Assessment of resistance to amoebic gill disease in the Tasmanian Atlantic salmon selective breeding population

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Declaration of originality

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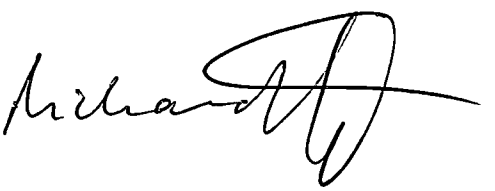

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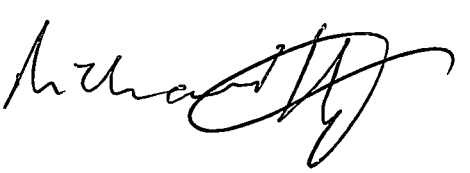

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Statement of co-authorship

The following people and institutions contributed to the publication of the work undertaken as part of this thesis:

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We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

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Abstract

The main priority in the breeding goal of the recently established Tasmanian Atlantic salmon selective breeding program is for resistance to amoebic gill disease (AGD), which is caused by the marine ectoparasite *Neoparamoeba perurans* and is characterised by hyperplastic changes to the gill tissue. A categorical gross 'gill score' is used to schedule freshwater bath treatments. Bathing and direct production losses to AGD are estimated to add over 20% to the cost of production. The purpose of this thesis was to (i) investigate whether genetic variation of resistance to AGD exists (ii) determine whether this variation can be adequately measured using gill score (iii) examine phenotypic trends for evidence of developing resistance (iv) assess whether serum antibodies to Neoparamoebae are related to AGD resistance and (v) establish the relationship between the selection trait (gill score) and the objective trait (time between freshwater baths). Initial evidence of a moderate broad scale genetic variation for AGD resistance was provided from a first infection tank challenge. This was adequately measured by gill score, which was moderately correlated with destructive histopathology and image analysis of affected gills. In a second trial, sea caged fish naturally developed AGD and were bathed over two infection rounds. On the third round, fish were gill scored and left unbathed so AGD related mortality occurred. Narrow sense heritability for AGD resistance, assessed by gill score, varied between 0.23 and 0.48 over the three rounds of infection. Heritability of AGD survival was 0.40 to 0.49. Estimates of genetic gains indicate that up to 82% of the expected gain in survival can be achieved utilising gill score breeding values. Phenotypic results were further examined, gill signs at the early onset of losses accurately predicted the incidence of mortality ($r = 0.96$). There was no relationship between gill score at first infection and the ultimate survival of each fish, though there was a marginal lower separation of the group that died early on. For later exposures, significant gill score differences ($P < 0.001$) were observed between fish that eventually survived or died in the challenge. The systemic immune reaction to Neoparamoebae was characterised, the proportion of seropositive fish increased from 46% to 77% with each infection. A measurable immune titre was seen in 13% of the survivors but there was no evidence that serum antibodies provided significant protection against AGD. The aim of the final chapter was to estimate whether gill score at a population average threshold, is under common

genetic control with the required period between individual freshwater baths. The results confirm that gill score closely predicts time to bath, a simple estimation of the response to gill score selection predicts that the period between freshwater baths can be increased by up to 9.3% per generation.

Together, these data have established that genetic variation for resistance to AGD exists and that this can be effectively measured by gill score. This non-destructive measure can be applied over reiterative rounds of AGD development and is closely linked to the required bathing frequency. Phenotypic trends and genetic parameters indicate that the nature of resistance changes after first infection. However, there is no evidence that the acquisition of resistance to AGD is linked to the development of serum antibody to Neoparamoebae.

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Explanatory note regarding thesis structure

This thesis is structured as a series of separately published manuscripts. Each research chapter comprises an already peer reviewed and published manuscript, with the exception of Chapter 6 which is in preparation for eventual submission. In consequence, some unavoidable textual overlap occurs, especially in the Introduction and Materials and Methods sections of the research chapters. The referencing style of this thesis follows the style of Aquaculture (Elsevier).

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1 : General Introduction

1.1 Atlantic salmon aquaculture in Tasmania

Aquaculture production accounts for almost 50 percent of the world's food fish and is perceived as having the greatest potential to meet the growing demand for aquatic food. World aquaculture production during 2004 was estimated at 59.4 million tonnes (FAO, 2007). International output of farmed salmon has increased rapidly from 500 tonnes in 1970 to over 1.3 million tonnes in 2005 (Liu and Sumaila, 2008) with over 85% coming from the top four producing nations (Norway, Chile, Scotland and Canada). This remarkable increase in salmon aquaculture has been achieved due to growing global markets, expansion of culture locations, improved productivity, enhanced husbandry and health management, development of new technologies and selective breeding for improved stock performance.

Atlantic salmon (*Salmo salar,* L.) are not native to Australia. They were first introduced to Tasmania in the 1800's but fish released into the River Derwent failed to reappear as breeding adults. In the mid 1960's, three individual imports of 100,000 ova from the River Philip, Nova Scotia, Canada were introduced to a landlocked freshwater hatchery at Gaden, New South Wales (Ward et al., 1994) as part of the Snowy Mountains hydroelectric power scheme. In the late 1960's the Commonwealth Government banned all imports of salmonid genetic material in order to prevent exotic diseases entering Australia. A Tasmanian Atlantic salmon stock was developed from three individual transfers of ova from Gaden in 1984 (115,000), 1985 (180,000) and 1986 (275,000) to the newly established Salmon Enterprises of Tasmania P/L (SALTAS) hatchery at Wayatinah. Through the 'Salt-Water Salmonid Culture Act 1985', SALTAS was granted a 10-year moratorium on the production of smolt. This monopoly enabled SALTAS to determine price levels on smolts that would create a sufficient surplus for funding research and to encourage the development of marine farming. Commercial culture commenced during 1985 with an initial harvest of 53 tonnes in 1987 (Munday et al., 1990). Tasmanian production of marine cage farmed salmon and trout has increased rapidly to reach 24,248 tonnes (AU $291 million) in 2008 (ABARE, 2009), accounting for 95% of Australia's salmonid production. The majority of Tasmania's marine farms

are located in the South East in the Huon River, Port Esperance, D'Entrecasteaux Channel and Tasman Peninsula. Other production sites are located in Macquarie Harbour on the West Coast, and in the Tamar estuary on the North Coast. Rainbow trout (*Oncorhynchus mykiss*, Walbaum) production occurs predominantly in Macquarie Harbour. Salmonid farming, processing and associated industries (feed, nets, cages, packaging and transport) provide significant employment in the State.

Although free of exotic bacterial and viral diseases, Tasmanian produced salmon are affected by a proliferative gill condition named amoebic gill disease (AGD). The disease is estimated to add up to 20% to the production cost (Munday et al., 2001) due to the reiterative need to monitor and treat the fish and the associated reduction in feed efficiency and growth. Key industry approaches to potentially minimise the impact of AGD include improved husbandry, development of AGD vaccine and selective breeding for disease resistance.

1.2 Amoebic gill disease

1.2.1 Historical background of AGD in Tasmania

Soon after commencement of marine caged culture of Atlantic salmon in Tasmania, the industry suffered devastating losses of fish (Munday et al., 2001). Clinical signs of respiratory distress and lethargy manifested as rising to the surface and increased rate of opercular movement (Kent et al., 1988; Munday et al., 1990). A severe mucoid branchialitis was observed upon the gills of afflicted fish and large numbers of an amoeboid protozoan were noted upon wet mounts of affected gill tissue (Munday, 1986). Mortalities of up to 10% per week occurred in affected Atlantic salmon and rainbow trout, with repeated outbreaks occurring during periods of elevated water temperature (> 15°C), possibly associated with net hygiene and crowding. As the industry grew and hatchery development of out-of-season and marine-premolt allowed the smolt input window to widen, AGD was no longer confined to summer months and became a year-round problem.

1.2.2 The aetiological agent

When first seen in the mid-1980's, AGD was believed to be caused by the amphizoic parasite *Paramoeba pemaquidensis* (Kent et al., 1988; Roubal et al., 1989) Due to a lack of surface microscales, the genus was reclassified as *Neoparamoeba* (Page, 1987), the major features of which included a nucleus, parasome(s) and hexagonal glycostyles. Species of marine *Neoparamoeba* are not known to have a resting stage or different life stages. Reproduction is by binary fission (Kent et al., 1988). Molecular analysis of cultured amoeba isolated from AGD affected fish using 18S rDNA supported the conclusion that *N. pemaquidensis* was the causative agent (Wong et al., 2004). Using amoeba cultured from AGD-affected gill tissues of Atlantic salmon, Dyková et al. (Dyková et al., 2005) demonstrated the presence of *N. branchiphila,* therefore it was considered possible that the disease was of mixed aetiology. However, cultured *Neoparamoeba* spp. fails to elicit AGD in Atlantic salmon (Kent et al., 1988; Morrison and Nowak, 2005; Vincent et al., 2007). More recently, analysis of 18S and 28S rDNA from gill derived ('wild-type') amoeba demonstrated that neither species is associated with gill lesions and that a new species, *N. perurans*, is the primary aetiological agent (Young et al., 2007).

1.2.3 Affected species and distribution

AGD is primarily a disease of marine cultured salmonids, affecting Atlantic salmon in Ireland, France, Chile, Spain and Norway (Rodger and McArdle, 1996; Clark et al., 2003; Steinum et al., 2008). Rainbow trout have been affected in Tasmania and France (Munday et al., 1990). The disease was first described in Coho salmon (*Onchorhynchus kisutch,* Walbaum) in Washington State and California (Kent et al., 1988) and has been seen in New Zealand Chinook salmon (*O. tshawytscha* Walbaum) (Munday et al., 2001). Amoeba associated losses of turbot (*Psetta maxima*, L.) were reported by Dyková et al. (1995) and *Neoparamoeba* spp. have been isolated from the gills of aquacultured European seabass (*Dicentrarchus labrax*, L.) and sharpsnout seabream (*Diplodus puntazzo*, Cetto) (Dyková et al., 2000; Dyková and Novoa, 2001). The majority of endemic teleosts captured around and within salmon cages in Tasmania do not appear to be susceptible to AGD, apart from an isolated case of a blue warehou (*Seriolella brama* Gűnther) reported by Adams

(2008). Douglas-Helders et al. (2002) were able to infect seahorse (*Hippocampus abdominalis* Lesson) and greenback flounder (*Rhombosolea tapirina* Gűnther) with wild-type Neoparamoebae but these animals failed to develop characteristic AGD gill pathology.

1.2.4 AGD signs and diagnosis

Microscopic examination reveals that AGD is initiated by the attachment of trophozoites to healthy gill tissue (Adams and Nowak, 2004b). Following attachment localised gill tissue changes occur, including desquamation and leukocyte infiltration within the central venous sinus. As the disease progresses, pronounced host cellular responses occur, including hyperplasia and hypertrophy of the gill epithelium and lamellar fusion, which may lead to the formation of interlamellar cysts. Progression of lesions along primary filaments most likely results from the migration of proliferating amoeba outward (Adams, 2003). In advanced lesions, an infiltration of leukocytes from the central venous sinus occurs (Adams and Nowak, 2004b). These leukocytes may be observed in close proximity to amoeba within the hyperplastic lesions (Bridle et al., 2003). Gross examination of AGD affected gill tissue reveals raised white multifocal mucoid lesions (Figure 1.1). The severity of AGD signs is dependent upon the concentration of invading trophozoites in the water column (Zilberg et al., 2001) and the development rate is faster at higher temperatures (Clark et al., 2003; Douglas-Helders et al., 2003; Adams and Nowak, 2004a).

AGD is broadly defined as gill lesions in the presence of Neoparamoebae, so full diagnosis of the disease is properly made by histological observation of the parasite in association with gill lesions (Clark and Nowak, 1999; Adams et al., 2004). The extent of AGD can be quantified by counting the percentage of gill filaments displaying hyperplastic lesions (Adams and Nowak, 2004a), but this technique is not suitable as a health management tool because it is slow, labour intensive , relatively expensive and it is destructive. Therefore, Tasmanian salmon farmers use a gross "gill score" to regularly assess the intensity and frequency of AGD signs from a random subsample of fish (n = 40) in each caged population, expressed as a

categorical scale between "clear" and "heavy". The results of each assessment are utilised to schedule proactive treatments. Reactive treatments may occur when gill scores are returned that are significantly higher than the predetermined threshold or upon observation of moribund or dead fish with AGD lesions. Gill score is low-cost, non-destructive and rapid, yet because it is a presumptive assessment, the presence of *Neoparamoeba* spp. should be confirmed by alternative means. The degree of conformity between clinical signs and histological lesions in commercial infections is moderate to good (Adams et al., 2004), though it may be less reliable in less severe cases (Clark and Nowak, 1999). It should be noted, however, that some disagreement may occur because histological diagnosis normally uses a single gill arch whereas gill score represents all eight gill arches (16 hemibranchs). Although histology remains the "gold standard" for confirmation of the presence of Neoparamoebae, the recent development of a *N. perurans* PCR test by Young et al. (2008b) lends itself to future development as a rapid test to confirm and quantify the presence of the parasite.

Figure 1.1 Gross gill inspection of heavily AGD affected Atlantic salmon, showing typical raised white mucoid spots, which have begun to coalesce into larger patches.

1.2.5 Commercial treatment of AGD

Following early observations that AGD gill lesions respond to towing to brackish water areas, freshwater bathing became recommended as the standard treatment for the disease (Foster and Percival, 1988). Commercially, this process involves the insertion of a large tarpaulin liner into an empty netpen and pre-filling it with freshwater, crowding the AGD affected fish, removing them from the donor pen by lifting or pumping, dewatering to prevent transfer of saltwater into the bath and then holding the population in oxygenated freshwater for a minimum of two hours. The liner is then removed, leaving the freshly bathed fish in a clean net. When initially developed, liners were typically 40m circumference, holding 200 t of water and 10 t of fish. Nowadays they are 120 – 160 m, holding 3 - 5 megalitres of freshwater and 180 t of fish. Bathing can occur 8 – 12 times for each pen of fish over a 15 - 18 month production cycle, this reiterative process is labour intensive and requires expensive and sophisticated equipment. The efficacy of freshwater bathing is highly dependant upon zero salinity being maintained, so low level ingress of saltwater can render a bath ineffective. Cameron (1993) recommends 4 ppt as the upper acceptable salinity limit, though in practice fish farmers today are unlikely to proceed with treatment if liner salinity exceeds 2 ppt. Limited sites for large dams, coupled with recent research suggesting that soft freshwater (< 40 mg l^{-1} $CaCO_3$) is more effective at clearing AGD (Roberts and Powell, 2003) has prompted the construction of large piped reticulation projects to supply riverine freshwater to farms in the Huon and Port Esperance.

Freshwater treatment has been shown to break up and remove mucus from the gills (Zilberg et al., 2000; Munday et al., 2001; Roberts and Powell, 2003), significantly reduce viable gill amoeba numbers and to cause shedding of hyperplastic tissue to assist in the resolution of gill lesions. A small proportion of viable amoebae may remain immediately post bathing (Clark et al., 2003; Roberts and Powell, 2003) and be involved in reinfection, though reinfection is primarily through trophozoite settlement on healthy gill tissue which can begin within 12 – 24 hours post treatment (Zilberg et al., 2000; Adams and Nowak, 2004a). Remaining areas of gill damage appear to exclude recolonisation by trophozoites (Adams and Nowak, 2004a), though

rapid epithelial repair soon renders them as suitable for reinfection as healthy gill tissue.

1.2.6 Host response to *N. perurans*

Different terms may be used to describe disease resistance; an animal may be resistant to the causative agent of the disease (it is less susceptible to infection) or it may be tolerant to the presence of the causative agent (it is clinically less affected by infection) (Gjøen et al., 1997). AGD is essentially a prolific gill disease in response to the presence of *N. perurans*. Therefore, resistance to AGD, measured by improved survival or reduced gill score, may include elements of resistance to the parasite or tolerance to its presence. In this thesis, the term *AGD resistance* refers to lower gill pathology which may include aspects of low susceptibility or high tolerance. Similarly, *AGD infection* refers to the initial infection with a parasite and subsequent host response that occurs with each wave of disease development following marine input or freshwater bathing.

Anecdotal evidence from commercial fish farms indicates that salmon that have been at sea for some time develop AGD signs at a slower rate than younger or more naïve fish and that some gills may remain remarkably clear of gross lesions. This resistance appears to be relative and may be easily overcome by excessive challenge (Munday et al., 2001).

Host defences against infection are classically differentiated into innate and adaptive (acquired) immunity though both are interconnected. A key feature of innate immunity is the ability to limit infectious challenge rapidly after recognition of pathogens as 'non-self', while adaptive immunity is characterised by the specificity of reaction to parasite antigens (Detilleux, 2001; Alvarez-Pellitero, 2008). Innate and adaptive responses are not independent pathways but mutually depend on one another, the adaptive immune system relies upon excitation of the innate system but can also regulate innate responses (Wegner et al., 2007). To date, the work conducted on parasite resistance in fish, although limited, has shown promising

results (Gleeson et al., 2000; Kolstad et al., 2005; Gilbey et al., 2006) and has mainly focussed on acquired resistance following initial exposure. For example, Karvonen et al. (2005) found that innate resistance of rainbow trout to the trematode parasite, *Diplostomum spathaceum* was minimal, but that resistance improved at the second exposure by up to 89%.

Innate responses of fish to a range of ectoparasites may involve the physico-chemical characeristics of mucus, the presence of bioactive substances including lysozyme, complement, C-reactive protein, haemolysins and lectins and the migration of inflammatory cells (Jones, 2001). Experimental evidence that a proportion of naïve fish were able to survive a highly aggressive first AGD infection with relatively minor gill pathology was presented by Bridle et al. (2005). Findlay and Munday (1998) suggested that protection is associated with stimulation or activation of the nonspecific immune system. Nonspecific reactions of salmonids to Neoparamoebae include increased head kidney phagocyte chemotactic response and a suppression of phagocyte respiratory burst, but plasma lysozyme and plasma protein are not affected (Gross et al., 2005). Further evidence for the role of innate immunity to AGD comes from the reported response to immunostimulants. For example, Atlantic salmon that were injected with CpG oligodeoxynucleotides demonstrated >30% higher survival rate than controls (Bridle et al., 2003). In-feed application of β-glucans and bath application of levamisole was reported to provide some protection against AGD related mortality by Zilberg et al. (2000) yet β-glucans failed to enhance AGD protection in a later study (Bridle et al., 2005).

There is increasing evidence that resistance to AGD improves after the first round of infection and freshwater bathing (Clark and Nowak, 1999). This acquired resistance may be due to stimulation of nonspecific immune responses or due to the development of adaptive immunity. Findlay (1995) and Findlay et al. (1995) observed that a proportion of fish previously exposed to AGD developed resistance to the disease, assessed as reduced gill pathology, following four weeks in freshwater. Findlay and Munday (1998) suggested that resistance did not appear until four weeks after bathing and that a fish exposed to two waves of infection and

industry simulated bathing were more resistant than fish that had received only a single bathing. However, in the study described by Gross et al. (2004) previous exposure to wild-type *Neoparamoeba* spp. did not influence the survival or gill pathology of Atlantic salmon subsequently challenged with AGD.

Evidence of a protective adaptive immune response to *N. perurans* would support the development of an AGD vaccine. Early studies documenting the development of serum antibody following inoculation, experimental infection or natural infection were assessed by binding to cultured *Neoparamoeba* sp. antigen (Akhlaghi et al., 1994; Akhlaghi et al., 1996; Howard and Carson, 1995; Findlay, 1995; Gross et al., 2004). These cultured amoebae have subsequently been shown to be avirulent (Morrison et al., 2005; Vincent et al., 2007). More recently, Vincent (2006) described the development of resistance to AGD, measured as improved survival, and found that systemic serum antibody to wild-type *Neoparamoeba* spp. could be detected by Western blot in 50% of these surviving fish. In most cases, the antibody response measured by enzyme-linked immunosorbent assay (ELISA) appears to be weak (Vincent et al., 2008). A study of cultured salmon that had been at sea for 8 to 15 months confirmed that anti wild-type antibodies can be detected following reiterative AGD development and freshwater bathing. However, there is no evidence that this weak response is protective (Vincent et al., 2009). Immunisation of Atlantic salmon with wild-type *Neoparamoeba* spp. antigens administered by bath (Morrison and Nowak, 2005), or by intraperitoneal (i.p) injection (Zilberg and Munday, 2001) failed to elicit resistance to AGD.

The host inflammatory response is a common pathology of many parasitic diseases of the gill and skin (Johnson and Albright, 1992; Jones, 2001; Lindenstrøm et al., 2004; Alvarez-Pellitero, 2008) and may be important in AGD pathogenesis. Morrison et al. (2006b) examined transcriptome responses in the gills of AGD affected fish and found a down-regulation of the *p53* tumour suppressor gene and up-regulation of Atlantic salmon anterior gradient-2 (an inhibitor of p53). The suppression of cellular proliferation and the up-regulation of tumour-suppressor loci in AGD resistant animals may be an important mechanism by which they can tolerate

the presence of the parasite (Wynne et al., 2008b). The presence of Neoparamoebae on the gill also triggers a variety of gene expression changes indicative of localised immune responses in both rainbow trout (Bridle et al., 2006a) and Atlantic salmon (Bridle et al., 2006b; Morrison et al., 2007; Young et al., 2008b; Wynne et al., 2007; Wynne et al., 2008a; Wynne et al., 2008b), although these changes appear to be relatively minor and many immune-related genes do not demonstrate increased expression following AGD infection. AGD affected rainbow trout demonstrate an up-regulation of the pro-inflamnatory cytokine interleukin-1 beta (IL-1ß) and inducible nitric oxide (iNOS) in the gills at 7 and 14 days post infection (Bridle et al., 2006a). Atlantic salmon gills display significant up-regulation of IL-1ß within gill lesions but no change in iNOS (Bridle et al., 2006a; Morrison et al., 2007). Wynne et al. (2008b) found that AGD resistant salmon displayed significantly higher expression of some genes involved in immune responses (immunoglobulin light chain, CD8 and granzyme-K), but it is unclear whether these gene expression changes represent an inherently superior immune system or are a specific response to AGD. What is evident, however, is that there are many genes involved in controlling proliferative and immune reactions and that gene expression may change between first infection and subsequent rounds of bathing and reinfection.

1.3 Selective Breeding

1.3.1 Aquaculture breeding programs

Animal breeding aims to improve productivity of production traits by selection of the best parents to produce the next generation. Selective breeding aims to produce animals that are more efficient in their use of available food, land, and water resources in the culture environment (Gjedrem, 2005). Livestock breeding programs have a long and continuing record of success at making useful change in commercially important traits, yet until recently the application of quantitative genetic principles to aquaculture has been very limited. The majority of improvements in aquaculture productivity has occurred through management; improved nutrition, health, welfare, and water quality, while only about 1 to 2% of aquaculture production is based on genetically improved fish and shellfish (Gjedrem, 1997; 2000). However, in recent years there has been a rapid increase in the number of aquaculture breeding programs, which are estimated to produce an annual economic gain of AU$ 136 million in Europe alone (FABRE-TP, 2006). Establishment of selective breeding in the 1970's was crucial to the development of Atlantic salmon farming in Norway (Thodesen and Gjedrem, 2006) and the industry is now dependant upon genetically improved stock to remain competitive. Selectively bred salmon of the 8th generation are now being disseminated to Norwegian farmers, results from the 5th generation suggest a selection response of 14% per generation for growth and a correlated response of 4-5% per generation for feed efficiency (Thodesen and Gjedrem, 2006) with resulting reduction in production time and cost of production.

1.3.2 Breeding goals

Breeders aim to maximise the profitability of aquaculture by deploying genetically improved stock from breeding programs, based on the selection, crossing and testing of a range of genotypes. For breeding to be worthwhile it should augment, or be more effective than modifying production technology or husbandry practices. Before selective breeding commences, breeders should (i) define a suite of biological traits that producers wish to improve (the 'breeding objective') that accounts for all inputs

and outputs in a production system (Ponzoni and Newman, 1989; Apiolaza and Garrick, 2001) (ii) weight objective traits according to their economic importance (iii) determine if there is exploitable genetic variation in objective traits; and (iv) identify suitable surrogate ('selection traits' or 'selection criteria') that reflect the traits in the breeding objective via genetic parameters (Ponzoni, 2006) and can be used indirectly to select for objective traits. It is preferable that traits are simple and cost effective to measure, as the difficulty and cost of measurement and selection may outweigh the benefits (Refstie, 1990). In reality, it is not uncommon for breeding programs to commence before the breeding objective is defined, in which case breeders are required to estimate preliminary economic and genetic parameters based upon their understanding of underlying economic drivers and empirical data from similar breeding programs or species.

1.3.3 Variation, the basis of selection

In order to find individuals which contribute to the breeding objective, knowledge of variation of objective traits within the breeding population is essential. Economically important traits are generally quantitative, thus cannot be segregated into distinct categories. They are assumed to be regulated by a large number of genes each with a small effect on the trait. The phenotypic value is partitioned into the influences of genotype and the environment (Falconer and Mackay, 1996), where genotype is the particular assemblage of genes possessed by the individual, and the environment is all non-genetic circumstances. Only the additive effects of genes are inherited quantitatively from one generation to the next, non-additive dominance variation (interaction between pairs of alleles at single loci) and epistasis (interaction of alleles between or within multiple loci) are not heritable. Additive genetic variance is the variance of true breeding values (Falconer and Mackay, 1996) and is the chief cause of resemblance between relatives and therefore the determinant of observational genetic properties of the population and of the response of the population to selection (Gjedrem and Olesen, 2005). As true breeding values are difficult to measure, predictions of their value are used to rank animals as candidate parents. In most breeding programs, potential broodstock are not directly tested, their breeding values are estimated from performance of their relatives. Utilising the degree of relatedness of relatives (pedigree), mixed linear models are used to account

for fixed effects and calculate best linear unbiased predictions (BLUPs) of breeding values. In BLUP analysis, variance and covariance parameters (including additive genetic variance components and additive genetic covariances/correlations) are estimated using restricted maximum likelihood (REML) procedures (Gilmour et al., 2006).

1.3.4 Genetic variation of disease resistance

Heritability is the proportion of the total phenotypic variation which is genetic in origin; the higher it is, the greater the genetic response that can be expected through selection. The heritability therefore acts as an indication of the potential success of selection for that trait within the breeding population. Heritability of disease resistance is commonly measured by progeny testing so that the risk of losing potential broodstock or introducing disease into the hatchery environment is minimised. Such progeny testing may occur in controlled challenge environments or on commercial fish farms. Challenge systems offer the advantage of controlled testing but may be expensive to set up to an appropriate scale, so are limited to larger or more established breeding programs. Challenge system results have been shown to be well correlated with natural field outbreaks (Gjøen et al., 1997; Ødegard et al., 2006; Storset et al., 2007). Field challenges rely on natural disease outbreaks to occur so may be reported as opportunistic observations (Guy et al., 2006) which may be difficult to interpret because of undesirable environmental influences (Norris et al., 2008). Because these challenges occur on commercial farms they may pose additional risk to the farmed fish in nearby tanks or cages. However, field challenges may be the only option where diseases are difficult to replicate artificially (Norris et al., 2008). They are favoured by smaller or less established breeding programs because they are cheaper to establish.

Additive genetic variation of disease resistance in fish is invariably measured as survival, a complex trait that may be due to low susceptibility or high tolerance (Gjøen et al., 1997) and can be affected by many biotic and abiotic factors. Survival of challenge is suitable for a range of acute fish diseases, which are typically viral or bacterial in origin, due to the short time between the onset of easily observed disease

signs (behavioural changes, lesions) and the onset of losses. Heritabilities for disease survival in Atlantic salmon are generally low to moderate (Table 1.1). However, destructive testing limits each test fish to a single trait measure when researchers are ethically bound to produce as much knowledge as possible from each test animal (Johansen et al., 2006). Some chronic diseases, especially those caused by ectoparasites, produce a range of easily observable pathologies that may lend themselves to non-destructive categorical scoring or continuous parasite counts (Mustafa and MacKinnon, 1999b; Kolstad et al., 2005) that are often utilised by growers in order to time disease treatments. Non-destructive measurement of genetic variation of disease signs allows animals to be retested, or to undergo a range of trait measurements, thus limiting the number of test animals required and providing a more cost effective result for breeding programs.

Disease	Type	h^2 (SE)	Scale	Authors
BKD	B	0.23 (0.10)	ULS	(Gjedrem and Gjøen, 1995)
Furunc	B	0.16 (0.12)	ULS	(Gjedrem and Gjøen, 1995)
Furunc	B	0.48 (0.17)	OBS	(Gjedrem et al., 1991)
Furunc	B	0.31 (0.03)	ULS	(Gjerde et al., 2009)
Furunc	B	0.53	ULS	(Gjøen et al., 1997)
Furunc	B	0.62	ULS	(Kjøglum et al., 2008)
Furunc	B	0.59 (0.14)	ULS	(Ødegard et al., 2006)
Furunc	B	0.43 (0.03)	ULS	(Ødegard et al., 2007a)
Hitra	B	0.13 (0.08)	ULS	(Gjedrem and Gjøen, 1995)
ISA	B	0.37	ULS	(Kjøglum et al., 2008)
Vibrio	B	0.69	ULS	(Gjøen et al., 1997)
SL(C)	E	0.22	LLC	(Mustafa and MacKinnon, 1999a)
SL(L)	E	0.25 (0.07)	LLC	(Kolstad et al., 2005)
IPN	V	0.08 to 0.28	OBS	(Guy et al., 2006)
IPN	V	0.07 to 0.56	RMS	(Guy et al., 2009)
IPN	V	0.55	ULS	(Kjøglum et al., 2008)
IPN	V	0.31 (0.06)	OBS	(Wetten et al., 2007)
ISA	V	0.26 (0.03)	ULS	(Gjerde et al., 2009)
ISA	V	0.19	ULS	(Gjøen et al., 1997)
ISA	V	0.32 (0.02)	ULS	(Ødegard et al., 2007b)
ISA	V	0.32 (0.02)	ULS	(Ødegard et al., 2007b)
PD	V	0.21 (0.01)	ULS	(Norris et al., 2008)

Table 1.1 Published heritabilities of disease resistance for Atlantic salmon to various diseases Diseases : BKD – Bacterial kidney disease, *Renibacterium salmoninarum*; Furunc - Furunculosis, *Aeromonas salmonicida*; Hitra - Cold water Vibriosis, *Vibrio anguillarum;*ISA - Infectious salmon anaemia, ISAV (ISA virus); IPN - Infectious pancreatic necrosis, IPNV (IPN virus); PD - Pancreas Disease, SPDV (Salmon PD virus); SL(C) – Sea lice (*Caligus elongatus*); SL(L) – Sea lice *Lepeophtheirus salmonis;* Vibrio – Vibriosis, *Vibrio anguillarum*. Type : B - Bacterial; E - Ectoparasitic; V - Viral. Scale : OBS - Observed binary (survival); LLC – Linear

lice count; RMS – Reduced mixed model (survival); ULS - Underlying liability (survival).

1.3.5 The Tasmanian Atlantic salmon selective breeding program

Although hatchery records from the 1970's and early 80's indicate that several hundred broodstock were used each year at the Gaden hatchery, anecdotal reports of low effective broodstock numbers in the 1960's suggested that moderate genetic bottlenecks may have occurred prior to the establishment of the Tasmanian salmon population. Therefore, SALTAS followed a 'no selection' policy whereby large numbers of broodstock were crossed each year to minimise inbreeding, though in practice some selection on 'appearance' and the avoidance of outliers occurred (Elliott and Kube, 2009). A family based selective breeding program to improve production traits was first considered in the mid-1990's, but this was not supported until Innes and Elliott (2006) published evidence that sufficient genetic diversity remained within the local population compared to archived scale samples from the ancestral River Philip population.

The SALTAS Atlantic salmon selective breeding program (SBP) commenced in 2004. The breeding goal was primarily to improve resistance to AGD but included additional breeding objectives of improving growth (reduced time to harvest weight), reducing the incidence of early maturation and maintaining harvest quality traits (flesh colour and fat). Traditional family based aquaculture selection relies upon individual family tanks for the hatchery and nursery phases to ensure that pedigree is known, following which individuals from each family are tagged and mixed to a common growout unit for performance testing. Individual family tanks are expensive to set up and manage and may introduce environmental (tank) bias that is difficult to separate from genetic influences (Kinghorn, 2006). The alternative option utilised by the SBP is DNA fingerprinting, whereby equal numbers of eyed eggs from each family are mixed into a common rearing tank prior to hatching, in order to avoid confounding family genetic merit with tank effect. One year later, at the presmolt stage, individuals are each tagged with a unique passive integrated transponder (PIT) tag and a fin-clip is taken for pedigree analysis using a microsatellite multiplex. Pedigree assignment success is in excess of 96% (Elliott and Kube, 2009).

The SBP revolves around a three-year production cycle (Figure 1.2), with three cycles operating at any time. Each autumn, approximately 150 full-sib families are spawned utilising a 2 x 2 factorial design whereby each sire is crossed with two dams and each dam crossed with two sires. Following tagging and genotyping at 12 months of age, up to 5,000 smolt are despatched to two marine test cages (Figure 1.3) and 2,500 retained in freshwater.

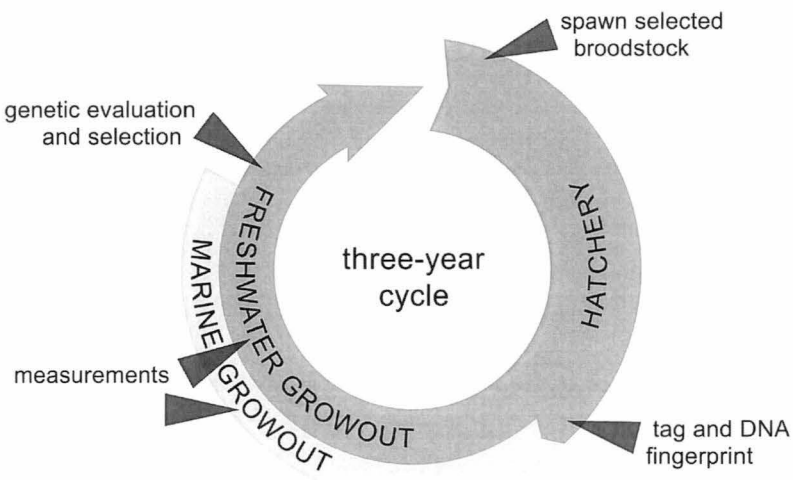

Figure 1.2 SBP year-class production cycle, showing cohort split at 14 months of age into the marine commercial growout and freshwater potential broodstock cohorts. Source : (Elliott and Kube, 2009)

Because large-scale seawater disease challenge facilities are unavailable in Tasmania and natural AGD development occurs year round, testing of sea-caged cohorts offers a cost-effective alternative for measuring a range of commercial traits through to harvest. However, due to biosecurity concerns, elite individuals cannot be returned to freshwater as broodstock. The freshwater cohort are measured for growth, maturation and some quality traits to establish genetic correlations with their marine siblings, but their primary function is as potential broodstock based upon family breeding values.

Figure 1.3 SBP marine cohort assessment unit, showing two 10 m x 10 m cages with central walkway, automated feeders and predator protection. This pen layout allows fish to be anaesthetised and assessed on the walkway platform before transferring to the second empty cage.

1.4 Aims of this thesis

The success of salmonid genetic improvement overseas indicates that it will be possible for the SBP to create gains in the Tasmanian industry. Identification of a genetic basis for resistance to a number of important fish diseases provides cause for optimism that the trait with the highest selection rating, resistance to AGD, will be improved through selective breeding. Although complete resistance to AGD is the ultimate goal, the initial focus will be to reduce the incidence of freshwater bathing with a view to reducing the cost of production.

The main aims of this thesis were to:

- Establish whether genetic variation of AGD resistance exists in the Tasmanian Atlantic salmon population. Results are presented in Chapters 2, 4 and 6.

- Compare the non-destructive gill score utilised by industry with some more rigorous destructive tests in order to assess whether gill score provides a robust

and cost effective selection trait for the SBP. Results are presented in Chapters 2 and 4.

- Examine phenotypic gill sign trends and serum antibody levels of tagged individuals, over a number of AGD development and bathing cycles, in order to examine the development of resistance and the potential development of protective antibody responses to wild-type Neoparamoeba spp.. Results are presented in Chapters 3 and 5.

- Examine the relationship between the selection trait (gill score) and the objective trait (increased period between freshwater baths in a production cycle). Chapter 6 compares genetic parameters of gill signs within the population at advanced average gill score against the required individual freshwater bath frequency.

2 : Genetic variation of resistance to amoebic gill disease in Atlantic salmon assessed in a challenge system

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2.1 Abstract

Amoebic gill disease (AGD) is the most serious health problem affecting the culturing of Atlantic salmon in Tasmania. A selective breeding project is selecting for AGD resistance amongst other important commercial traits. This paper presents the first assessment of genetic variation associated with resistance or susceptibility to AGD, and reports on a comparison of three measures of AGD infection leading to a recommended breeding trait measure. Naïve smolts from 30 full sibling families were communally challenged with *Neoparamoeba* spp., the agent of AGD, in a 19 day challenge in three replicate tanks. At termination of the challenge all fish were euthanased and the level of infection on the gills assessed through a gross gill score, histopathology and image gill score. Broad sense heritability estimates varied between 0.16 ± 0.07 for gross gill score and 0.35 ± 0.10 for image gill score. A high genetic correlation between image gill score and histopathology score was observed ($r_g = 0.90$) suggesting these are measurements of the same trait. A lower genetic correlation was observed for gross gill score with image gill score/histopathology ($rg = 0.65$). This study suggests a moderate proportion of genetic variance in AGD resistance exists within the Tasmanian Atlantic salmon population and provides good scope for selective breeding for AGD resistance.

2.2 Introduction

Amoebic gill disease (AGD) is the most significant disease affecting the culture of Atlantic salmon (*Salmo salar*) in Australia (Munday et al., 2001). At present the only successful treatment available is freshwater bathing (Powell et al., 2001). However, this process adds significant production costs for the industry due to its effect upon fish performance and the additional labour and freshwater requirements. Therefore, breeding for resistance (as measured by reducing the severity of infection or lowering the number of freshwater baths) has been identified as a high priority by the industry. This study is the first to examine whether there is any genetic basis to resistance to AGD.

AGD is believed to be caused by the protozoan parasites *Neoparamoeba* spp., which acting as the primary pathogen, infests the gills of marine cultured Atlantic salmon (Dyková et al., 2005). Clinical signs of AGD include lethargy, respiratory distress, rapid opercular movement, and ultimately, if not treated, death (Munday et al., 1990). Grossly, AGD is observed as white multifocal patches on the gills with an excess of mucous around the gill tissue (Adams et al., 2004). Histologically AGD is characterised by lamellar fusion, epithelial desquamation, oedema, epithelial hyperplasia and interlamellar vesicle formation (Adams and Nowak, 2003; 2004b). Moderate to good agreement between these gross signs of AGD and the histopathological diagnosis has been reported (Adams et al., 2004). Anecdotal evidence suggests considerable variation in AGD infection exists, quantified as both the severity of infection and survival. Indeed, Bridle et al. (2005) found considerable variation in survival following experimental infection with AGD and noted that surviving fish showed relatively minor gill pathology and even signs of possible lesion repair.

Current freshwater treatment protocols are not considered economically viable by the industry and attempts to improve bathing procedures are ongoing (Roberts and Powell, 2003). A number of alternative treatments have been investigated with some

success, including levamisole (Zilberg et al., 2000), chloramine-T (Harris et al., 2004), chlorine dioxide and hydrogen peroxide (Powell and Clark, 2003). Similarly, the use of immunostimulants such as CpG oligodeoxyncleotides have also shown some promise (Bridle et al., 2003).

A number of studies have reported genetically determined disease resistance in salmonids [see review by Chevassus and Dorson (1990)]. Heritability estimates as high as $h^2 = 0.48 \pm 0.17$ for resistance towards furunculosis (Gjedrem et al., 1991) and $h^2 = 0.23 \pm 0.10$ for resistance towards bacterial kidney disease (Gjedrem and Gjøen, 1995) have been reported in Atlantic salmon. Henryon et al. (2005), studying rainbow trout, found heritability for resistance to enteric redmouth, rainbow trout fry syndrome and viral haemorrhagic septicaemia to be 0.21 ± 0.05, 0.07 ± 0.02 and 0.11 ± 0.10 respectively. In general, heritability estimates for resistance to parasite infections are similar. For instance, estimates for resistance to sea lice infestation of Atlantic salmon range from $h^2 = 0.07 \pm 0.02$ (Glover et al., 2005) to $h^2 = 0.26 \pm 0.07$ (Kolstad et al., 2005).

This study was undertaken with two main aims. These were, firstly, to assess whether there was any genetic basis to AGD resistance and, secondly, to evaluate different AGD scoring methods with regard to their practicality and efficiency within a commercial selective breeding program. The three methods evaluated were histopathology, gross gill score and image gill score. Histopathology is the only method able to detect both the presence of the pathogen and resultant host response (Adams et al., 2004). However, this method may not be ideal for a selective breeding program because it requires destructive sampling and is costly and time consuming. Gross gill score is used by commercial growers to describe the prevalence and severity of spots and patches on the gills to determine the frequency of freshwater bathing (Clark and Nowak, 1999). This method is rapid and non-destructive but does not confirm the causative agent of the gross pathology. Image analysis is a novel method of photographing the gill surface in order to quantify the lesioned area. This is a destructive test that also fails to confirm the causative agent of the lesions.

2.3 Materials and Methods

2.3.1 Fish material

The Tasmanian Atlantic salmon stock originated from Western Canada and have been domesticated, but not selected, since the mid 1960's (Innes and Elliott, 2006). For this study, 30 full-sib families were randomly selected from the first generation (2004 year-class) of the Salmon Enterprises of Tasmania Pty Ltd (SALTAS) selective breeding population. Fertilization and egg incubation for each family were separate, and all families were combined in a communal tank after hatching. When fish reached the pre-smolt stage a random sample of individuals was anaesthetised, a fin-clip dissected and a PIT tag injected into the peritoneal cavity. After DNA extraction, genotyping and pedigree determination, a total of 24 pre-smolts were selected from each full sibling family and then divided into four equal groups of six fish per family and maintained in four replicate tanks at average weight 130.7 ± 41.2 g. There were no significant differences in average weight between the tanks.

2.3.2 AGD challenge design

Each group of 180 fish (30 families by 6 individuals) was transferred to a separate 3000 L seawater recirculation system, equipped with primary and biological filtration, a heat exchange and constant aeration. Temperature and salinity acclimation to 16°C and 35 ppt respectively was carried out over seven days each. Fish were fed commercial pellets at a restricted regime of approximately 1% body weight per day in a single daily feed. Each tank was then inoculated with 500 cells l^{-1} of *Neoparamoeba* spp. as described by Morrison et al. (2004). Unfortunately, one tank of fish was lost from the trial due to an equipment malfunction after inoculation, leaving 18 fish per family across three replicate tanks. Fish were monitored daily for signs of succumbing to AGD as described by Munday et al. (2001) until 19 days post-inoculation, at which point it was considered that an appropriate level of infection had occurred. At this point, all individuals were euthanased using 5 g l^{-1} Aqui-S (Aqui-S NZ Ltd, Lower Hutt, New Zealand).

2.3.3 AGD measurement

Three measures were used to quantify the level of infection by AGD on all surviving fish. These were gross gill score, image gill score and histopathology.

Gross gill score was measured using the standard industry 0 to 4 scoring method as described by Powell et al (2001). This method quantifies the gross number/coverage of white patches over all gill hemibranchs with score 0 as 'clear on gross examination' and score 4 as 'heavy' with extensive mucoid patches. All measurements were made by a single and experienced operator.

Image gill score was measured by dissecting the entire gill cage from each fish and fixing it in seawater Davidson's fixative for 24 hours. The first and second left anterior hemibranchs were dissected from the gill cage and photographed with a Canon digital camera (model EOS 350D) to produce digital images of 3456 x 2304 pixels. Light levels were not controlled beyond the cameras automatic flash, so images were sharpened and contrast increased before converting to grey scale with a standard 'blue separation' utilising Soft Imaging System GmbH analySIS® digital imaging software. Gill area was defined as a pixel area count by outlining the filament area above the arch on the visible (anterior/distal) side. With image enlarged to approximately x10, focal white spots or raised patches were interpreted as AGD lesions, which were outlined and expressed as pixel area counts. The area of gill affected by AGD was expressed as the ratio of lesion pixels to total hemibranch filament area pixels. A total of 503 fish were successfully sampled for gross gill score and image gill score.

Histopathology was performed on the second left anterior hemibranch. The gill arch was dehydrated, embedded in paraffin wax and sectioned at 5μm. Sections were stained with haematoxylin and eosin and viewed with a light microscope (Zeiss, Oberkochen, West Germany) at X400 magnification. The percentage of gill filaments (primary lamellae) displaying hyperplastic lesions were then counted for each section (Adams and Nowak, 2004a) and expressed as a percentage of the total number of useable filaments. Filaments were only counted when the central venous

sinus was visible in at least two-thirds of the total length (Adams and Nowak, 2003). Twenty three histology samples failed to meet the sampling standards, lowering the histology population to 480 fish. All sections were also examined for the presence of *Neoparamoeba* spp. to confirm clinical AGD.

2.3.4 Statistical analysis

For the genetic analysis, variance components, covariance components and correlations between gross gill score, histopathology and image gill score were calculated using the residual maximum likelihood methods of the program ASReml (Gilmour et al., 2006). Variances and covariances were calculated simultaneously by fitting the following multivariate mixed model:

$$Y_{ij} = \mu + T_i + F_j + \varepsilon \qquad (2.1)$$

Where Y is a vector of the observed values for gross gill score, histopathology (square root transformed) and image gill score; μ is the overall mean; T_i is the fixed effect of the i^{th} tank; F_j is the random effect of the j^{th} full-sib family; and ε is the residual random effect. The family and error terms included inter-trait variance and covariance matrices. The mating design did not include half-sib families and therefore additive and non-additive genetic variance components could not be separated. Consequently, only broad sense heritability could be calculated and this was done as:

$$H^2 = 2\sigma^2_f / (\sigma^2_f + \sigma^2_e) \qquad (2.2)$$

Where H^2 is the broad sense heritability; and σ^2_f and σ^2_e are, respectively, the variance components for F and ε estimated in the model above. Standard errors for heritabilities and genetic correlations were calculated routinely by ASReml. The histopathology data was transformed using a square root transformation due to the non-normality of the original data. The significance of fixed effects (tank effects)

was tested with conditional Wald F-statistics which are routinely calculated by ASReml.

Genetic correlations were not true genetic correlations because they are based on total genetic variance and not additive genetic variance. However, in this study it has been assumed non-additive effects are negligible and that these estimates are reasonable approximations of genetic correlations. This assumption is based upon data from a second and as yet unpublished genetic study of AGD gross gill scores in which half-sib families were made in a 2 by 2 mating design. In this study, non-additive genetic variance was small (less than 1% of total variation) and statistically insignificant.

To assess the variation between gill hemibranchs within fish the following univariate model was fitted to estimate variance components:

$$Y_{ijk} = \mu + T_i + F.T_{ij} + F_j + I_k + \epsilon \qquad (2.3)$$

Where Y is the vector of image gill score (both first and second left hemibranch), F and T are the random family by tank interaction effects, I_k is the random effect of the k^{th} individual within a family and e is the residual random effect (including hemibranch effects). This model was also fitted using ASReml. Phenotypic correlations were calculated using SPSS® (version 12.0, SPSS science).

2.4 Results

Pathology characteristic of AGD was observed within the sample material including hyperplasia, epithelial oedema and interlamellar vesicle formation. The presence of *Neoparamoeba* spp. was observed in all but one individual and was typically associated with the margins of hyperplastic lesions. A total of 503 fish (93.1%) survived to trial termination and AGD measurement. The final number of individuals within each family ranged from 14 to 18. Variation in all three measurement traits was observed across the sample material (Table 2.1). There were no significant differences between tanks one and two in each of the three measurements of AGD. However, tank three exhibited a significantly lower level of infection for each measurement score, the difference between this tank and the highest score being -0.3 for gross gill score, -8.6 % filaments infected for histopathology and -4.4 % area infected for image gill score. There were no tank x family interactions.

Trait	Units	N	Mean	SD	CV	Min. value	Max. value
Gross gill score	Scale 0 to 4	503	1.78	0.87	49%	0	4
Image gill score	%	503	10.07	4.91	49%	0.76	26.51
Histopathology	%	480	28.53	12.20	43%	0.00	85.45

Table 2.1 Summary statistics of measured traits (SD = standard deviation, CV = coefficient of variation).

There was significant genetic variation in AGD infection and differences in genetic variation of the different measures of AGD (Table 2.2). Gross gill score had a relatively low heritability (H^2 = 0.16) and was approximately half of the heritability of histopathology and image gill score (H^2 = 0.30 and 0.35 respectively).

Estimates of 'genetic correlations' between AGD measures are shown in Table 2.2. The correlation between histopathology and image gill score was high ($r_g = 0.90$). However, correlations between gross gill score and histopathology and between gross gill score and image gill score were lower ($r_g = 0.65$).

AGD trait measure	Heritability (H^2)	Gross gill score	Image gill score	Histopathology
Gross gill score	0.16 ± 0.07	-	0.40*	0.27*
Image gill score	0.35 ± 0.10	0.65 ± 0.18	-	0.64*
Histopathology	0.30 ± 0.09	0.65 ± 0.20	0.90 ± 0.17	-

Table 2.2 Estimates of broad sense heritability (± standard errors) for each measurement of AGD, with 'genetic correlations' (± standard errors) below the diagonal and phenotypic correlations above the diagonal (* indicates significant at $P<0.01$).

The relationship between gross gill score and the other two measures is, however, strongly and negatively influenced by one family which was notable in having the lowest average gross gill score (1.09 ± 0.77 SD). Removing this family from the analysis improved the 'genetic correlation' between gross gill score and histopathology to $r_g = 0.82 \pm 0.17$, and between gross gill score and image gill score to $r_g = 0.87 \pm 0.14$. There were no apparent reasons for the anomalies with this family.

There was high phenotypic variation between hemibranchs within individual fish and consequently measurements on different hemibranchs within the same fish were not highly repeatable. This can be seen in the partitioning of variation for image score (Table 2.3), where 55% of total variation was within fish variation. This was also

reflected in the poor phenotypic correlation of $r_p = 0.52$ between image gill score on the first and second hemibranch.

The high variation between hemibranchs affected the phenotypic correlation between histopathology (measured on a single hemibranch) and image gill score (measured on two hemibranchs). The correlation with image gill score measured on the same hemibranch was higher than that with measurements of different hemibranchs ($r_p = 0.61$ compared to $r_p = 0.48$). Combining measurements of image gill score for both hemibranchs improved the correlation with histology to $r_p = 0.63$ and has been utilised as the standard measurement in this paper.

It appears that small lesions are poorly accounted for with the gross gill scoring method. When image gill score was analysed as a simple count of AGD lesions rather than area infected, an increased phenotypic correlation with gross gill score was achieved by excluding image areas less than 1% of hemibranch area (approximately 3 mm^2). When this was done the phenotypic correlation increased from $r_p = 0.24$ to $r_p = 0.41$. However, when image gill score is expressed as an infected area there is little effect in removing the smaller lesions.

Source	Component	SE	% of total
Family	3.37	1.19	12%
Family x tank	0.45	0.65	2%
Individuals	8.47	1.22	31%
Within fish	15.32	0.97	55%

Table 2.3 Variance components for image gill score (SE = standard error)

2.5 Discussion

This work provides the first estimates of genetic variation in resistance to AGD in Atlantic salmon. These estimates are limited in that they do not estimate additive genetic variation which is the component of variation that can be captured by a breeding program. However, assuming non-additive effects are small, these results suggest sufficient variation exists within the population to consider inclusion of AGD resistance as a breeding program trait. All three methods of measuring the level of infection demonstrate significant genetic variation. Estimates of 'genetic correlations' between the AGD measurements were moderate to high, suggesting that gross gill score, histopathology and image gill score were similar traits over the duration of the trial. The moderate heritability estimates we have observed in this study suggest that it may be possible to increase resistance to AGD in the Tasmanian Atlantic salmon population through selective breeding.

The 'genetic correlation' between image gill score and histopathology was high in contrast to that between these measures and gross gill score. A major source of disagreement between the gross gill score and image gill score/histopathology may be attributed to the fact that gross gill score examines all gill hemibranchs (16 in total) whilst histopathology and image gill score only examine one and two hemibranchs respectively. Significant variation in image gill score was observed between the first and second left anterior hemibranchs within this study. Interestingly, the phenotypic correlation between gross gill score and image gill score was greatest when the two hemibranchs were considered as a composite within the image gill score, rather than a single hemibranch. Similarly, the phenotypic correlation between histopathology and image gill score also increased when the two hemibranchs were utilised for the image gill score. These results suggest that increasing the number of hemibranchs within the image gill score analysis may produce a better relationship with gross gill score.

A weakness of gross gill scoring is that small lesions are often unable to be visualised by the human eye. Image analysis was interpreted at approximately x10 magnification, allowing small lesions to be included in the overall lesion area. Analysis of image gill score as a count of AGD lesions >1% hemibranch area significantly enhanced the phenotypic correlation with gross gill score, compared to counting all lesions. However, this may be less of a problem during natural field infections compared to controlled challenge trials. The challenge infection differed from typical commercial field infections in having large numbers of small lesions that were occasionally coalesced into larger, more visible groups. In contrast, gross pathology in a field infection is generally characterised by a lower number of lesions that develop into larger mucoid patches (Adams et al., 2004).

Slower developing mucoid patches are typically produced in a natural infection from migration of proliferating amoebae out from the initial point of lesion formation with amoebae colonising the outer margins of the lesion (Adams and Nowak, 2003). Experimentally, AGD gross lesion numbers increase simultaneously with the inoculating amoeba concentration (Morrison et al., 2004; Zilberg et al., 2001). The levels used in this challenge (500 cells l^{-1}) were one to two magnitudes higher than the 10-50 cells l^{-1} reported in a natural infection in a commercial fish cage (Douglas-Helders et al., 2003). Further evidence of the acute nature of infection achieved in the challenge is shown by a comparison of the level of infected filaments scored by histopathology between the challenge and a natural infection. A 19 week natural infection in a commercial cage resulted in a mean farm score of 2.1 and a histology score of 17% (Adams and Nowak, 2003), while in our 19 day experimental challenge there was a similar mean farm score of 1.8 but a histopathology score of 28.6%

Broad sense heritability estimates for the different AGD scoring methods varied. The highest estimates were obtained using image gill score ($H^2 = 0.35$) and histopathology ($H^2 = 0.30$). Histopathology is the only method able to indicate both presence of the pathogen and host response, and is considered the 'gold standard' for AGD diagnosis (Adams et al., 2004). While these results suggest image gill score and histopathology may be the most appropriate traits for selection within the context

of a challenge trial, a number of disadvantages are associated with these methods when being considered for measurements outside the challenge system. Most importantly, both image gill score and histopathology are destructive techniques and therefore are not suitable to multiple sampling experimental designs. Furthermore, histopathology and to a lesser extent image gill score are both expensive and labour intensive. Heritability estimates based on gross gill score ($H^2 = 0.16$) were considerably lower than the other scoring methods but still indicated adequate genetic variation to support selective breeding. This method is the industry standard, is non-destructive and gives an overall impression of gill health as it relies on all 16 hemibranchs. The gross gill scoring method has been shown to be reliable in the field in moderate to advanced infections (Adams et al., 2004) but is less reliable for less severe cases (Clark and Nowak, 1999).

2.6 Acknowledgements

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3 : Gill observations in Atlantic salmon during repeated amoebic gill disease field exposure and survival challenge.

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3.1 Abstract

Amoebic gill disease (AGD) of Atlantic salmon in Tasmania is proactively treated by freshwater bathing when gross gill assessment ('gill score') indicates a moderate level of disease in a cage population. This generally ensures that few fish are exposed to severe disease symptoms and that few die, but also means that a proportion of the population shows little gross evidence of AGD. Individuals exhibiting few AGD symptoms at bath may be more resistant, or simply reflect an uneven spread of the disease through the population. This study had three main aims, firstly, to determine whether all fish in a cage population eventually require freshwater treatment after first infection; secondly, to ascertain whether there is any evidence of development of resistance to AGD; and thirdly, to see if there is a relationship between the level of proliferative gill reaction to the parasite, assessed by gill score, and time to mortality when the disease is left untreated. These aims were achieved by following gill score trajectories of individual fish through three rounds of natural AGD infection and relating these to the eventual fate of the fish in a natural AGD survival challenge after the second freshwater bath. There was no evidence of complete innate resistance to AGD as each fish eventually required a first freshwater bath. There was no relationship between the rate of first infection and the ultimate survival of each fish. For the second and third exposures, significant differences ($P < 0.001$) were observed between the surviving fish and those that died in the challenge. Individual gill scores at the latter measurements were suggestive of development of resistance to AGD. Mortality during a natural summer AGD challenge in an un-bathed population of fish, that had two previous treated exposures to the disease, was 67.7% and gill symptoms at the onset of losses accurately predicted the rate of mortality.

3.2 Introduction

Atlantic salmon have been farmed in sea cages in Tasmania, Australia since the mid 1980's and production has grown to 23,600 tonnes, worth AU$272 million in 2007 (ABARE, 2008). The most serious disease threat to the industry is amoebic gill disease (AGD), caused by an infection of fish gills by the protozoan ectoparasite *Neoparamoeba perurans*, see Young et al. (2007). AGD is not peculiar to Australia and has been reported to affect several temperate cultured teleost fish species around the world (Kent et al., 1988; Nowak et al., 2002; Munday et al., 2001). It is becoming more prevalent, possibly associated with higher water temperatures (Steinum et al., 2008). Mortality rates of 25 - 30% were recorded in Tasmanian salmon farms in the mid-1980's (M. Hortle, pers. comm.) and losses of between 12 - 82% have recently been reported from four Norwegian Atlantic salmon farms that had not previously encountered the disease (Steinum et al., 2008).

Colonisation of the gills by this parasite causes proliferative cell change reactions, including severe epithelial hyperplasia, hypertrophy, oedema and interlamellar vesicle formation (Adams and Nowak, 2001; 2003; 2004b; Adams et al., 2004). This can be seen grossly as the formation of white mucoid spots and plaques on the gill surface. Full diagnosis of AGD requires histopathology to confirm the presence of the parasite associated with damaged gill tissue (Adams and Nowak, 2001; 2003). However, the commercial producers utilise a categorical field evaluation of 'gross gill score' (hereafter termed 'gill score') that describes the extent of visible white patches on a scale of 'clear' to 'heavy' (Table 3.1) to schedule proactive freshwater bath treatments. In advanced infections, this presumptive scoring method is known to have a moderate to good agreement with histopathological diagnosis (Adams et al., 2004) but is less reliable for less severe cases (Clark and Nowak, 1999). Gill score is a gross measure of the degree of host response to the presence of *N. perurans*. The degree of lesion development is known to be in direct proportion to the infective parasite concentration and progression of the infection (Morrison et al., 2004). The primary infective role of *Neoparamoeba* spp. was confirmed by Adams and Nowak, (2004a) who demonstrated that trophozoite settlement occurs only on healthy gill

tissue. Subsequent lesion development and progression is then dependant upon proliferation and migration of amoeba along the filamental regions. The formation of hyperplastic lesions may be indicative of a fortification strategy adopted by teleosts against gill ectoparasites (Adams, 2003). Larger lesions are characterised by squamous epithelial and mucous cell stratification and are rarely colonised by trophozoites (Adams and Nowak, 2001). Filament regions with fully fused secondary lamellae deny trophozoites the opportunity to exploit interstitial mucous layer between lamellae. However, lesion margins exhibit concentrated trophozoite attachment. As larger AGD lesions develop, they coalesce and periodically slough away mucus and hyperplastic epithelium containing trophozoites, so the relationship between lesion area and parasite numbers presumably changes. It is conceivable that disparity between lesioned area and parasite mass could occur where the host is able to reduce parasite numbers with little concurrent lesion healing, or that hosts may vary in their proliferative response to the presence of *N. perurans*. However, the extent of AGD lesion generally reflects the degree of *N. perurans* infection. Indeed, preliminary evidence from Young et al. (2008a), utilising a PCR technique for the detection of *N. perurans*, demonstrated that the degree of amplification was consistent with the severity of AGD identified by histopathology of six fish from a first infection challenge, suggesting that the gill pathology reflects the degree of parasitism.

Infection Level	Gill Score	Gross Description
Clear	0	No sign of infection and healthy red colour
Very Light	1	1 white spot, light scarring or undefined necrotic streaking
Light	2	2-3 spots/small mucus patch
Moderate	3	Established thickened mucus patch or spot groupings up to 20% of gill area
Advanced	4	Established lesions covering up to 50% of gill area
Heavy	5	Extensive lesions covering most of the gill surface

Table 3.1 Gross gill score system to estimate the severity of AGD. (Adapted from Tasmanian Atlantic salmon farming company, Tassal Operations Pty Ltd).

Currently the only commercially effective treatment for AGD is freshwater bathing for a minimum of 2 hours (Powell et al., 2001). The osmotic effect of bathing removes gill mucus and gill-associated amoeba and promotes a rapid healing of gill lesions (Munday et al., 2001; Clark et al., 2003). Reinfection is primarily due to waterborne trophozoites attaching to healthy gill tissue, but may also occur from low numbers of amoebae remaining upon the gills post bathing (Adams and Nowak, 2004a). Pre-existing proliferative epithelial tissue appear to have an inhibitory effect upon trophozoite attachment (Adams and Nowak, 2004b), but these lesions heal rapidly and are then available for reinfection. The numbers of gill associated amoeba have been shown to return to prebath levels within 10 days (Clark et al., 2003). Lesion formation from reinfection typically begins between 1 and 2 weeks post bath (Adams and Nowak, 2004a). The reiterative process of freshwater bathing adds up to 20% to the cost of production (Munday et al., 2001) through increased farm infrastructure, added labour and operating costs. A typical farm of one million fish uses over 500 megalitres of freshwater in a 15 - 18 month production cycle, bathing each pen approximately 8 - 12 times (G.Purdon, Tassal, pers. comm.). With freshwater resources limited in Tasmania, bathing is not seen as a long-term solution for the industry.

The broad clinical definition of AGD is gill lesions in the presence of attached amoeba trophozoites. Therefore, resistance to AGD may include elements of both host control of the proliferative response and immune response to the parasite. Evidence of a level of innate immunity to AGD was provided by Bridle et al. (2005), who noted that a subpopulation of naïve fish exposed to a severe AGD infection were able to resist becoming heavily infected and furthermore survive the challenge. Evidence that fish with AGD that are bathed and then become reinfected appear more resistant to this subsequent infection compared to naïve fish and that resistance may increase with repeated exposures was presented by Findlay et al.(1995) and Vincent et al. (2006). This is suggestive of the acquisition of some type of response. Development of resistance to AGD may be associated with stimulation or activation of the nonspecific immune system (Findlay and Munday, 1998). Indeed, Bridle et al. (2003) demonstrated that immunostimulants could enhance the inflammatory response and increase survival to AGD challenge. There is empirical evidence for a

humoral antibody response with anti-*Neoparamoeba* antibodies measured in the serum of Atlantic salmon and rainbow trout (Findlay et al., 1995). However, they do not appear to elicit any specific protection. Gross et al.(2004) demonstrated that all fish develop a modest serum antibody activity to *Neoparamoeba* spp. when sent to sea and subjected to natural AGD infection and commercial bathing, but there was no evidence of protection. Similarly, Vincent et al. (2006) found serum antibodies in 50% of fish that had previously been exposed to *Neoparamoeba* spp.. To date there has been no systematic recording of tagged individuals over the production cycle to determine their response following multiple baths and reinfection events. Furthermore, the current laboratory-based challenge system developed to measure the immune response, and to trial AGD vaccines, relies upon survivability as the measure. This is an acute trial that is usually only run through first infection with limited capacity to simulate bathing and reinfection.

Currently there has been no definitive work linking AGD survival to gill score, the link between the measure used experimentally and the measure used practically is not known. The purpose of this study was to examine differences between individuals in their time to first bath (i.e. to determine if there is any innate resistance to AGD in the population), to track a cohort of tagged fish subjected to continual natural infection and observe fluctuations in gill score over a period of seven months as an indicator of AGD. Finally we wanted to determine whether gill score is a good predictor of survival if fish are left untreated.

3.3 Materials and Methods

3.3.1 Time to first bath trial

Mixed-sex Atlantic salmon spawned in 2006 were intramuscularly tagged with Passive Integrated Transponder (PIT) tags at the Salmon Enterprises of Tasmania Pty Ltd (SALTAS) Wayatinah hatchery in early June 2007. The fish were held in the hatchery for a further six weeks under lights (22L:2D) at ambient temperature. Once smoltified, 1830 fish (average weight 173 g ± 53) were transferred to a 10 x 10 x 8 m (800 m^3) marine fish pen in a commercially stocked lease at Tassal Operations Pty. Ltd., Dover, Tasmania on 31st July 2007 and fed commercial pellets *ad libitum.*

Following marine input, the development of AGD was monitored by fortnightly gill inspection of commercial standard subsamples (gill score of 40 randomly sampled fish) until 51 days post stocking, at which stage the remaining 1787 fish were gill scored by two experienced operators using a scale from 0 to 5 (Table 3.1). All fish of gill score 2 and above were bathed in soft riverine water (carbonate hardness and general hardness < 20 mg/l, pH 7.1) for a minimum of 2 hours and returned to the main 10 m net, while fish of low gill score (0 to 1) were returned to a 5 x 5 x 5 m (125 m^3) net suspended inside the main net. The fish in the 5m net were reassessed on a weekly basis and any individuals of gill score 2 or above were removed, bathed and returned to the main net.

3.3.2 Repeat AGD exposure and survival challenge

In a separate experiment utilising fish from the 2005 spawned cohort, 1504 mixed-sex PIT tagged Atlantic salmon (average weight 228 g ± 47) and one thousand untagged adipose clipped fish (167 g ± 38) were transferred to a 10 x 10 x 8 m marine fish pen at the Dover site on 17th August 2006. The un-tagged fish were included in the population to ensure a reasonable approximation to commercial stocking densities.

Following marine input, the development of AGD was monitored by commercial standard subsample on a fortnightly basis until a high infection rate, where in excess of 10% of the population was assessed as gill score 5, was achieved. All fish in the pen were then anaesthetised and the tagged fish weighed, measured and gill scored (Measure 1) by two experienced operators, before freshwater bathing for a minimum of 2 hours. In order to confirm clinical AGD, the second left anterior gill hemibranch was dissected from a random subsample of ten fish and fixed in seawater Davidson's fixative for 48 hours then transferred to 70% ethanol. The gill arch was dehydrated, embedded in paraffin wax and sectioned at 5 μm. Sections were stained with haematoxylin and eosin and viewed with a light microscope (Zeiss, Germany) at x400 magnification.

The redevelopment of AGD was monitored by gill inspection of 40 fish subsamples every fortnight for a further 70 days before reassessment (Measure 2) and bathing. Following the second bath there remained 1154 tagged (629 ± 246 g) and 942 untagged (518 ± 171 g) fish in the pen (Table 3.2), the reduction in tagged fish numbers being largely due to handling losses at each assessment, including 156 handling mortalities removed after bathing following Measure 2. The population was then monitored for the onset of AGD and after 50 days, when early low-level AGD losses had been confirmed by examination of mortalities, the fish were anaesthetised and the gill scores of the remaining 1105 tagged fish reassessed (Measure 3). At this stage, 274 tagged fish were removed from the population for unrelated experimental work; their selection was randomly chosen by PIT tag number and was independent of gill score. Following Measure 3 gill scoring, the 831 remaining tagged fish in the survival challenge cohort were left unbathed and the infection was allowed to continue. Mortalities were removed daily by a combination of diver and airlift retrieval and PIT tag ID recorded to monitor death date. The survival challenge trial was terminated fifty days later when losses had reached a consistent plateau, defined as mortality below 0.5% of the remaining population per day for a period of seven days. At the end of the trial all surviving tagged fish were anaesthetised and gill scored (Measure 4).

Date	Days post stocking	Survival trial day	Event	Action	No. tagged fish	Av. gill score (± SE of mean)	Av. Wt (g) (± SE of mean)
17/08/2006	0	N/A	Input	N/A	1504	-	227.6 ± 1.2
26/09/2006	40	N/A	Measure 1	Bathed	1374	3.46 ± 0.03	-
5/12/2006	110	0	Measure 2	Bathed	1310	3.27± 0.01	593.8 ± 7.2
24/01/2007	160	50	Measure 3	Not bathed	1105*	2.56 ± 0.04	897.0 ± 9.7
15/03/2007	210	100**	Measure 4	N/A	284	1.15 ± 0.07	1105.7 ± 16.7

Table 3.2 Measurements of PIT tagged fish at input and at AGD gross gill assessments before and during the survival trial. *Following Measure 3, 274 fish were randomly removed from the population for unrelated work. **The survival challenge cohort between Measure 2 and Measure 4 consisted of 880 tagged fish.

3.3.3 Animal Ethics

All animal procedures were approved by the University of Tasmania Animal Ethics Committee (Permit # A0009111) under the guidelines of the Australian Code of Practice. At all non-lethal samplings, fish were anaesthetised using 17 ppm Aqui-S (Aqui-S NZ Ltd, Lower Hutt, New Zealand). Lethal samplings were carried out at 100 ppm Aqui-S.

3.3.4 Statistical analysis

Trends in gill scores over the course of the repeat AGD challenge experiment in relation to the final fate of each fish were examined by defining 8 groups amongst the 880 survival challenge cohort individuals; 6 groups of surviving fish at Measure 4, with gill scores 0, 1, 2, 3, 4 and 5 respectively, one group of fish that died between Measure 3 and 4, and the final group of fish that died due to AGD before Measure 3. Gill scores obtained at Measures 1, 2 and 3 were tested for differences between these 8 groups (7 groups at Measure 3 as there were no gill scores for fish that died before Measure 3) by ordinal regression (McCullagh, 1980). The response variable is a gill score between 0 and 5 and the categorical predictor variable is group. Ordinal regression effectively tests for different distributions of the gill scores for different groups, whilst noting the natural ordering of the scores. We used a generalised linear model formulation of ordinal regression (McCullagh and Nelder, 1989) to fit the models.

Four individual AGD response trajectory categories were defined by the change in gill score between Measure 1 and Measure 3 as (a) Resistant – gill score at Measure 2 and Measure 3 less than or equal to one (b) Responding – gill score at Measure 3 at least two gill scores lower than Measure 1 (c) Non-responding – gill score at Measure 3 equal to or one lower than gill score at Measure 1 (d) Susceptible – gill score at Measure 3 greater than Measure 1. Where fish died prior to Measure 3 (Day 50 of trial) the category was determined by change in score between Measure 1

and Measure 2. The relationship between response trajectory category and days to death was examined.

For the 831 tagged survival challenge cohort fish alive at Measure 3, the effects of gill score, body weight and length, condition factor (Condition = weight (g) x 100/[body length (cm)]^3), and operator at Measure 3 on fish survival were assessed by Cox's proportional hazards model (Cox, 1972). The hazard of a fish is the instantaneous probability of it dying at time t, conditional upon survival to that time. The Cox model assumes the fish have a baseline hazard function that is modified proportionally by treatments and covariates, in this case the Measure 3 variables. Further, the model enables censored observations (fish that were alive at the end of the trial at Measure 4) to be included in the analysis. Maturation was not advanced through the period of the trial so the effects of sex and maturation were not considered.

Kaplan-Meier survival curves were calculated for days to death for each level of gill score at Measure 3 through to termination of the survival challenge at Measure 4. As the curves appeared to satisfy the proportional hazard assumption, differences between them were tested non-parametrically using the log-rank (or Mantel-Cox) test (Collett, 1994).

The relationships between gill score at Measure 2 and mortality due to handling at that measure, and between gill score at Measure 3 and ultimate mortality of the fish, were tested by performing the Cochran-Armitage chi-square test for trend (Armitage et al., 1994).

All analyses described in this section were performed in GenStat (GenStat for Windows, 2007)

3.4 Results

3.4.1 Time to first bath trial

The average gill score for the 'time to first bath' population at 51 days post input was 1.54 ± 0.02, with 54.5% of the population requiring bathing, having reached the treatment threshold of gill score ≥ 2 (Table 3.1). By day 77 only 0.6% of the population had not met the threshold for bathing and subsequently by 98 days post input all fish in the population had reached gill score 2. Therefore no fish was resistant to an initial natural chronic AGD infection.

3.4.2 Repeat AGD exposure

Average gill scores achieved at Measures 1 to 3 of the repeat AGD exposure experiment could be considered advanced (Table 3.2) compared to normal commercial levels that trigger a freshwater bath because we aimed for in excess of 10% of gill score 5 fish. To illustrate, at each of the measurements 1 through to 3, the proportion of gill score 5 fish was in excess of 13% (Figure 3.2) and low level mortality due to AGD had begun in the overall population. Because the disease was allowed to progress to a more severe state than during commercial production, handling losses following anaesthesia and bathing were consequently of a higher order than those normally experienced in commercial bathing. This contributed to the drop in overall numbers of fish between AGD assessments and was especially noticeable at Measure 2, where 11.9% mortality (156 fish) occurred following handling and bathing. The handling loss was positively correlated with gill score ($r = 0.59$; Cochran-Armitage chi-square = 34.35; d.f = 1; $P < 0.001$). However, this was due only to higher mortality (25%) of fish with gill score 5. Fish with gill scores 0 - 4 had 8% mortality, with no significant increase with gill score (Cochran-Armitage chi-square = 0.48; d.f. = 1; P = 0.487) and no significant difference between scores of 0 - 4 (chi-square = 4.32; d.f .= 4; P = 0.365).

Despite achieving a similar proportion of gill score 5 fish at each measure, the proportion of low scoring fish (gill score ≤ 1) increased from 0.7% at Measure 1 to 8.3% at Measure 2 and was 27.0% at Measure 3 (Figure 3.2).

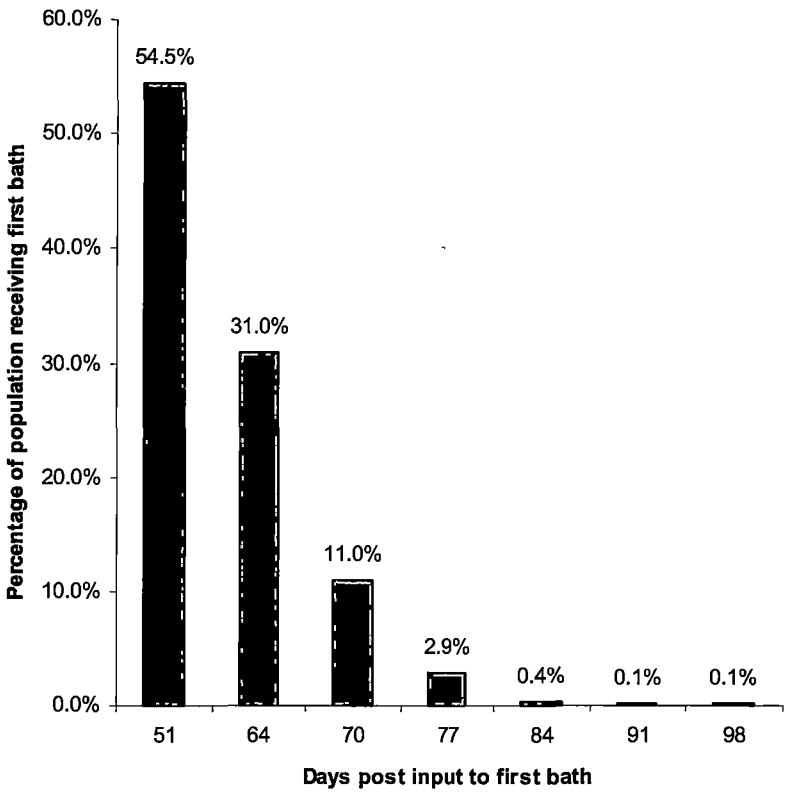

Figure 3.1 Time to bath at first infection, showing the percentage of the overall population (1787 fish) requiring first bath, i.e. with a gill score of 2 or above, at each assessment day post input.

3.4.3 Survival challenge

After adjusting for handling losses and the artificial removal of 274 fish for an unrelated experiment there were a total of 880 tagged fish available for inclusion in the analysis of the one hundred day AGD survival challenge trial that was undertaken following the second bath at Measure 2. Cumulative mortality from this bath (110 days post input) to the end of the survival trial (210 days post input) was 67.7%. Mortalities attributed to AGD began on day 141, losses then increased slowly (0.3%/day) until Measure 3 on day 160. Typical signs of morbidity included a rapid decrease in feeding response and dark, listless fish facing into the net in the upper three meters. Moribund fish exhibited characteristic gasping and 'coughing' opercular movements before rolling over and sinking to the base of the net. All mortalities and moribund fish inspected exhibited advanced macroscopic symptoms of AGD. Histopathology inspection of mortalities revealed substantial pathology including extensive fusion of lamellae; hyperplasia and hypertrophy of the epithelial cells; oedema; and interlamellar vesicle formation (data not shown).

From Measure 3 to Measure 4, the mortality rate ranged from 13.8% for fish with an initial (160 DPI) gill score of 0 to 100% for fish with a gill score of 5 (Table 3.3). Gill score at Measure 3 was highly correlated with the incidence of mortality ($r = 0.96$; Cochran-Armitage chi-square = 295.72; d.f = 1; $P < 0.001$). Survival modelling by Cox's proportional hazards model showed that the dominant predictor of survival was AGD score at Measure 3 (deviance = 597.2; $P < 0.001$). There was also a small but significant effect of fish length (deviance = 17.4; $P < 0.001$), with longer fish having a greater hazard, i.e. less likely to survive, and of fish weight (deviance = 5.4; $P = 0.020$), with heavier fish for a given length having a smaller hazard, i.e. more likely to survive. We only present the response to gill score, as the effects of length and weight are unimportant compared to the effect of gill score, seen from the relative sizes of the deviances.

Log-rank tests for equality of the six Kaplan-Meier survival curves (Table 3.3) confirmed that, when analysed in a pairwise fashion, they were significantly different

($P < 0.001$) except for the curves for gill score 0 and 1, which were not significantly different. Median time to death following Measure 3 was greater than 50 days for both gill scores 0 and 1. Fish with a moderate score (2) had a median time to death of 32 days whereas the median time to death for more highly infected fish (gill scores 3, 4 and 5) were 16, 11 and 3.5 days respectively.

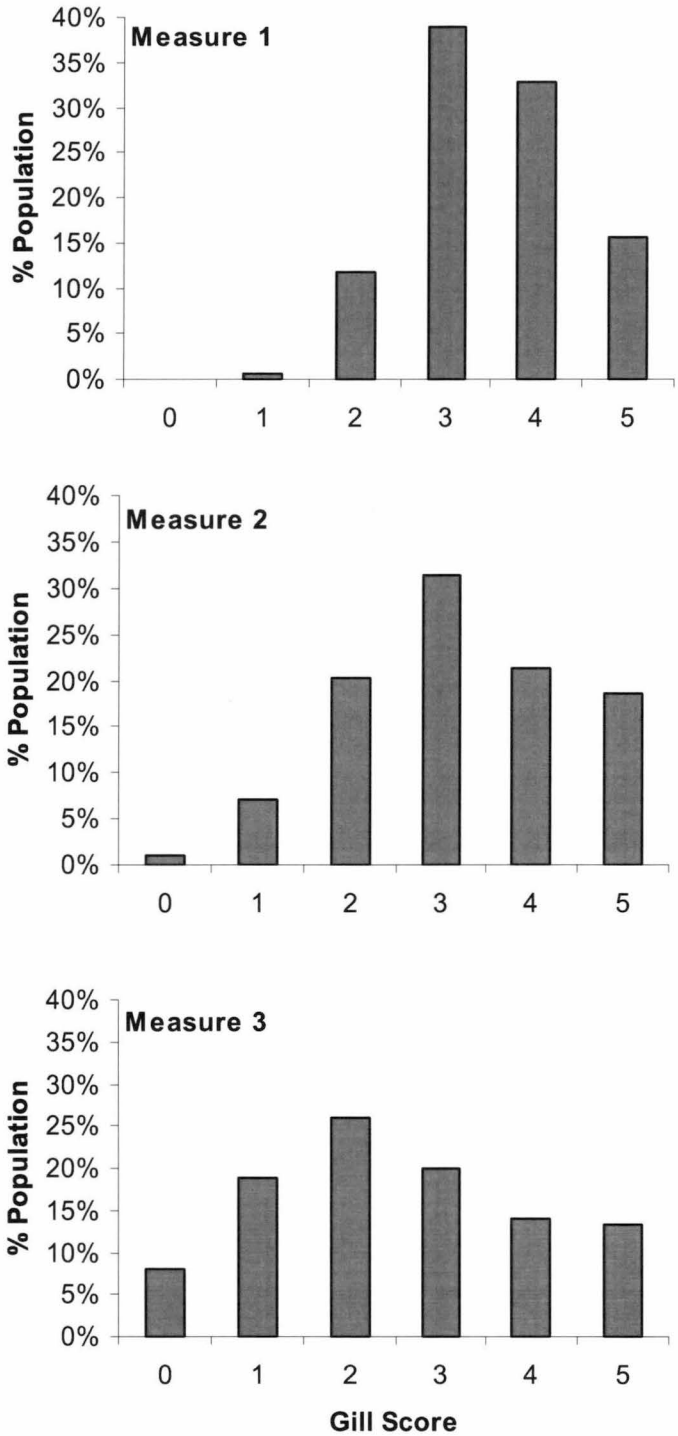

Figure 3.2 Gill score distribution in the 880 survival trial fish at each AGD measurement. Sample sizes were 869 (Measure 1), 879 (Measure 2) and 812 (Measure 3).

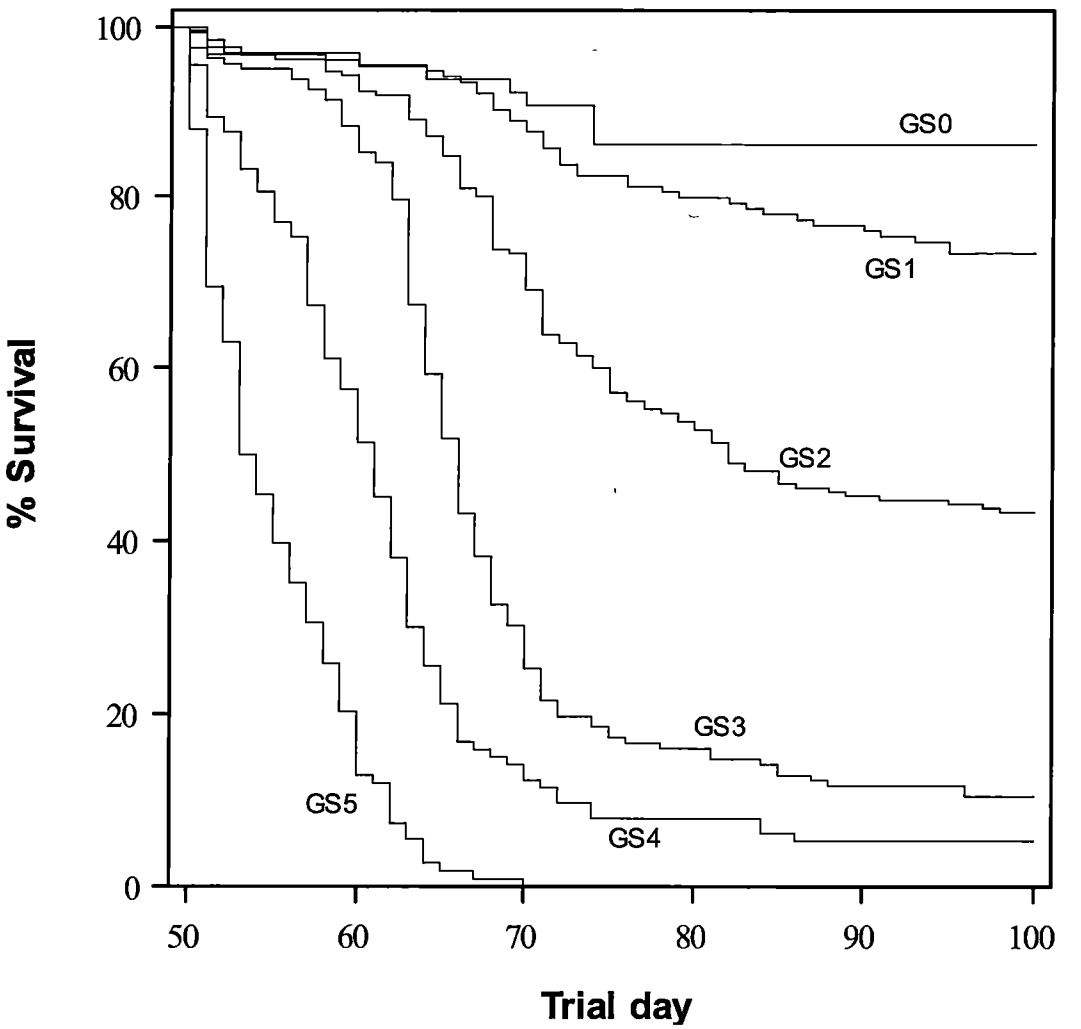

Figure 3.3 Kaplan Meier survival functions from Measure 3 (trial day 50) to Measure 4 (trial day 100) for fish with gill scores 0 (GS0 on figure) to 5 (GS5) at Measure 3. Numbers of fish for each gill score were 0 (65 fish), 1 (154), 2 (210), 3 (162), 4 (113), 5 (108).

3.4.4 Gill scores of surviving fish

By Measure 4, 72% of the remaining fish were at gill score 0 or 1 despite the lack of bathing intervention. Average gill score of the 284 surviving fish at Measure 4 was significantly lower (1.15 ± 0.07) than the Measure 3 average gill score (1.31 ± 0.06) for the same fish ($P < 0.001$) and when compared to the average for all 1086 fish alive at Measure 3 (2.56 ± 0.04, $P < 0.001$). The change in gill score from Measure 3 to Measure 4 is detailed in Table 3.3, indicating that only low numbers of the gill score 3 and 4 fish were able to reduce gross gill pathology and survive the trial. Gill score worsened in 63% of the gill score 2 fish, while 25% improved. Of the fish that were gill scored as 0 at Measure 3, 58% worsened as the trial continued, indicating that they continued to slowly develop the disease.

3.4.5 Gill score trajectories of the survival challenge cohort

Average gill score trajectories of the 880 survival challenge cohort fish are presented in Figure 3.4, grouped by survivor gill score and mortality prior to Measure 3 or Measure 4 of the survival trial. Ordinal regression showed that differences between the groups were marginally significant at the 5% level (deviance 14.2, $P = 0.047$, 7 d.f.) at Measure 1. However, by Measure 2 gill score was a significant predictor of the ultimate fate of each animal (deviance 137.4, $P < 0.001$, 7 d.f.) and the gill scores at Measure 2 of the groups that died prior to Measure 3 (GS 3.98 ± 0.16) and Measure 4 (GS 3.43 ± 0.05) were significantly higher than those of the survivors. Measure 3 was also a strong predictor of the fate of each fish (deviance 403.7, $P < 0.001$, 6 d.f); fish that died prior to Measure 4 had significantly higher gill score at Measure 3 (3.18 ± 0.06) than any of the survivors. The fish that survived the trial with gill scores of 4 and 5 only showed significantly higher gill scores at Measures 2 and 3 than the gill score 0 survivors, probably due to low numbers (12 fish) in this group. The surviving fish that ultimately had gill scores of 0 and 1 showed no difference to other fish at Measure 1, but by Measures 2 and 3 had clearly lower gill scores than surviving fish with gill scores of 2 and 3 and than both groups of fish that did not survive.

Individual infection trajectories revealed a broad range of responses. These are summarised in Figure 3.5 grouped by days to death following Measure 2. A high proportion of the fish that died early in the trial were classed as susceptible based on increasing gill score trajectories after Measure 1. These fish made up 77.8% of the population of the fish that died prior to day 40 but only 2.5% of the survivors on day 100. Conversely, in the cohort of the fish that died prior to day 40, there were no individuals that were classed as responding based on a rapid drop in gill score after Measure 1. Responding fish made up 60.6% of the population of survivors. The proportion of fish classed as resistant increased from 0% of fish that died prior to 60 days, to 14% of the survivors at day 100.

	Measure 4 distribution (percentages) across gill scores						
Measure 3	GS 0	GS 1	GS 2	GS 3	GS 4	GS 5	Dead
GS 0	41.5	38.5	4.6	1.5	0.0	0.0	13.8
GS 1	27.9	30.5	6.5	3.2	2.6	2.6	26.6
GS 2	7.1	17.6	12.4	4.8	0.5	1.0	56.7
GS 3	0.6	4.3	1.9	3.1	0.0	0.6	89.5
GS 4	2.7	0.0	0.9	1.8	0.0	0.0	94.7
GS 5	0.0	0.0	0.0	0.0	0.0	0.0	100.0
All fish	11.0	14.3	5.3	2.8	0.6	0.9	65.1

Table 3.3 Distribution of gill scores at Measure 4 for each Measure 3 gill score category and for all survival trial fish at Measure 3

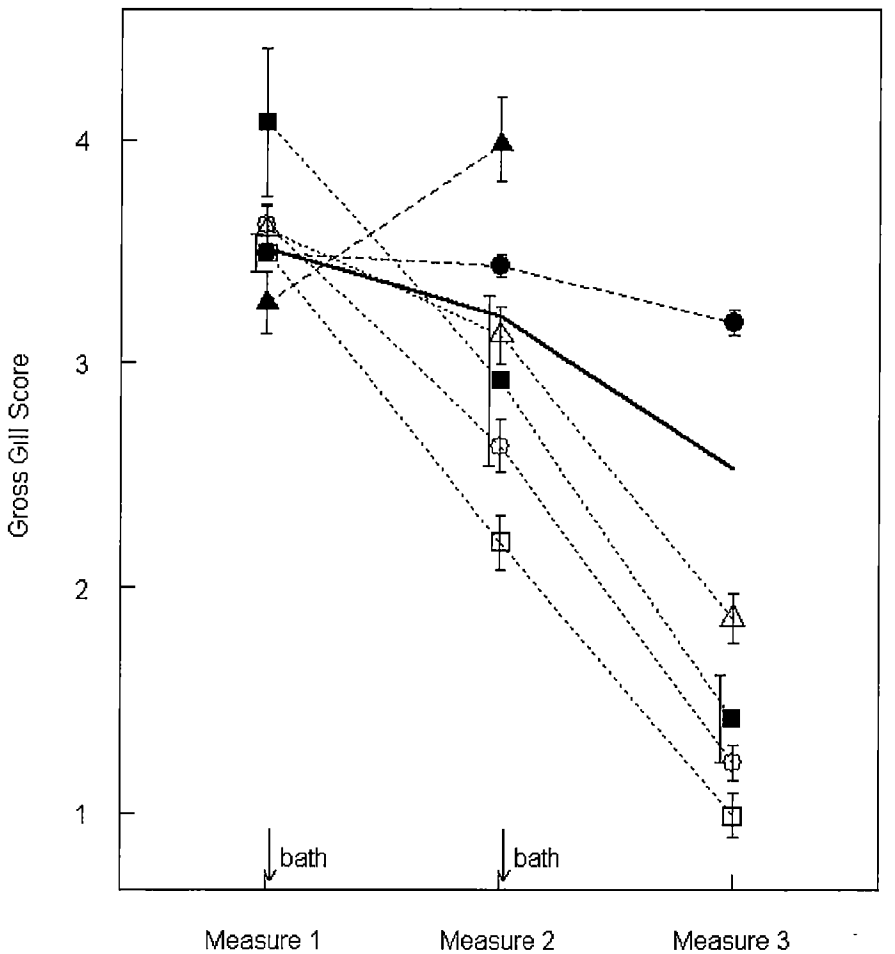

Figure 3.4 Average previous gill score history of 880 survival trial fish in 8 groups; ▲died before Measure 3 (49 fish), ● died before Measure 4 (547), □ survivor gill score 0 (90), ○ survivor gill score 1 (116), Δ survivor gill score 2 (43) and 3 (23), ■ survivor gill score 4 (5) and 5 (7). For clarity of presentation, the survivors have been grouped for gill scores 2-3 and gill scores 4-5. Population average gill scores are shown by solid black line. Points at successive measures have been joined by straight lines to indicate overall trends, however due to bathing gill scores are presumed to be zero after measures 1 and 2 and subsequently increase to the score at the next measure. Error bars are ± standard errors.

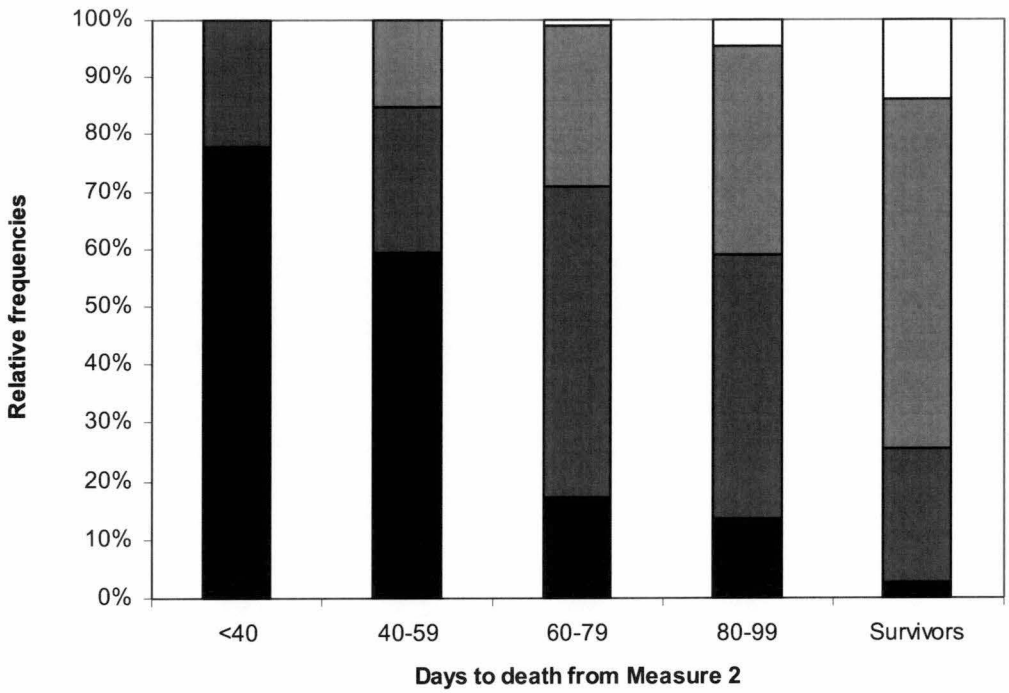

Figure 3.5 Distribution of individual AGD trajectories of four types (■ Susceptible, ■ Non-responding, ■ Responding, □ Resistant) within 5 groups based on days to death or survival following bathing at Measure 2. Numbers of fish in each group were < 40 (9), 40-59 (230), 60-79 (313), 80-99 (44), Survivors (284).

3.5 Discussion

This is the first study to explore the relationship between gill score and AGD in a cohort of tagged Atlantic salmon undergoing constant natural exposure to *Neoparamoeba perurans* in the field. Our results show that when fish are first exposed to AGD, no individuals are innately resistant as all fish eventually become infected and need bathing. The difference in individual times to first bath could be related to the spread of infection within the cage population or differing rates of susceptibility. Conversely, over a number of successive bathing and reinfection events, a proportion of the population develop resistance to AGD and this resistance is protective, as shown when the fish were left untreated. This work also confirms that gill score is an accurate predictor of the level of mortality and time to death in a natural summertime infection in fish that have previously been naturally infected with *N. perurans*.

Currently, from an economic and practical point of view, measurement of field survival to AGD is of limited value as the disease now seldom causes significant losses. However, it is not uncommon for farms to experience early low level losses to AGD, especially at the height of summer (Clark and Nowak, 1999) when the number of stocked pens is at a maximum and bathing intervals are as low as 18 - 21 days. Therefore, with knowledge of the average gill score and the frequency of high gill scores, a farmer can accurately schedule husbandry and bathing operations according to the degree of risk in each caged population.

The results of this trial provide further evidence that individual fish have the ability to limit AGD burden and that the duration or frequency of re-exposure may influence the ability to control the disease. In stating this, it must be understood that gross gill score is a measure of the fish's proliferative gill reaction to the parasite and does not directly measure parasite numbers. Low numbers of *Neoparamoeba* spp. were present on some of the gill score 0 survivors examined by histopathology, confirming that fish resistant to the disease were tolerant, but not necessarily resistant, to the

parasite. At first infection there was no significant separation of gill score of fish that eventually survived from those that died after three successive infections, thus Measure 1 was a poor predictor of the ultimate fate of each fish over successive rounds of reinfection and bathing. There was, however, a marginal separation of the fish that died early in the trial (prior to Measure 3) as the lowest gill score group at first infection (Figure 3.4), suggesting that a high degree of infection or proliferative response may be required to invoke some degree of resistance. By the second and third infections, eventual survivors had gill scores that were lower than the population average, while fish that eventually died had gill scores that were higher. Fish that eventually survived with gill scores of 0 or 1 also had significantly lower scores at Measures 2 and 3. From these trends it appears that a proportion of the population develop some form of AGD resistance with repeat exposure and that this is protective. The mechanisms of pathogen virulence and host resistance were not specifically examined in this study but there are indications in the gill score trajectories that different resistance strategies are utilised by the fish. There was little evidence of innate resistance in the population, expressed as fish that maintained gill score of clear to very light (gill score 0 - 1) at every AGD assessment. Only one fish consistently stayed within this narrow constraint. Indeed, in the "time to first bath" trial all of the fish were eventually bathed at gill score 2 or above.

The role of nonspecific immunity in resistance to AGD cannot be ruled out in our study. The innate immune or nonadaptive response of teleosts to ectoparasites is known to involve many pathways including complement, C-reactive protein, lectins, lysozyme and haemolysins and may be mediated through skin, blood or mucus. Mechanisms of innate resistance are known to increase in response to stimulation by reinfection or immunomodulators. Evidence that innate immunity may play a part in protective immune response to AGD has been suggested by a number of authors (Findlay and Munday, 1998; Zilberg et al., 2000; Bridle et al., 2003; Wynne et al., 2007). Gross et al. (2005) indicated that an increased phagocyte response may be important in innate immunity to AGD, but nonspecific humoral factors were unaffected. In our study, gross evidence of host-related hyperplasia and inflammation response was seen in all fish at first infection; these responses are known to be an important feature of nonadaptive immunity of fish to ectoparasites

(Jones et al., 2002; Buchmann and Bresciani, 1999). The innate hyperplastic reaction was shown to be critical in removing settled sea lice (*Lepeophtheirus salmonis*) copepods from salmon gills by Johnson and Albright (1992) and reduction of the gill hyperplastic reaction by cortisol implant caused increased susceptibility of coho salmon (*Oncorhynchus kisutch*).

Individual gill score trajectories identified in this study provide evidence of differing rates of development of AGD resistance with reinfection. This may indicate that the interplay of nonadaptive and adaptive responses varies within the population. Fish that had the ability to develop what we interpreted as a rapid resistance to AGD made up 5% of the survival trial population. These fish were successful at surviving three rounds of infection and the subsequent survival challenge for at least 80 days. The majority of these fish (87%) were alive at the end of the trial. There was evidence of development of a response to AGD in a further 35% of the survival challenge trial population based upon a drop of at least two gill scores between Measure 1 and Measure 3, and 55% of these responding fish survived until the end of the trial. These gill trajectories are suggestive of an adaptive response, although we are unable to demonstrate antigen specific memory within the confines of this field study. Wynne et al. (2008b), working on 28 fish that lived through our AGD survival trial, documented a large number of differentially expressed genes involved in adaptive immunity and cell cycle response and suggested that resistance to AGD is due to adaptive immune responses following multiple infection events. In this work, fish designated as resistant survivors (gill score 0) appeared to be able to control the proliferative reaction by dysregulation of the cell cycle and had an up-regulation of genes associated with adaptive immunity such as immunoglobulin light chain and MHC class II invariant chain-like protein. By comparing both resistant and susceptible groups with naïve individuals, it appears that change in cell cycle is probably a consequence of infection, rather than down-regulation in resistant animals. Conversely, the expression of transcripts involved in adaptive immunity is associated with resistance to the parasite. Functional evidence of an adaptive immune response (presence of serum antibodies) in AGD affected Atlantic salmon to *Neoparamoeba* spp. has been presented (Vincent et al., 2006; Vincent et al., 2008; Vincent et al., 2009), which occurs following exposure to multiple infections. This

antibody response is primarily to cell-surface carbohydrate antigens and, in only one documented case, a peptide epitope. Although there is qualitative evidence of an adaptive immune response via immunoblotting, antibody titres (determined by immunosorbent assays) have only been detected in two fish to date (Vincent et al., 2008). Logically, the duration or frequency of exposure to *N. perurans* may influence the development of this antibody response, with the current proactive bathing strategies employed by the Tasmanian industry possibly preventing a strong immune response by clearing infection prematurely. Evidence presented by gill score trajectories herein indicate that future work on the mechanisms responsible for the development of immunity to AGD should be targeted on fish that have been infected and bathed through at least two rounds of heavy infection.

Douglas-Helders et al. (2004) reported that prophylactic bathing gave no advantages in AGD prevalence or length of time between baths and that mean weight was significantly higher in pens that were not bathed too early. In our study, the pattern of AGD resistance exhibited by gill score trajectories of fish that survived two rounds of exposure to high average gill score and bathing, followed by prolonged survival through a natural summer infection, suggests that the degree of hyperplastic reaction at first infection is an important factor in entraining the host response to AGD. Therefore delaying bathing of newly input smolt may offer an opportunity to facilitate natural immune responses and should be further studied. Alternatively, the data presented herein, and those of Vincent et al. (2006), show that previously infected fish are more resistant to subsequent infection providing evidence that vaccination against AGD may be an avenue worth pursuing. However, most previous vaccination attempts have not provided an increase in protection against AGD (albeit in acute-to-morbidity laboratory challenge) despite the presence of serum antibodies.

We have shown that there is significant phenotypic variation in the Tasmanian Atlantic salmon population for AGD as measured by gill score in advanced infections. Variation in response to AGD increased over the first three infections indicating that different strategies of tolerance and resistance were utilised. Phenotypic variation is the cornerstone for selective breeding when it is coupled with

family information, allowing the calculation of the proportion of genetic variation within the population. Selection for resistance is likely to be successful even if the underlying mechanisms of resistance are unknown (Guy et al., 2006). Previously we have reported significant broad scale heritability for AGD resistance of 0.16 ± 0.07, measured by gross gill score at first infection, but this trial was terminated prior to subsequent bathing and reinfection (Taylor et al., 2007). Further work is in progress to assess whether genetic variation for AGD resistance exists in field infections and whether this variation increases with repeated exposures (P. Kube, pers. comm.)

In order to measure broad phenotypic variation in AGD resistance to support a selective breeding program, it is necessary to allow AGD to develop to high average gill score with 5 - 10% of the population at gill score 5 . This inevitably risks the loss of more susceptible fish from the population leading up to assessment or as a result of anaesthesia and handling. Future studies will need to examine whether adequate variability can be measured at slightly lower average gill scores, thus preserving susceptible fish for further assessment.

In summary, gill score trends of Atlantic salmon through subsequent rounds of heavy natural AGD infection, followed by a survival trial, provide evidence that : (1) There is no evidence for complete innate resistance of Tasmanian Atlantic salmon to AGD; all fish required bathing at first infection. There is, however, a range of responses at first infection that may indicate varying response or an uneven spread of the disease through the population. (2) AGD severity determined by gill score at first measure is a poor predictor of the ultimate response of each fish to subsequent infection. (3) following the first bath and through subsequent infections, segregation occurs, indicating that some fish develop a level of resistance to AGD; it is suggested that studies upon fish that have been subjected to multiple infection events will provide the best opportunity of understanding the immune mechanisms involved in AGD resistance. (4) Fish of lower gill score are less susceptible to dying from handling, and (5) AGD gill score is a good predictor of mortality rate if the disease is left untreated.

3.6 Acknowledgements

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4 : Genetic variation of gross gill pathology and survival of Atlantic salmon during natural amoebic gill disease challenge

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4.1 Abstract

Survival in an experimental disease challenge test or to natural disease challenge is utilised by aquaculture breeding programs as the selection trait for disease resistance. However, these trials are expensive and do not offer the ability to retest animals. The aim of this study was therefore to estimate genetic parameters for resistance to amoebic gill disease (AGD) measured by a categorical scale of gross gill signs ("gill score") and survival in a field challenge in order to establish whether gill score provides adequate measurement of genetic variation for AGD resistance compared to an AGD challenge survival. A total of 1504 Atlantic salmon smolt, representing 140 full-sib families, was transferred to a marine site in SE Tasmania. The gills were assessed by gill score prior to freshwater bathing on the first two rounds of infection, and then the disease was allowed to develop until mortalities began. Gill score was reassessed after 50 days and mortality was allowed to continue until it had reached a plateau at 100 days. The overall survival rate was 32.3% but varied from 0% to 69% between families. Estimated narrow sense heritability for AGD resistance assessed by gill score varied between 0.23 and 0.48 over the three rounds of infection. Heritability of AGD survival challenge was 0.40 to 0.49 on the observed scale using binary and longitudinal measures. Gill score and survival showed a weak (−0.19) to strong (−0.96) negative genetic correlation which improved when assessed closer to the survival challenge. Estimated genetic gains by selection of the top one hundred estimated breeding values for gill score indicated that up to 82% of the expected gain in survival can be achieved when compared to estimated gain by selection upon survival (days to death), thus minimising selection costs and improving fish welfare whilst allowing repeat measures to be made. The results show that genetic variation of gill score at the early onset of losses closely compares with survival results if the disease is allowed to progress without subsequent freshwater bathing. Gill score may therefore be utilised as a non-destructive and repeatable selection trait for breeding Atlantic salmon with greater resistance to AGD.

4.2 Introduction

Amoebic gill disease (AGD) is the main disease affecting marine Atlantic salmon aquaculture in Tasmania, Australia. The aetiological agent is the protozoan ectoparasite *Neoparamoeba perurans* (Young et al., 2007; Young et al., 2008c) which causes multifocal alterations in gill morphology, including severe epithelial hyperplasia, hypertrophy, lamellar fusion and interlamellar vesicle formation (Adams and Nowak, 2001; 2003; 2004b; a; Adams et al., 2004). Untreated, the disease causes inappetance, lethargy, respiratory distress, hypertension, cardiovascular compromise and eventual death (Munday et al., 1990; Powell et al., 2008) AGD is estimated to add up to 20% to the cost of production (Munday et al., 2001) due to growth loss, direct mortalities and the high infrastructure, labour and operating expenses of freshwater bathing to control the disease. The reiterative process of freshwater bathing each pen of fish eight to 12 times uses approximately 500 l freshwater per fish over the 15 to 18 month production cycle (Taylor et al., 2009b).

A recent approach to minimising the impact of AGD has been breeding for disease resistance. A prerequisite for a successful commercial selective breeding program is to establish that genetic variation of economically important traits exists. There is ample evidence that a significant genetic basis exists to resistance of many important viral, bacterial and parasitic Atlantic salmon diseases (Chevassus and Dorson, 1990; Gjedrem et al., 1991; Gjedrem and Gjøen, 1995; Mustafa and MacKinnon, 1999a; Kolstad et al., 2005; Guy et al., 2006; Ødegard et al., 2007a; Wetten et al., 2007; Kjøglum et al., 2008; Norris et al., 2008).

The characteristics assessed as a selection trait must adequately predict the objective trait and be cost effective to measure. In many aquaculture breeding programs, the selection trait for disease resistance is measured as survival to a controlled challenge or natural field infection. Lethal testing does not offer the ability to retest the same individuals. Considerable additive genetic variation in resistance to infectious finfish diseases has previously been measured through survival challenge tests in controlled

tank experiments (Gjedrem et al., 1991; Gjedrem and Gjøen, 1995; Gjøen et al., 1997; Henryon et al., 2002; Henryon et al., 2005; Kettunen et al., 2007; Ødegard et al., 2007b; Wetten et al., 2007; Silverstein et al., 2009). The advantage of these challenges is that the test environment is controlled and the host can be exposed to known quantities of a single pathogen, mortalities are easy to collect and the cause of death can be readily defined. However, due to space limitations, researchers are often curtailed in the number of animals they can trial, thus limiting the number and size of families that can be assessed. In addition, the facilities required for challenge assessment are expensive to establish and operate.

In breeding programs aimed at improving disease resistance in farmed fish, individuals and families should ideally be selected based on disease resistance in commercial production environments (Gjøen et al., 1997; Ødegard et al., 2006). Since the marine environment is an open system, field trials of aquatic animal diseases in aquaculture pens may be affected by environmental effects and non-target diseases, but are reflective of commercial infection conditions. The outcomes of disease outbreaks in the field have been shown to be highly correlated with those of tank challenge tests (Gjøen et al., 1997; Ødegard et al., 2006; Storset et al., 2007) though field genetic measures tend to be slightly lower due to higher error variance (Wetten et al., 2007). Using natural infections as a selection criterion is problematic due to a number of factors, including unpredictable timing and magnitude of infection (Kolstad et al., 2005); conversely, the biotic and abiotic stressors in the natural environment may be an essential factor in inducing typical field pathology that cannot be recreated in a tank challenge (Norris et al., 2008).

Measurement of genetic variation of disease resistance, as part of a selective breeding strategy, offers substantial economic benefits for industry and potential long-term welfare improvement for farmed fish. Although the number of test animals can be minimised in natural or controlled challenges, there are fish welfare concerns in testing fish to mortality. Researchers are ethically bound to produce as much knowledge as possible from each animal used (Johansen et al., 2006). The ability to test resistance to a field outbreak of disease using a non-destructive

assessment method would therefore offer significant cost saving and fish welfare benefits to the selection process as long as the accuracy of selection is maintained.

AGD is fully diagnosed by histopathology to confirm the presence of amoebae, containing a nucleus and symbiont parasome(s), in association with regions of hyperplastic gill (Adams and Nowak, 2001; 2003) that are formed by the host in response to the parasite. This method is destructive so is of limited value for selective breeding. The salmon farming industry utilises a simple presumptive gross "gill score" to schedule freshwater bath treatments. This categorical scale measures the prevalence and intensity of damaged gill which presents grossly as visible white mucoid spots and patches (Clark and Nowak, 1999; Adams and Nowak, 2001). This method is known to have a moderate to good agreement with histopathology in advanced infections (Adams et al., 2004) and a close phenotypic link between gill score and the level of mortalities was confirmed by Taylor et al. (2009b). Resistance to AGD, measured by variation in gill score, is presumed to relate to the degree of resistance to *N. perurans* infection, but may also include elements of host tolerance, differential exposure to the parasite or a refractory response to prior infection. Because the gill score method is non-destructive, rapid and utilised by industry to schedule freshwater bathing, it is favoured as a selection trait for the breeding program.

Evidence of varying levels of inherent resistance to AGD was suggested by Bridle et al. (2005) who noted that a subpopulation survived a severe first-infection of AGD in a challenge trial and showed relatively minor gill pathology. The first measure of genetic variation for resistance to AGD was provided by Taylor et al. (2007) reporting a broad sense heritability (H^2) of 0.16 ± 0.07, measured in a challenge-test at first infection. Resistance of Atlantic salmon to AGD after secondary exposure has previously been reported on the basis of gill pathology (Findlay and Munday, 1998). Vincent et al. (2006) presented evidence of enhanced survival of Atlantic salmon previously exposed to AGD and demonstrated that resistance is associated with systemic anti-*Neoparamoeba* spp. antibody development when compared to naïve control fish. The nature of this acquired response is poorly understood, but

future research may support development of a more specific measure of AGD resistance that can be exploited for selective breeding.

In this study, the aim was to establish the accuracy of the "gill score" as a selection trait for AGD resistance compared to survival to the disease in a natural challenge trial. Specifically, the aims were to (i) estimate additive (narrow sense) heritability for resistance to AGD assessed by gill score and survival challenge to a natural summertime AGD infection, (ii) establish whether gill score and AGD survival are under common genetic control, (iii) estimate the relative proportion of genetic gain in AGD survival that could be achieved by using selection strategies based upon different measurements of gill symptoms compared to survival data and (iv) compare relative sampling costs to the breeding program of gill score and survival challenge testing.

4.3 Materials and methods

4.3.1 Mating design, freshwater rearing and marine transfer

Broodstock (141 sires and 141 dams) were randomly selected from commercial stock as founder individuals to spawn the first generation offspring (2005 cohort) at the Salmon Enterprises of Tasmania Pty Ltd (SALTAS) Wayatinah hatchery in central Tasmania. Adipose fin samples were taken from all broodstock, stored in 95% ethanol and genotyped by a microsatellite multiplex by Landcatch Natural Selection (Scotland).

The 2005 cohort families were produced in May 2005 using a fractional factorial mating design, where each male was crossed with two females and each female with two males to create 282 full-sib families (i.e. 141 paternal and 141 maternal half-sib families). The performance of the brood fish was unknown so there was no intentional trait selection. The families of fertilised eggs were each allocated to separate egg tray compartments (two compartments per tray) and maintained there until just prior to hatching. Due to variable egg survival during incubation, 109 families were discarded, leaving 173 viable full-sib families (56 paternal and 70 maternal half-sib) from crosses between 115 males and 103 females. Eyed eggs were then transferred from each family to a communal tank to ensure a common environment for swim-up, early feeding and rearing through to pre-smolt stage under natural lighting and ambient water temperatures.

In June 2006, a random sample of pre-smolt (mean=158 g, SD=48 g) was anaesthetised, a caudal fin-clip dissected from each individual and a 12 mm x 2 mm passive integrated transponder (PIT, Sokymat, Switzerland) injected into the left flank muscle above the lateral line. Microsatellite genotyping and parentage determination was later performed on the fin-clips to allocate each tagged animal to family. The fish were held in the hatchery for six weeks under lights (22L:2D) at ambient temperature. On 17th August 2006, 1504 of these fish (mean=228 g, SD=47 g) and one thousand non-genotyped untagged adipose clipped fish (mean=167 g, SD=38 g) were transferred to a 10 m x 10 m x 8 m (800 m^3) marine fish cage on a

commercial lease operated by Tassal Operations Pty Ltd. (Dover, Tasmania). The un-tagged fish were included to ensure a reasonable approximation to commercial stocking densities.

4.3.2 AGD field challenges and subsequent survival trial

The fish were subjected to two rounds of natural AGD and subsequent freshwater bathing, followed by a further natural re-infection through to a 100 day AGD survival trial as described in Taylor et al. (2009b). A summary of the population structure at each measure is presented in Table 4.1. At **Measure 1** (40 days post smolt input to marine site (DPI)) and **Measure 2** (110 DPI), the fish were bathed in freshwater for a minimum of two hours immediately post gill assessment. Body weight and fork length were also recorded at Measure 2. The survival trial was deemed to have commenced at the completion of bathing following Measure 2; direct handling losses of 156 fish immediately following this measure were however not considered to be part of the survival trial. Soon after low level AGD mortalities had begun, fifty days into the AGD survival trial, the fish were again gill scored (**Measure 3**, 160 DPI) but not bathed.

Days post input	Event	No. fish	Av. Wt (g) ± SD	No. full-sib families	No. per full-sib family ± SD	No. paternal half-sib families	No. maternal half-sib families	Av. gill score ± SD
0	Input	1504	228 ± 47	-	-	-	-	-
40	Measure 1	1374	-	140	9.2 ± 14.1	44	57	3.5 ± 0.9
110	Measure 2	1310	594 ± 261	139	8.9 ± 13.6	44	55	3.3 ± 1.3
160	Measure 3	1105*	897 ± 322	132	7.8 ± 11.7	40	53	2.6 ± 1.5
110-210	Survival trial	880	-	75	10.9 ± 14.1	16	20	-

Table 4.1 Population and family structure of PIT tagged fish at input, AGD gill assessments and during the survival trial. (SD = standard deviation). * 274 fish from poorly represented families were removed from the population following Measure 3.

The gill score of each anaesthetised individual was subjectively measured by two experienced assessors on a categorical scale of zero (no visible lesions) to five (advanced lesions covering the majority of the gill surface) and clinical AGD was confirmed by histopathology of a random subsample of adipose clipped fish. Each AGD measure occurred when a random subsample showed at least 10% of the population was assessed as gill score 5. This level of gross symptoms is in excess of normal proactive bathing thresholds and is generally recognised as coinciding with the onset of early losses in commercial infections

At day 50 of the survival trial (Measure 3), 274 tagged fish were taken from the trial for unrelated work and to remove poorly represented families; their selection was random in terms of AGD traits. Approximately 812 un-tagged fish were retained in the pen to maintain a realistic stocking density. The infection was allowed to proceed and mortalities were removed daily by a combination of 'lift-up' airlift and diving. Dead fish were inspected to confirm the presence of gross gill lesions and PIT tag ID was recorded. The AGD survival trial was terminated after 100 days (210 DPI) when daily losses had consistently dropped below 0.5% of the remaining tagged population. At the end of the trial, all remaining fish were identified and gill scored.

All animal procedures were approved by the University of Tasmania Animal Ethics Committee (Permit # A0009111) under the guidelines of the Australian Code of Practice. At nonlethal samplings, fish were anaesthetised using 17 ppm Aqui-S (Aqui-S NZ Ltd, Lower Hutt, New Zealand). Lethal samplings were carried out at 100 ppm Aqui-S.

4.3.3 Statistical analysis

4.3.3.1 Definition of traits:

Analysis of gross gill changes was done using gill score at Measure 1, Measure 2 and Measure 3 as continuous variables measuring three separate traits. Analysis of

response to the AGD challenge during the one hundred day (110 to 210 DPI) AGD survival trial was performed using three different trait definitions:

1. Days to death (**DD**): A linear model of time (days) to death was applied to the observed continuous variable (0 to 100 days). Survivors were censored after 100 days (210 DPI) and have been scored as having died on that day.

2. Binary test-survival (**TS**): A linear model was applied to the observed binary response at median survival time (68 days, 178 DPI), where $y_i = 0$ if the fish died in the first 68 days and $y_i = 1$ if it survived beyond the 68^{th} day. An average mortality of 50% was chosen to obtain maximum variance and symmetric distribution of the observations. Genetic variation between families is expected to be at a maximum when a binary trait is terminated at about 50% mortality (Gjerde et al., 2009).

3. Survival category (**SC**): A linear model was applied to a composite ordered categorical variable with 13 categories, which incorporated information from both days to death (DD) and gill score of survivors at the end of the experiment; category 1 ($DD < 40$ days), category 2 ($40 \leq DD < 50$ days), category 3 ($50 \leq DD < 60$ days), category 4 ($60 \leq DD < 70$ days), category 5 ($70 \leq DD < 80$ days), category 6 ($80 \leq DD < 90$ days), category 7 ($90 \leq DD < 100$ days). As the surviving fish at day 100 (210 DPI) were gill scored, this score was used to further spread the survivors out with the assumption that higher gill scores carried a greater risk of subsequent mortality (Taylor et al., 2009b) such that category 8 = gill score 5, category 9 = gill score 4, category 10 = gill score 3, category 11 = gill score 2, category 12 = gill score 1, category 13 = gill score 0. The assumption of linearity for this trait is an approximation of the progression of the disease from those that died early in the challenge through to those fish that survived with low gill scores.

4.3.3.2 Analysis of fixed and random effects:

Each of the six traits was initially analysed by univariate analysis in ASReml (Gilmour et al., 2006) to determine the significance of fixed, random and regression effects of weight, length and condition factor (weight (g) x 100/[body length (cm)]^3) at the Measure 2, gill assessor at each AGD measure and time in freshwater baths at the previous bathing round. The significance of each variable was determined by

Wald F statistics in ASReml. Maturation was not advanced through the period of the trial so the effects of sex and maturation were not considered. Due to early feeding issues following smolt transfer, there was a noticeable level of fish with low condition factor evident at Measure 2, which appeared to have some effect on gill score and survival. The population was therefore divided into three Measure 2 condition factor classes (CF) of $CF < 0.85$ (10% of December population), $0.85 \leq CF < 0.95$ (13%) and $CF \geq 0.95$ (77%) to be used as a fixed effect for analyses. Although the fixed effect of CF was significant by the Wald F test ($P < 0.001$), including this factor in the model did not appreciably change the heritability estimate of each trait.

4.3.3.3 Additive genetic variation and genetic correlations:

Several papers have compared statistical analyses of challenge test data using linear and threshold models of bivariate survival, test-day survival and time until death assessed on a sire, animal or family basis on the observed and underlying scales (Henryon et al., 2005; Gitterle et al., 2006; Ødegard et al., 2006; 2007b; a; Kettunen et al., 2007; Kjøglum et al., 2008). Although the quantum of calculated heritability varies with method, these studies generally find a high correlation between EBVs or family rankings derived from the different methods of analysis. Therefore, it appears that a linear mixed model will give robust estimates providing genetic variation and disease incidence are not low.

In this study, binary data (TS), categorical (SC, Measures 1,2 and 3) and censored data (DD) were analysed as continuous data using a linear model. Binary linear models of survival are appropriate when all animals belong to the same environmental group (Kenway et al., 2008).

Variance components, covariance components and correlations between gill score (at Measure 1, Measure 2 and Measure 3) and the three measures of survival (DD, TS, SC) were calculated using the residual maximum likelihood methods of ASReml.

Variances and covariances were calculated simultaneously by fitting the following multivariate linear mixed animal model:

$$Yij = \mu + fi + aj + eij \quad (4.1)$$

where Y_{ij} is a vector of the observed values for gill score at each assessment and the three measures of survival; μ is the overall mean, f_i is the fixed effect for CF (i = 1,2,3); a_j is the random additive genetic effect of an individual and is distributed N(0, σ_a^2 A) where A is the numerator relationship matrix, and e_{ij} is the residual random effect (between fish within families). The error term includes inter-trait variance and covariance matrices. Inclusion of CF as fixed removes its effect from the data before the calculation of genetic parameters. Initial univariate analysis of each trait and bivariate analysis of each trait pairing included the random non-additive genetic effect of sire and dam interaction. In each case the non-additive effect was negligible (0% to 3.4% of total variation) and not significant, and therefore not fitted in the multivariate model.

Narrow sense heritability was estimated as the proportion of additive genetic variance to total variance as follows:

$$h_o^2 = (\sigma_a^2)/(\sigma_a^2 + \sigma_e^2) \quad (4.2)$$

where h_o^2 is the narrow sense heritability on the observed scale; σ_a^2 is the additive genetic variance; and σ_e^2 is residual variation. Approximate standard errors of variance components for random regression coefficients are automatically calculated in ASReml from the average information matrix obtained in the residual maximum likelihood procedure. Heritability of the binary variable TS was adjusted to the underlying liability scale using the method of Robertson and Lerner (1949):

$$h_u^2 = h_o^2(p(1-p))/z^2 \quad (4.3)$$

where h_u^2 is narrow sense heritability on the underlying liability scale, p is the proportion of affected individuals and z is the height of the standard normal curve at the threshold point. Heritability of the four categorical variables (SC, Measures 1,2 and 3) was adjusted to the underlying normal scale according to the method of Gianola (1979) for polychotomous characters :

$$h_u^2 = h_o^2(\sum_{i=1}^{m} a_i^2 \Pi_i[1-\Pi_i] - 2\sum_{i=1}^{m}\sum_{i<j=1}^{m} a_i a_j \Pi_i \Pi_j)/(\sum_{i=1}^{m-1} z_i[a_{i+1}-a_i])^2 \qquad (4.4)$$

where $(a_1, a_2, ...a_m)$ are the scores given to the m response categories, $(\Pi_1, \Pi_2, ...\Pi_m)$ are the m response probabilities, and z_i is the height of the standard normal curve at the boundary between category i and $i+$, *i.e.* where cumulative probability equals $\sum_{j=1}^{i} \Pi_i$.

Genetic correlations were calculated using the additive genetic components of covariance estimated by fitting the multivariate linear model with CF as fixed effect. Because genetic correlations are equal on the observed and underlying scale, there is no need to transform the calculated estimate (Ollausson and Rönningen, 1975; Gjerde and Schaeffer, 1989; Gjerde et al., 2009).

Phenotypic correlations between traits were obtained by firstly analysing each trait for the effect of CF by one-way ANOVA, then obtaining residuals which represent the traits adjusted for CF, and finally calculating correlations between the residuals. Approximate standard errors of these correlations were calculated by applying Fisher's z transformation, calculating SEs on the z scale, and back-transforming to the original scale (Fisher, 1950).

4.3.3.4 Estimation of genetic gains:

Taking daily survival under AGD challenge (DD) as the definitive standard, we examined the potential genetic gains that could be achieved based upon performance of individual fish at the three gill score assessments and by less frequent mortality retrieval during the AGD survival challenge. This was done in a three step process. Firstly, estimated breeding values (EBV) were calculated in ASReml from univariate linear animal model analyses of the 880 AGD survival cohort individuals for Measure 1, Measure 2, Measure 3, DD, TS and SC. Secondly, the fish were ranked by EBV for each trait. Thirdly, to illustrate a selection scenario producing 100 families in a mating design such as that used here, the top 100 individuals were selected for each trait and the EBV for DD for these individuals was returned. This scenario represents a selection intensity of 11.4% but does not allow for sex of the individuals or apply any restrictions on the number of fish per family that could be used for breeding to keep the rate of inbreeding at an acceptable level. The average gain of the top 100 fish was expressed as improved survival (days), converted to a percentage gain over the unselected population. Standard errors of mean improved survival were calculated from standard deviations of individual EBV's of DD for the top one hundred animals for each of the other five traits, divided by $\sqrt{100}$.

4.3.3.5 Comparison of sampling costs:

Progress for a breeding program is determined by its impact upon the breeding objective to improve economic margins for producers. This is determined by a linear combination of trait changes multiplied by their individual economic weights. However, it is also essential for the breeding program that selection traits be cost effective to assess. Therefore, relative costs of running an AGD field survival challenge with different sampling scenarios was compared with non-destructive gill score assessment.

An indicative technician and diver labour cost for a two thousand fish AGD survival trial with four sampling scenarios was calculated. The mortality rate was assumed to match the trend obtained in the current trial. The scenarios were : (1) Days to death :

diving every five days until the onset of losses at day 30 then diving and registering mortalities daily. (2) Survival 50%: diving and registering mortalities every five days until 50% loss is recorded, then registering all survivors. (3) Survival every 10 days: diving and registering mortalities every ten days, then gill score all surviving fish on the last day. (4) Gill score: diving every five days until the onset of AGD losses (~day 50), then gill scoring all remaining fish. Technicians were costed at AU $42 per man hour using two persons for registering and three for gill scoring; divers were costed at AU $60 per man hour, using a crew of three to dive, tend and supervise. Other feed and husbandry costs were considered equal and have been omitted from the calculation.

4.4 Results

4.4.1 Gill Score at Measure 1, 2 and 3

Due to the requirement to encourage a high expression of AGD, a gill score 5 was expressed in over 13% of the population at each measure and overall average gill scores were consequently high at over 2.50 (Table 4.1). Although this level of AGD is higher than normal industry bathing thresholds, it is not uncommon, occurring in 6.6% of industry data over 11 years (R.S.Taylor, unpublished data). The reduction in fish numbers between assessments (Table 4.1) was largely due to handling mortality of high gill score fish at each anaesthetised gill inspection and bathing event (Taylor et al., 2009b). It is possible that the loss of more susceptible animals at each handling could deflate subsequent estimates of additive genetic variation for AGD resistance though this may be accounted for in part by multitrait analysis. Discounting direct bath handling losses at Measure 2 and the 274 fish of poorly represented families that were removed at Measure 3, a total of 880 PIT tagged fish, representing 75 full-sib families, were subjected to the 100 day AGD survival trial between 110 and 210 DPI. Overall, mortality had reached 5.6% by Measure 3 (50 days into the survival challenge,160 DPI) and was 67.7% at termination of the trial (Figure 4.1) after 100 days (210 DPI). All mortalities were collected within 24 hours of death and displayed extensive gross lesions typical of advanced AGD. The presence of *Neoparamoeba* sp. in association with lesions was confirmed by histological observation of freshly dead fish. Environmental conditions were normal for Tasmanian summer time and do not appear to have unduly affected survival. Ambient five meter water temperatures rose from 14°C and peaked at 18°C by day 79 of the survival trial (Figure 4.1). Oxygen readings ranged between 6.4 and 8.6 mg/l (average 93.1 ± 6.1 percentage saturation).

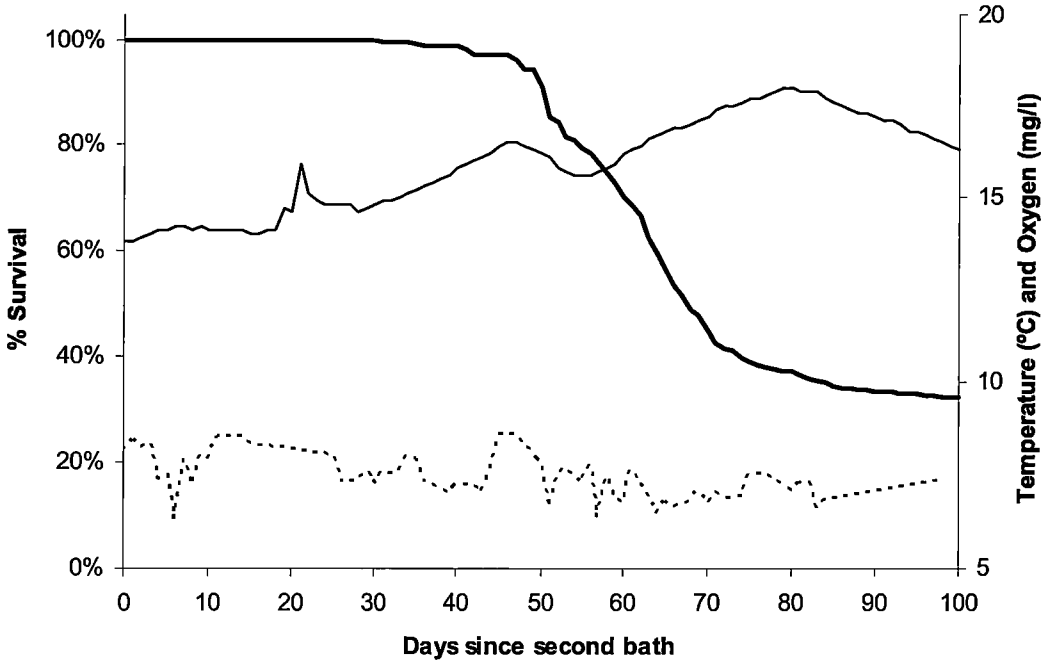

Figure 4.1 Kaplan-Meier Curve for AGD survival (—) from bathing at Measure 2 on 5 Dec 06 (day 0) to trial termination on 15 Mar 07 (day 100). Measure 3 (unbathed) was taken on day 50. Five meter water temperatures (–) and oxygen levels (--) for the period are shown on the secondary Y axis.

There were no losses attributed to phytoplankton, jellyfish, low oxygen or other fish diseases in commercial stocks on nearby leases throughout the period.

4.4.2 Heritabilities and correlations

Heritability of resistance to AGD, measured by gill score at Measure 1, 2 and 3, ranged from $h_o^2 = 0.23$ at Measure 1 to $h_o^2 = 0.48$ at Measure 3 ($h_u^2 = 0.26$ to 0.52, Table 4.2). Heritability for challenge survival (TS, SC, DD) on the observed scale was high for all three measures at $h_o^2 = 0.40$ - 0.49. After adjustment to the underlying scale, TS increased to $h_u^2 = 0.63$ and SC to $h_u^2 = 0.60$.

At the phenotypic level, Measure 1 was a poor predictor of gill score at Measure 2 ($r_p = 0.17$) and the correlation between gill score at Measure 1 and 3 and the correlation of gill score of Measure 1 with three survival traits (DD, TS, SC) was not significantly different from zero ($r_p = -0.04$ to 0.01; Table 4.3). Genetic correlations between gill score measurements were positive, being 0.63 between Measure 1 and Measure 2 and 0.66 between Measure 2 and Measure 3. However, this relationship weakened when assessments were further apart, being 0.26 between Measure 1 and Measure 3. Phenotypic and genetic correlations followed a similar pattern although the magnitude of genetic correlations was higher but with greater standard errors. Genetic correlations of AGD measures with the three survival traits (DD, TS, SC) were all negative, confirming that fish with a higher gill score tend to die earlier. This relationship becomes stronger when measured closer to the survival trial. Measure 1 had low (−0.19, −0.28, −0.23) correlations with the three survival measures. There was a moderate r_g of approximately −0.6 between Measure 2 and the three measures of survival. Measure 3, which was taken 50 days into the survival trial and thus missed the earliest 49 mortalities, was highly correlated with the survival measures ($r_g = -0.93$ to −0.96). Therefore, it appears that breeding for lower summer gill score will result in delayed losses during natural summer AGD. The three survival measures shared high positive genetic correlations of 0.96 to 0.98, indicating that the additional information gained from daily diving and registration of mortalities gave little additional benefit in measurement of genetic variation when

compared with a bivariate measure at 50% mortality or data grouped in 10 day categories with gill scored survivors.

4.4.3 Genetic gains

Estimated genetic gains in survival time from six selection strategies utilising the top one hundred fish (ranked by EBV) vary from 3% to 16% (Table 4.4). Compared to the strategy of breeding from the results of a full survival trial, selection by gill score is estimated to vary between 17% and 82% of the potential gain; this improved when the gill score was taken closer to the onset of AGD losses in the survival trial.

The effects of less frequent sampling upon the accuracy of selection are also estimated. A simple binary survival to 50% mortality provides 82% of the potential gain, while grouping samples every ten days and scoring the survivors gave an estimated 97% relative improvement.

4.4.4 Sampling costs

The assessment cost per fish in a two thousand fish trial was estimated at AU $11.80 for a full 'days to death' trial (DD), AU $3.98 for the ten day mortality grouping (SC), AU $2.98 for a binary survival (TS) and AU $2.28 for a nonlethal gill score.

Trait [a]	Va	Ve	h_o^2	h_u^2
Measure 1	0.19 ± 0.05	0.65 ± 0.04	0.23 ± 0.06	0.26
Measure 2	0.60 ± 0.13	0.94 ± 0.09	0.39 ± 0.07	0.43
Measure 3	0.96 ± 0.20	1.03 ± 0.13	0.48 ± 0.08	0.52
DD	169 ± 38	175.2 ± 24.2	0.49 ± 0.09	-
TS	0.09 ± 0.02	0.14 ± 0.01	0.40 ± 0.08	0.63
SC	6.74 ± 1.49	7.17 ± 0.96	0.48 ± 0.08	0.60

Table 4.2 Components of additive genetic variance (V_a), error variance (V_e) and heritabilities (h_o^2 = observed scale, h_u^2 = underlying scale) (± SE) for AGD measures (Measure 1, Measure 2, Measure 3) and field AGD survival challenge traits (DD, TS, SC).

[a] The traits were : Measure 1 = gill score 40 DPI, Measure 2 = gill score 110 DPI, Measure 3 = gill score 160 DPI, DD = linear days to death following Measure 2, censored at 100 days, TS = binary median test-survival, SC = survival category grouped by days to death and gill score at trial termination.

Trait [a]	Measure 1	Measure 2	Measure 3	DD	TS	SC
Measure 1		0.17 ± 0.03	0.01 ± 0.03	0.00 ± 0.03	-0.04 ± 0.03	0.00 ± 0.03
Measure 2	0.63 ± 0.13		0.42 ± 0.03	-0.36 ± 0.03	-0.32 ± 0.03	-0.36 ± 0.03
Measure 3	0.26 ± 0.16	0.66 ± 0.09		-0.69 ± 0.02	-0.66 ± 0.02	-0.66 ± 0.02
DD	-0.19 ± 0.17	-0.61 ± 0.11	-0.96 ± 0.03		0.84 ± 0.01	0.95 ± 0.00
TS	-0.28 ± 0.17	-0.57 ± 0.12	-0.93 ± 0.04	0.98 ± 0.02		0.75 ± 0.01
SC	-0.23 ± 0.17	-0.60 ± 0.11	-0.96 ± 0.03	0.98 ± 0.01	0.96 ± 0.03	

Table 4.3 Genetic correlations (r_g ± SE) below diagonal, phenotypic correlations (r_p ± SE) above diagonal. All correlations have been adjusted for the fixed effect of Condition Factor. Negative correlations of gill score measures with survival are considered favourable because they indicate that lower gill score equates to longer survival.

[a] The traits were : Measure 1 = gill score 40 DPI, Measure 2 = gill score 110 DPI, Measure 3 = gill score 160 DPI, DD = linear days to death following Measure 2, censored at 100 days, TS = binary median test-survival, SC = survival category grouped by days to death and gill score at trial termination.

Selection strategy [a]	Improved survival (days)	Improvement (%)	Relative Improvement (%)
Measure 1	2.09 ± 0.80	2.7	17
Measure 2	7.15 ± 0.60	9.4	58
Measure 3	10.09 ± 0.46	13.2	82
TS	10.03 ± 0.41	13.0	82
SC	11.85 ± 0.19	15.5	97
DD	12.27 ± 0.17	16.0	100

Table 4.4 Calculated genetic gain potential of survival time (trait average 76.7days ± 1.4 SE.) based on selection strategy of using the top 100 EBV ranked fish from TS, SC, DD and AGD Measure 1, Measure 2 and Measure 3; standard errors were calculated from individual EBV's of selected fish. Relative improvements are expressed compared to the calculated gain that can be achieved utilising breeding values for DD.

[a] The traits were : Measure 1 = gill score 40 DPI, Measure 2 = gill score 110 DPI, Measure 3 = gill score 160 DPI, DD = linear days to death following Measure 2, censored at 100 days, TS = binary median test-survival, SC = survival category grouped by days to death and gill score at trial termination.

4.5 Discussion

This study demonstrates that moderate to high genetic variation for resistance to AGD exists within the Tasmanian Atlantic salmon population, measured by both gill score and survival during a one hundred day natural summer AGD infection. Furthermore, there is a high genetic correlation between AGD gill symptoms and AGD related mortality, the most accurate measurement being obtained when gill score is taken at the outset of early losses within the same infection round. The ability to non-destructively measure genetic variation of AGD resistance by gross gill signs provides an economic and repeatable measure that mitigates the need for survival challenge assessment.

The additive genetic variation detected for AGD resistance through gill signs and mortality indicate that selection for increased resistance to AGD is possible within the selective breeding program, even though the underlying mechanisms of resistance are largely unknown. The moderate to high negative genetic correlation between gill score and the survival challenge measurements indicate that both gross gill signs and AGD survival were largely controlled by the same genes over the duration of the trial, though survival would also include elements of tolerance to the pathophysiological effects of the disease. Although relatively high levels of disease are required to accurately measure genetic variation of AGD resistance, the fish welfare implication in our breeding program is that gill score assessment provides a close estimate of survival breeding values without the need to trial fish to destruction. This finding also simplifies the breeding goal definition, in that decreased gill pathology, increased survival time and decreased bathing frequency are all inextricably linked.

In Tasmania, challenge to natural *Neoparamoeba perurans* infection and subsequent AGD development can be reliably achieved year round in marine sites, making this a robust test for our breeding program. However, survival trials in sea cages are costly and difficult to carry out because of the significant resources required to remove and

record mortalities on a daily basis. Our heritability for field AGD challenge survival on the observed scale varied between 0.40 and 0.49 in the three measures and all shared high positive genetic correlations. The survival trait was therefore also assessed on data grouped to simulate less frequent data gathering. While still recording the most susceptible fish, it is estimated that binary disease challenge to 50% mortality (TS) would reduce costs by 75% and still provide 82% of the potential gain in survival from breeding. The SC trait, which grouped the data into ten day blocks and added the relative 'risk' by gill score gave a similar result to the full 'days to death' selection scenario, reducing sampling costs by 66% while providing 97% of the potential gain. The slight differences in gain between the SC and DD models appear to be due to the additional information available from gill scoring the survivors. When we removed this additional information and simply grouped the data into ten day periods which were 'right censored' on day 100, an estimated 99.7% relative improvement in genetic gain was calculated. Our results therefore indicate that less frequent mortality removal will give a similar estimation of genetic variation in survival at a lower cost to the breeding program, though on a practical level this approach would be limited by rapid decomposition of dead fish, with some loss of PIT tags hence loss of individual fish identification.

Predicted genetic gains in survival to a natural AGD challenge through selection from gill score indicates that up to an 82% relative improvement can be achieved whilst reducing sampling costs by 81% on a single assessment. Compared to the binary survival (TS) trait, which is a widely accepted approach to disease testing in aquaculture breeding programs, gill score provided the same gains whilst costing approximately 23% less. The advantage of gill score is that AGD resistance can be assessed over multiple rounds of infection and fish are preserved for measurement of other selection traits such as growth, maturation and harvest quality. Gill score is a cheaper and simpler alternative to AGD survival challenges, with minimal loss of accuracy; it is therefore recommended as an effective option for the Tasmanian selective breeding program.

In comparison with the survival challenge approach adopted for bacterial and viral fish diseases, susceptibility to large ectoparasites is assessed non-destructively by parasite count (Bakke et al., 1999; Glover et al., 2004). Estimates of genetic variation for ectoparasitic disease resistance of farmed salmonids tend to be low. Glover et al. (2005) reported an approximated heritability of 0.074 for susceptibility of Atlantic salmon to sea lice (*Lepeophtheirus salmonis*) in a natural infection, while Kolstad et al. (2005) measured a log transformed heritability of up to 0.14 across three year classes. Both studies note that genetic variation in susceptibility is easier to measure at higher parasite intensities. Heritability for resistance to the sea louse *Caligus elongatus* was estimated at 0.22 (Mustafa and MacKinnon, 1999a) at a high infection prevalence and intensity. Our study of resistance to AGD differs in using an index based on the degree of reaction of the host to the parasite (grossly visible areas of white lesions due to hyperplasia and excess mucus) which is presumed to reflect parasite intensity, however low gill scores may reflect a degree of tolerance to the parasite rather than resistance. Never-the-less higher gill scores have been shown to correspond to higher mortality due to AGD (Taylor et al., 2009b). Although gill score is known to correspond with the level of gill filaments with AGD lesions (Adams et al., 2004; Taylor et al., 2007), there have been no studies linking the number or extent of lesions with parasite intensity in field outbreaks. Further studies of the relationship between intensity of infection and gill pathology are required to clarify whether the degree and nature of the host response is dependant upon the parasite load over the time course of infection, bathing and re-infection. A broad range of aquaculture diseases cause observable gross pathologies, these may be readily utilised by farmers as categorical scales to schedule husbandry treatments (Speare and Arsenault, 1997; Primavera and Quinitio, 2000; Kent and Poppe, 2002). It is probable that many of these presumptive scales would be suitable selection traits for breeding programs once the relationship to subsequent (untreated) mortality rates has been demonstrated.

Because the survival challenge was carried out in one pen over one round of a summer AGD infection it was not possible to consider the effect of site, cage, infection round, season or year on the outcome of the trial in relation to estimates of genetic variation or the correlation with gill score. The robustness of genetic

parameter estimates may be improved with a repeat of this trial for fish reared in more than one cage and/or location.

Due to the protozoan aetiology of AGD it is unclear whether selection for resistance to this disease would have any effect upon resistance to bacterial or viral fish diseases that may concern the Tasmanian industry in the future, as resistance to one disease may confer susceptibility to another (Stear et al., 2001). Gjøen et al. (1997) found a low to moderate positive genetic correlation between bacterial diseases (vibriosis and furunculosis) in Atlantic salmon but suggested negative correlations between viral infectious salmon anaemia (ISA) and these two diseases. More recently a low, but favourable, genetic correlation has been reported between bacterial furunculosis and ISA resistance in Atlantic salmon (Ødegard et al., 2007a) and between the bacterial diseases enteric redmouth and rainbow trout fry syndrome and viral haemorrhagic septicaemia in rainbow trout (Henryon et al., 2005). Although AGD remains the most economically damaging disease to the Tasmanian salmon industry, it may be necessary to test the genetic correlation between resistance to AGD and specific bacterial or viral diseases in future.

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5 : Amoebic gill disease resistance is not related to the systemic antibody response of Atlantic salmon

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5.1 Abstract

Amoebic gill disease (AGD) is a proliferative gill tissue response caused by *Neoparamoeba perurans* and is the main disease affecting Australian marine farmed Atlantic salmon. We have previously proposed that macroscopic gill health ('gill score') trajectories and challenge survival provide evidence of a change in the nature of resistance to AGD. In order to examine whether the apparent development of resistance was due to an adaptive response, serum was sequentially sampled from the same individuals over the first three rounds of natural AGD infection and from survivors of a subsequent non-intervention AGD survival challenge. The systemic immune reaction to 'wildtype' *Neoparamoeba* sp. was characterised by Western blot analysis and differentiated to putative carbohydrate or peptide epitopes by periodate oxidation reactions. The proportion of seropositive fish increased from 46% to 77% with each AGD round. Antibody response to carbohydrate epitope(s) was immunodominant, occurring in 43% to 64% of samples. Antibodies that bound peptide epitope were identified in 16% of the challenge survivors. A 1:50 (single-dilution) enzyme linked immunosorbent assay confirmed a measurable immune titre in 13% of the survivors. There was no evidence that antibodies recognising wildtype *Neoparamoeba* provided significant protection against AGD.

5.2 Introduction

Marine cage farmed Atlantic salmon are Australia's most valuable finfish species, worth AU$272 million in 2007 (ABARE, 2008). The most significant disease affecting the industry is amoebic gill disease (AGD), which causes lethargy, respiratory distress, rapid opercular gasping and, if not treated, death (Munday et al., 1990). Clinical and acute infections result in significant cardiovascular compromise (Powell et al., 2008). The disease is caused by the marine protozoan ectoparasite *Neoparamoeba perurans* (Young et al., 2007). Attachment of *N. perurans* to healthy gill tissue initiates localised proliferative host cell change response producing extensive alterations in gill morphology, including severe epithelial hyperplasia, hypertrophy, oedema and interlamellar vesicle formation (Adams and Nowak, 2003; 2004b). These inflammatory changes can be seen grossly as the formation of mucoid white spots and patches on the branchial filaments. The extent of macroscopic gill pathology is utilised by fish farmers as the basis of a presumptive categorical macroscopic 'gill score' to monitor the level of AGD in each caged population. Gill score has been shown to correlate well with histopathological diagnosis of the disease in more advanced infections (Adams et al., 2004) and is a good predictor of mortality rate if the disease is left untreated (Taylor et al., 2009b).

Although initially seen as a summer disease, AGD now occurs year-round and requires reiterative freshwater bath treatments, triggered proactively by gill health surveillance or reactively in response to early losses. AGD reinfection may occur from low numbers of Neoparamoebae remaining upon the gills post bathing (Adams and Nowak, 2004a). Due to the continual requirement for monitoring and treatment, the disease accounts for up to 20% of the cost of production (Munday et al., 2001) through increased farm infrastructure, staffing and operating costs and decreased feeding opportunity. Therefore, minimising or avoiding AGD through improved husbandry, selective breeding for AGD resistance and possible development of

vaccines and/or therapeutics are all considered necessary to the sustainability and future of the industry.

Resistance of a proportion of naïve fish was suggested by Bridle et al. (2005) based upon enhanced survival after 72 days of aggressive AGD laboratory challenge without bathing intervention. The authors noted that moribund fish had advanced AGD lesions covering the majority of the gill surface, while fish that survived had small focal lesions and few Neoparamoebae. Recent work on tagged fish held under commercial conditions in the Tasmanian Atlantic salmon selective breeding program has demonstrated that a moderate genetic variation for resistance to AGD at first infection exists, on the basis of gill score (Taylor et al., 2007). However, complete resistance does not exist in the initial round of infection as all fish eventually reach industry gill score threshold and require bathing (Taylor et al., 2009a; Taylor et al., 2009b). There is increasing evidence that resistance to AGD can be developed in some fish with repeat exposure. Findlay et al. (1995) showed that fish exposed to Neoparamoebae, kept in freshwater for four weeks and then re-exposed, displayed a high level of resistance to reinfection as assessed by gill pathology. Findlay and Munday (1998) reported that resistance did not appear to improve until four weeks after the first bathed infection and that salmon previously exposed to two waves of infection and industry simulated bathing were more resistant than naïve and first infection fish. Taylor et al.(2009b) reported that following the initial round of AGD and first bathing, a distinct subpopulation with lower gill score develops and remains largely consistent during the third round of disease development. This subpopulation is largely independent from the more resistant individuals from the first infection, therefore we hypothesised that there is a change in the nature of resistance. The change in gill score response between first and subsequent infections may indicate a strengthening of innate (nonadaptive) responses with repeat exposure or development of a level of adaptive resistance (for example an adaptive immune response) within the population.

Following on from our previous hypothesis (Taylor et al., 2009b) that changing gill score patterns indicate a change in the nature of resistance, this study examines the

effect of repeated natural AGD and subsequent freshwater bathing upon the development of detectable systemic (serum) antibodies. We followed the progression of gill score in a population of tagged salmon during two waves of bathed AGD and an eventual unbathed AGD survival challenge in order to identify resistant individuals. Our aim was to (i) characterise individual development of acquired immune response in the early rounds of infection, (ii) identify whether the development of antibody responses to wildtype *Neoparamoeba* spp. were related to gill score in a group of AGD survivors and to (iii) quantify antibody responses to wildtype Neoparamoebae using a rapid enzyme linked immunosorbent assay (ELISA).

5.3 Materials and Methods

5.3.1 Fish and AGD progression in the field

Mixed-sex Atlantic salmon smolts (approximately 2500 fish, mean = 212g, SD = 52), 1500 of which had previously been injected intramuscularly with passive integrated transponder (PIT) tags, were obtained from Salmon Enterprises of Tasmania (SALTAS) Wayatinah hatchery and stocked to a single 800 m^3 sea cage located at Tassal Operations Pty commercial salmon farm at Dover, Tasmania (Table 5.1). The development of AGD was monitored utilising the standard industry gill score on fortnightly random subsamples of 40 individuals which were removed by dipnet and anaesthetised. Gill score estimates the number of visible gross lesions on the gill surface and assigns a score of between 0 and 5 to each individual, where 0 represents no visible lesions and 5 represents heavily infected gills (Taylor et al., 2009b). At 40 days post input (DPI), an advanced infection had developed, with over 10% of the population presenting with a gill score of 5 (**Measure 1**). All tagged individuals were individually gill scored and the entire population was bathed in freshwater for a minimum of 2 h. Following bathing, fish were again monitored until advanced AGD had developed and then all tagged fish were gill scored and bathed (**Measure 2,** 110 DPI). After the second bathing, the disease progressed to in excess of 10% of tagged fish having a gill score 5 and the severity of AGD was assessed at 50 days post bath (160 DPI, **Measure 3**). The population was not bathed at this assessment, allowing AGD to continue without treatment so that resistant fish could be clearly identified by natural AGD survival challenge. The ensuing mortality to AGD was assessed by daily diver removal of dead fish and macroscopic examination of gills to confirm the presence of typical AGD lesions. In addition, at each daily monitoring, moribund fish were removed by dipnet and euthanased. These individuals were classed as 'moribund' because they were dark, not actively swimming, losing equilibrium, facing into the net corners and gasping with flared operculae. The AGD survival challenge was terminated after 100 days (210 DPI, **Measure 4**) when the total mortality had reached a consistent plateau. All remaining individuals (tagged and untagged) were gill scored.

Date	DPI	Event	Action	Population	tagged	untagged	Av. gill score of tagged fish
17/8/06	0	Input	N/A	2504	1504	1000[a]	-
26/9/06	40	Measure 1	bathed	2374	1374	1000[a]	3.46 ± 0.03
5/12/06	110	Measure 2	bathed	2287[b]	1310	977	3.27 ± 0.01
24/1/07	160	Measure 3	not bathed	1917	1105[c]	812	2.56 ± 0.04
15/3/07	210	Measure 4	survivors sampled	784	284	500	1.15 ± 0.07

Table 5.1 Measurement and freshwater bathing events during repeated natural AGD infection and subsequent AGD survival challenge, showing numbers of PIT tagged and untagged (adipose fin clipped) fish present at each measure and the average gill score of the tagged population (± SE of mean). DPI = Days post input to sea [a]approximate [b]following bathing losses after Measure 2, there remained 1154 tagged and 942 untagged fish [c]274 tagged fish were removed for unrelated work following Measure 3, this was independant of gill score

All animal procedures were approved by the University of Tasmania (UTAS) Animal Ethics Committee (Permit # A0009111) under the guidelines of the Australian code of practice. At all nonlethal samplings, fish were anaesthetised using 17 ppm Aqui-S (Aqui-S NZ Ltd, Lower Hutt, New Zealand). Lethal samplings were carried out at 100 ppm Aqui-S.

5.3.2 Gill score trajectory of tagged fish

In order to describe the AGD progression of each tagged survivor, individual response trajectory categories were defined by the change in gill score of the tagged fish between Measure 1 and Measure 3. These were (a) Resistant – gill score at Measure 2 and Measure 3 less than or equal to one (b) Responding – gill score at Measure 3 at least two gill scores lower than Measure 1 (c) Non-responding – gill score at Measure 3 equal to or one lower than gill score at Measure 1 (d) Susceptible – gill score at Measure 3 greater than Measure 1

5.3.3 Serum sampling and AGD assessment

5.3.3.1 Sequentially bled fish, Measures 1, 2 and 3

At Measure 1, 76 tagged fish were randomly selected and 500 µl blood collected from the caudal sinus with a 21 guage x 1½" needle and 5 ml syringe. Each sample was held on ice overnight to maximise clot retraction. On the next day, the tubes were centrifuged (10,000 x *g* for 10 min at 4°C) and the serum drawn off and stored at −80°C in 1.5 ml eppendorf tubes. Working stocks of serum for storage at −20°C were mixed 1:1 with glycerol (Estévez et al., 1994) to minimise chemical and physical degradation during freeze-thaw. At Measures 2 and 3, the remaining individuals from this group (identified by tag number) were bled again as described above. A final group of 28 individuals, which had been sampled at all three Measures, were selected for further analysis (Table 5.2).

5.3.3.2 Moribund fish sampling

Between days 173-181 DPI, ten moribund fish were taken for histology and serum sampling (Table 5.2). The first and second left anterior gill hemibranch was dissected and fixed in seawater-Davidson's fixative for 48 h, then transferred to 70% ethanol until processed for histology. Blood (500 – 1000 µl) was sampled and serum extracted and stored as described above.

DPI	Sequential bleed	Moribunds	LGS Survivors	HGS Survivors
40	28	-	-	-
110	28	-	-	-
160	28	-	-	-
173-181	-	10	-	-
210	-	-	70[b]	31[b]
Western blot	28[a]	10	70	31
ELISA	-	10[c]	70[c]	31[c]

Table 5.2 Summary of sampling groups and serum antibody analysis. DPI = Days post input to sea. LGS = Low gill score (0-1). HGS = High gill score (4-5) [a]serum samples from the same 28 individuals, which had been sampled at each of the measures (40, 110 and 160 DPI) were assessed by Western blot for antibody binding to wildtype NP These 28 fish came from an initial group of 76 individuals at Measure 1. [b]of the total 101 sampled survivors, 82 were tagged individuals. Gill score trajectories, between 40 DPI and 160 DPI, were compared with the anti-wildtype NP responses of these 82 fish. [c]serum samples assessed as responding by ELISA were further examined by sodium periodate oxidation and Western blot to describe putative epitope antibody activity

5.3.3.3 Survivors

At the completion of the AGD survival challenge, a sample of 101 fish (82 tagged, 19 untagged) was selected for further analysis on the basis of gill score (Table 5.2). This consisted of 70 fish of low gill score 0 to 1 (54 and 16 fish respectively, 'LGS' group) and 31 fish of high gill score 4 to 5 (17 and 14 fish respectively, 'HGS' group). Gills and serum samples were collected and stored as previously described. All other survivors were bathed and retained for unrelated work

5.3.3.4 Wildtype NP antigen preparation for immune assays

Attempts at eliciting AGD with cultured Neoparamoebae have been unsuccessful (Morrison et al., 2005; Vincent et al., 2007). Therefore, 'wildtype' amoebae obtained from the gills of infected fish were utilised for testing the immune response to infective *Neoparamoeba* spp.. The term "wildtype NP" is therefore used throughout this publication to describe a mixed assemblage of amoebae species which consists mainly of *N. perurans*. Wildtype NP were isolated from the gills of AGD affected Atlantic salmon housed at the UTAS Aquatic Centre, Launceston, Tasmania, using the petri dish adherence and washing procedure described by Morrison et al. (2004). Cells were concentrated by centrifuge at 500 x *g* for 5 min and enumerated using a haemocytometer. The amoebae were washed twice with PBS and the cell pellet stored at −80°C until required.

5.3.3.5 SDS-Page and Western blotting

Amoeba pellets were resuspended at 8,000 cells μl^{-1} in Tris-HCl buffer containing 10% β-mercaptoethanol, centrifuged at 16,000 x *g* for 5 min and boiled for 10 min. The reduced amoeba antigen preparations were stored at −20°C and 8 µl (~64,000 cells) per well separated in NuPAGE® Novex 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA). Electrophoresis was run at 200 V for approximately 50 min in an X-Cell SureLock™ Mini-Cell (Invitrogen) and NuPAGE® MES SDS running buffer (Invitrogen). The antigens were transferred to nitrocellulose Trans-Blot® membrane, pore size 0.2 µm (Bio-Rad, Hercules, CA, USA) using a *Mighty Small*

transphor electrophoresis unit (Amersham Biosciences San Francisco, CA, USA) and NuPage® transfer buffer (Invitrogen) with 20% methanol, run at 150 mA for 90 minutes. Broad molecular weight range (2-212 kDa) protein standards (BioLabs, Ipswich, MA, USA) were included on the gels. Individual lanes were visualised with ponceau stain (Sigma-Aldrich, Saint Louis, MO, USA) and cut into separate strips prior to destaining with 0.1 M NaOH for 2 min, washing in sterile water and blocking for 30 min in casein solution (Vector, Burlingame, CA, USA). Between incubation steps, individual strips were washed 3 x 5 min with tris-buffered saline (TBS, pH 7.2). The strips were probed for 1 h with individual fish sera diluted 1:100 in casein solution. Blots were probed with monoclonal anti-salmonid IgM antibody (Immuno-Precise Victoria, BC, Canada) diluted 1:500 in casein for 30 min and antibody binding was visualised using BCIP/NBT (Sigma-Aldrich) developed for approximately 20 min and then rinsed in sterile water. All incubation and wash steps were at room temperature. Positive control sera were chosen from individuals found to produce intense reactions to wildtype NP antigen(s). The negative control was pooled sera from five AGD naïve smolt sampled in fresh water at the hatchery.

The Western blot profiles produced by binding of serum anti-wildtype NP antibodies were broadly defined into two reactions as described by Vincent et al.(2008), these were (i) 'clear' (seronegative), (ii) a seropositive 'smear'across a broad molecular range or (iii) seropositive 'bands'at M_r > 200 kDa.

5.3.3.6 Western blot discrimination between carbohydrate and peptide epitopes

Identification of binding to carbohydrate or peptide epitopes was carried out on a subsample of a variety of sera that produced positive reactions to wildtype NP antigen that were measurable by ELISA. Bound antigens on adjacent strips were either incubated for 1 h in 50 mM sodium acetate (BDH Chemicals, Australia) pH 4.5 or oxidised with 20 mM sodium periodate (Sigma-Aldrich) as described by Woodward, Young & Bloodgood (1985). Following a 3 x 5 min TBS wash step, all strips were exposed to 50 mM sodium borohydride (Sigma-Aldrich) for 30 min. Membranes were then washed, blocked and probed as outlined above. Loss of

reactivity of the periodate-treated antigen was interpreted as indicating recognition of carbohydrate epitope(s) by individual fish sera. Retention of reactivity indicated antibody response to putative peptide epitope(s).

5.3.3.7 Indirect enzyme-linked immunosorbent assay (ELISA)

The general methodology of each ELISA was as follows. Wildtype NP antigen was prepared from pellets previously frozen in PBS. Each pellet was suspended in 1ml PBS, sonicated on ice, centrifuged at 16,000 x g for 10 min and the protein concentration of the supernatant was determined by QubitTM fluorometric assay (Invitrogen). The wildtype NP supernatant was then aliquoted into coating buffer (50 mM NaHCO3, pH 9.5) at 24 µg total protein per 5 ml and stored at −20°C. Flat bottom 96 well MaxiSorp ELISA microplates (Nunc, Rochester, NY, USA) were coated with 0.24 µg protein per well in coating buffer (50 nM NaHCO3, pH 9.5) and left overnight at 4°C. The unbound protein was removed by a wash-step (3 x with 300 µl PBS per well). Wells were then blocked for 30 min at 37°C with 300 µl 0.3% casein-PBS. Following a further wash step, serum samples at test dilutions were applied in 0.3% casein-PBS at 50 µl per well for 1 h at room temperature and washed. Bound antigen was detected with monoclonal anti-salmonid IgM antibody #IPA006A (Immuno-Precise, Canada) diluted at 1:500 in 0.3% casein-PBS, applied at 50 µl per well for 30 min at 37°C. Following another wash step, the reaction was developed at room temperature with 100 µl alkaline phosphotase yellow (pNPP) liquid substrate (Sigma-Aldrich) for 20 min in the dark and stopped with an equal volume of 2 M NaOH. Immediately after this, optical density (OD) was read using a Lucy 2 Luminometer (Anthos Labtec, Cambridge, UK) at 405 nm.

When estimating antibody levels in an ELISA, several dilutions of each serum sample are usually analysed to arrive at an endpoint dilution or units of antibody (Alcorn and Pascho, 2000). Replicate analysis of a dilution series of each test serum limits the number of samples that can be tested on a microplate and is costly because it requires larger volumes of antigen, serum and secondary antibody. Therefore, we developed a single-dilution ELISA test which included a positive standard curve and

replicate negative standards to account for inter-plate and day-to-day variation (Miura et al., 2008). Similar single-dilution ELISA approaches have been used in numerous studies (Estévez et al., 1994; Dey et al., 2004; Rogers-Lowery et al., 2007).

In order to optimise the single-dilution assay, ELISA control background activity was assessed by running the assay with each reagent in turn omitted. The OD values were determined for doubling dilutions of a subsample of sera known to have varying Western blot responses (negative, positive/smear and positive/bands). One sample (#387) was selected as a serum standard because it showed a high anti-wildtype NP antibody concentration and a linear relation between the mean OD and the serum dilution in a significant proportion of the curve (Figure 5.1). Because this sample produced a curve that was significantly higher than the unknown samples, and in order to conserve serum stocks, it was diluted 1:8 in pooled serum from naive (fresh water) smolt to create the test standard. On each test plate, a duplicate doubling dilution (1:25 to 1:3200) of this standard (1/8 #387) was used. This gave a consistent linear relation ($r^2 \geq 0.975$) between the reciprocal dilution and the OD from 1:25 to 1:200. The optimal test concentration at which to measure the relative titre of unknown samples compared to this positive standard was 1:50, this was chosen as the highest dilution that did not show nonspecific binding and for the convenience of processing and the economic use of the original sample. All test sera were assayed in triplicate, allowing 24 samples to be evaluated per plate. In addition, each sample was retested in triplicate on at least one other plate. Internal negative controls of pooled naïve sera were randomly distributed to eight individual wells on each plate at 1:50 dilution.

Samples were considered to be above the positive-negative threshold (PNT) baseline if their mean OD on a plate was at least twice the average OD of the eight negative control wells. Entire plates were discarded if the coefficient of variation (CV) of the negative controls was $\geq 15\%$. The absorbance of these test sample dilutions was corrected by subtracting the average absorbance of the internal negative control serum dilutions from the same plate.

The results of an ELISA for antibody may be reported in several forms, such as OD values or as arbitrary units on the basis of the reactivity of the standard or dilution of the test serum (Alcorn and Pascho, 2000). We therefore expressed the OD values of our test sera as antibody units compared to the serum standard to correct for inter-plate variation. The dilutions 1:25, 1:50, 1:100, 1:200 and 1:400 were therefore expressed relative to the 1:50 dilution and respectively assigned 200, 100, 50, 25 and 12.5 relative antibody units. A standard line was created for each plate by plotting the reciprocal of each dilution against the mean OD within this dilution range. The results of the entire microplate were disregarded if the correlation between the reciprocal dilution and OD was $r^2 < 0.975$.

5.3.4 Data Analysis

The relationships between gill score at Measure 1 and Measure 2 and mortality due to sequential bleed handling at each measure were tested by performing the Cochran-Armitage test for trend using gill score as an ordered variable (Armitage et al., 1994). The same test was used to explore the relationship between gill score trajectories of fish surviving the AGD survival challenge and anti-wildtype NP antibody reaction. The relationship between gill score group (LGS or HGS) of survivors and the antibody reaction category was tested by chi-square tests of association. Where the results of chi-square tests were marginal, data was further assessed by Fisher's exact test (Yates, 1984) for association in a 2 x 2 contingency table. The overall relationship between gill score of survivors and relative antibody units was assessed using the Spearman's rank correlation test.

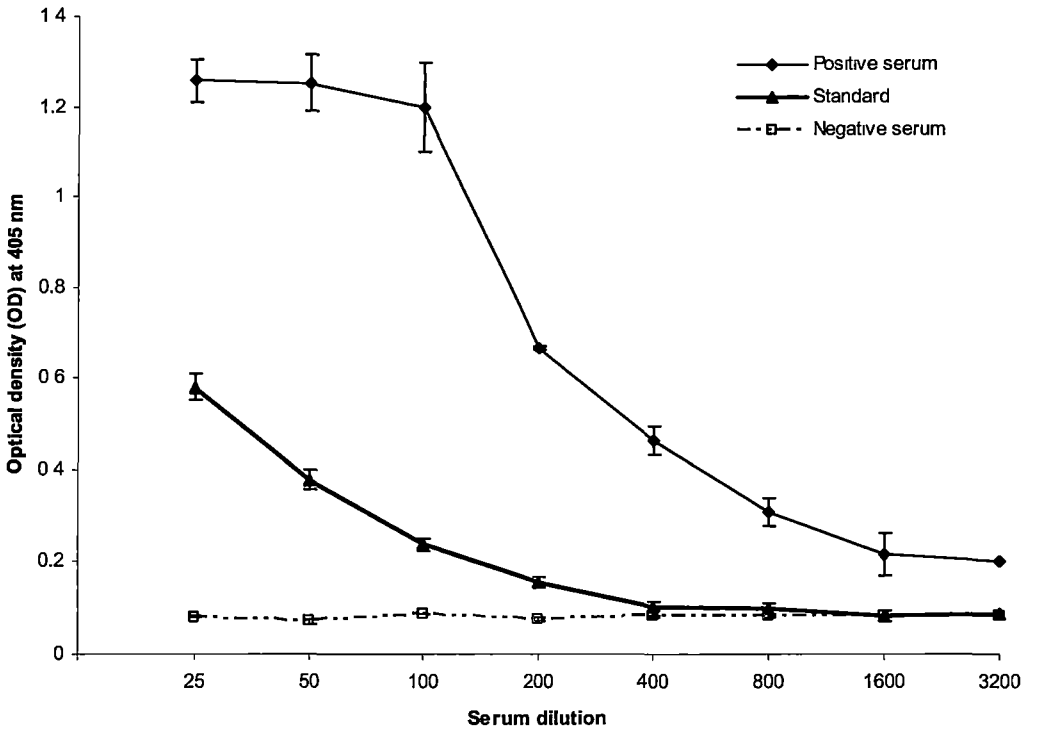

Figure 5.1 ELISA OD values for dilutions of positive control serum #387. Because this control appeared to have stronger antibody binding than other samples and nonspecific binding occurred when dilution was below 1:100, it was diluted 1:8 with naïve control serum to create a standard that was within range of other samples. The '1/8 #387' standard was included as a doubling dilution series in duplicate on each single-dilution ELISA 96 well plate (1:25 to 1:3200) and naïve control serum was included on eight random wells. Unknown samples were tested in triplicate at 1:50 dilution.

5.4 Results

5.4.1 Antibody response in sequentially bled fish, Measures 1,2 and 3

Of the 76 sequentially bled fish that were followed from Measure 1, thirty six survived to the third sampling. Mortality between Measure 1 and Measure 3 was higher in this group (52.6%) than in the overall tagged population (19.6%), presumably due to the additional stress of handling and blood sampling. Mortality (20 fish) following Measure 1 bleed and bathing was not related to gill score prior to bathing (Cochran-Armitage chi-square = 1.68: d.f. = 1; P = 0.195). However, losses between Measure 2 and Measure 3 (20 fish) were closely related to gill score at Measure 2 ($r^2 = 0.71$, Cochran-Armitage chi-square = 8.67; d.f. = 1, $P = 0.003$). Therefore, it is likely that the process of handling and bleeding selected for more robust or disease resistant fish. Western blot analyses showed that the proportion of these fish that were seronegative to wildtype NP decreased from 54% (at Measure 1) to 32% (at Measure 3). The majority of seropositive samples gave a broad molecular range smear, while a small proportion (3.6% at each measure) reacted with two distinct bands at $M_r > 200$ kDa.

5.4.2 Antibody response in AGD survival challenge fish

Following removal of handling mortalities after Measure 2 and 274 tagged fish at Measure 3 (Table 5.1), the AGD survival challenge population was 1822 fish (880 tagged and 942 untagged). Mortalities attributed to AGD began 31 days after the Measure 2 bath and increased slowly (average 0.3% per day) prior to Measure 3 at 160 DPI (Figure 5.2). Mortalities peaked between 169 and 182 DPI (averaging 2.8% per day) before abating. During this period, ten moribund fish were culled and sampled for antibody response, one was seronegative by Western blot, six showed broad molecular range smears and three displayed double banding at $M_r > 200$ kDa. All moribund fish exhibited advanced macroscopic signs of AGD.

Overall mortality after 100 days of non-intervention AGD survival challenge was 57%. Gills of dead fish were examined daily, all of which exhibited extensive macroscopic signs of AGD. Although the low and high gill score survivors showed markedly different gross signs of AGD at the conclusion of the survival challenge, both groups had demonstrated remarkable tolerance to AGD or resistance to the parasite by surviving 100 days of untreated natural summertime AGD infection. Histopathology of gill sections from the LGS and HGS survivors confirmed significant differences in the severity of infection that were consistent with gill score observations. The HGS survivor group showed large numbers of attached *Neoparamoeba* spp. and associated pathology, including fusion of the lamellae, hyperplasia and hypertrophy. In contrast, the LGS survivors displayed a low level of AGD pathology with few, or no, Neoparamoebae. The absence of *Neoparamoeba spp.* on some LGS fish may simply be an artifact of histology rather than confirming complete resistance to the parasite. Inflammatory cells (neutrophils and macrophages) were observed within lesions and the adjacent central venous sinus of both LGS and HGS survivors, though the inflammatory response was more pronounced in the HGS group.

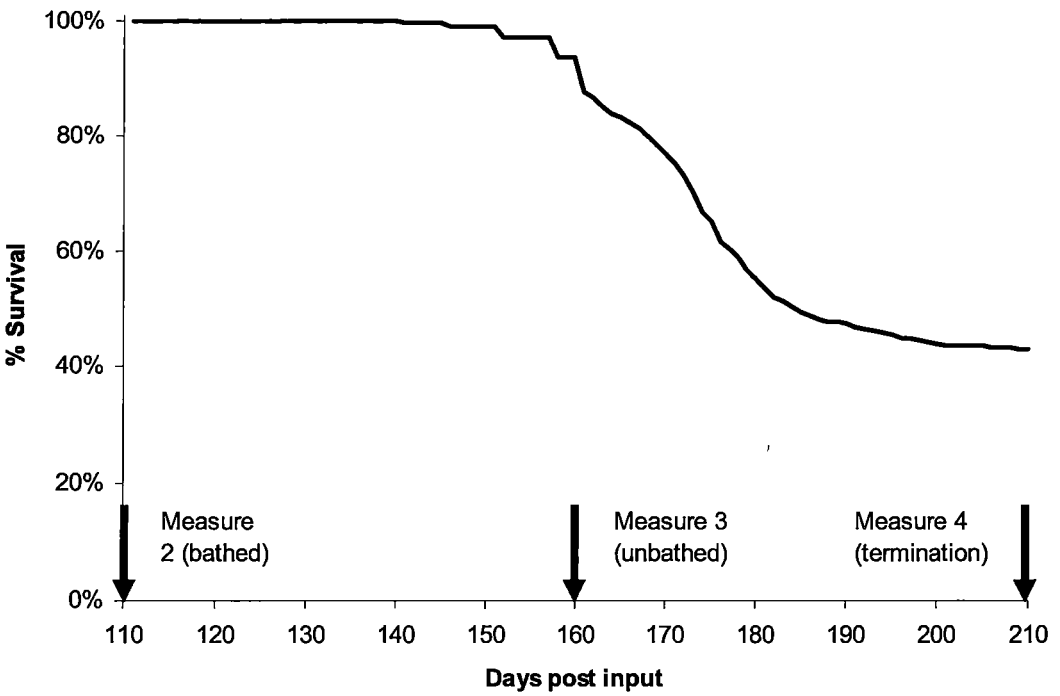

Figure 5.2 Kaplan-Meier Curve for AGD survival of all fish (—) from bathing at Measure 2 (110 DPI) until challenge termination at Measure 4 (210 DPI). Key measurement events are indicated by bold arrows. Fish that were classed as 'handling mortalities', or were removed for unrelated studies, are not included in the data.

Anti-wildtype NP antibodies were undetectable by Western blot in 23/101 (22.8%) of the AGD challenge survivors (Table 5.3 and Figure 5.3). Seropositive antibodies were further characterised as smears (60.4% of sample) or bands (16.8%). There were no significant differences between the LGS and HGS groups in terms of the proportions of seronegative samples, seropositive smear samples and seropositive band samples (Pearson chi-square = 1.28; d.f. = 2; $P = 0.527$). Therefore, gill score of the survivors was not linked to the presence of antibodies recognising wildtype NP.

5.4.3 Antibody response and gill score trajectory of survivors

The Measure 1 to Measure 3 gill score trajectories of the 82 tagged survivors were predominantly 'responding' (72%) or 'resistant' (21%) (Table 5.4). Due to mortality of 'susceptible' fish earlier in the survival trial, only 3.7% were seen in the survivor sample. The proportion of 'non-responding' fish (3.7%) was lower than the overall survivor population (23%) described in Taylor et al. (2009b). There were no significant differences between gill score trajectory response and the proportions of seronegative, seropositive smear and seropositive band fish (Pearson chi-square = 4.49; d.f. = 6; $P = 0.610$). There were no significant differences between the 'responding' and 'resistant' groups, recording 20% and 29% seronegative, 61% and 53% smears and 19% and 18% bands respectively (Pearson chi-square = 0.64; d.f. = 2; $P = 0.727$).

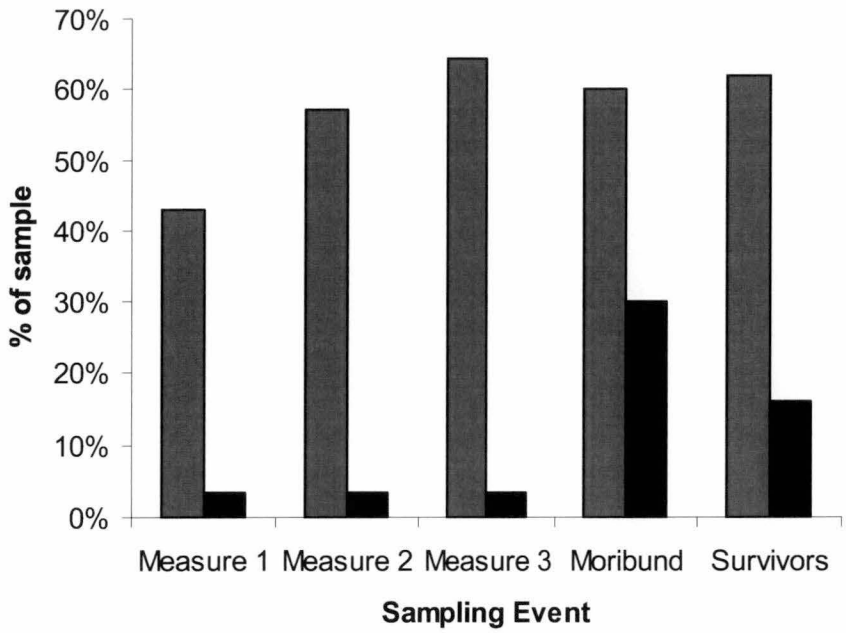

Figure 5.3 Serum antibodies that bind to wildtype NP were detected by Western blot in sea-caged salmon. ■ seropositive - broad molecular weight smear, ■ seropositive - bands, M_r > 200 kDa. The samples at Measures 1, 2 and 3 were from a group of 28 tagged fish that were randomly selected at 40DPI then sequentially bled. The survivor samples were from 101 fish that survived 100 days of natural summer AGD infection between 110 and 210 DPI, these were selected as low gill score (0-1) survivors (LGS, n = 70) or high gill score (4-5) survivors (HGS, n = 31). The moribund sample (n = 10) were taken between 173 and 181 DPI during the peak of losses from the AGD survival challenge.

Antibody detection	LGS		HGS		Total	
Seronegativer	18	(25.7%)	5	(16.1%)	23	(22.8%)
smear (ELISA –ve)	39	(55.7%)	17	(54.8%)	56	(55.4%)
smear (ELISA +ve)	1	(1.4%)	4	(12.9%)	5	(5.0%)
bands (ELISA –ve)	6	(8.6%)	3	(9.7%)	9	(8.9%)
bands (ELISA +ve)	6	(8.6%)	2	(6.5%)	8	(7.9%)
Total	70	(100%)	31	(100%)	101	(100%)

Table 5.3 Results of Western blot and 1:50 (single-dilution) ELISA analysis oflow gill score (0-1) survivors (LGS) and high gill score (4-5) survivors (HGS) of 100 day non-intervention AGD survival challenge. Seronegative samples were undetectable by Western blot. Broad molecular weight smears were detectable by Western blot only (ELISA –ve) or were 'strong responders' detectable by Western blot and ELISA. Samples producing banding at $M_r > 200$ kDa were detectable by Western blot only (ELISA –ve) or were 'strong responders' detectable by Western blot and ELISA.

Gill score trajectory	Seronegative		Seropositive (Smear) ELISA -ve		Seropositive (Smear) ELISA +ve		Seropositive (Bands) ELISA -ve		Seropositive (Bands) ELISAa +ve		Total	
Susceptible	2	(66.7%)	1	(33.3%)	0	(0.0%)	0	(0.0%)	0	(0.0%)	3	(100%)
Non-Responding	1	(33.3%)	2	(66.7%)	0	(0.0%)	0	(0.0%)	0	(0.0%)	3	(100%)
Responding	12	(20.3%)	33	(55.9%)	3	(5.1%)	4	(6.8%)	7	(11.9%)	59	(100%)
Resistant	5	(29.4%)	9	(52.9%)	0	(0.0%)	3	(17.6%)	0	(0.0%)	17	(100%)
Total	20	(24.4%)	45	(54.9%)	3	(3.7%)	7	(8.5%)	7	(8.5%)	82	(100%)

Table 5.4 Detection of anti-wildtype NP antigens by Western blot and 1:50 (single-dilution) ELISA of surviving tagged fish, grouped by gill score trajectory between Measure 1 and Measure 3. Gill score trajectories were (a) Resistant – gill score at Measure 2 and Measure 3 less than or equal to one (b) Responding – gill score at Measure 3 at least two gill scores lower than Measure 1 (c) Non-responding – gill score at Measure 3 equal to or one lower than gill score at Measure 1 (d) Susceptible – gill score at Measure 3 greater than Measure 1

5.4.4 ELISA of moribund and surviving fish

Two moribund fish tested positive by the 1:50 (single-dilution) ELISA (data not shown), one sample had displayed a seropositive smear (36.4 relative antibody units) by Western blot and the second sample (42.5 relative antibody units) produced banding at *Mr* > 200 kDa. This confirms that strong antibody responses to wildtype NP can be measured in some fish that are dying from AGD.

The mean OD of the 101 challenge survivors tested was 0.23 (SD = 0.08) but OD's can not be directly compared due to inter-plate variation. When OD is converted to relative antibody units and compared to gill score using Spearman's rank correlation, there was no significant correlation between serum anti-wildtype NP antibody levels and gill score at Measure 1 (Spearman's rho = 0.13, $P = 0.26$), Measure 2 (rho = 0.11, $P = 0.31$), Measure 3 (rho = 0.05, $P = 0.63$) or Measure 4 (rho = 0.15, $P = 0.14$). Thirteen samples (12.9%) were positive by ELISA (Table 5.3). Comparing the LGS and HGS survivor groups, only 1/40 (2.5%) of the LGS smear samples were positive by ELISA, compared to 4/21 (19%) of the HGS smear samples, this difference was significant (Pearson chi-square = 5.01; d.f. = 1; $P = 0.025$) and for Fisher's exact test $P = 0.044$). The proportion of ELISA positive banding samples was not significantly different between the LGS (6/12) and HGS (2/5) groups (Pearson chi-square = 0.14; d.f. = 1; $P = 0.707$). Excluding the standard sample #387 from the HGS group, there was no significant difference in the strength in relative antibody units of the positive ELISA samples between the LGS (mean = 49.1, SD = 22.2) and the HGS (mean = 47.7, SD = 24.0) groups or between the samples producing smears (mean = 53.9, SD = 27.2) and bands (mean = 44.7, SD = 19.2).

When compared to their previous gill score trajectories, for the 82 tagged survivors, positive ELISA results were only seen in the responding group (Table 5.4) with 3/36 smears (8%) and 7/11 bands (64%). The difference in proportion of ELISA positive smears between the responding and resistant groups was not significant (Pearson chi-square = 0.80; d.f. = 1; $P = 0.370$). There was, however, a marginal difference in

the proportion of ELISA positive bands between these two groups (Pearson chi-square = 3.82; d.f. = 1; $P = 0.051$). The suggested higher proportion of ELISA positive banding samples in the responding group was not significant when further tested by Fisher's exact test ($P = 0.096$), probably due to the low sample numbers in the 'resistant' group. The ELISA positive samples showed similar strength responses between the responding group (mean = 46.6, SD = 22.9) and resistant group (mean = 44.7, SD = 17.2).

5.4.5 Western blot discrimination between carbohydrate and peptide epitopes in ELISA positive samples

Samples that were positive by ELISA were broadly separated into those that produced a broad molecular range smear and those that produced bands at $Mr > 200$ kDa (Table 5.4). In Western blots that displayed high molecular range bands, binding was not affected by sodium periodate oxidation though some clearing of background smears was noted. This suggests that anti-wildtype NP antibodies on these blots were directed towards putative protein epitope(s) with a low level of reaction to carbohydrate epitope(s). In contrast, the Western blots displaying broad molecular weight smears were cleared by periodate oxidation, though in some cases two faint bands of $Mr > 200$ kDa were evident following this treatment. In these cases it appears that anti-wildtype NP antibodies that reacted to carbohydrate epitope(s) were immunodominant but there was also a low level of antibody present in some samples that bound to putative protein epitope(s).

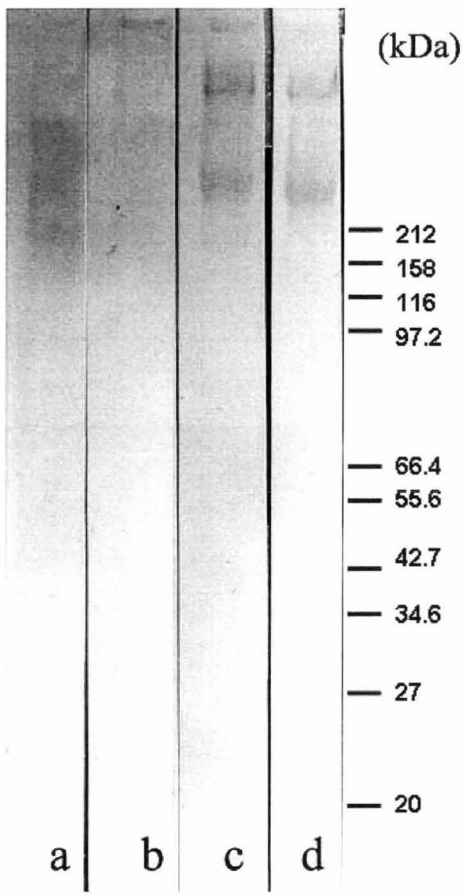

Figure 5.4 Binding of serum anti-wildtype *Neoparamoeba* sp. antibodies from fish exposed to two rounds of natural AGD and freshwater bathing, followed by a third non-intervention natural AGD survival challenge, produces two distinct profiles. A serodominant broad molecular range response (a) was sensitive to periodate oxidation (b), indicating reaction to putative carbohydrate epitope(s). In contrast, the double banded reaction at M_r > 200 kDa (c) was not sensitive to periodate oxidation (d), indicating recognition of putative peptide epitope(s).

5.5 Discussion

We previously hypothesised that gill-score trends of Atlantic salmon, through three rounds of advanced natural AGD infection and a subsequent non-intervention survival challenge, were suggestive of the development of a protective response (Taylor et al., 2009b). We then postulated that this may be due to circulating antibodies. We therefore examined serum samples that were taken from known individuals during the multiple AGD infection and survival challenge trial. Our results confirm that the proportion of seropositive salmon within the study population increased with repeated exposure to AGD. There are, however, no apparent links between high or low gill score of fish surviving AGD challenge and the presence of anti-wildtype NP antibodies. Comparison of gill score trajectories suggest that strong serum antibody responses (measurable by ELISA at a single-dilution of 1:50) may only be seen in surviving individuals that were considered to be 'responding', as evidenced by a drop of two or more gill scores between the first and third rounds of infection. Our results agree with previous observations (Vincent et al., 2009) that serum antibodies recognising carbohydrate epitope(s) are immunodominant. We also report the first observation of antibodies specific to wildtype NP peptide epitopes following natural AGD infection.

The presence of anti-*Neoparamoeba* antibodies was demonstrated by Findlay et al. (1995), Akhlaghi et al .(1996) and Gross et al. (2004) who all found that the presence of antibodies does not confer protection to AGD. However, in each case this activity was directed towards cultured *Neoparamoeba* spp.. The first evidence of a serum antibody response to wild-type *Neoparamoeba* sp. antigens was provided by Vincent et al. (2006) who challenged low numbers of Atlantic salmon with gill-derived amoebae and demonstrated that enhanced survival at second infection was associated with anti-wildtype NP antibodies detectable in serum of 50% of surviving fish. In the current study we observed an increase in the proportion of seropositive fish with each round of advanced natural AGD infection and bathing (from 46% to 78%) over the first 210 days post input. This gradual increase in the frequency of seropositive

fish with reiterative exposure to AGD and freshwater bathing is in broad agreement with the observations of Vincent et al. (2009), who found an increase in the frequency of anti-wildtype NP antibodies in commercially sea-reared fish from 12.5% at 8 months to 57.5% at 13 months post input. The higher levels of seropositive fish in our study may reflect the selection of more robust individuals by handling and AGD survival challenge. In addition, our population was repeatedly exposed to more advanced AGD by deliberately delaying bathing to beyond normal commercial thresholds.

Western blotting revealed two distinct profiles at each AGD measurement, either a defined smear of broad molecular weight or a paired banding at M_r > 200 kDa. The observation of two separate antibody binding profiles to wildtype NP agrees with Vincent et al. (2008). The broad molecular range smear was immunodominant during repeated natural AGD events, though the proportion of fish with this response remained relatively unchanged at 57% to 64% of the population from Measure 2 onwards. As shown in earlier studies (Villavedra et al., 2007; Vincent et al., 2008) these smears were sensitive to periodate oxidation, indicating antibody binding to carbohydrate epitope(s) of wildtype NP. Carbohydrate antigens are abundant on the cell-surface of many protozoan parasites and may be involved in host attachment and immune evasion (Kaattari et al., 2002). It is possible that the presence of these epitopes acts to sub-divert the Atlantic salmon's immune response toward non-protective antigens thus allowing survival of the parasite on the host gill tissue.

The paired banding response (M_r > 200 kDa) was not sensitive to periodate oxidation, which is suggestive of a putative peptide antigen(s) response. A similar observation was previously reported from the isolated case of one individual which was held in a laboratory cohabitation tank for approximately 168 days (Vincent et al., 2008), this fish was histologically clear of AGD when sampled. Average survival times of fish in the laboratory system range from 7-10 days therefore it could be postulated that this specific peptide antibody response was protective. However, the earlier gill history of this fish was not known, making this hypothesis difficult to test. In our study, antibodies that bound wildtype NP peptide antigen(s) were seen at a

low level (3.6%) in a small sample over three rounds of advanced AGD but were found in 16.8% of samples from survivors of the 100 day non-intervention natural AGD survival challenge. This higher proportion of seropositive fish recognising peptide epitope(s) in the surviving group suggests that this type of antibody response may be immunoprotective. However, there were no differences in the proportion of LGS and HGS survivors with a peptide response and the highest frequency (30%) of antibody response to peptide epitope(s) was seen in the sample of moribund fish that was taken during the peak period of AGD mortality. In order to determine whether the peptide response is protective, the epitopes to which this sera binds would need to be determined and the corresponding synthetic peptide(s) made before performing comprehensive vaccination and challenge trials.

Despite the high incidence of fish with antibody response to wildtype NP seen by Western blot in this study, only 15 fish (2 moribund and 13 survivors) were judged to be positive by ELISA, these 'strong responders' were independent of antibody type and gill score. This apparent paucity of samples with measurable antibody activity was also seen by Vincent (2008), who found only two in 330 samples (0.6%) that were positive over a range of serum dilutions. The lower ELISA sensitivity may indicate that salmon antibodies are not directed against cell-surface antigens of Neoparamoebae, which should be largely unchanged in ELISA coating buffer. Increased reactivity following exposure to β-mercaptoethanol and separation through SDS-polyacrylamide prior to blotting may indicate that cell-surface antigenic determinants are altered by denaturation or that internal antigens are released from the Neoparamoebae. Alternatively, the lower sensitivity of the ELISA test may simply reflect that anti-wildtype NP antibody levels are generally low, because our ELISA positive samples also produced the most intense smears or banding when visualised by Western blot. The higher incidence of ELISA positives in our study may reflect greater exposure to wildtype antigens after a long period (210 days) of reiterative advanced natural AGD as the two strong responders sampled from a laboratory cohabitation tank by Vincent et al.(2008) had also been constantly exposed to AGD for an extended period. In our study, high antibody titre and gill score of survivors were not linked. High antibody titre may therefore reflect the degree of previous exposure to Neoparamoebae, rather than current levels of the

disease. Indeed, in comparing previous gill score trajectories of survivors we only saw ELISA positives in the responding group. High antibody titre may bear little relevance to antigen affinity (Kaattari et al., 2002) so may not indicate increased protection potential. Indeed, the high titre of fish #387 (which was at gill score 5 when sampled) may indicate a high antibody response with low affinity directed against wild-type Neoparamoebae during extensive AGD.

Although our fish had demonstrated resistance or tolerance to AGD by enduring 100 days of an AGD survival challenge, we were unable to provide definitive evidence that anti-wildtype NP antibodies confer protection against AGD. This is in part due to the fact that the presence and type of antibodies was not related to the gross gill pathology at the time of sampling. Furthermore, the presence of anti-wildtype NP antibodies in high gill score moribund fish during the peak period of mortalities suggests that the presence of anti-wildtype NP antibodies does not confer protection. A number of other studies have demonstrated an increasing antibody response to fish gill parasites following prolonged or repeated infections. However the evidence on whether this antibody response may be protective is mixed. For instance, rainbow trout *Onchorhynchus mykiss* (Walbaum) and brown trout *Salmo trutta* L. naturally infected with the monogenean gill parasite *Discocotyle sagittata* show elevated humoral antibody levels to the pathogen but without increased protection (Rubio-Godoy et al., 2003; Rubio-Godoy and Tinsley, 2004). Conversely, two-year-old tiger puffer *Takifuga rubripes* (Temminck & Schlegel) had lower levels of the diclidophorid monogenean *Heterobothrium okamotoi* when compared to naïve individuals and this was related to the presence of specific antibodies (Nakane et al., 2005). Although the development of a systemic antibody response following prolonged exposure to gill ectoparasites may not be surprising, the development of a protective immune response may depend on a number of yet undescribed factors contributing to the host-pathogen relationship. Bricknell, Bisset & Bowden (2002) demonstrated that the binding affinity of Atlantic salmon serum antibodies is markedly reduced above physiological osmolality (300 to 400 $mOsml^{-1}$), so would be minimal in seawater (1000 $mOsml^{-1}$). A feature of AGD is the increased production of mucus and increasing mucous cell numbers (Powell et al., 2008), therefore mucosal antibodies may be more effective against Neoparamoebae. Maki &

Dickerson (2003) found that cutaneous mucus antibody levels were lower than serum antibody responses in Channel catfish *Ictalurus punctatus* (Rafinesque) immunised against the protozoan ciliate *Ichthyophthirius multifiliis*. The mucus and serum antibody concentrations did not increase concomitantly, suggesting that mucus antibodies do not arise by passive diffusion from the blood. Initial attempts to isolate anti-wildtype NP antibodies from cutaneous mucus of *S. salar* were unsuccessful (Vincent et al., 2006). Future research on gill mucus and antibody secreting cells in the gills of salmon that have survived reiterative advanced AGD for a prolonged period may determine whether the production or presence of mucus antibodies is related to the acquisition of resistance.

Although there appears to be little correlation between the presence and/or type (recognising carbohydrate or peptide) of systemic antibody response to wildtype NP and resistance to AGD, it is possible that other adaptive immune mechanisms may be responsible for resistance. Previous studies have shown that MHC class II^+ cells are present in the gill epithelium of AGD-affected Atlantic salmon, indicating immune cell trafficking and antigen presentation (Morrison et al., 2006a). Wynne et al. (2008b), working on 28 survivors of our non-intervention AGD survival challenge, classed gill score 0 and gill score 5 survivors as 'resistant' and 'susceptible' respectively (i.e. fish from our LGS and HGS groups) and demonstrated that 'resistant' animals displayed significantly higher expression of immunoglobulin light chain and MHC class II invariant chain-like protein genes involved in adaptive immunity, when compared to 'susceptible' and naïve fish. The transmembrane glycoprotein CD8 (which serves as a T-cell co-receptor) and granzyme K (which is implicated in T- and natural killer cell-mediated cytotoxic defense reactions) were also up-regulated. However, other genes involved in the adaptive immune pathway, such as CD3 (T-cell receptor) and immunoglobulin heavy chain (large antibody polypeptide subunits) were not differentially expressed. Therefore further investigation into other immune mechanisms, such as T-cell mediated immunity, is warranted in relation to AGD resistance.

In recent years there has been increasing evidence that innate and adaptive mechanisms of teleost immunity to parasites are integrated into a multilevel network (Alvarez-Pellitero, 2008) so effective immunity may result from a combination of immune factors. Immune responses are initiated by parasite/host interactions and can include immunosuppression by the parasite, such as the down-regulation of genes involved in MH class I and class II pathways at first exposure of salmon to AGD (Young et al., 2008b). The main feature of AGD is the proliferative host response to the parasite; AGD-susceptible survivors sampled from our trial were shown to have higher expression of acute phase proteins (APP) and positive regulators of cell cycle (Wynne et al., 2008b). Innate immune recognition relies on pathogen recognising receptors (PRR), including C-type lectins which recognise pathogen carbohydrate structures. These receptors initiate the inflammatory response including production of inflammatory cytokines to release complement and opsonise pathogens for presentation to macrophages and neutrophils. Innate mechanisms of resistance to AGD are undoubtedly involved after exposure to *Neoparamoeba* spp., with an increased phagocyte response but no effects on plasma lysozyme or total plasma protein (Gross et al., 2005). Nonspecific responses may be heightened by repeat exposure to the parasite or other environmental stressors, which could account for the changing patterns of resistance observed in our population (Taylor et al., 2009a). The complex nonadaptive/adaptive immune cascade is undoubtedly controlled by many genes. Therefore, the most effective route to developing resistance in our stocks appears to be through quantitative selective breeding techniques which do not require direct knowledge of the genes or processes that afford resistance. As molecular genetic techniques improve, it may be possible to develop a whole-genome association approach whereby the statistical association of single nucleotide polymorphisms (SNPs) with AGD resistance observations is tested, allowing the development of marker assisted selection.

5.6 Acknowledgements

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6 : Genetic variation of amoebic gill disease gross pathology and required disease treatment frequency in Atlantic salmon

6.1 Abstract

Amoebic gill disease (AGD) continues to have a significant economic impact upon production of sea-farmed Atlantic salmon in Tasmania, Australia. The disease is a proliferative reaction at the gill that occurs in response to attached trophozoites of marine ectoparasitic Neoparamoebae. The only effective treatment is regular freshwater bathing, which is expensive and labour intensive. The highest selection weighting in the breeding goal of the Tasmanian salmon breeding program is on resistance to AGD, where the objective is to increase the time between freshwater baths, thus reducing the number of treatments required. The selection trait is a simple measure of gross gill signs ("gill score" scale 0 to 5) taken when the population average score has advanced beyond commercial bathing thresholds. The aims of this study were to establish whether recurrent high average levels of AGD predisposes fish to refractory responses that bias the gill score selection trait and to establish the genetic relationship of gill score with the objective trait (time between baths). Following marine transfer, individual fish were allowed to develop AGD signs over a 190 day period and were bathed when assessed at gill score ≥ 2, while sibling fish in a second population were gill scored and bathed when that population average gill score exceeded 2.0. Time to individual bath at gill score 2 was calculated and all periods were expressed as thermal time (degree days). Heritability of resistance to AGD, assessed by gill score was $h^2_o = 0.09$ to 0.48 depending upon the number of infections experienced. There was a moderate to strong negative relationship between the period of individual AGD infection and the average gill score measures. These results indicate that exposure to AGD at advanced population average does not cause undue bias in subsequent gill score measurements. Genetic parameters of gill score at population average threshold are closely related to the time between individual baths. Therefore selection by gill score will result in reduced bathing frequency. A simple selection scenario indicates that the interval between baths can be increased by 5.4 to 9.3% through gill score selection, providing a reduction of at least one freshwater bath in a production cycle.

6.2 Introduction

Amoebic gill disease (AGD) of marine farmed salmon is emerging as a significant issue worldwide, having been reported in Australia, Chile, France, New Zealand, Scotland and the United States (Kent et al., 1988; Munday et al., 2001; Nowak et al., 2002). In Ireland, AGD occurred during periods of record sea temperatures at sites experiencing oceanic salinities (Rodger and McArdle, 1996). Recently, AGD outbreaks causing up to 82% mortality have been described in Norway following a prolonged period of higher than average seawater temperatures (Steinum et al., 2008). However, the production area most impacted by AGD is Tasmania (Australia), where average water temperatures are higher than other growing areas and reiterative freshwater bathing is required to treat the disease year-round. Due to the high infrastructure and labour costs associated with freshwater bathing, AGD is estimated to add up to 20% to the cost of production (Munday et al., 2001).

The aetiological agent of AGD is *Neoparamoeba perurans* (Young et al., 2007), a free living, amphizoic marine amoeba. Primary attachment of this ectoparasite to salmon gill epithelium causes a progressive host response including lamellar fusion, epithelial hyperplasia, hypertrophy, oedema and interlamellar vesicle formation (Adams and Nowak, 2001; 2003). In the early stages, the disease presents grossly as small raised white spots on the gills, but can rapidly develop to large mucoid patches as the parasite proliferates and lesions enlarge and coalesce. Microscopically, trophozoites blanket smaller lesions but then migrate out and are found only on the edges of larger lesions (Adams and Nowak, 2001). The visible progression of gross signs, from 'clear' (no visible signs) to 'heavy' (large patches), is utilised by farm managers as a simple categorical 'gill score' to assess the prevalence and intensity of AGD through regular subsampling of individuals from each production cage. The information gathered is used to schedule proactive freshwater bath treatments at low to moderate disease thresholds. Adams et al. (2004) demonstrated that macroscopic gill assessment of a single gill hemibranch has a moderate to good agreement with histopathology in advanced cases but in less severe cases it is less accurate.

Selective breeding for enhanced disease resistance or tolerance is becoming more common in aquaculture production. There is considerable evidence of a significant underlying genetic component to resistance of Atlantic salmon to a range of viral, bacterial and parasitic diseases (Gjøen et al., 1997; Mustafa and MacKinnon, 1999b; Kolstad et al., 2005; Glover et al., 2005; Guy et al., 2006; Guy et al., 2009; Ødegard et al., 2006; 2007a; b; Storset et al., 2007; Wetten et al., 2007; Kjøglum et al., 2008; Norris et al., 2008). The primary breeding goal of the recently established Tasmanian salmon selective breeding program includes a number of economically important commercial traits, the most valuable of which is resistance to AGD. The objective trait is defined as 'a reduction in the frequency of freshwater bathing'. Gill score is used as a simple and cost effective selection trait for measurement of AGD resistance in a marine challenge. Gill score shows a moderate genetic correlation (r_g = 0.65) with histopathology and lesioned image area measurements (Taylor et al., 2007) and is closely related to natural field survival to AGD when the disease is allowed to progress unbathed (r_g = -0.96) (Taylor et al., 2009a). Gill score in the breeding program marine challenge cohort is allowed to express to a higher average bathing threshold than is commonly targeted in commercial cages to ensure that genetic variation is measured adequately.

In this study, the aim was to establish that selection for lower gill score will result in a reduced freshwater bathing requirement. It is possible that the pattern of disease development in the population reflects differential exposure (time and dose) to the parasite and that severe infection experienced by some individuals predisposes them to a refractory response in subsequent infection rounds. Therefore, the specific aims were to (i) compare genetic parameters for the variation of gill score at population average bath thresholds with the disease development time to individual gill score threshold, (ii) establish whether gill score and required bathing frequency are under common genetic control and to estimate the genetic gain in freshwater bathing frequency possible when selecting on gill score.

6.3 Materials and Methods

6.3.1 Mating design, freshwater rearing and marine transfer

141 full-sib families were produced by crosses between 78 males and 71 females in May 2006. The mating design was a 2 x 2 factorial, whereby each sire was crossed with two dams and each dam with two sires. Some families were discarded due to poor fertilisation or low egg survival, achieving a final design of 63 paternal half-sib families and 70 maternal half-sib families. Immediately prior to hatching, 500 eyed eggs from each family were mixed into a single tank to ensure a common environment for swim-up, feeding and rearing. In June 2007, a random sample of 4500 pre-smolt from the combined tank were individually weighed (mean = 130 g, SD = 40 g) and intramuscularly tagged with Passive Integrated Transponder (PIT, Sokymat, Switzerland) tags and a caudal fin clip dissected from each individual and stored in 95% ethanol for eventual DNA extraction, genotyping and pedigree determination by Landcatch Natural Selection (Scotland) using a microsatellite multiplex. The fish were held in the hatchery for a further six weeks under lights (22L:2D) at ambient temperature. On 31st July 2007, the smolt (mean weight 173 g, SD = 53) were randomly stocked to two 10 x 10 x 8 m (800 m3) marine fish pens moored 100 m apart on a commercial lease at Tassal Operations Pty. Ltd., Dover, Tasmania. The two pens contained 2615 and 1830 fish (Table 6.1), results of pedigree analysis confirmed that all families were represented in both pens and in proportion to the stocked populations. The average number of animals per full-sib family in each population were 17.4 ± 9.7 (range 3 - 48) and 12.3 ± 6.1 (range 1 - 36). Both stocked pens were fed commercial pellets ad libitum throughout the trial period.

6.3.2 Reiterative natural AGD development and bathing

6.3.2.1 Bathing at population average gill score (PGS) threshold:

In one pen (containing 2615 fish), AGD bathing was scheduled according to the population average gill score. This was repeated over multiple rounds of natural AGD development, bathing and reinfection over 184 days. The development of

AGD in the population was monitored fortnightly by randomly subsampling 40 fish and recording gill scores from 0 to 5 (Taylor et al., 2009b). This continued until an average score in excess of 2.0, with a few fish at gill score 5, was achieved. All fish in the pen were then anaesthetised using 17 ppm Aqui-S (Aqui-SNZ Ltd, Lower Hutt, New Zealand) and gill scored (Infection 1, 51 days post input (DPI)) by two experienced assessors prior to bathing in soft riverine freshwater for a minimum of 2 h. The redevelopment of the disease was monitored in the same way until a similar expression of gross gill lesions was achieved. Each fish was again anaesthetised and gill scored prior to bathing (Infection 2, 105 DPI). This process of monitoring, scoring and bathing continued through two more waves of reinfection and disease development (Infection 3 at 140 DPI and Infection 4 at 184 DPI).

6.3.2.2 Bathing at individual gill score (IGS) threshold:

The second cage, containing 1830 fish, had a similar family structure to the PGS group (Table 6.1). This pen was stocked in order to measure variation in individual time to bath at an individual bathing threshold of gill score 2. This population was monitored fortnightly by gill inspection of subsamples (40 fish) until approximately half of the population had developed to gill score 2 or above (51 DPI). Each fish was then gill scored by two experienced assessors and each individual that was judged to have reached the individual bathing threshold (≥ 2) was freshwater bathed for a minimum of 2 h and returned to the main (800 m^3) net, while fish of low gill score (0 and 1) were returned unbathed to a 5 x 5 x 5 m (125 m^3) inner net suspended within the main net. The continued development of AGD on fish in the 125 m^3 net was reassessed on a weekly basis and any individuals that had reached the gill score 2 threshold were removed, bathed and returned to the main net while fish below the individual threshold were retained unbathed. Gill score of the population in the main net was monitored by fortnightly subsamples until there was substantial expression of fish of gill score 2 and minimal expression of gill scores > 2. At this stage (91 DPI), the entire population was reassessed (main net and inner net) and individuals bathed if they had reached the threshold. Fish of low gill score (0 and 1) were placed back in the inner net and reassessed on a weekly basis until they had reached the bathing threshold. At 126 DPI, the entire population was reassessed, sorted and

individuals were bathed if they had reached the individual gill score threshold. The process of assessment, sorting and bathing continued until most individuals had been bathed at least three times. The trial was terminated at 190 DPI by gill scoring and bathing the entire population.

The data gathered from the IGS group was initially expressed as 'days between baths' for each individual. However, in order to minimise fish handling, it was inevitable that some fish were bathed at above the gill score 2 threshold. In these cases, the 'virtual bath date' at which gill score 2 is estimated to have occurred was back-calculated linearly. If a fish was bathed at gill score 2, the actual and virtual bath dates are equal. The assumption of linearity was based upon a study of 11 years of commercial pen average gill score records, which demonstrate that a linear approximation of gill score development is relevant to the Spring-Summer time period through which this trial ran (Muller and Taylor, 2009).

Neoparamoebae are known to be more abundant in summer (Douglas-Helders et al., 2003) and temperature significantly influences the severity and timing of AGD outbreaks in the field (Adams and Nowak, 2001; 2003; 2004a). Therefore, days between baths was further adjusted to recognise the increasing temperature throughout the study period (Figure 1.1) by summing daily temperatures between the actual start date and individual virtual bath date for each infection round. For the first infection this is expressed as degree days from input to first virtual bath (**°Days 0-1**). For the second infection, **°Days 1-2** is degree days between the first bath (actual) and the second bath (virtual). Similarly, the third infection (**°Days 2-3**) is degree days from the second bath (actual) to the third bath (virtual).

Longer-term individual AGD development was expressed as **°Days 0-3** (average degree days per infection round between input and the third bath). This was calculated as the average of °Days 0-1, °Days 1-2 and °Days 2-3; any fish that died prior to the third bath were excluded from the data. Previous studies (Taylor et al., 2009a; 2009b) and the data from the PGS group (Table 6.4) indicate that resistance to first AGD infection differs from AGD reinfection (the second round onwards).

Therefore, long-term reinfection development was expressed as **°Days 1-3** (average degree days per infection between the first actual bath and the third virtual bath), calculated as the average of °Days 1-3 and °Days 1-3 with dead fish removed.

6.3.3 Animal Ethics

All animal procedures were approved by the University of Tasmania Animal Ethics Committee (Permit # A0009111) under the guidelines of the Australian Code of Practice.

6.3.4 Statistical analysis

6.3.4.1 Genetic parameter estimation:

Each of the nine traits (Infection 1, 2, 3 and 4 from the PGS group; °Days 0-1, 1-2, 2-3, 0-3 and 1-3 from the IGS group) were initially analysed as continuous variables with a univariate linear mixed animal model in ASReml (Gilmour et al., 2006). The significance of weight at tagging, time in freshwater at the previous bath round and of assessor at each bathing was determined by Wald F statistics. Freshwater tagging weight and time in bath were found to be non-significant and were dropped from the model. Assessor effect was only significant at Infection 1 ($P < 0.001$). The random non-additive effect of sire by dam interaction was included in this initial analysis but was found to be negligible (< 4% of total variation) and not significant, and therefore not fitted in the final model.

Using starting values from the initial univariate analysis, variance components for the random effects of the nine studied traits were estimated more accurately by fitting a multivariate linear mixed animal model using ASReml. Use of a multivariate model also allows covariance between traits to be estimated. The following model was used for all traits:

$$Y_{ij} = \mu + f_i + a_j + e_{ij} \qquad (6.1)$$

where Y_{ij} is a vector of the observed values for gill score at each PGS assessment, the five measures of degree days to bath in the IGS group; μ is the overall mean, f_i is the fixed effect for assessor (i = 1,2) at each gill scoring event; a_j is the random additive genetic effect of an individual, and e_{ij} is the residual random effect (between fish within families). The error term includes inter-trait variance and covariance matrices. Narrow sense heritability was estimated as the proportion of additive genetic variance to total variance as follows:

$$h_o^2 = (\sigma_a^2)/(\sigma_a^2 + \sigma_e^2) \qquad (6.2)$$

where h_o^2 is the narrow sense heritability on the observed scale; σ_a^2 is the additive genetic variance; and σ_e^2 is residual variation. Approximate standard errors of variance components for random regression coefficients are automatically calculated in ASReml from the average information matrix obtained in the residual maximum likelihood procedure.

Heritability of the four categorical variables (Infections 1, 2, 3 and 4 for the PGS group) was adjusted to the underlying normal scale (Falconer and Mackay, 1996). The observed phenotype (gill scores or number of baths) was assumed to be related to an underlying, unobservable continuous variate through a set of fixed thresholds. The cumulative proportion of individuals that were accounted for at each threshold was assumed to follow a standard normal distribution, described on a liability scale. As liability increases, the observed phenotype changes from one threshold (e.g. gill score 1) to the next (e.g. gill score 2). These thresholds are defined as deviations from the mean as follows (Gianola, 1979):

$$h_u^2 = h_o^2 \left(\sum_{i=1}^{m} a_i^2 \Pi_i [1 - \Pi_i] - 2 \sum_{i=1<j=1}^{m} \sum^{m} a_i a_j \Pi_i \Pi_j\right) / \left(\sum_{i=1}^{m-1} z_i [a_{i+1} - a_i]\right)^2 \qquad (6.3)$$

where $(a_1, a_2, \ldots a_m)$ are the scores given to the m response categories of gill score at Infections 1,2,3 and 4, $(\Pi_1, \Pi_2, \ldots \Pi_m)$ are the m response probabilities, and z_i is the

height of the standard normal curve at the boundary between category i and $i+$, *i.e.* where cumulative probability equals $\sum_{j=1}^{i} \Pi_j$.

The phenotypic correlation (r_p) between traits within each pen were obtained by firstly analysing each trait for the effect of assessor by one-way ANOVA, then obtaining residuals which represent the traits adjusted for assessor, and finally calculating correlations between the residuals. By applying Fisher's z transformation, approximate standard errors were calculated on the z scale, and back-transformed to the original scale (Fisher, 1950). The genetic correlation (r_g) between each trait pair was calculated using model 6.1. The r_g estimates do not need to be transformed because they are equal on the observed and underlying scale (Ollausson and Rönningen, 1975; Gjerde and Schaeffer, 1989; Gjerde et al., 2009).

6.3.4.2 Estimation of response to selection:

The predicted increase in bath interval (the objective trait) when selecting for gill score (the selection trait) was estimated. Separate estimations were made for Infections 1, 2, 3 and 4 using the correlated response to selection relationship of Falconer and Mackay (1996):

$$G_x = ih_x h_y r_g \sigma_x \qquad (6.4)$$

Where G_x is the gain in bath interval, expressed as °Days 1-3, i is the selection intensity, h_x is the square root of the heritability of °Days 1-3, h_y is the square root of the heritability of gill score at each population average assessment (Infection 1, 2, 3 and 4), r_g is the genetic correlation between °Days 1-3 and each Infection (1,2,3 and 4) and σ_x is the phenotypic SD of °Days 1-3. The selection was made on the best 200 individuals from a population of 2500 (selection of the top 8%, $i = 1.86$). This scenario does not allow for sex of the individuals or apply any restrictions on the number of fish per family that could be used for breeding to keep the rate of inbreeding at an acceptable level. For each selection, the calculated improvement to

the population mean of the next generation was expresses as percentage improvement in °Days 1-3.

				Distribution (percentages) across gill scores						
Event	Days Post Input	No. fish	Av.Wt.(g) (SD)	GS 0	GS 1	GS 2	GS 3	GS 4	GS 5	Av. gill score (SD)
PGS group										
Input	0	2615	171 (55)	-	-	-	-	-	-	-
Infection 1	56	2532	316 (96)	4.7	15	43	30	7	0.4	2.2 (1.0)
Infection 2	105	2489	663 (152)	2.4	11.5	34.2	34.2	15.1	2.5	2.6 (1.0)
Infection 3	140	2301	922 (210)	3	14	32.9	28.6	15.9	5.6	2.6 (1.2)
Infection 4	184	1875	-	8.1	27	26.9	20.7	12.2	5	2.2 (1.3)
IGS group										
Input	0	1830	171 (55)	-	-	-	-	-	-	-
1st bath	51-98	1777	-	0	0	74.9	22.1	2.9	0.2	2.3 (0.5)
2nd bath	77-146	1735	-	0	0	86.9	11.8	1.2	0.1	2.1 (0.4)
3rd bath	104-190	1652	-	0	0	73.4	23	3.4	0.2	2.3 (0.5)

Table 6.1 Sampling schedule and gill score distribution at each sampling. All fish in the PGS group were bathed at population average gill score threshold of > 2; each fish in the IGS group was bathed at an individual gill score threshold of ≥ 2. (SD = standard deviation). Gill score 0 (GS 0) to gill score 5 (GS 5)

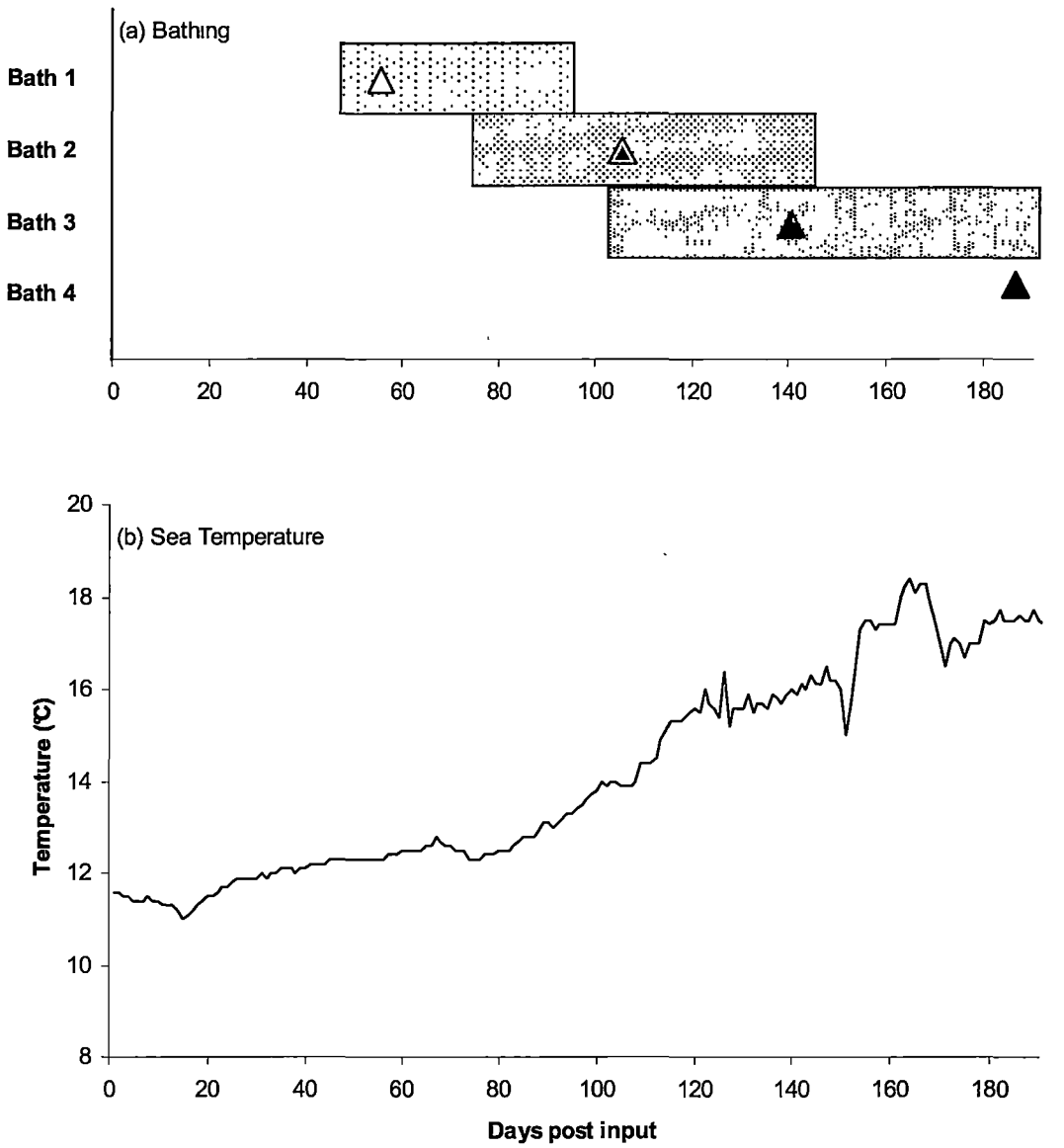

Figure 6.1 Freshwater bathing history following smolt input. (a) PGS gill scores were measured immediately prior to bathing, triggered at pen average gill score threshold of ≥2 (Δ Infection 1, ▲ Infection 2, ▲ Infection 3 and ▲ Infection 4). Fish in the IGS group were bathed at individual gill score threshold ≥ 2, bars represent the range of actual bathing dates (▭ Bath 1, ▭ Bath 2, ▭ Bath 3). (b) Daily water temperatures (—) for the period at five meters depth.

6.4 Results

6.4.1 Bathing at population average gill score threshold

Gill score averages prior to bathing the Pen 1 population ranged from 2.2 to 2.6 (Table 6.1) over the four rounds of AGD and each gill score category (0 to 5) was represented at each measure. The reduction in fish numbers from 2532 at the Infection 1 measure to 1875 at Infection 4 is largely due to mortalities of heavily infected individuals prior to bathing or during anaesthesia, handling and bathing. This effect was exacerbated between Infection 3 and Infection 4 (422 fish, 18.5% mortality) when temperatures were over 16°C (Figure 1.1). Losses between these two measures were highly correlated with gill score at Infection 3 ($r = 0.86$, Cochran-Armitage chi-square = 391.46; d.f. = 1; $P<0.001$).

6.4.2 Bathing at individual gill score threshold

The reiterative process of gill scoring 1777 fish and bathing at the individual gill score threshold of ≥ 2 required 12090 individual gill scores (mean = 6.8 ±2.6 assessments per fish) and 6106 individual bath events over 139 days between 51 DPI and 190 DPI. This strategy resulted in lower proportions of high gill score (4-5) fish, contributing to the lower handling mortality (18.5%) during this period, compared with 25.9% in Pen 1 (56 DPI to 184 DPI, 7322 individual gill score events).

The time for individuals to reach the bathing threshold for the first infection ranged from 20 days to 98 days after adjustment to a constant gill score. This was expressed (Table 6.2) as degree days from input to first bath (°Days 0-1, mean = 661, SD = 145, range = 239-1212).

For the second infection, the time to a bathing threshold was between 14 and 89 days (mean = 44.1, SD = 12.6) after the first bath (also adjusted to a constant gill score). Expressed as degree days this range was 175 to 1246 (mean = 577, SD = 178). The time between the second bathing and the third infection was 14 to 49 days (mean = 26.6, SD = 7.1) following constant gill score adjustment. Expressed as

degree days the range was 196 to 814 (average = 408, SD = 105, range = 196-814). Only 10 surviving fish (0.7%) did not require a third bath within the 190 day trial period.

Average degree days per infection, between input and the third corrected bath (°Days 0-3) ranged between 276 and 880 (mean = 546, SD = 60). Average degree days for the two reinfection rounds between the first bath and third corrected bath (°Days 1-3) was between 211 and 970 (mean = 489, SD = 86).

Mean weight in the two study pens, soon after the completion of the AGD trials was significantly higher ($t = 5.26$, d.f. = 3029, $P<0.001$) in the individually bathed pen (IGS mean = 1701 g, SD = 358) than in the high population average bathed pen (PGS mean = 1631.9 g, SD = 357). Therefore it is possible that the differing AGD bathing and handling strategies employed in the two study cages may have influenced relative growth performance to some extent. However, other potential cage effects independent of AGD and handling can not be ruled out.

Trait	Group	Trait description	Average (SD)	CV	Range
Infection 1	PGS	Population average gill score 56 DPI	2.2 (1.0)	0.43	0-5
Infection 2	PGS	Population average gill score 105 DPI	2.6 (1.0)	0.41	0-5
Infection 3	PGS	Population average gill score 140 DPI	2.6 (1.2)	0.46	0-5
Infection 4	PGS	Population average gill score 184 DPI	2.2 (1.3)	0.6	0-5
°Days 0-1	IGS	Degree days from input to the first corrected bath date[a]	661 (145)	0.22	239-1212
°Days 1-2	IGS	Degree days from the first bath (actual) to the second corrected bath date[a]	577 (177)	0.31	175-1246
°Days 2-3	IGS	Degree days from the second bath (actual) to the third corrected bath date[a]	408 (105)	0.26	196-814
°Days 0-3	IGS	Average °days per infection between input and the third bath	546 (60)	0.11	276-880
°Days 1-3	IGS	Average °days per reinfection between the first and the third bath	489 (86)	0.18	211-970

Table 6.2 Summary statistics and trait descriptions for average gill score measures in PGS group and degree days between individual baths in the IGS group. (SD = standard deviation). [a]corrected by linear back-calculation if gill score > 2.

6.4.3 Heritabilities and correlations

Heritability of resistance to AGD (Table 6.3) measured in the PGS group, was low at Infection 1 ($h^2_o = 0.09$) and moderate at Infection 2, 3 and 4 ($h^2_o = 0.36$, 0.48 and 0.27, respectively). Transformation to the underlying scale increased heritability estimates of each of these traits by 7-11%. Heritability of the rate of individual gill score development on each infection round, measured as degree days between bath events in the IGS group, were low and significant, ranging from $h^2_o = 0.06$ to 0.18. The heritability of the average bathing interval between input and the third bath was $h^2_o = 0.35$ while h^2_o for the average period of reinfection between the first and the third bath was 0.24. The large increase in h^2_o for the combined measures suggests a single measure of the rate of disease development is imprecise and estimates can be greatly improved with replication.

The phenotypic correlations between Infection 1 and the three later infections (2, 3 and 4) were low ($r_p \leq 0.10$) although significantly different from zero (Table 6.4). Genetic correlations of Infection 1 with Infections 2 and 3 were moderate ($r_g = 0.41$ and 0.38 respectively) and significant, but r_g with Infection 4 was not significant, suggesting that the relationship from first to later infections declines over subsequent infection rounds. The phenotypic correlations between Infections 2, 3 and 4 were moderate ($r_p = 0.22$ to 0.52) and significant, while the corresponding genetic correlations were high ($r_g = 0.75$ to 0.88).

Measures of degree days between individual baths had low to moderate negative phenotypic correlations ($r_p = -0.12$ to -0.30). There were no significant genetic correlations between the first individual bathing (°Days 0-1) and the two later rounds (°Days 1-2 and °Days 2-3). The relationship between the second and third individual bath periods was moderate ($r_g = 0.53$). It is possible that these relationships were influenced by the linear back-calculation of high gill scores (> 2). However, the heritabilities of each degree day measure were unchanged and the correlations were largely unaltered when cross-checked with a restricted dataset of fish that were consistently bathed at gill score 2 or when no back-calculation was performed on individuals of gill score > 2 (data not shown).

At each round of PGS infection, there was a high negative genetic correlation with the corresponding rate of development to IGS bath threshold (Infection 1 and °Days 0-1 = —1.31; Infection 2 and °Days 1-2 = —0.97; Infection 3 and °Days 2-3 = —0.65) suggesting that selection for low gill score will result in extended time to bathing. Genetic variation at first infection appears to be a different trait to later (re)infections, Infection 1 was not significantly correlated with °Days 1-2 or °Days 2-3; °Days 0-1 showed a moderate relationship with Infection 2 and 3 (*rg* = —0.47, —0.39 respectively) and was not significantly correlated with Infection 4. During the reinfection rounds, there was moderate to high genetic correlation between °Days 1-2 and Infections 3 and 4 (*rg* = —0.76 and —0.67 respectively) and between °Days 2-3 and Infection 4 (*rg* = —0.43). The relationship between °Days 2-3 and the previous PGS infection round (Infection 2) was low (*rg* = —0.28). The combined measure of IGS infection period from input to the third bath (°Days 0-3) had a moderate to high correlation with each PGS infection (*rg* = —0.46 to —0.87). The combined IGS reinfection measure (°Days 1-3) was not significantly correlated with Infection 1 but was highly correlated with each PGS reinfection (Infection 2,3 and 4; *rg* = —0.89, —0.82 and —0.64 respectively).

Trait	V_a	V_e	h^2_o	h^2_u
Infection 1	0.08 (0.02)	0.79 (0.03)	0.09 (0.02)	0.1 (0.03)
Infection 2	0.39 (0.07)	0.68 (0.04)	0.36 (0.05)	0.39 (0.06)
Infection 3	0.66 (0.11)	0.72 (0.06)	0.48 (0.06)	0.51 (0.06)
Infection 4	0.48 (0.09)	1.29 (0.07)	0.27 (0.05)	0.29 (0.05)
°Days 0-1	1379 (528)	19606 (794)	0.07 (0.02)	-
°Days 1-2	5787 (1312)	25870 (1259)	0.18 (0.04)	-
°Days 2-3	629 (271)	10458 (431)	0.06 (0.02)	-
°Days 0-3	1313 (244)	2389 (171)	0.35 (0.06)	-
°Days 1-3	1786 (393)	5599 (323)	0.24 (0.05)	-

Table 6.3 Components of additive genetic variance (V_a), error variance (V_e) and heritabilities (h^2_o = observed scale, h^2_u = underlying scale) for average gill score measures and degree days between individual baths. Trait descriptions are detailed in Table 6.2. Standard errors are shown in parentheses.

Trait	Infection 1	Infection 2	Infection 3	Infection 4	°Days 0-1	°Days 1-2	°Days 2-3	°Days 0-3	°Days 1-3
Infection 1	-	0.1 (0.02)	0.09 (0.02)	0.07 (0.02)	NA	NA	NA	NA	NA
Infection 2	0.41 (0.14)	-	0.52 (0.02)	0.22 (0.02)	NA	NA	NA	NA	NA
Infection 3	0.38 (0.14)	0.85 (0.04)	-	0.33 (0.02)	NA	NA	NA	NA	NA
Infection 4	0.29 (0.16)	0.75 (0.07)	0.88 (0.05)	-	NA	NA	NA	NA	NA
°Days 0-1	-1.31 (0.17)	-0.47 (0.17)	-0.39 (0.17)	-0.09 (0.2)	-	-0.26 (0.02)	-0.12 (0.02)	0.46 (0.02)	-0.36 (0.02)
°Days 1-2	-0.16 (0.17)	-0.97 (0.06)	-0.76 (0.09)	-0.67 (0.11)	0.37 (0.21)	-	-0.3 (0.02)	0.54 (0.02)	0.81 (0.01)
°Days 2-3	-0.04 (0.25)	-0.28 (0.2)	-0.65 (0.17)	-0.43 (0.2)	-0.07 (0.3)	0.53 (0.24)	-	0.2 (0.02)	0.32 (0.02)
°Days 0-3	-0.52 (0.14)	-0.87 (0.06)	-0.78 (0.07)	-0.46 (0.13)	0.65 (0.13)	0.95 (0.04)	0.62 (0.18)	-	0.66 (0.01)
°Days 1-3	-0.16 (0.17)	-0.89 (0.06)	-0.82 (0.07)	-0.64 (0.12)	0.33 (0.21)	0.98 (0.02)	0.83 (0.15)	0.93 (0.03)	-

Table 6.4 Intertrait genetic (below diagonal) and phenotypic correlations (above diagonal). Trait descriptions are detailed in Table 6.2. Standard errors are shown in parentheses. NA not available.

6.4.4 Genetic gains

An increase in the period between AGD infection (the objective trait) of between 5.4% and 9.3% was estimated (Table 6.5) by selecting the top 200 ranked individuals from the gill score at population average (Infection 2, 3 and 4, the selection traits) The effect of selection from Infection 1 could not be estimated because this trait was not significantly correlated with °Days0-1 (Table 6.4).

Selection strategy	Calculated response in °Days 1-3	Improvement (%)
Infection 1	0	0
Infection 2	85.9	8.8
Infection 3	90.8	9.3
Infection 4	53.1	5.4

Table 6.5 Predicted response in the period between baths (°Days 1-3) from selection on gill score at Infections 1 to 4. Assumes selection the top 200 candidates from a population of 2,500 fish (i = 1.86). The selection traits were : Infection 1 = gill score 56 DPI, Infection 2 = gill score 105 DPI, Infection 3 = gill score 140 DPI, Infection 4 = gill score 184 DPI. The response trait was °Days 1-3.

6.5 Discussion

Gross gill scores is utilised as the selection trait for resistance to AGD in the Tasmanian Atlantic salmon breeding program. The disease is allowed to progress to an advanced average gill score prior to freshwater bathing so that gill signs are approximately normally distributed and genetic evaluation and segregation of the most susceptible and resistant animals within the population is clear. It is assumed that selection for lower gill score will result in reduced freshwater bathing frequency. However, it is possible that gill score measurement may be biased by the degree of previous exposure to the disease. The objective of this study was therefore to compare genetic parameters of gill score at a population average and individual level in sibling populations to assess whether results are biased by high exposure to the disease and establish the relationship of the selection trait (gill score) with the objective trait (period between freshwater baths).

6.5.1 Gill score bias due to prior AGD infection

A potential issue with measurement of gill score at advanced population average is that refractory responses to previous AGD infection may bias measurement of additive genetic variation of the disease in subsequent reinfection rounds. This bias may be because fish of low gill score have not been exposed to adequate parasite loads to trigger or strengthen immune responses. Conversely, large gill lesions, or the trophozoites associated with them, may be more resilient to bath treatment thus rendering the fish more likely to redevelop AGD signs. Freshwater is known to remove the majority of attached trophozoites, to clear tissue debris and excess mucus and to augment rapid healing of hyperplastic lesions (Munday et al., 2001; Clark et al., 2003; Adams and Nowak, 2003; Roberts and Powell, 2003), though the primary stimulus for lesion repair is the removal of amoebae. Reinfection may be initiated by amoebae that remain post-treatment but is mainly due to waterborne trophozoites attaching to healthy gill tissue (Adams and Nowak, 2004a) which can begin within 12-24 h post bathing (Zilberg et al., 2000; Adams and Nowak, 2004b). Cellular proliferation within the gill is controlled by a range of differentially expressed genes involved in cell cycle pathways (Wynne et al., 2008b), therefore lesions that are

more resilient to freshwater may be predisposed to rapidly enlarge in association with further parasite insult. However, it is known that trophozoite attachment to pre-existing areas of injured gill tissue is inhibited during infection (Adams et al., 2009) and residual post bath lesions are not recolonised by amoebae until they have healed over a few days (Adams and Nowak, 2004a). This localised resistance may be due to cellular priming of inhibitory factors or the physical disruption of attachment due to damaged epithelial structure.

This study compared the variation of gill score at advanced population average (2.0) with the period between baths at a low individual threshold (2). The high correlation between each round of PGS bathing and the corresponding IGS infection round (Infection 1 and °Days 0-1, Infection 2 and °Days 1-2; Infection 3 and °Days 2-3) indicates that genetic control of AGD progression was similar despite the differing levels of disease exposure in the two treatments. Potential bias due to low gill score fish failing to develop a response to Neoparamoebae was avoided because each individual was infected to at least a gill score 2. Some bias in the IGS group may have occurred because gill score 2 was frequently exceeded (Table 6.1). However, there was little change in genetic parameters when these fish were removed from the data. The moderate to high genetic correlation between average infection period and each of the PGS measures (Infection 1, 2, 3 and 4) indicates that the time between baths is adequately predicted by our measurement of gill score at population average. Genetic variation of resistance at first infection was moderately to poorly correlated with later rounds of reinfection. This confirms previous observations (Taylor et al., 2009a; 2009b) and suggests a change in the nature of resistance with repeat exposure to AGD. The average period of reinfection (°Days 1-3) was unrelated to the first infection (Infection 1) in the PGS group but was highly correlated with each reinfection round (Infection 2, 3 and 4).

The disadvantage of measuring variation of gill score at advanced population average threshold is that the onset of losses of high gill score (4-5) fish may begin in the days prior to a measurement and further mortalities of susceptible fish may be associated with anaesthesia and bath handling, which is intensified at high water temperatures. It is possible that the lower heritability at Infection 4 that we report here is due to the

cumulative effect of susceptible fish losses over previous infection rounds. However, when reassessed using only data from fish that had survived to 184 DPI, there was only a slight reduction in heritability at earlier rounds of infection and minor increase in genetic correlations. Therefore, the apparent drop in heritability from Infection 3 to Infection 4 may be due to other effects, such as a genetically linked stress response during periods of high water temperature. Further work is required to establish whether this apparent drop in heritability in mid summer is related to cumulative losses of previously susceptible fish or an increase in error variance in response to environmental stressors. As a selection trait for the breeding program, it appears there is a balance to be struck between allowing expression of advanced average gill score and the risk of losing information on the most susceptible fish.

6.5.2 Gill score selection for freshwater bathing frequency

The reported genetic variation of gill score over reiterative rounds of natural AGD challenge agrees with our earlier findings (Taylor et al., 2009a). The studies were carried out in different years on the same site, following mid-winter input using unrelated fish. The heritability of gill score at first infection ($h^2_o = 0.09$) in the current study is lower than our previous value ($h^2_o = 0.23$), possibly reflecting the lower average gill score in the current trial (2.2 compared to 3.5). Heritability of gill score in the second and third rounds of infection is comparable to our earlier values ($h^2_o = 0.39$ and 0.48).

Selection for resistance to AGD is based upon variation of gill score over reiterative natural infection and bathing cycles. This selection assumes that breeding for lowered gill score will result in an increased period between freshwater baths. The results of this trial confirm that the selection trait (gill score) closely predicts the objective trait (time to bath). The gill score at Infection 1 was highly correlated with the time to first infection and each of the reinfection rounds (Infection 2, 3 and 4) were highly correlated with the average reinfection period (°Days 1-3). The predicted response of selection based upon gill score suggests that the period between freshwater baths can be increased by 5.4 to 9.3% per generation. Based

upon current industry expectations of 13 baths per production pen (Tassal Group Limited, 2009a), an 8% increase in bathing period will result in a reduction of one bath per production pen over a typical 15 month production cycle.

Our previous studies (Taylor et al., 2009a; 2009b) have indicated that, following first infection, some fish are able to maintain a gill score of '0' when assessed at population average bathing threshold, suggesting that they develop complete resistance to AGD. However, all fish in the IGS group reached an individual bathing threshold of 2 over the first two individual bathing rounds. Only 0.7% of the fish did not require a third bath within the study period. This suggests that resistance is relative and not absolute, at least in the first few rounds of natural AGD infection. Fish that show no gross gill lesions are simply slower to infect and are bathed before they develop gross gill signs. From the overall patterns of additive genetic variation and correlation, it is reasonable to conclude that measurement of gill score at advanced population average reflects the range in AGD development rates. Therefore, it is reasonable to predict that selection for lower gill score will result in offspring that have a slower rate of AGD development.

Compared to direct measurement of 'time to bath', gill score is a simpler method for the breeding program. The higher levels of additive genetic variation and larger coefficient of variation associated with gill score supports a more accurate selection for AGD resistance. Gill score is highly correlated with mortality due to AGD (Taylor et al., 2009a; 2009b), so a reduced rate of AGD development will directly benefit the salmon industry by retarding the onset of AGD related mortalities. Slower disease development will also improve opportunities for bathing to be applied proactively at low to moderate gill score, rather than reactively at moderate to high gill score averages, thus reducing handling losses and simplifying farm resource planning. The ultimate effect of slower AGD onset will be to reduce the number of freshwater bath treatments required during a production cycle.

6.5.3 Conclusion

Gill score at population average freshwater bathing threshold is the selection trait for AGD resistance utilised in the Tasmanian salmon breeding program. The trait is measured at above normal commercial disease thresholds in order to clearly separate the most susceptible and the most resistant animals. When compared to individual time to bath, reiterative development to advanced average gill score does not appear to unduly bias subsequent measures. However, cumulative losses of the most susceptible fish may reduce subsequent estimates of additive genetic variation of AGD resistance. As a selection trait, gill score is highly correlated to the objective trait (time between baths). Selection for lower gill score is therefore likely to lower the required bathing frequency.

6.6 Acknowledgements

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7 : General Discussion

7.1 Preamble

Amoebic gill disease causes cardiovascular and metabolic compromise in affected fish (Powell et al., 2008). Although direct mortalities to the disease are largely controlled by regular monitoring and proactive freshwater treatment, low level losses may occur in the period immediately preceding bathing or as a direct result of crowding and transfer of diseased fish during the bathing process. The main economic impact of AGD is the high cost of freshwater bathing, which has recently been estimated at AU $0.08/kg per bath from AU $5.00/kg production cost (Tassal Group Limited, 2009b), with 13 baths required in a production cycle (Tassal Group Limited, 2009a). Therefore, the highest selection weighting in the breeding goal of the recently established SALTAS Tasmanian Atlantic salmon selective breeding program (SBP) is for resistance to AGD (Elliott and Kube, 2009). The focus of selection is to increase the time between treatments and therefore reduce the incidence of freshwater bathing.

The primary aims of this thesis were to determine whether genetic variation of AGD resistance exists within the Tasmanian Atlantic salmon population and how to assess this variation within the context of a breeding program. The Tasmanian salmon farming industry uses a categorical gill score in order to schedule freshwater bathing of caged populations at predetermined average threshold. This simple method of disease assessment is attractive to the SBP as a selection trait because it is non-destructive and likely to be cost-effective. Gill score was therefore examined as a selection trait in comparison to some potentially more rigorous destructive sampling methods, including histopathology and survival in a natural AGD challenge. It is presumed that selection for lower gill pathology will increase AGD resistance and thus increase the required time between treatments and therefore the selection trait (gill score) was therefore compared with the objective trait (period between freshwater bathing). In addition, phenotypic gill score trends were examined for evidence of developing resistance over reiterative rounds of natural AGD infection. The relationship between this developing resistance and the serum antibody response to wildtype Neoparamoebae was also examined.

7.2 Genetic variation of AGD signs

Histopathology has been the only true diagnostic method indicating a diseased state and its causative agent (Adams and Nowak, 2004b) and can also be used to quantify the degree of AGD pathology (Adams and Nowak, 2001). Image analysis of preserved gills is another quantitative method utilised by researchers (Adams and Nowak, 2004a), but it is a presumptive test that requires confirmation of the presence of Neoparamoebae. In Chapter 2, genetic variation of an industry standard five point (0 to 4) gill score (Powell et al., 2001) was compared with histopathology and gill image measurements following an AGD tank challenge on naïve smolt. Due to the full-sib mating design that had been used to create the cohort, only broad-scale heritability could be estimated. Subsequent AGD measures have confirmed that non-additive effects are negligible, suggesting that these initial estimates ($H^2 = 0.30$ for histopathology, 0.35 for image analysis and 0.16 for gill score) primarily represented moderate additive genetic variation of gill pathology. These levels of genetic variation gave the first indication that significant gains can be made towards the genetic improvement of AGD resistance. The results suggest that histopathology provides a more accurate measure of genetic variation of AGD pathology in a single infection experiment as used in research trials where fish are to be euthanased. The phenotypic disparity between gill score and histopathology ($r_p = 0.27$) recorded in Chapter 2 is consistent with previous reports (Clark et al., 2003; Zilberg et al., 2001; Adams and Nowak, 2001; 2003; 2004a) and results because gross examination may miss smaller lesions or misdiagnose lesions that are not AGD related. Conversely, disagreement may also occur because histopathology and image analysis are generally limited to one or two gill arches so gill pathology on other hemibranchs is overlooked. It is likely that the level of agreement between the assessment methods would be higher from a natural AGD infection because the natural inoculating amoeba density is lower (Douglas-Helders et al., 2003), resulting in slower AGD development to more discrete spots and patches. 'Genetic correlations' between gill score and the two destructive measures were high ($r_g = 0.65$), indicating that gill score provides an adequate estimate of genetic variation that support its use as a simple and non-destructive field measure.

Due to the high cost of rearing, tagging and genotyping to the SBP, it is preferable to keep fish alive through to the final harvest measurement in order to maximise the information on key selection traits that can be returned. From an animal ethics viewpoint it is also advantageous to minimise the number of test animals and optimise the knowledge returned from them (Johansen et al., 2006). In most aquaculture breeding programs, measurement of variation in resistance to specific diseases is primarily achieved by survival testing in tank challenge or following natural disease outbreak. However, because AGD is reiteratively treated on commercial fish farms, a non-destructive method of measuring AGD variation is required in order to determine whether genetic variation of AGD resistance remains consistent with each reinfection. This information will enable development of AGD trait assessment strategies that provide adequate information to support selection decisions whilst minimising the number of measures.

Following the preliminary estimates of genetic parameters of gill score presented in Chapter 2, genetic variation of gill score and survival were compared in Chapter 4. This trial relied upon natural infection because the biotic and abiotic stressors in the natural environment may be an essential factor in inducing typical field pathology that cannot be easily recreated in tank challenge (Norris et al., 2008). The gill score categorical scale was extended to six points (0-5, Table 3.1) in order to give closer approximation to a continuous distribution and more precisely estimate the phenotype of each individual. The trial followed fish through two rounds of advanced natural AGD and freshwater bathing, followed by a third round in which gill scores were recorded after 50 days at the onset of low-level losses and the disease allowed to continue untreated for a further 50 days until mortalities reached a plateau. Heritability of survival time following the second freshwater bath was high ($h^2_o = 0.49$) indicating that selection would yield slower onset of losses and improved survival. However, the need to remove dead fish from the cage on a daily basis represents a significant added cost to the breeding program. When compared to less frequent mortality collection including a binary measure to 50% mortality, genetic parameters were highly correlated, suggesting that sampling costs can be minimised. However, there is added risk of a loss of information through tag loss from uncollected mortalities. These would also present additional hygiene risks to the

farm and act as a reservoir of infective Neoparamoebae (Douglas-Helders et al., 2000). Heritability of AGD resistance measured by gill score improved from moderate ($h^2_u = 0.26$) to high ($h^2_u = 0.52$) over the three rounds of infection. The first infection was not significantly genetically correlated with the later survival trial, while the second and third gill score measures were highly correlated with survival ($r_g = -0.61$ to -0.96). As a selection trait, gill score is estimated to improve AGD survival time by 13.2%. Although AGD losses are largely controlled by proactive freshwater bathing, the improved survivability achieved through selection for lower gill score will directly benefit the industry in delaying the onset of AGD related mortality. Furthermore, non-destructive gill score measures were shown to be more cost effective for the breeding program for a single measure, with the added advantage of preserving animals for additional commercial trait measurements.

7.3 AGD assessment at advanced population average gill score

Genetic variation in disease susceptibility is more readily measured at high infection prevalence and intensity (Glover et al., 2005) because a low infection level increases the number of non-informative families (Kolstad et al., 2005). Throughout this thesis AGD was allowed to develop to an advanced average gill score (≥ 2.0) to ensure that fish with 'clear' and 'heavy' gill signs were well separated and gill score distribution was approximately normal. Although average gill scores at bathing in excess of 2.0 are above commercial target treatment thresholds of 1.0 to 1.5, they have occurred in 17.6% of 2437 baths recorded in over 11 years of industry data (Muller and Taylor, 2009), so should not be considered as unusually high. The disadvantage of allowing advanced average gill score to develop is that the risk of death of AGD susceptible fish increases. High gill score is phenotypically correlated with the rapid onset of mortality (Chapter 3) and was the main factor explaining losses following anaesthesia and handling in the AGD survival trial (Chapter 3 and 4) and in the Chapter 6 PGS group. In both cases, losses were highest during summer handling events, illustrating the interplay of high gill score with environmental stressors. This

suggests that rigorous fish handling protocols (crowding, oxygenation, anaesthesia) during assessments will help to minimise unnecessary loss of susceptible fish.

In developing a disease resistance test for breeding selection, it is necessary to gauge whether the test environment causes bias that will influence selection decisions. Exposure to high pathogen loads or advanced disease levels is likely to cause a range of individual responses within a population that may range from increased susceptibility to increased resistance. It is necessary to establish whether reiterative exposure of the test population to high levels of AGD predisposes fish to be more resistant or susceptible to subsequent AGD challenge than would be expected at lower commercial bathing thresholds. Therefore, in Chapter 6 gill score variation at average population threshold was compared with a sibling group of fish that were bathed at an individual threshold. Although there were limitations on the frequency at which individuals could be sampled, estimates of genetic variation were closely linked in the two trial environments indicating that similar patterns of disease expression developed. Therefore, repeated exposure to advanced population average gill score appears to provide an unbiased estimate of the range of individual responses that result within a population exposed to commercial AGD management strategies. Similar questions about the effect of testing environment are faced by breeding programs wishing to develop controlled tank challenge to pathogens. These generally report a high genetic correlation between different testing strategies (Gjøen et al., 1997; Kolstad et al., 2005; Storset et al., 2007; Wetten et al., 2007). However, there have been no studies examining whether reiterative exposure to higher intensity of disease will alter selection decisions. Future research is warranted within the SBP to compare genetic variation of AGD in populations exposed to either commercial bath treatment or regular bathing at advanced average gill score. In addition, it is suggested that the relationship between gill score and *N. perurans* abundance on the gills should be established, using the quantitative PCR technique developed by Young et al. (2008a), in order to understand whether amoebae reduction at freshwater bathing is independent of gill score.

7.4 Genetic correlation between selection traits and the objective trait

The findings of Chapter 6 examining the relationship between gill score and the objective trait (time between freshwater baths) generally supported the assumptions underlying the use of gill score at population average bathing threshold as the selection trait. Significant genetic variation was reported in both gill score and the period (°Days) between individual baths. Genetic correlations were high between gill score at first infection and the time to first individual bath; and between the reinfection rounds (Infection 2, 3 and 4) and the average period (°Days) between individual reinfections. The results of this study indicate that selection for lower gill score will result in an increase in time between freshwater baths (reduced bathing frequency). However, the measure of 190 days of individual interbath period may not fully reflect population bathing frequencies over a full production cycle (~450 days). Therefore, a larger scale study is required to assess the realised selection gains of bathing frequency of the offspring. It was not possible to measure the response to selection within the timeframe of this thesis because the generation interval of the SBP is three years (Figure 1.2) and measurements were limited to fish from the founder generation of the program.

7.5 The nature of resistance to AGD

In animal breeding programs, disease resistance is generally treated as a quantitative trait under the control of many genes (Detilleux, 2001). Selection for resistance is likely to be successful even if the underlying mechanisms are unknown (Guy et al., 2006). However, knowledge on the major genes or immunological processes responsible for resistance may enable selection to be enhanced. Furthermore, evidence for the role of adaptive immunity in protection against *N. perurans* would provide encouragement for the development of an AGD vaccine.

The phenotypic results from Chapter 3 indicate that there is a change in the nature of resistance following the initial *N. perurans* infection and subsequent AGD

development and freshwater bathing. At first exposure, no fish were innately resistant but some were able to limit the extent of gross gill lesions. Over subsequent rounds of bathing many fish developed a level of resistance as defined by reducing gill score trends. It is possible that a high AGD exposure is necessary to 'prime' the immune response as it was noted that individuals that experienced lower gill score at first infection were poor at surviving reiterative challenge, while higher initial gill score predicted improved survival success and lower gill pathology in later infection rounds. It is unknown whether a fully protective resistance can develop with repeat exposure, though gill score was seen to reduce during the last 50 days of the third (unbathed) infection in fish that survived 210 days of repetitive natural AGD.

One aspect of adaptive immunity, the serum antibody response to *N. perurans*, was studied in Chapter 5. This study confirmed earlier observations (Vincent et al., 2009) of an increase in the frequency of anti-wildtype NP antibodies following repeated waves of natural AGD and freshwater bathing. Despite the high incidence of fish showing visible evidence of antibodies by Western blot, few were 'strong responders' when measured by ELISA. There was no definitive evidence that the presence of either type of anti-wildtype NP antibodies or the antibody titre were related to individual gill score, suggesting that serum antibodies are not protective. This apparent lack of protection is not surprising because *N. perurans* is an ectoparasite that is seldom seen associated with blood cells, thus limiting the presentation of antigens to the systemic immune system. Furthermore, the binding affinity of serum antibodies at the gill-seawater interface is minimal (Bricknell et al., 2002). It is possible that mucus antibodies would be more effective, but initial efforts to detect antibodies in cutaneous mucus of AGD affected Atlantic salmon were unsuccessful (Vincent et al., 2006). However, there have been no attempts to measure mucosal antibodies in AGD resistant fish following repeated natural AGD development and freshwater treatment. Rubio-Godoy and Tinsley (2004) suggested that fish immunity to monogenean ectoparasites is multifactorial and combinatorial in nature, hence the difficulty of correlating protection with one particular immune component. It is likely that a similar situation exists with regard to the development of acquired resistance to *N. perurans*. This could be due to a combination of reiterative stimulation of the innate immune system by interaction of cells from the

adaptive immune system and their products. It is also possible that the physical expression of AGD, lesions caused by an host response to *N. perurans*, could also be affected by variation in the degree of host tolerance to the presence of the parasite. These processes appear to be under the control of a wide variety of genes, as outlined in Chapter 1.

Evidence of a strong genetic basis to the change in resistance following first infection is provided in Chapters 4 and 6. These two trials were conducted one year apart, following winter smolt input to the same location. Both trials confirm significant genetic variation of AGD resistance over multiple infection and bathing rounds (Table 4.2, Table 6.3). However, there is a low genetic correlation between first and subsequent infections (Table 4.3, Table 6.4) which suggests that they are different traits. The genetic correlation between AGD reinfections (from the second round onwards) was high, suggesting that the processes of resistance to reinfection are largely under common genetic control. A similar trend of low initial heritability of innate resistance of sheep to endoparasitic nematodes (*Haemonchus contortus* and *Ostertagia circumcincta*), followed by a well correlated genetic component of resistance over multiple reinfections was reported by Stear et al. (1999). This suggests that genetic resistance to reinfection operates through control of adaptive responses. Wynne et al.(2008b), working on a subset of survivors of the natural AGD survival challenge described in Chapters 3, 4 and 5, found an upregulation of genes involved in adaptive immunity. It is possible that resistance may not be associated with an AGD-specific mechanism but simply reflect an inherently superior immune system in these fish, giving them a superior "general" disease resistant phenotype compared to susceptible animals.

This work has confirmed previous reports of increased resistance to AGD following multiple rounds of disease development and freshwater bathing. The host response includes the development of serum antibodies directed against carbohydrate epitope(s) and peptide epitope(s). Although these serum antibody responses are attenuated and not protective, they may play a role in directing non-adaptive and adaptive immune responses that are stimulated by repeat infection. The

identification of a number of strong antibody responders from this research indicates that informative results may be obtained in future from studies of fish that have survived unbathed AGD reinfections.

7.6 Summary

Following the establishment of the Tasmanian Atlantic salmon selective breeding program in 2004, the initial aims were to establish breeding systems, stock management protocols and trait measurements of the founder generations. With a three year production period, the first selected stock are now beginning to enter the measurement and breeding cycle. Selections are based upon a variety of commercial traits as defined in the overall breeding goal. The findings of this thesis will contribute to the future measurement and selection for AGD resistance within the program.

The main findings from this project are:

1. Resistance to AGD is a quantitative trait that can be measured in a variety of ways.
2. Gill score provides a cost-effective and rapid measure of AGD resistance. The main advantage of this method is that it is non-destructive, thus allowing fish to be measured sequentially over multiple rounds of AGD development and freshwater bathing.
3. The dynamics of commercial AGD and freshwater bathing cycles were reliably created in the SBP marine cohort challenges. Marine cage challenge is therefore a suitable model for future AGD trait measures within the SBP.
4. The selection trait (gill score) closely reflects the objective trait (time between baths). Selection for lower gill score is therefore expected to result in a reduction in bathing frequency in future generations.
5. Potential bias induced by repeated incidences of advanced average gill score does not appear to differ from bias related to normal commercial conditions.
6. There is a strong phenotypic and genetic link between gill score and subsequent survival in an unbathed AGD challenge. Selection for lower gill score will

therefore result in delayed onset of AGD mortalities or lower losses if bathing is delayed.

7. Handling of fish at advanced average gill score may introduce bias through sequential loss of susceptible fish and handling protocols are needed to minimise handling stress.
8. First AGD infection appears to be a separate trait to subsequent reinfections, this has the potential to reduce the number of gill score events necessary for selection trait measurements. AGD 'reinfection' should be treated as a separate selection trait from 'first infection' and the two traits weighted accordingly in the selection index.
9. The change in the nature of resistance between first and subsequent AGD development rounds cannot be explained by the development of serum antibodies directed against Neoparamoebae. It is suggested that resistance is multifactorial and under polygenic control.

Due to the short time period and the limited stocking strategies available for this research, the following restrictions apply to the results:

1. The natural AGD resistance results discussed in this thesis are from mixed-sex salmon put to sea in mid to late winter at a single commercial marine site. However, the Tasmanian industry is largely based upon earlier input of all-female stocks. Limited testing of genetic correlations between different sites and late input times indicates favourable correlations (P. Kube, pers. comm.).
2. This thesis does not address the genetic relationship of AGD resistance to other important commercial traits. There is a favourable genetic correlation between growth and AGD resistance (P. Kube, pers. comm.), yet these results appear to be confounded by measurement of growth in populations exposed to advanced average AGD levels.
3. Due to the protozoan aetiology of AGD, it is unclear whether selection for resistance to this disease would confer susceptibility to other fish diseases that may threaten the Tasmanian salmon industry in the future. It is suggested that the development of controlled marine challenge facilities will be necessary to tackle these issues as the SBP develops.

7.7 Further research

This thesis has demonstrated that a significant heritable component in AGD resistance exists in the Tasmanian Atlantic salmon population and that this can be readily measured using gill score. However, additional research is required to confirm that similar variation exists across a variety of stocking strategies and sites and to correlate disease resistance against other commercially important traits. Furthermore, estimates of response to selection presented in this work should be tested once the progeny of selections are available. Additional work is required to understand the nature of resistance in order to fine tune selection and understand potential genetic interactions with vaccines. Finally, in common with other animal breeding programs, it may be possible to develop whole genome selection. This will reduce the need for marine disease testing and lead to improved genetic gains through direct selection of broodstock in the freshwater environment.

Specific suggestions from this project are to:

1. Compare genetic variation of AGD resistance of a population reiteratively bathed at advanced average gill score against a population with a previous history of low to moderate commercial gill score treatments. This will enable potential bias at advanced average gill score to be further gauged.
2. Establish whether adequate genetic variation of AGD resistance can be measured at lower disease incidence, thus minimising the risk of losing susceptible animals.
3. Measure genetic parameters for AGD resistance across a wide variety of stocking strategies and sites. This will establish whether the patterns of AGD resistance described are consistent.
4. Establish growth parameters in genotyped fish held in marine sites with little or no AGD so that potential compounding effects of disease upon growth are separated.
5. Apply the recently developed PCR test to measure the relationship between *N. perurans* abundance and gross gill score. This may establish whether gross gill pathology varies according to individual host tolerance to the presence of the parasite.

6. Carry out future research on mechanisms of immunity from individuals that have been subjected to multiple infection and bathing events. The majority of previous research into mechanisms of AGD resistance has been based upon first infection trials.
7. Breed synthetic lines of AGD 'resistant' and 'susceptible' animals to assist in future understanding of the immune and genetic mechanisms of AGD resistance.
8. Map the genes associated with AGD resistance to enable whole genome selection.

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