Why fish die - the treatment and pathophysiology of AGD

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2001/205	Why fish die – treatment and pathophysiology of amoebic gill disease

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OBJECTIVES:

1. To establish an on-going laboratory source of AGD affected fish
2. To establish and validate a controlled infection/challenge system
3. To understand how AGD affects the respiratory and cardiovascular systems of Atlantic salmon.
4. To determine how environmental parameters interact with AGD pathophysiology. In particular the interaction of temperature, oxygen, salinity and carbon dioxide.
5. To develop and test new chemical or pharmaceutical treatments for the control of AGD.
6. To optimise the efficacy of current treatments by minimising the physiological impact on the fish.
7. To determine the efficacy of water softening agents and repeated bathing of fish in artificially softened water on the control of AGD compared with conventional (hard) freshwater water bathing practices.
8. To examine the potential for short-term high concentration treatments of AGD affected salmon using hydrogen peroxide under laboratory conditions.

NON-TECHNICAL SUMMARY:

OUTCOMES ACHIEVED

There has been partial adoption by the aquaculture industry of some aspect of this project such as the use of soft freshwater sources. Other aspects of this project have identified several treatments with potential for commercialisation for the control of AGD. These treatments include improvements in freshwater bathing, novel chemical disinfectants and potential in–feed treatment compounds to alleviate the impact of AGD. This project has developed a reproducible laboratory challenge protocol for studying amoebic gill disease. In addition the project has identified a primary cause for AGD-related mortality of Atlantic salmon. Two PhD students and a post-doctoral fellow were involved in this project.

Prior to this project, the study of amoebic gill disease patho-physiology was confined to using either fish infected by cohabitation or else clinically infected on fish farms under commercial conditions. This project developed a standardised process for acclimating Atlantic salmon smolts to seawater and infecting those salmon with *Neoparamoeba* sp. in the laboratory. This produced a reliable and repeatable challenge that could re-create AGD in the laboratory within 2-3 weeks. Although it developed more quickly than infections under field conditions, this condensed infection provides a tool with which to study the physiology of the disease and the efficacy of candidate treatments.

This project characterised the pathogenesis of AGD under laboratory conditions and supported suggestions that respiratory compromise was a minor cause of mortality in AGD affected salmon. Although the resting metabolic rate of the fish is increased with infection, probably reflecting an increased energetic cost of disease, the main cause of mortality appears to be due to acute cardiovascular compromise. Studies showed that increased vascular resistance (hypertension: high blood pressure) resulted in circulatory failure in AGD affected salmon. This same pathology was not however, seen in less susceptible salmonids such as rainbow trout. The effects of the hypertension could be partially reversed using drugs that lower blood pressure.

The role of mucus in AGD was closely studied. Fish affected by AGD showed a reduction in the viscosity of mucus, analogous to a “runny nose” which would slough off the offending *Neoparamoeba* sp. parasite from the gills. This was reflected in biochemical and histochemical changes in the composition of the mucus both during seawater acclimation and in response to infection in salmonids.

The project identified several potential improvements to bathing as a treatment for AGD. The use of softened water enhances mucus sloughing and is more effective at killing *Neoparamoeba* sp. Similarly, replacement of freshwater with a chloramine-T treatment in seawater may offer an alternative to freshwater bathing, especially as an emergency treatment or for farms that have limited access to freshwater. The use of artificially softened water is now at the point of commercial adoption by the aquaculture industry, and the use of chloramine-T in seawater is close to that point

The use of in feed amoebocides or treatments that help to overcome the effects of AGD were also tested. *Neoparamoeba* sp. are resistant to many families of anti-protozoal drugs, although at least in vitro, *Neoparamoeba* sp. was sensitive to the drug bithionol. In addition, the use of mucolytic drugs to enhance mucus sloughing and reduced mucus viscosity showed promise in retarding the onset of AGD. Also, the use of nutritional supplements Aquacite and Betabec, maintained feed intake and growth in AGD affected fish under laboratory conditions.

In conclusion, this project has identified a primary cause of AGD related mortality and several potential treatment options. This has significantly increased our understanding of the pathophysiology of the disease as well has providing avenues for improvements in the commercial control of the disease.

KEYWORDS: Amoebic Gill Disease, *Neoparamoeba* sp. Atlantic salmon, aquaculture, physiology, treatment.

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Photographs and diagrams courtesy of Dr. Mark Powell, Dr. James Harris, Dr. Rick Butler or Mr. Tim Green unless otherwise indicated.

BACKGROUND

Amoebic Gill disease

Amoebic gill disease (AGD) affects sea-caged salmonids (Atlantic salmon, *Salmo salar* and rainbow trout, *Oncorhynchus mykiss*) in Tasmania, Australia (Munday et al 1990), Ireland (Roger and McArdle 1996; Palmer et al. 1997), France and New Zealand (J. Carson, Tasmania DPIWE pers. comm.). AGD has also been reported in Chile, Canada and the Pacific Northwest of the United States (Nowak et al. 2002) where it continues to be a significant problem to the industry since changing from growing Chinook salmon (*Oncorhynchus tshawytscha*) to growing Atlantic salmon. A single outbreak was originally reported in coho salmon, *Oncorhynchus kisutch* in Washington State and California in the USA (Kent et al. 1988) and ongoing disease and mortalities have been reported (Douglas-Helders et al. 2001). AGD has also been diagnosed in turbot, *Scophthalamus maximus* as well as sea bream, and sea bass, *Dicentrarchus labrax* in the Mediterranean (Dykovà et al. 2000).

Pathophysiology of AGD

The amphizoic amoeba *Neoparamoeba pemaquidensis* and the newly described species *N. branchiphila* (Dykovà et al. 2005) are believed to attach to the surface of the gills and in doing so cause a localised irritation of the gill epithelium. In response to this irritation, the fish produces large amounts of mucus and a localised hyperplastic response of the gill epithelium occurs. This hyperplasia leads to a fusion of adjacent gill lamellae and a localised reduction in the functional gill surface area. Theoretically, this would lead to a subsequent hypoxaemia that may account for mortality due to AGD. However, this is not the case! Powell et al. (2000) have shown that fish with AGD appear to have a reduced arterial oxygen tension ($P_{a}O_{2}$), but that oxygen uptake capacity does not appear to be adversely affected under normoxic conditions. Interestingly, carbon dioxide excretion across the gill appears to be adversely affected, a function of carbon dioxide excretion being more diffusion limited compared with perfusion limited oxygen uptake (Powell and Perry 1999). This accumulation of carbon dioxide results in a respiratory acidosis (lowering of blood pH due to carbonic acid accumulation) (Powell et al. 2000, Powell et al. 2001). Even under hypoxic conditions, where oxygen uptake is lightly impaired in AGD

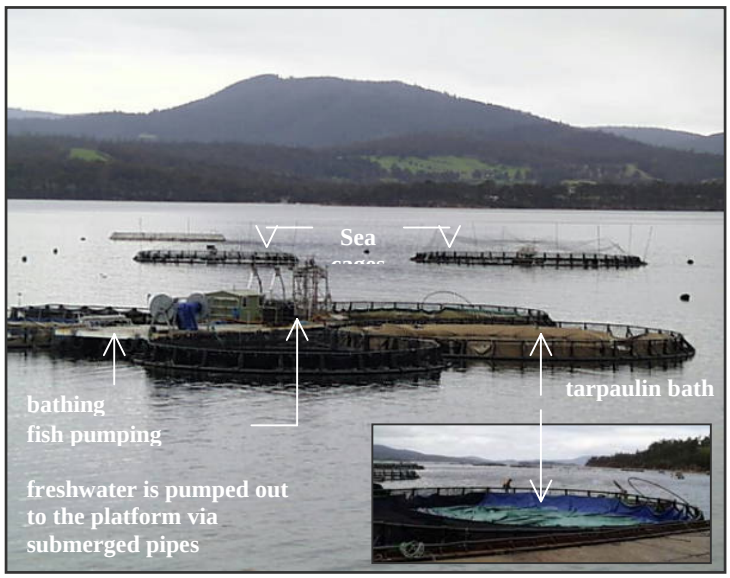
affected fish (Fisk et al. 2002), the physiological responses of the salmon appear to mitigate the effects of AGD in terms of blood gas transport (Powell et al. 2000). So then why do fish die? AGD affected salmon, when challenged by subjecting them to hypoxia (25% air saturation) for 20 min, had poor survival in the subsequent 24 h in normoxic water compared with unaffected fish (20% versus 90% respectively) (Fisk et al. 2002). This suggested that respiratory failure was not a primary cause of mortality, rather survival from stress in AGD affected fish may be linked to cardiovascular or neurological dysfunction.

It is possible that AGD affects the cardiovascular system of fish. The respiratory and cardiovascular systems of fish are intimately linked. Small changes in vascular resistance or in cardiac performance can have dramatic effects on the amount of blood flowing through the systemic or branchial vasculature. Indeed, AGD does appear to have some impact upon the cardiovascular system. Powell et al (2002a) showed that Atlantic salmon with a history of heavy AGD have altered cardiac morphology with more elongate ventricles and a thicker compact muscle layer. However, there was no overall increase in cardiac somatic index or ventricular mass compared with fish from the same cohort but that had experienced less AGD. This suggests that the changes in shape and muscle distribution occur at the expense of the internal mass (trabeculae), potentially increasing the internal volume of the blood being held in the ventricle. How this may impact upon the physiological performance of the heart in light of a *Neoparamoeba* sp. infection (AGD) is unknown. In another study, Powell et al (2002b) found that fish clinically affected by AGD were hypertensive, with significantly higher dorsal and ventral aortic blood pressures than reported for other salmonids. After freshwater bathing, the blood pressures returned to within a normal range. This suggests that AGD does have an impact upon the cardiovascular system and raises the possibility that the presence of *Neoparamoeba* sp. may contribute to the pathology, perhaps through the production of a systemic exotoxin. However, this remains to be investigated further.

Freshwater bathing treatments for AGD control

To date the most effective control method for AGD is a 2-3 h freshwater bath. Cages of fish are towed from their grow-out location to a bathing site. The fish are transferred (by silkstream pump, airlift or fish elevator) from their holding cage to a

tarpaulin-lined cage containing up to 1 ML of freshwater (stocking densities of 30-45 kg m^{-3}). Initial oxygen concentrations vary within the industry (normally 110-150% air saturation) but may approach 200% air saturation. The oxygen level is either maintained throughout the bath or allowed to slowly decline to levels approaching those of ambient (90-100%) by the end of the bath. After a 2-3 h bathing period, the tarpaulin liner is pulled away and the fish are released into the cage which is then towed back to its original grow-out location (see Fig. 1). The fish appear to tolerate the osmotic and hyperoxic shock physiologically (Powell et al. 2001) that appears to remove gill amoebae (Parsons et al. 2001). Clark et al. (2003) demonstrated that although freshwater bathing does appear to remove amoebae (most of which are *Neoparamoeba* sp.) from the gill and the incidence of viable cells is reduced, recolonisation of the gills can occur in as little as 10 days post-bathing. This is comparable with previous data that suggested that re-infection occurred within a month (Clark and Nowak 1999).



Schematic diagram of freshwater bathing

AGD-affected fish (sea-pen) —*fish pump*→ freshwater bath (tarpaulin lined sea-pen) —*~3 hours*→ liner pulled from under sea-pen to release freshwater

Figure 1. Infrastructure involved in commercial freshwater bathing fish on an Atlantic salmon farm lease in southern Tasmania, and schematic diagram of the process involved in freshwater bathing (after Roberts 2004).

Water chemistry and its impact on freshwater bathing

The basis for freshwater bathing is believed to be that *Neoparamoeba* sp. cannot tolerate freshwater, is osmotically compromised and subsequently killed. However, it has been shown that water from some freshwater dams (the same water used by the fish farms for freshwater bathing) was ineffective at killing *Neoparamoeba* sp. in crude gill isolates *in vitro* (Clark et al. 2003). The high ionic content of the dam water was believed to cause this result. The ionic environment of the freshwater has a significant impact upon the potential efficacy of the freshwater bath to kill *Neoparamoeba* sp. In addition, Best (2002) showed that the cultured strain of *Neoparamoeba pemaquidensis* (PA027) could tolerate dilute seawater (10%) for up to 9 h *in vitro*. This suggests that even if amoebae are remove from the gill of AGD affected fish, they may survive in the bath and resultant dispersed bathing water for a prolonged period of time, with the potential to re-infect the same or other fish in the vicinity of the bathing site.

Investigations have shown that the calcium and magnesium concentrations of the bathing water are primary factors facilitating the survival of *Neoparamoeba* sp. in freshwater (Powell and Clark 2003). Factors such as pH and oxygen content do not appear to play significant roles. However, these tests were conducted on freshly isolated gill amoebae from AGD affected fish and mucus-free samples were not obtainable. The influence of gill mucus on the protection of *Neoparamoeba* sp. from osmotic or ionic stress in freshwater baths is not clear. Best (2002) suggested that mucus may afford some protection of *Neoparamoeba* sp. with fish mucus binding divalent cations (such as calcium and magnesium) that may provide a semi-protective microenvironment for the amoebae once removed from the fish.

Current bathing practices appear to be inadequate for the sustained control of AGD. The large degree of variability in the chemistry of the source water used by farms for freshwater bathing (Parsons et al. 2001) would suggest that the efficacy of freshwater bathing may vary from farm to farm. Water chemistry must be considered when developing improved strategies for improved use of freshwater for AGD control.

Chemical treatments for AGD control

A number of chemical treatments have been screened in search for treatments for AGD (Howard and Carson 1994). These studies yielded a number of potential candidates but concern was expressed with regard to target animal toxicity (salmon toxicity) or potential environmental impacts. Freshwater appeared to be the most suitable treatment, however, its efficacy and suitability for use in the present day is being questioned (see above).

Hydrogen peroxide proved useful as a potential chemical treatment at concentrations known to have low toxicity to salmonids in seawater and freshwater (Howard and Carson 1994; Cameron 1994a; Powell and Perry 1997a). However, this treatment strategy was abandoned because it was believed to have a low margin of safety to smolt in seawater (Cameron 1993; 1994b). The use of hydrogen peroxide as well as chlorine-based oxidising disinfectants (chlorine dioxide and chloramine-T) has been examined as potential additives to freshwater baths (Clark and Powell 2004). Under the treatment conditions tested, hydrogen peroxide appeared to be the least effective treatment although both chlorine dioxide and chloramine-T showed promise in terms of their efficacy at removing gill amoebae from bathed salmon in freshwater. Both unactivated chlorine dioxide (25 ppm) and chloramine-T (10 ppm) reduced the gill amoebae numbers of AGD affected salmon by 50% of that which could be achieved by freshwater alone (Powell and Clark 2004). However, at higher concentrations potential toxicity issues may limit their use under the same experimental conditions (3 h exposure at ~19^{o}C and 150% air saturation). Chlorine dioxide is approved for use in the seafood processing industry as a biocide. Residue levels of 10 ppm are permitted in packaged water and 1 ppm in food, the same as for free chlorine. Since chlorine dioxide is activated to liberate reactive oxygen and chlorine when the pH is lowered, its potential for use in aquaculture is high since freshwater lower the water pH by over 1 pH unit (Parsons et al. 2001).

Chloramine-T (n-chloro-*para*toluenesulphonamide) is a biocidal disinfectant that breaks down by hydrolysis and photolysis to *para*toluenesulphonamide and a hypochlorite (OCl^-) ion (Booth and McDonald 1988). As such, the hypochlorite ion exerts its biocidal activity similarly to that of hypochlorite where prokaryote and eukaryote cell membranes are damaged through lipid peroxidation (Venkobacker et

al. 1977). Chloramine-T is widely used as a disinfectant, being more stable that hypochlorite in solution and thus its disinfectant properties are more sustained. Unlike hypochlorite, which readily reacts with organic material, chloramine-T degradation is exacerbated by organic matter (termed the chlorine demand) and the resultant biocidal activity is mitigated. In freshwater the toxicity of chloramine-T to fish including salmonids is low and is enhanced by low pH and low water hardness (Bills et al. 1988a and b). Although the impact on fish physiology is minor, Powell and Perry (1996; 1998) demonstrated that chloramine-T and the degradation product *para*toluenesulphonamide caused transient respiratory and acid-base disturbances as well as ionic disturbances in the gills of trout at therapeutic concentrations (9 mg L^{-1}). However, these ionic disturbances could be rectified by low concentrations of salt in the environment (Powell and Perry 1997b). The facts that chloramine-T kills *Neoparamoeba* sp. *in vitro* at 10 mg L^{-1} and is widely used even as a disinfectant for drinking water with low toxicity to target and non-target animals including humans, suggest that it is a potential candidate for use in AGD control in salmon.

NEED

There is an urgent need to develop novel treatments that would reduce the impact of AGD on the Atlantic salmon aquaculture industry in Tasmania. A detailed cost/benefit analysis for the Aquafin CRC AGD project was undertaken that gave a Net Present Value of the economic benefit of $21.6M, benefit/cost ratio of 5.3. The current project was designed to deliver short-term solutions and provide information and advice to the industry on a regular basis.

This project is part of an integrated research program, with deliverables not only aligned with the development of novel treatments, but also with effective vaccine development, management and other control methods. Developing and maintaining standardised infection is included in this subproject, and it will be crucial for vaccine testing and experimental work within the host-pathogen interaction project (2001-244) and the model development – epidemiology of AGD project (2001-245). The development of quantitative experimental challenge model is an essential prerequisite to vaccine research and novel treatment testing. Without the quantitative experimental model it is very difficult to compare the effectiveness of treatments or vaccines tested in separate experiments. Additionally, the controlled laboratory infection will provide crude gill isolates to researchers working in other projects.

The growth of the industry and apparently declining effectiveness of freshwater bathing have resulted in the need for the development of new treatments that will either aid in improving current freshwater bathing technology, or offer completely new avenues for the treatment of AGD in Atlantic salmon. Effective treatment of the disease can only be achieved if we understand the physiological and pathological processes at work. To this end it is imperative that we understand:

1. The effect of the parasite on the normal physiology of the salmon resulting in mortality
2. The effect of the treatment on the parasite
3. The effects of the treatment on fish parasitised to different degrees (ie potential toxicity side effects of treatments).

Successful treatments will be rapidly adopted by the industry given the magnitude of the problem and cost of freshwater bathing technology. Advances in the development of either additives to the current freshwater baths or freshwater bath replacement by a less time consuming chemical treatment will ensure that treatment and control of AGD as a constraint to Atlantic salmon production in Tasmania is reduced.

OBJECTIVES

1. To establish an on-going laboratory source of AGD affected fish
2. To establish and validate a controlled infection/challenge system
3. To understand how AGD affects the respiratory and cardiovascular systems of Atlantic salmon.
4. To determine how environmental parameters interact with AGD pathophysiology. In particular the interaction of temperature, oxygen, salinity and carbon dioxide.
5. To develop and test new chemical or pharmaceutical treatments for the control of AGD.
6. To optimise the efficacy of current treatments by minimising the physiological impact on the fish.
7. To determine the efficacy of water softening agents and repeated bathing of fish in artificially softened water on the control of AGD compared with conventional (hard) freshwater water bathing practices.
8. To examine the potential for short-term high concentration treatments of GAD affected salmon using hydrogen peroxide under laboratory conditions.

SECTION 1: LABORATORY INFECTION AND INFECTION CHALLENGE

METHODS

Laboratory source infection

In order to provide a regular supply of infective gill amoebae (*Neoparamoeba* sp.) it was necessary to set up an active infection that would continue by cohabitation of AGD affected and non-affected fish. The laboratory source infection was established in a recirculating 3000 L seawater system comprising of a 2 m^3 Rathburn tank, a trickle tower biofilter and sump (approximate volume 1000 L). The water was recirculated with temperature control to ensure a temperature of 15-18^oC could be achieved (Fig. 2).

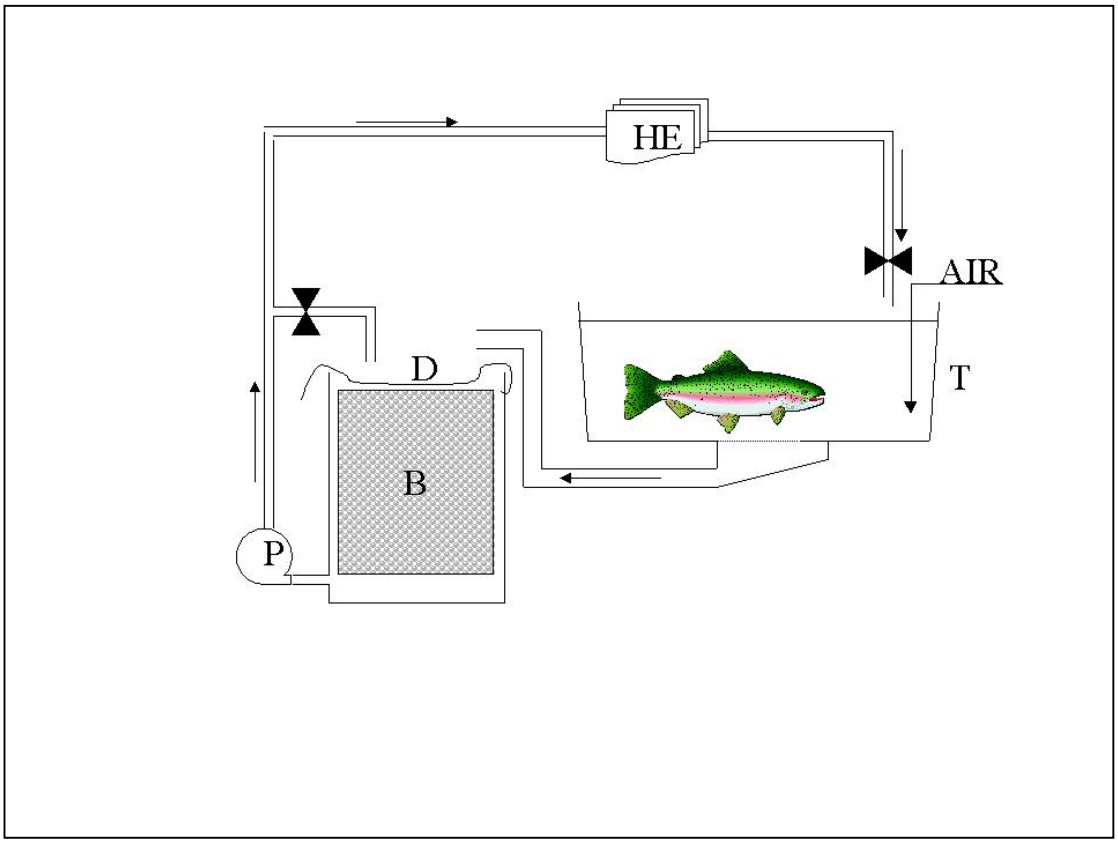


Figure 2. Schematic of the recirculating water fish culture unit for maintaining the laboratory infection. T represents a Rathburn culture tank, D is Dacron^{TM} matting for solid filtration, B is a biological filter containing biological filter media ("Bioballs"), P represents the circulating pump (Onga 413) and HE represents a 10 kW heat exchanger.

Seawater acclimated salmon (see below) were infected with raw gill isolates from AGD affected salmon obtained as described by Powell and Clark (2003) from the Huon Aquaculture Company. The initial infective dose was in excess of 2500 cells L^{-1}.

Rearing and seawater acclimation of fish

Diploid, mixed sex Atlantic salmon smolts and post-smolts of between 80g and 300g were most frequently used for the laboratory source infection. Similarly fish of a similar size were used in challenge experiments (see below). During a period of April-August each year, the availability of spring smolts and post-smolts was limited due to the commercial production cycle. Consequently, during this period, diploid, out-of-season photo-manipulated smolts or marine pre-smolts were used.

Laboratory challenge

Diploid spring smolt fish used in all experiments of this project and fish that were used to maintain the infection source tank were acclimated according to the following scheme. Fish were transferred to a 4000 L acclimation tank of identical configuration to that in figure 1. The only exception was that the recirculating system also incorporated a Venturi-driven foam fractionator. The water salinity was increased daily 5 g L^{-1} over a period of 7 days by gradually adding seawater and seasalt. During this acclimation period fish were offered food. Total ammonia-nitrogen (NH_3-N) concentrations were monitored in the acclimation tanks system twice weekly using a poolside color test kit (Australian Pet Supplies Pty Ltd, Smithfield NSW). If NH_3-H concentrations exceeded 1.0 mg L^{-1}, then a 50% exchange of the water was made and the acclimation procedure continued. After 7 days of acclimation, (salinity 35 g L^{-1}), fish were maintained for a further 3 days prior to experimentation.

Laboratory challenges consisted of maintaining fish in replicated recirculating water systems (Fig 2). Each module consisted of a biological filter and sump (300L), circulating pump (Onga 413), heat exchanger (chilled freshwater line passed through a 23kW heater chiller unit), header tank (300L) and three culture tanks (700L). A foam fractionator was attached to each module as necessary to ensure optimal water quality (colour). Suspended solids were removed by Dacron™ matting laid over the surface of the biological filter. The water level in each module was regulated by an external standpipe (not shown) and each culture tank was supplied with an individual air line and diffuser. Tank oxygen levels were maintained in excess of 90% air saturation.

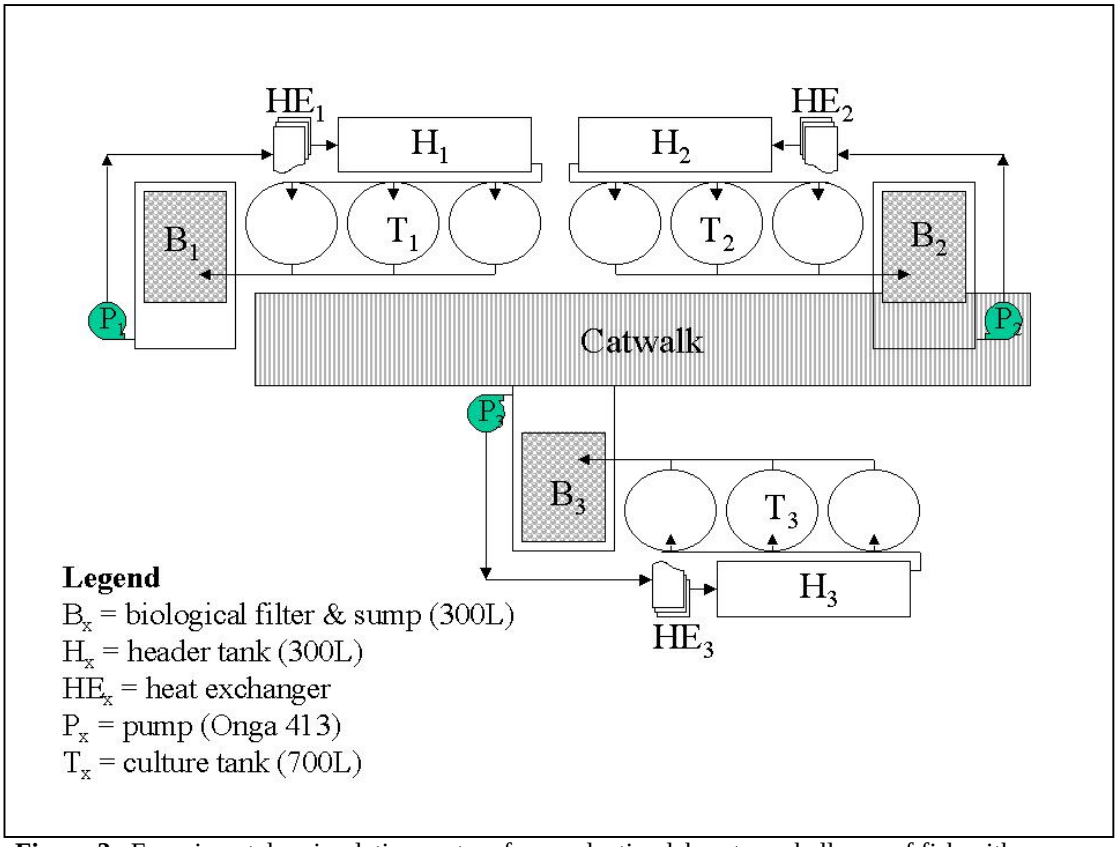


Figure 3. Experimental recirculation system for conducting laboratory challenge of fish with *Neoparamoeba* sp.

Fish were transferred from the acclimation tank (see above) to the experimental recirculating challenge system and maintained at an initial stocking density of 4.25-8.5 kg m^3 (depending upon required experimental conditions). Fish were fed either to satiation or a fixed ration as the experimental conditions dictated. Water quality was measured according to table 1.

Table 1. Water quality parameters measured and acceptable limits during experimental infections of Atlantic salmon with *Neoparamoeba* sp.

Water quality parameter	Limits	Frequency	Instrumentation
Dissolved oxygen (mg L^{-1})		daily	Oxygard handy gamma
Dissolved oxygen (% sat)	>80%	daily	Oxygard handy gamma
Temperature	17^{o}C	daily	Oxygard handy gamma
Salinity	35 g L^{-1}	daily	Iwaki TD508 refractometer
pH	7.8-8.1	2x weekly	Hannah H9017pH meter
Ammonia-N	0.25 mg L^{-1}	weekly	Australian Pet Supplies Test kit
Nitrite-N	0.5 mg L^{-1}	weekly	Australian Pet Supplies Test kit
Nitrate-N	100 mg L^{-1}	weekly	Australian Pet Supplies Test kit
Water exchanges	20%	1-2x weekly	

At the start of the infection challenge foam fractionators were stopped and a seawater suspension (35 g L^{-1}) of isolated amoebae was added to each system. The concentration of amoebae was based on each particular study. In 2001-2002 crude gill isolates of amoebae were obtained according to methods described in Powell and Clark (2003). In these studies a standard challenge of 2500 amoebae L^{-1} was used. However, after 2002, with the development of a more refined amoeba isolation method (Powell et al. 2003; Morrison et al. 2004) a lower number of amoebae were used (100-500 cells L^{-1}).

Variations to acclimation and challenge

The availability of spring smolts was limited during the early-mid part of the year (typically March-September) and as a consequence out of season (photomanipulated) smolts or marine pre-smolts were used. Acclimation of these fish to seawater (35 g L^{-1}) was problematic under the 7day regime as indicated above. Consequently, fish were acclimated to the same salinity increments but over a 10-14 day period. Upon transfer of these fish to the experimental challenge units, these fish would often not recover their feeding response quickly (taking up to a week compared with 1-3 days for spring smolts). Bacterial infections were common in these out-of-season fish. Mixed flexibacteria and *Vibrio* spp. infections were diagnosed (Fish Health Unit, Tasmanian Department of Primary Industries, Water and Environment). These

infections were controlled by removing moribund fish (fish showing visible scale loss and skin lesions), reducing the salinity in the experimental challenge units and re-establishing a strong feeding response, then gradually increasing salinity to complete the *Neoparamoeba* sp. challenge.

In order to accelerate the induction of AGD for the examination of the pathophysiology in surgically cannulated fish (see below), the challenge protocol was modified. Fish were exposed to an acute concentration of 10^4 cells L^{-1} for 6 h by recirculating a 200 L volume of aerated seawater containing the amoebae. After the initial 6 h exposure period, the challenge water was allowed to mix with the remaining system water to provide a background concentration of 2500 cells L^{-1}. This protocol was successful at inducing the onset of gill lesions within 24 h with progression of the disease over a subsequent 96 h period (see below).

Assessment of AGD

Gill samples (whole gill arches) were fixed for 24 h in Davidson's seawater fixative then transferred to and stored in 70% ethanol. The cartilagenous gill arch was removed from the filaments and the filaments placed flat in Tissue-Tek™ cassettes and processed for wax histology using a Tissue Tek II automatic processing unit. Gills were embedded flat in paraffin wax on a Shandon Histocentre 2 and sectioned at 5 μm on a Microm microtome. The resultant sections were stained with haematoxylin and eosin (Thompson and Hunt 1966).

Assessment of the severity of AGD was made at 10x objective magnification. The total number of well orientated gill filaments were counted along with the number of those filaments that had at least one AGD lesion (Nowak and Munday 1994) associated with them. A well oriented filament was defined as having >3/4 of the filament with a central venous sinus showing and lamellae evenly sized along both sides of the filament (Speare et al. 1997). An AGD lesion was defined as having more than 5 adjacent lamellae fused by hyperplastic filamental epithelium and in the absence of any treatment, amoebae with a parasome could be seen associated with the lesions. This gave an estimation of the disease severity in percentage of lesions gill filaments.

RESULTS AND DISCUSSION

Laboratory challenges

The progression of the laboratory disease model has been repeatable with characteristic stages of disease development (± 1 day). Despite the difference in the amoeba concentration between the two challenge methods, a similar progression of pathology occurred (Table 2).

Table 2. Characteristic disease milestones for the laboratory AGD model

Day post challenge	Response (gross pathology)
Day zero	Amoebae added to the system
Day 3-4	Fish ventilation is pronounced (physiological effects occur see later)
Day 5-7	Appearance of gill patches (some minor decrease in feeding)
Day 10-12	Patches more pronounced (feeding sometimes decreased)
Day >15	Patches prolific and mortalities begin occurring (feeding significantly decreased).

This corresponded well with the percent of lesioned gill filaments used as the primary method for assessing AGD severity (Fig. 4). This method for assessment of AGD severity is used because of the recognition of histology as the gold standard for assessing AGD. The presence of lesions and characteristic Neoparamoebae associated with the lesions forms the basis of one of the case definitions proposed for this disease (Nowak et al. 2004).

Despite the different amoeba concentrations used in the challenges, the development of lesions (and severity of the disease) was very similar over the first 10 days of the challenge (Fig. 4). Thereafter, the disease progressed more quickly where higher numbers of amoebae were used (Fig. 5). It was also noteworthy that at 100 cells L^{-1}, there was considerable variability in the severity of AGD (percent lesioned filaments) at the same time point between different experiments (Fig. 5). This suggests that 100 cells L^{-1} should represent a minimum number of cells used in the infection challenge rather than a standard challenge model. This result is consistent with the observations

of Morrison et al. (2004) where 100 cells L^{-1} induced approximately the same proportion of gill lesions after 14 days.

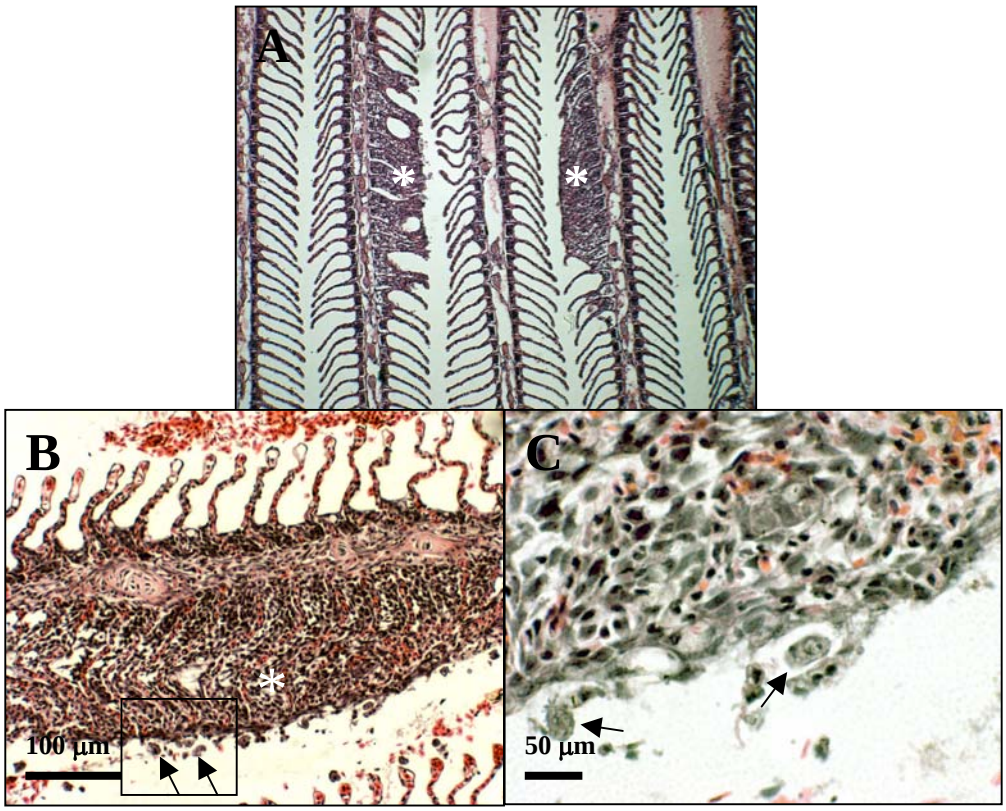


Figure 4. A. Low power (4x obj) of Atlantic salmon gill with characteristic AGD-type hyperplastic epithelial lesions (asterisk). B. AGD lesion with amoebae associated (arrows). C. Inset showing characteristic Neoparamoebae associated with the lesions (arrows) (H&E).

These results indicate that the laboratory challenge developed for experimental use is effective and although inducing AGD more rapidly than seen in the field (where AGD can take several weeks to eventuate) it offers a useful model that presents pathology similar to that seen in "wild-type" AGD from farms.

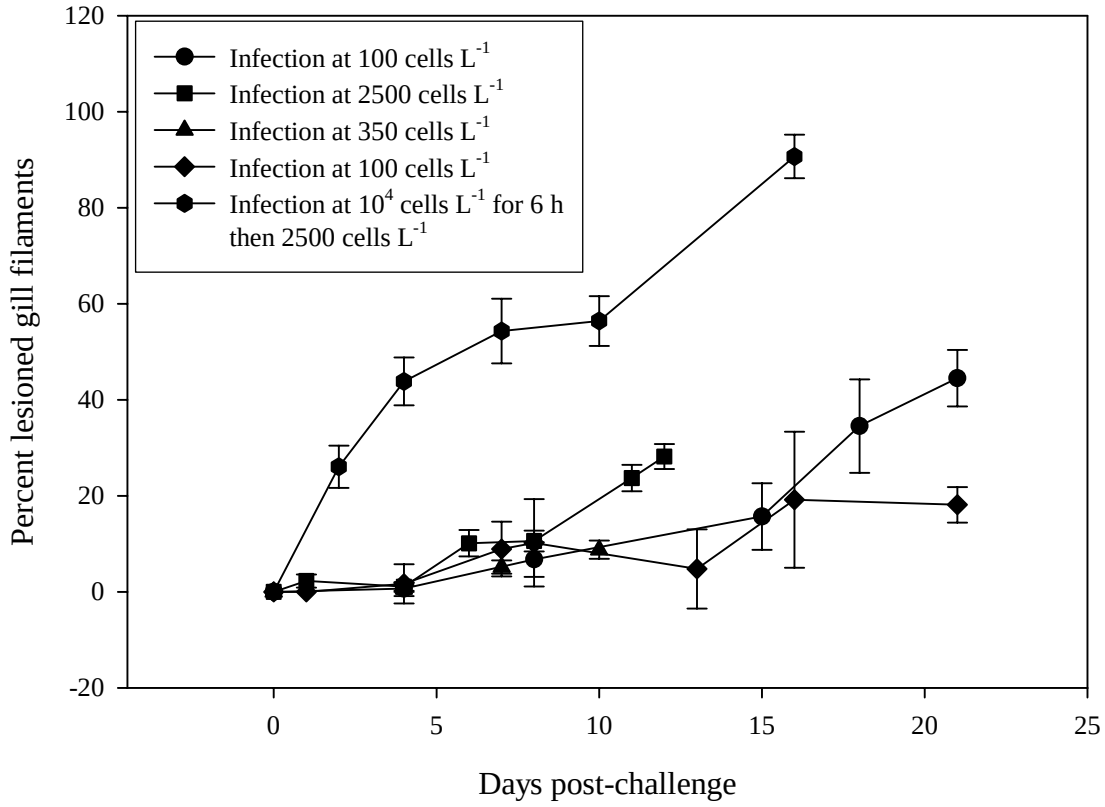


Figure 5. Progression of AGD severity in Atlantic salmon smolts challenged with *Neoparamoeba* sp. at different concentrations as determined by wax embedded histology. All challenges are individual experiments (details described below) at 17°C and 35 g L^{-1} salinity.

The reason that the disease progresses more quickly in the laboratory may be based upon primarily 3 factors:

1 The infective dose given to the fish at the start of the infection challenge is higher than that seen in wild-type infections.

2 The system is recirculated such that fish can readily re-infect themselves as time progresses. This is in contrast to the flow-through type situation seen on fish farms where the infection progresses in an open culture system of sea cages.

3 The *Neoparamoeba* sp. used for the challenges has been repeatedly passaged through fish within the infection tank. As a consequence the potential for increasing virulence exists as has been demonstrated with other diseases.

SECTION 2: PATHOPHYSIOLOGY OF AGD

METHODS

AGD respiratory pathogenesis

The assessment of respiratory pathogenesis was undertaken at two levels. The first level was that of cannulated large fish (approx 700-900 g) that were infected with *Neoparamoeba* sp. and subsequent assessments made of respiratory blood gases and acid-base balance over a subsequent 96 h period. Secondly, smaller fish (approx. 100 g) fish were infected with *Neoparamoeba* sp. and the blood biochemistry and gill disease progression assessed over a subsequent 10 day period.

Surgical procedures

Atlantic salmon of approximately 700 g were anaesthetised in aerated seawater with clove oil (0.1 mL L^{-1}) until equilibrium was lost and opercular movements ceased. The fish were then transferred to a surgical table and supported inverted with the gills constantly irrigated with flowing chilled seawater containing 0.01 mL L^{-1} clove oil. The dorsal aorta was cannulated according to the method of Soivio et al. (1975), the cannula secured with silk sutures and the fish recovered in flowing seawater and placed into a black acrylic respirometer box supplied with flowing air-saturated seawater. The surgical procedure typically took 15 min per fish. Cannulae were flushed periodically with heparinised modified Cortland's saline (Wolf 1963; Milligan et al. 1993, 160 mM NaCl) containing 100 IU mL^{-1} ammonium heparin (Sigma-Aldrich).

Fish were challenged with *Neoparamoeba* sp. using a modified acute exposure of 10^4 cells L^{-1} for 6 h followed by a background of 2500 cells L^{-1} as described above. At intervals post-challenge blood samples were withdrawn from the cannula and analysed for respiratory variables.

Blood analysis

Blood samples (500 μL) were withdrawn anaerobically from the cannulae and replaced with an equivalent volume of modified Cortland's saline. Whole blood haemoglobin concentration was measured using the cyanmethaemoglobin method of a

commercially available a spectrophotometric assay (Sigma Procedure 525). Haematocrit was determined using duplicate microhaematocrit tubes of whole blood centrifuged at 10 000x g for 5 min and the packed cell volume read manually. Arterial oxygen tension (P_{aO_2}) was determined by injecting the blood sample into a thermostatically controlled BMS Mk 2 blood gas analyser (Radiometer Copenhagen) fitted with Cameron Instrument Company E101 oxygen electrodes. Oxygen electrodes were calibrated against a 2% $NaSO_4$ solution (zero) and air-saturated seawater (~166 mmHg adjusted for atmospheric pressure). Whole blood pH was determined using an Activon AE333 mini electrode calibrated to ± 0.005 using radiometer precision buffers (Radiometer Copenhagen). The remaining blood was centrifuged at 8000 x g for 1 min and the plasma decanted and analysed for total carbon dioxide content using a Cameron Instrument Company (Port Aransas, Texas) Capni-con 5 total carbon dioxide analyser. Plasma lactate was analysed using a commercially available spectrophotometric assay (Sigma procedure 735).

Metabolic rate determination

Fisk et al. (2002) demonstrated that Atlantic salmon heavily affected by AGD did not show elevations in routine metabolic rate compared with lightly affected fish under normoxic conditions. However, under hypoxic conditions, heavily affected fish showed a suppression of metabolic rate suggesting that fish were unable to maintain oxygen uptake under hypoxic conditions compared with lightly affected animals. This means that two things were probably occurring in these fish:

1. Oxygen uptake was limiting metabolic rate – resulting in a phenomenon known as metabolic suppression.
2. The scope for aerobic activity was being limited because the basal metabolic processes were increased and thus under hypoxic conditions, there was insufficient scope to increase oxygen uptake.

Atlantic salmon (mean weight 118.1g ± SE 7.0) were maintained in 3 replicated circular 350 L open, flow-through respirometers (Fig. 7) at a density of 11.8 kg m^{-3}. A fourth respirometer served as a blank to account for background oxygen consumption due to the biochemical oxygen demand of the water. Water within the respirometer was recirculated through a biological filter and sump with continuous aeration of the sump water. By measuring the effluent oxygen content via Oxygard orbisphere 2432

oxygen electrodes (Oxygard, Birkerod, Denmark) interfaced with an AD Instruments PowerLab™ and personal laptop computer it was possible to record effluent dissolved oxygen content of the water at a rate of 4 samples min^{-1} continuously. Oxygen levels were monitored continuously and by comparing to influent oxygen concentrations (measured manually) it was possible to calculate the oxygen consumption rate of the fish. The lowest oxygen consumption rate over a 24 h period was considered equivalent to the standard (basal) metabolic rate (Cech 1991).

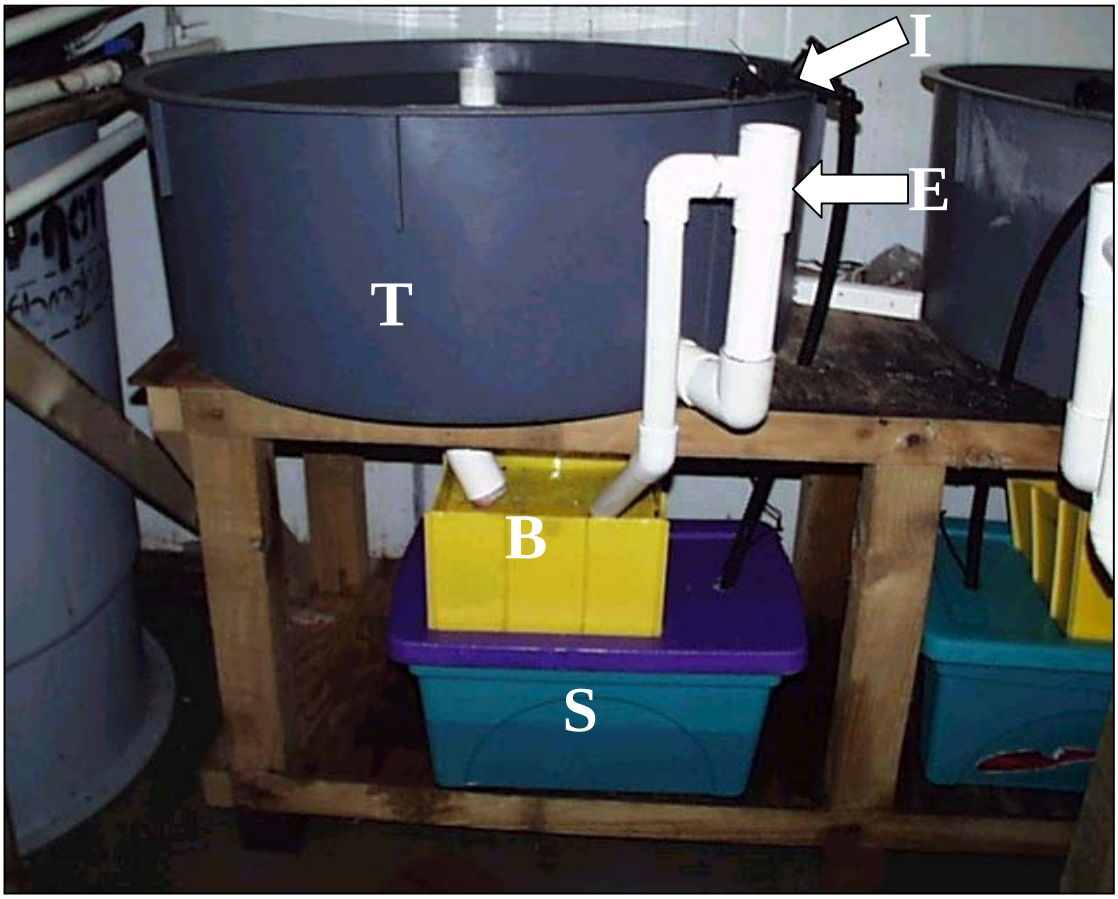


Figure 6. Flow-through bioassay respirometer comprising of a 350 L tank (T), biological filter (B), sump (S). Dissolved oxygen of the effluent water is measured at outflow (E) and at the inflow manifold (I).

To achieve a maximal metabolic rate, fish were removed from the respirometer and placed into a bucket of isothermic oxygenated seawater (120% air saturation) and chased manually for 10 min or until fish were no longer responsive (as the method described by Altimiras et al. 2002). After the chasing, the fish were immediately returned to the respirometer and the rate of oxygen consumption measured as described above. It is important to note that due to the flow-through lag of the respirometer, reading of oxygen consumption began 20 min from the transfer of fish back into the respirometer. The metabolic rate measured was then an "actively recovered rate" realising that some recovery of metabolic rate would have taken place during this period.

Each respirometer was then inoculated with gill amoebae isolated from AGD affected salmon from the infection source (see above) at a concentration of 2300 cells L^{-1} and measurements of oxygen consumption were recorded continually for 5 days. After 5 days the fish were removed and chased as described above and the actively recovered metabolic rate determined.

Standard oxygen consumption rates (metabolic rates) were compared on day 5 to the pre-inoculation rate using repeated measures ANOVA and Tukeys as a planned contrast post-hoc analysis. The difference between standard metabolic rate and active metabolic rate (aerobic metabolic scope) was compared between the pre-inoculation and post-inoculation condition using paired t tests.

Ionoregulatory effects of AGD

Experimental series 1: Disease progression, blood osmolality and involvement of the natriuretic peptide hormone system.

Atlantic salmon smolts (128 g ± SE 33 g) were infected at 2500 cells L^{-1} in the experimental challenge system (see above). Controls consisted of uninfected fish at the same stocking density. Fish were sampled (n = 12 per treatment) by dip net at 0, 1, 4, 6, 8, and 11 days post-challenge. A caudal blood sample (heparinised with 100 IU mL^{-1} ammonium heparin) was taken and the blood centrifuged at 8000 x g for 1 min and the plasma decanted. The gills were fixed for histology as detailed above. After 11 days the remaining fish were bathed in soft freshwater (ca 30 mg L^{-1} $CaCO_3$ hardness) and blood and gill samples (n = 12 per treatment) taken 0, 1, 3 and 6 days

post-bathe. The plasma osmolality was determined using a Wescor Vapro 5520 vapour pressure osmometer (Helena Laboratories, Columbus, Ohio USA).

Gill filaments from each fish were frozen in liquid nitrogen and transported to Deakin University where membrane preparations were made and assayed for cyclic GMP activity following stimulation with trout atrial natriuretic peptide (tANP) and c-type natriuretic peptide (tCNP) using radioimmunoassay (McWilliam 2003).

Experimental series 2: Effects of freshwater on net branchial ionic fluxes

Seawater acclimated Atlantic salmon of mean mass 323g ± SE 12g were either infected with *Neoparamoeba* sp. as described above (n = 17) or left uninfected (controls, n = 16). Fish lightly anaesthetised with clove oil 0.05 mL L^{-1} and placed into closed acrylic respirometer boxes supplied with flowing, air-saturated, seawater (17^{o}C and 35 g L^{-1} salinity). Following recovery overnight, the respirometers were flushed with fresh water (17^{o}C; pH 7.4; 260 mg L^{-1} $CaCO_3$ hardness). Whole body net Na^+, K^+, Cl^- and NH_4^+ fluxes were then determined over a subsequent 3 h period as described by Roberts and Powell (2003).

The role of mucus in AGD: a potential target for therapeutic intervention

Experimental series 1: Effects of salinity and AGD on the mucus of different salmonids

Fish body (cutaneous) mucus is often used as a proxy for gill mucus because of its ease of collection, lack of contamination from blood and other tissue fluids and its biochemical similarity to branchial mucus. The visco-elastic properties of fish mucus, which behaves as a non-Newtonian fluid, have been characterised recently (Roberts and Powell 2004). In this work, body mucus was gently scraped from Atlantic salmon, rainbow trout or brown trout that had been acclimated to fresh or salt water and affected or naïve of AGD. The viscosity and crude biochemical composition of the mucus was analysed using a model LVT-C/P 42 Brookfield Engineering Laboratories viscometer with a cone angle of 1.565^{o}. Crude biochemical analysis of the mucus was made by measuring the total protein and glucose components of the mucus (Roberts and Powell 2004), the ratio of which, gives an estimate of the glycosylation of the mucoproteins.

The gills from the same fish from which body mucus was taken, were fixed for histology as described previously. The tissue sections from the gills were stained with a combination of Alcian blue and Periodic Acid-Schiff (AB/PAS) at a pH of 1.0 or 2.5. The differential staining of tissue with Alcian blue at pH 1.0 or 2.5 allows differentiation of Alcian blue positive (mucous) cells as containing either predominantly sulphated or carboxylated mucins respectively (as used by Roberts and Powell 2004). The distribution of mucous cells based upon their histochemical characteristics was determined according to established methods (Powell et al. 1995; Speare et al. 1997; Roberts and Powell 2003a).

Experimental series 2: Effects of mucolytic drugs on mucus viscosity in vitro.
Superficial cutaneous mucus was collected according to Roberts (2004) and incubated with 0, 8, 12, 100 or 200 mg L^{-1} L-cysteine ethyl ester for 2h at 17^{o}C. The viscosity of the mucus solutions was determined using a model LVT-C/P 42 Brookfield Engineering Laboratories viscometer with a cone angle of 1.565^{o} as described above.

Experimental series 3: The effects of oral mucolytic treatment
Seawater acclimated Atlantic salmon smolts (mean weight 303.8 ± SE 12.2 g) were fed either L–cysteine ethyl ester (LCEE) coated salmon pellets or control (untreated) pellets for a 2 week period similar to the experimental design used by Stone et al. (2000). LCEE was top-dressed onto the feed daily at a concentration of 8 g kg^{-1} feed in cod liver oil. Over the 2 week feeding period the calculated mean daily dose was 52.7 ± 3.5 mg LCEE kg^{-1} fish day^{-1} (Roberts and Powell in preparation). Control fish received cod liver oil-coated pellets only. A sample of fish (n = 6) was taken from both LCEE and control fed fish prior to and after 2 weeks of feeding, a blood sample taken from the caudal vessels and the gills processed for histology (as described previously). Tissue sections were differentially stained with AB/PAS as described above.

After the 2 week feeding period, 6 fish from each treatment were transferred to individual flux chambers (as described by Roberts and Powell 2003a) and whole body net H^{+} and NH_4^{+} fluxes determined (Roberts and Powell in preparation, Powell et al 2004). The remaining fish were individually identified according to treatment (LCEE or control fed) using a PanJet tag and transferred to the infection source tank

(see section 1). After 3 and 5 days fish (n = 6 per treatment) were lethally sampled and the gills processed for histology as described above.

Cardiovascular effects of AGD

Experimental series 1: The post-surgical cardiovascular effects of AGD in different salmonids Atlantic salmon, Salmo salar, brown trout, S. trutta, and rainbow trout, Oncorhynchus mykiss

It was apparent from previous work (Munday et al 2001 and Fry 2003) that there appeared to be differences in the susceptibility of different salmonid species to AGD. These differences manifest themselves in the presentation of the gross pathology as well as the immunological responses. This study specifically was designed to examine the cardiovascular effect of AGD in three salmonid species since Powell et al (2002) demonstrated that AGD related pathology appeared to be associated with a systemic hypertension. For this study fish were seawater acclimated and then used for experimentation prior to or after 3 days of co-habitation with AGD affected fish in the infection tank (as described above).

Surgical procedures

Sea water (35 g L^{-1} salinity) acclimated Atlantic salmon, *Salmo salar* (mean weight 516 g ± 35 g SE), brown trout, *Salmo trutta* (mean weight 872 g ± 31 g SE) and rainbow trout, *Oncorhynchus mykiss* (mean weight 965 g ± 39 g SE) were anaesthetised with clove oil and fitted with dorsal aortic cannulae as described above. Following placement of a DA catheter, an ultrasonic cuff-type 2.5PSL flow probe (Transonic Systems Inc., Ithaca, NY, USA) was placed non-occlusively around the bulbus arteriosus to permit measurement of cardiac output (Q). In brief, a midline ventral incision was made to expose the pericardial cavity, which was then dissected to expose the bulbus arteriosus (Fig. 7). Lubricating jelly (K-Y, lubricant jelly) was used with the perivascular probe as an acoustic couplant. Black braided 2.0 silk sutures were then used to close the ventral incision and to anchor the probe output lead to the skin (Fig. 7). Following surgery fish were transferred to individual black acrylic 5 L boxes supplied with flow-through seawater (95 - 100 % O_2 saturation) at 16 ± 0.5 ºC. Surgical procedures, including anesthesia, took between 20 - 30 min.

Cardiovascular measurements

To allow measurement of dorsal aortic pressure (P_{DA}) the dorsal aorta catheter was attached to a disposable pressure transducer (DPT-60003, Peter von Berg, Eglharting,

Germany) that was connected to a PowerLab® data acquisition system (AD Instruments, Castle Hill, Australia) interfaced to a portable personal computer (Fig. 8). The transducers were calibrated against a column of water in 10 cm steps. P_{DA} and Q were measured continuously during the course of the experiment at a sampling rate of 4 samples s^{-1} (Fig. 8). The perivascular flow probes used to measure Q were connected to a pre-calibrated Transonic T106 small animal blood flow meter (Transonic Systems, Inc., Ithaca, NY, USA) (Fig 3). Systemic vascular resistance was calculated as mean P_{DA} divided by Q (i.e. $R_S = P_{DA} / Q$). Mean values for each previously described parameter were calculated from 10 min recordings at 6 h post-surgery.

Experimental series 2: Partial characterisation of AGD-associated cardiovascular effects in Atlantic salmon

Using the same surgical and experimental procedures as described above, seawater acclimated Atlantic salmon were co-habited with AGD affected fish in the infection tank for 3 days.

Experimental design and delivery of anti-hypertensive agents

Experiments commenced at 6 h post-surgery with a pre blood sample taken via the DA catheter. Twenty minutes after the pre blood sample, DAP and cardiac output were recorded for a 10 min period to establish a stable baseline, following which, fish were injected with both saline and sodium nitroprusside (SNP at 40 µg kg^{-1}) or saline and captopril (10^{-4} mol L^{-1} at 1 mL kg^{-1}). Each injection was delivered within 1 min via the DA catheter and recording of DAP and cardiac output was continuous for 60 min (Fig. 8). Mean values for each measured parameter were taken from the average of a 1 min period at 2, 5, 10, 15, 30 and 60 min post-injection. The mean value for the baseline data was taken as the average of the 10 min period preceding each injection. At 60 min post each injection, a blood sample was taken via the DA. The fish was then left undisturbed for a 20 min period before recording re-commenced for 10 min to establish the baseline data for the next injection. R_s was calculated as previously described.

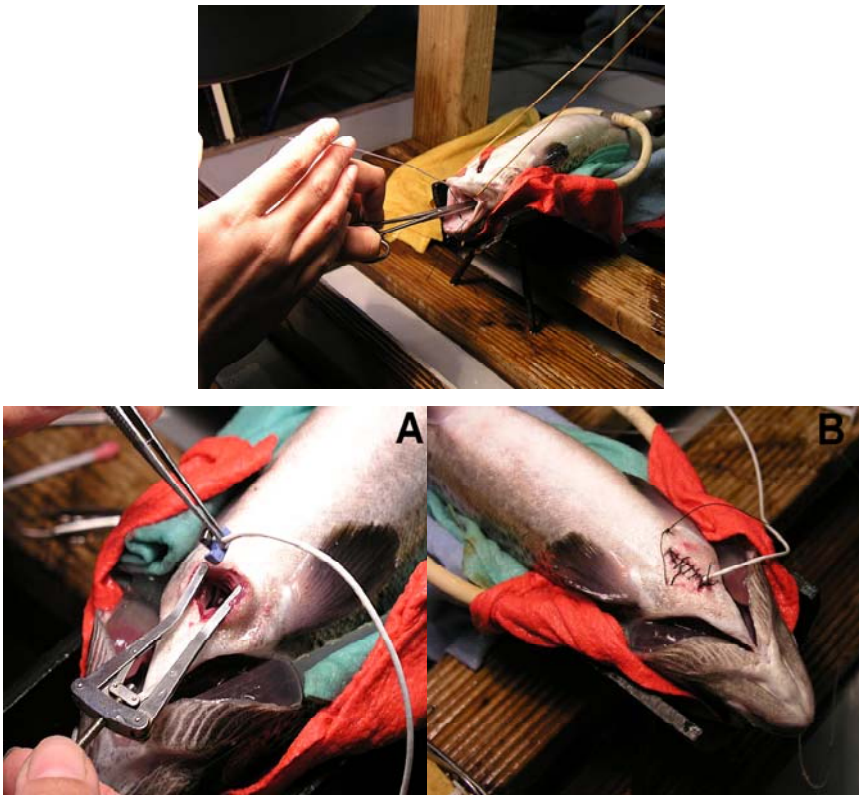


Figure 7. Insertion of a dorsal aortic cannula (upper picture) and placement of cardiac flow probe into the pericardial cavity (A) around the bulbus arteriosus and closure of incision (B).

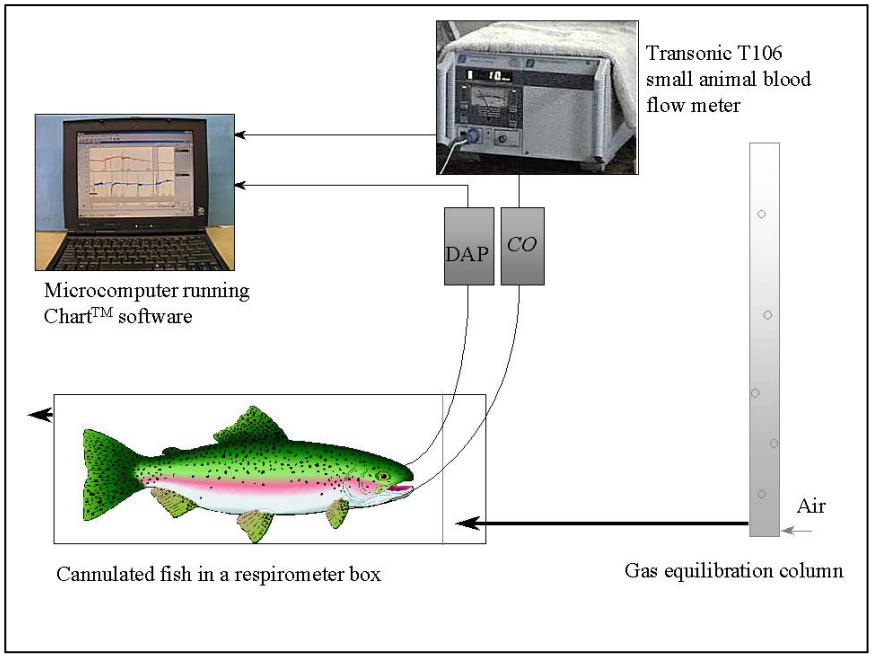


Figure 8. Schematic representation of the equipment used to measure cardiovascular parameters in fish.

RESULTS AND DISCUSSION

AGD respiratory pathogenesis

There was a progressive acidosis that developed 3 days post-infection. There was no significant effect on blood oxygen tensions (Leef et al. 2005 in press). This suggested that the onset of the respiratory acidosis as seen previously in clinically infected fish (Powell et al. 2000) occurs early with the disease and corresponds to the onset of the first branchial lesions.

Metabolic rate determination.

There was a small but significant increase in standard metabolic rate of fish over the time course of the AGD infection day 4 compared with day 7 (Fig. 9). There was no significant difference in actively recovering metabolic rate (R_{active}) between the pre and post-inoculated groups. This resulted in no significant difference in the relative aerobic metabolic scope between the pre and post-inoculation groups (Harris et al. 2003; Leef et al. in prep).

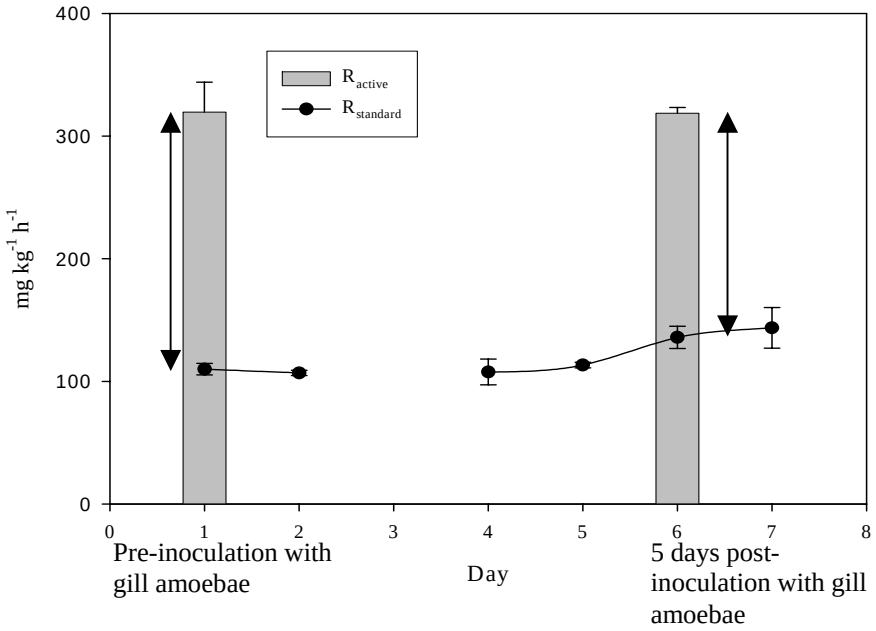


Figure 9. Standard metabolic rate ($R_{standard}$) and actively recovered metabolic rate (R_{active}) for Atlantic salmon prior to and 5 days post-inoculation with gill amoebae. Vertical arrows represent a relative aerobic metabolic scope.

Histological examination of the gills after 5 days post-inoculation revealed that all of the fish exhibited AGD type lesions with a mean of 44.8% (± SE 4.3%) of gill filaments lesioned.

This study suggests that standard metabolic rate does increase during the onset of an AGD infection. Although Fisk et al. (2002) did not see differences in routine metabolic rate, standard metabolic rate is a more reliable measure of metabolism since it is not subject to small variations in activity as with routine. However, standard metabolic rate can only be measured by continuous measurement of oxygen consumption over a 24 h period. Despite an increase in standard metabolism, there was no significant decrease in metabolic scope. There are two possibilities for this. One is that AGD results in a true decrease in metabolic scope due to the limitation of oxygen transfer across the gill and that this is not detectable because of the mechanisms for maintaining gill perfusion so oxygen uptake does not become oxygen limited (Powell and Perry 1999). The second hypothesis is that metabolic scope may be slightly decreased but not detected statistically in this study because of insufficient statistical power. Since 3 respirometers were used to gather these data, the latter is plausible. Irrespective of whether metabolic scope is affected by AGD or not, it is clear that standard metabolic rate was increased which partially explains an observed decrease in growth of fish suffering from heavy AGD compared with lightly affected fish (Powell et al. 2002). An increase in standard metabolic rate will mean an increase in the energy required for basal metabolic processes such as ion and acid-base regulation as well as the growth and repair of tissues such as gills. These physiological process are likely to be affected by AGD (Powell et al. 2000; Roberts and Powell 2003a) as well as increased tissue metabolism as a result of the development of hyperplastic gill lesions.

Ionoregulatory effects of AGD

Experimental series 1: Disease progression, blood osmolality and involvement of the natriuretic peptide hormone system

There was a significant increase in plasma osmolality as AGD progressed in severity (McWilliam 2003) and this was seen by the weak but significant ($P < 0.05$) correlation between the number of lesioned filaments and plasma osmolality (Fig. 9). Following the freshwater bath, there was a significant but transient decrease in plasma osmolality in both AGD and control fish that recovered to pre-treatment levels after 3 days (controls) and 6 days (AGD) (McWilliam 2003). The proportion of lesioned filaments declined following the freshwater bath but remained significantly elevated over pre-challenge or control levels (McWilliam et al. in preparation).

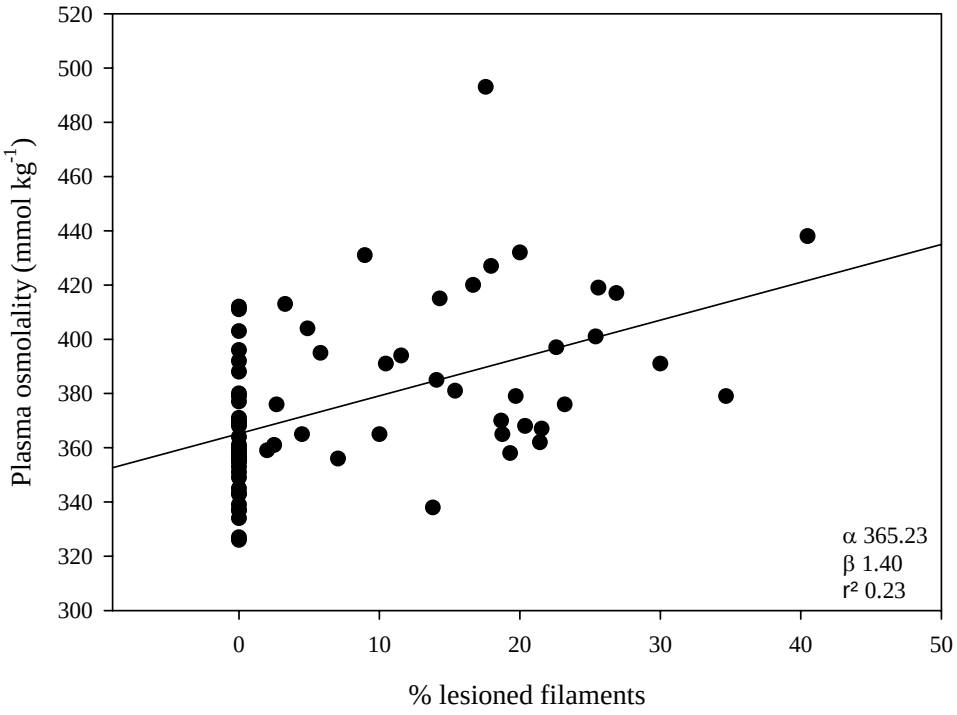


Figure 10. Relationship between percent of AGD lesioned gill filaments and plasma osmolality of Atlantic salmon over an 11 day AGD-challenge.

Cyclic GMP activities of gill membrane preparations stimulated by both tANP and tCNP were elevated over baseline levels for control and AGD-affected salmon throughout the progression of the AGD challenge period. However, there were no significant differences in cGMP production between control and AGD-affected fish

with regard to the tANP or tCNP stimulation of gill membrane preparations over the duration of the AGD challenge (McWilliam 2003). Interestingly, immediately following the freshwater bath at the end of the challenge period, there was a significant increase in cGMP production, in response to both tANP and tCNP stimulation in AGD-affected fish. A more gradual increase in cGMP production occurred in response to both hormones in controls 3 days after freshwater bathing (McWilliam 2003). This result suggests that the progression of AGD does not up regulate natriuretic peptide receptor hormone expression in salmon gills. However, in an AGD-affected fish, the "stress" of the disease may compromise the cardiovascular capabilities (see below). The natriuretic peptide hormone system has been implicated in modulating vascular hypertension and blood volume as well as salt balance in fishes (Takei and Hirose 2002). That the natriuretic peptide receptor expressions (as shown by cGMP production) was acutely "up regulated" following a stressor such as freshwater bathing suggests that the natriuretic peptide hormone system may be important in accommodating the additional "stress" in an AGD-affected fish. That natriuretic peptide hormone expression was also up regulated in controls following freshwater bathing but over a longer time frame suggested that natriuretic peptide hormones are potentially involved in modulating the stress response of teleost fishes. This represents a tantalizing paradigm shift in the current understanding of stress in fishes that should be investigated further.

Experimental series 2: Effects of freshwater on net branchial ionic fluxes

AGD affected fish had a significantly greater proportion of the gill filaments with lesions than controls. There were no significant differences in the whole body net Na^+ flux of AGD affected fish compared with that of controls. There was, however, a significant negative net Cl^- flux in both control and AGD affected fish. The net flux (a net loss of Cl^-) was greater in AGD affected fish than that seen in controls (Roberts and Powell 2003). There were no differences in the net K^+ flux for either controls or AGD affected fish. However, there were significant negative net NH_4^+ fluxes in both control and AGD affected fish. The net loss of ammonium (negative net NH_4^+ flux) in AGD affected fish was significantly greater than that seen in controls suggesting a higher ammonium excretion rate in AGD affected fish during bathing compared with control fish (Roberts and Powell 2003). Atlantic salmon appeared to be more resilient to sudden changes in salinity in terms of their ionoregulatory capacity compared with

previously published data on other salmonids (Roberts and Powell 2003). This result suggests that, even with AGD, Atlantic salmon are capable for tolerating freshwater baths for several hours; consistent with our previous work (Powell et al 2001).

The role of mucus in AGD: a potential target for therapeutic intervention

Experimental series 1: Effects of salinity and AGD on the mucus of different salmonids

Freshwater salmonids have a lower cutanneous mucus viscosity, osmolality, protein and glucose content compared with seawater acclimated or AGD affected fish (Table 3). With the exception of rainbow trout, the number of mucous cells on the gills was also lower in freshwater. The relative viscosity of cutaneous mucus was lowered in response to AGD infection for salmon and brown trout but not rainbow trout. A reduction in the protein/glucose ratio was evident for salmon cutaneous mucus but not for either trout species (Table 3). In AGD affected fish the branchial mucous cell density increased compared to AGD-free seawater acclimated fish on both the non-lesioned and lesioned parts of the gill (Table 3). These results suggest that in freshwater the mucous coat of fish is less viscous than in seawater (related to the hydration of mucus being dictated by the ionic Donnan potential across the mucous cell membrane (Roberts and Powell 2004)). However, there are significant differences in the mucus characteristics between salmonid species, with rainbow trout having a less viscous mucus than either *Salmo* species. In terms of AGD, the effect of the disease is reduced mucus viscosity in both *Salmo* species although no effect was detected in rainbow trout. Since all fish were infected for the same amount of time (held in the infection tank), the differences are likely to be due to the severity of infection reflecting specific differences in disease susceptibility.

In seawater, the protein/glucose ratio is reduced indicating a reduction in the degree of glycosylation of the mucus (Roberts and Powell 2004). This corresponded to changes in mucous cell histochemistry in the gills (data not shown see Roberts and Powell (2004) for detailed discussion). However, with AGD affected fish, only Atlantic salmon showed a significant reduction in the protein/glucose ration of cutaneous mucus. This is analogous to the fish having a “runny nose” in response to a branchial infection. The consequence of reducing mucus viscosity and corresponding

glycosylation will ensure that the mucus will be readily stripped from the fish so ensuring a less stable environment for the AGD-causing *Neoparamoeba* sp. to attach.

Experimental series 2: Effects of mucolytic drugs on mucus viscosity in vitro.
Mucus viscosity shows a shear-dependant behaviour where viscosity decreases with shear rate and the greatest differences between treatments occur at the lowest shear rates (Roberts 2004). Mucus viscosity significantly decreased with increasing concentration of L-cysteine ethyl ester *in vitro* (Fig. 11).

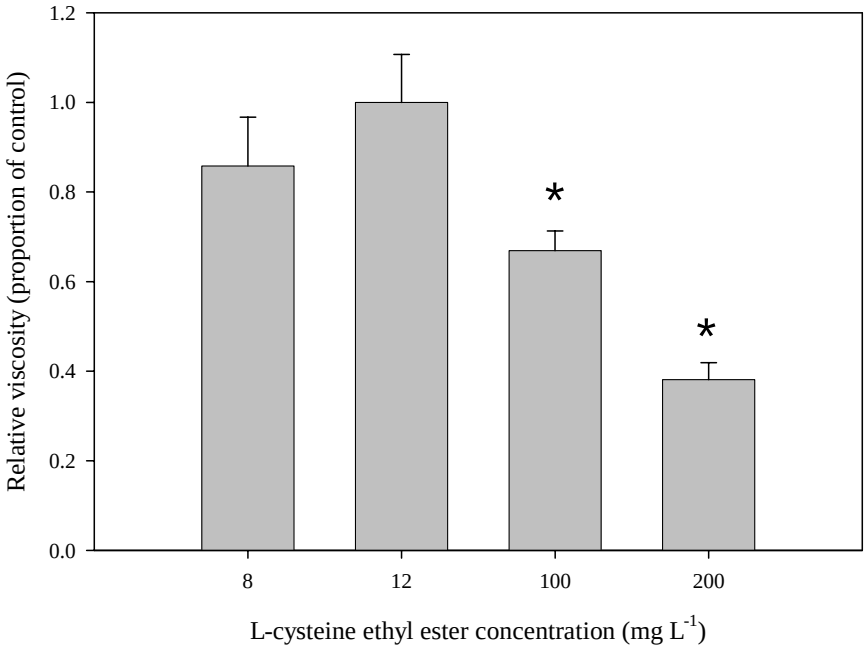


Figure 11. Relative viscosity of cutaneous Atlantic salmon mucus incubated the L-cysteine ethyl ester for 2 h at 17°C. Asterisks indicate significant difference from control values (1.0).

Table 3. Comparison of body mucus relative viscosity and composition as well as gill mucous cell number for three species of salmonids in freshwater (FW), seawater (SW) and affected by AGD (AGD). All data are means (± SE), superscript letters indicate statistical differences, capitals indicate differences across species, lower case differences between species (modified from Roberts and Powell 2004; 2003).

Species	Treatment	Rel. viscosity (cps at 11.5 s^{-1})	Osmolality (mmol kg^{-1})	Protein (mg L^{-1})	Glucose (g L^{-1})	Prot/Gluc	Total cell (cells ILU^{-1}) non-lesion	Total cell (cells ILU^{-1}) lesion
Atl. salmon	FW	3.3 (0.3)^{d}	70.3 (1.1)^{Aa}	320 (10)^{Aa}	24 (1)^{A}	13.7 (0.7)^{A}	0.26 (0.05)^{a}	--
(*S. salar*)	SW	20.3 (0.3)^{a}	988.9 (4.8)^{Bb}	470 (50)^{Ba}	66 (4)^{B}	7.2 (0.4)^{B}	1.94 (0.14)^{b}	--
	AGD	15.7 (1.0)^{b}	982.0 (4.0)^{Bb}	580 (50)^{Ca}	145 (8)^{C} 3.8 (0.2)^{C}	3.46 (0.30)^{b}	5.15 (0.65)^{c}	
Brown trout	FW	9.9 (1.2)^{c}	71.2 (1.3)^{Aa}	290 (20)^{Ab}	22 (7)^{A}	14.8 (3.0)^{A}	0.12 (0.01)^{a}	--
(*S. trutta*)	SW	19.4 (1.1)^{a}	934.1 (7.8)^{Cb}	370 (20)^{Bb}	64 (4)^{B}	5.9 (0.4)^{BC}	0.21 (0.06)^{ab}	--
	AGD	14.2 (0.4)^{b}	912.4 (9.5)^{Cb}	400 (40)^{Cb}	145 (8)^{B}	6.0 (0.9)^{BC}	0.34 (0.04)^{b}	0.70 (0.05)^{c}
Rainb. Trout	FW	1.0 (0.1)^{e}	70.0 (0.8)^{Aa}	330 (20)^{Ab}	30 (6)^{A}	10.9 (1.5)^{A}	1.59 (0.10)^{a}	--
(*O. mykiss*)	SW	8.8 (0.5)^{c}	900.9 (9.6)^{Cb}	400 (40)^{Bb}	71 (5)^{B}	5.6 (0.4)^{BC}	1.04 (0.15)^{a}	--
	AGD	8.6 (0.4)^{c}	975.3 (19.0)^{Bc}	420 (70)^{Cb}	65 (5)^{B}	6.3 (0.6)^{B}	1.23 (0.20)^{a}	2.64 (0.37)^{b}

LCEE interacts with the cysteine disulphide bridges within the protein strands that make up the mucus. The result is to separate the protein strands and thus allow for greater slippage of the mucus proteins over each other and hence lower viscosity (Roberts 20004). This work suggested that LCEE could reduce salmon cutaneous mucus viscosity, albeit at high concentrations in vitro. Potential therefore exists for the use of LCEE as a mucolytic treatment in vivo for the control of AGD.

Experimental series 3: The effects of oral mucolytic treatment

There was an approximate 40% decrease in feed intake of fish fed L-cysteine ethyl ester compared with controls. However, over the 2 week feeding period, there was no difference in the final weight of the fish between medicated (LCEE) or controls but controls had a slightly but significantly higher condition index (Roberts 2004). There was a significant reduction in the caudal blood pH of fish fed LCEE compared with controls that corresponded with a significant increase in a positive net whole body acid flux (net uptake of H^+). There were no significant differences in plasma protein or osmolality between controls or LCEE fed fish. Net ammonia excretion rates were also not significantly different between control and LCEE treated fish (Roberts 2004).

After 3 days co-habitation with AGD affected fish in the infection tank, there were more AGD lesions on control fish compared with LCEE fed fish (38% compared with 19% respectively). A similar pattern continued for fish co-habited with other AGD affected fish for 5 days (Roberts 2004). This suggested that LCEE pre-feeding retarded the onset of AGD. The use of an in feed retardant for AGD is attractive, since it would be relatively easy to deliver. However, it is important that issues such as reduced feed intake and reduced fish condition as a consequence of this be addressed. Similarly, the cost of the mucolytic agent must be sufficiently low or used in sufficiently low concentrations to make its use financially viable. Some of these issues are addressed below (Section 3).

Cardiovascular effects of AGD

Experimental series 1: The post-surgical cardiovascular effects of AGD in different salmonids: Atlantic salmon, Salmo salar, brown trout, S. trutta, and rainbow trout, Oncorhynchus mykiss

Examination of histological gill sections showed that there were no significant differences between the species in terms of the appearance of, nor statistical difference in the number of AGD-associated lesions. Unlike both rainbow and brown trout, AGD-affected Atlantic salmon displayed significantly lowered cardiac output (Q) and elevated systemic vascular resistance (R_S) compared to the control counterparts (Fig. 12). Elevated R_S was previously suspected to be the cause of an apparent hypertension reported for pre-bathed AGD-affected Atlantic salmon (Powell et al. 2002), and although no significant differences were seen in dorsal aortic pressure (DAP), the results from this study do suggest that there may be a cardiovascular dysfunction associated with AGD, at least in Atlantic salmon (Leef et al. in prep.). This finding may also explain why Atlantic salmon appear to be the most susceptible salmonid to the infection (Munday et al. 2001).

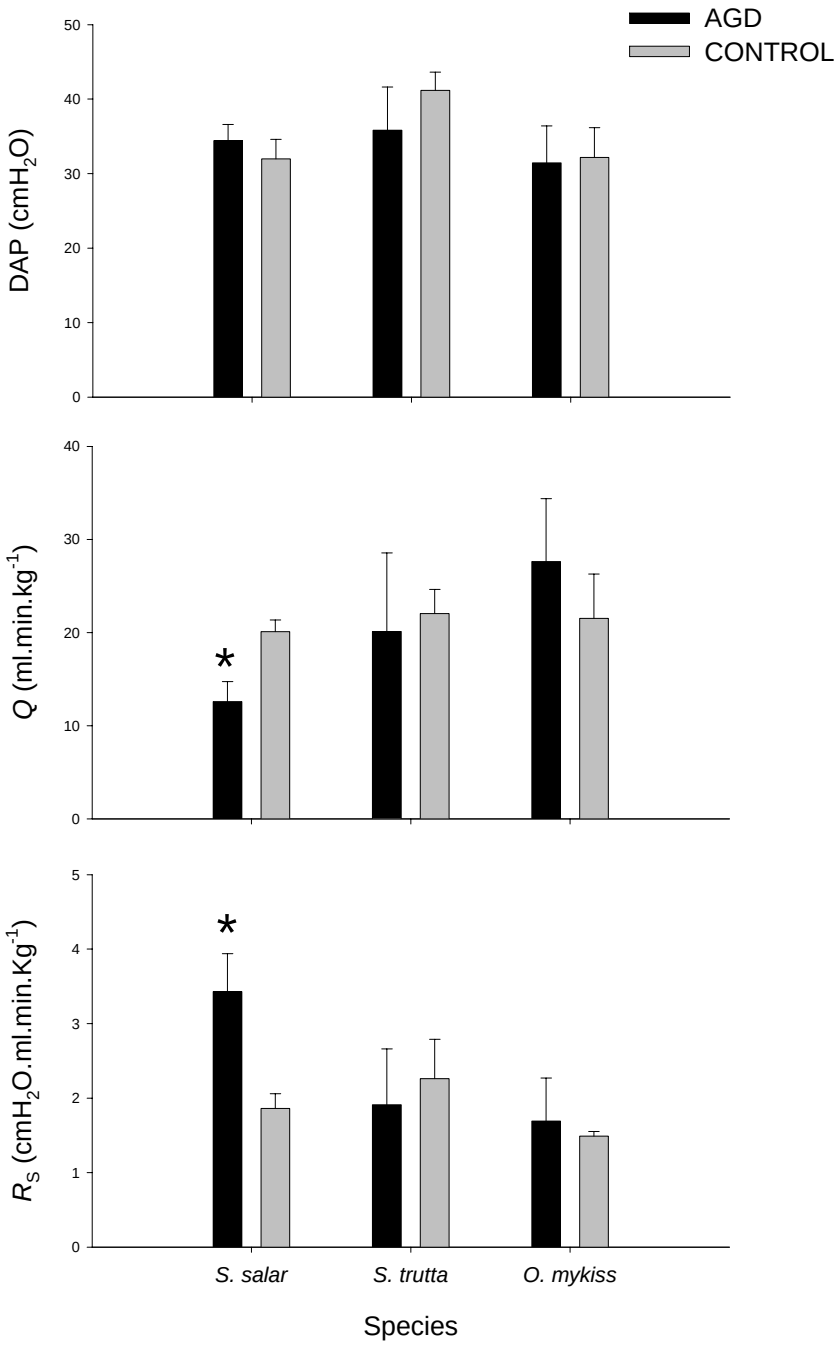


Figure 12. Comparative effects of AGD on dorsal aortic pressure (DAP), cardiac output (Q) and systemic vascular resistance (R_s) in Atlantic salmon, brown trout and rainbow trout. Asterisks represent significant differences between AGD affected and non-affected (control) fish.

Experimental series 2: Partial characterisation of AGD-associated cardiovascular effects in Atlantic salmon.

While both sodium nitroprusside (SNP) and captopril had no significant effects on Q or R_S, results showed that the drop in DAP at approximately 2-5 min following SNP administration, was significantly greater in AGD-affected salmon compared to control fish (Fig. 13). Comparatively, captopril administration produced a greater effect on DAP for both AGD-affected and control salmon, with maximal effects occurring at approximately 60 min post administration. These results suggest that while captopril, an angiotensin converting enzyme (ACE) inhibitor exhibited a definitive hypotensive action in salmon, AGD-affected fish may have a greater scope for vasodilation as demonstrated with SNP, a potent vasodilator known to be effective in both *in vivo* and *in vitro* experiments with rainbow trout. Potentially these results suggest that the previously demonstrated lowered cardiac output in AGD-affected fish is due to a vasoconstriction of systemic vessels (Leef et al. in prep.).

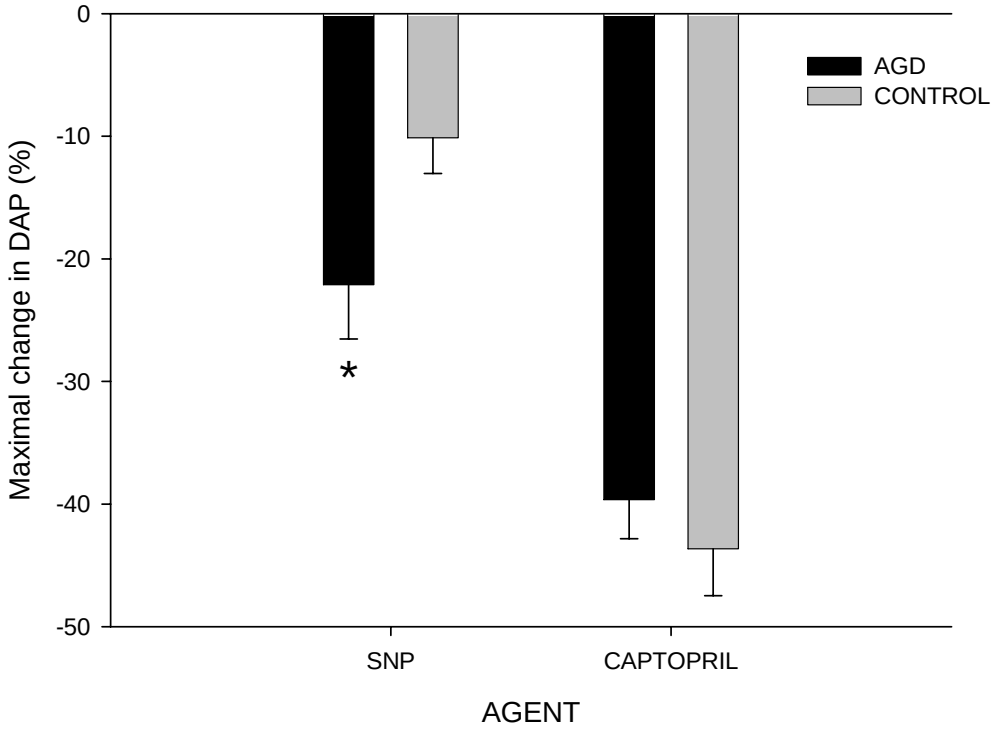


Figure 13. Maximal change in dorsal aortic pressure (DAP) following administration of sodium nitroprusside (SNP) and captopril. Values are mean ± 1 S.E.M. An * denotes a significant difference between AGD-affected (AGD) and respective control ($P < 0.05$).

SECTION 3: TREATMENTS FOR AGD

METHODS

Screening of potential amoebocides and disinfectants

Identification of potential compounds for toxicity screening

Previous studies have screened a large range of different anti-protozoal compounds and antibiotic compounds for their toxicity to *Neoparamoeba* sp. (Howard and Carson 1994, Powell and Clark 2003). Of these compounds screened, a few candidate compounds had been identified, but further development was limited due to either potential target animal (salmon) toxicity or the potential cost of treatment. In addition, some of the treatment options identified were not considered worth exploring further through consultation with the Tasmanian Aquaculture Industry (eg. the addition of disinfectants to improve freshwater bathing efficacy).

Review of the published scientific literature revealed compounds not previously tested that may have potential for use as amoebocides or amoebostats if used as either bath treatments or as an in-feed delivery treatment (Table 4).

Crude whole gill amoeba isolates

Gill mucus was extracted from the gills of AGD affected salmon according to the methods described by Powell and Clark (2003). The number of viable amoebae was determined using a haemocytometer and staining with trypan blue (Powell and Clark 2003) then the raw mucus/amoeba suspension was aliquoted at a concentration of 10 000 cells mL^{-1} in a 25 well Replidish^{TM}. A stock solution of the chemical being tested was then added to each replicated well to yield the final test concentration (Table 5) and a total test volume of 5 mL. The number of viable amoebae within the well was counted each successive hour for 3 h using the trypan blue method of Powell and Clark (2003). All test concentrations were derived from effective concentrations for other parasites (see table 4) or based upon their solubility in seawater. The efficacy of treatments to kill amoebae were tested using ANOVA following testing for normality of the data. Where the data were not normally distributed nor variance non-homogeneous, a log or square root transformation was used.

Table 4. Summary of potential compounds for further toxicity testing against *Neoparamoeba* sp.

Compound	Registered Ag/Vet use[1]	Mode of Action[2]	Pathogens tested against	Type of test	Relative Efficacy	Relative toxicity[3]	Reference
Amprolium	Y	A	*Ichthyophthirius* sp	in vivo	++	low	Rahkonen and Koski (2002)
			Ichthyobodo sp	in vivo	+		Tojo and Santamarina (1998a)
			Gyrodactylus sp	in vivo	+		Tojo and Santamarina (1998b)
			Hexamita sp.	in vivo	+		Tojo and Santamarina (1998c)
Toltrazuril	Y	A	*Ichthyobodo* sp	in vivo	+	low	Tojo and Santamarina (1998a)
			Gyrodactylus sp	in vivo	+		Tojo and Santamarina (1998b)
			Hexamita sp.	in vivo	+		Tojo and Santamarina (1998c)
Bithionol	Y	A	*Ichthyobodo* sp	in vivo	+++		Tojo and Santamarina (1998a
			Gyrodacylus sp	in vivo	+++		Tojo and Santamarina (1998b)
			Hexamita sp.	in vivo	+++		Tojo and Santamarina (1998c)
Albendazole	Y	A	*Ichthyobodo* sp	in vivo	+		Tojo and Santamarina (1998a)
			Gyrodacylus sp	in vivo	+		Tojo and Santamarina (1998b)
			Hexamita sp.	in vivo	+		Tojo and Santamarina (1998c)

Table 4 continued

Compound	Registered Ag/Vet use[1]	Mode of Action[2]	Pathogens tested against	Type of test	Relative Efficacy	Relative toxicity3	Reference
Caprylic acid	?	?	*Cryptocaryon* sp.	in vivo	++	low	Hirazawa et al. (2001a)
			Benedenia sp.	in vitro	++	low	Hirazawa et al. (2001b)
			Pseudocaligus sp.	in vitro	++	low	Hirazawa et al. (2001b)
			Kudoa sp.	in vitro	++	low	Hirazawa et al. (2001b)
Chloramine-T	Y	D	*Neoparamoeba* sp.	3h test	++	low-mod	Powell and Clark (2003)
				Agar plate	++		Howard and Carson (1994)
			Ichthyophthirius sp.	in vivo	++	low	Cross and Hursey (1973)
			Icthyobodo sp.	in vivo	++		Ostland et al (1995)
Chlorine Dioxide	N	D	*Neoparamoeba* sp	3h	++	low-mod	Powell and Clark (2003)

Table 4 continued

Compound	Registered Ag/Vet use[1]	Mode of Action[2]	Pathogens tested against	Type of test	Relative Efficacy	Relative toxicity[3]	Reference
Hydrogen Peroxide	N	D	*Neoparamoeba* sp.	3h test	++	mod	Powell and Clark (2003)
				Agar plate	++		Howard and Carson (1994)

[1] registered for agricultural or veterinary use in Australia

[2] A = antiprotzoal, D = disinfectant biocide, ? = unknown

[3] Toxicity to target (host) animal, low, moderate or high

Table 5. Summary of test concentrations examined using the gill isolate *in vitro* assay

Compound	Tradename	Test conditions				
		Test concentration (mg L^{-1})				
		100	10	1	0.1	0.01
Amprolium	Amprolium200™	+	+	+	+	
	Coccivet™	+	+	+		
Toltrazuril	Baycox™		+	+	+	
Albendazole	Alben™		+	+	+	
Bithionol	--			+	+	+
		Test concentration				
--	Citrox™	1%	0.1%			
		Test concentration				
		1mM		0.1mM		0.01mM
Caprylic acid		+		+		
Caprylic acid in ETOH		+		+		+

Individual differences in means were compared using a Bonferroni planned contrast post-hoc analysis relative to seawater controls.

5-day toxicity assay

Acute toxicity tests using either crude gill isolates or isolated adherent *Neoparamoeba* sp. (3 h tests) are suitable for the testing of toxicity over short duration and identifying candidates suitable for bath-type treatments. However, in order to test for candidate compound toxicity to *Neoparamoeba* sp. for in-feed delivery, a 5 day continuous exposure toxicity assay was developed (Powell et al. 2003). Using this method, Adherent isolated *Neoparamoeba* sp. were incubated in a 96-well microtitre plate in sterile filtered seawater at 18^{o}C for 5 days at a concentration of 10^4 cells $well^{-1}$ (total volume 200 μL). The test chemical was added to the culture at the start of the experiment. Replicate wells (minimum of n = 8) were destructively sampled daily, amoebae removed by the addition of 40 μL of 1% trypsin-EDTA and the viable amoebae counted using the trypan blue method (Powell et al. 2003). Efficacy of treatments could be compared against control wells (containing no test compound).

Artificial softened freshwater.

Experimental series 1: Water hardness and the influence on AGD

Water hardness was proposed by Powell and Clark (2003) as a major contributor to the survival of gill amoebae in freshwater in vitro. Indeed, Best (2002) demonstrated that even under brackish water conditions, the cationic environment of the cultured strain of *Neoparamoeba pemaquidensis* (PA027) was important for survival. More recently, we have shown that water hardness is a more dominant effect on the survival of *Neoparamoeba* sp. in freshwater than the dissolved organic carbon and tannin concentrations (Green et al. 2004). Even under seawater conditions, calcium is essential for the survival of *Neoparamoeba* sp. *in vitro* (Green et al. in prep).

To assess the effects of water hardness on the efficacy of freshwater bathing, two experiments were conducted. The first was a laboratory-based study where seawater acclimated Atlantic salmon (mean weight 323 g ± SE 12 g) were infected with *Neoparamoeba* sp. Once the infection was established, the fish were bathed in either seawater (control), soft freshwater (mean total hardness 37.4 ± SE 5.4 mg L^{-1} $CaCO_3$ equivalents) or hard freshwater (mean total hardness 236.2 ± SE 11.9 mg L^{-1} $CaCO_3$ equivalents) for 3 h. The number of viable amoebae on the gills and severity of AGD was measured using techniques described above (Robert and Powell 2003b).

In the second experiment the effects of reduced water hardness on marine reared Atlantic salmon on a fish farm were assessed. Two 34 m^3 pens of clinically AGD affected salmon (mean mass 1.46 ± SE 0.08 kg) were split to provide 4 pens (2 per treatment) and bathed in either oxygenated dam water (173 mg L^{-1} $CaCO_3$ equivalent total hardness) or artificially softened dam water (19.3 mg L^{-1} $CaCO_3$ equivalent total hardness) (Roberts and Powell 2003b). To artificially soften the water, dam water was first flocculated with 100 mg L^{-1} $Al_2(SO_4)_3$ and then the clear water was passed through a Series 255 Valve/440i Control water conditioner (Filterworks Holden Hill, SA) containing a Purolite C-100E cation exchange resin where multivalent ions were exchanged for Na^+.

A lethal sample of fish (n = 6 per pen) was taken immediately following bathing and again at 8 weeks post-bathe. Gross gill pathology was assessed for all fish according

to the standard gross gill pathology scoring method (modified form Powell et al 2000) (Table 6):

Table 6. Gross gill pathology scoring scheme (after Roberts 2004).

Score	Descriptor	Description
0	Clear	Gills appear clean, healthy red colour
1	Faint spots	1-3 faint spots and slight increase in mucous coat
2	Spots	>3 clearly defined spots and thickened mucous coat
3	Patches	Established, thickened and numerous patches

Gross gill score and mucus samples for iFAT (indirect fluorescent antibody test) were taken from a sample of fish (n = 10 per pen) every 2 weeks for up to 8 weeks post-bath (Roberts and Powell 2003b).

Alternatives to ion exchange water softening

Following the publication of the CRC report commissioned to examine the availability and potential for commercial application of water softening technology in freshwater bathing (Koehnken 2003), a compound known as Calgon T (sodium hexametaphosphate) was identified. Calgon T (Albright and Wilson (Australia) Pty Ltd) is a polyphosphate compound that chelates divalent cations (primarily Ca^{2+}) from freshwater. The efficacy of Calgon T was tested in hard and soft freshwater (225 and 22.5 mg L^{-1} $CaCO_3$ equivalent hardness respectively) at concentrations representing the range of concentrations recommended by the manufacturer (3-6 mg L^{-1} Calgon T for each mg L^{-1} total hardness). To test the efficacy of Calgon T at killing amoebae (due to chelation of Ca^{2+}), a minor modification of the *Neoparamoeba* sp. primary culture acute 3 h toxicity assay as described above was used (Green 2003).

Experimental series 2: Artificially softened water in semi-commercial field trials

Following the one-off use of artificially softened water above, a second semi-commercial scale study was designed with the intent of repeat bathing experimental cages of Atlantic salmon.

Atlantic salmon (average weight 697g) from a commercial fish farm were allocated to one of 4 replicated 5 x 5 x 5 m net-pens (125 m^3 volume) at a stocking density of 2.8

kg m^{-3} (500 fish per pen). The experimental net-pen system (Fig. 14) was moored at a commercial fish farm site and surrounded by a predator net, bird net and electric fence to deter predation from seals and birds. There was no evidence of predation by seals throughout the duration of the study. The fish were stocked into the system at the time when the parent population was determined as requiring a commercial freshwater bath for the control of AGD. Each experimental net pen was then bathed individually as described below.

For the purposes of this study, the source water used for freshwater bathing was from a freshwater dam that had characteristically hard water (Clark 2002). Two pens, designated as hard water pens, were bathed with the source water directly. Two pens, referred to as soft water pens, were bathed with source water that had been passed through a Series 255 Valve/440i Control water conditioner (Filterworks, Holden Hill, SA) containing a Purolite C-100E cation exchange for exchange of divalent cations with Na^+. Bathes were carried out in a 9000 L tarpaulin pulled over one corner of the cage. After the end of the bathe, the tarpaulin was pulled away and the fish returned to their source cage underneath (Fig. 14). Water quality for each bathe was monitored continuously using a YSI 556 water quality analyser. The water hardness and quality for the bathes are shown in table 7.

Table 7. Mean (± SE) water quality parameters for hard and soft water bathes

Bathe event	1	1	2	2
Treatment	Soft	Hard	Hard	Soft
Hardness (mg L^{-1})	51.0	259.8	ND	ND
Temperature (^oC)	18.8 (0.01)	19.6 (0.00)	14.5 (0.01)	12.5 (0.00)
Salinity (g L^{-1})	1.33 (0.002)	1.24 (0.01)	8.25 (0.40)	1.85 (0.00)
DO (% air saturation)	122.7 (0.9)	163.1 (1.6)	116.4 (0.7)	149.9 (0.8)
DO (mg L^{-1})	11.3 (0.1)	14.9 (0.1)	11.4 (0.1)	15.8 (0.1)
pH	7.76 (0.01)	7.54 (0.00)	8.36 (0.02)	7.41 (0.01)
ORP (mV)	51.0 (0.01)	50.4 (0.02)	41.3 (0.1)	36.4 (0.01)

ND = not determined

Prior to and immediately following the bath a sample of 20 fish was dip-netted from each cage and lightly anaesthetised with AQUI-S. The gross gill pathology was scored according to a semi-quantitative scale developed in conjunction with the Tasmanian salmon aquaculture industry and modified from the method used by Aquatas Pty Ltd for gill health assessment (Fig. 15). Ten fish from each cage, were lethally sampled and killed with a overdose of AQUI-S anaesthetic. Fish were weighed, measured and the gills were removed. Total viable amoeba counts were made according to established methods (Powell and Clark 2004; Harris et al 2004a; 2004b in press) from one half of the basket. The number of viable gill amoebae per fish was determined and corrected for the mass of the fish by dividing by the natural log mass since log gill surface area scales with log mass of fish (Palzenberg and Phola 1992). The remaining half of the gill basket was fixed in Davidson's seawater fixative for histology as described above.

Fish were fed once daily according to satiation (visual cessation of feeding activity). After 7 days and thereafter every 2 weeks, the above sampling regime was repeated until the gross gill pathology score trigger point was reached at which point the required cages were bathed. If hard and soft water cages differed in their scores, then they were bathed according to their individual score, which meant that hard and soft water treatments became out of synchrony (see results). The data for individual cages were pooled and treatments compared over time using ANOVA.

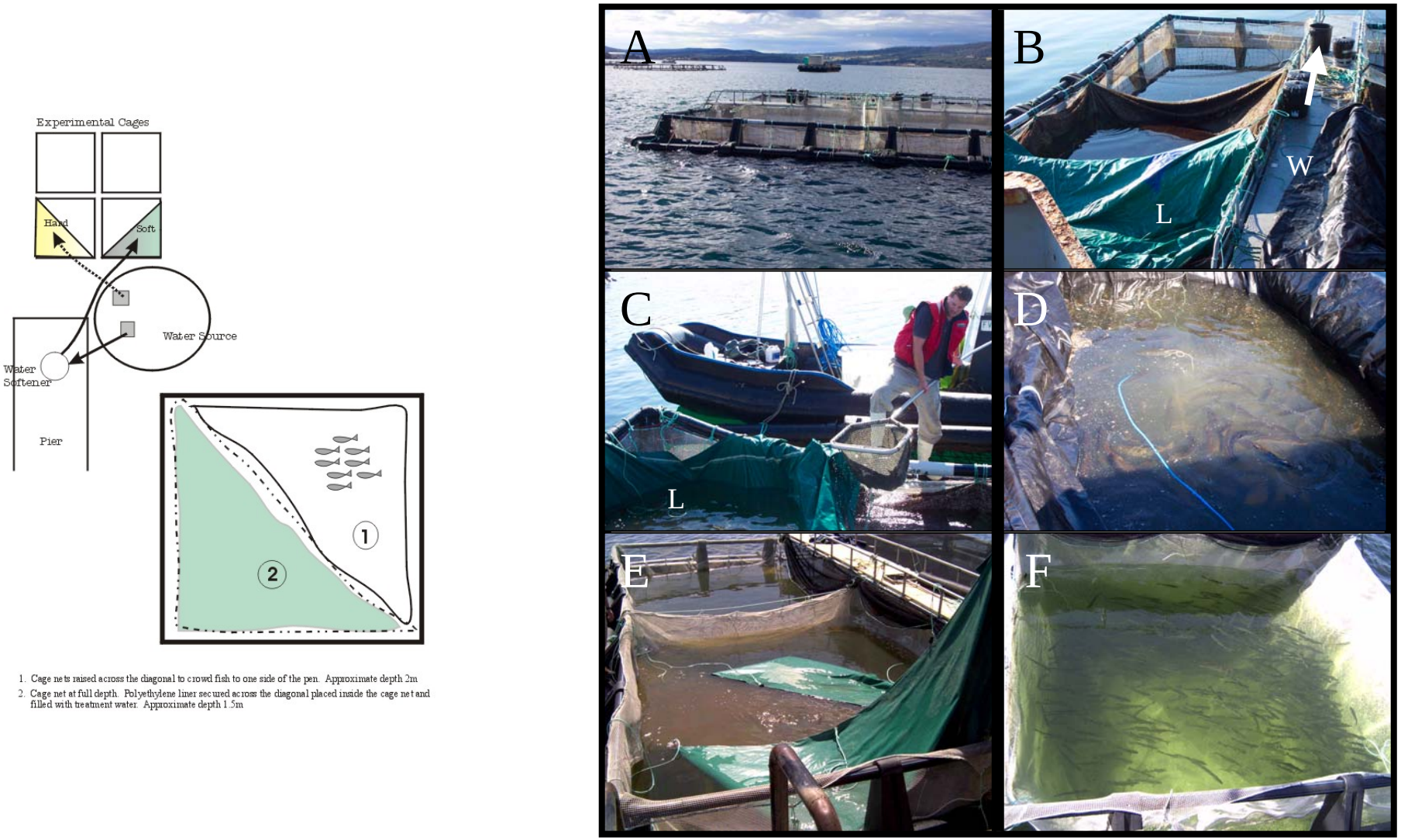


Figure 14. Left hand panel. Schematic representation of the bathing process for the experimental cages. Right hand panel. (A) Experimental net-pen cage. (B) tarpaulin bathing liner (L) secured across the diagonal of the pen to the walkway (W). Each pen has its individual feed bins (arrow). (C)Fish are dip neted from the net-pen into the bathing liner (L) and C) bathed in freshwater with oxygen for 3 h fore the bathing liner is pulled away (D) and the fish remain in the cage (E).

Gill assessment criteria

Gill will be assessed for gross score at the time of bathing and sampling (or in exceptional circumstances in an intervening period).

20 fish will be removed from each experimental cage for gill assessment (4% of the population based on cage numbers of 500 fish). Note commercial practice would assesses at least 50 fish from at least 25000 fish per cage which represents 0.2% of the cage population. Thus the experimental criteria assess a greater proportion of the cage population.

Gill will be graded according to the Aquatas scheme:

Clear – All gills appear clean, healthy red colour, no white patches or excessive mucus present
Very Light – 1 amoeba spot, not raised, infrequent white or peppered markings, light mucus or thin mucus strings
Light – 2 or 3 small white spots per fish, extra mucus, several areas of white or peppered markings, usually only 1-2 affected gill arches
Medium – Clusters of 3 or more raised spots or 1 or 2 established patches or plaques, excess mucus, usually 2 or more affected gill arches
Heavy – 3 or more established patches or plaques, many clusters of small raised spots, heavy mucus and gill thickening, gills may appear pale (wishy washy), usually 3 or more gill arches affected

The gross gill score will then be calculated according to the Aquatas calculations:

$$\text{Cage Score} = \frac{(\#\text{very light} \times 1) + (\#\text{light} \times 2) + (\#\text{medium} \times 4) + (\#\text{heavy} \times 6)}{\text{Total no. checked}}$$

The cage score will therefore range from 0 (*ie*. all fish clear) to 6 (*ie*. all fish heavy).

Trigger points for bathing

The need to bath a cage in the experimental system will depend upon the severity of gross signs. The following trigger points will act as a guide:

Gross score	**Action**
>1.5	Bathe regardless
0.75-1.0	Bath in 1 week
< 0.75	Reassess in 2 weeks with likelihood of a bathe being required (ie score will be >1.5)

Deviations

- If mortalities increase or feeding response declines markedly, then UTAS staff will be immediately contacted and a bath (and associated sampling) will be implemented immediately (within 48h).
- The cage score should be considered in conjunction the actual individual scores as sometimes the cage score may not reflect the number of fish in the cage with heavy AGD. In some cases a significant percentage of fish (*ie*. say 20%+) may be heavy, but the rest are clear.
- In exceptional circumstances (eg jellyfish swarm or algal bloom) decision to bathe will be made between Aquatas management staff and UTAS staff.

Figure15. Gross gill pathology assessment criteria used for artificial soft water bathing experiment.

Chloramine-T disinfection of salmon in seawater

Chloramine-T has been shown to be effective at reducing viable amoeba numbers on the gills of salmon bathed in freshwater (Powell and Clark 2004). The progression of this work was to examine the efficacy of chloramine-T on the viability of gill amoebae in seawater. In total three sets of experiments were conducted. The first involved the bathing of AGD affected Atlantic salmon from a commercial fish farm, in tanks containing either seawater only (control), freshwater only, freshwater and 10 mg L^{-1} chloramine-T, or seawater and 10 mg L^{-1} chloramine-T for 1, 3 or 6 h (Harris et al. 2004a). The viable number of gill amoebae on the gills was determined as described above and gills were examined histologically for the proportion of lesioned gill filaments as described above (Harris et al. 2004).

In the second set of experiments, AGD affected Atlantic salmon were bathed in a $1m^3$ tank of oxygenated seawater containing 10 mg L^{-1} chloramine-T or freshwater for 1 h (Harris et al. 2004a). Following the bath, fish were transferred to pocket nets on the side of commercial seacages where they were held for a subsequent 3 weeks. A weekly sample of 10 fish was taken from each of the nets (2 nets per treatment, 10 fish per net), and the gills removed for histological examination of the proportion of lesioned filaments as described above (Harris et al. 2004).

In the third set of experiments, four groups of 500 AGD affected Atlantic salmon were maintained in an experimental replicated cage unit (see Fig. 14) in 125 m^3 nets at a commercial fish farm site. Fish were either bathed using a tarpaulin liner slung across the corner of the cage unit (see Fig. 14) containing freshwater or seawater containing 10 mg L^{-1} chloramine-T for 1 h under slightly hyperoxic conditions. Oxygen concentration was carefully monitored in the bathes since research has demonstrated that oxygenation influences the toxicity of chloramine-T in fresh and salt water (Powell and Harris 2004). To maintain oxygen levels, air was pumped into the bath liner using a compressor and oxygen titrated from oxygen cylinders to maintain a dissolved oxygen level of between 90 and 110% air saturation (Harris et al. 2004b in press). Fish were bathed for 1 h in either freshwater (local dam water) (2 cages) or seawater containing 10 mg L^{-1} chloramine-T (as Halamid^{TM}), (2 cages). A sample of 10 fish were removed from each cage prior to and immediately following the bathe. The fish were lethally anaesthetised with Aqui-S or clove oil, weighed and

measured and the gross gill score determined according to industry standards. The gills were then removed and half of the gill basket incubated in sterile seawater and the number of viable amoebae determined as described above. The other half fixed in seawater Davidson's fixative for histological examination as determined above. The number of viable gill amoebae per fish was determined and corrected for the mass of the fish by dividing by the natural log mass since log gill surface area scales with log mass of fish (Palzenberg and Phola 1992). Every subsequent 2 weeks following the bathe, 10 fish were removed from each cage and sampled as described. After 6 weeks the bathing process was repeated and a second cycle of fortnightly sampling continued (Harris et al. 2005).

Toxicity of chloramine-T to Atlantic salmon smolts

Oxygen and salinity influences

Pilot investigations into the efficacy of chloramine-T in seawater compared with freshwater suggested that there may be influences of salinity in the toxicity of chloramine-T . In similar tests, high levels of oxygen appeared to enhance the redox potential of the treatment bath (J. Harris unpublished pilot data). As a consequence of these apparent phenomena, a short term acute static toxicity experiment was devised.

Atlantic salmon smolts (mean mass 47.8 g ± SE 0.7g) were maintained in freshwater or acclimated to 30 g L^{-1} seawater. Fish were allocated to round plastic tanks at a density of 9.5 kg m^{-3} at 17^{o}C ± 1^{o}C with either aerated freshwater (100% air saturation), oxygenated freshwater (200% air saturation), aerated seawater (100% air saturation) or oxygenated seawater (200% air saturation). Chloramine-T (as Halamid^{TM}) was added to duplicate tanks at 0 (control), 5, 10, 25,or 50 mg L^{-1} and the fish maintained for 12 h until sampling (Powell and Harris 2004). Where fish became moribund prior to the 12 h sampling prior (moribund defined as loss of equilibrium, sporadic ventilation and unresponsive to the touch), fish were killed with an overdose of clove oil anaesthetic. A caudal blood sample taken and the gills removed for histological examination (Powell and Harris 2004).

Toxicity in terms of the median lethal time (LT50) were calculated from log transformed plots of the morbidity data (Sprague 1969). Blood samples were analysed for plasma chloride concentration (Zall et al. 1956), plasma haemoglobin

(Sigma procedure 525) and osmolality using a Wescor Vapro 5520 vapour pressure osmometer (Powell and Harris 2004).

Physiological effects of chloramine-T exposure in salmonids

Previous studies on the physiological effects of chloramine-T on salmonids have shown that acute exposure results in a transient internal hypercapnia due to the diffusion limitation of carbon dioxide excretion across the fish gill (Powell and Perry 1996; 1999). However, the effects of chloramine-T exposure seawater and in Atlantic salmon have not been investigated.

Atlantic salmon (mean weight 775.7 g ± SE 31.7 g), showing no signs of AGD were cannulated under clove oil anaesthetic via the dorsal aorta according to the method of Soivio et al (1975) (see above). Following a recovery period of approximately 24 h in black acrylic respirometer boxes supplied with flowing recirculated seawater (35 g L^{-1} salinity, 17^{o}C and 90-100% air saturation), the 750-L system containing the respirometer boxes was partially closed for 6 h to create a recirculating volume of 100 L. The fish were then exposed to a 1 h pulse of seawater containing 10 mg L^{-1} chloramine-T while control animals were supplied continuously with recirculated (chloramine-T free) seawater. Arterial blood samples (500 μL) were anaerobically withdrawn from the cannula at 0, 1, 3, 6, 12 and 24 h and the removed volume replaced with modified Cortland's saline (Wolf 1963; Milligan et al. 1991).

From the whole blood sample, haematocrit was determined using a microhaematocrit method (Statspin®III, Norfolk Scientific), whole blood haemoglobin concentration measured using a cyanomethaemoglobin method (Sigma procedure 525) and the arterial oxygen tension was measured using a Cameron Instrument Company (Port Aransas, Texas, USA) polarographic oxygen electrode in a thermostatically controlled Radiometer BMSIII blood gas analyser (Radiometer, Copenhagen Denmark) connected to a Cameron Instrument Company BGM 200 blood gas meter. Blood pH was measured using an Activon (AEP333) combination electrode calibrated to ± 0.005 using Radiometer precision buffers and interfaced with the Cameron Instruments BGM 200 blood gas meter. The remaining blood was centrifuged at 8000 x g and the plasma analysed for total carbon dioxide content using a Cameron

Instrument Company Capni-con 5 total CO_2 analyser calibrated against a 20 mM $NaHCO_3$ standard.

Changes in respiratory and acid-base parameters were analysed using a repeated measures two-way ANOVA with a Dunnett's post-hoc planned contrast.

Other oxidative disinfectants

Previous studies have investigated the efficacy of both hydrogen peroxide and chlorine dioxide as potential additives to freshwater (Powell and Clark 2004) or as disinfectants in seawater (Cameron 1994ab). Despite these previous studies that showed that the margin of safety for hydrogen peroxide (and to a limited extent chlorine dioxide) was narrow, there was interest from the Salmon aquaculture industry to examine the potential of using hydrogen peroxide as a bath treatment. In contrast to Cameron (1994ab), the potential efficacy of hydrogen peroxide in a bath treatment was tested at lower water temperatures (10^{o}C and 15^{o}C), similar to those experienced during the winter months in Tasmania.

Atlantic salmon smolts were infected on two occasions with *Neoparamoeba* sp. in a 3000 L recirculating seawater tank at 18^{o}C, salinity 35 g L^{-1}. After approximately 10 days the infection had progressed with all of the fish examined having a medium grade infection according to industry standards. The water temperature was then reduced 2^{o}C per day over the following 4 days (to 10^{o}C) or 2 days (15^{o}C).

A sample of fish (n = 9) was removed from the source population (+ve control) and killed with an overdose of clove oil anaesthetic, the gills dissected and half of the branchial basket transferred to sterile seawater for counting the number of viable gill amoebae (see details above). The remaining half of the brachial basket was fixed in seawater Davidson's solution for histological examination (see above). Fish were transferred to 60 L plastic tanks containing constantly aerated seawater at either 10 or 15^{o}C depending upon the temperature of the fish used. Hydrogen peroxide (Merck) was added to each tank at the following concentrations (Table 8).

Table 8. Study design for assessing hydrogen peroxide efficacy at removing gill amoebae (n = 9 per treatment, per time period).

H_2O_2 conc^n	10^{o}C	15^{o}C
+ve control	+	+
0 ppm	ND	20, 40, 60 min
500 ppm	20, 40, 60 min	20, 40, 60 min
1000 ppm	20, 40, 60 min	20, 40, 60 min
1500 ppm	20, 40, 60, min	ND

ND = not determined

Fish were removed from each treatment after 20, 40 or 60 min of exposure, lightly anaesthetised with clove oil anaesthetic (0.03 mL L^{-1}), marked on the under belly with a PanJet and transferred to a 3000 L recirculating water tank where they were allowed to recover. After 18 h of recovery, fish were removed from the recovery tank and killed with an overdose of clove oil anaesthetic, then the gills removed for determination of the number of viable gill amoebae, or fixed for histological examination.

The number of viable gill amoebae were compared between hydrogen peroxide concentrations and times using two-way ANOVA with differences between groups determined using a Tukey's test.

Mucolytic treatments

The addition of the mucolytic agent L-cysteine ethyl ester (LCEE) to cutaneous mucus resulted in a significant reduction in viscosity. Moreover, when LCEE was fed to salmon, AGD appeared to be retarded (see above, Roberts 2004). This work suggested that feeding LCEE to salmon either prior to or at the point of infection could potentially retard the onset of AGD and could therefore be a pharmaceutical method for the control of the disease in feed.

Atlantic salmon smolts (mean weight 150.9 g ± SE 8.3 g) were acclimated to seawater and randomly allocated to each of nine challenge tanks (see above). One group of three tanks was maintained as controls and fed oil coated feed but no LCEE. A second group was fed control diet up to the point of infection and thereafter LCEE medicated feed (therapeutic group). A third group was fed LCEE coated feed (8 g LCEE kg^{-1} feed) as described previously (Robert 2004). All fish were fed daily to satiation. Previous studies with LCEE (see above, Roberts 2004) indicated that feed intake was reduced in the LCEE fed group. To account for reduced feed intake, LCEE fed fish were fed first to satiation, thereafter the remaining groups were fed the same mount as that taken by the LCEE fed group. Thus each group received the same ration of feed. Fish were fed control or LCEE coated feed for 14 days prior to challenge with 356 amoebae L^{-1}. Over the pre-feeding period, LCEE fed fish consumed an average of 44.9 mg ± SE 1.9 mg LCEE kg^{-1} fish day^{-1}) (Roberts 2004).

A sample of fish was removed from each treatment (n = 9) immediately prior to pre-feeding, at the point of challenge (day 0), 4, 7, 10, 11, 14 and 18 days post-challenge. The fish were killed with an overdose of clove oil and a caudal blood sample taken, heparinised (100 IU mL^{-1} lithium heparin) and centrifuged at 10 000 x g and the plasma removed and frozen for biochemical analysis. The gills were removed and fixed in seawater Davidson's fixative for histological examination (as described above). Ten days post-challenge, the remaining fish in each treatment were removed from their tanks and bathed in soft freshwater (37.4 mg L^{-1} $CaCO_3$ total hardness) for 3 h and returned to clean (amoeba free) seawater. Therefore the rate of recovery from AGD could be determined in fish fed LCEE.

Plasma was analysed for osmolality (using a Vapro 5520 vapour pressure osmometer (Wescor, Utah, USA)), chloride concentration (using the method of Zall et al. 1956), total protein (using a commercial assay (Sigma-Aldrich) and total plasma ammonium (using the salicylate-hypochlorite method by Verdouw et al. (1978)) (Roberts 2004). All data were analysed by two-way ANOVA with a Student-Newman-Keuls post-hoc analysis to identify statistical differences (Roberts 2004).

Non-parasiticide approaches to AGD: the nutritional supplement Aquacite

One of the effects of AGD is the progressive loss of appetite of the fish and the subsequent reduced growth rate. The reduction in growth rate can be partially explained in terms of increased standard metabolic rate of AGD affected fish (see above). In commercial terms, a reduction in feed in take and growth rate compounds any days of lost feeding due to handling and bathing AGD affected salmon. This is all assuming that no mortalities occur due to AGD.

Aquacite and Betabec are nutritional supplements provided by James Mackie (Agricultural) in Scotland. Aquacite is a proprietary blend of bioflavenoids, beta 1,3 and 1,6 glucans, B vitamins, amino acids, calcified seaweed and vitamin C. Betabec is a proprietary blend of vitamins C, E, B1, B2, B6, B12 and extract of dried brewers yeast. According to the company, Aquacite and Betabec have the potential to bolster the immune system, and improve the general well being of the fish. Indeed data provided by the company suggest that Aquacite has some potential value in the control of lomiasis in Atlantic cod (*Gadus morhua*) and ichthyophthiriasis in rainbow trout (J. Mackie pers comm.). In order to test the efficacy of Aquacite and Betabec against AGD, the following studies were developed.

It was decided that with an in-feed study, the development and severity of AGD lesions as determined by histology was the most reliable indicator of efficacy. In order to assess the potential effects of the nutritional supplements on growth and feed intake, fish were fed to satiation by hand twice daily in the experimental challenge system (see above). Uneaten pellets were collected and the amount of feed consumed calculated on a daily basis corrected for the biomass of fish in each treatment.

Experimental series 1: Aquacite and Betabec fed in combination at the point of ***Neoparamoeba*** sp. *challenge.*

Seawater acclimated Atlantic salmon smolt (mean weight 164.4 g ± SE 9.5 g) were maintained in the experimental challenge system at 35 g L^{-1} salinity, 17^{o}C and fed a standard commercial pelleted diet to satiation. The systems were and inoculated with 100 cells L^{-1} *Neoparamoeba* sp. At the point of inoculation, 3 tanks received Aquacite and Betabec top-dressed onto the feed pellets in gelatin at a concentration of 2.5 g kg^{-1} and 8 g kg^{-1} respectively according to the manufacturers recommendations.

Controls consisted of fish fed gelatin coated commercial pellets only. Fish were fed twice daily by hand to satiation and any uneaten pellets collected and weighed. Thus it was possible to determine the amount of food ingested at each feeding. A sample of 6 fish from each treatment (2 fish per tank) was removed, killed with an overdose of clove oil anaesthetic, weighed and measured then the gills dissected and fixed in seawater Davidson's fixative for quantitative histological assessment of AGD (see above). After 21 days post-challenge, the remaining fish were killed with an overdose of clove oil anaesthetic, weighed and measured. Thus from the initial tank weights and final tank weights absolute and specific growth rates could be calculated after Busaker et al. (1991).

Experimental series 2: Aquacite and Betabec fed in combination for 14 days prior to Neoparamoeba sp. *challenge.*

As in experimental series 1, seawater acclimated Atlantic salmon (mean weight 109.7 ± SE 3.8 g) were maintained in the challenge system at 35 g L^{-1} salinity and 17^{o}C. These fish were fed Aquacite and Betabec top-dressed onto the commercial feed pellets according to manufacturers instructions (2.5 g kg^{-1} Aquacite, 8 g kg^{-1} Betabec). Controls consisted of fish fed gelatin coated pellets only. Fish were maintained on these diets for 14 days. As in experimental series 1, daily feed intake was monitored by feeding fish to satiation and collecting and weighing uneaten pellets. After 14 days of pre-feeding both Aquacite and Betabec, fish were fed on pellets on to which Aquacite only (2.5 g kg^{-1}) was top-dressed in gelatin. This was in accordance with the recommendations of the manufacturer who suggested that Betabec be fed on a cycle of 2 weeks out of 8 weeks (J. Mackie pers. comm).

After 14 days of pre-feeding, all fish were challenged by inoculating the tanks with *Neoparamoeba* sp. at 100 cells L^{-1}. Feeding to satiation and monitoring of feed intake was continued throughout the challenge. On days 0, 10, 15, 18, 21, 24, 27, 30 and 35 post-inoculation, 6 fish per treatment (2 per tank) were removed, killed with a overdose of clove oil, weighed and measured and the gills removed for histological examination (see above). As with experimental series 1, 35 days post-inoculation, all of the fish were killed, weighed and measured for the calculation of absolute and specific growth rates according to Busacker et al. (1991).

Experimental series 3: Aquacite and Betabec fed independently 14 days prior to ***Neoparamoeba*** sp. *challenge.*

As described for the previous two experiments, seawater acclimated Atlantic salmon (mean weight 111.9 g ± SE 13.4 g) were maintained in the challenge system at 35 g L^{-1} salinity and 17^{o}C. In this experiment, groups of fish (3 tanks per treatment) received one of 3 treatments. The first group were fed Aquacite top-dressed onto commercial pellets with gelatin at 2.5 g kg^{-1}. The second group were fed Betabec top-dressed on to commercial pellets with gelatin at 8 g kg^{-1}. The third group of controls received pellets that were coated with gelatin only. As described above, all fish were fed twice daily to satiation and the remaining pellets collected and weighed to determine daily feed intake. Fish were maintained on these diets for 14 days. After 14 days, the Betabec group received gelatin only (control) coated pellets. After the initial 14 day pre-feeding period, all tanks were inoculated with *Neoparamoeba* sp. at 100 cells L^{-1} and the fish continued to be fed and daily feed intake monitored during the resultant AGD challenge. As before on days 5, 7, 9, 12, 16, 21,23,26,28 and 30 post-inoculation a sample of 6 fish per treatment (2 fish per tank) were killed with clove oil anaesthetic, weighed and measured and the gills removed for quantitative histological assessment of AGD.

RESULTS AND DISCUSSION

Screening of potential amoebocides and disinfectants

Crude whole gill amoeba isolates

Disinfectants such as chloramine-T and citrox™ were effective at killing amoebae in vitro at the recommended test concentrations or below within the 3 h *in vitro* assay duration (chloramine-T recommended concentration 10 mg L^{-1} , citrox™ manufacturer's recommended concentrations 2% solution) (Fig. 16 and 17). Of the two disinfectants tested using this assay, only chloramine-T was effective at a concentration that would be practical for use under commercial conditions.

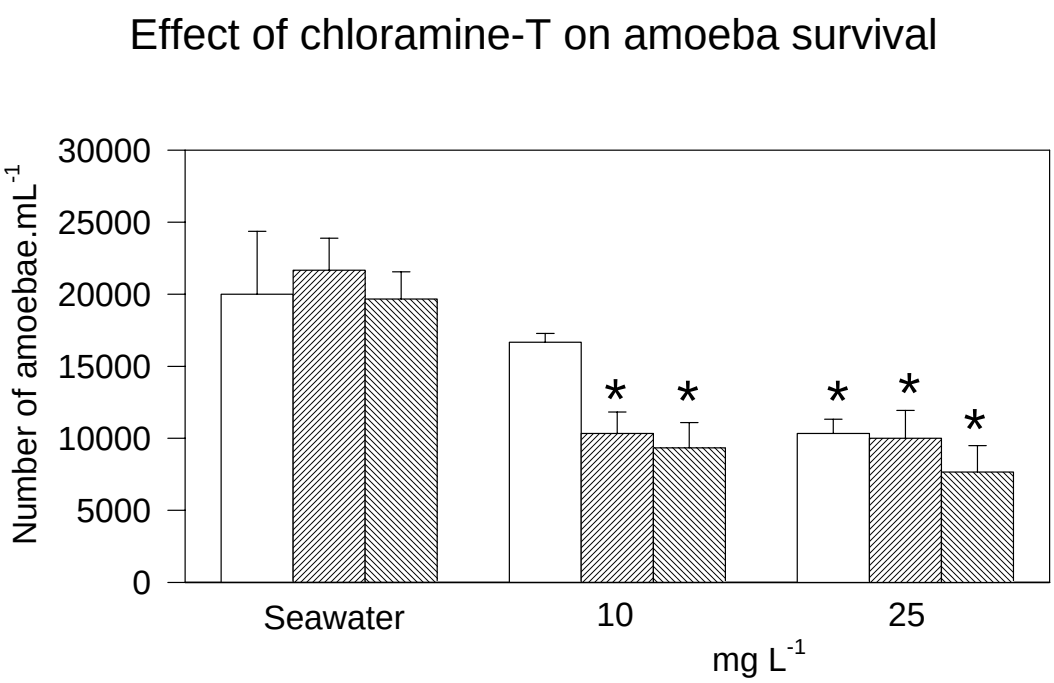


Figure 16. The effects of chloramine-T at two test concentrations on the survival of gill amoebae following a 1 (open bar), 2 (left hatch) or 3 h (right hatch) exposure *in vitro*. Asterisks represent significant difference from seawater control.

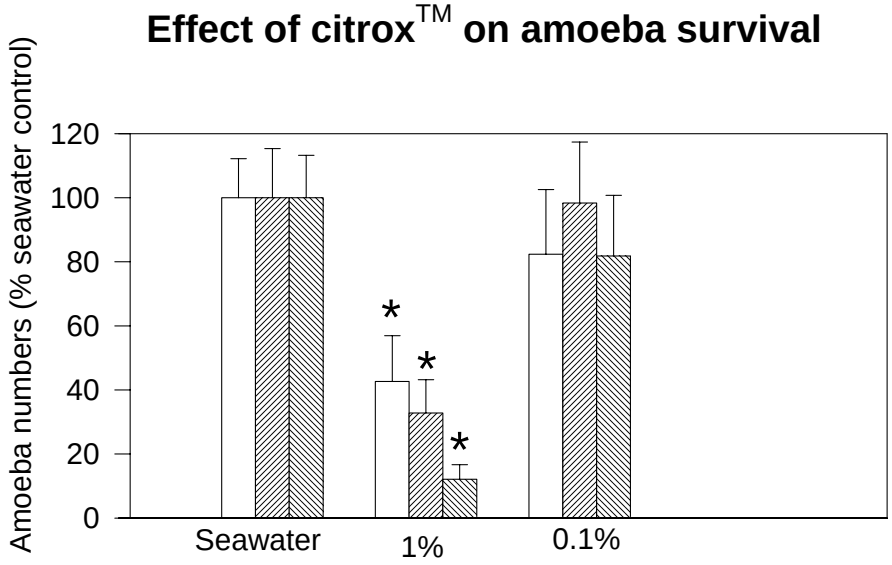


Figure 17. The effects of citrox™ at two test concentrations on the survival of gill amoebae following a 1 (open bar), 2 (left hatch) or 3h (right hatch) exposure *in vitro*. Asterisks represent significant difference from seawater control.

There was no significant effect of either of the amprolium containing preparations (Amprolium 200™ and Coccivet™) at any of the concentrations tested (Fig. 18). Toltrazuril, at 10 mg L^{-1} showed a reduced survival of amoebae by 3 h. However, the results were marginal (Fig 19). Bithionol had no effect on amoeba survival over the 3 h test period (Fig. 19). Albendazole (as Alben™) had a marginal effect on amoeba survival but only at the highest concentration tested and after 2 h of exposure (Fig. 20). Caprylic acid, as a raw organic fatty acid or pre dissolved in ethanol to give a final concentration of 1%, reduced amoeba survival at 1 mM, but not at lower concentrations (Fig. 21).

Of the non-disinfectant type compounds tested, only toltrazuril, albendazole and caprylic acid had any significant effect on amoeba survival. There was a trend for reduced survival with bithionol but this was not statistically significant.

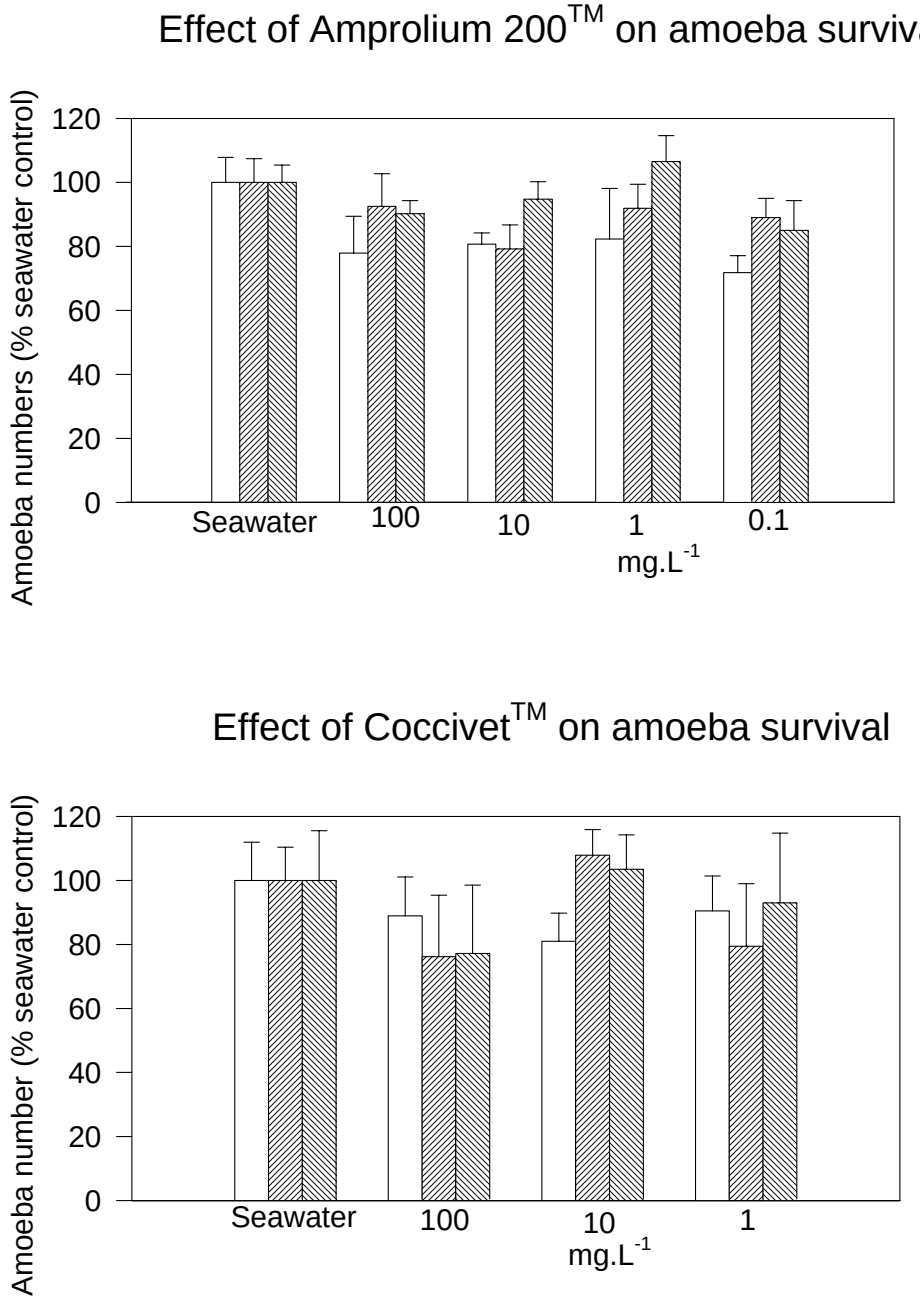


Figure 18. The effects of Amprolium 200™ and Coccivet™ at a range of test concentrations on the survival of gill amoebae following a 1 (open bar), 2 (left hatch) or 3h (right hatch) exposure *in vitro*.

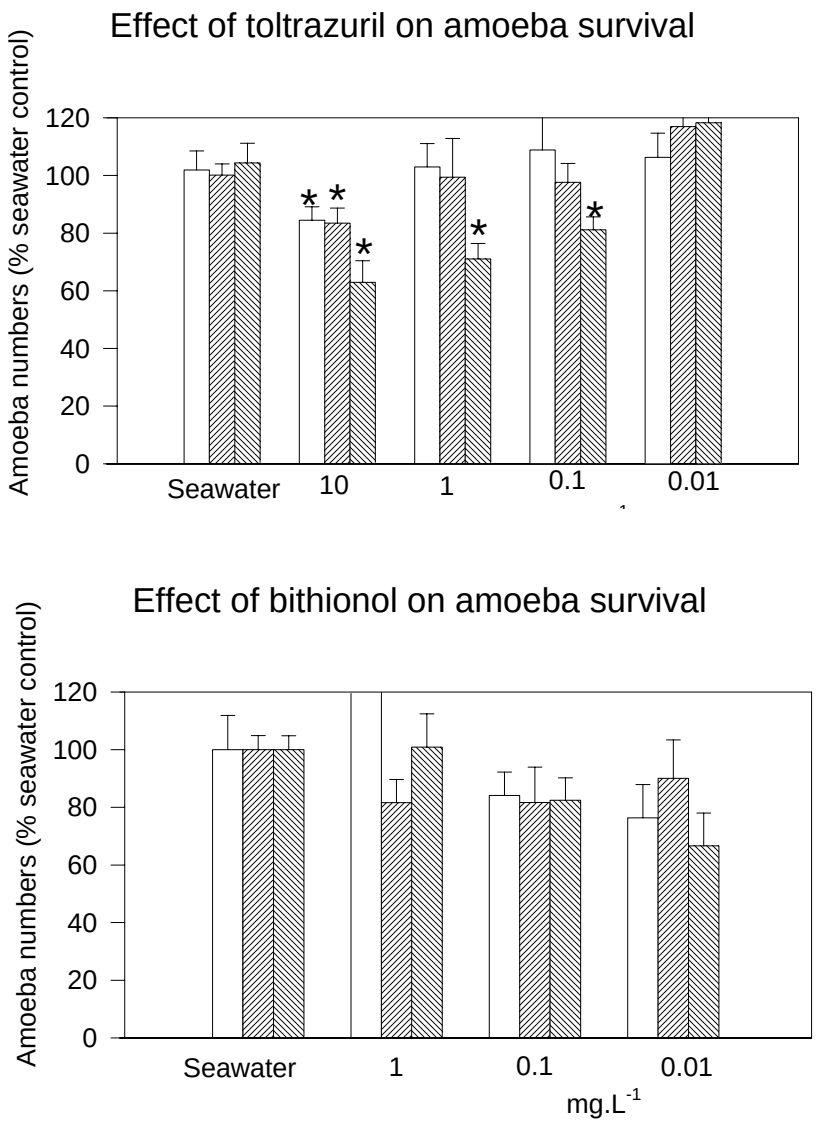


Figure 19. The effects of Amprolium 200™ and Coccivet™ at a range of test concentrations on the survival of gill amoebae following a 1 (open bar), 2 (left hatch) or 3h (right hatch) exposure *in vitro*.

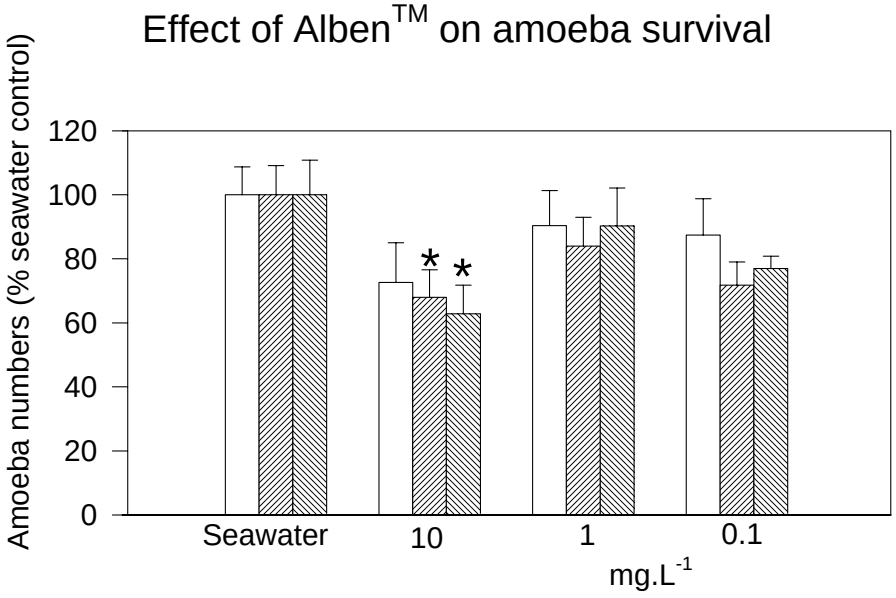
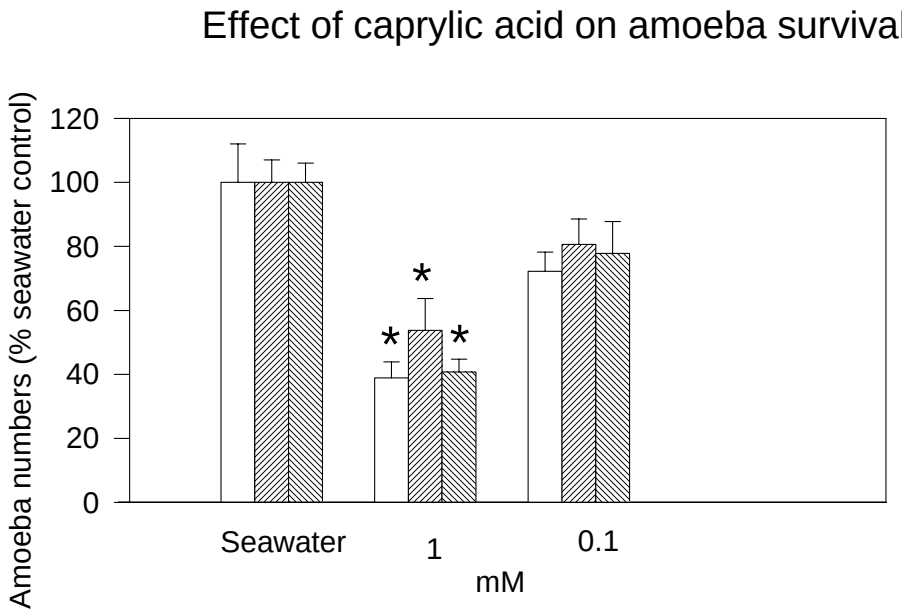


Figure 20. The effects of albendazole (as Alben™) at a range of test concentrations on the survival of gill amoebae following a 1 (open bar), 2 (left hatch) or 3h (right hatch) exposure *in vitro*. Asterisks indicate significant differences from seawater control.

The effects seen were only marginal with a 20-30% reduction in amoeba survival. Anecdotal evidence from one fish farm that has included caprylic acid into the diet of AGD affected salmon suggested that caprylic acid may be useful to prolong the inter-bath interval with AGD when infection rates are low such as in the early spring and later autumn (D. Mitchell pers. comm). Of those compounds showing some toxicity to amoebae, while it may not be feasible to consider a bath-type treatment with these compounds (due to cost or environmental release concerns), it may be feasible to include these compounds into the diet. A longer term toxicity assay was therefore developed and used to assess the specific toxicity to *Neoparamoeba* sp. and at continuous exposure for several days (see below).



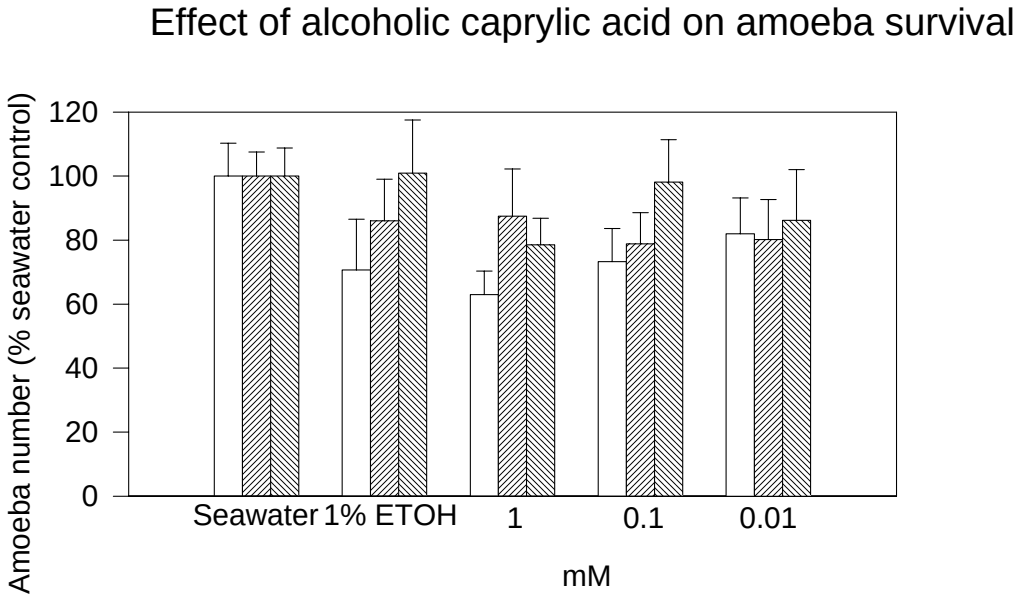


Figure 21. The effects of caprylic acid either as fatty acid (upper panel) or pre-dissolved in ethanol (lower panel) at a range of test concentrations on the survival of gill amoebae following a 1 (open bar), 2 (left hatch) or 3h (right hatch) exposure *in vitro*. Asterisk indicates significant differences from seawater control.

5-day toxicity assay

Using the 5-day toxicity assay, it was clear that *Neoparamoeba* sp. were insensitive to toltrazuril, amprolium and albendazole at 1 mg L^{-1} (Powell et al. 2003). However, bithionol was toxic to cultured *Neoparamoba* sp. in a concentration dependent manner (Powell et al. 2003). The superiority of bithionol as an oral treatment against other epithelial protozoal infections in fish has been demonstrated by Tojo et al. (1994) and Tojo and Santamarina (1998c). These results would suggest that bithionol is an ideal candidate for an in-feed amoebocide treatment for the control of AGD in salmon.

Artificial softened freshwater.

Experimental series 1: Water hardness and the influence on AGD

Bathing fish in soft water resulted in significant reductions in the number of viable amoebae on the gills of salmon held under laboratory conditions (Roberts and Powell 2003b). Under semi-commercial conditions, using a one-off bath of artificially softened farm dam water, there was a significant retardation of reinfection. Based upon gross gill score, the fish bathed in artificially softened water lagged approximately 2 weeks behind fish bathed in normal dam water (hard water) in terms of reinfection (Roberts and Powell 2003b).

Alternatives to ion exchange water softening

Calgon T significantly reduced the viability of *Neoparamoeba* sp. in the 3 h culture assay (Fig. 22). However, the feasibility of the use of this compound must also be considered due to its price and phosphate content. Calgon T costs \$1.99 kg^{-1} AUD and therefore to treat a 1ML freshwater bath at a hardness of 300 mg L^{-1} would cost \$3600 AUD. This represents a significant additional cost to freshwater bathing. Based on the fact that Calgon T is approximately 74% phosphate, so a 1ML bath with a hardness of 300 mg L^{-1} would release approximately 1.3 tonnes of phosphate into the environment, which would increase phosphate concentrations above acceptable limits, potentially promoting eutrophication. The use of Calgon T under these conditions would therefore not be environmentally sustainable.

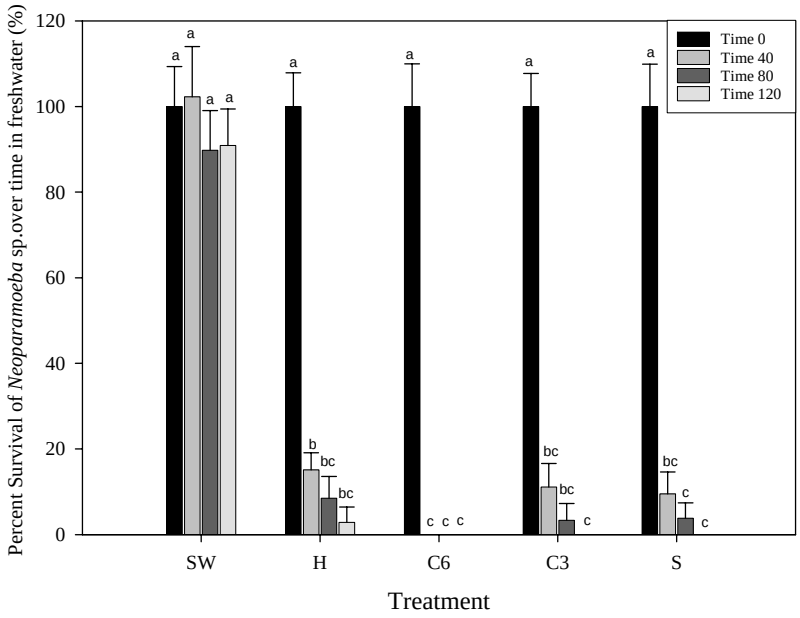


Figure 22. Differences in mean survival of *Neoparamoeba* sp. bathed in hard freshwater with varying concentrations of Calgon T (+ SE). Superscripts indicate significant differences between treatments ($P < 0.05$). SW = seawater control. H = hard freshwater (225 mg L^{-1} $CaCO_3$). C6 = hard freshwater with 6 mg L^{-1} Calgon T per mg L^{-1} of hardness. C3 = hard freshwater with 3 mg.l^{-1} Calgon T per mg L^{-1} of hardness. S = soft freshwater (22.5 mg L^{-1} $CaCO_3$).

Experimental series 2: Artificially softened water in semi-commercial field trials

There were no significant differences between replicate cages for each treatment with regard to weight, gill score and amoeba density so data were collapsed for both cages within each treatment. There was an improvement in condition and growth of the fish over the duration of the study (Fig. 23). Similarly, there were significant differences in the mass of the fish from hard and soft water groups only at 2 sample points reflective of the relatively small sample size used for these calculations (n=10 fish per cage). There were apparent differences in growth rate over the study period with hard water fish having absolute and specific growth rates of 7.31 g day^{-1} and 0.64% respectively compared with soft water bathed fish of 5.50 g day^{-1} and 0.48% respectively.

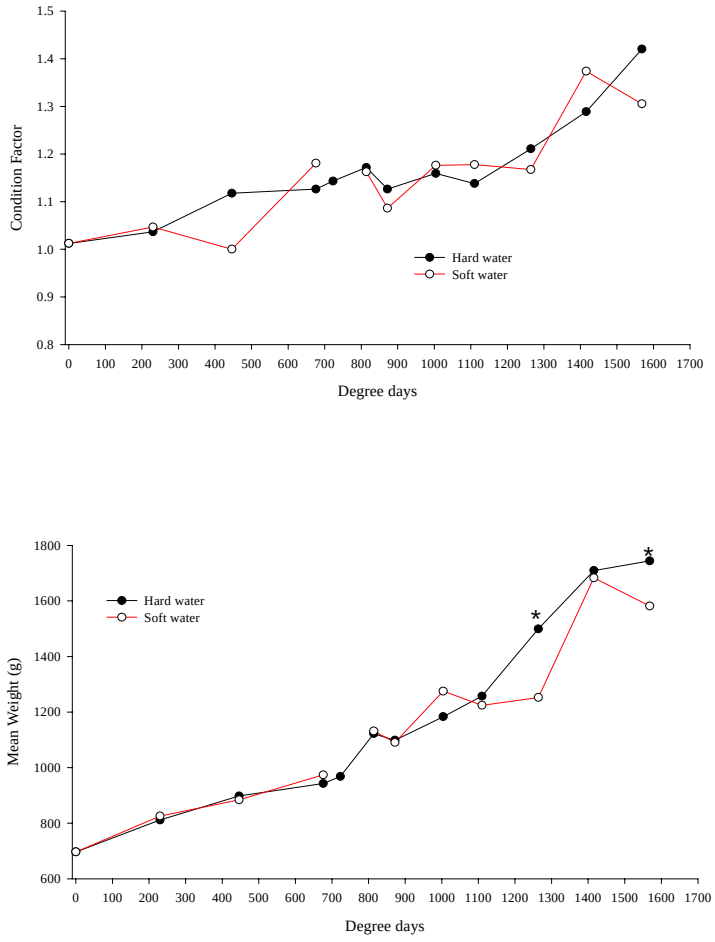


Figure 23. Changes in condition factor and mean weight of fish bathed in either hard water (solid symbols) or artificial soft water (open symbols). Asterisks indicate significant differences between hard and soft water groups. Error bars have been omitted for clarity.

Both hard and soft water bathing reduced the gross gill score one week following the bath after which the gross gill score steadily increased (Fig. 24). The hard water bathed fish reached the second bath trigger point 113 degree days before the soft water bathed fish. This represented a 13% increase in the inter-bath interval (Fig. 24). Gill amoeba density increased following the first bath treatment for both hard and soft water bathed fish peaking at approximately 695 degree days post-bath for both treatments (Fig. 24). Subsequent to the second bath there was a decrease in gill amoeba density albeit not to the low initial levels seen after the initial bath (Fig. 25 and 26). Subsequent to the second bath, although gill amoeba numbers increased to re-bath levels, gross gill score remained below the bathing trigger threshold throughout the remainder of the study. Unfortunately the gross gill score never reached the trigger threshold again before the end of the study. It was not possible

therefore, to examine whether there was any compounded effect of soft water bathing. However, it is apparent that during periods of an "active" AGD outbreak, bathing fish in soft water does extend the inter-bath interval significantly, re-affirming the results of Roberts and Powell (2003b).

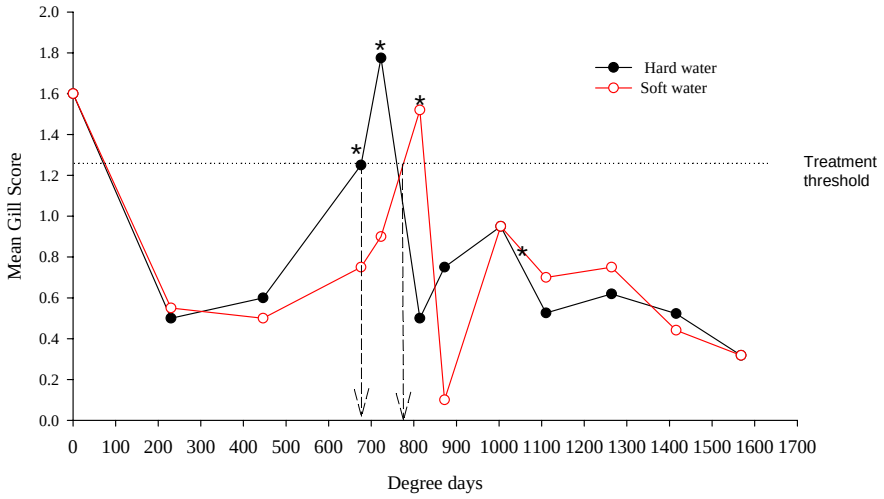


Figure 24. Change in mean gill score and viable amoeba density following an initial freshwater bath (Time 0) for fish bathed in either hard water (solid symbols) or artificial soft water (open symbols). Arrows indicate the inter-bath interval. Asterisks indicate significant differences between hard and soft water groups. Error bars have been omitted for clarity.

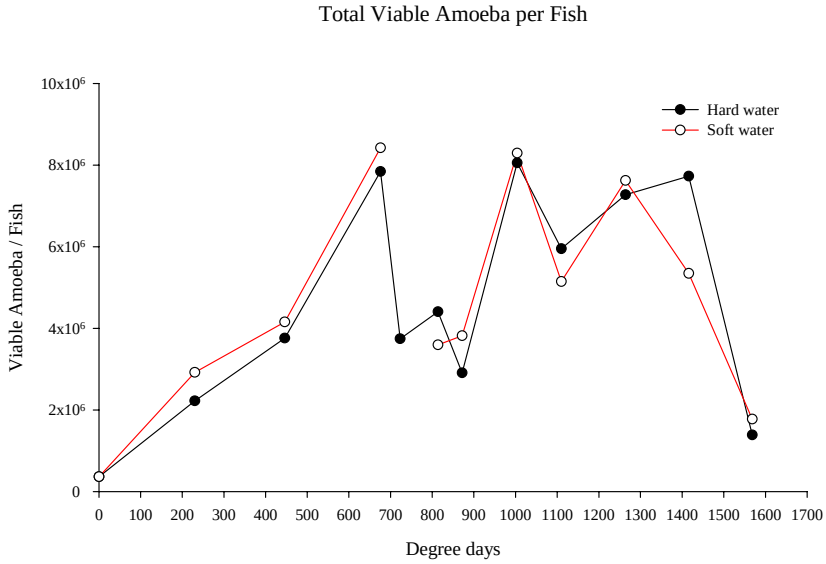


Figure 25. Change in viable amoeba density following an initial freshwater bath (Time 0) for fish bathed in either hard water (solid symbols) or artificial soft water (open symbols). Error bars have been omitted for clarity.

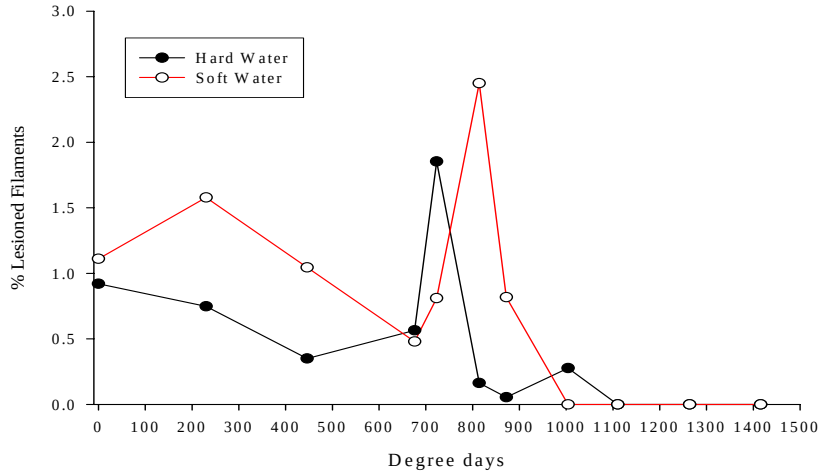


Figure 26. Change in the percentage of lesioned gill filaments following an initial freshwater bath (Time 0) for fish bathed in either hard water (solid symbols) or artificial soft water (open symbols). Error bars have been omitted for clarity.

Chloramine-T disinfection of salmon in seawater

Chloramine-T (10 mg L^{-1}) in seawater alone, significantly reduced the number of viable gill amoebae on the salmon gills with respect to the pre-exposure condition compared with freshwater alone, seawater control or freshwater with 10 mg L^{-1} chloramine-T added. Interestingly, there was no effect of the duration of exposure, such that a 1 h exposure was as equally effective as a 6 h exposure. (Harris et al 2004). In the second experiment, where fish were bathed then translocated into nets and held for a further 3 weeks, seawater and chloramine-T baths reduced gill amoebae numbers significantly compared with freshwater baths (Harris et al 2004). However, the gill lesions failed to resolve in seawater chloramine-T treated fish compared with freshwater treated fish in the subsequent 3 weeks post-bath holding period (Harris et al. 2004).

In the third set of experiments, chloramine-T in seawater bathes were as effective at removing gill amoebae as freshwater bathes. However, as with experiment 2, the histological lesions failed to resolve as quickly in chloramine-T treated fish compared with freshwater treated fish (Harris et al 2005). Nonetheless, there were no significant differences in the severity of AGD (as determined by histological lesion

counts) between freshwater and seawater chloramine-T treated fish (Harris et al 2005).

This suggested that chloramine-T at 10 mg L^{-1} in seawater was a least as effective as freshwater at disinfecting the gills of salmon of gill amoebae. Chloramine-T has been used as a treatment for bacterial and parasitic gill diseases of fish in freshwater for decades (Cross and Hursey 1973; From, 1980; Bullock et al. 1991; Ostland et al. 1995; Bowker and Erdahl 1998) and is widely used in hatcheries in Canada (Thorburn and Moccia 1993). This is the first time chloramine-T has been reported to have successfully used in seawater to treat a parasitic infection of salmon.

The use of a 1 h chloramine-T treatment in seawater has great potential for a cost effective control of AGD. To begin with freshwater is replaced with seawater. For farms that have limited access to freshwater, this may represent a significant saving specially where city water was previously purchased (G. Westbrook, Tassal Pty Ltd. pers comm). Secondly, the reduction of bath duration from a standard 2-4 h, to a 1 h bath represents a significant labour saving. Thirdly, the use of seawater and a disinfective treatment for AGD would mean that bathing liners could be lighter and deployed around or under the cages rather than over the top of cages. This would mean that fish could be bathed in situ rather than having to be transferred for the bathing process. This would potentially reduce the stress of bathing and labour costs due the minimal transfer of fish. The lighter liners may represent cheaper bathing infrastructure as the need for a highly impermeable tarpaulin is reduced.

There are however, issues that remain to be addressed. The use of a disinfective treatment in the ocean does represent the input of a chemical into the marine environment. Chloramine-T however, degrades quickly (to hypochlorite and para-toluenesulphonamide, pTSA). The hypochlorite ion is further reacted to chlorine and water whereas the pTSA is slowly degraded. A great deal of work has been conducted upon chloramine-T and its breakdown products since it has been a candidate for registration around the world for aquaculture. An experimental use permit has been obtained by the author for further investigations in salmon aquaculture for the Australian Pesticides and Veterinary Medicines Authority. It is

anticipated that research into the efficacy of chloramine-T and its commercialisation into Australian aquaculture will continue.

Toxicity of chloramine-T to Atlantic salmon smolts

Influences of salinity and oxygen

Both salinity and oxygenation status had significant influence on the toxicity of chloramine-T to Atlantic salmon smolts. Chloramine-T was less toxic in freshwater than in salt water. Chloramine-T was also more toxic under hyperoxia (200% air saturation) compared with normoxia (100% air saturation) (Powell and Harris 2004). Toxicity occurred as a result of an osmoregulatory disturbance that resulted in acute haemolysis. Histological examination of the gills indicated that moribund fish had extensive lamellar epithelial necrosis and congestion of blood within the central venous sinus. Fish exposed to 10 mg L^{-1} or less survived the 12 h static chloramine-T exposure. Surviving fish had little haemolysis or osmoregulatory disturbance although there was hypertrophy of branchial chloride cells consistent with ionoregulatory or acid-base disturbances (Powell and Harris 2004) that have been previously reported for chloramine-T exposure of rainbow trout in freshwater (Powell et al. 1994; 1995; Powell and Perry 1998).

At the recommended therapeutic concentration (10 mg L^{-1}) for 1 h, chloramine-T has low toxicity and minimal detectable pathological effect to Atlantic salmon smolts. Nevertheless, it is essential that the exposure concentration and duration be carefully monitored. Errors in the estimating the volume of the bath liner could lead to significant errors in estimating the exposure concentration. Under these conditions, it may be possible to approach the threshold for toxicity of chloramine-T especially if this is error is coupled with hyperoxia of the bath.

Physiological effects of chloramine-T exposure in salmonids

Results showed that there were no significant treatment effects between naive control and experimental fish; however, significant time effects were observed (Leef et al. in prep). As a result of repeated sampling regime, naive fish had significantly lower haematocrit values at 12 and 24 h and significantly lower whole blood haemoglobin concentration at 24 h compared to the time zero baseline. Additionally, arterial oxygen tension increased immediately post-exposure becoming significant at both 3

and 12 h (Leef et al. 2005 in press). In light of these results it is suggested that treatment of chloramine-T at the therapeutic concentration of 10 mg L^{-1} for 1 h has no significant respiratory effect in healthy (non-diseased) Atlantic salmon. These results are in conflict with previous published results where there were transient effects of chloramine-T exposure on respiratory and acid-base parameters in rainbow trout in freshwater (Powell and Perry 1996). However, seawater has a reduced capacity for oxygen dissolution and as such, marine acclimated fish tend to have higher ventilation rates compared with their freshwater counterparts. Since carbon dioxide excretion is sensitive to water flow over the gill (Iwama et al. 1987), it is possible that any transient effects of chloramine-T exposure would be immediately offset by the slightly increased ventilatory water flow over the gills. Alternatively, the chemistry of chloramine-T in seawater is likely to differ from that in freshwater due to the high concentration of chloride in seawater that may affect the hydrolysis of the chloramine-T molecule to its breakdown products sodium hypochlorite and paratoluenesulphonamide. Powell and Perry (1996) showed that sublethal levels of acute hypochlorite exposure of trout resulted in similar effects on respiratory and acid-base blood parameters as seen with chloramine-T and thus this was suggested as the primary mechanism by which chloramine-T exerted its effects on trout. If the degradation of the parent compound (chloramine-T) is not the same in seawater compared with freshwater then it is possible that the effects on fish may be different, consistent with our findings in the present study.

Other oxidative disinfectants

At the highest concentrations tested, hydrogen peroxide was acutely toxic to AGD affected Atlantic salmon smolts. The higher concentrations (1500 ppm) were not tested at 15^{o}C given the unexpected 100% mortality that occurred at 10^{o}C (Table 9). However, there was no mortality at 10^{o}C at 500 and 1000 ppm for up to 60 min exposure and at 15^{o}C at 500 ppm for up to 40 min exposure (Table 9).

The number of viable amoebae on the gills prior to the bath treatment differed between experiments (temperature). The reasons for this were unclear but it would appear that the infection of fish that were tested at 10^{o}C was more aggressive than that used to infect fish for the 15^{o}C experiment (Fig. 28).

There was a significant reduction in the number of viable amoebae on the gills of fish after the bath treatment with hydrogen peroxide at 10°C, however, this was not observed in fish bathed at equivalent concentrations at 15°C. There was no effect of bath duration on the efficacy of hydrogen peroxide at either experimental temperature (Fig. 27).

Table 9. Percent survival of salmon following a H_2O_2 bath treatment (18 h post-treatment)

Temp	H_2O_2 conc^n	Exposure time		
		20 min	40 min	60 min
10°C	0 ppm	ND	ND	ND
	500 ppm	100	100	100
	1000 ppm	100	100	100
	1500 ppm	89	78	0*
15°C	0 ppm	100	100	100
	500 ppm	100	100	89
	1000 ppm	100	11	0
	1500 ppm	ND	ND	ND

ND = not determined

* moribund fish were sampled

these results confirm previous studies with hydrogen peroxide that the margin for safety is low and highly temperature dependent for AGD affected salmon smolts (Cameron 1994ab). Moreover, the efficacy at disinfecting the gill of amoebae is low when low numbers of gill amoebae were present. Given that the salmon aquaculture industry currently bathe fish when gross gill pathology scores are low (light) (T. Dix pers. comm), it would be difficult to appreciate any advantage using hydrogen peroxide as a gill disinfectant. Hydrogen peroxide could potentially be used if water temperatures were low. Under such conditions a maximal concentration of 1000 ppm for 20 min would be recommended.

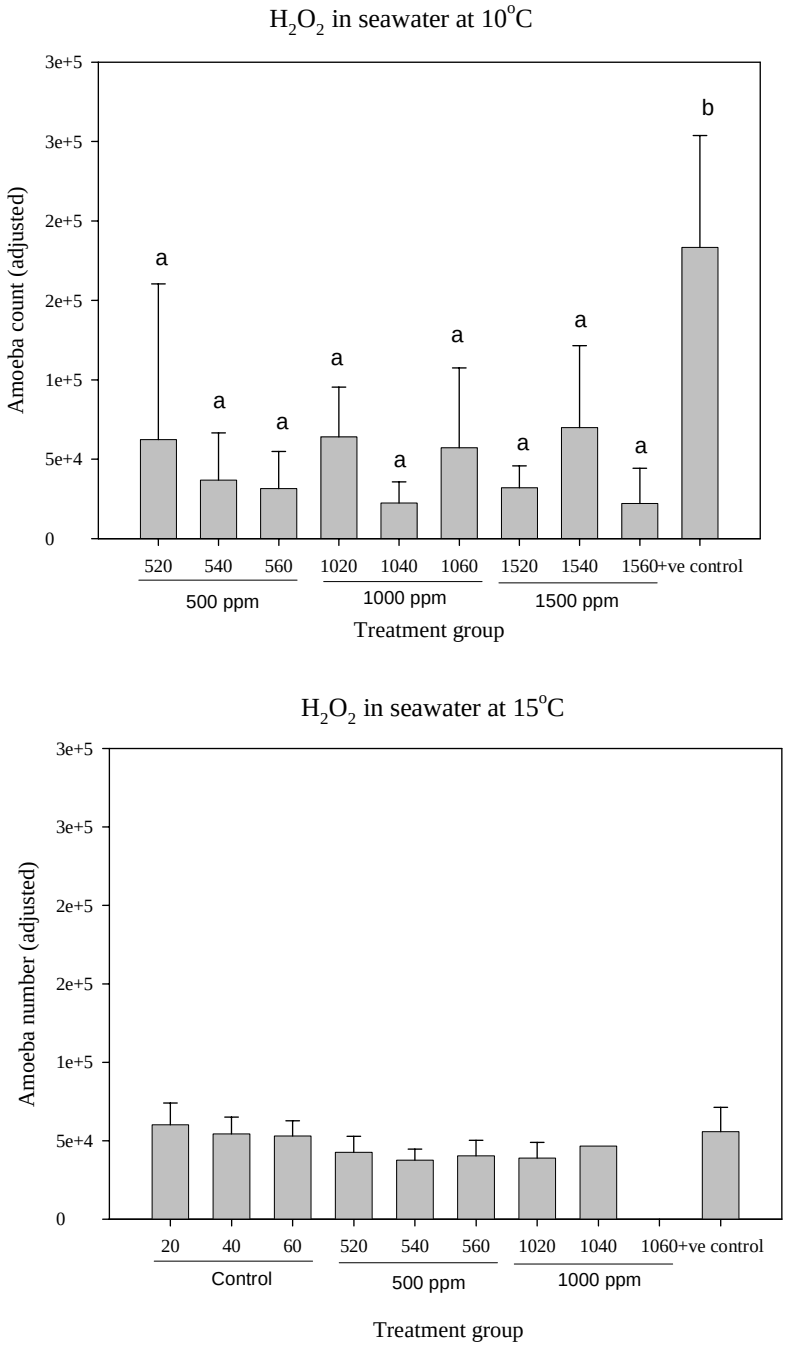


Figure 27. The mean number of viable amoebae (+ SE) isolated form the gills of AGD affected salmon before (+ve control) and after bathing with H_2O_2 at different concentrations for 20, 40 or 60 min at 10°C and 15°C.

Mucolytic treatments

Pre-feeding Atlantic salmon smolts for 14 days prior to challenge resulted in a significant reduction in the severity of AGD compared with controls (Fig. 28).

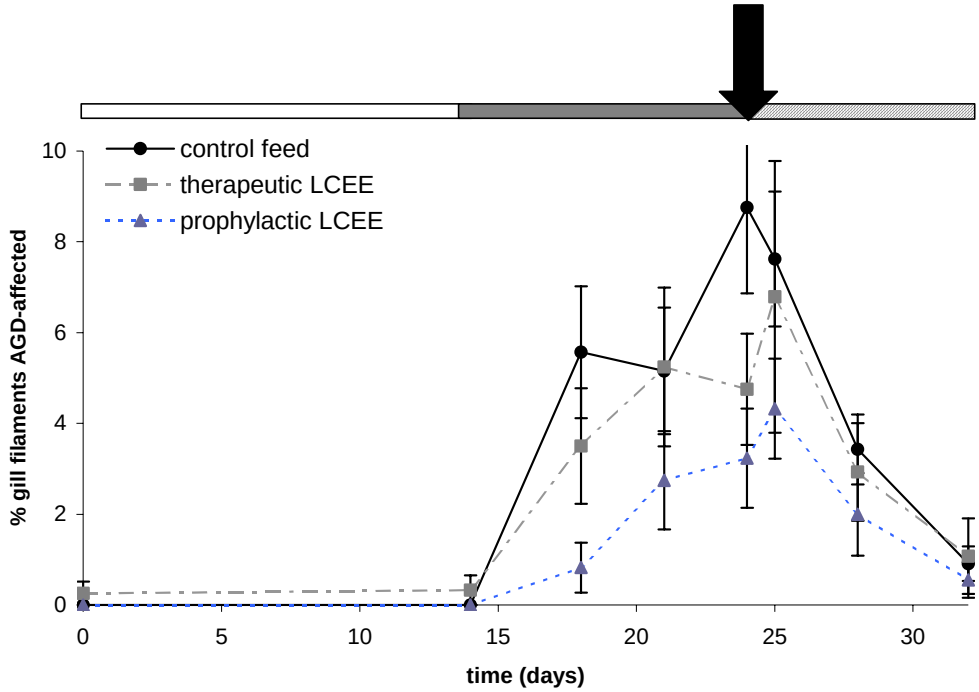


Figure 28. Percent of lesioned gill filaments over the duration of the experiment as defined by the upper bar, open bar = pre-feeding period, solid bar = *Neoparamoba* sp. challenge and development of AGD, hatched bar = post-bathe recovery from AGD. Arrow represents point at which soft water bathing was undertaken (from Roberts 2004).

Feeding LCEE at the point of challenge (day 0) did not significantly retard the severity of AGD in the subsequent infection (Fig. 29). There was also no difference in the rate of recovery of fish following a freshwater bath (Fig. 28). There were no significant effects of feeding LCEE on any of the blood variables tested (Roberts 2004).

These data suggest that pre-feeding fish a mucolytic agent may have potential as a method to reduce the severity of AGD, potentially increasing the interval between freshwater baths required commercially. However, LCEE is an analogue of N-acetyl cysteine and was chosen because of reported success feeding to fish affected by harmful phytoplankters (Yang and Albright 1994). At the time of going to press, no commercial supplier of LCEE has been found and the cost of using LCEE in commercial quantities (~$1540 AUD kg^{-1}) would be prohibitive. However, this work

demonstrated that in-feed mucolytic agents can be used to reduce the severity of AGD. With further study, perhaps examining more commercially available analogues of LCEE, and shortening the pre-feeding period, a commercially viable treatment for AGD may be developed.

Non-parasiticide approaches to AGD: the nutritional supplement Aquacite

The fish used in these studies all varied in their quality prior to the experiment. In experimental series 1 and 2, the smolts were out–of-season and marine pre-smolts respectively. As a consequence of poor acclimation to seawater, the seawater acclimation protocol was adapted with a more gradual (10-14 day compared with 7-10 day) acclimation protocol (see above).

In all of the experiments, Aquacite (with or without Betabec) facilitated a comparable or increased feed intake compared with controls. When Aquacite supplemented feed was pre-fed to fish, there were increases in both absolute and specific growth rates (Table 10). Control growth rates (AGR and SGR) in experimental series 2 and 3 were either negative or close to zero. This reflected the fact that AGD affected fish generally cease feeding as the disease advances. The feed intake of experimental series 3 was low compared with the other trials. The reason for this is not clear. Nevertheless, the effect of supplementation of fish with Aquacite or Betabec resulted in improved performance compared with controls. It is interesting that there was no apparent difference in the performance of fish supplemented with Aquacite alone compared with Betabec alone (Table 10). Although it is tempting to speculate that the common denominator in the two supplements were β-glucans and vitamin C and that these were responsible for the improved performance in these fish, this requires significant further research.

Table 10. Average feed intake as percent body weight per day (%BW), absolute growth rate (AGR) and specific growth rate (SGR) for fish fed control or Aquacite/Betabec supplemented diets at the point of *Neoparamoeba* sp. challenge (experimental series 1), 14 days prior to and during *Neoparamoeba* sp. challenge (experimental series 2 and 3).

Experimental series	Treatment	daily feed intake (%BW)	AGR (g day^{-1})	SGR (% day^{-1})
1	Control	1.22	1.96	0.18
	Aquacite/Betabec	1.42	2.21	0.18
2	Control	0.81	-0.32	-0.33
	Aquacite/Betabec	0.98	0.56	0.51
3	Control	0.36	-0.08	-0.07
	Aquacite	0.35	0.37	0.30
	Betabec	0.38	0.37	0.30

BENEFITS AND ADOPTION

This project has directly benefited the Atlantic salmon aquaculture industry in Tasmania by providing primarily two commercialisable strategies for improved control of AGD.

1. Use of soft or artificially softened water for bathing

This research has built on our previous studies that suggested that soft water was more efficacious at controlling AGD than hard water. This project has demonstrated unequivocally that soft water promotes a more efficacious removal of gill amoebae as well as prolonging the interval between baths. As a result of this research, there has been a shift in the Atlantic salmon aquaculture industry to use soft water sources for freshwater bathing operations.

The efficacy of artificially softened freshwater for bathing has been clearly demonstrated. Further research on the commercial implications of the use of artificially softened water is under consideration by the Atlantic salmon aquaculture industry. The availability of naturally relatively soft water sources from which water for freshwater bathing can be sourced would suggest that further development of artificially softened water on a commercial scale is unlikely in the near future.

2. Use of chloramine-T for emergency seawater control of AGD

The replacement of freshwater bathing with a treatment that allows fish to be bathed in seawater for a short duration is attractive to some companies within the Atlantic salmon aquaculture industry, particularly at those sites where supplies of freshwater for bathing are limited. Further semi-commercial and full scale commercial trials are required to fully develop this treatment for commercial integration into normal Atlantic salmon aquaculture practice in Tasmania. There are two issues that need to be addressed. Firstly the image of Tasmanian salmon may be affected by the use of a disinfective treatment (other than freshwater) for the control of AGD. Secondly, for commercial use of chloramine-T as a treatment control strategy for AGD, a minor use permit or full drug registration would be required by the Australian Pesticides and Veterinary Medicines Authority. This registration would include the satisfaction of environmental release requirements imposed by the Tasmanian Department of Primary Industries, Water and Environment. However, at the time of publication, an

experimental use permit for further commercial trials with chloramine-T for the control of AGD has been approved.

This project has provided a useful research tool in an acclimation and challenge protocol for laboratory efficacy testing of treatments and understanding the pathophysiology of AGD. In addition we have developed assay tools for the *in vitro* assessment/screening of potential amoebocidal compounds. This will allow the assessment of new compounds for their potential as either in-feed or bath-type treatments. By developing the tools for studying AGD in the laboratory, we have directly advanced an understanding of the physiological basis behind AGD and its impact on salmon (and other salmonid species). This advance is not trivial and has begun to shift the paradigm of how "gill diseases" are viewed and the pathology addressed. From the pathophysiology research, the option for further development of treatments that address the clinical signs of AGD such as mucolytic agents or pharamceutical agents that control hypertension is now possible. Aquatic animal health research in Australia as overall benefited from the training of 2 PhD students and a post-doctoral fellow from this project.

FURTHER DEVELOPMENT

This research project has highlighted several potential avenues for further development at different levels of commercialisation for articulation into the Atlantic salmon aquaculture industry. Treatments like artificially softened freshwater will probably not be further examined unless developed by individual companies wishing to adopt this technology. However, the commercialisation of seawater chloramine-T treatments remains a challenge for future projects.

Using our existing tools of *in vitro* toxicity assays and laboratory disease challenge protocols it will be possible to further investigate potential treatment strategies for the chemical control of AGD. These treatments should include pharmaceutical treatments that address the causes of AGD pathology, namely the systemic hypertension. The use of bath type treatments is limited to those that can be practically administered under commercial conditions and should only be further developed if sufficient quantities of the treatment is available at a reasonable (as defined by the current cost of AGD freshwater treatments) cost. All bath treatments should also be environmentally acceptable.

The most cost-effective approach to the future control of AGD is likely to be the development of in-feed treatments. These could take the form of amoebocides where the metabolised drug is excreted across the gills and thus kills/controls the population of gill amoebae. This is a particularly attractive strategy although primarily limited by the necessary branchial excretion of the drug rather than the more conventional biliary and renal excretion pathways. Alternatively, treatments that affect the physiology of the fish such that the onset of AGD is retarded or the signs of disease (such as hypertension) are reduced may be more feasible strategies. This would reduce the impact of the disease. Although the necessity of bathing fish would not be removed, the impact on the overall health of the fish would most likely be lessened. To this extent the use of feed additive compounds and nutritional supplements such as Aquacite may prove useful. If fish growth and appetite continues throughout the onset of the disease, then the impact of the disease on the farmer is reduced.

Finally, despite the research conducted to date, we still understand little about how the disease occurs in salmonids. For example, what attracts *Neoparamoeba* sp. to the gills? Why are some fish affected more than others? How do gill lesions form and what are the inflammatory processes controlling their formation? Although these questions target fundamental processes, their answers may open up new avenues for AGD control beyond those limited to gill disinfection and oral amoebocides.

PLANNED OUTCOMES

The outcomes of this project have both directly and indirectly benefited the Atlantic salmon aquaculture industry as well as providing a fundamental understanding of AGD pathology and physiology. The project has also provided material, methods and information to other sub projects. The technology developed in this project has been transferred to the industry and the research groups. The following planned outcomes have been addressed as follows:

1. **To have an improved understanding of the underlying pathophysiological effects of *Neoparamoeba* infection on salmon gills and how resultant AGD results in mortality.**

We have identified underlying physiological effects (hypertension and changes in mucus viscosity) that promote AGD pathology and ultimately result in mortality. This in formation is particularly valuable in understanding the causes of AGD related mortality.

2. **To have a more effective and cost-effective treatment for the removal of *Neoparamoeba* sp. from the gills of farmed salmon**

Two treatment strategies have been developed using existing technology (freshwater bathing) and the use of chloramine-T in seawater. The use of soft water (natural or artificially softened) ensures a more efficacious treatment, whereas the use of chloramine-T in seawater removes the requirement for freshwater resources for bathing. In addition the treatment time when using chloramine-T is reduced by at least half so promoting a potential treatment labour cost saving.

3. **That any new treatments are environmentally safe and acceptable to the public.**

Softened freshwater when produced by ion-exchange produces as a by product only small volumes of calcium enriched water. This is preferable to the use of chelating agents (eg Clagon-T) that could potentially produce tonnes of phosphate. At the time of publication, chloramine-T has been approved for experimental use and discharge on fish farms. As a disinfectant chloramines are widely used in treating drinking water and are safe for human consumption. Chloramine-T specifically is also used as a disinfectant for drinking water as well as in the food processing industry.

4. **Establishment of a validated controlled infection challenge system for use by other CRC AGD projects.**

We have developed and used an acclimation and challenge protocol that is reliable and repeatable throughout this project. The protocol is such that a laboratory infection can be reliably reproduced within 14 days of challenge. Although this is considerably quicker than occurs with natural (wild-type) infection on farms, it means that experimental investigations can be reliably reproduced in the laboratory over a time frame that is practical for assessing the efficacy of potential AGD control treatments.

5. **Reduced cost to industry of controlling AGD and hence improved profit margins.**

Although the Atlantic salmon aquaculture industry does not have a reliable measure for the cost of AGD in different parts of its production, the use of more efficacious treatments will have a follow-on impact in terms of the cost effectiveness of treatments. Those treatments that are at the point of commercial (soft water and chloramine-T) adoption offer potential cost savings due to increased efficacy (extended inter-bath interval) or reduced bathing time.

CONCLUSION

Objective 1: Establish an on-going laboratory source of AGD affected fish

This project has established from a crude isolate of gill amoebae from a clinical outbreak and has been an ongoing laboratory infection source that has since been adopted by other projects within the Aquafin CRC. The infection has been continuously passaged through Atlantic salmon since 2001.

Objective 2: Establish and validate a controlled infection/challenge system

We have developed protocols for the reliable challenge of Atlantic salmon for experimental induction of AGD and use in treatment efficacy studies. These challenge protocols have proved reliable and have been used throughout this project. Future projects examining the efficacy of treatments will employ a similar challenge protocol.

Objective 3: To understand how AGD affects the respiratory and cardiovascular systems of Atlantic salmon.

Studies of the respiratory physiology and progressive pathology of an AGD infection have revealed that the clinical signs of AGD occur after approximately 3-4 days post challenge. Moreover, the respiratory effects of AGD are relatively minor compared with the effect that AGD appears to exert on the cardiovascular system. Cardiovascular effects are characterised by a systemic hypertension that primarily manifests as a significant increasing in systemic vascular resistance. Moreover, vasodilators and angiotensin converting enzyme inhibitors can partially reverse the effects.

Objective 4: To determine how environmental parameters interact with AGD pathophysiology.

Given the limited effect of AGD on respiratory physiology, there appears to be little impact on gas transfer across the gills. However, basal metabolic rate is significantly increased in fish with AGD suggesting that if oxygen were limiting in the

environment, transitory hypoxia may occur. The implications of an elevated basal metabolic rate is that energy that would otherwise be used for growth is being used to maintain basal metabolic functions such as ion regulation, acid-base regulation etc.

Objective 5: To develop and test new chemical or pharmaceutical treatments for the control of AGD.

We have tested a range of drugs and compounds for their amoebocidal activity over a short duration of exposure (3 h) as well as over prolonged continuous exposure (5 days). With the exception of 5 day continuous exposure to bithionol, most of the compounds tested were in effective at killing *Neoparamoeba* sp. The concentrations at which bithionol killed amoebae *in vitro* suggested possible use as an in-feed amoebocide. The disinfectant chloramine-T has been successfully developed for use in seawater. In tank and experimental cage trials, chloramine-T at 10 mg L^{-1} was found to be as effective as freshwater at removing gill amoebae. However, the characteristic gill lesions resolved at a slower rate.

Objective 6: To optimise the efficacy of current treatments by minimising the physiological impact on the fish.

We have successfully used artificially softened water using an ion exchange column to enhance the efficacy of freshwater bathing and extend the inter-bath interval by approximately 2 weeks or 113 degree days under field conditions. This represents a significant improvement in bathing efficacy. The industry has been adopting naturally softer water supplies for freshwater bathing based upon the results from this and the previous FRDC project (FRDC 2000/266).

Objective 7: To determine the efficacy of water softening agents and repeated bathing of fish in artificially softened water on the control of AGD compared with conventional (hard) freshwater water bathing practices.

As described above.

Objective 8: To examine the potential for short-term high concentration treatments of AGD affected salmon using hydrogen peroxide under laboratory conditions.

Hydrogen peroxide is an effective amoebocide. When used on AGD affected fish under laboratory conditions the toxicity and efficacy were temperature dependant. Hydrogen peroxide was more effective at 10^{o}C compared with 15^{o}C. It would appear that hydrogen peroxide may have a potential as a treatment for AGD but only under cool temperature conditions such as winter. Given that AGD is primarily a disease that affects Atlantic salmon in the spring and summer, the need for a winter treatment only occurs when occasional outbreaks occur at low water temperatures.

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APPENDIX 1: INTELLECTUAL PROPERTY

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1. Copyright of this report

APPENDIX 2: STAFF

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APPENDIX 3: CONFERENCE PRESENTATIONS AND ABSTRACTS

Roberts, S and Powell, M. (2001). Effects of freshwater exposure on marine Atlantic salmon: implications for disease status and water hardness. *Proceedings of the Australia-New Zealand Society for Comparative Physiology and Biochemistry Conference, Adelaide, SA, Australia, Dec. 6-9.*

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APPENDIX 4: PUBLICATIONS FROM THIS PROJECT

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Leef, M., Harris, J.O. and Powell, M.D. The respiratory effects of chloramine-T exposure in seawater acclimated Atlantic salmon, *Salmo salar* L., affected with amoebic gill disease (AGD). *Aquaculture (to be submitted)*

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