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Reduced total hardness of fresh water enhances the efficacy of bathing as a treatment for amoebic gill disease in Atlantic salmon, *Salmo salar* L.

S D Roberts and M D Powell

The Cooperative Research Centre for Sustainable Aquaculture of Finfish, School of Aquaculture, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania, Launceston, Tasmania, Australia

Abstract

The current treatment for amoebic gill disease (AGD)-affected Atlantic salmon involves bathing sea-caged fish in fresh water, often sourced from local dams, for 3–4 h. In both a small-scale laboratory and an on-farm field experiment, the effects of water hardness on the efficacy of freshwater bathing were assessed. Results showed that soft fresh water (19.3–37.4 mg L^{-1} $CaCO_3$), whether it be naturally soft city mains water or artificially softened dam water, was more efficacious at alleviating AGD in affected fish than hard fresh water (173–236.3 mg L^{-1} $CaCO_3$). Soft freshwater bathing significantly reduced viable gill amoebae numbers (from 73.9 to 40.9% of total count) and significantly alleviated gill pathology, both gross and histological. Following bathing, gross gill pathological scores of soft freshwater bathed fish lagged 2 weeks behind hard freshwater bathed fish. Significant gill lesion fragmentation, and shedding of lesion-associated hyperplastic tissue, was accompanied by a significant reduction in AGD-affected gill filaments in soft freshwater bathed fish. Furthermore, soft freshwater bathing alleviated the blood plasma electrolyte imbalance seen in control (sea water) and hard freshwater bathed fish. This study showed that the use of soft fresh water for bathing AGD-affected Atlantic salmon could be an improvement to the current method of treatment. Not only does it reduce gill amoeba numbers, but also, it is of a therapeutic advantage with the potential to reduce bathing frequency.

Keywords: Atlantic salmon, freshwater bathing, amoebic gill disease, treatment.

Correspondence *Dr S D Roberts, School of Aquaculture, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania, Locked Bag 1-370, Launceston, Tasmania 7250, Australia*
(e-mail: shaner@utas.edu.au)

Introduction

The development of improved therapeutic treatments for amoebic gill disease (AGD) caused by *Neoparamoeba pemaquidensis* (see reviews by Munday, Zilberg & Findlay 2001; Nowak, Carson, Powell & Dykova 2002) is imperative for the continued sustainability of the Tasmanian Atlantic salmon aquaculture industry. In the absence of suitable chemotherapeutic regimes, the current treatment of AGD involves bathing fish in fresh water, often sourced from local dams (Parsons, Nowak, Powell & Dix 2001b). However, as a treatment, freshwater bathing is variable and increasingly less effective, thus there is a demand from industry to improve its efficacy (Parsons, Nowak, Fisk & Powell 2001a; Clark, Powell & Nowak 2003). Short-term freshwater bathing of AGD-affected Atlantic salmon seemingly has little direct physiological consequences, with no effect on plasma ions and branchial chloride cells (Powell, Parsons & Nowak 2001, water hardness not documented), although a reduced ionoregulatory capacity is evident compared with unaffected controls (Powell *et al.* 2001; Roberts & Powell 2003, used hard fresh water).

Variations in water chemistry between bath water sources have previously been suggested to influence the efficacy of commercial freshwater bathing (Clark 2002). Powell & Clark (2003) investigated the effects

of divalent cations on the survival of gill isolated amoebae *in vitro* and found that amoebae survival was prolonged in Ca^{2+} and Mg^{2+} freshwater treatments compared with deionized water controls. In addition, it is well documented that mucus, a highly polyanionic glycoprotein (Verdugo 1984), has a high binding affinity for divalent cations (Zuchelkowski, Pinkstaff & Hinton 1985; Gupta 1989). As mucus hydration and expansion is dependent upon the Donnan potential, its physical characteristics are heavily reliant on the ionic composition of the bathing medium (Gupta 1989). Thus, in a low cation concentration, the polyanionic driving force of mucin molecules could potentially generate mucus of greater hydration and thus a reduced viscous nature. Changing the mucous layer in this way would not only allow the exposure of *N. pemaquidensis* to the harmful effects of lowered cationic fresh water, but may also increase the sloughing and production of gill mucus, subsequently aiding the fish to shed its gill pathogens. Consequently, there is possible scope for improving the efficacy of current bathing treatments by using softened (reduced divalent cation concentration) fresh water.

This study aimed to assess the effects of water hardness on the efficacy of freshwater bathing as a treatment for AGD in Atlantic salmon. The hypothesis tested was that soft fresh water would be more efficacious than hard fresh water at alleviating AGD in affected fish.

Materials and methods

Laboratory fish

Atlantic salmon, *Salmo salar* L., of mean weight (±SE) 322.9 ± 12.2 g were maintained in 4000 L closed recirculating freshwater systems, each consisting of a fibreglass Rathburn tank connected to a biofilter, at 15 °C. Prior to experimentation, fish were acclimatized to 35 ‰ 1 μm filtered sea water over 7 days and maintained at 17 °C in a closed recirculating system that included a foam fractionator. Water pH and total ammonia were maintained at 7.7 ± 0.03 (±SE) and < 0.25 mg L^{-1}, respectively. All fish were fed commercial salmon feed daily to satiation.

Laboratory infection

Seawater acclimatized fish were infected with amoebae isolated from the gills, as described by Howard & Carson (1994), of commercially farmed Atlantic salmon experiencing a clinical outbreak of AGD. This was achieved by adding the source amoebae to the tank water at a concentration of 3000 amoebae L^{-1} and switching off the foam fractionator of the system. The presence of *N. pemaquidensis* on the gills of infected laboratory fish was later confirmed using an indirect fluorescent antibody test (IFAT, Howard & Carson 1993) and dotblot (Douglas-Helders, Carson, Howard & Nowak 2001). An infection was established within 3 weeks of inoculation.

Experimental protocol

Laboratory experiment

AGD-affected marine Atlantic salmon ($n = 36$) were bathed in plastic 500 L aerated tanks containing a static water treatment in groups of three fish over a 3-h period (three replicates). Fish were starved for 24 h prior to the experiment. Treatments included a bath control (seawater bathed fish), soft freshwater bath (mean total hardness ± SE = 37.4 ± 5.4 mg L^{-1} $CaCO_3$; $[Ca^{2+}]$, $[Mg^{2+}]$ = 0.24, 0.08 mM), hard freshwater bath (mean total hardness ± SE = 236.3 ± 11.9 mg L^{-1} $CaCO_3$; $[Ca^{2+}]$, $[Mg^{2+}]$ = 1.49, 0.53 mM), as well as a pre-treatment control group where fish were sampled from the holding tank. The boundary between hard and soft water is considered to be about 0.4 mM $[Ca^{2+}]$ (McDonald & Rogano 1986). Soft fresh water used was Launceston city mains water, de-chlorinated by bubbling air for at least 24 h, and hard fresh water was mains water artificially hardened by the addition of $MgSO_4$ and $CaCl_2$, both at a concentration of 200 mg L^{-1}. All experimental fish were killed with a lethal concentration of clove oil (0.02%) and weighed. Fish were bled from the caudal vessels using a heparinized syringe (ammonium heparin 100 IU mL^{-1} Sigma, Aldrich Pty Ltd, Castlehill, Australia) and the blood centrifuged at 10 000 ***g*** for 90 s and the plasma refrigerated (4 °C) then frozen at −20 °C for further analysis. The right branchial chamber, consisting of all four gill arches, was removed and placed in a plastic vial containing 0.2 μm filtered sea water and kept on ice for an immediate total amoeba cell count (see below). The second left gill arch was excised and fixed in Davidson's fresh or seawater fixative for at least 72 h, then transferred to 70% ethanol for storage and later histological examination (see below).

On-farm field experiment

The effects of total hardness of fresh water used during bathing was assessed on a commercial salmon lease in the Huon estuary, southern Tasmania. Two trial sea-pens (34 m^3) of AGD-affected Atlantic salmon of mean mass (±SE) 1.47 ± 0.08 kg were experimentally exposed to either oxygenated dam water (173 mg L^{-1} $CaCO_3$; $[Ca^{2+}]$, $[Mg^{2+}] = 0.63$, 0.67 mM) or artificially softened dam water (19.3 mg L^{-1} $CaCO_3$; $[Ca^{2+}]$, $[Mg^{2+}] = 0.03$, 0.10 mM) for 3 h. A pre-treatment group of six fish was sampled from both pens prior to the commencement of bathing. Source dam water was flocculated using 100 ppm of aluminium sulphate [$Al_2(SO_4)_3$] prior to the experiment. $Al_2(SO_4)_3$ at 100 ppm over 3 h does not affect isolated gill amoeba survival *in vitro* (S. Roberts, unpublished observations). Water for the soft freshwater treatment was artificially softened using a Series 255 Valve/440i Control Water Conditioner (Filter-Works, Holden Hill, SA) containing Purolite C-100E cation exchange resin, where multivalent cations were exchanged for Na^+. The fish in each sea-pen were randomly divided into two equal groups. Half of the fish from each pen were bathed in the softened dam water treatment (mean water quality ± SE: 15.5 ± 0.1 °C; DO 12.8 ± 1.6 mg L^{-1}; pH 6.0 ± 0.1), while the other half from each pen were bathed in the normal hard dam water treatment (mean water quality ± SE: 15.4 ± 0.1 °C; DO 12.6 ± 1.8 mg L^{-1}; pH 6.3 ± 0.1). A tarpaulin liner was fitted inside the sea-pen to hold the bath water. After the 3-h bath, six fish were sampled from each of the four groups, and the remaining fish were transferred from the bathing liner to four separate sea-pens (34 m^3 sea-pens, mean stocking density ± SE = 8.0 ± 0.1 kg m^{-3}) to be maintained for an assessment of time to re-infection over an 8-week period. Daily feed intake for all pens did not significantly differ over this period. Six fish were lethally sampled from each of four pens at 24 h and 8 weeks postbath. The gross pathology score, IFAT smears and total amoeba cell counts were determined and histological samples were taken as described below. Gross pathology was scored according to the visibility of white mucous patches (characteristic of AGD) on the gills of fish. Gross scores were ranked similar to those of Powell *et al.* (2001) and Fisk, Powell & Nowak (2002), however, we grouped scores 1 and 2 (very light and light) to achieve a four score system (clear, faint spots, spots and patches). Being a non-destructive method, gross pathology is routinely used by commercial farms for quantifying pathological severity during an AGD outbreak. In addition, 10 fish were non-lethally sampled (using 0.005% clove oil) from each of four pens to monitor gross pathology and IFAT at 2, 4 and 6 weeks postbath.

Plasma and water analysis

Blood plasma was analysed as per the protocol of Powell *et al.* (2001). Ion concentrations of water samples (to calculate hardness) were determined using a Varian SpectrAA.300 flame atomic absorbance spectrophotometer (Varian Australia Pty Ltd, Mulgrave, Australia).

Total amoeba cell count

Gill amoebae were harvested from the gills by a method modified from Howard & Carson (1994). Gills were removed from their sample vials, washed in filtered sea water and lightly scraped to remove their mucous coating. A mucous solution was made up with filtered sea water and homogenized using a 1 mL pipette. Gill amoebae were counted from a 100 μL sample of the preparation added to a solution of 0.5% trypan blue, using a haemocytometer. Viable and non-viable cells were counted for the laboratory experiment, while viable cells only were counted for the field experiment. Raw data from the counts were normalized to $\log_{10}$ mass to account for the allometric relationship that gill surface area has with body weight (Palzenberger & Pohla 1992).

Indirect fluorescent antibody test

Gill mucus smears were taken and allowed to air dry for up to 2 h before being refrigerated. Smears were later heat fixed and stained with an IFAT, using a sheep antiserum to *N. pemaquidensis* (strain PA-027), adapted from Howard & Carson (1993). The procedure differed only by the addition of Evans Blue counterstain (Sigma, E 0133). Before applying the secondary antibody to samples, it was diluted at 1:40 in Evans Blue solution. Evans Blue solution was made up as 1% Evans Blue (Sigma) in phosphate-buffered saline. This step allowed an enhanced contrast between green fluorescent *N. pemaquidensis* cells and other gill smear debris that fluoresced red. In addition, when comparing positive control smears of

N. pemaquidensis (PA-027), Evans Blue faintly stained the nucleus and other various internal bodies red without affecting the green fluorescence. This produced a distinctive characteristic in *N. pemaquidensis* cells, allowing easier identification in the face of other autofluorescent material.

Histology and staining

Gill samples were processed, stained and their pathology quantified (per cent of gill filaments with an AGD lesion) according to the methods of Roberts & Powell (2003).

To quantify the therapeutic effect of treatment, the proportion of AGD lesions that were fragmented and the degree of fragmentation was recorded. A fragmented AGD lesion was defined as one that had two or more breaks in the hyperplastic tissue between lamellae, where a break was defined as a separation in the hyperplastic tissue of at least 75% of the depth of surrounding lamellae. Fused lamellae in between breaks were termed a section, and the number of sections per lesion gave an estimate of the degree of fragmentation. If a section was separated by more than three non-fused lamellae, it was considered to be a separate lesion (see Fig. 1).

Statistical analysis

All laboratory data and lesion and total amoeba cell count data from the field experiment were analysed using a one-way ANOVA followed by a planned contrast using a Dunnett (two-sided) *t*-test comparing treatment means to that of the pre-treatment. Homoscedasticity and normality of the data were checked by both Levene's test and visually assessing residual plots. Where necessary, square root $(n + 1)$ and Log_{10} $(n + 10)$ transformations of data were employed to ensure homogeneity. Gross gill score and IFAT data were analysed using Chi-square cross tabulation. As a *post hoc* test, standard residuals between groups were compared and deemed significantly different from the expected mean if > 1.9. In all statistical analyses used, significance was accepted for $P < 0.05$. Data analysis and statistical testing were performed using SPSS 10.0 software (SPSS Inc., Chicago, IL, USA). Values are given as mean ± standard error (SE).

Results

Laboratory experiment

All fish used in this experiment were AGD-affected and had 65.2 ± 3.2% of gill filaments with AGD type lesions. Total gill amoeba data was calculated from viable and non-viable cells and expressed as percentage of viable cells for each treatment (Fig. 2). A statistically significant difference was found between treatments ($F_{3,35} = 8.18$, $P < 0.001$). A *post hoc* test revealed soft freshwater bathed fish had a significantly lower per cent of viable cells present compared with the prebath controls (40.9 ± 6.6, 73.9 ± 5.7% viable cells, respectively, $P < 0.01$).

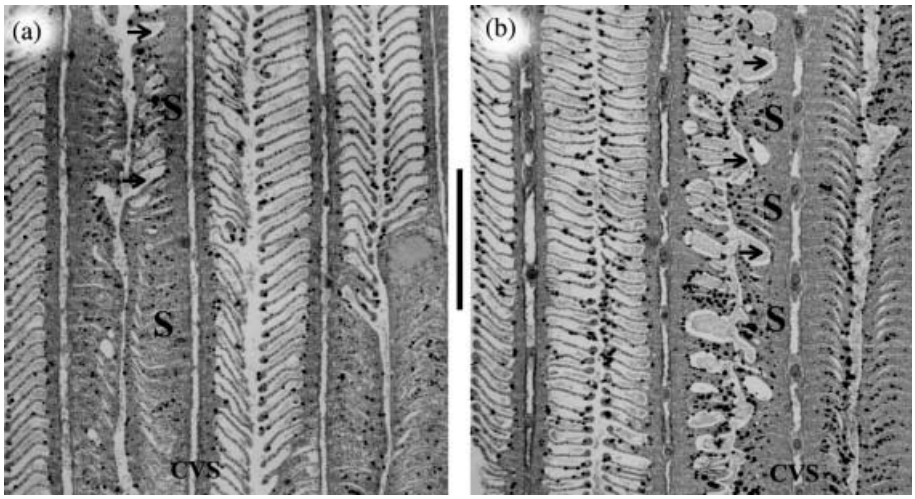

Figure 1 Periodic acid-Shiff/Alcian blue (PAS/AB) stained histological sections of Atlantic salmon gill filaments showing amoebic gill disease lesion fragmentation in (a) pre-bathed fish and (b) soft water bathed fish from the laboratory experiment. CVS = central venous sinus, S = section of a lesion, arrows indicate defined breaks within a lesion (bar = 500 μm).

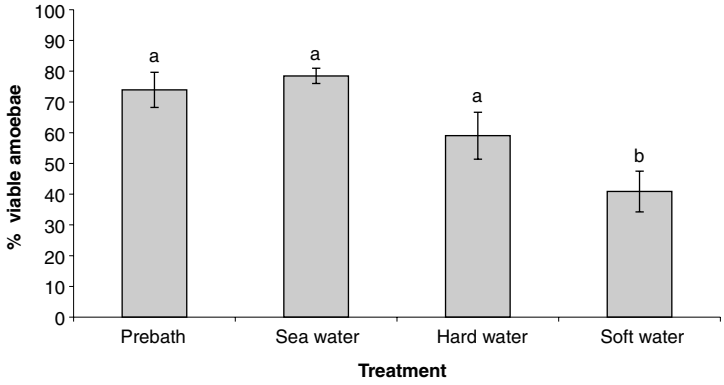

Figure 2 Per cent viable amoebae of total amoebae cell count (mean ± SE) from the gills of Atlantic salmon pre- and post-3-h bathing ($n = 9$ per treatment). This data is from the laboratory experiment. Letters indicate significant differences when compared with pre-bath controls ($P < 0.05$).

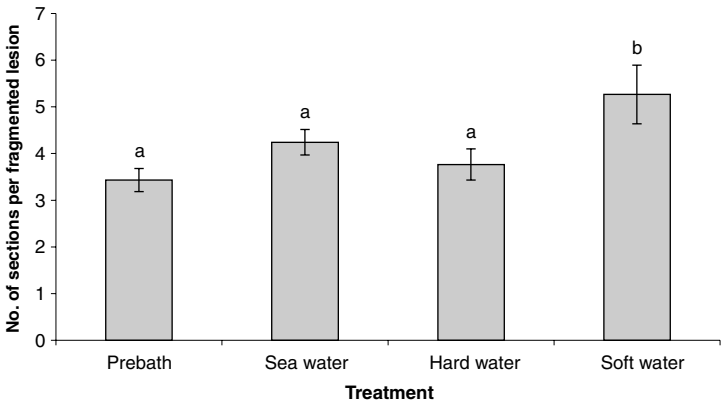

Figure 3 Degree of lesion fragmentation (no. sections per fragmented lesion) (mean ± SE) on the gills of Atlantic salmon pre- and post-3-h bathing ($n = 9$ per treatment). This data is from the laboratory experiment. Letters indicate significant differences when compared with pre-bath controls ($P < 0.05$).

No statistically significant difference was found between treatments for the number of lesions per AGD-affected gill filament ($\mu = 1.55 \pm 0.05$), or for the number of these that were fragmented ($\mu = 0.42 \pm 0.05$). However, there was a significant difference between treatments for the degree of lesion fragmentation ($F_{3,34} = 4.14$, $P < 0.05$) (Fig. 3). A *post hoc* test revealed that soft freshwater bathed fish had a significantly greater degree of fragmentation when compared with the prebath control group (5.27 ± 0.63, 3.43 ± 0.25 sections/fragmented lesion respectively, $P < 0.01$) (see Fig. 1 for histological comparison).

Plasma protein and Na^+ concentrations did not significantly differ between treatments when statistically analysed (Table 1). Plasma K^+ concentrations differed significantly between treatments ($F_{3,35} = 3.41$, $P < 0.05$). A *post hoc* test revealed that both control and hard freshwater bathed fish differed significantly from prebath control fish (2.6 ± 0.3, 2.6 ± 0.2, 4.8 ± 1.0 mM, respectively, $P < 0.05$). Plasma Cl^- concentrations also differed significantly between treatments ($F_{3,35} = 7.35$, $P < 0.001$). A *post hoc* test revealed that control bathed fish differed significantly from prebath control fish (216.9 ± 2.9, 198.0 ± 5.5 mM, respectively, $P < 0.05$).

Table 1 Blood plasma protein, Na^+, K^+, and Cl^- concentrations (mean ± SE) for amoebic gill disease affected Atlantic salmon pre- and post-3-h bathing in the laboratory ($n = 9$ per treatment)

Plasma analysis	Prebath	Control	Hard water	Soft water
Protein (g L^{-1})	43.0 (2.3)	46.3 (2.7)	50.4 (2.2)	49.8 (2.8)
[Na^+] (mM)	147.9 (6.4)	162.9 (6.5)	147.9 (7.4)	151.1 (4.6)
[K^+] (mM)	4.8 (1.0)	2.6 (0.3)*	2.6 (0.2)*	3.0 (0.5)
[Cl^-] (mM)	198.0 (5.5)	216.9 (2.9)*	190.0 (5.4)	191.9 (3.9)

* Significant difference from prebath fish ($P < 0.05$).

On-farm field experiment

All fish used in this experiment were AGD-affected and had 5.2 ± 1.1% of gill filaments with AGD type lesions. Viable gill amoebae significantly differed across treatments ($F_{6,83} = 6.34$,

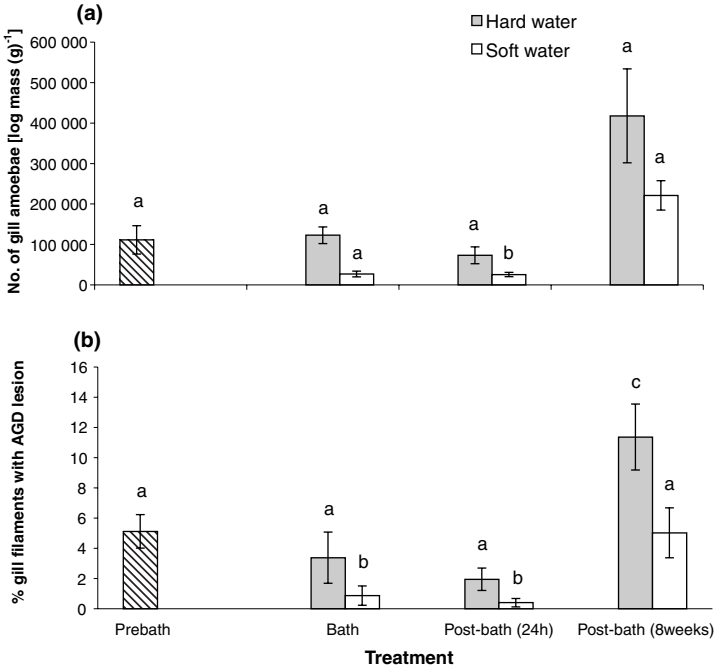

Figure 4 On-farm field data of (a) the number of gill amoebae and (b) the per cent of gill filaments with an AGD type lesion (mean ± SE) in Atlantic salmon pre- and post-3-h bathing treatments ($n = 12$ per treatment). Letters indicate significant differences when compared with prebath controls ($P < 0.05$).

$P < 0.001$) (Fig. 4a). A *post hoc* test revealed that gill amoebae on soft freshwater bathed fish significantly decreased at 24 h postbath compared with prebath controls ($P < 0.05$). The data for percentage gill filaments with an AGD lesion differed significantly across treatments ($F_{6,83} = 8.97$, $P < 0.001$) (Fig. 4b). A *post hoc* test revealed that soft freshwater bathed fish had a significantly lower proportion of AGD-affected filaments at bath and 24 h postbath (0.9 ± 0.6, 0.4 ± 0.3% filaments AGD-affected, $P < 0.05$, $P < 0.01$, respectively) compared with prebath controls (5.1 ± 1.1% filaments AGD-affected). Also, at 8 weeks posthard freshwater bath, fish had a significantly greater proportion of AGD-affected filaments (11.4 ± 2.2% filaments AGD-affected, $P < 0.05$) compared with prebath controls.

Gross gill score ratios across treatments were compared with expected means for both hard and soft freshwater bathed fish (Fig. 5). To satisfy the assumptions of the statistical test, spots and patches were grouped together to increase the count for this score. The prebath gill score ratio shows 50% of fish having either spots or patches, which was significantly different from the expected mean score (standard residual > 1.9) for both the hard and soft freshwater data sets. Clear scores for both groups of fish were greatest at weeks 2, 4 and 6. However, for hard freshwater bathed fish they were of a lower proportion, thus lowering the expected mean, than soft freshwater bathed fish. This explains why weeks 2 and 4 for hard freshwater bathed fish had significantly greater clear scores compared with the expected mean (standard residual > 1.9). Soft freshwater bathed fish had observed clear scores greater than the expected mean at 4 weeks (standard residual > 1.9). At 8 weeks postbath for the hard freshwater treatment, the gill score ratio returned to that of the prebath ratio with spots and patches being significantly different from the expected mean (standard residual > 1.9). However, for the soft freshwater treatment the gill score ratio did not return to the prebath ratio at 8 weeks, but more resembled the hard freshwater gill score ratio at 6 weeks.

The proportion of IFAT +ve scores for both hard and soft freshwater bathed fish were compared with expected means. The proportion of prebath fish that were IFAT +ve was 100%. Group means for both hard and soft freshwater bathed fish were at their lowest at 2 weeks postbath (65 and 35%, respectively). However, only soft freshwater bathed fish were significantly different from the expected mean (standard residual > 1.9).

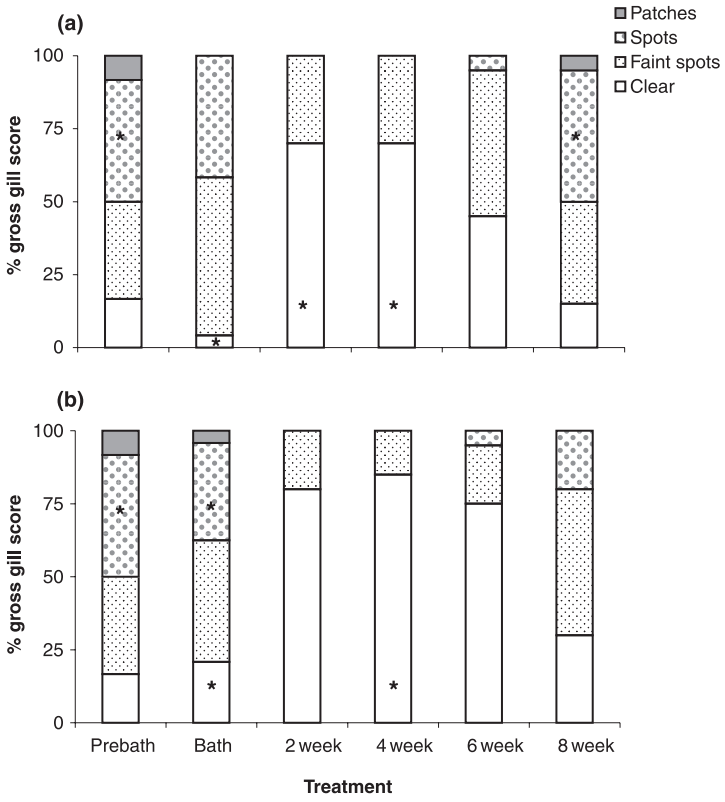

Figure 5 On-farm field data of percent gross gill score of Atlantic salmon for (a) hard and (b) soft water bathed fish. Gill score ratios are graphed for pre- and post-3-h bathing. Asterisks indicate a significant difference from an expected mean score (standard residual >1.9).

Discussion

Experiments conducted in the laboratory and on-farm showed that soft fresh water (19.3–37.4 mg L^{-1} $CaCO_3$) was more efficacious for bathing marine AGD-affected Atlantic salmon than hard fresh water (173–236.3 mg L^{-1} $CaCO_3$). This improved bathing method significantly reduced viable gill amoebae and proved to be of a therapeutic benefit by alleviating the pathological signs of AGD.

Viable gill amoeba numbers and IFAT +ve scores both significantly decreased following soft freshwater bathing only. Total gill amoebae included the causative agent of AGD, *N. pemaquidensis*, as the laboratory experiment used a confirmed infection (IFAT and dot blot +ve) and a high proportion of fish were IFAT +ve during the field experiment. These results are similar to those of Parsons *et al.* (2001a) who also found a significant decrease in gill amoebae post-commercial freshwater bath, however, water hardness was not tested.

Soft freshwater bathing also showed significant therapeutic benefits not seen in hard freshwater bathed fish. The degree of AGD lesion fragmentation significantly increased and the per cent of gill filaments with an AGD lesion significantly decreased following soft freshwater bathing. Additionally, postbath gross gill score ratios for soft freshwater fish lagged approximately 2 weeks behind hard freshwater bathed fish with a large proportion of fish exhibiting clear and faint spotted gills. Only hard freshwater bathed fish returned to prebath ratios by 8 weeks. These results suggest that soft fresh water, compared with hard fresh water, has a greater ability to increase the fragmentation and shedding of gill lesion-associated hyperplastic tissue in AGD-affected marine salmon, probably a direct consequence of both osmotic pressure and changes to the mucous layer.

The physiochemical effect that soft fresh water has on the mucous layer of a marine fish *in vivo*, compared with hard fresh water, is to our knowledge not fully documented. However, common theory of mucus hydration and expansion related to the Donnan potential (Gupta 1989) suggests that mucus subjected to a low cationic medium would have a greater hydration and expansion, thus lowering its viscosity. Thus, the reduced viability of gill amoebae and therapeutic benefit in soft fresh

water was possibly, in part at least, attributed to a greatly reduced mucus viscosity. This effect on the mucous layer would result in the subsequent harmful exposure of amoebae to soft fresh water (Martin 1987; Powell & Clark 2003) and result in an increased sloughing of mucus.

No physiological consequences of soft freshwater bathing were found, a conclusion elucidated from the blood parameters measured. In fact, bathing AGD-affected marine salmon in soft fresh water seemed to alleviate the blood ion disturbances seen in control (sea water) and hard freshwater bathed fish. Blood $[K^+]$ and $[Cl^-]$ of control bathed fish and blood $[K^+]$ of hard freshwater bathed fish significantly differed from prebathed fish. This is suggestive of an osmoregulatory disturbance, which is associated with stress in fish (Wood, Turner & Graham 1983; Ackerman, Forsyth, Mazur & Iwama 2000). Similarly, Roberts & Powell (2003) found that both AGD-affected and unaffected Atlantic salmon experience a minor ionoregulatory dysfunction when exposed to hard fresh water. Powell *et al.* (2001) found no significant effect on blood plasma ions after a commercial freshwater bath, however water hardness was not documented.

The frequency of freshwater bathing conducted by the Tasmanian Atlantic salmon industry is determined by the gross examination of gills. Our results for gross gill scores are encouraging, as the frequency of bathing could be reduced with the use of soft fresh water. Furthermore, soft fresh water was shown to both reduce viable gill amoeba numbers and to be of a therapeutic benefit. Water hardness varies markedly between farm freshwater sources (Parsons *et al.* 2001b), so to improve the efficacy of freshwater bathing commercial farms should seek to maintain accurate water quality data and use fresh water with the lowest total hardness (mg L^{-1} $CaCO_3$). Alternatively, in the absence of suitable soft freshwater sources, this study has shown that artificially softened dam water is as effective as naturally soft mains water.

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