Synopsis of AGD research to date and review/recommendation of future AGD related R&D directions including the development of a vaccine for AGD.

Dr Mathew Cook

Project No. 2010/741

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This review was undertaken by Dr Paul Hardy-Smith from PanAquatic Health Solutions, on behalf of the TSGA and Seafood CRC

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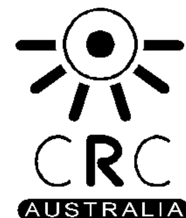

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Non-Technical Summary

2010/741 – Synopsis of AGD research to date and review/recommendation of future AGD related R&D directions including the development of a vaccine for AGD.

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OBJECTIVES:

1. Comprehensive review/synopsis of AGD research undertaken to date
2. Decision as to whether a vaccine for AGD is worth pursuing
3. Development of a short, medium and long term R&D plan for AGD

NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED TO DATE
The major outcome of this project was the development of a 2011 R&D strategy by the TSGA. This included a list of R&D priorities for 2011, including AGD related research, and a specific list of areas of interest for 2011. Subsequent to this the TSGA received 7 project proposals pertaining to their R&D priorities and areas of interest for 2011. The final outcome was the formation of the AGD Working Group (AGDWG) consisting of representatives from Industry, the key research providers and the funding agencies (FRDC and Seafood CRC). The role of this group is to review and provide advice on AGD R&D. This group met on the 21st of June 2011 to discuss and consider these research proposals

The following summarises the key points made in this review:

- In early 1987 the amoebae closely associated with AGD was identified as a Paramoeba. They contained parasomes which are small, self contained bodies that lie in close proximity to the nucleus of the amoebae. The paramoeba not only affected Atlantic Salmon but also Rainbow Trout (*Onchorhynchus mykiss*) which were at that time being grown in sea cages in south east Tasmania. The disease manifested differently in Rainbow Trout. Around the same time, AGD was described in the gills of Coho Salmon (*Oncorhynchus kisutch*) in Washington and California, USA.
- The amoeba species causing AGD acts as a primary pathogen. For quite a while it was named *Neoparamoeba pemaquidensis.* Differences between the pathogenic amoebae found on the gills (often referred to as the "wild" type) and amoebae taken from gills and cultured *in vitro* were noted very early into the research on AGD. This has been one of the most frustrating issues. Loss of virulence during culture could be due to (1) selection of non-virulent strains

of amoebae during the culture process, or (2) by "down regulation" of virulent factors.

- In 2007, the pathogenic amoeba was identified as *N. perurans* through sequencing of its ribosomal DNA. Identification of *N. perurans* may indicate that it has been the selection of non-virulent strains that has been the problem – this review also considers the possibility of down regulation of virulent factors, e.g. through the use of specific classes of antibiotics during culture.
- Currently, all "virulent" amoebae used in challenge type models are collected from the gills of infected salmon. Significant improvements have been made in the collection of such amoebae. However, the reliance on use of amoebae isolated from AGD infected salmon for clinical trials has hampered research. An urgent need exists for a virulent axenic cultured strain of virulent amoeba to underpin future experimental studies.
- The response of the gill to amoebic infection is characterised by a marked increase in numbers (hyperplasia) and size (hypertrophy) of the epithelium lining the secondary gill lamellae, resulting in extensive thickening of the lining epithelium. There is obliteration of normal gill structure and increase in mucous cells accompanied by an inflammatory reaction. This reaction is not dissimilar to the reaction of gills to other parasites such as Amyloodinium, Cryptocaryon, Chilodonella and Ichthyobodo and appears to represent a general response of the gill to irritation.
- Fundamental information on the pathophysiology of AGD was found to be lacking, especially on the ability of amoebae to produce toxins, on specific adhesion mechanisms and the biochemical or pathophysiological basis whereby hyperplasia and hypertrophy of the lamellar epithelium are induced. The characterisation of such factors would provide valuable information on the pathogenesis of infection likely providing information by which practical control mechanism might be developed.
- Information relating to physical and chemical stability of the causal amoeba is well described. Salinities equal to or below 15‰ are detrimental to the survival of the AGD amoebae and a salinity of 4‰ or less is required to achieve a satisfactory bathe, with the hardness of the water influencing the effectiveness of the bathe. Some amoebae will survive bathing. Early in its history AGD occurred during the warmer months of the year. However, AGD now occurs throughout the year, suggesting a shift in the host-parasite-environment interaction.
- Fish to fish transmission of this organism has been established. It is possible that the amoebae that infected salmon and trout in the early years came from wild fish. Now, it is likely that the key reservoir of virulent amoebae is the farmed salmon themselves. Although wild fish may harbour or have harboured the amoebae causing AGD, they do not appear to be regularly affected by the disease.
- Experimental transmission studies in the tank environment have demonstrated that as few as 10 amoebae per litre of water may be infectious. It is not known whether increasing infection rates on individual fish occurs through replication of the amoeba within the host or whether it is progressive accumulation of infective amoeba from external sources. The finding that only 21.4% of AGD affected fish survived stresses which included exposure to a 50% reduction in dissolved oxygen compared to a survival rate of 88.9% in non-affected fish would tend to support the view that respiratory dysfunction, which is affected by the availability of oxygen, is important in the

pathogenesis of AGD. There is some ambiguity in the findings of research on this point.

- There has been considerable research conducted on treatment of AGD but the industry still relies on freshwater bathing, a technique developed over 20 years ago. Gaining further fundamental understanding of this parasite is likely to be the most worthwhile mechanism to assist in the identification of possible alternative treatments to freshwater, if such treatments exist.
- No direct unequivocal evidence in research conducted to date could be identified that shows that it is actually the adaptive immune response that is influencing the ability of the salmon to survive challenge with amoebae causing AGD. Further studies that demonstrate processing of antigen of amoebic origin and a cell mediated or humoral immunological response against such antigen that affords demonstrable protection against amoebic colonisation of the gill surface would appear appropriate.
- Critical to vaccine development is the ability of the adaptive immune response to develop protection against subsequent infection. A greater understanding of the fundamental aspects of AGD would assist in being able to better understand whether vaccination of fish is a viable possibility to protect against AGD. It would also greatly assist in better defining what research is needed to develop such a vaccine, should it be considered viable.
- The identification and selection of fish with resistance to disease would appear to be best implemented through the selective breeding program. Thought should be given to breeding from individuals or their cohorts following experimental or natural challenge. Such programs afford major potential for the control of AGD.
- The use of such traits as survival and gill scores and whole genome approaches for genetic selection provides a reasonable and logical basis for selection. The possibility that an exuberant host response to infection may be the primary cause of disease is noted though. Modification of the exuberant host response to infection with AGD amoeba might actually be the most important trait to capture genetically.

KEYWORDS: AGD, Review, amoebic gill disease, research

1. Background

Since the early 1990's and through the life of two previous CRC's and this current Seafood CRC much research, both fundamental and applied, has been undertaken into amoebic gill disease (AGD) in Atlantic salmon. This research has been undertaken by a number of research organisations, the industry itself, and a large number of individual researchers from students through to professorial level staff. Much knowledge has been gained regarding host pathogen interactions, the epidemiology of disease and many treatment options have been trialled. Much of the knowledge regarding AGD is retained in peer reviewed journal articles, student thesis, confidential and non-confidential reports and some is retained by individual researchers. In order to best move forward on AGD research options and to provide a single repository for AGD research outcomes there is a need to undertake a review and compile a synopsis of AGD research undertaken to date. There is also a need to identify major advances and/or possible gaps in knowledge regarding the issue of AGD. Furthermore, there is a need to develop a short, medium and long term strategic research strategy for AGD. As part of this there is a need to correctly identify the avenues/methods that are most worthy of pursuing.

2. Need

This project was required in order to provide funds to undertake a comprehensive review of AGD research conducted to date, to produce a single document encapsulating what has been attempted and what has worked. This review will be used by the industry and research providers to formulate a short, medium and long term R&D strategy for AGD.

3. Review Terms of Reference and Methodology

Dr Paul Hardy-Smith from Panaquatic Health Solutions undertook the review. The intention was for the review to provide a generalised synopsis of the research undertaken to date with a key focus on the areas of;

- Biology of the causative agent
- Epidemiology
- The host response to disease
- Environmental factors contributing to disease
- The treatments tested and their success/failure,
- History of vaccine development and its success/failure
- Breeding for resistance to AGD
- Determination of the true costs of production related to AGD

Methods used by Dr Hardy-Smith, included;

- Assembling and collating all relevant information (including scientific literature, FRDC reports, conference proceedings, PhD theses) pertaining to research on AGD in Tasmanian salmon.
- Review all information gathered and summarise key advances in knowledge on AGD identified in this information.
- Identify key researchers and discuss key advances based on their research.
- Compile information gained from above into a comprehensive report.

This synopsis was then be used by the TSGA to formulate a short, medium and long term R&D strategy with relation to AGD.

4. Outcomes

4.1 TSGA Technical Committee 2011 R&D Strategy - AGD

PURPOSE:

The purpose of this Research and Development (R&D) Strategy is to:

- encourage the Tasmanian Salmonid industry to think strategically about their R&D activities as a critical and ongoing part of their business
- support the successful management of R&D projects, providing focus and structure to R&D activities and thereby enhancing the likelihood of successful outcomes

FUNDAMENTAL PRINCIPLES:

- The TSGA TC R&D strategy will be fluid; evolving and adapting to changes in the industry. The strategic process should be a living, breathing process that allows industry to take facts and observations, understand them and develop insights. These insights are translated to implications for R&D, generating a portfolio of R&D projects and programs with maximum positive impact on business performance
- The TSGA TC R&D strategy provides a basis for an appropriately balanced portfolio of R&D programs in alignment with the Tasmanian Salmon and Trout Industry Strategic Plan: now to FY2030
- Intelligence gathering will include emerging trends, consumer needs, technical advances, and changes to the competitive and customer landscapes
- The business strategic planning process and the R&D strategic planning process will occur simultaneously once an ongoing process is established. R&D strategy must be linked to the Tasmanian Salmon & Trout Industry Strategic Plan

SCOPE:

In addition to tactical development activities associated with fish health, production and the environment, the TSGA TC R&D strategy will address major non-development activities including;

- Organisational change (design, staffing, etc.)
- System implementation (project management, performance management, etc.)
- Skill and capability improvements (training, software/tools, facility upgrades, etc.)
- Cultural initiatives (innovation, teaming/high-performance, etc.)

These activities are critical to building and maintaining competitive advantage in a changing environment.

R&D PRIORITIES FOR 2011 - AGD

Note: Amoebic Gill Disease (AGD) is the greatest health problem for the Atlantic Salmon industry in Tasmania, imposing a significant financial burden on our industry. It is a priority across all the tactical development areas. The following table has been extracted directly from a document entitled 'TSGA TC 2011 R&D Strategy-Research Agency' which was prepared by the TSGA Technical committee to address tactical and strategic R&D priorities and sent to research providers by Dr Adam Main, CEO, TSGA on the 27th of April 2011. The table summarizes the key R&D themes for fish health and in particular in relation to AGD

Tactical Development Area	Strategic R&D Themes
Fish Health	• Fish health surveillance/investigation/diagnosis- (FHU) • Vaccine development- RLO; Reo virus; Aquabirna virus • AGD- Treatments • AGD- Selective Breeding Program • AGD- Epidemiology and pathobiology • AGD- determination of what is actually killing the fish • AGD- Culture AGD amoebae and maintain infectivity • AGD- Comparing the response/mechanisms of salmon to native fish

AREAS OF INTEREST FOR 2011

In addition to the R&D priorities for 2011, there are a range of other 'areas of interest' that the Tasmanian Salmonid industry would encourage input and comment on. Like the above table the following was extracted from the 'TSGA TC 2011 R&D Strategy-Research Agency' document

Tactical Development Area	Area of Interest
Fish Health	• AGD- Vaccine • AGD- comparing response between salmon and trout • AGD- determination of virulence factors • AGD- development of in-vitro assay systems

4.2 Project Proposals pertaining to AGD R&D received as part of May 2011 call

Project Number	Tactical Development Area	Title	Principal Investigator	Budget
1105-4	AGD	Survivorship and comparative responses of endemic fishes and salmonids to amoebic gill disease	Mark Adams, UTAS	$100,001-250,000
1105-5	AGD	Revisiting alternative treatments for AGD	Mark Adams, UTAS	$0-50,000
1105-15	AGD	The effects of AGD on gill function: Use of a perfused gill model.	Melanie Leef, UTAS	$50,001-75,000
1105-16	AGD	Relationship between the presence of the infective amoeba, Neoparamoeba perurans, in the environment and AGD outbreaks	Barbara Nowak, UTAS	$0-50,000
1105-19	AGD	Genetic selection for AGD resilience in the SALTAS salmon breeding program	Richard Taylor, CSIRO Marine and Atmospheric Research	$75,001-100,000
1105-25	AGD	Amoebae virulence: determination of the differences between infective Neoparamoeba perurans and other Neoparamoeba species	Mathew Cook & Ben Maynard, CSIRO Food Futures Flagship	$250,001+
1105-26	AGD	AGD resistance: learning from other species to bolster the natural Atlantic salmon response	Mathew Cook & Ben Maynard, CSIRO Food Futures Flagship	$250,001+

APPENDIX 1: AGD Review Compiled by Dr Paul Hardy-Smith and Dr John Humphrey from Panaquatic Health Solutions.

Research on Amoebic Gill Disease of salmonids in Tasmania 1985 -2010 – a review.

FINAL REPORT

Prepared for the AGD Vaccine Management Advisory Committee

by

Drs Paul Hardy-Smith and John Humphrey

Panaquatic® Health Solutions Pty Ltd

March 18th, 2011

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Summary of Findings

Amoebic Gill Disease (AGD) is a parasitic disease affecting Atlantic salmon (*Salmo salar*) grown on marine sites in the south east of Tasmania. The treatment for the disease is to bathe affected salmon in freshwater for 2-3 hours – numerous bathes may be required through a production cycle and this has a major adverse impact on the cost of production.

AGD was first identified shortly after the first Atlantic salmon were introduced into marine sites in Tasmania in 1985. Since then, there has been a considerable amount of effort put into researching AGD to determine how industry can better manage and treat this disease.

Drs Paul Hardy Smith and John Humphrey of Panaquatic Health Solutions Pty Ltd ("Panaquatic") were commissioned by the AGD Vaccine Management Advisory Committee (AVMAC) to review the research conducted on AGD in Tasmanian Atlantic salmon to date. The review was to provide a generalised synopsis of the research undertaken to date with a key focus on a number of specific areas, including biology of the causative agent, epidemiology, the history of vaccine development and its success/failure, and breeding for resistance to AGD. The review was not to provide recommendations for going forward, but was to provide general information to assist AVMAC in the development of short and medium term AGD research plans.

The approach taken by the authors was a combination of systematically reviewing published (and some unpublished) literature on AGD research and personal discussions with a number of key researchers and industry members within a framework of those factors necessary for the development of disease, specifically addressing the key issues within the scope of the review.

The review also sought to present information that might assist companies farming Atlantic salmon in marine sites in Tasmania developing better control and/or treatment options.

Providing a synopsis on over 25 years of research and its attendant voluminous literature is not an easy or simple task, and the authors acknowledge that some information may have been overlooked and some considered not to have been adequately addressed by researchers who have significantly more familiarity in their specific area of expertise and research. The authors also acknowledge though that should omissions have inadvertently occurred, then the healthy debate and discussion which may result will hopefully itself be beneficial in assisting the decisions on how best to go forward in both the short and long term.

The following summarises the key points made in this review:

- In early 1987 the amoebae closely associated with AGD was identified as a Paramoeba. They contained parasomes which are small, self contained bodies that lie in close proximity to the nucleus of the amoebae. It not only affected Atlantic salmon but also rainbow trout (*Onchorhynchus mykiss*) which were at that time being grown in sea cages in south east Tasmania. The disease manifested differently in rainbow trout. Around the same time, AGD was described in the gills of coho salmon (*Oncorhynchus kisutch*) in Washington and California, USA.

- The amoeba species causing AGD act as a primary pathogen. For quite a while it was named *Neoparamoeba pemaquidensis.* Differences between the pathogenic amoebae found on the gills (often referred to as the "wild" type) and amoebae taken from gills and cultured *in vitro* were noted very early into the research on AGD. This has been one of the most frustrating issues. Loss of virulence during culture could be due to (1) selection of non-virulent strains of amoebae during the culture process, or (2) by "down regulation" of virulent factors.
- In 2007, the pathogenic amoeba was identified as *N. perurans* through sequencing of its ribosomal DNA. Identification of *N. perurans* may indicate that it has been the selection of non-virulent strains that has been the problem – this review also considers the possibility of down regulation of virulent factors, e.g. through the use of specific classes of antibiotics during culture.
- Currently, all "virulent" amoebae used in challenge type models are collected from the gills of infected salmon. Significant improvements have been made in the collection of such amoebae. However, the reliance on use of amoebae isolated from AGD infected salmon for clinical trials has hampered research. An urgent need exists for a virulent axenic cultured strain of virulent amoeba to underpin future experimental studies.
- The response of the gill to amoebic infection is characterised by a marked increase in numbers (hyperplasia) and size (hypertrophy) of the epithelium lining the secondary gill lamellae, resulting in extensive thickening of the lining epithelium. There is obliteration of normal gill structure and increase in mucous cells accompanied by an inflammatory reaction. This reaction is not dissimilar to the reaction of gills to other parasites such as Amyloodinium, Cryptocaryon, Chilodonella and Ichthyobodo and appears to represent a general response of the gill to irritation.
- Fundamental information on the pathophysiology of AGD was found to be lacking, especially on the ability of amoebae to produce toxins, on specific adhesion mechanisms and the biochemical or pathophysiological basis whereby hyperplasia and hypertrophy of the lamellar epithelium are induced. The characterisation of such factors would provide valuable information on the pathogenesis of infection likely providing information by which practical control mechanism might be developed.
- Information relating to physical and chemical stability of the causal amoeba is well described. Salinities equal to or below 15‰ are detrimental to the survival of the AGD amoebae and a salinity of 4‰ or less is required to achieve a satisfactory bathe, with the hardness of the water influencing the effectiveness of the bathe. Some amoebae will survive bathing. Early in its history AGD occurred during the warmer months of the year. However, AGD now occurs throughout the year, suggesting a shift in the host-parasite-environment interaction.
- Fish to fish transmission of this organism has been established. It is possible that the amoebae that infected salmon and trout in the early years came from wild fish. Now, it is likely that the key reservoir of virulent amoebae is the farmed salmon themselves.

Although wild fish may harbour or have harboured the amoebae causing AGD, they do not appear to be regularly affected by the disease.

- Experimental transmission studies in the tank environment have demonstrated that as few as 10 amoebae per litre of water may be infectious. How quickly amoebae replicate on the gills of fish under different environmental conditions has not been established but would be useful to understand. The finding that only 21.4% of AGD affected fish survived stresses which included exposure to a 50% reduction in dissolved oxygen compared to a survival rate of 88.9% in non-affected fish would tend to support the view that respiratory dysfunction, which is affected by the availability of oxygen, is important in the pathogenesis of AGD. There is some ambiguity in the findings of research on this point.
- There has been considerable research conducted on treatment of AGD but the industry still relies on freshwater bathing, a technique developed over 20 years ago. Gaining further fundamental understanding of this parasite is likely to be the most worthwhile mechanism to assist in the identification of possible alternative treatments to freshwater, if such treatments exist.
- No direct unequivocal evidence in research conducted to date could be identified that shows that it is actually the adaptive immune response that is influencing the ability of the salmon to survive challenge with amoebae causing AGD. Further studies that demonstrate processing of antigen of amoebic origin and a cell mediated or humoral immunological response against such antigen that affords demonstrable protection against amoebic colonisation of the gill surface would appear appropriate.
- Critical to vaccine development is the ability of the adaptive immune response to develop protection against subsequent infection. A greater understanding of the fundamental aspects of AGD would assist in being able to better understand whether vaccination of fish is a viable possibility to protect against AGD. It would also greatly assist in better defining what research is needed to develop such a vaccine, should it be considered viable.
- The identification and selection of fish with resistance to disease would appear to be best implemented through a prospective programs selecting for survival or resistance in experimental or natural challenge, subsequent breeding from these individuals or their cohorts and an assessment of the resistance of the progeny to the same disease, as is being done in current programs. Such programs afford major potential for the control of AGD.
- The use of such traits as survival and gill scores and whole genome approaches for genetic selection provides a reasonable and logical basis for selection. The possibility that an exuberant host response to infection may be the primary cause of disease is noted though. Modification of the exuberant host response to infection with AGD amoeba might actually be the most important trait to capture genetically.

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Consultative Process on Review of this Report

Introduction

The AGD Vaccine Management Advisory Committee (AVMAC) commissioned Drs Paul Hardy Smith and John Humphrey of Panaquatic Health Solutions Pty Ltd ("Panaquatic") to review the research conducted on AGD in Tasmanian Atlantic salmon and to provide a provide a generalised synopsis of the research on AGD undertaken to date. This in essence included all research conducted since AGD was first identified in Tasmania in 1985. The review was not to include recommendations but to provide information which could be utilised by the AVMAC to develop short and medium term goals for further AGD research.

Circulation of Draft Review and AVMAC Meeting

A "preliminary draft" of this review was prepared and forwarded to the project coordinator for comment. On receival of these comments, a "draft for circulation" was then prepared and circulated to members of AVMAC in February, 2011 one week prior to an AVMAC meeting held on February 23, 2011. One of the authors of the report, Dr Hardy-Smith, attended the meeting. A major component and key agenda item of the meeting was discussion and feedback on this draft review. This had been circulated as a draft specifically to allow AVMAC members and other researchers who had been involved in AGD research an opportunity to provide constructive comment on the draft review and in particular identify, if necessary, literature or papers that the authors may have not adequately addressed or indeed were not aware of prior to the final review being distributed.

Research Priorities identified

In addition, as the draft review was discussed section by section, the AVMAC identified a number of research priorities during this meeting. These priorities were, in the order recorded:

i. To be able to culture of the AGD amoebae and maintain infectivity;
ii. The need to go back to basics, i.e. understand more about the fundamental aspects of the pathobiology of AGD, specifically understanding why the gills of salmon seem to be so attractive to the amoebae;
iii. Comparing the response/mechanisms of salmon to trout noting that trout have a more diffuse response;
iv. Comparing the response/mechanisms of salmon to native fish, noting that native fish seem resistant and raising the question as to whether amoebae are actually present but without eliciting a reaction?
v. Determination of virulence factors, for example through comparisons between N. perurans and N. pemaquidensis;
vi. Determination of what is actually killing the fish, especially studying the role of the exuberant host response; and
vii. Development of in-vitro assay systems, e.g., gill cell culture, salmon cell/amoebae assay systems, to underpin future research.

Subsequent to the meeting the authors were presented with a summary of the comments made on the draft review by members of AVMAC and other researchers. The reviewers provided a considerable number of constructive and most helpful comments in the 22 pages of comment received.

The authors welcomed such critique and recognised differences of opinion evident between the different researchers on a number of research issues when providing comment.

Initially, on receiving the comments, the authors had considered spending the time if necessary to go back through the draft review and determine whether any of the comments warranted inclusion or alteration of what had already been presented.

However, after consultation with members of AVMAC it was decided that a more useful and expedient approach was to simply include the summary of comments reviewed as an Appendix to this final version of the review (Appendix B). The inclusion of the comments will allow those reading over the review to also read over and reflect on the various additional comments made on its draft version and use the additional information for further reference as may be necessary.

1 Introduction

Amoebic Gill Disease (AGD) is a parasitic disease affecting Atlantic salmon grown on marine sites in the south east of Tasmania. The treatment for the disease is to bathe affected salmon in freshwater for 2-3 hours – numerous bathes may be required through a production cycle. This treatment was developed and first recommended in 1988 (Foster and Percival 1988a), relatively soon after the disease was identified in Tasmania in 1985. The industry today still uses freshwater bathing to manage this disease.

Understandably, the resources required to bathe Atlantic salmon using freshwater is significant and managing this disease has a major adverse impact on the cost of production. The disease also affects production by having an impact on the salmon themselves; signficant morbidity and mortality can occur if AGD is not treated.

Considerable research has been conducted on the disease since its discovery in 1985. A key area of this research has been the development of a vaccine for the disease with the hope that a vaccine may prevent the occurrence of AGD. To date, the quest for a vaccine has proven elusive.

The AGD Vaccine Management Advisory Committee (AVMAC) has a role in overseeing AGD research. AVMAC commissioned Drs Paul Hardy Smith and John Humphrey of Panaquatic Health Solutions Pty Ltd ("Panaquatic") to review the research conducted on AGD in Tasmanian Atlantic salmon to date and provide a report identifying key advances in knowledge. The AVMAC will use this report to assist in the development of short and medium term AGD research plans going forward.

1.1 Scope and purpose

The scope of this review is to provide a generalised synopsis of the research on AGD undertaken to date with a key focus on the areas of:

- Biology of the causative agent
- Epidemiology
- The host response to disease
- Environmental factors contributing to disease
- The treatments tested and their success/failure,
- History of vaccine development and its success/failure
- Breeding for resistance to AGD
- Determination of the true costs of production related to AGD

With respect to the final point, the true costs of the impact of AGD on production, we elected not to focus on this issue in this review. While it is an important aspect of AGD, there is no doubt that the true cost to industry is significant and we considered it more beneficial to focus on the other areas of research.

By doing this, in addition to assisting in the development of short and medium term AGD research, the authors sought to present information that might assist those farming Atlantic salmon in marine sites in Tasmania to devise better control and/or treatment options with the ultimate aim of minimising the impact AGD has on this industry and these true costs.

1.2 Methodology and approach

The approach taken in considering and reviewing research undertaken on AGD was based on the following:

- A systematic review of the comprehensive published literature on AGD research, including research publications in refereed journals, research reports and conference and workshop proceedings;
- Personal discussions with a number of research scientists and industry representatives currently or previously involved with AGD;
- Consideration of the above literature and personal discussions within a framework of those factors necessary for the development of disease, specifically addressing the key issues within the scope of the review (above) and providing guidelines for further research directions

The terms of reference for this review were to consider the research conducted to date under various areas, outlined in Section 1.1. However, to assist in providing industry with information that allows significant improvements in its ability to manage and control this disease, this review has also considered the research according to the disease process at both the individual (fish) or population level i.e. the steps necessary for AGD to develop in salmon and the current understanding of each of these steps as identified by the research.

The critical steps identified in the disease process are:

i. A requirement for the AGD amoeba to be present in the environment;

ii. The presence of susceptible host species;

iii. Specific mechanisms of attachment of the amoeba to the host;

iv. An infectious dose of organisms sufficient to induce infection;

v. The host response to the organism; and

vi. Outcome of infection : Death or resolution.

This approach will hopefully ensure that we have identified key areas of understanding on AGD and key areas of deficiencies in our understanding which will allow better evaluation of what research may be beneficial to industry going forward.

1.3 The independence of reviewers

Dr Paul Hardy-Smith and Dr John Humphrey of Panaquatic conducted this review.

Dr Hardy-Smith qualified as a veterinarian in 1987, and has been working full time in the aquatic animals industries since 1995. Positions he has worked in since then include the Aquaculture Veterinarian for the Tasmanian Department of Primary Industries (where he had hands-on experience of AGD) and the Production Veterinarian for Heritage Salmon Limited, a Canadian company producing around 15,000 tonnes of salmon annually.

Dr Hardy-Smith set up Panaquatic in 2003 and is the Managing Director of the company. Panaquatic is independent of any government, research or industry organisations. It is though important to note that one regular client of Dr Hardy-Smith is a company in Tasmania that farms Atlantic salmon and rainbow trout. However, none of the fish farmed by this company are affected by Amoebic Gill Disease.

Dr Humphrey is the veterinary pathologist of Panaquatic and is a recognised authority on disease and pathology of aquatic animals. He helped establish the first Australian Fish Diseases Laboratory in Victoria in the 1980s and is the author or co-author of over 50 publications encompassing health and disease issues in terrestrial, avian and aquatic animal species. In addition, he has significant hands-on experience in aquatic animal disease diagnosis and research, health certification and the development and implementation of biosecurity strategies.

1.4 Confidentiality and disclosure

This consultancy has been undertaken by Panaquatic on an independent basis. It has been written to provide the AGD Vaccine Management Advisory Committee with an independent health assessment of of research into AGD from 1985 to 2010 based on the Scope as outlined above. Panaquatic has charged a fixed fee to undertake this consultancy and prepare this report.

On nearing completion of this consultancy though, Panaquatic was requested by the Project Coordinator to provide an estimate of the additional time over budget that was spent in writing this report with the understanding that further additional funding may be made available. Panaquatic acquiesced to this request, but did not initiate it.

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1.5 Acknowledgements

We would like to thank the AGD Vaccine Management Advisory Committee for providing us with this opportunity to review the research on AGD. We would also like to thank those researchers and industry members who assisted us in undertaking this review and others who were happy to assist even though they no longer are involved in this issue, particularly Drs Bret Robinson and Professor Bob Lester.

We also acknowledge the assistance of Dr Cassandra Ypelaan, who provided assistance in collating the information on treatment research.

1.6 Report dates

1.6.1 Preliminary Report

February 5th, 2011

1.6.2 Draft Report for Circulation

February 16th, 2011

1.6.3 Final Report

March 18th, 2011

2 Amoebic Gill Disease – a brief history focusing on the early years in Tasmania

2.1 Introduction

Amoebic Gill Disease (AGD) was first identified in Tasmania over 25 years ago, which is likely to be before quite a few current industry members and researchers were born.

As some of this information is not readily available we thought it useful to include a brief account of the early history of this disease. It will we hope also provide background and context for the rest of the review which focuses more on specific aspects of AGD.

2.2 First sea cage farming of Atlantic salmon in Tasmania - 1985

The present Tasmanian salmonid aquaculture industry has its origins in the establishment of a freshwater rainbow trout farm at Bridport in the north of the state in 1964. A second farm was established in 1978 at Russell Falls - in central Tasmania. The first successful seawater rainbow trout trial was conducted in 1981 at Nubeena in the south east of Tasmania as a result of collaboration between Japanese experts, the Tasmanian Fisheries Development Authority and a local company[1]. The authors are not aware of whether these fish were affected by disease and more particularly by gill disease.

Atlantic salmon eggs were introduced into Tasmania from the New South Wales freshwater hatchery at Gaden in 1984[2,] and further transfers took place during the following two years. All introductions were made under strict quarantine controls and the hatched fingerlings were held in quarantine at the Taroona Fisheries Research Laboratory (now part of the Tasmanian Aquaculture and Fisheries Institute).

In 1985, progeny of those fish introduced from Gaden were transferred to a sea site.

2.3 First report of Amoebic Gill Disease and associated pathology - 1985

According to Munday (1988a) and Foster and Percival (1988a), amoebic gill disease (AGD) was first diagnosed in smolt located in cages off Bruny Island in December, 1985 i.e. the same year as the first introductions of Atlantic salmon into sea water in Tasmania occurred. Hence there was no "*lag phase*" with this disease – it had an immediate impact from the very start of commercial Atlantic salmon industry in Tasmania.

Munday (1988a) in a presentation given at the *First Australian Workshop on Diseases of Fish and Shellfish*, held at Benalla, Victoria in May, 1986 noted that in affected fish there was a "severe branchialitis with considerable mucus production" and histologically there was "metaplasia of the gill epithelium with lamellar fusion". He also noted that fish "*do not cease feeding*" and that "*scrapings of gills reveals numerous parasites, which may show typical amoeboid features*".

[1] http://www.utas.edu.au/library/companion_to_tasmanian_history/A/Aquaculture.htm, viewed January 10, 2011

[2] Atlantic salmon had been previously imported into Tasmania in the 1800's, but the translocations from Gaden, NSW in 1984, 1985 and 1986 were the original stock from which the current salmon industry was derived.

We could find no reference to gill disease affecting the rainbow trout introduced into net pens in Tasmania a few years earlier than the salmon. This could be either because indeed they were not affected or it could be that because of the more diffuse reaction of the rainbow trout gills to AGD (as identified in later research) it may not have been recognised.

2.4 First identification of amoeba species and some epidemiological features of AGD - 1987

In early 1987 the amoebae associated with AGD was identified as a Paramoeba by Bret Robinson, from the South Australian Engineering and Water Supply Department (Foster and Percival, 1988b). Around the same time, affected smolt were transferred to a brackish water site at Port Huon. Prior to transfer, smolt had been dying at a rate of close to 1% per day. Recovery was reported to occur overnight with the mortality rate per day reduced to 0%, and hence it was quickly established that fresh water was the key to treatment of the problem.

At this time rainbow trout were also being grown in sea cages in the south east of Tasmania. They also were affected by AGD, and it was determined that trout >2kg were less susceptible to AGD (suffering around 1-2% mortality per week) compared to trout <1kg (where mortality rates could be as high as 10% per week).

Foster and Percival (1988b) also identified that:

- The paramoeba take approximately 2 months to infect new fish on a site;
- The disease usually only occurs during summer months and usually between 12° and 20°C, but that paramoeba can be found on gills during the winter;
- Overcrowding “encourages” the disease, but that low stocking will not prevent fish from developing AGD; and
- Paramoeba could be found in the water column at a concentration of one amoeba per litre of water.

The above findings were all based on clinical assessment and visual investigation, grossly and microscopically.

2.5 Finding of AGD in wild fish - 1988

Interestingly, the Foster and Percival (1988b) also noted that large numbers of Paramoeba can be found on wild fish, particularly couta (*Thyrsites atun*). This was based on research conducted by Timothy Jones for his Bachelor of Science Honours Thesis (Jones, 1988). The couta examined had been captured outside the cages in the general lease area as had species of tiger flathead (*Neoplatycephalus macrodon*) and a single Bastard trumpeter (*Latridopsis forsteri*) on which Paramoeba were also identified. Yellow-eyed mullet *(Aldrichetta forsteri)* caught within the cages were also found to have Paramoeba on their gills. This is further discussed in Section 4.3.

2.6 Recommendation that fresh water bathing was an effective treatment - 1988

On May 16th, 1988, Foster and Percival (1988a) published a paper titled *"Treatment of Paramoebic Gill Disease in Salmon and Trout"*. This paper recommended a "*simple, full strength freshwater bath for 2-6 hours*" as the treatment for AGD which will result in "*an immediate recovery*" and would likely provide at least 4 months protection from AGD. The authors of this paper noted that the fact that this treatment provided at least 4 months protection and suggested that the fish had developed an "*immune defence"* against the paramoeba. The authors also noted that while on full salinity sites disease would develop within two months of smolt entry (hence usually in mid-December for smolt delivered mid-October), in brackish water sites disease may develop at any time between December and May depending on the freshwater influence, disease developing two months after the last major freshwater occurrence. They reported that in the laboratory a one hour bath in freshwater would kill amoebae.

As far as the authors are aware, the industry quickly adopted the treatment recommended in Foster and Percival (1988a) and incorporated freshwater bathing of smolt as a standard practice in the production cycle. It remains as a standard treatment today, over 20 years later.

2.7 Finding of AGD on coho salmon in USA - 1988

Around the same time that AGD was identified in Tasmania, an infestation of *Paramoeba pemaquidensis* on the gills of coho salmon (*Oncorhynchus kisutch*) had been identified in Washington and California, USA. A report on this was also published in 1988 (Kent et al 1988). Gill disease associated with the infestation was observed in coho salmon reared in a marine site in net pens (Washington) in 1985, 1986 and 1987 and in land based tanks (California) in 1987. The amoebae elicited a prominent epithelial hyperplasia on the gills of affected fish. Visualisation of the amoebae in the mucus taken from gills revealed a prominent parasome and vesicular nucleus. The parasome, an endosymbiont, is a prominent distinguishing feature of the genus Paramoeba Schaudinn, 1896.

Kent et al (1988) noted that examination of histological material from AGD affected salmon in Tasmania indicated that the disease in Washington and California appeared identical to the Tasmanian AGD. The authors of this paper cultured the organisms and determined that exposure of salmon to cultured amoebae did not elicit disease. Through electron microscopic examination they also identified morphological differences between the cultured organism and the amoebae taken directly from gills, finding that cultured amoebae did not have the numerous filaments which extended approximately 350 nm from the surface of the plasmalemma of the non-cultured amoebae.

2.8 Increase in understanding of AGD - 1989-1990

Roubal et al (1989) published a paper where they also examined in detail, by light and electron microscopy, the amoebae associated with AGD in Tasmania. As with Kent et al (1988), the

authors of this paper examined both cultured amoebae and amoebae still attached to the gills of salmon.

Amoebae used in the Roubal study were isolated by Bret Robinson from infected salmon gill provided by Craig Foster, by culturing on seawater agar seeded with *Pseudomonas maltophilia*, without incorporating antibacterials or antifungals. Bret was at that time working for the South Australian Water Authority in Salisbury, South Australia. From the primary culture, amoebae with a morphology consistent with 'Paramoeba', i.e. possessing a DNA-containing body adjoining the nucleus (the parasome), were cloned by micromanipulation onto similar media. TEMs published in the Roubal study are consistent with this identification, but this strain cannot be identified with any of the three *Neoparamoeba spp* linked to the aetiology of AGD as it is no longer available for molecular studies (Bret Robinson, personal communication).

Differences were identified between cultured and gill associated amoebae, including the average size (gill associated amoebae on average were approximately twice the diameter of cultured amoebae) and the presence of pseudopodia that passed into cavities at the surface of degenerating surface epithelial cells or passed between surface epithelial cells. Cultured amoebae lacked any surface hairs or protuberances and there were small, electron dense deposits present in vesicles in the gill associated amoebae which were not present in cultured amoebae.

In 1990, Munday et al (1990) provided further detail regarding the histopathology, morphology, clinical manifestation, epidemiology and treatment of the disease. The fact that fish may still be eating up to the time of death (as dead fish still had feed in their stomachs) was again noted, and the description of affected fish being sluggish and swimming with open operculae suggested that there was a respiratory component to the pathogenesis of the disease. The authors noted that excessive mucus production was a feature of the disease and that the disease manifested differently in rainbow trout compared to Atlantic salmon. Affected trout tended to have a diffuse mucoid branchitis i.e. the inflammation in the gills was more general across the gills compared to salmon where the inflammation was more "*patchy*" (confined to discrete areas). The Paramoebae were associated with regions of hyperplastic epithelium where it was also noted that there were above average numbers of mucous cells. Chloride cells were rare in these regions, as compared to the unaffected regions of gills in salmon living in marine environments where they are numerous. It was noted that affected fish are generally hypernatraemic (increased levels of sodium in the blood). One of the key functions of chloride cells is the active secretion of sodium.

Based on all investigations conducted prior to that time Munday et al (1990) noted that under "appropriate" environmental conditions (*"water temperature ≥12°C, high salinity, poor water exchange"*), the Paramoebae act as a primary pathogen. The authors again note that the only effective treatment appears to be freshwater baths, and suggest that the treatment has its effect in three ways:

1. By greatly reducing the number of amoebae on the gills;

2. By removing the mucus cover on the gills, which is stable in sea water but removed by fresh water; and
3. By reducing, at least temporarily, the hypernatraemia.

2.9 Conclusion

In the few years after AGD was first diagnosed in Tasmania researchers and industry members had quickly determined the causative agent of the disease, had developed a sound but basic understanding of the disease and had an effective, though resource hungry treatment. The following chapters focus on specific aspects of the disease, its causative agent and the host as identified by research. Interestingly, significant aspects of current understanding are based on some of the original work outlined above.

3 The causative agent of AGD – its identification and biology

3.1 Introduction

Amoebic Gill Disease (AGD) has a considerable adverse impact on the Tasmanian salmon industry growing Atlantic salmon in marine sites. There has been an enormous amount of effort put into researching AGD over the past 25 years. Despite this, the primary control of the disease still relies on a treatment method developed over 20 years ago i.e. the bathing of affected fish in freshwater. At the farm level it would thus appear that the significant amount of research has not yet been able to provide the industry with better options to manage and control AGD.

However, while no doubt it has been frustrating for both industry and researchers that better treatment options have not been identified, there is still a lot that has been learnt regarding the disease AGD. We hope that this review will allow both industry and researchers to refresh and possibly renew their understanding of the disease and what is known and not known about it.

3.2 Early amoebae isolates

While Paramoebae species were quickly identified as the most likely cause of AGD, these species were not the only amoebae living on the gills of the salmon. In the early 1990s Howard and Carson (1993a) prepared a total of 680 gill cultures in the field from which they collected 61 amoeba isolates. Of the 30 samples the researchers examined further, only 5 were identified as Paramoebae species (considered to be "*likely pemaquidensis*"). Hence very early it was clear that there were mixed populations of amoebae on the gills of Atlantic salmon affected by AGD.

The amoebae cultures Howard and Carson (1993a) established through this research were used extensively in research conducted through the 1990s and into the early years of this century. For example, the Paramoeba culture named "PA-016" is frequently mentioned from papers published during this period as the culture used to conduct research. This culture was one of the isolates collected from an AGD- affected Atlantic salmon being farmed at Dover in March 1992. It was though, non-infectious as noted by researchers who have attempted to infect salmon using this strain.

3.3 Naming the agent of Amoebic Gill Disease

The actual name given to the causal agent of AGD has undergone a number of changes during the period of this review i.e. 1985 to 2010. The evidence that the cause is an amoebae species is convincing and even though Koch's postulates[3] have not been satisfied, we are comfortable

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[3] Koch's postulates are four criteria designed to establish a causal relationship between a causative microbe and a disease (Seal et al 2010). They state that:

1. The microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy animals.
2. The microorganism must be isolated from a diseased organism and grown in pure culture.
3. The cultured microorganism should cause disease when introduced into a healthy organism.
4. The microorganism must be reisolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.

with the cause of the disease being an amoeba(e) which contains one or more parasomes or endosymbionts. Parasomes are small, self contained bodies that lie in close proximity to the nucleus of the amoebae. There is enough research, in our opinion, showing that isolation of amoebae from infected fish in a relatively pure manner and the use of the isolated amoebae to inoculate that water containing healthy, naïve fish induces typical AGD in the naive fish: Little possibility exists of any other organism (e.g. virus or bacteria) being involved. Consistent visualisation of the amoebae attached to gill epithelium in diseased fish also strengthens the case that amoebae are the causal agent.

We consider the amoebae is capable of causing disease in salmonids in its own right, in the absence of other stressing factors or intercurrent disease.

3.3.1 Microscopic identification

Initially, identification of the amoebae isolated from AGD-affected fish was by visually examining the amoebae (using light or electron microscopy) and classifying the amoebae on its morphological characteristics e.g. the presence of absence of parasomes, whether or not the external surface of the amoebae had scales and the average size of the amoebae. Polyclonal antiserum was created (by injecting amoebae into sheep or rabbits) and this antisera used to confirm the presence of amoebae in affected gill tissue of fish suffering from AGD.

As such, the amoeba causing disease was thought to be a *Paramoeba* species. This then quickly changed to it being a *Neoparamoeba* species, most likely *N. pemaquidensis*. Differences between the “wild” type of amoebae (i.e. that associated with the gill) and the “cultured” type of amoebae (that taken from gills of affected fish and cultured) were noted.

3.3.2 Molecular characterisation and taxonomy

More recently, molecular technology has been used to examine the molecular structure of the amoebae and its parasome(s), focusing on the ribosomal RNA gene. Comparison of the nuclear genome of both the host (amoeba) and parasomes has been used to establish both species concepts and phylogenetic positions of the organisms (as noted in Caraguel et al 2007). This has led to the reporting of new species as being involved in AGD (e.g. *N. branchiphila* as reported in Dykova et al 2005).

Based on the research conducted by Young et al (2007a) which sequenced ribosomal DNA of a number of different amoebae[4] reported at various times to be the causative agent of AGD, the cause of AGD was determined to be different and was called *Neoparamoeba perurans*. Discussion with researchers conducted as part of this review indicated that this finding has been a major breakthrough in the investigation of AGD.

[4] In Young et al (2007a), full-length PCR amplification of the 18S rRNA gene was conducted on seven non-cultured amoeba (i.e. taken directly off gills) and 36 strains of Neoparamoeba from GenBank and PCR amplification of a portion of the amoeba 28S rRNA and six (28S rRNA) non-cultured amoeba and four strains of cultured, purified *N. pemaquidensis*. 18S and 28S rRNA from "wild" strain (i.e. non-cultured strain) were found to be different from rRNA from cultured strain

It is though reasonable for industry to have some degree of scepticism with respect to this finding, considering the number of times they have been informed of “new” species being identified. This scepticism was raised by some industry members, again during discussions conducted as part of this review.

From an industry perspective, the actual name given to the amoebae causing disease is not that important – what is important is having the ability to clearly and unambiguously differentiate between infective and non-infective forms of the causative amoebae agent. It would be highly useful if markers developed by Young et al (2007a) could do this. However, these researchers base their conclusions that there is one specific infective form of causative amoeba agent on ribosomal RNA differences about which we raise some issues in Section 3.8. We are at this stage reluctant to use *N. perurans* as the name of the infective form of the causative amoebae agent in this review, though we agree that the research of Young et al (2007a) is significant.

Hence to avoid ambiguity in this review by applying a specific name that may be considered subjective by some, or that may be redundant in a number of years, we have used the term *“AGD amoeba”* throughout this review to denote the infectious parasome containing amoeba(e) that is the causal agent of AGD in Tasmanian salmon.

3.4 Amoeba in the environment

Clearly, in order to initiate infection, AGD amoeba must be present in the marine environment. However, little is known regarding the occurrence or biology of the AGD amoeba in the environment when not on infected fish.

3.5 Life cycle

As far as we are aware, the only lifecycle stage of the AGD amoeba that has been identified is the trophozoite stage. Our review of the literature would suggest that this is the only stage this organism possesses, but we were unable to confirm this conclusion.

The finding of the AGD amoebae on both the gills of fish and in the environment also suggests that the organism has both a free living stage and parasitic stage. However, we were unable to find in our review of the research any definitive information on generation time, potential for sporulation, survival and replication off the host, nutritional requirements and ability to survive on other potential non-living substrates. Hence the complete life cycle of AGD amoeba remains uncertain.

We do note the precedent with other amoebae e.g. *Naegleri fowleri*, an amoeba that is pathogenic with a free living stage (Cervantes-Sandoval et al 2008).

It is feasible that the AGD amoeba is a saprophytic organism which utilises salmonid gills as another suitable substrate. In fact, gill tissue may be more suitable than many of the other environmental subtrates available and infection of salmon gills may be opportunistic. As such, the amoebae may simply regard the gill environment of the fish as another suitable substrate.

We consider that characterisation of the life cycle is essential in understanding the biology of the organisms. We also consider that knowledge of the life cycle is fundamental to the development of control or mitigation strategies for the disease

3.6 Physical and chemical stability

The influence of salinity on the severity of the disease AGD in fish populations has been examined in some detail and it can be reasonably concluded from the research that salinity has a direct effect on the AGD amoebae, with salinities equal to or below 15‰ being detrimental to the survival of the AGD amoebae (Douglas-Helders et al, 2005b) and a salinity of 4‰ or less required to achieve a satisfactory bathe (Cameron 1993).

However, an important finding of Findlay and Munday (1998) was that some pathogenic amoebae survived the two hour bath treatment (but not a four week freshwater period) and subsequently were able to initiate infection in both bathed and naïve fish post bathing. Hence it appeared that the gill environment in some way protected a proportion of the amoebae from the effects of freshwater when exposed for a short period.

Temperature has also been considered a key factor influencing AGD amoebae survival. It it is more likely, however, that the effect temperature has on AGD is not so much a direct effect on the AGD amoebae but more an effect on the host, with higher temperatures influencing host response (Douglas Helders et al 2005b).

We searched the research to see if we could determine the generation time of AGD amoebae at different temperatures. Unfortunately, we were unable to find any reference to such studies conducted on wild type strains of AGD amoeba. Determining if replication time is shortened as water temperatures rise would seem reasonable, given other parasite lifecycles. Confirmation of this would be useful.

It has though been difficult on occasion to determine the significance of some of the research which has examined physical and chemical stability *in vitro* of amoebae species, primarily because much of the research has been done on cultured strains of the amoebae, and not on AGD amoebae. The use of culture strains of amoebae may not be truely representative of wild type strains.

For example, Howard and Carson (1991a) showed that cultured amoebae grew at 10°C and that growth was enhanced at 20°C with viability being lost at 25°C, and that cultured amoebae grew equally well at 33ppt and 16.5ppt. It is not known whether the AGD amoebae (i.e. the infective form) have the same characteristics.

Some papers made attempts to examine the susceptibility of amoebae harvested directly from the gills but the range of parameters these “freshly infected” amoebae were exposed to was limited (e.g. Douglas Helders 2005b).

There have also been a number of studies which have examined the influence of different culture environments on survival of amoebae (e.g. Howard and Carson 1991a). Unfortunately, as far as we can ascertain all such studies have been conducted on strains of amoebae that have lost virulence and again may not be representative of wild type strains. The finding of a high

molecular weight glycoprotein (HMWG) on the surface of AGD amoeba but not on cultured (non-infectious) strains of the organism (Villavedra et al 2010) also tends to support the potential that tolerance of AGD amoebae to various chemicals/altered water quality parameters may be different than the tolerance of cultured amoebae given these differences in surface properties. An example of this may have inadvertently been seen in Douglas-Helders et al (2005b) who showed a difference in the susceptibility of AGD amoebae (more sensitive) to copper sulphate than cultured amoebae (less sensitive).

Hence we are reluctant to comment on the findings of these early trials that used cultured amoebae for studies on physical and chemical stability. Such data is, however, critical to understanding the biology or the organisms and underpinning the development of control strategies.

The ability to culture AGD amoebae and retain its virulence through the culture process would greatly assist researchers in determining its susceptibility to various physical and chemical parameters of this organism.

3.7 Role and significance of the parasome

AGD amoebae have, as a distinguishing feature, one or more eukaryotic endosymbionts, or parasomes, in close association with the nucleus of the amoeba (Roubal et al 1989). The involvement or otherwise of the parasome(s) in the pathogenesis of AGD has not yet been clearly defined although there are a number of good discussions on this issue in the literature (e.g. Dykova et al, 2003, Caraguel et al 2007).

Discussion with researchers on this issue did raise the question – is the parasome in fact that parasite? We note the comment made in Steinum et al (2008) of the similarity between the pathology noted in AGD in salmon in Norway and that induced by the kinetplastid *Ichthyobodo necator*, a well recognised protozoan parasite of the skin and gills of fish. Interestingly, the various discussions on the parasomes consider this organisms to be more closely related to *I. necator* (e.g. Dykova et al 2003), one paper even suggesting that the parasome be called *I. necator*-related organism, or IRO (Caraguel et al 2007).

We do not think the the parasome is the primary parasite initiating AGD (we consider the amoeba to be this) but if it can be confirmed that the parasome is involved in the pathogenesis of AGD then it potentially provides another area to target, for example, in the development of treatments.

3.8 Loss of virulence during culture

Howard, Carson and Lewis (1993) showed that rainbow trout exposed to a cultured Paramoeba ("PA-016") (with or without prior gill irritation using a chemical irritant) at a concentration of approximately 9,000 amoeba /ml for an hour did not develop AGD yet rainbow trout placed into a tank with Atlantic salmon affected with severe AGD did within 7 days. They had shown previously as discussed above that Paramoebae associated with gill lesions could be immunostained using antiserum developed to PA-016. These researchers were using polyclonal

antiserum and hence it is quite possible that such antiserum may stain up both infective and non-infective amoebae.

Back then, though, it would therefore have seemed reasonable to consider that this particular strain of Paramoebae was indeed the pathogen. The fact that they could not infect healthy fish with it when the fish were exposed to large numbers of the cultured organism was to be possibly the most vexing problem for researchers for many years to come. It was even more frustrating that cohabitation with infected fish caused healthy fish to develop disease within a week – the pathogenic amoeba was definitely there but whether they were working with the correct organism that was somehow changed during the culture process of whether they still had not managed to actually culture the pathogenic organism the researchers could not say.

The potential loss of virulence with *in vitro* culture is well recognised with a range of micro-organisms. The inability to culture the AGD amoebae and then use cultured amoebae to re-infect fish and cause disease has been a major stumbling block limiting research of this organism. Unfortunately, there is still no standardised method available to isolate amoebae from fish affected by AGD, purify and culture the amoebae in large quantities and then use these cultured amoebae in infection models. We say this acknowledging the efforts of many researchers, including Crosbie et al (2010) to develop such a method. Researchers have also been unable to cryo-preserve amoebae to allow long term storage.

We would like to discuss the loss of virulence in cultured strains and the designation of new "species" of amoebae based on molecular analysis further as it is so important to going forward with research into AGD.

Loss of virulence is likely due to two possibilities as noted by a number of researchers including Villavedra et al (2010):

1. The initial inoculums consists of more than one species of amoeba and the culture process selects for the non-virulent specie(s) while impeding the growth of the AGD amoebae i.e. there is a selective pressure applied during the culture process;
2. The culture process modifies the AGD amoebae ("downregulates") virulence factors such that cultured amoebae no longer possess the attributes that underpin pathogenicity.

3.8.1 Neoparamoeba perurans

The finding of Young et al (2007a) would tend to suggest that the reason for non-virulence of culture strains is because of reason (1) above – culture has selected non-virulent strains of the amoebae and the virulent strain (identified by these researchers as *Neoparamoeba perurans*) was selected against. They base this in large part on molecular examination of the SS rDNA sequence of "wild" strains of amoebae (gill associated strains or what we have called "AGD amoeba" in this review) compared to cultured strains of amoeba. The cultured strains were themselves originally harvested from the gills of AGD affected fish.

This would appear to be a pivotal finding in the research on this disease and we are hopeful that the lack of virulence in cultured strains to date may be explained by this mechanism. However, Young et al (2007a) based their finding on differences in ribosomal RNA.

3.8.2 Virulence determinates

Determinants of virulence are those attributes possessed by a micro-organism that allow them to coloniuse, invade and cause disease in the host. Common determinants of virulence include the production of toxins, the presence of specific adhesions and the ability to resist the host inflammatory or immune responses.

The determination of virulence factors provide essential knowledge in understanding how disease develops and how strategies might be developed to mitigate disease. As far as we are aware no specific virulence determinants have been identified for the AGD amoebae. This is a very important area of research which should be pursued.

Having said this, we do though acknowledge the work conducted by groups involved in the development of an AGD vaccine where differences in both gene transcripts (which may themselves code for virulence determinants) and surface glycoproteins are being identified.

3.8.3 Surface glycoproteins and other structures

Villavedra et al (2010) identified a high molecular weight glycoprotein (HMWG) on the surface of AGD amoeba but not on non-infectious strains. It is possible that HMWG is a mucin. Chavez et al (1986) noted differences in the cell coat of amoebae when cultured with or without antibiotics. 67-182nm which was lacking in amoebae cultured with antibioticdifferences in the cell coat between cultured and non-cultured strains of amoeba. Kent et al (1988) noted the presence of plasmalemmal surface filaments on amoebae taken from gills but not on cultured amoebae.

We make these points for reasons that will be explained below.

3.8.4 Culture methodology

Chavez et al (1986), on examination of the free living, polymorphic amoeba *Phreatamoeba balamuthi* noted that the culture of amoebae had a "profound" effect on the cell coat of this amoeba species. They noted that amoebae grown in the presence of bacteria (E. coli) were covered by an amorphous cell coat 67-182 nm thick. This cell coat was absent in cultured strains of the same amoebae. The culture process used by these researchers involved washing the amoebae, packing them and treating for one hour with an antibiotic mix consisting of 500 units/ml Penicillin G, 250 units/ml Dihydrostreptomycin sulphate and Neomycin sulphate. The amoebae were then put onto agar that contained the same antibiotics at half the strength. It is not known whether the lack of cell coat influenced the virulence of the amoeba – we use this example though as part of our consideration of this issue, as will be explained.

We note the presence of the High Molecular Weight Glycoprotein on AGD amoeba but not on cultured amoeba as noted above (Villavedra et al 2010) and speculate that there may be similarities in what these two groups of researchers have discovered.

Kent et al (1988) was another early paper noting that cultured amoebae from fish affected by AGD lost virulence. Interestingly, the medium used by Kent at al (1988) to culture the amoebae consisted of Medium 199 (Sigma, St. Louis, MO., USA), filtered sea water at 25 ppt, 10 % fetal calf serum, 5 % chicken serum, and 100μg ml-1 of the following antimicrobial compounds: neomycin, kanamycin, novobiocin, penicillin, streptomycin and nystatin. Through electron microscopic examination they also identified morphological differences between the cultured organism and the amoebae taken directly from gills, finding that cultured amoebae did not have the numerous filaments which extended ca 350 nm from the surface of the plasmalemma of the non-cultured amoebae (Figure 1).

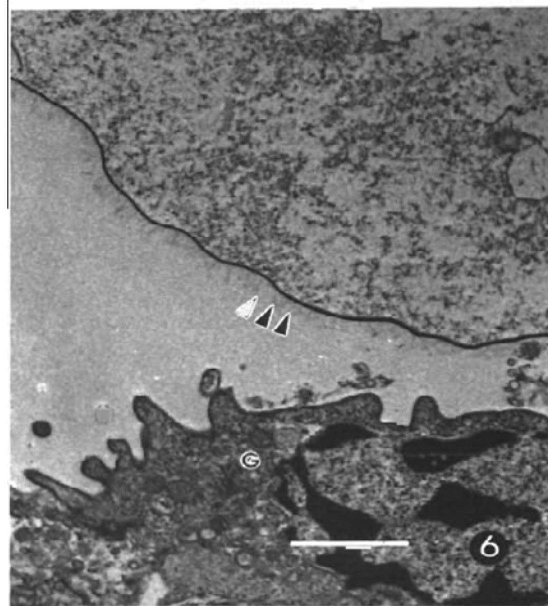

Figure 1 Electron microscopy of amoebae on coho salmon gills (from Kent et al 1988). G=gill epithelial cell, Arrows = plasmalemmal surface filaments on amoebae (not seen in cultured amoebae). Bar = 2 μm

Roubal *et al* (1989) published a paper where they also examined in detail, by light and electron microscopy, the morphological characteristics of amoebae associated with AGD in Tasmania. As with Kent *et al* (1988), the authors of this paper examined both cultured amoebae and amoebae still attached to the gills of salmon. This was though an early study where, as far as we can ascertain, antibiotics were not used in the culturing process. The amoebae used in the Roubal study were isolated by Bret Robinson[5] from infected salmon gill provided by Craig Foster. Discussions with Dr Robinson have confirmed that isolation of the amoeba was by culturing on

[5] Bret Robinson was at that time working for the South Australian Water Authority in Salisbury, South Australia. From the primary culture, amoebae with a morphology consistent with 'Paramoeba', i.e. possessing a DNA-containing body adjoining the nucleus (the parasome), were cloned by micromanipulation onto similar media. Transmission Electron Micrographs published in the Roubal study are consistent with this identification, but this strain cannot be identified with any of the three Neoparamoeba spp linked to the aetiology of AGD as it is no longer available for molecular studies (Bret Robinson, personal communication).

seawater agar seeded with *Pseudomonas maltophilia*, without incorporating antibacterials or antifungals.

The paper by Roubal *et al* (1989), when carefully examined, did note that the gill associated ("wild type") amoeba had small cell wall projections at the parasite-host interface but admitted that such projections may have been artefactual. There was no comment in this paper of differences between the cell walls of cultured versus non-cultured amoeba, but we have not been able to go back to the original authors to confirm this.

3.8.5 Use of antibiotics in culture media

Generally, culture techniques have included antibacterials (+/- antifungals) to a lesser or greater degree which is understandable, as the aim has been to try to get pure cultures of the amoebae, and to minimise bacterial contamination/overgrowth.

This is not unprecedented – the culture of *Entamoeba histolytica*, the human pathogen has also utilised antibiotics to get axenic cultures (Mirelman et al 1986).

An important example with regard to AGD amoebae is the culture process developed and routinely used by Howard and Carson (1991a) as the technique was also used by other researchers in subsequent years (e.g. Akhlaghi et al 1996) or amoebae derived from this culture technique (e.g. Douglas Helders et al 2001a, Elliot et al 2001, Parsons et al 2001). This technique used as the medium a sea water agar which was first autoclaved to ensure sterility. Antibacterials (10µg/ml of streptomycin, penicillin, kanamycin and novobiocin) and an antifungal (5µg/ml of amphotericin) were added to the molten agar and then the surface of the plates were covered with a thin layer of the bacterium *Flexibacter* species. Cultures were maintained at 20°C and subcultured every 2 to 4 weeks.

Even when antibiotics were taken out of the culture medium itself, isolation techniques often incorporated a stage where gill tissue was placed into a medium containing antibiotics. For example, Zilberg et al (2001) used the following isolation technique when removing gill arches from fish: the arches were dissected from euthanased fish and then put in an antibiotic mix which contained 0.5 mg streptomycin sulphate, 0.5 mg penicillin, 0.5 mg carbenicillin, 1.25 mg ampicillin and 0.5 mg erythromycin in 50ml of solution prior to storage of the arches overnight at 4°C. Mucus from these gills was then scraped off.

Villavedra et al (2005) showed a change in antigenic properties of "infectious" amoebae collected from the gills of AGD affected fish occurring over approximately 15 days. The "infectious" amoebae were cultured in medium containing a number of different antibiotics.

We make the point about antibiotics used for harvesting and culturing amoebae for 2 reasons:

1. We could find little reference in any research discussing the use of antibiotics and the effect they may have on amoeba except for one trial conducted in Howard and Carson (1991). These researchers examined the growth of an amoebae species when exposed to various antibiotics. The isolate they used (UQ-1) was donated to them by the University of Queensland. Growth of the amoeba was not inhibited when the

antibacterials penicillin, streptomycin, kanamycin and novobiocin and the fungicide amphotericin were added to the culture medium. However, from comment made in a number of reports it is unlikely that this isolate was AGD amoebae but a cultured, non-infectious strain[6]. and

2. A key class of antibiotic being used in these cultures are the aminoglycosides: Streptomycin, dihydrostreptomycin, spectinomycin, neomycin, kanamycin and gentamicin all belong to this class of antibiotics. The antibiotics target ribosomal RNA as discussed below.

The scope of this review was not to review literature pertaining to the potential effects of different classes of antibiotics on amoeba. However, we consider the issue of using antibiotic "cocktails", particularly those containing aminoglycosides, in the culture medium as warranting of further discussion and investigation, in part due to the fact that we could find no discussion on this issue in any of the research papers and reports that we examined. We accept that it may have been discussed and even researched in laboratories and the results of that research not considered signficiant enough to warrant publication. If this is the case we would welcome reviewing any such work, even if it were not published.

We do note that culture techniques developed for *Entamoeba histolytica*, the parasite causing Amoebiasis in humans, also utilised antibiotics, including aminoglycosides (Mirelman et al 1986).

The reason we are particularly concerned about aminoglycosides in relation to this issue is because:

1. Aminoglycosides can be amoebicidal and indeed are used in some circumstances as the drug of choice in amoebic disease (Loebenberg et al 1975, Staufer and Ravdin 2003);
2. Their action is targeted against ribosomal RNA, the region of focus for genetic phenotyping;
3. The polycationic chemical structure of aminoglycosides facilitates passage across the cell wall of bacteria but impedes passage through the hydrophobic lipid bilayer of eukaryotic cell membranes. While it may be purely coincidental, we raise this point due to the finding by Chavez et al (1986) that amoeba exposed to aminoglycosides in the culture process lacked a thick cell coat which was evident in amoeba that weren't exposed to the antibiotics. We also note the research conducted by Villavedra et al (2010) that identified the presence of high molecular weight glycoprotein (HMWG) on the surface of AGD amoeba but not on cultured amoebae. The authors of this paper considered the HMWG likely to be a mucin. We consider it plausible that the presence of aminoglycosides in the culture medium could induce metabolic changes within the amoeba resulting in absence or modification of HMWG or could rapidly select for varients that do not have on their surface the HMWG or other specific peptides, directly resulting in loss of virulence and invalidating much of the research conducted on the

[6] UQ-1 is noted in Howard and Carson 1993a as being *Platyamoeba plurinucleolus*

amoeba cultured *in vitro*. The mechanism of this selection may have been the potential that the HMWG to facilitate entry of the aminoglycoside hence leading to bioassumulation of these antibiotics in these amoebae[7].

3.8.6 Further comment on antibiotics and culture methods

We consider the isolation technique developed and described in Morrison et al (2004) to be significant as it utilises the adherent property of the amoebae in the process of isolation. These researchers have shown that amoebae isolated from affected fish in this manner retain pathogenicity when used in inoculation trials. What is more, they have shown that there is a linear association between the inoculating amoebae concentration and the mean number of AGD-like gill lesions and AGD-affected gill filaments detected by histology. This would suggest that the isolation technique ensured isolation of a relatively pure mix of AGD amoebae – if the isolation technique resulted in a mixed population of both infective and non-infective amoebae, then one would logically expect to find variation in the association between the inoculum concentration and the response, unless the proportion of pathogenic to non-pathogenic amoebae remains constant.

We consider this to be supported by the comment made in a number of papers including Villavedra et al (2010) and Crosbie et al (2010) on analysis of amoebae collected from AGD affected salmon in the culture tank at the University of Tasmania. The PCR analyses of the isolates have shown that over at least 2 years all isolates appear to be AGD amoeba, indicating that either other species are in very low (undetectable) numbers or simply are not present and that the amoebae on the gills of these fish is a culture containing only AGD amoebae.

What is also significant with regards to the discussion above is the finding that isolating amoebae from the gills of affected salmon using the technique described in Morrison et al (2004) and then culturing the amoebae also caused cessation of pathogenicity. These are amoebae that have been selected on the basis of their adherence. Again we note that the amoebae, once isolated, were resuspended in sterile seawater (SS) with 5.5 × 108 heat-killed E. Coli ml–1 (ATCC strain 25922) and streptomycin sulphate (0.001%) (Sigma), benzylpenicillin (0.001%) (CSL), carbenicillin (0.001%) (Sigma), ampicillin (0.0025%) (Sigma), erythromycin (0.001%) (Sigma), sulphadiazine (0.63%) (Sigma) and trimethoprim (0.13%) (Sigma) i.e. a cocktail of antibiotics including two aminoglycosides.

We also identified one other interesting finding that may or may not be related to this issue. Throughout the literature there are variations in findings between different research conducted at different times. One such variation relates to the infective dose required in the water column to cause AGD in susceptible salmon. Zilberg et al (2001c) found that it required 230 Neoparamoeba per litre to initiate AGD after 7 days whereas Morrison et al (2004) could initiate AGD in 14 days with as few as 10 cells per litre of water. While there are a number of other variants in the methodology of these two trials, we do note that there was an isolation (but not

[7] Comparisons between the cell wall of prokaryotes and eucaryotes are beyond the scope of this review, however, given the suggested changes in cell wall structure between wild-stran and cultures amoeba, further research inthhe areas would appear appropriate in understanding the pathogenesis of AGD.

culture) step using aminoglycosides in the methodology outlined in Zilberg et al (2001c) but not as far as we could determine in Morrison et al (2004). While it is possible that the plastic adherence step used in Morrison et al (2004) could itself be selecting for pathogenic amoebae, it is also worth questioning as to whether the short period of exposure to the aminoglycosides (overnight at 4°C) in Zilberg et al's method was sufficient to impact on the virulence of the amoebae inocula, or select for non-virulent strains.

We would suggest that at the very least, culturing gill derived amoebae in a number of replicates with each replicate exposing the amoebae to perhaps only one class of antibiotic and determining whether pathogenicity is retained depending on the antibiotic class used may be a worthwhile area of research.

3.8.7 Loss of virulence due to other factors

We do also accept the other possibility that AGD amoebae utilise products produced by the fish and incorporate these products into their own cell surface and it is the lack of these products in the culture media that lead to a loss of virulence. This has been suggested, for example, by Villavedra et al (2005). If this were the case, identifying compounds present in the mucus of salmon and incorporating them into the culture media may allow assemblage of mucin on the surface of amoebae which allows retention of pathogenicity. We do though also note that Douglas Helders et al (2003) found that amoebae could retain pathogenicity in sea water for up to two weeks without contact with fish. There are two possibilitites here:

1. That sufficient numbers of AGD amoebae survive this period to remain infectious and cause disease in salmon; and/or
2. That AGD amoebae replicate during this period ensuring sufficient numbers remain in the water column to establish infection and disease on salmon

If AGD amoebae are replicating during this period and retaining virulence, then it would discount the assertion that they need specific fish products in the culture media to retain virulence. This is of course dependent on knowing what the replication cycle is in the amoebae which, as far as we are aware, is not known. Further studies in this area appear warranted.

Finally, as noted by Howard and Carson (1994):

"Propagation of a virulent Paramoeba, able to infect native fish, is one of the most important goals for AGD research…the development of a vaccine relies heavily on the cultivation of a virulent paramoeba isolate…"

4 Epidemiology

4.1 Introduction

Epidemiology may be defined as the study of the patterns of disease that exist under field conditions. More specifically, it considers the frequency, distribution and determinants of disease in populations (Martin et al 1987) which includes the spatial and temporal attributes of the disease.

The major purpose of considering the epidemiology of a disease like AGD is to provide information on which a rational decision(s) for the prevention and/or control of a disease in an animal population can be based. This is why the study of the epidemiology of AGD is so important for the industry as its objective is to provide answers to industry on how to improve control and prevention.

It is unfortunate and frustrating that research conducted on the epidemiology of AGD over the past 25 years does not appear to have provided industry with information that has significantly improved its control or prevention of AGD. This is based on the fact that treatment still relies on a method developed over 20 years ago with very little change in the approach taken to how and when bathing of fish populations occurred, apart from modifications in the actual process of bathing used at different farms. In reality, it appears that despite the considerable research undertaken, industry's understanding of AGD has progressed little.

Epidemiology is in part dependent on an understanding of the biology of the agent including such factors as host range, physical and chemical tolerances, lifecycle, geographic distribution and means of transmission, some of which are discussed elsewhere.

4.2 Transmission of disease

There is ample research confirming that the cohabitation of salmon affected with AGD with naive salmon results in AGD in the naive fish hence fish to fish transmission of this organism is established.

In a cage situation, where there are high densities of susceptible fish such as salmon, it is likely that the most dominant means of transmission is via this horizontal route and we agree with Munday et al (2001) that the primary source of infectious amoebae are fish and at least in later years likely in most instances to be farmed fish i.e. the salmon themselves.

There is also evidence that bathing in a 2 to 3 hour bath does not destroy all amoebae on fish, and that these surviving amoebae a likely to the main source of infection immediately post bathing (Parsons et al 2001) further supporting the assumption that the main source of infection in salmon cages are the salmon themselves.

As noted, based on experimental transmission studies, we also consider it highly unlikely that another pathogen (e.g. of bacterial or viral origin) is involved in the epidemiology of AGD.

It is noteworthy, however, that in the early days of the industry naïve fish were placed in cages in an area likely to have little if any other salmonids and somehow transmission occurred in a

very short space of time. Also, in salmon growing regions such as Norway where wild salmon naturally occur, AGD has occurred with again no indiction of how initial transmission happened.

4.3 Reservoirs of the amoebae – where does it come from?

4.3.1 Initial infection of salmon in 1985

The primary source of the AGD amoebae which initially infected Atlantic salmon and rainbow trout in Tasmania in the early days of the industry, and how it infected the salmon, remains uncertain. It is likely however that this initial source continues to exist but may not be as dominant a factor as it was originally.

It is feasible that the AGD amoeba is a saprophytic organism which utilises salmonid gills as another suitable substrate. In fact, it may be more suitable that many of the other putative subtrates it has available to it.

Fundamental to understanding the epidemiology of AGD is an understanding of the susceptibility of other common fish species to AGD especially those found around cages. Douglas-Helders et al (2002) argue that wild fish are not a signficiant reservoir for *Neoparamoeba pemaquidensis*, the name given at that time to the AGD amoeba.

This research is cited in a number of subsequent papers. However, logically we can find no reason why, if salmonid gills are such a suitable substrate for this organism, then so too to a greater or lesser degree are gills of other fish.

The fact that AGD was detected very soon after the introduction of Atlantic salmon into marine sites in Tasmania would logically tend to suggest that the in the early days of salmon farming amoeba were either in relatively large numbers in the water column or that the transfer of pathogenic amoeba to the salmon was in some way facilitated.

Zilberg et al (2001c) showed that a level of around 230 amoebae per litre of saltwater could cause AGD to develop in salmon within 7 days and Morrison et al (2004) showed that as few as 10 amoebae per litre of saltwater could initiate disease in tank environments. It is though difficult to be convinced that in Tasmania in the mid 1980's there was such a level of pathogenic amoebae residing in the water column and that it was simply a matter of the Atlantic salmon being introduced into the marine sites that allowed exposure and subsequent establishment of amoeba on the gills of the fish, resulting in AGD.

There has been considerable effort gone into determining what the reservoirs of amoebae in the environment. Initially the research used technology based on polyclonal antisera, generally developed towards cultured *Neoparamoebae* species (e.g. Douglas-Helders et al 2003). Later research used molecular technology to develop specific PCR protocols for the detection of amoebae in the environment (e.g. Wong et al 2004). This research, focusing on strains of *N. pemaquidensis*, was conducted prior to Young et al (2007a) indicating that the infectious amoebae may not be *N. pemaquidensis* i.e. much of the this work was conducted on strains of amoebae other than what is likely to have been AGD amoebae.

4.3.2 Wild fish as a reservoir

Work conducted very early in the history of AGD in Tasmania by a University of Queensland student, Timothy Jones, for his Honours thesis and published in 1988 (Jones, 1988) identified large numbers of what then were considered paramoebae type species on the gills of wild fish, particularly couta (*Thyrsites atun)*. The couta, an active pelagic species, had been captured outside the cages in the general lease area as had species of tiger flathead (*Neoplatycephalus macrodon*) and a single Bastard trumpeter (*Latridopsis forsteri*) on which Paramoeba sp. were also identified. Yellow-eyed mullet (*Aldrichetta forsteri*) caught within the cages were also found to have *Paramoeba* on their gills. Jones (1988) also found AGD like lesions on the gills of a number of these wild fish.

We consider this to be an important finding and were surprised when researchers investigating the prevalence of amoebae on wild fish in later research did not make reference to these findings. The presence of AGD amoeba on non-salmonid species has major implications for disease control and management. As noted above Douglas-Helders et al (2002) concluded that AGD does not appear in wild fish and wild fish do not seem to be a reservoir of the pathogen. However, no couta were examined in their investigation and they make no reference to Jones (1988) in their paper.

We questioned this apparent omission with one of the co-authors of Douglas-Helders (2002) who indicated that the information provided in Jones (1988) was not valid to cite in a scientific article due to information being anecdotal and quite possibly incorrect due to the possibility that Jones had used antibodies against the wrong type of amoeba.

Unfortunately, we only had excerpts from a draft of the honours thesis to review. A supervisor of Jones at the time he conducted his research was Professor Bob Lester, an eminent parasitologist who is an expert in fish parasites. We contacted Professor Lester regarding Jones' work. Professor Lester was fortunate to still be in contact with Timothy Jones who provided an original copy of the thesis for Professor Lester to examine.

The method used in Jones (1988) to isolate and identify amoebae from the gills of farmed and wild fish was as follows- the gill was carefully washed and wet mounts prepared from both the washings and the gill filaments. The wet mounts were then examined for the presence of amoebae. *Paramoeba* sp. were directly identified by the presence of a parasome, which was made more visible in the wet mount by applying gluteraldehyde, a method advised by Dr Brett Robinson, SA Water. (Professor Bob Lester, personal communication). The photos in figure 3 are taken directly from the honours thesis.

Importantly, Jones (1988) used a direct method of examination of the gills which minimised the possibility that amoeba on the gills would be lost through processing. The methodology used in Douglas Helders et al (2002) placed gill tissues into fixative with examination only after processing through alcohols and other reagants, a procedure that may have dislodged loosely attached amoebae.

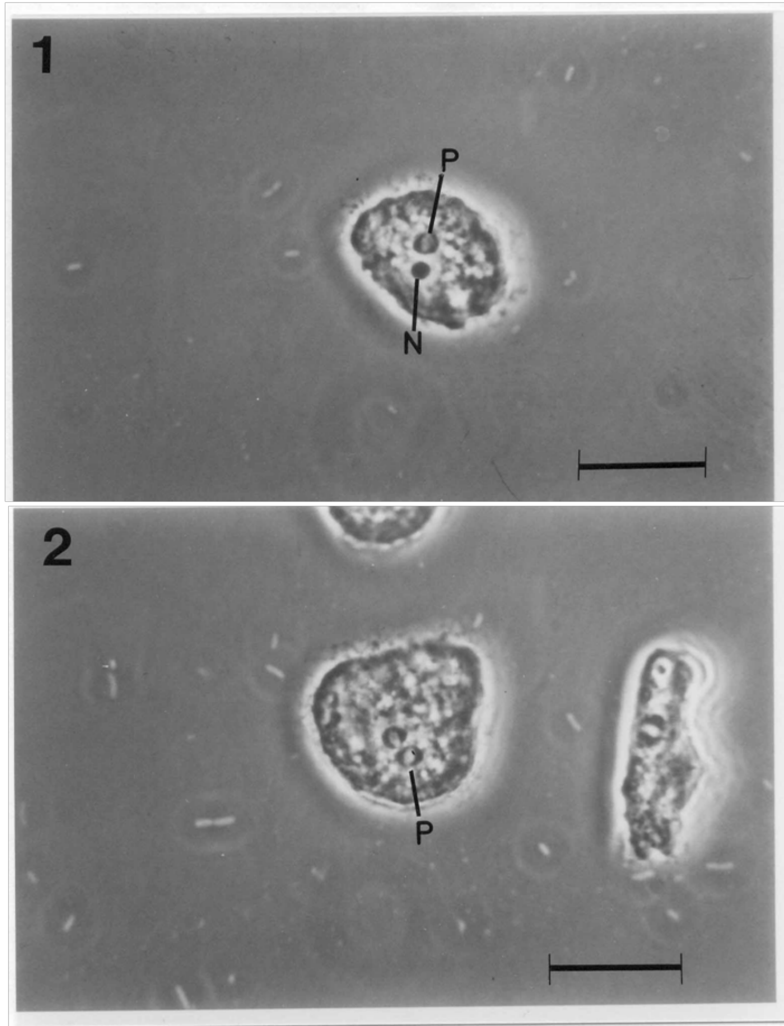

Figure 3 Photomicrographs from Jones (1988) Locomotive form from culture showing P - bipolar parasome and N - nucleus with visible nucleolus. (Gluteraldehyde, 100X phase contrast) Bar = 25 µm

Logically, it would make sense that the first salmon introduced in net pens in Tasmania came in contact with wild fish and this was how infection established in the salmon. It is postulated that the activity around these early net pens, particularly feeding, was an attractant to local fish species (and predatory pelagic species as well) and this facilitated transfer of pathogenic amoebae species over to what then (and still are) highly susceptible salmonid species.

By 2002, when Douglas-Helders et al (2002) published their paper, it is likely that the key reservoir for the amoeba were the salmon themselves as by this time the amoeba had been co-habiting with the salmonids for 17 years or so. Wild fish may not therefore have been as important in the epidemiology of AGD as they had back in the 1980's.

We are therefore reluctant to discount the possibility that in the early years of the Atlantic salmon industry in Tasmania wild fish were the reservoirs of the pathogenic amoebae that led to the establishment of the disease in salmon. We consider it a logical and feasible explanation of

pathogen transfer and note the importance of identifying wild fish as reservoirts of infection in the epidemiology of the disease.

4.4 Infective dose

The difference between one amoebae being able to infect a salmon and then multiplying rapidly compared to the increase in amoebae being due to recruitment in the gills is very important. We could not find any information indicating which of the two mechanisms of increase in number are predominant.

The infective model in a tank is different from what would occur in a sea cage. Hence while 10 amoebae per litre may be infectious in the laboratory, what the infectious dose is in a salmon cage under field conditions is not known.

Munday et al (2001) pointed out in research conducted by Zilberg et al (2001) that the minimum infective dose for salmon is around 230 amoebae per litre of water. This was when *N. pemequidensis* was considered the pathogen. Antibiotics were also added to the culture in the isolation technique used by these researchers, as noted previously.

Morrison et al (2004) showed that as few as 10 amoebae per litre of water could be infectious in a tank environment. These researchers refined the isolation of amoebae using a plate adherence stage to separate out amoebae and as far as we are aware used no antibiotics in the culture process. It is possible that incorporating the adherence step may increase selection for pathogenic amoebae although this is not proven. What these observations do emphasise is the need for research in the mechanisms by which AGD amoeba adhere and colonise the gill surface, a fundamental step in the pathogenesis AGD.

We do not know how a finding of an infective dose in a tank environment translates to an infective dose in the sea water net pen. Having said that, we agree with Munday et al (2001) who noted that the danger posed by a cage of fish affected with AGD is considerable given these levels of infectious dose identified in the laboratory.

The number of amoebae in the water column has not been a simple task to assess, though we do note that Jones (1988) examined amoebae in the water column around net pens and found a level of 3.4 parasites (range 2.4-7.9) per litre within the net pens but could not detect the parasite outside the net pens.

Douglas-Helders et al (2003) used an immunodot blot method to look for amoebae in the water column and a “most probable number” technique to quantify levels. While they did find levels of amoebae reduced the further the distance from cages, there is a possibility that not all amoebae identified were viable and it was noted that there was room for refinement in the techniques used.

4.5 Susceptibility of non-salmonid species to AGD

The variation in the pathological response of rainbow trout, which show a diffuse mucoid bronchitis (Munday et al 1990) compared to salmon, which have a more discrete and distinctive focal reaction is noted. The difference may explain the abscence of any reports on AGD in the

populations of rainbow trout entered into marine sites earlier than the salmon as it is quite conceivable that rainbow trout were exposed to AGD amoebae when first introduced into net pens in Tasmania for reasons discussed above.

Rainbow trout have been shown subsequently to be susceptible to the disease (Foster and Percival, 1988). So too have turbot (*Scophthalmus maximus*) and it has been possible to experimentally produce gill infection, but not AGD, with AGD amoebae in greenback flounder, *Rhombosolesa tapirina* andbig-bellied seahorses, *Hippocampus abdominalis* (Nowak et al 2002).

Critical to understanding the epidemiology of AGD may be the understanding of the relationship of AGD amoebae with the gills of wild fish species. One opportunity that may have provided some insight was the culturing by one salmon company of striped trumpeter (*Latris forsterił*) in close proximity to its salmon pens. While there was no apparent indication that the striped trumpeter developed AGD (S. Percival, personal communication) the fish were not examined to confirm whether AGD amoebae could be isolated from their gills.

We make this point as we could find no research that had looked at whether there were attributes of the gill structure of salmonids that make them more susceptible to development of the disease compared to wild fish. If such research were to be conducted, we would suggest that the differences in gill structure associated with the fact that salmon have tolerance to freshwater (which generally wild marine species in Tasmania do not) should be considered.

4.6 Host factors

The response of the host to infestation with amoebae is a crital aspect for epidemiology and potential control mechanisms.

The host response can be thought of in three levels – the protective physical barriers of the host, its innate (or non-specific) immune defences and its acquired immunity. We discuss these specific host factors at the individual fish level in Section 7.

Epidemiology considers factors at the population level, hence host factors in this section concern the dynamics of fish populations and its impact on development and severity of disease.

4.6.1 Previous exposure.

Laboratory trials have identified differences in the susceptibility to infection in fish that have previously developed AGD and recovered. Findlay et al 1995 and Vincent et al 2006 showed that previous exposure decreased susceptibility whereas Gross et al 2004 disagreed with these other researchers.

From an industry perspective, our understanding is that in the field fish previously exposed to AGD do not show a decrease in susceptibility, having to be treated on multiple occasions through their lifecycle.

We understand the difficulties of attempting to study the differences in the epidemiology of AGD in the field with much of the fundamental understanding of this organism still lacking.

Attempts however have been made to gain some understanding of differences observed in the field e.g. Clark and Nowak (1999) found differences in seasonality and prevalence of AGD in specific salmon populations at three different sites during a period of two years. In a sense, this research reinforced what was already known at the farm level and unfortunately, the information gained from this research did not assist industry in its ability to control or manage disease.

4.7 Environmental factors

Environmental factors clearly play a major role in the occurrence and distribution of AGD. The estuarine habitats supporting the Atlantic salmon industry in Tasmania show considerable variation within which the AGD amoeba may or may not survive and regions of Tasmania exist where AGD has not been reported (Douglas Helders et al 2005). The reasons for these differences remain uncertain. Salinity and temperature have long been associated with the occurrence of AGD, with increases prevalence associated with increased water temperatures and amelioration of disease associated with decreased salinities

There ares some studies that specifically address the influence of environment on the development of AGD. Nowak et al (2007) reported that the infectivity of amoeba and its ability to cause AGD is reduced at salinities below 35ppt and described morphological changes to the amoeba, likely affecting infectivity, with lysis, rounding up and detachment at salinities below approximately 9ppt over a 24hr period.

In addition to temperature and salinity, the influence of a number of other environmental parameters on the development of AGD has been investigated. In a major study of environmental influences on AGD (Nowak et al 2007), cages of Atlantic salmon exposed to increased artificial lighting were shown to require earlier bathing for treatment of AGD compared to fish not subject to artificial lighting, although a possible role of a halocline was evident. Supplemental oxygenation and high energy diets were found not to affect AGD occurrence. Exposure to freshwater was identified as inactivating amoeba, as no trophozoites were recovered either by adherence to petri dishes or by invitro culture. Douglas Helders et al (2005) reported decreased amoeba survival at salinities of 15ppt, low amoebic densities and water sourced from Macquarie harbour, in which lower dissolved calcium and magnesium concentrations were identified.

It would appear that there is still much to be learnt about the influence of environmental factors on the development of AGD and definitive research in this area may be of benefit in developing disease mitigation strategies. This may be helped by the ability to specifically identify AGD amoebae.

5 The host response to infection

5.1 Introduction

Disease entails a host response and indeed it is sometimes the host response that is most damaging to the host. The only factor in this sequence of events leading to AGD that has been reasonably described in AGD is the response of the host at the cellular level. Even at this level we do not think the inflammory/immune response has been satisfactorily characterised particularly with respect to clear designation of the cell types involved.

It is the inflammatory/immune process that is critical to determination of whether the host is overwhelmed by the organism or indeed survives infection.

In AGD, it is possible that at some point the tissue damage caused in the disease process exceeds the functional capacity of the gill of the fish to support life, directly and indirectly contributing to death of the affected individual.

5.2 Gross pathology

Reference has been made to the fact that affected fish are frequently in good body condition and may have food in the gastro-intestinal tract, evidence of recent feeding activity (Munday 1988).

Significant gross pathology in AGD is restricted to the gills (Munday et al 1990) and is characterised by a severe mucoid branchiitis (Munday 1988). Gross lesions may be seen within 2 days exposure to amoeba (Zillberg and Munday 2000). Typically, irregular, raised, multifocal pale mucoid patches are seen on the gill filaments varying from discrete focal spots to extensive regions of coalescing mucoid patches (Munday et al 1990, Harris et al 2004, Adams et al 2004, Munday et al 2001). In Atlantic salmon, the lesions tend to be irregular or patchy in distribution whereas in rainbow trout, the lesions are relatively diffuse (Munday et al 1990). Early lesions of AGD may not be detected grossly (Adams and Nowak 2004b) and gross assessments of infection (gross scores) correlate well, but not exactly with histological lesions (Adams and Nowak 2004a). Smaller lesions not visible grossly may be visualised by stereomicroscopic (sub-gross) examination (Adams et al 2004).

Subgross observations of grossly detectable AGD lesions show a distinctive protrusion of tissue upon the leading edge of filaments extending deep into the interlamellar regions (Adams et al 2004a).

The concept of “gill score” has been used to describe the lesion severity of gills affected by AGD at the gross level and is regularly used by industry. Gill score has also been used as trait on which selective breeding may be based. The actual method used to evaluate AGD severity uses a scale, either from 0 to 4 (e.g. as noted in Powell et al 2001 and shown in Table 1) or 0 to 5 (e.g. as noted in Taylor et al 2009). A score of “0” reflects no lesions and a score of “4”or “5” reflected advanced lesions covering the majority of the gill surface.

Infection level	Score	Gross signs
Clear	0	Gills appear clean, healthy red colour
Very light	1	1 mucoid patch, light mucus accumulations
Light	2	2-3 mucoid patches, some paling colour
Medium	3	Established thickened mucoid patches and mucus
Heavy	4	>3 mucoid pathes or a single large patch resulting from patch accumulation

Table 1 - Industry gross gill scoring mechanism as identified in Powell et al 2001

5.3 Histopathology

The response of the gill at the cellular level in natural and experimental infections of Atlantic salmon with AGD is well described (for example, Munday 1988, Adams and Nowak 2001, Adams and Nowak 2003, Adams and Nowak 2004a, Harris et al 2004 Munday et al 1990, Roubal et al 1989, Munday et al 2001). No differences are reported between natural and experimental lesions. The lesions are characterised by variable hyperplasia of the secondary lamellar epithelium with fusion of adjacent lamellae and obliteration of normal gill structure, spherical or ovate cystic cavitation (also described as inter-lamellae vesicles or lacunae) within the hyperplastic epithelium, mucous cell metaplasia or hyperplasia and an inflammatory cell reaction. Hyperplastic epithelial cells were described by Roubal et al (1989) as being hypertrophied with a rounded nucleus, with scattered clumps of heterochromatin and a distinct nucleolus; In some cells, heterochromatin may be inapparent and the nucleolus indistinct.

5.3.1 Histological development of AGD lesions

The progression of lesions associated with AGD are described in natural and experimental cases by Zilberg and Munday (2000) and Adams and Nowak (2003, 2004a, 2004b). Attachment of amoeba to healthy gill epithelial tissue occurs within 12 hrs of exposure and by 48 hours, markedly increased numbers of amoebae are seen associated with developing lesions and with normal epithelium. Attachment of amoeba was described by Adams and Nowak (2004a) as occurring generally at the base of the secondary lamellae, sometimes associated with a leucocytic response within the central venous sinus.

The initial response to attachment of trophozoites to normal gill epithelium results in localized epithelial desquamation and oedema in juxtaposed regions of the filament, with a hyperplastic reaction to attached trophozoites and the presence of leucocytes. Within 24 hours exposure, lamellar fusion occurs in response to attached amoebic trophozoites, with localized more pronounced epithelial desquamation and presence of leucocytes within the central venous sinus. By 48 hr exposure, lamellar fusion affecting multiple filaments is seen, with many amoebae adhered to these regions. Lamellar fusion appears facilitated by recruitment of undifferentiated epithelial cells in the affected region.

By 4 days, multifocal hyperplasia and lamellar fusion is seen, progressing to moderate to severe multifocal hyperplasia involving multiple lamellae and with hypertrophic and spongiotic

epithelial cells evident by day 7. At this time, amoeba become increasingly abundant and are associated with the surface epithelium and are found between the lamellae of the hyperplastic epithelium. Amoeba may also be seen in the process of exfoliation, surrounded by mucus and cellular debris and in areas, segments of hyperplastic tissue with attached amoeba sloughing off the gill, leaving damaged gill tissue and exposed vasculature.

Infiltration of leucocytes via the central venous sinus into fused lamellae with macrophages seen apically on lesions is also described. Lesions are reported to be similar but more extensive and larger within three weeks of initial exposure. The presence of inter-lamellae cysts or vesicles is associated with advanced lesions. By 4 weeks up to 75% of fish may show severe lesions involving some 50% of the gill surface epithelial hyperplasia and lamellar fusion were extremely extensive, occupying most of the gill lamellae and . At this time, affected fish may appear moribund, at which stage, and “extreme” numbers of amoebae on the gill lamellae (Zilberg and Munday 2000).

Zilberg and Munday (2000) described a marked increase in the number of mucous cells at 14 days post exposure, however, when the hyperplastic regions of the gill filaments were compared with the normal regions of AGD-infected fish at 14 days post-exposure, the number of mucous cells was significantly lower on the hyperplastic regions

Although the histopathology of AGD has been reported by numerous researchers, descriptions focus on morphology on H&E stained histological sections which provide little other information on which an understanding of the pathogenesis of AGD can be further understood.

Although Morrison et al (2006) identified Major Histocompatibility Antigen Class ll, an antigen presentation molecule on cells associated with the host immune reposnse, the further use of such *in situ* techniques, including *in situ* hybridisation, immunoperoxidase staining for localisation of antigens and especially the use of markers to characterise the nature of the cell types involved in the immune and inflammatory responses (macrophages and immunocytes) would greatly assist in understanding the host response to infection and may provide information on which decisions relating to further research on vaccines may be based. It is noteworthy in this regard that gill tissue of clinically normal fish frequently show increased numbers of inflammatory cells, including immunocytes.

Further, automated technology is readily available for quantitative histological assessments of tissue components. This technology might well be used to quantify mucous cells and chloride cells, as well as other cell types involved in the host response to AGD amoeba, again contributing to an understanding of the host-parasite relationship.

5.3.2 Distribution of amoeba

Adams et al (2004) reported that amoebae were more numerous on the protruding face of most lesions compared with the inter-filament regions in cases of AGD.

5.3.3 Distribution and severity of lesions

The extent and severity of the lesions varies with time and with the number of amoeba present (Roubal et al 1989). Lesions may vary in size, with fusion of two or three lamellae up to involvement of some 50% of lamellae in an affected primary filament (Adams and Nowak 2004a). Gill lesions may be found at any location along the length of the primary lamellae. Although Roubal et al (1989) reported that the lesions of AGD do not appear to have a specific distribution on the gills, Adams and Nowak (2001) determined that lesion numbers and the severity of lesions were significantly higher in the dorsal region of the second left gill arch compared to the ventral region.

5.3.4 Mucous cells

The presence of markedly increased mucous cell numbers, representing mucous cell metaplasia or hyperplasia, is a commonly reported histological feature of the response to infection. Roubal et al (1989) described the frequent occurrence of large, actively secreting mucous cells on the surface of the hyperplastic epithelium, between the lamellae adjacent to the hyperplastic regions and deep within the hyperplastic epithelium. Cells showed conspicuous rough endoplasmic reticulum between the nucleus and secretory vesicle. Mucous cells opening into subsurface lacunae or cystic cavities formed by the hyperplastic epithelium were also described. Adams et al (2004) described mucous cells as extremely numerous and hypertrophic in close proximity to or within a lesion and within the deeper interfilamental regions, noting that hyperplastic epithelia heavily populated with mucous cells were rarely colonized by amoebae.

5.3.5 Chloride cells

An abundance of chloride cells in unaffected regions of gill is described (Roubal et al 1989), consistent with normal gill histology. Chloride cells were reported by Roubal et al (1989) to be rare, or reduced in number at the surface of the hyperplastic epithelium, although some were deeply buried within the hyperplastic epithelium. Adams and Nowak (2003) describe chloride cells being sloughed off the forming lesion along with a proteinaceous necrotic exudate containing amoebae, epithelial cells and leucocytes as the lesion matured.

Reference is made to chloride cells in decreased numbers associated with the lesions and image analysis of branchial mucous cells and chloride cells were reported by Adams and Nowak (2004a) to show no temporal differences between gills examined over the period of their study, ie, presumably between histologically normal gills and gills with developing AGD. Adams and Nowak (2003) also reported no significant differences in chloride cell numbers in early infections (up to approximately 2 weeks), however, these authors noted that a lower number of chloride cells was associated with larger hyperplastic lesions, presumably in later stages of infection.

5.3.6 Inflammatory cells

A range of inflammatory cells is recorded in association with gill lesions of AGD: Abundant neutrophil-like cells, identified by the presence of rod-shaped granules were described in the filament connective tissue, within vascular elements and within the hyperplastic epithelium by

Roubal et al (1989) and Adams and Nowak (2003). Adams and Nowak (2004a) described macrophages, neutrophils and eosinophilic granule cells within the venous sinus and within the hyperplastic epithelial tissue, with macrophages on the surface of the lesions. High numbers of eosinophilic granule cells were also associated with swollen vasculature in cases of AGD by Adams and Nowak (2003). In infections of longer duration, infiltrating cells are reported as mainly mononuclear in nature and described as macrophage-like cells and lymphocyte- like cells (Munday et al 1990). Adams and Nowak (2001) reported a localised inflammatory response associated with lesions with migration of neutrophils, macrophages and lymphocytes along the central venous sinus. These cells were reportedly adhering and migrating through the endothelia of the central venous sinus into hyperplastic tissues and in some cases eosinophilic granule cells were seen in close association with the connective tissues of the primary lamellae. Inflammatory cells, morphologically, identifed as neutrophils and macrophages were occasionally reported by Adams and Nowak (2001) infiltrating medium sized intra-epithelial cysts.

A reported feature of recovered cases is the presence of possible lymphoid nodules in the primary lamellae and in the basal interlamellae tissues (Roubal et al 1989). These reactive nodules were described as more conspicuous in rainbow trout compared with Atlantic salmon.

5.4 Cellular evidence of an immune response in the host

Adams and Nowak (2003) reported that "*A marked innate cellular immunological response was a secondary feature observed histopathologically during lesion formation and along the margin of larger lesions*" with *"Macrophages and neutrophils were the most abundant leucocytes identified throughout hyperplastic regions"* suggesting that this was a precursor to an acquired immune response. This could though also be a feature of a purely innate immune response to infection with AGD amoeba and gill tissue damage.

The cellular basis of resistance and immunity warrants comment. Although a leucocytic response to infection is well described, further, as noted above, it is not uncommon to visualise inflammatory cells within the stroma of the primary lamella in the gills of clinically healthy fish. No compelling morphological descriptions of lymphocytic responses induced by AGD within the gill lamellae can be identified. Although the presence of an acquired immune response to AGD cannot be excluded, as there is histological evidence of macrophages presumably phagocytising amoebic antigens, it is less than certain that such macrophages actually process antigen for subsequent presentation to immunocytes and mediating an acquired cell mediated or humoural immune response. It is possible that the inflammatory cell response to infection is just that, with sloughing of cells and no processing of antigen, or processing of antigen from non-amoeba sources. Reports of resistance in previously exposed fish may be due to age. It would seem appropriate to investigate antigen processing, the presence of specific antibody in gill mucous and possible age acquired resistance to infection as a basis for immunological research and attempts at vaccine development.

We do note the research reported in Wynne et al (2008) which showed a difference in expression of various genes (including some associated with the adaptive immune response) in

the gills of “resistant” and “susceptible” salnon as being potentially indicative of the ability, in the resistant fish, of enhanced antigen presentation and immunoglobulin production. We consider there to be a number of unresolved issues that may pose some uncertainty with this potential conclusion. Firstly, Wynne et al (2008) cites research reported in Vincent et al (2006) as demonstrating a functional relevance of anti-AGD amoebae antibodies to AGD resistance. In our opinion, Vincent et al (2006) did show variability in the production of anti-AGD amoebae antibodies between fish identified as “resistant” to infection, but did not show any evidence that the antibodies produced by 50% of the “resistant” fish were in any way responsible for that resistance.

In addition, Wynne et al (2008) admits that the “resistant” salmon and “susceptible”salmon had all survived three challenges with AGD amoebae so were all, in some way, “resistant” or “tolerant” to AGD. Also, both “resistant” and “susceptible” salmon showed up regulation of some of the adaptive immunity genes (e.g. the immune gene Ig and MH Ic), compared to naive fish, though at a variable rate. Though difficult, we think it may be of more interest to compare the gene profile of salmon that have survived three challengs of AGD with those that have succumbed to AGD on both the first and second challenge.

While we consider the research conducted by Wynne et al (2008) to provide evidence of a variability of gene expression in salmon categorised as varying in susceptibility to AGD, it does not, in our opinion, provide direct unequivocal evidence that it is actually the adaptive immune response that is influencing the ability of the salmon to survive challenge with AGD amoebae. The possibility that the expression of genes described is a non-specific response to gill protozoan parasitism and is not specific for AGD amoeba should be considered. Further studies that demonstrate processing of antigen of amoebic origin and a cell mediated or humoral immunological response against such antigen that affords demonstrable protection against amoebic colonisation of the gill surface would appear appropriate.

5.5 Regression of lesions

At the histological level regression of lesions appears to occur rapidly. Adams and Nowak (2004a) described an absence of histological lesions of AGD one week following freshwater baths of previously infected fish.

5.6 Biochemical abnormalities

Markedly elevated blood sodium levels are recorded in Atlantic salmon with clinical AGD, with lower, but still elevated levels in sub-clinically infected fish (Munday 1988, Munday et al 1990), possibly due to reductions in chloride cell numbers (Adams and Nowak 2003).

Powell et al (2001) cannulated AGD affected and unaffected salmon (assessed by scoring gills) and examined the differences in arterial oxygen and CO_2 tension between the two groups. They determined that AGD affected fish had persistent respiratory acidosis both under normoxic conditions and during hyoxia. This was not observed in unaffected fish. However, while they concluded that their work suggested that infection of salmon with AGD results in an impediment of gas transfer under normoxic conditions - AGD affected fish showing significantly lower arterial

partial pressures of oxygen, increased partial pressures of CO2 and reduced pH- they conceded that when challenged with hypoxia, physiological mechanisms such as increases in gill perfusion or blood flow redistribution within the gill were likely to compensate for any impediment in gas exchange, so allowing the fish to compensate. Their conclusion was that it seems unlikely that AGD related mortality can be attributed to acute respiratory failure or to asphyxiation or hypoxaemia.

It was interesting to note that no mention was made of the Bohr effect in this paper – the Bohr effect describes the property of haemoglobin such that in the presence of an increasing CO_2 concentration the affinity of haemoglobin for oxygen decreases. This is an important mechanism to assist in the delivery of oxygen to tissues where it is needed as indicated by the increase in CO2 levels due to metabolic function. The capacity for oxygen uptake at the gill epithelium/water interface may be impacted where there is a systemic respiratory acidosis. If no compensatory mechanisms are enacted, this may results in reduced oxygen delivery to tissues.

Considering at the severity of gill pathology induced by AGD amoeba, inescapably, the presence of gill dysfunction with respect to oxygen uptake, CO_2 excretion and osmoregulatory homeostasis cannot be disregarded. The authors could find no references whereby deaths due to AGD occurred in the absence of severe gill pathology.

Paradoxically, Powell et al (2002) state that AGD causes only minor respiratory disturbances to the fish, although substantial acid–base disturbances arise from a persistent respiratory acidosis. The same authors then describe a much higher 24 hr mortality in fish exposed to acute hypoxia compared a control group (21% survival c/f 90% survival). In contrast, Leef et al (2005a) failed to demonstrate short term (48hr) changes in blood PO2 in experimentally infected Atlantic salmon.

A short term (48hr) increase in aortic blood pH was reported by Leef et al (2005a) following experimental challenge in surgically catheterised fish when compared with pre-challenge levels: No difference was reported between control and challenged fish. In contrast, longer term (16days) exposure in smaller experimentally infected fish showed an increase in caudal blood pH compared to non-infected control fish, suggesting the inductiuon of a metabolic alkalosis.

5.7 Haematological changes

Short term (48hr) changes in blood parameters (haematocrit and haemoglobin) were also reported by Leef et al (2005a) with time in surgically catherterised and experimentally challenged Atlantic salmon, however, no differences were noted between controls and challenged fish, and MCHC remained constant, suggesting changes due to loss of blood at sampling.

We could not find reference to the possibility that the respiratory acidosis may cause a reduction in the affinitiy of haemoglobin for oxygen (Bohr effect) and whether such a mechanism may have caused a reduction in the ability of red blood cells to take fully load with oxygen when passing through the gills.

5.8 Cardiovascular dysfunction

Elevated systemic vascular resistance in Atlantic salmon experimentally infected with AGD, accompanied by significantly reduced cardiac output are reported, suggesting that cardiac dysfunction may be a mechanism by which AGD causes or contributes to mortality in Atlantic salmon (Leef et al 2005b): No differences were found in Rainbow trout or brown trout, however, possibly explaining why Atlantic salmon are more severely affected by AGD.

5.9 Experimental models of disease

Determining the process or processes involved in the pathogenesis of AGD, particularly the host-parasite relationship has proven difficult. An understanding of the mechanisms by which amoebae elicit disease underpins research aimed at providing industry with tools to more effectively manageor prevent AGD.

Critical to this research is having a solid and reliable challenge model. Unfortunately, a reliable model that has access to a ready, consistent supply of AGD amoebae (i.e. the infective strain) is not possible while there remains the inability to culture the infective strain. Improvement in isolation techniques (Morrison et al 2004) and identification of the infectious amoebae as being a distinct species (Young et al 2007) has facilitated refinement of the challenge model. However, even with these refinements Crosbie et al (2010) found inter-batch variation in the response of salmon to infectious dose of exactly the same concentration under their challenge model[8]. The authors of this paper cite a number of possible reasons behind this variation, including the potential variability in the cell division rate of amoebae after harvest or viability of the amoebae once removed from the gills. They do not consider the proportions of infectious amoebae between batches to have altered as has been discussed elsewhere in this review.

It would appear that the cell replication time from AGD amoeba has not been determined under different environmental conditions.

We note that Crosbie et al (2010) in their trial design used a watering can to distribute inocula across the surface of tanks. We assume that the watering can was plastic as a metal watering can may have caused potential toxicity to the amoebae. If, however, a plastic watering can was used, we assume a very specific protocol was developed which ensured uniformity in the time taken from when the inoculum was put into the watering can to when it was dispersed into the tanks and in agitation of that inocula once in the watering can. We assume this as variability in this process could potentially result in variability of the number of amoebae being delivered due to the adherence of amoebae to the plastic in the water can?

It would appear that the inability to culture infectious amoebae and hence the reliance on use of amoebae isolated from AGD infected salmon for clinical trials has seriously hampered research and will continue to hamper research until the ability to reliably culture an infectious form of the amoebae is identifed.

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[8] We do note the good summary table in Crosbie et al (2010) of experimental challenge methods for induction of AGD in Atlantic salmon which goes back to the work conducted by Howard et al (2003).

An urgent need exists for a virulent axenic cultured strain of AGD amoeba to underpin future experimental studies.

5.10 The role of AGD amoeba in the disease process

As noted, possibly the most frustrating aspect of AGD has been determining the process or processes involved in the development or pathogenesis of AGD, particularly the host-parasite relationship, and how infection with AGD amoebae ultimately results in the death of a high proportion of affected fish if left untreated. An understanding of the mechanisms by which AGD amoebae elicit disease underpins research aimed at providing industry with tools to more effectively manage or prevent AGD.

As noted in Section 4 pathology related to AGD is confined to the gills of affected fish with only a single report of changes in myocardial morphology based on examinations of hearts from Atlantic salmon of uncertain individual AGD status (Powell et al 2002). Although numerous researchers have described the pathology and histopathology of amoebic gill infections Munday 1988, Roubal et al 1989, Munday et al 1990, Adams and Nowak 2001, Munday et al 2001, Adams and Nowak 2003, Adams and Nowak 2004a, Harris et al 2004), less attention has been paid to the underlying mechanism that elicit the gill lesions and how these are associated with disease and death of the fish.

AGD amoeba are primary pathogens, i.e., are capable of inducing disease in healthy fish and do not require the fish to be stressed or otherwise compromised by intercurrent disease (Adams and Nowak 2004b). Following exposure to AGD amoeba, attachment to healthy epithelium occurs within 12 hours, with pseudopodia infiltrating the epithelial cells or cell junctions. The presence of AGD amoeba appears to stimulate epithelial hyperplasia and hypertrophy, resulting in early lamellar fusion within 24 hours and severe multifocal hyperplasia described above within one week of exposure. At 3-4 weeks, gill lesions may be severe with a high proportion of fish affected.

The response of the gill to amoebic infection is characterised by a marked increase in numbers (hyperplasia) and size (hypertrophy) of the epithelium lining the secondary gill lamellae, resulting in extensive thickening of the lining epithelium. Associated with this hyperplasia and hypertrophy is fusion of adjacent lamellae, the occurrence of cystic cavities within the thickened epithelium, increased numbers of mucous cells, decreased abundance of chloride cells in affected areas and an infiltration of inflammatory cells. AGD amoeba are invariably attached to the surface of the thickened epithelium or are entrapped between gill filaments. The normal histological structure of gill lamellae characterised by a thin, single layer of epithelium overlying the blood sinuses is largely obliterated due to the massive thickening of the gill epithelium: In advanced cases, approximately 50% of the gill lamella in a primary filament may be involved (Adams and Nowak 2004a) and lesions may be found at any location on the gill filament.

Gill-attached amoeba were described by Roubal et al (1989) as invasive with pseudopodia penetrating into and between epithelial cells, possibly reflecting a primary virulence determinant. Mechanical damage caused by the AGD amoebae was considered to be possibly

the primary cause of epithelial hyperplasia: it remains unknown if parasite excretory factors are involved.

It is noteworthy, that attachment of amoeba to experimentally abraded filaments was less than on healthy filaments (Adams and Nowak 2004b). Munday et al (2001) confirmed that the amoeba is capable of colonizing the normal gill epithelium, likely utilising a lectin/glycoconjugate bond, and indicating that the glycoconjugate is galactose or N-acetylgalactosamine.

Munday et al (2001) also suggest that the hyperplastic epithelium is more attractive to the amoeba, thus promoting a positive feedback whereby increasing numbers of amoeba are attracted to the epithelium, resulting in more increased hyperplasia. Severe gill lesions resulting from other causes including jellyfish damage, lamellar clubbing and necrotic gill syndrome are reported to be rapidly colonised by amoeba (Munday et al 2001).

The underlying pathophysiological cause of death remains enigmatic. Although it is claimed by Powell et al (2002) that mortalities in affected fish are not due to a respiratory cause and are due to compromised cardiac function, this statement is inconsistent with reports by the same authors of compromised respiratory function in fish with AGD under conditions of reduced oxygen. It is, however, feasible that the AGD amoeba itself induces an exuberant host response characterised by proliferation of gill epitheklium cells and it is this response itself that is the ultimate cause of death.

Fundamental information on the AGD amoeba is lacking, for example, its ability to produce toxins, specific adhesion mechanisms and the biochemical or pathophysiological basis whereby hyperplasia and hypertrophy of the lamellar epithelium are induced. The characterisation of such factors would provide valuable information on the pathogenesis of infection.

5.11 Intercurrent lesions

A range of histological lesions intercurrent with AGD lesions have been identified including haemorrhage, epithelial necrosis, telangiectiasis, lamellar epithelial hyperplasia and hypertrophy, resolving vascular thrombosis, shortening or “clubbing” of distal filaments and in more pronounced cases, filamental endothelial and epithelial hyperplasia resulting in significant distal filamental restructuring (Adams and Nowak 2004a). Adams et al (2004a) observed that some of these lesions were similar to those induced in AGD. It is difficult to exclude absolutely that these lesions may have been caused by AGD amoeba which were lost in processing.

Although the presence of intercurrent gill lesions was earlier suggested as a pre-disposing factor for the development of AGD by Nowak and Munday (1994), studies by Adams and Nowak (2004b) demonstrated that no predisposing gill lesions are necessary for infection.

5.12 Intercurrent infections

Zilberg and Munday (2000) found no evidence of intercurrent bacterial or parasitic infection of the gills of Atlantic salmon experimentally infected with AGD. The results of a study by Powell et al (2005) indicated that abrasion and gills and experimental *Tenacibaculum marinum* infections did not influence the severity of development of AGD in concurrently infected Atlantic salmon.

5.13 Discussion

We have been surprised, when reviewing the literature on the host response to infection, that histopathological comparisons between gills in cases of AGD and gills affected by other protozoan pathogens have received scant attention. Although the histological response of the gill in AGD is dramatic, at the histological level it is no more so than the response to a number of other protozoan infections including the common pathogens Amyloodinium, Cryptocaryon, Chilodonella and Ichthyobodo. One reference that does comment on this aspect is in Steinum et al (2008), who notes the similarity between the response of salmon to AGD and their response indeed to Ichthyobodo.

These other common pathogens may also induce profound epithelial hyperplasia and fusion of lamellae and at least in the case of Amyloodinium and Cryptocaryon, intraepithelial cyst development. A histological feature of AGD does, however, appear to be the intense mucous cell response. It remains unclear if this response is metaplastic, i.e., derived from lamellar epithelial cells, or whether it is due to proliferation of existent mucous cells. Nevertheless, the gill response should better be considered a response to irritation and as such is not unexpected in gill protozoan infections.

We do acknowledge the finding of Villavedra at al (2010) of differences at the cell surface of AGD amoeba that are not present in cultured amoebae and hope that this finding can assist in further elucidation of the pathogenesis, particularly the identification of virulence determinates on the AGD amoeba. Clarification of changes in chloride cell populations and their role in modulating serum electrolyte levels appear warranted.

The finding that only 21.4% of AGD affected fish survived stresses which included exposure to a 50% reduction in dissolved oxygen compared to a survival rate of 88.9% in non-affected fish (Fisk et al 2002) would tend to support the view that respiratory dysfunction, which is affected by the availability of oxygen, is important in the pathogenesis of AGD.

6 Treatments for AGD

6.1 The first report recommending freshwater bathing

On May 16th, 1988, Foster and Percival (1988b) published a paper titled "*Treatment of Paramoebic Gill Disease in Salmon and Trout*". This paper recommended a "*simple, full strength freshwater bath for 2-6 hours*" as the treatment for AGD which will result in "*an immediate recovery*" and would likely provide at least 4 months protection from AGD. The authors of this paper noted that the fact that this treatment provided at least 4 months protection and suggested that the fish had developed an "*immune defence*" against the AGD amoeba. The authors also noted that while on full salinity sites disease would develop within two months of smolt entry (hence usually in mid-December for smolt delivered mid-October), in brackish water sites disease may develop at any time between December and May depending on the freshwater influence, disease developing two months after the last major freshwater occurrence. They reported that in the laboratory a one hour bath in freshwater would kill AGD amoebae[9].

As far as the authors are aware, the industry quickly adopted the treatment recommended in Foster and Percival (1988b) and incorporated freshwater bathing of smolt as a standard practice in the production cycle. It remains as a standard treatment today, over 20 years later.

6.1.1 Soft and hard freshwater

We consider it important to mention here the findings of Roberts and Powell (2003), who showed that soft fresh water (19.3–37.4 mgL^{-1}.CaCO3) was more efficacious at alleviating AGD in affected fish than hard fresh water (173–236. mgL^{-1}.CaCO3) and that bathing fish in soft freshwater significantly reduced viable gill amoebae numbers and significantly alleviated gill pathology, both gross and histological.

The exact reason for this difference in efficacy is an area worth further investigation, in our opinion.

6.2 Research into other treatments

During the early 1990s, there was considerable research devoted to finding alternative treatments to freshwater baths. Much of this research was conducted and/or funded through the Research Division of Salmon Enterprises of Tasmania Pty Ltd (SALTAS) and published in the SALTAS Research and Development Review Seminars, held annually.

Key papers presented at the 1991 Review Seminar were those by Howard and Carson (1991a and 1991b). These authors continued to provide papers detailing the findings of their research at the 1992, 1993 and 1994 seminars; much of the findings of research conducted on AGD during this period was presented at these seminars.

Howard and Carson (1991) looked to optimize culture methods for AGD amoebae in order to better study the organisms and the effect various chemicals and treatments may have *in vitro*

[9] We are still to determine how

on it. The method they developed and routinely used to culture and maintain amoeba species used as the medium a sea water agar which was first autoclaved to ensure sterility. Antibacterials (10µg/ml of streptomycin, penicillin, kanamycin and novobiocin) and an antifungal (5µg/ml of amphotericin) were added to the molten agar and then the surface of the plates was covered with a thin layer of a Flexibacter species. Cultures were maintained at 20°C and subcultured every 2 to 4 weeks.

Using an isolate that was donated by the University of Queensland (UQ-1, a cultured organism originally considered the pathogen[10]), Howard and Carson (1991) tested 32 chemotherapeutants in vitro by testing the ability of the cultured amoebae to grow or survive in the presence of various concentrations of the drug. Ten of the drugs had an inhibitory effect, however as it is quite possible the paramoebae being tested was a cultured strain and not the "wild" strain how relevant the results of these tests are is difficult to determine. These authors also concluded that freshwater from five sources (dams, bores and rivers) did not kill the cultured Paramoebae after 4 hours. This is an unusual finding and worthy of further investigation. A summary of these trials is presented in Appendix A.

As noted in Chapter 3, Howard and Carson (1991) did determine that the growth of the amoebae isolate donated to them by the University of Queensland was not inhibited when the antibacterials penicillin, streptomycin, kanamycin and novobiocin and the fungicide amphotericin were added to the culture medium.

At this stage, identification of amoebae species identity was by confirming the presence of parasomes in the amoebae either using phase contrast microscopy on wet mounts fixed in 1% gluteraldehyde or on air dried smears, using fixatives and stains that dyed the nucleus and parasome of the Paramoeba dark purple. The specificity of molecular diagnostics was still a number of years away.

Alexander (1991) trialed a variety of drugs in the field by either in-feed medication (5 medicants trialed) or medicated seawater bath treatments (5 medicants, 3 detergents) using freshwater baths as a control. The antibiotic Romet 30 (consisting of ormetoprim and sulfadimethoxine was combined with trimethoprim (which is in the same class of antibiotics as ormetoprim) and used as the only antibiotic treatment. A summary of these treatments is provided in Appendix A.

No treatments were successful in reversing or stopping the progress of the disease. Alexander also determined that there was no beneficial or detrimental effects to prolonging bathing past 2-3 hours duration.

Further medicated feed and medicated bathing trials were conducted and the results presented in Cameron (1992). Levamisole was included in those compounds tested but there was no significance in the results obtained.

Cameron (1994a, 1994b and 1994c) details trials conducted using hydrogen peroxide. Previous research by Howard and Carson (1993) showed that hydrogen peroxide totally inactivated

[10] UQ-1 is noted in Howard and Carson 1993a as being *Platyamoeba plurinucleolus*

cultured Paramoeba at concentrations as low as 100ppm after 2 and 4 hours11. Cameron (1994a) then determined that solutions of 200ppm or greater of hydrogen peroxide were relatively stable over three hour period in saltwater at a water temperature of between 13° and 14°C. Solutions of 100ppm were reduced to 90ppm after 150 minutes. In general, the higher the temperature, the quicker was the breakdown of hydrogen peroxide.

Atlantic salmon smolt were found to be able to tolerate exposure to 100ppm hydrogen peroxide for up to three hours "*without serious consequences*" between 12 and 19 degrees (Cameron 1994b).

Unfortunately, bathing trials where hydrogen peroxide was added to a saltwater baths at various concentration did not control developing AGD in Atlantic salmon and Cameron (1994c) concluded that "hydrogen peroxide has little potential as a routine therapy for the control of AGD and is unlikely to replace freshwater bathing" in part due to the narrow margin of safety and temperature dependent toxicity of this chemical[12].

Cameron (1993) reported that the maximum salinity threshold to resolve AGD lesions was 4‰ when fish were bathed for 2.5 hours. Bathing in a salinity of 8‰ had little effect in reducing the average number of patches on gills. A comment made in this paper is that an important aspect of the bath is the dramatic change in salinity the amoebae is exposed to (i.e. from 35‰ to 0) in a very short time period. The author recommends the change needs to be abrupt to maximise the effect of the osmotic shock to the amoebae and the sloughing effect on mucus. A slow drop in salinity (e.g. by adding freshwater gradually to the saltwater in a liner containing fish) was considered not to be a valid treatment method.

Clark and Nowak (1999) reported that the addition of levamisole to the freshwater in the bath (used at concentrations of 2.5 - 5 mg/L was found to have an influence on prevalence of AGD lesions in fish post bathing, but no effect on prevalence of amoeba. The authors did admit though that replication was low and the power of analysis also low.

Zilberg et al (2000) showed an interesting response to the addition of levamisole to the freshwater in which fish were bathed. Levamisole at 5mg/l in freshwater bath was found to significantly reduce mortalities in 4 week period post bathing in (1) naïve fish given first bathe and (2) fish that were being given their second bath then cohabited with AGD affected fish, but no effect on fish that had been exposed to AGD, treated and then put in fresh water for 4 weeks. It is possible that the levamisole was providing an immunostimulatory effect to the naïve and single bathed fish which assisted in their ability to cope with AGD, with the effect not being required in the older fish.

Findlay et al (2000) complemented this work by showing that levamisole had significant effect on the number of gill patches that developed in groups of fish given either a freshwater bath or

[11] Howard and Carson (1993) also determined that ozone totally inactivated Paramoeba at concentrations above 0.1ppm with significant inhibition occurring at ozone concentrations between 0.02-0.08ppm.
[12] Hydrogen peroxide is currently available as a bath treatment of both freshwater and marine food production finfish under the APVMA issued Minor Use Permit 12169

a freshwater bath containing 1.25, 2.5 and 5mg/L of levamisole, again depending on the previous history of exposure to AGD of each group of fish. The authors summed up their thoughts on the effect of levamisole as follows:

1. Fish infected for the first time and given a 2 hour fresh water bath only will develop a moderate increase in their non-specific immunity and will have mucus and amoebae removed from their gills. However, the lesions of gill hyperplasia and inflammation will remain to attract amoebae (Nowak and Munday 1994) and some amoebae may survive within cystic lesions present in the gills (Munday etal. 1990).
2. Fish infected for the first time and given a 2 hour fresh water bath containing levamisole will experience a very much enhanced non-specific immune response and, therefore, there will more likely be resolution, rather than persistence, of infection and resultant lesions.
3. In fish that have been previously exposed on two occasions and given two industry-simulated baths lesions are still present, but the nonspecific immune response has been augmented to a sufficient level to allow recovery. In this instance levamisole provides only a temporary advantage of a slightly higher resistance to reinfection in the early weeks of exposure.
4. Fish that have been infected for the first time and allowed to recover in fresh water for 4 weeks have gills that are in excellent condition when re-exposed, so while their immune response may not remain at a high level, the condition of the gills compensates for this.

There have been a number of other treatments tested both in the laboratory and in the field. The full list of these is summarised in Appendix A

6.3 Conclusion

The fact that industry still relies on freshwater bathing as its main treatment of AGD is probably the best indication of the how successful research has been at finding other viable alternatives. We are certain that, given time and dedication, other treatments will be found.

Being able to identify a suitable compound and devise a treatment strategy whereby that compound is effective is greatly assisted by a sound understanding of the organism to be treated and the pathogenesis of the disease it causes. As has been highlighted in this review, there is still much that is not understood about AGD amoebae.

Gaining further understanding of this parasite will, in our opinion, be the most worthwhile mechanisms to assist in the identification of possible alternative treatments to freshwater, if such treatments exist. We do though hope that summarising all the treatments that have so far been researched (Appendix A) will greatly help in reducing research on products that may already have been investigated or provide valuable background information to assist in improving on previous work.

7 Vaccination and its potential to prevent AGD

7.1 Introduction

The immune system of fish is a system of biological structures and processes within the fish that protects it against disease by identifying and killing pathogens. Critically, this system needs to differentiate disease agents from itself. The system can be thought of as a series of layers with increasing specificity.

The first layer is the physical barriers e.g. the skin and gills of fish, which physically prevent easy entry of disease agents. The next layer of defence is the innate immune system which provides an immediate, but non-specific response both on the surface of the skin and gills (e.g. through products released in mucus) and under the surface (e.g. through the presence of cells capable of ingesting foreign matter). Fish have a well developed innate immune system.

The final layer is the adaptive, or acquired immune system, which is activated by the innate immune system. Acquired immunity relies on certain cells in the fish being "presented" with the disease agent. These cells then develop processes to improve the response to the disease agent should the fish be exposed to it again. The processes can either be in the development of antibodies against the disease ("humoral immunity") or the development of cells that can recognise and destroy disease agents far quicker if exposed again then when exposure occurred for the first time ("cell mediated immunity"). This latter process is known as cell-mediated immunity.

A successful vaccine relies on stimulating the acquired immune system by using a product that will not cause significant disease but will have attributes of the real disease. Vaccinating the fish with this product ensures that, should the fish be exposed to the real disease some time after being vaccinated, the fish will be able to respond to it much faster than if it had not been previously not been exposed. Hence to create a vaccine the fish must have the ability to develop an effective acquired immunity to the disease agent of concern, and be able to utilise that immunity if exposed to the disease agent.

The development of a vaccine for AGD has been a focus of research for nearly as long as the disease has been known. Attempts to show the presence of antibody levels in the serum and mucus of salmon (i.e. humoral response) have been numerous as there is no doubt that there would be great benefit to the industry if a vaccine were developed.

7.2 The early research

The first vaccine trial for AGD was conducted in conjunction with the University of Queensland in November, 1991 when 12 Atlantic salmon were given an intraperitonal injection of 150-300µl of a sonicated amoeba homogenate. According to Cameron (1992) though, there were no Paramoeba isolates included in the homogenate. The vaccine trial proved unsuccessful.

Akhlaghi et al (1994) then investigated the effect of injecting fish with amoeba antigen using both "wild" type amoeba ("AGD amoebae") and cultured amoeba. Both formalin killed and sonicated amoebae were injected. The cultured amoeaba used in this research was the isolate

PA-016. Collection of the "wild" amoeba was by scrubbing the mucus from the gills of 40 severely infected fish, homogenizing the suspension by adding normal saline, then filtering and inactivating with formalin.

These researchers also injected fish with antisera to amoeba (created by injecting sheep with amoeba antigen and harvesting the antiserum) and challenged fish with infection to determine if passive immunization using this method would provide any protection. While the sheep antibodies were found to be long lasting in the fish (up to 8 weeks) they did not protect the fish from disease when injected fish were cohabited with infected fish.

Akhlaghi et al (1994) did show a humoral response to amoeba which could be detected in 35% of salmon naturally infected with AGD and 48% of salmon infected after cohabitation with infected fish.

The critical aspect to this research is that researchers found no protective immunity conferred by any method used to immunize salmon. Passive immunity using sheep anti-amoeba antibodies did not confer protection and the injection of various forms of wild and cultured amoeba did not offer protection. The researchers did acknowledge that due to there being 100% mortality in in the cohabitation trial to determine whether there was protection resulted, it is possible any small effect of the immunization may have been masked.

Critically, Akhlaghi et al (1994) showed that the fish responded to the injection of antigen actively by producing antibodies which were quantifiable in the serum. However, this did not extend to the gill mucus, where no antibodies could be detected.

Howard and Carson (1994) also examined gill and serum antibody levels in Atlantic salmon naturally exposed to Paramoeba species. Using ELISA, they showed that there was a marked difference in humoral antibody reponse between salmon to injection of Paramoeba antigen, although all salmon that were injected had some response.

The level of anti-amoeba antibody in production fish varied depending on the group of fish. 100% of harvest fish had anti-amoeba antibodies in their serum, 50% of "pinheads" and 57% of smolt which had been in seawater for approximately 8 months and had remained unbathed.

The antibody levels in naturally infected fish were found to be considerably lower than those measured in fish injected with antigen.

Critically, none of the naturally infected or immunized fish produced detectable gill antibody levels[13].

[13] Antibody extraction was from frozen gills, which were first defrosted and then soaked in a cocktail of 0.85% saline, 2mM phenylmethylsulphonyl fluoride (PMSF), 2mM N-ethylmalemide (NEM), 10mM EDTA and 0.02% sodium azide for 2 hours at 4°C with shaking. The gill tissue was then removed and the mucus preparations centrifuged at 30,000g for 30 minutes at 4°C. The resulting supernatants were dialysed against distilled water containing PMSF and sodium azide. The dialysate was then centrifuged at 1000g for 15 mins to remove additional debris. The mucus preparations were lyophilised until use. Detection of antibodies was by ELISA after resuspending the preparations in 1ml PBS, with all samples being absorbed with *S. malophilia*.

Work conducted by Findlay et al (1995) initially exposed salmon to infection by cohabitation of naïve fish with infected fish. Over four weeks of cohabitation all fish had lesions and mortalities had occurred. Surviving fish were then allowed to recover by being returned to freshwater for four weeks. Interestingly, when the survivors were returned to saltwater and challenged again by cohabitation with infected fish, their response was different from naïve fish which had also been placed in the tank. Both previously exposed and infected fish and naïve fish developed patches after a week. In the majority of the previously infected salmon (85%), the average number of patches then declined. In the naïve fish being exposed for the first time, the average numbers of patches increased over the next few weeks.

The authors of this paper did conclude that their work suggested that surface antibodies on the gills of fish are not involved in natural immunity to AGD but did admit that due to limitations with detection methods for these antibodies further work in this area was warranted.

The work began and reported on in Findlay et al (1995) was continued and further results published in Findlay et al (1998). This was interesting work, as it showed differences in the response of salmon to re-infection depending on how they were treated. Salmon which were affected by AGD and given a standard two hour freshwater bath responded to re-infection in the same manner that they had responded as naïve fish. However, if the salmon that were infected were then placed in freshwater for a period of 4 weeks OR allowed to remain in saltwater and given a second freshwater bath after four weeks their response to subsequent challenge is moderated. It may be that the moderation of the response to AGD challenge in these fish may be not only due to the bathing impacting on infection but also a function of time which has allowed the fish to make changes that assist it in dealing with the amoebae challenge.

During the late 1990s, research was in part focusing on the potential for there to be resistance in fish to AGD. The finding of “resistance” to re-infection could indicate that there was an acquired immunity.

The research conducted by Clark and Nowak (1999) confirmed the fact that salmon being entered into a seawater environment developed AGD and continued to be affected by the disease throughout their time in seawater, despite being bathed in freshwater on up to three occasions. This would then suggest that salmon exposed to infection do not develop a resistance to re-infection under natural conditions even after multiple exposures.

7.3 Later research

There are a number of other researchers that considered the possibility of acquired immunity in salmon (and rainbow trout) to AGD (e.g. Zilberg and Munday 2001, Gross et al 2004, Morrison and Nowak 2005). We note the relatively frequent reference to the work of He (1997), which details the experimental recombinant vaccine against the ecto-parasitic ciliate *Ichthyopthirius multifiliis* which is based on a surface immobilization antigen. However, we also note that natural infection with *I. multifiliis* elicits a protective immunity in fish that survive infection, something that AGD does not.

A critical aspect of the vaccine research is the failure to identify AGD amoebae antibodies in the mucus of gills. This was not, it would seem, from want of looking. For example, Vincent et al (2006) found no IgM in any mucus in any salmon during their trials. Antibodies produced systemically by fish were reasonably frequently shown, but as one of the researchers concluded systemic antibodies do not provide protection against AGD. Villavedra et al (2010) demonstrated the presence of antibodies in skin mucus of fish vaccinated with the HMWA. Using ELISA, they identified antibodies 39 days post infection against the HMWA. However, it was only in three out of five fish that had survived challenge.

Other work conducted on the issue of immunity in Atlantic salmon to AGD included Bridle et al (2006), who demonstrated the up regulation of a specific immune response gene (Interleukin-1β (IL-1β)) in the gills of Atlantic salmon infected with AGD amoebae, and stressed the importance of the host response at the site of infection. This gene is involved in the inflammatory response which is part of the innate immune system.

7.4 The "protein" and "DNA" approach

Since 2004, research into the development of an AGD vaccine has been conducted along two broad approaches under essentially two camps, one being headed by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) and the other being headed by the University of Technology, Sydney (UTS). As far as we can determine, both camps have operated autonomously with no specific formal collaboration between the two camps. We understand that there has been a degree of informal and more ad hoc communication. Both have been operating under the Aquafin Cooperative Research Centre (CRC) and both have received funding through the CRC and the Fisheries Research and Development Coorporation (FRDC).

The two broad approaches are:

1. The "protein" approach (UTS), which aims to identify the specific AGD amoebae surface molecules (lectins) that mediate attachment to gill tissue. These molecules can then be used to induce an immune response at the gill surface by vaccination of fish with the specific peptide antigens identified. This strategy is based on the hypothesis that only wild type, infective paramoebae (what we have termed "AGD amoebae" in this review) contain the molecules for binding to host cells, whereas the non-infective cultured strains of paramoebae lack these attachments.
2. The "DNA" approach (CSIRO) which utilises molecular technology to create as complete a library as possible of the genes of AGD amoebae and by comparing the genes of the infectious "wild type" (or AGD amoebae) compared to the non-infective cultured strain determine which genes may be related to virulence. These genes could then be used either as a DNA type vaccine using a plasmid vector or as a sub unit vaccine.

Both approaches look for differences between the infective and non-infective strains of the amoebae and hope to find a difference from which a vaccine can be developed. Both approaches have, in their own way, had some success as both have run vaccine trials utilizing the High Molecular Weight Antigen (HMWA) fraction (UTS) and sub-libraries of potential

antigenic DNA molecules (CSIRO). In the UTS trials, while the HMWA elicited significant serum antibody production, it failed, like many vaccine trials before it, to elicit a specific antibody response at the gills. Indeed, as noted in Villavedra (2010):

"Preliminary results indicate that immunisation of salmon with HMWA does not lead to protection against challenge infection; rather it may even have an immunosuppressive effect".

Possibly the most significant finding under this approach was that the best protection given to the fish in any of the trials conducted was when Freuds Complete Adjuvant (FCA) was injected alone. FCA consists of a dried mycobacterium derivative and mineral oil and is considered highly antigenic. It provides significant stimulation of the innate immune response. It is quite therefore likely that injection of this adjuvant was providing subsequent protection not through an adaptive immunity but through a boosting of the innate immune system of the fish.

In the CSIRO approach, library fraction #2, consisting of approximately 284 clones, showed a significant improvement in relative percent survival in an initial trial (25% improvement over control). However, it would seem that luck was not with the CSIRO approach as subsequent vaccine trials suffered a series of setbacks and the initial finding was not, as far as the authors can determine, repeated.

Through the research conducted, both approaches have delivered some interesting findings with regards tothe differences between the AGD amoebae (infective strains) and non-infective strains. This includes identifying differences in both surface coat structure and gene expression.

However, neither approach has convincingly showed that Atlantic salmon have the ability to produce specific antibodies to AGD amoebae that provide a commercially feasible level of protection. Neither has either approach clearly identified the nature of the difference between infective and non-infective strains and exactly how it leads to virulence. This is unfortunate, given the effort obviously put into both approaches by the researchers involved.

The protein approach has provided information which tends to confirm what many researchers have been saying throughout and that is that it is the innate, or non-specific immune system that likely plays a key role in protection against AGD. The DNA (genomic) approach has shown a slight improvement in relative percent survival but when using a sub-library consisting of hundreds of clones. Given that virulence phenotype may not simply be due to a single gene but many, determining the specific suite of genes that may be involved in virulence could prove to be a very big task, given the possible number of permutations and combinations that would need to be examined.

7.5 Conclusion

As far as we can ascertain populations of Atlantic salmon being farmed in marine sites in south east Tasmania do not develop immunity to AGD during the course of a production cycle. Hence in the field it appears that natural infection with AGD amoebae does not lead to immunity.

Immunity does develop under field conditions to other parasitic infections, for example *Ichthyopthirius multifiliis*. We do note that even with this disease where natural infection leads to immunity there is still no commercially available vaccine.

Hence to develop a vaccine against AGD amoebae is essentially trying to “improve upon nature”. This is a very difficult task, particularly when there are fundamental gaps in the understanding of AGD, as has been highlighted in this review.

These gaps mean that the research on development of a vaccine has been based in part on assumptions, including the key assumption that fish do indeed have the ability to develop an adaptive immune response to AGD that is protective. Another assumption that underlies current research is that a key step in the development of disease is the ability of AGD amoebae to attach to the gill epithelium. This would seem a reasonable assumption, but as far as we are aware this has not been proven. A further assumption is that by developing an antibody that in some way can block this attachment fish will be protected. Again a reasonable assumption, but we are now starting to build assumption on assumption.

This is not to say that there are some important findings coming out of the work on vaccine development. A worthwhile area that recent research has concentrated on is identifying differences in both gene transcripts (which may themselves code for virulence determinants) and surface glycoproteins between infective (“AGD amoebae”) and non-infective strains of amoebae. We fully support this area of research but caution that the findings may not necessarily identify a potential vaccine but may provide more insight into the nature of the disease and how infective amoebae are able to elicit disease.

In essence however, we consider that the significant gaps in our fundamental understanding of AGD and how amoebae cause disease are limiting the effectiveness of AGD vaccine research.

A greater understanding of the fundamental aspects of AGD would assist in being able to better understand whether vaccination of fish is a viable possibility to protect against AGD. It would also greatly assist in better defining what research is needed to develop such a vaccine, should it be considered viable.

Some of the current work being conducted by researchers working on the development of an AGD vaccine may assist in the understanding of AGD. If so, we would support its continuation. If not, we would suggest that research to better understand some of the fundamental aspects of AGD as discussed elsewhere in this review, take priority at least for the short and medium term.

Gaining this understanding may greatly assist in the development of an AGD vaccine in the long term.

8 Breeding for resistance to AGD

Papers describing research to investigate the potential and feasibility of selecting and breeding Atlantic salmon for resistance to AGD have been published in recent years (Taylor et al 2007,2009, 2010; Dominik et al 2008, Wynne et al 2008) focussing on the identification of phenotypic traits as potential markers of genetic resistance.

In 2007, the Tasmanian Atlantic Salmon Breeding Program (TASBP) commenced.

The potential for genetic selection from resistance to AGD was demonstrated by Wynne et al (2008) through the identification of multiple genes involved in immune and cell cycle reponses at the level of the gill. Resistance to AGD in individual fish was associated with a higher expression of genes involving adaptive immunity and negative regulation of the cell cycle compared with higher expression of acute phase proteins and positive regulation of the cell cycle in susceptible individuals. These observations support the concept that disease may be related to an exuberant host response and that fish that do not exhibit such a response, although infected by amoeba, may not develop such severe disease[14].

The genetic basis of the systemic antibody response to AGD was assessed by Taylor et al (2010). Although these authors demonstrated an antibody response in fish surviving amoebic challenge, and although the proportion of seropositive fish increased with multiple challenges, there was no evidence that antibodies provided protection against AGD. . As such, selection of fish for resistance to AGD on the basis of systemic antibody appears invalid. The findings suggest that resistance to AGD (or perhaps more correctly the ability to cope with infection by AGD amoebae) may be genetic in nature, but unlikely to be related to systemic immune function.

Dominik et al (2009) proposed a whole genome approach for selection of resistance to AGD, in Atlantic salmon, noting substantial potential gains compared to traditional genetic improvement programs.

Using gross gill score, histopathology and gill image scores as potential markers for resistance to AGD in Atlantic salmon affected by AGD under field conditions, Taylor et al (2009) suggested that gill score may be used as a non-destructive selection trait, providing scope for selective breeding.

The use of gill score as discussed by Taylor et al (2009) is essentially what has happened in the TABP where gross gill score is used as the key determinant for assessing “resistance” to AGD in family cohorts. While it may be considered an indirect mechanism to measure the response to infection with AGD amoebae, its use as a marker would appear sound as it is gill score that is used by industry to determine when fresh water bathing is necessary. It is also reasonable that increasing gill score in fish is correlated with decreasing survival.

Selecting for fish that have maintain a lower gill score in the face of challenge should ultimately mean that the length of time between bathes will increase and survival should increase, other factors being equal.

[14] The response of wild non salmonid fish to challenge by amoebae is an area worthy of further research

8.1 Discussion

The identification and selection of fish with resistance to disease would appear to be best implemented through a prospective programs selecting for survival or resistance in experimental or natural challenge, subsequent breeding from these individuals and an assessment of the resistance of the progeny to the same disease.

From our understanding this is what is happening in the TABP and we support this approach.

It has been interesting to note that the ability to cope with the first exposure to AGD amoebae and infection is different (and has different genetic traits) to the ability to cope with subsequent exposure. The potential role of gill pathophysiology influencing AGD susceptibility of smolts to their initial exposure to AGD amoebae when first acclimated to sea-water is still an area warranting further investigation given this difference.

Based on the research undertaken to date, we conclude that selective breeding affords major potential for the control of AGD. We also consider the use of such traits as survival and gill scores and whole genome approaches for genetic selection provides a reasonable and logical basis for selection.

We note, however, the possibility that an exuberant host response to infection may be the primary cause of disease and that modification of the host response to infection with AGD amoeba might be the most important trait to capture genetically.

While we are well aware of the need for strict biosecurity between hatcheries and sea sites to minimise the transmission of other diseases between freshwater and saltwater, we are of the understanding that the ability to breed directly from fish that have been transferred to saltwater and exhibited an ability to cope with AGD can signficiantly increase the speed of improvement in this trait. Currently, this does not occur. We would therefore suggest that a mechanism that would minimise any risk of transfer of disease but that could use the fish sent to sea as breeders should be investigated as a means of increasing the rate of improvement year to year.

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Appendix A – A summary of treatments trialed on AGD

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Reference	Species	Treatment	Fish used	Method	Result
Alexander J.M (1990-91) Note: Natural infection by amoeba progressed throughout the experiment in two waves	Atlantic salmon	**Various treatments were examined in this research as summarised below:** Medicated feed: Exp 1 5 medications given in-feed: Romet 30/Trimethoprim (50/22mg/kg/fish/day) Albendazole (10 & 50mg/kg/fish/day) Amproloim (50mg/kg/fish/day) Quinacrine dihydrochloride (50mg/kg/fish/day) Above were coated onto feed pellets with 5% gelatin solution = increased moisture content by 6.5% - feeds made up every 3-4 days Fumagillin (0.1% and 0.3% in feed) made up daily by combining feed with drug dissolved in methylated spirits Medicated and detergent seawater baths: Exp 2 Mebendazole (1ppm) $CuSO_4$ in citric acid (2ppm) Toltrazuril (10ppm) Mal green/formalin (0.1/50ppm and 0.2/100ppm) Quinacrine (30ppm) LWA (10ppm) Tween 20 (100ppm) Alkadet (25ppm) 100 fish per treatment per replicate 1st bath treatment: 15.5°C temp – medicated bath for 4 hrs then returned to net 2nd bath treatment: 16-20°C for 3 hrs (groups of 20 fish – had been previously treated with Rom/TMP then used for controls in Exp 1) Evaluated for amoeba 1 and 3 weeks post bathing Fresh water bath duration: Exp 3 Six groups of 21 fish (medium stage amoeba) bathed in oxygenated fresh water for 0, 0.5, 1, 2, 3 and 4hrs in 100L bins (density = 42kg/m^3) – This experiment was done 3 times in total A large sample of fish examined 1 and 4 weeks post bathing Fresh water bath at increasing levels of infection: Exp 4 Only carried out once Fish used were previously treated with Rom/TMP Five groups of 80 fish bathed in 4 x 100L bins (density = 40kg/m^3) of oxygenated water for 3 hrs 20 fish evaluated for amoeba (gill patches)	65.4g 10 groups of 500 fish	White patches per gill were counted: Low = 0-3 patches/fish Mod = 3-5 Mod high = 5-7 High = 7-10 Very high = 10-15 Heavy = >15 Maximum number of patches able to be counted was 15 Quantitative evaluation of amoeba infection initially monitored by a gill washing technique using ammonium chloride developed by Jones (1977)	Medicated feed: Exp 1 Medications not successful in reversing progress of AGD Gross pathology used to determine progress of disease 1st trial: Before medication (28.11.90): all fish showed little evidence of AGD, by 6.12.90 approx 20% of all fish had gill patches (i.e med and control groups). By 17.12.90 this increased to 50% in each case. All medications were stopped on 20.12.90 (3 weeks after medication). Fish were freshwater bathed 28th and 31.12.90 Fumagillin: given 14.12.90, fish had mod level of infection. After 3 weeks of medication, infection had not abated, med was increased 3 fold for 1 week with no change. 2nd trial: 3 weeks of medication, Quinacrine fish were infected with amoeba but at a lower rate cf controls but further testing shows it did not slow the progress of infection Rom/TMP did not slow or reverse progress of infection Mortality due to medication was not apparent and growth rates were effect most likely due to palatability. Medicated and detergent seawater baths: Exp 2 Amoeba infection: evaluated (patches per gill) 1 and 3 weeks after baths Fresh water bathed fish recovered from infection, whereas other treatments the disease progressed. Gills examined under the microscope confirmed presence of amoeba in medicated seawater baths but not fresh water baths. Histo of gills showed fresh water baths were least after 1 and 3 weeks. Detergent baths did not show significant changes in gill pathology (histology immediately post bathing). Toltrazurill showed prominent and numerous chloride cells and Tween 20 showed some swelling of chloride cells, disturbance of capillary blood flow and osmotic balance. Alkadet showed severe disturbance of capillary blood flow and epithelial cell swelling. Little changes were seen with LWA. Fresh water bathing duration: Exp 3 Minimum safe bathing time is 2-3hrs, after this there is no beneficial effect. No significant damage to gill epithelial tissue after 3-4hrs bathing however some damage to inflammatory cells after this time. Fresh water bath increasing levels of infection: Exp 4 Shows a decrease in Amoeba levels

Reference	Species	Treatment	# fish used	Method	Result
Howard T. & Carson J. (1991) Note: Amoeba used in these trials was a cultured amoeba hence unlikely to be the AGD amoeba		**Various treatments including a large number of anti-amoebic/malarial/coccidials were examined in this research as summarised below:** Anti-amoebic drugs: *Emetine dihydrochloride* *Berberine* *Fumagillan* *8-hydroxyquinoline, 8-hydroxyquinoline (copper salt), 5-chloro-7-iodo-8-hydroxyquinoline Chloroquine diphosphate* *Niridazole* *Metronidazole, Tinidazole* Anti-malarials: *Pyrimethamine* *Sulfadioxine* and *Pyrimethamine* *Quinine sulphate* *Mefloquine hydrochloride* *Primaquine diphosphate* Anti-coccidials: *Quinoline* *Monensin, Narasin* *Juglone* *Toltrazuril* *Phthalylsulphathiazole, sulphaquinoxaline* *Amprolium* *Quinacrine hydrochloride* Anthelminthics: *Praziquantel* *Pyrantel (citrate salt)* *Mebendazole* *Levamisole* Miscellaneous: *Nitrothiazole* DP483 *Carnidazole*	No fish used – *Paramoeba* were tested for their ability to grow or survive in the presence of the drugs for a period of 7 days	All drugs were solubilised using appropriate solvents. Solvents used were: Water, ethanol, sodium hydroxide, acetone, dimethyl formamide Fumagillan and mebendazole were used in suspension Qualitative tests: Drugs were incorporated into agar at 5 concentrations 5µg, 10µg, 15µg, 20µg and 30µg per ml A suspension containing approx 5000 *Paramoeba* per ml was serially diluted 1:2, 1:4, 1:16 to 1:64. For each dilution, 0.1ml aliquots were added to each drug concentration. Negative controls were run as well as controls containing the drug solvents to ensure an inhibition was from drug and not solvent.	*Growth studies:* *Paramoeba* isolate grew at 10°C, but was enhanced at 20°C, viability was lost at 25°C *Paramoeba* grew equally well at both 33ppt and 16.5ppt salinity. No effect on *Paramoeba* growth was observed when the following antibacterials and fungicide were incorporated into the medium: 10 and 100µg/ml of penicillin, streptomycin, kanamycin, novobiocin and 5µg/ml amphotericin Drug trials: *Qualitative tests:* 10 had an inhibitory effect on *Paramoeba* isolate and 2 drugs are yet to be tested. Following drugs had an inhibitory effect: 8-hydroxyquinoline, 8-hydroxyquinoline (copper salt), 5-chloro-7-iodo-8-hydroxyquinoline, Pyrimethamine, Quinoline, Narasin, Juglone, Quinacrine hydrochloride, Levamisole and DP483. *Quantitative tests:* Preliminary test carried out using quinacrine at 15, 30 and 50µg/ml, sampling *Paramoeba* at 1, 2, 3 and 4hrs. Quinacrine showed an estimated >90% kill rate at 15µg at 4 hrs, 30µg at 3hrs and 50µg at 1hr. Results of the quantitative tests for the remaining drugs have not yet been finalized.

Reference	Species	Treatment	# fish used	Method	Result
Howard T. & Carson J. (1991) (cont.) Note: Amoeba used in these trials was a cultured amoeba hence unlikely to be the AGD amoeba		Anti-amoebic drugs (cont)	No fish used – *Paramoeba* were tested for their ability to grow or survive in the presence of the drugs for a period of 7 days	lates incubated for 7 days at 20°C and scored according to amount of growth: 4+ = ≥ 400 *Paramoeba*, 3+ = 200 - 400 2+ = 100 - 200 1+ = 20 – 100 +/- = < 20 Inhibition was indicated by direct comparison of the neg control score and the drug score. Plates incubated for a further 7 days and any futher changes noted Quantitative tests: Drugs that showed some inhibition of *Paramoeba* growth were tested using the MPN method of estimating cell numbers: Approx 100,000 amoeba were suspended in 10mls of sterile sea water containing 5µg, 15µg and 30µg per ml of dissolved drug Samples of 2mls were taken at 3 time intervals (0, 4 and 8hrs). All samples were washed and centrifuged twice to eliminate the drug, then diluted and inoculated onto "Repli dishes" for counting by the MPN method. Plates were inoculated at 20°C for 7 days	Actual drug results: Inhibition: *Emetine dihydrochloride* not at ≤30µg/ml *Berberine hydrochloride* not at ≤30µg/ml *Fumagillan* not at ≤30µg/ml *8-hydroxyquinolin* ≥ 10µg/ml *8-hydroxyquinoline (copper salt)* ≥ 15µg/ml *5-chloro-7-iodo-8-hydroxyquinoline* ≥ 10µg/ml *Chloroquine diphosphate* not at ≤30µg/ml *Niridazole* not at ≤30µg/ml *Metronidazol* not at ≤30µg/ml *Tinidazole* not at ≤30µg/ml *Pyrimethamine* ≥ 5µg/ml *Sulfadioxine and Pyrimethamine* not at ≤30µg/ml *Quinine sulphate* not at ≤30µg/ml *Mefloquine hydrochloride* not completed *Primaquine diphosphate* not at ≤30µg/ml *Quinoline* ≥ 20µg/ml *Monensin* not at ≤30µg/ml *Narasin* ≥ 20µg/ml *Juglone (5-hydroxy-1,4, napthoquinone)* ≥ 5µg/ml *Toltrazuril* not at ≤30µg/ml *Phthalylsulphathiazole* not at ≤30µg/ml *Sulphaquinoxaline* not completed *Amprolium* not at ≤30µg/ml *Quinacrine hydrochloride* ≥ 10µg/ml *Praziquantel* not at ≤30µg/ml *Pyrantel (citrate salt)* not at ≤30µg/ml *Mebendazole* not at ≤30µg/ml *Levamisole* ≥ 5µg/ml *Carnidazole* not at ≤30µg/ml *Nitrothiazole* not at ≤30µg/ml DP483 ≥ 60µg/ml Controls: Ethanol no inhibition detected NAOH no inhibition detected Acetone no inhibition detected Dimethyl formamide no inhibition detected

Reference	Species	Treatment	# fish used	Method	Result
Howard T. & Carson J. (1991) (cont.) Note: Amoeba used in these trials was a cultured amoeba hence unlikely to be the AGD amoeba		**The effects of freshwater on amoebae were examined in this trial.** Cells were suspended in 10ml of freshwater type and sampled at 2 and 4hrs. Samples were washed and centrifuged once.	No fish used – *Paramoeba* were tested for their ability to grow or survive in the presence of freshwater		Fresh water bathing: No changes were observed in *Paramoeba* survival and viability between the 3 fresh water samples and sea water over a 2 and 4hr bathing period. Further work was being conducted
Cameron D.E. (1992)	Atlantic salmon	**Various treatments were examined in this research as summarised below:** 3 sets of medicated feed trials with 4 chemicals: Juglone, Narasin, Levamisole, 8-Hydroxyquinoline Feed requirements based on feed rate of 2.5% Levamisole and Narasin were applied to the feed in 5% gelatin solution 8-Hydroxyquinoline and Juglone were applied in an ethanolic solution Bathing trials: Fresh and seawater (20 fish per treatment – 5 treatments per trial). Level of infection assessed 1 & 2 weeks post bathing Medicated baths: Juglone 15ppm, Narasin 30ppm, Levamisole 25ppm, 8-Hydroxyquinoline 15ppm Bathing for 2.5hrs Detergent baths: Anionic detergentsL Sodium dodecyl sulphate (20ppm) and Sodium choleate (80ppm) in seawater for 2.5hrs Other bath treatments: Re-evaluation of min time for freshwater bathing Maximum salinity of bathing before effectiveness is reduced Effect of rapid transfer to freshwater cf gradual decrease in salinity	Five groups of 265 fish at average weight of 104g 5mx5m cages	Degree of infection assessed by counting white patches on all gills on both sides described by Alexander (1991)	Medicated feed: 1st trial inconclusive data (level of infection insufficient) 2nd trial (15 days): Only Narasin had any effect in reduction of infection cf control group. Lev was not significantly different cf control. Jug and 8-Hydroxy were both more heavily infected cf controls at end of trial 3rd trial (15 days): Narasin reduced level of infection but did not clear gills completely of amoeba patches. (palatability problems with Narasin feed for 3rd trial – 53mg/kg/fish/day instead of 60mg) Bathing trials: Not completed at time of writing so no data available

Reference	Species	Treatment	# fish used	Method	Result
Cameron D.E. (1993)	Atlantic salmon	**Varying the salinity (maximum salinity of 4%) in which fish were bathed was examined in this research**	Smolts 10-15 fish per group	Bathing for 2.5hrs in 2.2-4.1% salinity Gill samples in Davidsons fixative made with seawater, once fixed samples were transferred to 70% ethanol	Established AGD infections were completely cleared in water with salinity of 2.2% and almost completely cleared at 4.1% Moderately brackish water (8-13%) was ineffective in clearing AGD Histology showed progressive development of nodules and plaques on the secondary lamellae, which appeared to be preferentially colonized by amoebae
Howard T. & Carson J. (1993) Note: PA-016 non-infective amoeba	*Paramoeba* taken from infected Atlantic salmon	**Hydrogen peroxide:** Hydrogen peroxide added to sea water (100ppm, 250ppm, 500ppm, 1000ppm and 1500ppm) pH lowered to 6 (using hydrochloric acid and acetic acid) Freshwater bathing (sterilized reagent water <2µSm)	Approx 150,000 *Paramoeba*	*Paramoeba* isolate (PA-016) taken from infected atlantic salmon MPN method used	Hydrogen peroxide totally inactivated *Paramoeba* at concentrations as low as 100ppm at 2 and 4hrs exposure Lowering the pH to 6 for 4 hrs had little effect on viability cf controls Freshwater bathing totally inactivated *Paramoeba* after 2hrs of exposure (repeated and reproducible)
Cameron D.E. (1994) Note: Fish were low-mod infected at time of research	Atlantic salmon	**Hydrogen peroxide:** 100ppm (3hrs) @ 15°C 200ppm (1, 2 & 3hrs) @ 17°C 300ppm (30min, 1 & 2hrs) @ 15°C	Post-smolt (480g) 15-25 fish per group	750L with fish density of $50kgm^{-3}$ Lovibond method Groups examined at day 6 and 12 post treatment Gill samples fixed in neutral buffered formalin for processing	Hydrogen peroxide did not eliminate or reduce AGD infection levels in treated fish at any concentration or exposure times No mortality for 100ppm Mortalities for 200ppm: 1hr = 21%, 2hrs = 47% and 3hrs = 76% morts Mortalities of 300ppm were 50% for those bathed for 2hrs, none for the 30min or 1hr groups

Reference	Species	Treatment	# fish used	Method	Result
Findlay V.L. & Munday B.L. (1998)	Atlantic salmon	**Effects of freshwater bathing frequency and intervals were examined with respect to immunity/resistance in this research:** Trial one: 40 naïve fish were placed in seawater for 4 weeks with 15 infected fish 20 fish were placed in fresh water for 4 weeks 20 fish were placed in fresh water bath for 2-3 hrs then returned to seawater 20 new naïve fish were added in with fish to seawater and exposed The 40 fish in seawater were then placed in a fresh water bath for 2-3 hours then returned to seawater At the end of the 4 weeks the 20 fish in freshwater were returned with the 40 fish in seawater along with 20 new naïve fish for a further 4 weeks Trial two: Four groups of fish which had been infected for differeing periods of time and treated for AGD in different ways. Into each replica tank, 15 naïve fish, 15 fish that have been infected once and given a 2hr bath, 15 fish that had been infected twice and given a 2hr bath at the end of each 4 week period and 15 fish that had been infected once and kept in fresh water for 4 weeks.	100-200g 40 smolts All fish had not been exposed to AGD and therefore naïve. Temp 14°C All fish were infected with *Paramoeba* sp. by horizontal transmission after mixing with AGD-infected fish for 4 weeks.	Trial one: At the end of first 4 weeks, the naïve smolts had severe gill lesions and large numbers of *Paramoeba* after running with infected post-smolts (only time in the experiment that infected fish were run with naïve fish). Weekly monitoring by counting number of lesions on the first gill arch of both sides using the method described by Alexander (1991). Trial two: No infected donor fish were added for this trial. For 4 weeks, weekly checks for severity of infection was recorded by counting number of lesions on both side of the first gill arch.	Trial one: Fish treated with 2hr fresh water bath displayed little more resistance to re-infection than previously naïve (unexposed) fish. There was no significant difference between the previously exposed groups and the naïve groups for the first 3 weeks. However, by week 4 the previously exposed fish displayed significant fewer lesions than the naïve fish. Fish given a 4 week fresh water bath only a small number ever displayed lesions, and even than it was at low levels. Trial two: The group of fish that had experienced two waves of infection and had been treated with a 2hr fresh water bath displayed only a moderate level of disease 1 week after challenge. In summary it appears that fish given one fresh water bath for 2-3 hrs do not show the same level of resistance to fish given two fresh water baths for 2-3 hrs each time. After 3 and 4 weeks after treatment there was no difference between 2-3 hr fresh water baths and those fish which remained in fresh water for 4 weeks.
Findlay V.L. et al (2001)	Atlantic salmon	**The use of levamisole was examined in this research** Trial one groups: Lev dose = 5mg/L 1. Previously exposed (once) + Lev + 2-3hr fresh water bath 2. Previously exposed (once) + 2-3hr fresh water bath 3. Naïve + Lev + 2-3hr fresh water bath 4. Naïve + 2-3hr fresh water bath	100-200g Temp 14°C 14-20 fish/tank 3 trials Trial one: 18 fish/group	Monitoring infection: Counting number of mucoid patches on the first gill arch of both left and right sides using the method described by Alexander (1991)	Trial one: 5mg/L dose Number of lesions due to AGD were significantly reduced among levamisole-treated salmon experiencing their 2nd wave of infection cf fish treated with fresh water only. Most apparent at 3 and 4 weeks post exposure. Naïve fish treated with lev experiencing their first wave of infection were variably significantly lower cf those fish treated with fresh water only.

Reference	Species	Treatment	# fish used	Method	Result
Findlay V.L. et al (2001) (cont.)	Atlantic salmon	**The use of levamisole was examined in this research** Trial two groups: Lev dose = 5mg/L 1. Previously exposed + 4 weeks in fresh water before re-exposure + Lev + 2-3hr fresh water bath 2. Previously exposed + 4 weeks in fresh water before re-exposure + 2-3hr fresh water bath 3. Previously exposed (twice) + Lev + 2-3hr fresh water bath 4. Previously exposed (twice) + 2-3hr fresh water bath 5. Previously exposed (once) + Lev + 2-3hr fresh water bath 6. Previously exposed (once) + 2-3hr fresh water bath 7. Naïve + Lev + 2-3hr fresh water bath 8. Naïve + 2-3hr fresh water bath Trial three groups: 1. Previously exposed (once) + Lev (5mg/L) + 2-3hr fresh water bath 2. Previously exposed (once) + Lev (2.5mg/L) + 2-3hr fresh water bath 3. Previously exposed (once) + Lev (1.25mg/L) + 2-3hr fresh water bath 4. Previously exposed (once) + 2-3hr fresh water bath 5. Naïve + 2-3hr fresh water bath	Trial two: 15 fish/group Trial three: 14-20 fish/group		Trial two: 5mg/L dose Continual passage of AGD through susceptible fish raised the virulence of *Paramoeba* sp. The pattern of infection remained similar to trial one, the magnitude of infection was greater which affected the outcome cf trial one. (Mortalities in naïve fish and fish exposed to AGD once (no lev treatment) were prematurely withdrawn from the trial by 4 weeks post exposure). Levamisole treatment appeared to help naïve fish and those exposed to one wave of infection and immediately returned to sea water, by significantly reducing the number of gill lesions at 2-4 weeks post exposure. Levamisole significantly reduced number of patches in fish exposed to two waves of infection at 1-2 weeks post exposure, but not later in the trial. Treatment did not affect the outcome for fish that had been returned to fresh water for 4 weeks after initial infection Trial three: Groups of fish were treated with different concentrations of levamisole (1.25, 2.5 and 5ppm). There were no significant differences between lesion numbers for any of the groups treated with levamisole. The groups given fresh water bath only had significantly more lesions at weeks 3 and 4 post exposure. Fish previously exposed once and treated with fresh water only had fewer lesions cf naïve fish on weeks 3 and 4. Result: While there were significant decreases in lesion numbers in most of the groups treated with levamisole, the fish that benefited were those fish experiencing their 2nd wave of infection (had been previously been infected and given a fresh water bath before being re-exposed).

Reference	Species	Treatment	# fish used	Method	Result
Parsons H et al (2001)	Atlantic salmon	**The ffect of fresh water bathing on gross lesions and AGD amoebae was examined in this research** Three fresh water baths investigated. Bathed for approx 3hrs Temp 20-22.6°C pH 8.3 before pH 6.7 after Salinity 0 ppt Weight: 280-800g		Samples collected from three groups before and after bathing. Howard and Carson (1993) method for sampling. Samples fixed in seawater Davidson's fixative.	This study showed that mucoid patches associated with AGD was higher before fresh water bathing cf after bathing. Before fresh water bathing less than 10% of fish sampled were clear of AGD patches. After bathing 45% of fish sampled showed no signs of AGD patches. A greater percentage of alive paramoebae were present before (86%) than after (27%) fresh water bathing. This study demonstrated that fresh water bathing killed or removed the majority of *Paramoeba* present on gills, however some survived and could be a source of re-infection.
Clark G. et al (2003)	Atlantic salmon	**The effects of fresh water bathing on re-infection with AGD was examined in this research** No untreated controls could be included, results were compared to each other Oxygen maintained at over 120% saturation Temp ranged from 14.9-16.4°C Fish bathed once before, 3 weeks before study 2 hour bathing duration	3 baths - fish biomass: 1. 16,590kg 2. 23,023 kg 3. 24,296kg	Fish with AGD were examined over a 10 day period following fresh water bathing to assess the time taken to re-infection. Samples taken before fresh water bathing and then 1, 3, 5 and 10 days post-bathing to determine number of amoebae on gills Efficacy was evaluated by monitoring changes in amoeba numbers on the gills and histological lesions over time after bath.	Fresh water bathing significantly reduced the number of amoebae on the gills, with an 86 ± 9.1% reduction. Amoeba numbers returned to pre-bath levels 10 days after bathing. The number of *Neoparamoeba pemaquidensis* dramatically dropped in histological sections from 0.53/AGD lesions before bath to 0/AGD lesions 1 day after bathing and then remained significantly lower, reaching 0.08/AGD lesion 10 days post bathing. This did not correlate with the amoebae counted in gill isolates (which returned to pre-bath levels 10 days post bathing). It is possible that after fresh water bathing, amoebae are not attached to the gills and could be lost during fixation. This study showed that fresh water bathing is effective at removing amoebae from gills of fish; however, re-infection can occur within a week as not all amoebae are removed during fresh water bathing.

Reference	Species	Treatment	# fish used	Method	Result
Douglas-Helders G.M. et al (2003)	Atlantic salmon	**Copper oxide-based antifouling paint treatment was examined in this research** Two out of four nets were treated with copper-based antifouling paint and soaked in seawater for 72 hrs before stocking. The other two nets were washed with fresh water in a netwasher and also soaked for 72 hrs in seawater before stocking. Sampling took place at day 0 (before fresh water bathing and introduction to the treated/untreated net) and on days 15, 30, 45, 58 and 71, when fresh water bath treatment was needed.	Four sea cages stocked with 5-9 kg/m^3	Presence of *N. pemaquidensis* on gills was assessed using immuno-dot blot while presence of excess mucus, used as an indicator for infection, was assessed using gross gill scores.	Results suggest that copper paint treated cages had significantly higher paramoeba and AGD prevalence cf control cages. No treatment effect was found on the intensity of infection, determined by gross gill scores. At the end of the study, paramoebae prevalence of net samples was 58.5% and AGD prevalence was 42.5% for copper treated nets. No paramoebae were found on control nets and AGD prevalence was 35%, nets could be the source of *N. pemaquidensis* infection of fish with AGD and therefore copper paint treated nets could be a risk factor for AGD.
Roberts S.D. & Powell M.D. (2003) Note: Fish infected with amoebae isolated from the gills of commercially farmed Atlantic salmon during an outbreak of AGD (Howard & Carson method)	Atlantic salmon	**The softness and hardness of water was examined in this research** <u>Lab experiment Groups</u>: Bath control Soft fresh water bath (37.4 ± 5.4 mg/L $CaCO_3$, 0.24mM Ca and 0.08mM Mg) Hard fresh water bath (236 ± 11.9 mg/L $CaCO_3$, 1.49mM Ca and 0.53mM Mg) Pre-treatment control group Hard water was artificially hardened by adding $MgSO_4$ and $CaCl_2$ both at a concentration of 200mg/L. <u>On-farm experiment:</u> exposed for 3hrs Soft fresh water bath (19.3 mg/L $CaCO_3$, 0.03mM Ca and 0.10mM Mg) artificially softened with water conditioners Hard fresh water bath (173mg/L $CaCO_3$, 0.63mM Ca and 0.67mM Mg) sourced from dam Fish were returned to seawater and assessed over 8 weeks. *Neoparamoeba pemaquidensis* infection was established within 3 weeks of inoculation Gill samples were placed in Davidson's fixation for 72hrs then transferred to 70% ethanol	Lab test: 322.9g ± 12.2g Temp 15°C pH 7.7 ± 0.03 Total ammonia < 0.25mg/L On farm test: 1.47 ± 0.08kg	Gross pathology score according to visibility of white mucous patches on gills (ranked similar to Powell et al (2001)). Gill amoebae were harvested from the gills by a method modified from Howard & Carson (1994). Lab experiment: All fish in the experiment were AGD-affected and had 65.2 ± 3.2% of gill filaments with AGD type lesions. On-farm experiment: All fish used in this experiment were AGD-affected and had 5.2 ± 1.1% of gill filaments with AGD type lesions.	Results showed that soft fresh water (19.3-37.4mg/L $CaCO_3$) was more effective at alleviating AGD in affected fish than hard fresh water (173-236.3mg/L $CaCO_3$). At 8 weeks post hard water bath fish had significantly greater proportion of AGD-affected filaments cf pre-bath controls. Soft water significantly reduced viable gill amoebae numbers from 73.9 to 40.9% of total count. It also significantly alleviated gill pathology, both gross and histological. This study showed not only does soft water reduce gill amoeba numbers but it also is of a therapeutic advantage with the potential to reduce bathing frequency. This study also showed that artificially softened water is as effective as naturally soft water.

Reference	Species	Treatment	# fish used	Method	Result
Adams M.B & Nowak B.F. (2004) Note: naturally infected salmon	Atlantic salmon	**The effects of freshwater bathing was examined in this research**	2 pens	Sampling commenced immediately before and after initial bathing then on a weekly basis until a second bath was required to treat re-infection One pen was re-bathed after week 4 sampling and the other pen was was re-bathed after week 5. Gills were excised, rinsed gently in 0.22μm filtered sea water and fixed for 1-2hrs in seawater Davidson's fixative.	Infection level / Description Clear: Gills are healthy, clean and red Faint spots: Small discrete spots, not raised, translucent appearance Spots: Raised opaque spots on single filaments, spots display a distinct white colouration on a red gill background Patches: Raised white patch affecting two or more filaments, excessive mucus production Initial fresh water bathing treatment reduced the percentage of AGD lesion affected filaments by 48% from 8.3 to 4.3%. No histological signs of AGD were apparent at 1 week post-bathing. By 2 weeks post-bathing, light levels of infection were present in 15% of sampled fish. In the final sampling week 75% of fish displayed signs of infection which were identical to those described prior to bathing. Re-infection is driven primarily by salinity and temperature.
Powell M.D. Clark G.A. (2004)	Atlantic salmon	**Two oxidative disinfectants were examined in this research. These were:** **Chlorine dioxide** (0, 10, 25 and 50mg/L), **chloramine-T** (0, 10, 25 and 50mg/L) or hydrogen peroxide (0, 10, 50 and 100μL/L).	6 fish per treatment per concentration	Amoebae were isolated from the gills using a technique modified from Jones (1988). Experiment was carried out on two separate farms. Each chemical was pre-dissolved or mixed with 100ml of tank water and added to each tank and mixed with fresh water. Fish were exposed to chemical treatments for a total of 6 hours. After 3 hours fish from each tank were sampled, smear from second gill arch for indirect fluorescent antibody test. The remaining 3 fish were maintained for a further 3 hours (total of 6 hours).	Chlorine dioxide (25 & 50mg/L) and Chloramine-T (10-50mg/L) reduced the number of amoebae on the gills by approx 50% cf pre-exposure numbers. The results from the hydrogen peroxide treatment were equivocal and the toxicity of hydrogen peroxide was high.

Reference	Species	Treatment	# fish used	Method	Result
Green T.J. et al (2005)	Atlantic salmon	**Dissolved organic carbon (DOC) and its interaction with water hardness was examined during this research** Humic acid = HA	123.5g ± 4g Temp 17°C pH 7.7 Fish infected with isolate at 230 cells/L (Modified Morrison et al (2004) technique)	Once fish were identified as AGD infected they were bathed in 1µm filtered seawater (control) or in one of 4 fresh water treatments: 1. Hard fresh water with high tannic acid (TA) 2. Soft fresh water with high TA concentration 3. Hard fresh water with low TA concentration 4. Soft fresh water with low TA concentration Fish were bathed for 2.5 hrs High DOC treatments contained 20mg/L HA Low DOC treatments contained 5mg/L HA Experiment was repeated 3 times 12, 13 and 14 days post infection Form of dissolved organic carbon used was humic acid (HA). Hard fresh water was artificially hardened with the addition of calcium chloride dehydrate ($CaCl_2$ $2H_2O$) and magnesium chloride hexahydrate ($MgCl_2$ $6H_2O$) to mains water.	A clinical infection was observed 11 days post infection. This study showed that the concentration of DOC had no significant effect on efficacy of fresh water as a treatment for AGD at concentrations commonly found in water used for bathing around SE Tasmania. The experiment did, however, provide further support that soft fresh water (<50mg/L) is more effective as a treatment for AGD cf hard fresh water (>50mg/L).

Reference	Species	Treatment	# fish used	Method	Result
Harris J.O. et al (2005)	Atlantic salmon	**Chloramine-T (Cl-T) and fresh water was examined during this treatment**	2,000 clinically AGD affected Atlantic salmon	Conducted over 3 months with an initial bath in either fresh or seawater with Cl-T, followed by a second bath 6 weeks later. One cage of 500 fish was bathed in freshwater and one cage of 500 fish was bathed in seawater and Cl-T (10mg/L) for 1 hour. This was repeated the next day with another sample of fish. 10 fish sampled at 14 day intervals. At 6 weeks from the initial bath, the fish were bathed again in the same treatments and conditions at the first bath. The fish within the cages were maintained for another 6 weeks for a total of 12 weeks.	According to this paper, amoeba densities were reduced to 54% (Cl-T and seawater) and 80% (freshwater) of original values. *Neoparamoeba* sp. density was not affected by bathing and was not significantly different over the course of the experiment. Lesion prevalence was higher for Cl-T treated fish (14.30 ± 1%) than for freshwater treated fish (8.03 ± 0.57%). This was also seen for gross gill scores. In the fortnight after each of the two baths, Cl-T treated fish had significantly higher lesion levels, although this difference was then resolved by 4 weeks post bathing. The use of short Cl-T in seawater is at least as effective as freshwater at reducing amoebae density. Note: Majority of amoebae observed on the salmon in this study were not *Neoparamoeba* sp.
Florent R.L. et al (2007)	Atlantic salmon	**Bithionol as an oral treatment was examined in this research** Feeding AGD-affected Atlantic salmon twice daily to saturation with bithionol at 25mg/kg feed. Three feeds examined: 1. Bithionol 2. Plain commercial (control) 3. Fish oil coated commercial (control)	288 fish allocated to 9 tanks (density 6.6g/L) Atlantic salmon smolts 90.4g (± 5.2g)	Feeding started 2 weeks prior to exposure of *Neoparamoeba* sp. at 300 cells/L and continued for 28 days post exposure. Efficacy was examined by gross gill score twice weekly for 4 weeks post exposure	According to this paper, when bithionol was fed as a two week prophylactic treatment at 25mg/kg feed it delayed the onset of AGD pathology and significantly reduced the percent lesion gill filaments by 53% and halved gill score from 2 to 1 cf with both the plain and oil control groups. There was no palatability problems observed during the study. Bithionol was also consumed more throughout the trial than the control feeds.

Reference	Species	Treatment	# fish used	Method	Result
Florent R.L et al (2007)	Atlantic salmon and rainbow trout	**Bithionol as a bath treatment was examined in this research** AGD-affected fish were esposed to either a 1hr seawater bath containing bithionol concentrations of 0, 1, 5, 10 or 25mg/L or a 3hr fresh water bath or a 1hr seawater bath containing alumina (25mg/L)	Juvenile diploid rainbow trout (133.9g ± 6g) Atlantic salmon diploid spring smolts (92g ± 2.4g) 6 fish in each group	Fish housed separately in seawater tanks. Following the baths the fish were returned to seawater. Sampling occurred immediately after bathing and 24 hours post bathing. Exposure to *Neoparamoeba* sp. was according to Morrison et al (2004). Isolation concentration was 300 cells/L/system	During the 1hr bath both species exhibited 100% morbidity at 25mg/L. During the 1hr bath for 10mg/L, Atlantic salmon exhibited 44% and Rainbow trout exhibited 16% morbidity. No morbidity was noted for the 1 and 5mg/L concentrations or freshwater groups. 24hrs post bath Atlantic salmon in the 10mg/L treatment exhibited 44% morbidity, no other Atlantic salmon showed morbidity for the other treatments. Whereas Rainbow trout had morbidity across all bithionol treatments, with 16% (one fish) morbid fish in both the 1 and 5mg/L treatments and 66% (four fish) in the 10mg/L treatment. There was no morbidity observed for either species in the 0mg/L or freshwater treatments. The lowest concentration (1mg/L) of bithionol tested significantly reduced percent lesioned gill filaments to similar levels of the freshwater control, and all other bithionol concentrations having significantly reduced percent lesioned gill filaments cf the seawater control. A similar pattern was observed in the Rainbow trout, with all bithionol concentrations significantly reducing percent lesioned gill filaments cf the seawater control. Atlantic salmon exposed to a 1hr bithionol bath at 1, 5, 10 or 25mg/L had percent reduction in amoeba numbers of 33, 46, 47 and 60% respectively when cf seawater control. Rainbow trout exposed to a 1hr bithionol bath at 1, 5, 10 or 25mg/L had percent reduction in amoeba numbers of 43, 49, 56 and 60% respectively. Concentrations of 1, 5 and 10mg/L in Atlantic salmon were equal to that of the current industry standard of fresh water bathing. In summary, results of this experiment showed that bathing fish in seawater with bithionol at 1mg/L for 1 hr is as effective as fresh water bathing to treat AGD. A minimum crude amoeba reduction of 33% was seen cf no treatment.

Reference	Species	Treatment	# fish used	Method	Result
Powell M.D. et al (2007)	Atlantic salmon and Rainbow trout	**N-acetyl cysteine (NAC) as a feed treatment was examined in this research** Atlantic salmon had 200µg/mL of NAC Rainbow trout had 100µg/mL of NAC	Rainbow trout (124.4g ± 3.5) Atlantic salmon smolts (78.5g ± 19.1) Stocking density of 34kg/m^3	Fish fed an oil-incorporated, NAC-medicated diet (8kg NAC/kg diet) for up to 24 days and challenged with inoculation of 300 cells/L *Neoparamoeba* sp. (Morrison et al (2004) method). Control fish were fed normal oil-coated pellets Rainbow trout were fed NAC for 4 days and 2 days prior to infection and a third group not fed NAC (control). Each group was inoculated with 300 cells/L of *Neoparamoeba* sp. At days 0, 4, 7, 11 and 14 post exposure fish were sampled. NAC was continued to be fed to treated groups during 14 days. Atlantic salmon were fed for 5 days and 3 days prior to infection and a third group were feed no NAC (control). Each group was inoculated with 300 cells/L and NAC was continued to be fed to treatment groups throughout the 19 day exposure. At days 0, 5, 7, 12, 15 and 19 post exposure fish were sampled from each group (2 tank).	According to this paper, NAC medication failed to reduce the severity of gill lesions associated with AGD even though the mucus viscosity from medicated fish was less than that of controls. Oral NAC medication does not appear to be an effective method for controlling AGD in salmonids despite reducing cutaneous mucus viscosity.

Reference	Species	Treatment	# fish used	Method	Result
Florent R.L. (2009)	Atlantic salmon	**Bithionol as a bath and oral treatment was examined in this research** Bithionol (oral treatment)at 25mg/kg and biothionol with fresh water bath for 3hrs	396 fish into 9 tanks (density of 9.9g/l)	Feed: 3mm Atlantic salmon grower LE pellets was used for all treatments Feed was prepared in 1kg batches and stored in 4°C Control feed: moistened feed with 60ml distilled water, then evenly coated with 30ml of fish oil. Fed was air dried for 24hrs then stored at 4°C Medicated feed: same as control but bithionol was added to fish oil prior to coating moistened pellets. Stocking density of 30g/l. Once 3hr bath was done, fish were returned to the original tank Bithionol feed alone: Fish were fed at a maximum of 1% bw/day equally dispersed over 12hrs All treatment was fed to fish 14 days before infection with *Neoparamoeba* spp Isolation delivered approx 300 cells/l/system Bithionol with freshwater bath: Following 28 days of exposure of *Neoparamoeba*a 3hr freshwater (17°C, pH 7.2, treated with sodium thiosulphate at 0.005mg/l to remove chlorine) bath was administered.	According to this paper, bithionol when fed as a 2 week prophylactic or therapeutic treatment at 25mg/kg feed delayed the onset of AGD pathology and reduced the percentage of gill filaments with lesions cf controls Treated fish 14 days had significantly less affected filaments cf control Bithionol was effective as a 14 day prophylactic treatment for AGD with gross gill score halved and a 53% reduction in percent lesions over 28 days

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Appendix B: Comments received on draft of AGD

Comments: Aquatic Animal Health group, University of Tasmania
Summary Barbara Nowak (full comments from other group members attached below)

This review has attempted to cover all AGD publications ever published until the end of 2010, which would have been quite a daunting task, particularly for someone not familiar with the literature. I fully agree with all the comments listed by Mark Adams, Melanie Leef and Phil Crosbie (all attached below), as well as the issues raised by Richard Morrison in his response to the review. I accept that the review represents the views of the authors based on their reading and understanding of the literature as well as discussions with various people involved at different stages in AGD research and salmon industry. This review is more a historical document listing chronologically different research than it is a synthesis of AGD research. The choice of literature used and cited was done by the authors and does not fully reflect AGD research. There is a big emphasis on the early work and not much on more recent publications. This review contains a number of errors (where the literature is misrepresented) and omissions and I will only address the major ones and give examples of others, the attached comments from the group members outline other examples. Additionally, the authors made the decision not to review unpublished research done by Honours and PhD students and yet spent a lot of time discussing Honours thesis from 1988, if this thesis is included the other theses should be discussed as well. To be consistent it would be best to remove the section addressing this Honours thesis, unless all other Honours and PhD theses were covered.

Despite these flaws I agree with some of the report's conclusions about future research, in particular "The inability to culture AGD amoebae (.. .) has been a major stumbling block" and that there is "an urgent need to develop axenic cultured strain of AGD amoeba", which is our current research direction. I also agree that DNA vaccine research should be stopped at the end of the current project.

The next sections cover issues, which are additional to those already covered by other comments, including:
- AGD aetiology
- culture methodology, in particular the use of antibiotics in culture
I fully support those and other comments.

Issues

Amoeba taxonomy

"The parasome, an endosymbiont, is a prominent distinguishing feature of the genus Paramoeba Schaudinn, 1896." – this is incorrect - the parasome is characteristic for other genera, including *Neoparamoeba* and *Janickina* (Dyková *et al*.,. 2003, Adl *et al*., 2005).

"As such, the amoeba causing disease was thought to be a *Paramoeba* species. This then quickly changed to it being a *Neoparamoeba* species, most likely *N. pemaquidensis*." *Paramoeba pemaquidensis* was moved to the genus *Neoparamoeba* (so it became *N. pemaquidensis*) before AGD was first described, but incorrectly named *Paramoeba pemaquidensis* in the first

description of AGD and then the incorrect name was carried through scientific literature until it was finally corrected. This species was transferred (together with *P. aestuarina*) to genus *Neoparamoeba* due to the absence of microscales on the surface of the trophozoites (Page 1987, Dyková *et al*., 2000).

Amoeba biology

"However, we were unable to find in our review of the research any definitive information on generation time, potential for sporulation, survival and replication off the host"

Douglas-Helders et al 2003 left gill isolated amoebae in water for 14 days without any fish, the amoebae infected Atlantic salmon introduced to the tanks after that time

Based on our experience and preliminary experiments, as well as all work done by Dr Iva Dyková in her laboratory, the trophozoite is the only stage and N. perurans similarly to other *Neoparamoeba* spp and *Paramoeba* spp do not form spores. The trophozoites appear to double in numbers overnight (12-24 hours) and undergo 4-7 divisions in a week (room temperature). Light does not seem to have any effect on the generation time. Furthermore, exposure to ammonia (up to 100 mg/L of ammonium sulphate) did not affect numbers of gill isolated amoebae, however exposure to copper (10, 100, 1000, 10 000 and 100 000 µm) reduced gill isolated amoebae numbers over time (Douglas-Helders et al 2005). Using PCR tests, *N. perurans* has been detected in water from cages containing farmed Atlantic salmon affected by AGD in Tasmania and from freshwater used to bathe fish on the same farm (Bridle *et al*., 2010).

"The infective model in a tank is different from what would occur in a sea cage. Hence while 10 amoebae per litre may be infectious in the laboratory, what the infectious dose is in a salmon cage under field conditions is not known. " Current and previous estimates suggest around 20-60 amoebae/L in the farming environment (based on PCR and MPN – Douglas-Helders et al 2003, Bridle et al 2010), this is consistent with the laboratory results .

Susceptibility of non-salmonid species to AGD

"Rainbow trout have been shown subsequently to be susceptible to the disease (Foster and Percival, 1988). So too have turbot (*Scophthalmus maximus*) and it has been possible to experimentally produce gill infection, but not AGD, with AGD amoebae in greenback flounder, *Rhombosolesa tapirina* andbig-bellied seahorses, *Hippocampus abdominalis* (Nowak et al 2002)."

The important point missed here that all these species in which AGD was reported were cultured (including turbot and ayu (Crosbie et al 2010) – not mentioned here).

"One opportunity that may have provided some insight was the culturing by one salmon company of striped trumpeter (*Latris forsteriI*) in close proximity to its salmon pens. While there was no apparent indication that the striped trumpeter developed AGD (S. Percival, personal communication) the fish were not examined to confirm whether AGD amoebae could be isolated from their gills." While this is true, the other fact not mentioned here is that both striped trumpeter and Atlantic salmon have been maintained at MRL Taroona – while Atlantic salmon suffered incidental AGD outbreaks, striped trumpeter did not. Gill histology from striped trumpeter never indicated any amoebic infections or AGD-like lesions.

Host response

"The only factor in this sequence of events leading to AGD that has been reasonably described in AGD is the response of the host at the cellular level. Even at this level we do not think the inflammory/immune response has been satisfactorily characterised particularly with respect to clear designation of the cell types involved."

Results from numerous publications by Richard Morrison, Andrew Bridle and Neil Young as well as Kally Gross and Benita Vincent (including but not limited to Morrison et al 2006, Morrison et al 2007 and Young et al 2008) are not mentioned. These results provide further information about the host response and propose how the disease affects fish. Also, see my later comment about identification of different cell types in AGD lesions.

"Laboratory trials have identified differences in the susceptibility to infection in fish that have previously developed AGD and recovered. Findlay et al 1995 and Vincent et al 2006 showed that previous exposure decreased susceptibility whereas Gross et al 2004 disagreed with these other researchers."

Table 1 summarises these trials (and others), it does show that different conditions were used which can explain the differences in the results.

Table 1. Experimental evidence for resistance to subsequent AGD infections following previous exposures. * treatment protected from subsequent infection. FW – fresh water, SW – sea water. Salinity given for SW. Adapted from Gross 2007 and Vincent 2008.

	Findlay *et al.*, 1995	Findlay & Munday 1998 Trial 1	Findlay & Munday 1998 Trial 2	Gross *et al.,* 2004a	Vincent *et al.*, 2006
Treatment groups	FW maintained* FW bathed/SW maintained Naïve	FW bathed* Naive	FW maintained x2 FW bath x1 FW bath Naive	FW bathed/SW maintained* FW maintained Naive	FW bathed* Naive
Infection method	cohabitation	cohabitation	cohabitation	Inoculation (3300 cells L^{-1})	Inoculation (500 cells L^{-1})
Salinity	unknown	unknown	unknown	36	35
Temperature	14°	14°C	14°C	17°C	12° /16°C
First exposure (weeks)	4	4	4	2	4
FW bath (h)	None	2	2	4	24
Resolution (weeks)	4	4	4	4	5
Second exposure (weeks)	4	4	4	4	5
Assessment of infection	Gross gill score	Gross gill score	Gross gill score	Cumulative mortality, histology	Cumulative mortality, histology

"It would seem appropriate to investigate antigen processing"

That was done in a lot of detail by Neil Young, unfortunately his results suggesting that *N. perurans* can evade host immune response due to disruption of molecular mechanisms essential for activation of an effector T-cell mediated responses are not mentioned in this review.

Experimental model of disease

"Critical to this research is having a solid and reliable challenge model. Unfortunately, a reliable model that has access to a ready, consistent supply of AGD amoebae (i.e. the infective strain) is not possible" This is currently achieved by the use of infection tank. While having in vitro culture of virulent amoebae will take it one step further, it is incorrect to say that there is no reliable model to access supply of amoebae. Even with in vitro culture and cryopreservation there maybe a batch effect, as shown for other pathogens. This means that it is crucial to use control treatments to show consistent experimental performance.

Examples of omissions leading to incorrect statements

"Although the histopathology of AGD has been reported by numerous researchers, descriptions focus on morphology on H&E stained histological sections which provide little other information on which an understanding of the pathogenesis of AGD can be further understood."

This statement is incorrect. The table below lists different stains used, their results and references. There is a lack of reagents for salmon (Prof Bob Raison, UTS applied for funding to develop salmon cell markers, unfortunately without any luck), we use the cell markers as they become available. Furthermore we characterize the lesions and different cells by investigating expression of various genes, despite most of these results being published this area has not been addressed in the review.

Stain	Target cell	Result	Reference
PCNA	proliferating	increased	Adams and Nowak 2003
PAS/Alcian Blue	mucous	increased	Many for example: Adams and Nowak 2003, Adams and Nowak 2004
Anterior gradient	mucous	increased	Morrison and Nowak 2008
NaKATPase	chloride	reduced	Adams and Nowak 2003
MHC class II	MHC class II +ve cells, mononuclear cells, possibly macrophages, epithelial and/or dendritic cells (on the basis of morphology)	No difference in normal gill, present in lesions	Morrison et al 2006
Ig	Ig +ve cells	Present but no difference	Gross 2007
iNOS		No binding in Western Blot – not used	Gross 2007
T cell marker	T cells	Present but no difference to controls	Koppang et al in prep, presented at EAFP conference 2009

"Further, automated technology is readily available for quantitative histological assessments of tissue components. This technology might well be used to quantify mucous cells and chloride cells, as well as other cell types involved in the host response to AGD amoeba, again contributing to an understanding of the host-parasite relationship. "

This statement is incorrect. Automated image analyses are used when appropriate (for example by Adams and Nowak 2001, Adams and Nowak 2003).

Other comments

Role and the significance of the parasome

"We note the comment made in Steinum et al (2008) of the similarity between the pathology noted in AGD in salmon in Norway and that induced by the kinetplastid *Ichthyobodo necator*, a well recognised protozoan parasite of the skin and gills of fish."“The pathology seen associated with the amoebae was similar to that induced by *Ichthyobodo necator*, and neither of these two ectoparasites seem to cause the vascular lesions and extensive inflammation and necrosis typical for PGI, which is caused by a variety of more tissue invasive pathogens.” (Steinum et al 2008).So this suggest that there is no extensive inflammation in either infection.

Wild fish as a reservoir

We are very impressed by the amount of effort which went into getting a copy of Timothy Jones' Honours thesis, if histology blocks from this study are still available we could determine the species of the amoebae present on the gills of wild fish.

"Logically, it would make sense that the first salmon introduced in net pens in Tasmania came in contact with wild fish and this was how infection established in the salmon." Alternatively, *N.perurans* are amphizoic species (like many other amoebae species which infect humans or fish) and were always present in the environment but moved to salmon only when the farming started.Adams et al 2008 (*Neoparamoeba* detection in wild blue warehou present in salmon cage) not mentioned.

"We (...) note the importance of identifying wild fish as reservoirts of infection in the epidemiology of the disease." I disagree, while this is interesting it's not urgent or important research.

Treatment of AGD

This section contains only references up to 2000 (so the last 10 years of research is missed). While there is a table attached at the end it is difficult to follow as it mixes up in vitro and in vivo treatments and treatments applied in fresh water and sea water. I was surprised that neither L-cysteine or bitionol treatments were not mentioned, while they were not followed up by the industry (I understand at least partly on the basis of treatment costs) the results were promising at the time.

"some amoebae may survive within cystic lesions present in the gills" Subsequently it was shown that the lesions are all closed and not open to the environment and thus would not facilitate survival of amoebae (Adams and Nowak 2001).

Vaccine

"Other work conducted on the issue of immunity in Atlantic salmon to AGD included Bridle et al (2006), who demonstrated the up regulation of a specific immune response gene (Interleukin-1β (IL-1β)) in the gills of Atlantic salmon infected with AGD amoebae, and stressed the importance of the host response at the site of infection. This gene is involved in the inflammatory response which is part of the innate immune system." The point which is being missed here that the IL-1β upregulation is not followed by upregulation of other inflammatory cytokines.

Selective breeding

"we are of the understanding that the ability to breed directly from fish that have been transferred to saltwater and exhibited an ability to cope with AGD can signficiantly increase the speed of improvement in this trait. Currently, this does not occur." This is incorrect, it is being done by HAC since 2002.

Comments from Phil Crosbie

Review on Research on Amoebic Gill Disease : Review Comments Phil Crosbie

I agree with the majority of comments made about the review by Richard Morrison and Mark Adams and so I will not repeat their concerns.

In my opinion the review failed in its primary objective of providing a synopsis of research carried out into amoebic gill disease. Although the review charts the historical route of the research too much emphasis has been placed on some of the original work whilst some recent work is neglected. Much of the work carried out as part of recent FRDC and CRC projects and post graduate research projects and detailed in respective reports and theses has not been included. For example there is no mention of much of Benita Vincent's work on amoeba-salmon attachment factors, nor are many of the studies dealing with immunological perspectives of AGD included.

There appears to be some confusion regarding the use of antibiotics in amoebae isolations and culture. On some occasions in the past a cocktail of antibiotics (including streptomycin, erythromycin, benzylpenicillin, carbenicillin and ampicillin) has been used when transporting AGD-affected gill arches from salmon farms back to the University to instigate amoebae infections and subsequent disease. In my experience, and from subsequently published results, AGD was instigated in all cases where this occurred after material scraped from the gills was added to a tank of seawater acclimated salmon which were naive to AGD. Since the development of the adherence isolation technique of Morrison et al. (2004) where amoebae are isolated, partially purified and accurately counted AGD has been initiated by adding known numbers of cells to tanks and again, on some occasions antibiotics have been used. Since 2007 all vaccine/challenge experiments performed at Utas to test efficacy of DNA vaccines developed by the CSIRO oxolinic acid and ampicillin have been used to "disinfect" amoebae which were used to instigate AGD. The exposure period is 18-24h and for every experiment AGD has been successfully instigated. In a smaller scale pilot study these antibiotics have been shown to have no impact on virulence of *N. perurans* (see Crosbie et al., 2007). Suggestions in the review that some antibiotics could somehow alter part of the genome, namely the 18s rRNA gene, over a few weeks seem very speculative if not impossible. All our evidence regarding the use of antibiotics and *N. perurans* suggests that they have no impact on the virulence of the amoeba, at least in the short term.

During initial attempts to culture *N. perurans* various combinations of antibiotics were used to control contaminating organisms, however recent attempts have excluded the use of antibiotics. Current developing culture techniques involve growing isolated *N. perurans* on low nutrient agar plates with an overlay of seawater with no antibiotics added. We do not exclude the use of antibiotics in the successful development of culture methods for this amoeba in future. However when and if antibiotics are used full appraisals of the impact on growth and maintenance of virulence and indeed the identity of the amoeba will be tested in appropriately controlled experiments.

Comments from Mark Adams

AGD DRAFT REVIEW COMMENTS (Mark Adams Feb 2011)

The current draft review on AGD research represents a solid synopsis of scientific literature, grey literature and current opinion from selected salmon industry personnel and scientific researchers alike and I appreciate the opportunity to comment. Overall the review presents an informative history of AGD in Tasmania and describes in most part the key advances in our collective knowledge of this disease. The draft review presently provides direction for future research based on the author's interpretations of past research. The review suggests that research scope be aligned with understanding some fundamental questions still remaining with regards to AGD and its infective agent, particularly its biology, pathogenicity, epidemiology and treatment. It is suggested that future work in these areas will provide further grounding to provide solutions for future management of AGD in Tasmania. I concur with the reviewer's overall theme for future research directions albeit with some minor exceptions. Without being overly critical, these exceptions relate to a number of issues regarding some of the content within this review. Furthermore, I would note the limited assessment given in the review (in terms of scope and synthesis) afforded to immunological and patho-physiological research conducted over the last 15 years.

Comments regarding these issues and other discussion points are given below under the relevant review topics:

3.3.2 Molecular characterisation and taxonomy

This section in essence compares the naming of *N.perurans* as the causative agent to be potentially as misguided as the previously held assumption that *N.pemaquidensis* was the sole aetiological agent of AGD. The reviewers have overlooked the stark differences between the techniques used and assumptions made when attributing causality to the two species. The molecular tools used to identify *N. Perurans* as the aetiological agent of AGD provided a directly observable result. In subsequent sections, the reviewers argue that this result may be in error due to a speculative theory regarding an antibiotic mediated alteration to rRNA sequences. I would suggest that the reviewers seek opinion from molecular taxonomists before proceeding with this assumption. The reviewers' insistence that the term "AGD amoeba" be used is confusing and counter-productive. Considering the reviewers have perused literature regarding the culture of *Entamoeba* sp. it is surprising that they have not acknowledged that a similar error was made in the early years of attributing causality of amoebic dysentery to the correct species, studies that I am confident would have had substantially more funding and resources available.

3.8.4 Culture methodology

Much work, as the reviewers rightly conclude, remains to be done on successfully mass culturing pathogenic *Neoparamoeba*. Future research investigating the differences between pathogenic and non-pathogenic species of *Neoparamoeba* could also lead to further understanding of the pathogenic mechanisms of this amoeba. However, speculation regarding the use of antibiotics and loss of virulence, given our collective research experience and observation (both published and unpublished) seems unwarranted as the rationale for pursuing these lines of investigation are highly speculative and not supported by the broader literature pertaining to other amoebic pathogens. The reviewers' do mention that it was not within the scope of this review to examine the broader literature; however this should have been requisite when proposing such a hypothesis.

3.8.6 Further comment on antibiotics and culture methods

The reviewers raise the issue that Zilberg etal (2001) reported a much higher infective dose than Morrison etal (2004) and suggested this maybe due to the use of antibiotics. Although they acknowledge a different methodology, no credence is given to this acknowledgment. From personal experience (using both methods many times) I have noted that Zilberg's method produces an extremely high percentage of non-viable amoebae (based on a rounded morphology and use of either exclusion or inclusion dyes) and is hence reflected in the subsequent huge variation of disease development rate. Zilberg's method (adapted by Clark etal 2003 and others) has also been used on several occasions for *in vitro* experiments producing similarly high variability in viability (or percentage of dead versus live amoebae). It is more likely in my opinion that Zilberg's minimum infective dose was severely overstated possibly by an order of magnitude. In addition, amoebae isolated from the gills using Morrison's method are routinely incubated overnight in an antibiotic cocktail (doubling the population of the attached cells) and used to infect fish and initiate disease successfully.

4.3 Reservoirs of amoebae – where does it come from?

The authors suggest that wild fish may have attributed to the initial spread of AGD in the formative years of salmon culture under marine conditions. Although this hypothesis is quite plausible it is equally as likely that due to the organism's free living ubiquity and the capacity of just a single trophozoite to attach, divide, initiate a hyperplastic reaction and elicit subsequent disease within the gills and presumably a population is equally as feasible. There seems little point in producing a retrospective assumption regarding the original mode of infection form the early years of salmon culture. As the reviewers suggest, it is likely that the salmon themselves have become the principal reservoir (see Bridle etal 2010). The review provides a substantial discussion to the presence or absence of Neoparamoeba in wild fish. However the argument for or against is largely circular; the methodologies used by the two authors mentioned are diagnostically incompatible and/or incomplete in detailed substantiation. There is no mention of a histologically definitive detection in of AGD in blue warehou published in 2008.

There is much interesting discussion regarding transmission of the infective agent, however an issue overlooked is the logistical difficulty of containing *N.perurans* within the culture environment. It is little wonder that considering the agent's ability to transfer location via tidal flows and other currents (somewhat analogous to the difficulty in containing a terrestrial airborne pathogen) results in the reviewers' asserting that "*research conducted on the epidemiology of AGD over the past 25 years does not appear to have provided industry with information that has significantly improved its control or prevention of AGD* ".

4.5 Susceptibility of non-salmonid species to AGD

Rainbow trout are broadly classified as a salmonid, suggest rewording the title of this section. Additionally the reviewers should note that the infection of seahorses and greenback flounder was not substantiated diagnostically in the sense that a clear histology figure depicting the presence of amoeboid cells displaying a nucleus and parasome (endosymbiont) in association with the aforementioned species gill tissues.

5.3.1 Histological Development of AGD lesions

The review suggests that automated technology is readily available for quantitative histological assessment. I would refer the reviewers to Adams etal 2003 where such methodology was employed. Fully automated image analysis on gill tissue sections is extremely difficult owing to the significant variability of cell distributions and cut depth within gill sections.

5.4 Cellular evidence of an immune response in the host

The first paragraph quotes an observation from Adams and Nowak (2003) regarding the presence of macrophages and neutrophils within gill lesions. Following the quotation it is written that "suggesting that this was a precursor to an acquired immune response.". Adams and Nowak (2003) makes absolutely no assumptions or suggestions regarding the potential for acquired immunity of salmon to AGD. I would insist this is rewritten or omitted.
5.5 *Regression of lesions*
I would refer the authors to the Aquafin CRC report on the effects of husbandry on AGD (Nowak 2007). Significant discussion is given to regression of lesions in a study on immature versus mature salmon which is also a relevant epidemiological study along with many other experiments reported therein.
7.4 *The "protein" and "DNA" approach*
To my knowledge no fish from any vaccine trial has ever presented without signs of clinical disease.

A further comment regarding the tone of the review
Although likely to be unintentional, there is an underlying tone of failure conveyed by this review pertaining to research performed in the last 15 years. Some examples are seen in on page 15, last sentence; p16, 3.1 Introduction; p28 paragraph 4. Although I concur that research has not been able to substantially change the principal form of AGD management (freshwater bathing), the fundamental knowledge base and the multitude of approaches attempted by researchers (both past and present) to provide answers to industry is formidable. This should be duly acknowledged by the review as the apparent "failures" along with the successes are all results that can be built upon. Fundamental research that develops sometimes over decades is an extremely common occurrence within the realm of protozoan disease research (eg malaria, amoebic dysentery, guardaisis); diseases that are all treatable but not always preventable. It is essential this review does not unintentionally isolate the industry from further research investment by conveying a tone of failure of previously completed work.

Comments from Melanie Leef (focused on the sections citing her work)
Report authors comments in Blue and responses in Black

5.6 Biochemical abnormalities
"Markedly elevated blood sodium levels are recorded in Atlantic salmon with clinical AGD, with lower, but still elevated levels in sub-clinically infected fish (Munday 1988, Munday et al 1990), possibly due to reductions in chloride cell numbers (Adams and Nowak 2003). Powell et al (2001)" I don't think this is the correct ref? Should be Powell et al., 2000 I think Powell et al., 2001 describes effect of hyperoxia. From Powell et al., 2001 – "Acute exposure of AGD affected salmon to hyperoxic freshwater for at least 2 h resulted in .....respiratory and acid–base variables were unaffected by acute hyperoxic and normoxic exposure."

" It was interesting to note that no mention was made of the Bohr effect in this paper" Maybe because Hb and intracellular RBC pH were not investigated.
"The capacity for oxygen uptake at the gill epithelium/water interface may be impacted where there is a systemic respiratory acidosis." The partial pressure gradient for oxygen across the gill, and the oxygenation status of blood both dictate oxygen uptake rates in fish as opposed to the diffusion limited excretion of CO2 which involves the movement of HCO_3^- from plasma to RBC

(rate limiting steprate limiting step). Therefore CO2 is more likely to be of consequence for AGD affected fish. Faster transit times of blood through the gills may result in a decrease in O2 uptake however the primary driver for ventilation in fish is O2 not CO2(resp acidosis).
"Paradoxically, Powell et al (2002) state that AGD causes only minor respiratory disturbances to the fish, although substantial acid–base disturbances arise from a persistent respiratory acidosis." This ref is for the cardiovascular paper, it should be Powell et al., 2000 (Powell, Fisk and Nowak paper).
"The same authors then describe a much higher 24 hr mortality in fish exposed to acute hypoxia compared a control group (21% survival c/f 90% survival)." Suggested by Powell et al., 2002 to possibly relate cardiovascular compromise
"In contrast, Leef et al (2005a) failed to demonstrate short term (48hr) changes in blood PO2 in experimentally infected Atlantic salmon." Not in contrast – Leef et al., 2005a described changes following exposure to amoebae – initial pathogenesis whereas the Powell et al., 2000 paper described changes in fish that had established AGD. Changes in PaO2 would not have been expected (initial exposure, to amoebae)

"A short term (48hr) increase in aortic blood pH was reported by Leef et al (2005a) following experimental challenge in surgically catheterised fish when compared with pre-challenge levels" Thought to be associated with hyperventilation resulting from attachment of amoeba (effectively acting as an irritant)
"No difference was reported between control and challenged fish." Leef et al. (2005a) found that within 48 h of initial exposure to Neoparamoeba spp., Salmo salar displayed a significantly elevated arterial blood pH indicative of a respiratory alkalosis that was most likely related to hyperventilation. From day 7 post-exposure onwards, arterial blood pH decreased indicating the development of the characteristic respiratory acidosis reported previously (Powell et al., 2000; Powell & Nowak, 2003). The onset of the acidosis coincided with a significant increase in the number of affected gill filaments (Leef et al., 2005a).

"In contrast, longer term (16days) exposure in smaller experimentally infected fish showed an increase in caudal blood pH compared to non-infected control fish, suggesting the inductiuon of a metabolic alkalosis." Not correct, increase in pH was seen at day 2 (compared to 0 day) in AGD affected fish only (in agreement with respiratory alkalosis due to intiail exposure to amoebae, amoebae attachment and hyperventilation. At day 7 pH was seen to decrease consistently. This was thought to be indicative of the onset of the characteristic respiratory acidosis previously observed by Powell et al. 2000, Powell & Nowak 2003

5.7 Haematological changes

"Short term (48hr) changes in blood parameters (haematocrit and haemoglobin) were also reported by Leef et al (2005a) with time in surgically catherterised and experimentally challenged Atlantic salmon, however, no differences were noted between controls and challenged fish, and MCHC remained constant, suggesting changes due to loss of blood at sampling. We could not find reference to the possibility that the respiratory acidosis" In Leef et al., 2005a? – there was no acidosis observed in the catheterised fish in this study.

"may cause a reduction in the affinitiy of haemoglobin for oxygen (Bohr effect) and whether such a mechanism may have caused a reduction in the ability of red blood cells to take fully load with oxygen when passing through the gills." Differences in PaO2 have either not been observed in respiratory studies (see Leef et al., 2005a) or have been minimal (slight depression - see Powell et al., 2000)

5.8 Cardiovascular dysfunction

"Elevated systemic vascular resistance in Atlantic salmon experimentally infected with AGD, accompanied by significantly reduced cardiac output are reported, suggesting that cardiac dysfunction may be a mechanism by which AGD causes or contributes to mortality in Atlantic salmon (Leef et al 2005b)" Should be Leef et al., 2007?
"No differences were found in Rainbow trout or brown trout, however, possibly explaining why Atlantic salmon are more severely affected by AGD." In terms of susceptibility not gill pathology

5.10 The role of AGD amoeba in the disease process

"The underlying pathophysiological cause of death remains enigmatic. Although it is claimed by Powell et al (2002) that mortalities in affected fish are not due to a respiratory cause and are due to compromised cardiac function, this statement is inconsistent with reports by the same authors of compromised respiratory function in fish with AGD under conditions of reduced oxygen." Is this refering to Powell et al., 2000? "The elevated levels of *P*CO2 in fish affected by AGD resulted in a persistent respiratory acidosis even during hypoxic challenge. These data suggest that even though the fish were severely affected by AGD, the presence of AGD while impairing gas transfer under normoxic conditions, did not contribute to respiratory failure during hypoxia."

Comments - CSIRO research group collective Response

General Comments on the Review:

- We believe the reviewers have done a good job at pulling together the information though in places they are a little over critical of some of the outcomes; for instance we are not sure we were paying them for their opinion on the speed of progress etc.
- In general we feel the reviewers have captured most of the key areas but as a piece of science literature there are considerable gaps.
- It would have been good to see a separate section that looked at the tank challenge model in its entirety; individual limitations are picked up such as the need to use fresh gill samples for challenge which makes reproducibility an issue. We feel this is important as we believe the development of a better system (*in vitro or in vivo*) to evaluate potential products is essential in going forward. The lack of an analysis of the challenge system means this has not been fully teased out. It would also be good to recognise the limitations of only having fish available at a restricted number of times during the year and the small number of experiments that can be conducted in 1 year; all of which have limited progress. We are keen to also point out that we are not singling out this system. One of the major shortcomings of the research (and definitely not the fault of researchers) was the push to develop such a system while at the same time using it to test various things (eg vaccines, breeding strategies and treatments). One of the key areas moving forward is the development of a suite of sound and reliable tools that can underpin AGD research.
- In the vaccine section the reviewers appear to be unaware that there is an adaptive cellular arm to the immune system (e.g. if Ab's are not produced then there is no memory); not that it changes the conclusions though leaves the report with a gap. The review also runs into the trap of not differentiating quality of immune response from level of immune response in some areas i.e. not all Ab's are equal. The review needs to highlight the difference between adaptive immune response and protective immunity. It is clear that many fish develop an adaptive immune response to AGD. What is yet to be conclusively proven (there is some anecdotal evidence) is a comprehensive **protective** immunity to AGD (natural or otherwise).
- The reviewers seem to feel they have it upon the mechanism for switching from virulent to avirulent form of the amoeba i.e. the presence of antibiotics. Surely antibiotics have been used in challenge systems that have resulted in disease. We point the reviewers to the comments by Phil Crosbie regarding the use of antibiotics in the inoculums used for the vaccine challenge trials.
- Determination of true cost of AGD – note that this is being considered by industry through the Saltas Selective Breeding Program to place an economic value to this selection trait. The review does not address this cost, however without this cost it is difficult to do a cost benefit analysis on any treatments and/or selective breeding that may be applied to the industry (highlighted by the L-cysteine and bitionol research and lack of take up from industry)

Some more Specific comments relating directly to the document:

- Page 11 section 2.2. The smolt sent to sea in 1984 would not have been progeny from the Gaden introductions, they would have been smolt from the fertilised eggs brought over from Gaden in 1984. The 1984 introduction of fish would then have been spawned in 1987 and smolt produced in 1988.
- Naming of causative agent – We would suggest that it has not yet been 100% proven that *N. peruans* is a new species and that it causes AGD. This is a point of conjecture between the key research groups and therefore speculation will remain until conclusive research is undertaken to look at *N. perurans* compared

to the non-infective *neoparamoebea* such as *N. pemaquidensis*. Indeed this along with a reliable culture of infective *N. perurans* is a key R&D area moving forward.

- Page 33 2nd last para; pg 52 5th para; page 54 last para – It is clear from the work with the selective breeding program that there are two different resistant traits or responses; an innate response to a first infection followed by an acquired (specific or non-specific) response to subsequent infections. A proportion of the population does show a decrease in susceptibility to subsequent infections. The issue currently for the industry is that they treat based on the more susceptible members of a commercial cohort.
- Page 43 para 3 – We would suggest that it has not been proven that there is no link between AGD and stressed fish, particularly for fish under natural multiple challenges and on commercial farms.

Comments specifically on pursuit of an AGD Vaccine:

- The reviewers do mention the 'two camps' approach to vaccine development, particularly post 2000. This is correct, however it should be highlighted that this was at the bequest of the funding agency. The two groups did attempt to collaborate, however their research directions did not have much overlap.
- With regard to the DNA approach the reviewers stop dead at the development of a 284 clone vaccine. Much research has been undertaken following the 284 clone trials which resulted in a 6 clone experimental vaccine (for which a provisional patent was granted). We would like to highlight the following with regard to vaccine development and would strongly urge the reviewers to include this in their document.
    - Work following on from the 284 clone vaccine and utilizing a subtractive RNA approach (directly targeting differences between 'gill isolated AGD infective amobae' and cultured N. pemaquidensis) developed a six clone vaccine that resulted in on average (a total of 12 tanks over 3 separate trials) an RPS of 40% (range 24-53%) in the laboratory tank challenge system when using morbidity as the measure. We do note some issues with the system but these results were obtained from multiple tanks during multiple experiments
    - A recombinant protein vaccine (representing the 6 antigens) resulted in no decrease in RPS (over 3 tanks in 1 trial)
    - One trial in which gill score was used as the measure resulted in a reduction of between 21 and 33% in gross gill score. However, in all subsequent trials (4 in total) there was no difference in gill score between vaccinated and control groups.
    - The DNA vaccine was trialed in the sea on 3 occasions, which is highlighted in the AGD Review Document prepared for the MAC by CSIRO, which we believe the reviewers have a copy of, but to summarise;
        - Year 1 resulted in a diminishing positive effect on gill score over 3 measures; measure one a 27% improvement; Measure two a 22% improvement; measure three a 11% improvement. The major outcome was that there seemed to be an improvement but that this diminished overtime. Unsure whether this was due to decrease in efficacy of the vaccine or natural resistance.
        - Year 2 had a difference at first measure but no real differences after that. A note on this trial is the significant loss of PIT tags which resulted in lost data and may have compromised the trial
        - Year 3 trial resulted in no improvement in gill score over 4 measures
- The take home message from the DNA vaccine work was that a modest RPS did not mean that fish were unaffected by AGD and hence there was no real decrease in gill score (the measure used to trigger a bath commercially). One can postulate that the vaccine is working through non-specific mechanisms and

allowing fish to remain alive or that there is a component of non-specific ‘resistance’ or ‘tolerance’. However, the overriding message is that the current 6 clone DNA vaccine it is not a commercial viability using the current antigens and/or vaccination strategy. This does not mean that a vaccine is not achievable it just means that the one pursued here has not come to fruition as a treatment or cure for AGD.

- Whether a vaccine should be pursued remains a pertinent question. However, as highlighted by the review there are a number of fundamental questions that probably need to be addressed in order to make a more informed (less assumptions) decision on this avenue.

Comments specifically on Section 8 – Breeding for Resistance:

- See attached paper (Elliott and Kube, 2009 *Proc. Assoc. Advmt. Anim. Breed. Genet.* 18: 362-365), and in press article in Global Aquaculture Advocate. Shows heritability of selection for AGD, and predicted reduction in treatments of 25% over six tears of selection.
- Whole genome selection to increase selection intensity for AGD, was considered in the pilot study (Dominik et al 2009) as feasible, and this line of research is currently being investigated by CSIRO in collaboration with Norwegian colleagues. If an association between AGD resistance and genome markers is demonstrated this will allow more direct selection on broodstock while minimising biosecurity risks in moving broodstock.
- The phenotypic measure of ‘gill score’ is at present the only reliable (rapid, repeatable, cheap, in-situ) measure of the host response to the amoeba, this has been shown through the strong correlation between gill score and time to death.
- The question that needs considering for potential improvement is whether the tolerance shown by some fish to the presence of the amoeba can be measured and exploited through selection.
- Replace Tasmanian Atlantic Salmon Breeding Program (TASBP) with SALTAS Selective Breeding Program
- Another source for industry view on selection assisting in AGD is: Tassal 2009. Tassal Group Limited, Merrill Lynch Emerging Companies Conference (Sydney), 19th June 2009. http://www.tassal.com.au/index.php?/company-announcements.html

Comments -Industry Response – Steve Percival, Huon Aquaculture

- In late 1980's we had a population of smolt delivered to Saltas at Hawkers Point, Dover. This cage of fish was tied up directly onto a cage with moderate-heavy AGD. This population of fish was very heavy with AGD in 10 days post-transfer to sea. The normal pattern at that stage across industry was that smolt became heavy after 2 months. Normally cages were about 50M apart on grids. Indicated that even 50 M made a significant difference. Effectively like a cohabitation, just that fish were in a separate cage. Progression of infection similar to tank cohabitation trials.
- One thing I've noticed over the years is that fish seem to be dying from AGD with very different gross severity of disease. It was not unusual in the early days for fish to still be alive with almost complete obliteration of all gill arches, whereas these days fish can seem to die with much level gross infection. Suggest that other factors contributing to death, not always just gross gill patches.
- There were several cases in the early days of Jeremy's IFAT test where the IFAT was negative despite gross signs in gills suggestive of AGD. There was one case where Nortas delayed bathing because the IFAT was negative in the face of gill patches and they ended up losing a lot of fish. The report mentions the IFAT testing positive for infectious and non-infectious amoebae, but in these cases the IFAT didn't pick up anything, including the amoebae causing severe infection and mortality.
- P 22. – last sentence 1st paragraph – which means?
- In early days when almost everything was a spring smolt the fish needed a bath approx. 8 weeks post transfer to sea. Then again in around 8 weeks but often even quicker, then a third bath in some populations late March early April. Not all populations needed the 3rd bath. No baths required after that. Some populations would have moderate gill scores in Mar/Apr, but would resolve to being clear once water temperature declined and without any bath. Once they passed this period they were fine right through to harvest 12 months later.
- Over time there have been increasing requirements for, now up to 13 or 14 in the production cycle. However, once it reached this threshold it doesn't seem to have increased significantly from this over many years. Probable reasons for increased bath requirements right through to harvest now include the fact there are many more susceptible hosts (ie. salmon) breeding up the numbers of amoebae in the salmon and the environment, plus there is a continual supply of hosts, including the very susceptible fish post transfer. Both assisting the breeding up of numbers but possibly also increasing pathogenicity because of continual passage through salmon.
- I agree with the wild fish being initial reservoir – they don't need to have many, just need to transfer to salmon where they go gangbusters – wild fish then irrelevant. I actually caught fish off the barge at Saltas Dover which had AGD lesions. Back in those days there were a lot more seasonal fish like couta, mackerel, salmon.
- Dynamics of AGD infection very different in younger salmon versus older salmon. In younger fish once the infection comes on it comes on quickly pretty much in the whole population, whereas in harvest size fish there can be 5-10% heavy infections (fish dying) and there be 80% which are clear. Unfortunately we have to bath for the 5-10% because the cost of not doing so is too great.
- Smolt that go into Pillings Bay (brackish water site) can be there for months, but as soon as they get transferred to fully marine sites they come on very quickly. We believe this is because they already have a population of amoebae on the gills which is suppressed enough not to cause heavy AGD, but as soon as the brackish disappears they go gangbusters. Interestingly, the low level exposure of fish at Pillings Bay sometimes over many months doesn't appear to give them any immunity once they go to full strength seawater.

- As you say the dynamics of first infection and second infection onwards is quite different in terms of heritability etc in the breeding program. One cause of better effects after second infection could be a booster like effect of the second infection.
- In early days it was common to have all the pinheads clear of infection in amongst the good condition fish which were heavy. This is not so clear cut any more and there have been reports of pinheads being heavily infected.
- We know there are lots of different amoebae on the gills of salmon. One possibility over the years, if wild fish are a source of the infective amoebae is that the amoebae involved in the infection have changed with the introduction of a different species at some stage by seasonal fish. This is what happened with sealice in Chile where the original species was largely replaced by a different species which is thought to have come in one year with wild fish that didn't normally pass through the salmon growing areas, but did when the environmental conditions were different.
- HAC has a different AGD scoring system to what's mentioned but this doesn't matter. Just to be aware of.
- At gill checking the patches start first and are most severe at the ventral and dorsal posterior extremities of the 2nd. 3rd and 4th gill arch. Often worst on posterior aspect of 4th gill arch.
- P. 41 - We've done a lot of work on post-harvest rigor mortis including muscle pH. We also consistently find a short increase in pH before the usual decline in pH through rigor mortis. Internationally people are unsure of the cause of this, but probably relates to an initial release of alkaline compounds from cells before the whole lactic acid process kicks in.
- Also a clear association with more rapid onset of AGD when a lot of algae around, in particular spiky algae like Chaetocerus/Pseudonitschia.
- The work done by Barbara where they scraped the gills with a scalpel blade to cause damage to see if damage predisposed to AGD and they found that AGD didn't come on as quickly as undamaged gill – I believe is misleading. The process caused by scraping the gill with scalpel blade to cause bleeding is very different for example to the process ensuing if there are lots of spike algae, or jellyfish toxin/tentacles that cause damage to he gill epithelium. Scraping the gill is likely to result in blood and associated white blood c ells etc to be present (probably not conducive to the amoebae), whereas the reaction to algae eg. mucus production/necrosis may well be quite attractive to the amoebae.
- Barbara is currently undertaking work n H2O2 for industry and there have been some preliminary results circulated to industry which seem to indicate some promise. Best to get the info from Barbara.
- P. 57 – mass selection at HAC using broodstock from sea is occurring (3rd last line)

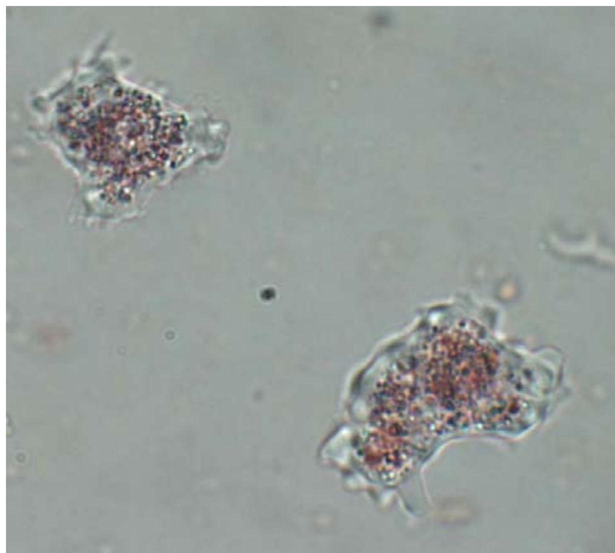
Comments - Dr Richard Morrison

The review of research on amoebic gill disease is comprehensive and the authors are to be praised for their efforts in the face of a considerable literature base. The ultimate goal of AGD research is to develop a practical on-farm method of ameliorating or perhaps eliminating the disease. On that front, I was particularly interested in the section on AGD vaccine research, where a positive outcome could arguably have the most direct, long-term benefit to industry. The reviewers have commendably recognised that the AGD vaccine development program was established in the absence of any evidence that AGD-affected fish develop a protective adaptive immune response and this is still highly relevant to discussions on AGD vaccine research. It is also commendable that the reviewers have identified the assumptions on which the vaccine R&D has been based. One such assumption, that a key step in the disease process is an ability of amoeba to attach to the gill epithelium, is indeed a concern. However, I believe that it can be assumed that attachment is necessary. But what cannot be assumed is that the process is mediated by a specific receptor or receptors. Whether attachment is non-specific, such as when cells attach to glass or plastic by electrostatic interaction has not been excluded.

In regard to the question of AGD aetiology, it is unfortunate that the review does not clearly reflect our current understanding. This area of AGD research is complex and would have been difficult to piece together through the short period of investigation. I would like to clarify the pertinent issues so that there is no misunderstanding of the term “AGD amoeba”. Over the past 30 or so years, molecular phylogenetic techniques have become an invaluable tool for the description of eukaryotic and prokaryotic taxa. These are widely accepted and to a certain extent, standardised techniques are used to infer relationships amongst organisms. DNA or RNA (cDNA) sequences are obtained, aligned and processed using specialised computer software. The final output of this process is the so-called phylogenetic tree. When Neoparamoeba perurans was described, stable relationships were observed in phylogenetic trees erected using two sequence loci (18S and 28S rRNA genes) and three different methods of analysis. The fact that ribosomal RNA gene sequences were used in this process should not create any doubt over the validity of the result. The link the reviewers attempt to make between aminoglycoside antibiotics and ribosomal RNA sequences used in phylogenetic analyses is fundamentally flawed. In turn, the discussion on the use of antibiotics in culture media is of little relevance. It is strongly recommended that the reviewers take expert advice so that they can be guided through the aetiological aspects of AGD research during revisions of the review. The most important and compelling aspect of this research (describing N. perurans) was that amoebae were fully identified in histological sections, thus establishing disease causality. While this may not fulfil the traditional method of establishing disease causality (Koch’s postulates), the process was certainly in accordance with Fredricks and Relman (1996), who devised a contemporary alternative to Koch’s postulates using modern techniques. To date, N. perurans remains the only detectable member of the Neoparamoeba genus directly associated with AGD lesions and it is therefore important to highlight that at this point in time, N. perurans is unquestionably a synonym of “AGD amoeba”.

For many years it had been broadly speculated that cultured Neoparamoeba may “lose virulence”. I too had speculated about this loss of virulence, particularly in the paper describing the N. pemaquidensis isolate NP251002 (Morrison et al., 2005). Not surprisingly, the reviewers have addressed this issue in the review. However, it must be recognised that there is no such evidence that Neoparamoeba lose virulence: It was a hypothesis based entirely on the assumption that N. pemaquidensis is the agent of AGD. Knowing that N. perurans is (at the very least) the predominant agent of AGD diminishes the relevance of this issue. The discussion regarding the “loss of virulence” in the review will create confusion and further research in this area is needless. Rather, it seems reasonable to focus attention upon what is already known as an agent of AGD (N. perurans) and the axenic in vitro culture of this species would appear to be a priority. In regard to the involvement of other members of the Neoparamoeba genus in AGD, the absence of evidence to date (using in situ hybridisation) suggests otherwise. However, further exhaustive testing, including ISH, is still required for total exclusion.

Finally, one other technical issue that has not been directly addressed in the review is the assessment of post-harvest amoeba viability. This is worth consideration as it has a bearing on the research discussed in section 4.4 (Infective dose), section 5.9 (Experimental models of disease) and section 3.8.6. As far as I’m aware, there is no method available to make a valid assessment of postharvest viability. Dye exclusion assays (eg. trypan blue and neutral red) are not appropriate as both viable and non-viable amoeba take up dye into the cytoplasm. In the absence of a valid assay, plastic adherence is arguably the best proxy for determining viability. Not only that, plastic adherence is selective for viable cells and a skilled technician should obtain a very high proportion of viable cells using this technique. For research pre-2004 (when the plastic adherence method was developed) the amoebae harvesting techniques were crude and accurate cell counts were difficult. Variability introduced by post-harvest amoebae viability and cell counting would have almost certainly introduced variability into the down-stream observations.



Free-floating neutral red-stained amoebae harvested from the gills of

an AGD-affected fish. The cells are viable, yet they contain dye.

References

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