2001, Morrison 2003, unpublished data).

Chloride cell populations did not change during the course of re-infection consistent with findings during AGD progression in naïve fish (Adams & Nowak

2003). Mucous cell populations, were also unchanged, contrasting with reported increases to this cell type concurrent with initial infection. The mean values throughout the re-infection period were comparable to later stages of initial infection. Clark *et al.* (2003) also found no changes to total mucous cell numbers during re-infection. It is possible that after recruitment in response to infection, higher populations of mucous cells remain for some time after disease alleviation. Unfortunately no published literature is available either refuting or confirming this hypothesis. The presence of other potential irritants, most notably moon jellyfish (*Aurelia aurita*) may have also influenced this finding.

The pathological features described for non AGD type gill lesions, noted during this study, bore some similarity to those described by Clark, Nowak, Handlinger, Munday & Percival (1997). Similarly, we were unable to ascertain the causative agent of this type of pathological change, although Clark *et al.* (1997) suggested coelenterates as a possible risk factor. In this study, moon jellyfish were present during the corresponding sampling weeks and a tentative causality is assumed. To the best of our knowledge, there is no published information describing gill damage due to coelenterate envenomation.

There was no evidence during this study that other gill lesions were parasitized by *Neoparamoeba* sp. trophozoites. However, it has been suggested that lesions resulting from jellyfish damage and clubbing and necrosis gill syndrome are sometimes rapidly colonized by *Neoparamoeba* sp. (J. Handlinger pers. comm. cited by Munday *et al.* (2001). Predisposing gill lesions were not a prerequisite for pathological development of AGD during field infection of naïve salmon prior to initial freshwater treatment (Adams & Nowak 2003). Jellyfish were not considered a risk factor for AGD by farm managers (Douglas-Helders, Saksida & Nowak 2003).

The apparent contrast in findings would suggest further work is required to resolve this issue.

As indicated, gross AGD pathology was reasonably consistent with that of histopathological diagnosis within the sample populations although some discrepancies in statistical significance were apparent. It is likely that non AGD histopathological lesions, that were evident macroscopically, incorrectly designated AGD lesions increasing the proportion of AGD grossly diagnosed cases during weeks one to three. In most cases, non AGD hyperplastic lesions were indicative of a regenerative process in response to necrosis and hemorrhaging as described above. Disparity between gross and histological diagnosis, during light level AGD infection, was initially described by Clark & Nowak (1999). The presence of non-AGD type lesions were identified as one of many sources of disagreement between gross and histopathological diagnosis of AGD (Adams *et. al.* 2004).

In summary, pathogenesis of gill re-infection by *Neoparamoeba* sp. mirrors that of initial infection and is driven primarily by salinity and temperature. Residual post-bath AGD lesions were not re-colonized by amoebae and lesions arising from non-amoebic insults did not influence amoebic re-infection observed within this study.

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CHAPTER 7 - General Discussion

7.1 Preamble

The broad aim of the research described within the previous chapters was to address the need for an improved understanding of AGD associated pathology during commercial culture of Atlantic salmon in Tasmania. Our previous understanding encompassed the major pathological features but not the more discrete aspects of gill pathology in terms of disease progression. A more resolute understanding provides the basis for not only differential diagnosis per se but also a foundation and/or reference for future research which is often dependent upon histopathological outcomes as an evaluative endpoint. As such, the context of this discussion is to relate the major findings from the previous chapters within the broader context (past, present & future) of AGD and to some extent other ectoparasitic fish diseases.

7.2 Re-defining the pathological model for AGD

7.2.1 Initial Host-Pathogen Interactions

The gill is a complex sieve-like structure designed to channel water through the confined interstitial regions between the secondary lamellae (Gilmour 1998). Initial colonization of the gill occurs subsequent to contact between *Neoparamoeba* sp. trophozoites present within the water column and the gill environment. As chapter 1 demonstrated, lesion distribution is apparently favoured within the low flow areas of the dorsal gill region. This indicates that disease progression may be influenced by flow dynamics within the opercular cavity. Recovery of bacteria from the gills was

more reliable when samples were collected from the dorsal gill region, the result was also attributed to reduced flow patterns (Ferguson 1989). Reductions to flow velocity, across the gills, may serve to not only increase contact time between the pathogen and gill but also influence the ongoing adherence of established amoebae populations. *In vitro* observations have shown that liquid cultures subjected to perpetual motion prevent the adherence of trophozoites to substratum (Martin 1985). It is therefore likely that due to the gills sieve-like structural complexity, amoebae are in effect filtrated from passing ventilatory waters. Prior to hyperplasia, amoebae were adhered to the proximal regions of the secondary lamellae within the relatively sheltered interlamellar spaces (Chapter 4). A similar, though more dramatic retention effect is seen during algal blooms where interlamellar spaces of exposed fish accumulate large numbers of cells (Jones & Rhodes 1994). When structural complexity of the gill arrangement was reduced due to advanced lamellar fusion, a reduction of numbers of adhered trophozoites was apparent (chapter 3), an observation consistent with other studies (Dyková *et. al.* 2001). The gill is the sole organ affected by AGD (Dyková *et. al.* 1995; Zilberg & Munday 2000) and to the best of this author's knowledge there are no published reports of epithelial hyperplasia of high flow/smooth structures such as the skin. *Neoparamoeba* sp. requires a surface negative charge for adhesion (Martin 1987; Adams, pers. ob.), therefore it is possible that the polyanionic properties (Shephard 1994) of mucus may play a role during initial adherence. Gill explants, treated with a mucolytic agent (hyaluronidase), had lower amoeba loads than untreated explants after trophozoite exposure both *in vitro* and *in vivo* (Butler and Nowak, submitted). This suggests that *Neoparamoeba* sp. may indeed have a requirement for the presence of mucus during adherence to the epithelium. *Acanthamoeba* sp. and *Entamoeba histolytica* (causative agents of *Acanthamoeba*

keratitis and enteric amoebiasis in humans), bind to host cells by lectin attachment subsequently resulting in cytolysis of the affected cells (Kain & Ravdin 1995; Petri and Shnaar 1995; Cao *et. al.* 1998; Petri *et. al.* 2002). It is presently unclear whether *Neoparamoeba* sp. possesses a similar carbohydrate mediated ligand-receptor attachment mechanism. Ultimately however, the physical introduction of *Neoparamoeba* sp. to the gill environment provides the opportunity for adherence to the gill epithelium to occur, leading to an initial host response.

7.2.2 Early AGD progression post-trophozoite attachment

Although the mechanism(s) for attachment remain unresolved, the preceding chapters clearly identified the requirement for trophozoite adherence as a precursor to subsequent lesion development (Chapter 4, 5 & 6). Importantly, this result was observed in the field where no previous observations of primary interactions, prior to epithelial hyperplasia, had been made. Field observations of early attachment were reproduced experimentally (chapter 4) and concur with previous experimental co-habitation infection which noted attachment to normal gill epithelium (Zilberg & Munday 2000). The pathogenic model is further refined by the observation of localized tissue responses (epithelial oedema and desquamation) evident at the point of trophozoite attachment. Such changes are consistent with that of amoebic infections in higher vertebrates such as *Acanthamoeba* keratitis and enteric amoebiasis in humans (Niederkorn, Alizadeh, Leher and McCulley 1999; Espinosa-Cantellano & Martinez-Palomo 2000). These features will assist histopathological interpretation of developing AGD lesions distinct from other stages of AGD lesions and/or non AGD lesions.

7.2.3 Hyperplasia and leucocytic infiltration

The results (Chapters 4, 5 &6), suggest that attachment of individual amoebae results in a cumulative hyperplastic response juxtaposed to amoebic attachment. As noted during chapter 4, amoeba numbers at locations of lesion development were increasing with time. This suggests amoebic proliferation occurs once attachment is established. Field observations (Chapters 4 & 5) indicated smaller lesions (at approx. 10 interlamellar units) were uniformly covered in trophozoites. Yet upon larger lesions, that occupy greater lengths along the filament, a pattern of peripheral attachment was evident. This was not considered a fixation artefact as localized changes indicative of amoebic attachment were not present centrally upon larger lesions (Chapter 5). Additionally, SEM observations showed healthy squamous epithelium with well defined micro-ridges in regions devoid of attached trophozoites (Adams & Nowak 2002 unpublished data). It was at the locations of marginal trophozoite-lesion attachment where desquamation and oedema were apparent indicating hyperplastic induction. Therefore it is concluded that lesion development is dependent upon proliferation and migration of trophozoites along the filamental regions. This concurs with *in vitro* studies upon *Neoparamoeba* sp. where both proliferation and migration only occurs when attachment is facilitated (Martin 1985; Martin 1987). During development of human amoebiasis, *E. histolytica* exploits its motility to advance infection and proliferation amongst surrounding tissues and their densities are generally concentrated at lesion peripheries (Sehgal, Bhattacharya & Bhattacharya 1996; Espinosa-Cantellano & Martinez- Palomo 2000). Further studies *in vivo* of the gill micro-environment will validate the hypothesis of an attachment-proliferation-migration strategy most likely responsible for lesion development in AGD.

As hyperplasia of the lamellar epithelium developed, leucocytes were observed infiltrating the central venous sinus (CVS) and regions of lamellar fusion. In previous studies the involvement of these cells was only briefly mentioned (Roubal *et. al.* 1989; Munday *et. al.* 1990). Chapters 1, 4 & 5 provide some clarification for not only the types of leucocytes present but also some indications pertaining to their respective roles and initial appearances. Macrophages had either migrated or differentiated to or at lesion surfaces by 24 – 48 hours and appeared to play a front line defensive role concordant with their primarily phagocytic nature (Secombes 1996). This is consistent with macrophage behaviour noted in other ectoparasitic studies (Barmanrokh & Woo 2001; Bennett and Bennett 2001; Colquitt, Munday and Daintith 2001). Neutrophilic granulocytes (referred to as neutrophils in the preceding chapters) were also consistently present during lesion formation; these cells were confined within the gill as there was little evidence of migration to lesion surfaces. Their major function appeared to involve infiltration into interamellar vesicles and surrounding hyperplastic tissues for removal (either directly or adjunctly) of trophozoites. Such a role was also observed by Bridle *et. al.* (2003) during experimental AGD. The systemic antibody response to AGD (Bryant *et. al.*1995) may arise from antigen presentation by macrophages and neutrophils (Secombes 1996; Dalmo *et. al.* 1997) that have interacted with trophozoites at lesion surfaces or trapped within hyperplastic tissue. Neutrophils have demonstrated a prominent role in defence against amoebic infections of higher vertebrates (Niederkorn *et. al.* 1999; Espinosa-Cantellano& Martinez-Palomo 2000). Another potentially important inflammatory cell detected but not previously reported for AGD, were eosinophilic granule cells (EGCs) (Chapters 2, 5 & 6). The presence of these cells has been reported for other persistent parasitic insults (Reite 1998; Treasurer and Turnbull 2000; Barmanrokh &

Woo 2001; Colquitt *et al.* 2001). It was apparent that the recruitment and activity of these cells was influenced by the adherence of *Neoparamoeba* sp. to gill tissue although their exact role and activation timing were not clear. Much conjecture is apparent within the literature regarding the morphology and functional role of these cells in fish (reviewed by Reite 1998).

Gauging the effectiveness of the innate immune response against gill associated *Neoparamoeba* sp. was beyond the scope of this study. However, chapter 6 indicated rapid re-infection with *Neoparamoeba* sp. following freshwater treatment suggesting resistance was not apparent. Likewise, laboratory trials showed that fish infected with *Neoparamoeba* sp. and subsequently treated with a commercially simulated freshwater treatment were re-infected without any apparent resistance being evident (Gross *et al* 2003; Adams & Nowak unpublished data). Manipulation of the innate immune response has been trialled in the field using the immune modulator levamisole although the results were largely inconclusive (Findlay & Munday 2000; Findlay, Zilberg & Munday 2000; Zilberg, Findlay, Girling & Munday 2000). A recent laboratory experiment demonstrated manipulation of the innate immune response by oligodeoxynucleotides, containing CpG motifs, which improved the survival of salmon affected with AGD (Bridle *et al.* 2003). Clearly the role of the immune response to AGD requires further investigation inclusive of cellular and non cellular processes.

7.2.4 Advanced infection

Advanced AGD lesions displayed a consistent pattern of squamation of the superficial epithelium upon lesion surfaces (less pronounced or absent at lesion peripheries) and recruitment of mucous cells to these regions (chapters 3, 5 and 6). Additionally, these areas of substantial epithelial rearrangement were rarely colonised by trophozoites.

This is likely to be indicative of a reparative "fortification" strategy adopted by teleosts against pathogenic or physical insults to epithelial integument. Re-epithelialization with squamous epithelial cells, upon the gills, was described by Bennet and Bennet (2001) around sites of attachment by caligid copepods. Treasurer and Turnbull (2000) observed areas of gill epithelial hyperplasia, where glochidia had attached, that were distinctly demarcated from normal gill tissue. The strategy employed bears some resemblance to the latter stages of wound healing (re-epithelialization response) of the skin integument shown by many fish species (Roberts 1989). Squamation of epithelial tissue within interlamellar cysts where trophozoites are internalized and destroyed was described in chapter 1. The desired objective of gill epithelial rearrangement, as a defence against AGD is the internalization/exclusion and destruction/isolation of the pathogen from susceptible tissue. The mid to late stages of AGD are likely the most important stages in terms of disease horizontal transmission. Chapters 5 & 6 described rapid increases to the severity and prevalence of AGD amongst the sampled pen populations prior to bathing. The shedding of trophozoites into the water column may result from sloughing of hyperplastic epithelia and mucus from the gills (Munday & Zilberg 2000) although this was not a prominent observation in the field (Chapters 5 & 6). Larger lesions deny trophozoites the opportunity to inhabit the sheltered interstitial space between secondary lamellae possibly resulting in removal due purely to the impact of water flow. Alternatively (or perhaps congruently), squamous epithelia in combination with concentrated mucous cell recruitment, may be an unsuitable domain for trophozoite adherence, an observation consistent with other ecto-parasitic infections (Urawa 1992; Buchmann & Bresciani 1998). Increases to mucous cell populations (chapter 5) were indicative of advanced infection and are consistent with

both field and experimental infections (Roubal *et al.* 1989; Munday *et al.* 1990; Munday & Nowak 1994; Zilberg & Munday 2000; Roberts & Powell 2003b). However, the role of mucus in not only AGD but other ecto-parasitic conditions is somewhat perplexing. As mentioned previously (7.2.1), it is possible that mucus may be a component of the adherence process for *Neoparamoeba* sp., yet in regions rich in mucous cells, trophozoite attachment was clearly diminished (Chapters 5 & 6). Also, the mucus layer is considered a barrier to infection (Shephard 1994) and contains a range of protective substances including lysozyme, lectins, proteinases and immunoglobulins (Alexander & Ingram 1992; Buchmann & Lindestrom 2002) that may influence the course of infection (Buchmann & Bresciani 1998; Buchmann 1999, Buchmann & Lindestrom 2002). As AGD develops, lesion surfaces periodically slough away mucus and hyperplastic epithelium (Zilberg & Munday 2000) and the viscosity of the mucus layer decreases (Roberts & Powell 2003b). The answer may lie in the fact that prior to infection the physical forces of removal (higher water flow exposure, sloughing and defensive mucosal substances) are not stimulated. Additionally, it is feasible that like *E. hystolytica*, *Neoparamoeba* sp. possess cysteine proteinases that degrade mucus thus facilitating attachment (Moncada *et. al.* 2003). The complexities of these aspects are considerable and require elucidation.

Although it would appear that the gills are able to defend to some extent against infection, the required hyperplastic response is ultimately counter productive leading to losses of respiratory surface area. The clinical severity of the observed pathology in chapter 5 (approx. 15% of AGD lesion affected filaments) was relatively minor and indicative of a proactive treatment regime. However, laboratory infections have often produced substantial lesions affecting up to 100% of filaments (Roberts and Powell 2003; Gross *et. al.* 2003, submitted; Adams & Nowak, unpublished data).

Since the advent of freshwater bathing and its resultant control of explosive mortality patterns and clinical respiratory distress, advanced cases of this nature are rarely seen during commercial culture (Munday *et. al.* 2001), However possible causes of mortality still warrant consideration. An interesting feature of larger lesions (Chapter 5) was the almost complete disappearance of chloride cells; the principal cell responsible for iono-regulation (Jurss & Bastrop 1995). It stands to reason that in fish with severe hyperplasia physiological disturbance would be apparent. Such disturbances were reported from field and laboratory infected fish which were found to be hypernatraemic (Munday *et. al.* 1990; Findlay 2001). The notion of hypoxic death due to respiratory surface area reduction has been rebuked (Powell *et. al.* 2000). However this notion remains untested upon fish exhibiting advanced hyperplasia attributable to AGD. The production of excess mucus may have detrimental physiological consequences. Powell *et. al.* (2000) observed a respiratory acidosis which was attributed to CO_2 diffusion inhibition from the gill as a result of excess mucus production. Chronically affected fish were hypertensive (Powell *et. al.* 2002a) and displayed altered cardiac morphology (Powell *et. al.* 2002b). It is likely that a combination/interaction of the above factors and possibly others not yet considered, both intrinsic and extrinsic, contribute to death.

7.2.5 Environmental Influences

Salinity and temperature were found to play an important role that appreciably influenced the severity and timing of AGD outbreaks (Chapters 2, 5 & 6). Clark & Nowak (1999) identified salinity and temperature as the primary and secondary environmental factor influencing AGD outbreaks. Outbreaks of AGD in Ireland were also simultaneous to increased salinity and record high temperatures (Rodger &

McArdle 1996). Densities of *Neoparamoeba* sp. in the water column, at commercial culture sites in the Huon estuary (Southern Tasmania), are higher in summer (Douglas-Helders *et. al.* 2003). During summer, farmed salmonids in Tasmania are exposed to high temperatures (17 – 20°C) above the optimal physiological range (Plumb 1994). Changing environmental circumstances can affect the ability of the host to resist disease or promote the pathogens ability to cause disease (Slauson & Cooper 1982). The immuno-regulatory capacity of fish is well documented as being temperature dependant and detrimentally affected by stress induced by thermal challenge (Corbell 1975; Rijkers 1982; Blazer 1991; Plumb 1994). Unfortunately, there is no published evidence detailing the effects of salinity and temperature upon the growth, survival or pathogenicity of Tasmanian isolates of *Neoparamoeba* sp. under controlled conditions. Such research is warranted and would provide a basis for predictive modelling at the production level.

7.2.6 A secondary or commensal pathogenic role for *Neoparamoeba* sp.?

The formative pathological model of AGD development suggested that *Neoparamoeba* sp. may preferentially colonize hyperplastic tissue (Nowak & Munday 1994, Zilberg & Munday 2000). The production of further hyperplastic tissue then encourages further attachment of amoebae and so on (Munday *et. al.* 2001). In contrast, the observed pathogenic role of gill infection with *Neoparamoeba* sp. in this study was of a primary nature as demonstrated by a confluent hyperplastic reaction in direct response to trophozoite adherence. Experimentally, pre-existing physical gill injury did not influence initial attachment of trophozoites (Chapter 4). Field results suggested there was no evidence of a commensal opportunism for *Neoparamoeba* sp. occurring where gill lesions were induced by other aetiological agents during infection (Chapter 6). Additionally, following treatment for AGD, amoebae were not

observed colonizing pre-existing gill lesions. Indeed, the associated severity of AGD infection is dependant upon the concentration of *Neoparamoeba* sp. within the water column (Zilberg *et. al.* 2001; Morrison & Nowak 2003, submitted). The accumulated evidence leaves little doubt of a primary role for *Neoparamoeba* sp. in amoebic gill disease.

However, the outcome of these studies does not necessarily exclude a secondary or commensal role as being biologically plausible. Occurrences of severe blooms of noxious aetiological agents such as harmful algae or jellyfish capable of inducing significant fish kills did not occur during field studies. It was upon histological examination of such events that suggestion of secondary pathogenic opportunism was made for *Neoparamoeba* sp. (Munday *et. al.* 2001). However this type of opportunistic behaviour was inferred from one off sampling during routine disease investigation; no sequential evidence has been forthcoming. The presence of bacteria, other amoebae or protozoa (ciliates, flagellates) have been noted during examination of infected fish (Dyková *et. al.* 1998; Dyková *et. al.* 1999). It was suggested that the presence of other microorganisms may possibly impart some influence upon the progression of AGD and is an aspect warranting further investigation.

7.2.7 Treatment & re-infection

Results from chapter 5 indicated that freshwater treatment successfully removed the offending parasite and partially augmented resolution of hyperplastic lesions. As for initial AGD development in naïve fish, subsequent reinfection displayed identical pathological progression post-treatment.

During freshwater bathing, the majority of trophozoites are most likely flushed from the gills and into the treatment water column as indicated by reductions in total

gill amoebae post-bath in other studies (Parsons *et al.* 2001; Clark *et al.* 2003; Roberts & Powell 2003a). However, Parsons *et. al.* (2001) and Clark *et. al.* (2003) observed survival of amoebae post bath and AGD has been experimentally shown to return post-bathing without re-exposure to seawater dispersed amoebae (Gross, Butler & Nowak 2003; Adams & Nowak, unpublished data). Salmon gills are capable of harboring amoebae with a population magnitude of 10^5 -10^6 cells (Clark *et. al.* 2003; Roberts & Powell 2003a). It is probable that vast numbers of surviving amoebae are released back to the environment post treatment, possibly providing another mode of re-infection.

Water hardness is the primary determinant of freshwater bathing efficacy and manipulation has shown to be effective in augmenting reductions to gill associated amoebae populations post bath (Powell & Clark 2003; Roberts & Powell 2003a). Interestingly, population estimates of gill isolated amoebae did not correlate with histological severity assessed over a ten day period post bath (Clark & Powell 2003). However, the methods adopted for quantification (trypan blue exclusion staining upon gill mucous scrapings) are not specific for *Neoparamoaba* sp. Other non-pathogenic amoeba have been found associated with the gills of both healthy and infected Atlantic salmon and other fish species (Howard & Carson 1993; Dyková *et. al.* 1999).

As mentioned during chapter 1, bathing frequency has increased 5 fold over the last decade. It has been hypothesised that survival of amoebae post-bath may be selecting for a freshwater tolerant strain(s) (Powell & Clark 2003) or that perhaps continued renewal of infection has increased the virulence of the organism's population (Findlay 2001). However, from a historical perspective, the most striking trend in Tasmanian salmonid aquaculture has been the cumulative increases to salmon biomass at the farms. It is now clear that salmon are the primary host for proliferation

and subsequent transmission of the pathogen and that AGD severity is cell concentration dependent (Zilberg *et. al.* 2001; Morrison *et. al.* 2003). Therefore it is extremely likely that the underlying reason for an increased mitigation frequency is that environmental populations of *Neoparamoeba* sp. have likewise increased. As it is unlikely that host-pathogen contact can be prevented at the commercial level, it is imperative that future treatment strategies are developed to produce a "total kill" of amoebae, both associated with the gill and those liberated during bathing.

7.3 Future Considerations

7.3.1 Diagnostic implications

Chapter 3 specifically described the appearance of gross lesions upon the gills of AGD affected fish. This has improved our understanding of the condition macroscopically and provides for a more accurate pathological description of gross changes other than "white mucoid patches or spots". Additionally, the reasons for agreement (or discrepancy) between gross assessment and histopathology have been clearly defined. The latter result in particular may have implications for other forms of AGD assessment such as IFAT and immuno-dotblot used for non-destructive sampling. The aforementioned methods indicate either pathogen presence or antigen presence but do not indicate that the disease itself has manifested. Both methods, which require scraping mucus from the gills, may be enhanced by targeting grossly affected regions (which have been verified as the sites of hyperplastic lesions and trophozoite attachment). Where destructive sampling is not possible, an indication of gross change combined with detection of the pathogen or antigen would provide an enhanced diagnosis as opposed to pathogen/antigen presence as a sole indicator of

disease. Additionally, it is essential that due consideration is given to extrinsic factors preceding sampling to further assist diagnostic interpretation (eg. prevailing environmental circumstances, disease stage, time from last treatment & presence of other irritants). It would be beneficial that a case definition is provided when making an inference from observed results, particularly when multiple methods of disease assessment are used simultaneously.

7.3.2 Refined Methodologies

To re-define the pathological model of AGD, some novel and refined investigative approaches were utilised to quantitatively and qualitatively describe the progressive pathology associated with AGD. Some of these adopted approaches may well serve to enhance the usefulness and accuracy of further fish disease research.

The use of temporal sampling strategies is a key component to understanding disease progression (Slauson & Cooper 1982) and formed a crucial part of the previously described research (Chapters 4, 5 & 6). Image analysis provided an accurate, reproducible, less subjective and more powerful method of quantifying host cell populations and has much potential for future development. Adaption of immuno-markers (eg. PCNA & M30 [Chapter 5]) provided new information pertaining to cellular responses of the diseased state. Mammalian immuno-marker availability is rapidly expanding and provides a host of options for future research particularly immunological aspects and cell cycle processes. Some such markers have been found to cross react with peripheral blood leucocyte populations from fish (Cook *et. al.* 2001) and further scope for this type of work is limitless.

7.3.3 Further research

The potential for future research into AGD is seemingly limitless, particularly in view of the extensive amount of literature that has been generated for amoebic infections of higher vertebrates. For this reason, suggested topics for future research are confined to that which may further improve our understanding of AGD pathogenesis.

Substantial information within the literature pertaining to amoebic infections of higher vertebrates is readily available (eg. acantha keratitis and enteric amoebiasis). As discussed, the adherence mechanism(s) for *Neoparamoeba* sp. is poorly understood, using the above diseases as a comparison would provide a useful starting point for further understanding of host-pathogen primary interactions in AGD. The perplexing role of mucus in AGD development was mentioned and is another aspect which requires investigation.

To date the production of a cultured pathogenic strain of *Neoparamoeba* sp. has not been forthcoming. It is likely that alterations to the nutritional profile of the organism's food source play an important role in its virulence as is the case for *Paramoeba invadens* (Jellet & Scheibling 1988). It is possible that mucus and associated cellular debris are utilised as an attachment component or food source prior to, or during infection (Roberts 1989, Padilla-Vaca, Ankri, Bracha, Koole & Mirelman 1999). The development of a pathogenic cultured strain of *Neoparamoeba* sp. would be a vital step in vaccine development (work in progress) and a crucial advance toward understanding the precise mechanisms of pathogenesis for this organism.

There is an inherent need for further investigation of factors (both extrinsic and intrinsic) that potentially influence the course/severity of AGD progression.

Such factors include salinity, temperature, immune status, husbandry practices and the presence of other gill-microorganisms.

Collectively, further knowledge of the above facets of AGD development would place researchers in a better position for future mitigation of the condition and provide valuable scientific information for fish disease studies in general.

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