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Effects of gill abrasion and experimental infection with *Tenacibaculum maritimum* on the respiratory physiology of Atlantic salmon *Salmo salar* affected by amoebic gill disease

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ABSTRACT: The effects of gill abrasion and experimental infection with *Tenacibaculum maritimum* were assessed in Atlantic salmon *Salmo salar* with underlying amoebic gill disease. The respiratory and acid-base parameters arterial oxygen tension (P_aO_2), arterial whole blood oxygen content (C_aO_2), arterial pH (pH_a), haematocrit and haemoglobin concentrations were measured at intervals over a 48 h recovery period following surgical cannulation of the dorsal aorta. Mortality rates over the recovery period were variable, with gill abrasion and inoculation with *T. maritimum* causing the highest initial mortality rate and unabraded, uninoculated controls showing the lowest overall mortality rate. Fish with abraded gills tended to show reduced P_aO_2 and lower C_aO_2 compared with unabraded fish. Infection with *T. maritimum* had no effect on P_aO_2 or C_aO_2. All fish showed an initial alkalosis at 24 h post-surgery/inoculation which was more pronounced in fish inoculated with *T. maritimum*. There were no significant effects of gill abrasion or infection upon the ratio of oxygen specifically bound to haemoglobin or mean cellular haemoglobin concentration. Histologically, 48 h following surgery, abraded gills showed multifocal hyperplastic lesions with pronounced branchial congestion and telangiectasis, and those inoculated with *T. maritimum* exhibited focal areas of branchial necrosis and erosion associated with filamentous bacterial mats. All fish examined showed signs of amoebic gill disease with multifocal hyperplastic and spongious lesions with parasome-containing amoeba associated with the gill epithelium. The results suggest that respiratory compromise occurred as a consequence of gill abrasion rather than infection with *T. maritimum*.

KEY WORDS: Atlantic salmon · *Tenacibaculum maritimum* · Respiration · Pathophysiology · Gill disease · Gill abrasion

INTRODUCTION

Amoebic gill disease (AGD), putatively caused by the amphizoic amoeba *Neoparamoeba pemaquidensis*, is a significant disease for marine aquaculture affecting salmonids and non-salmonid species such as turbot, sea bass and sea bream (for review see Nowak et al. 2002). Although known to cause acute multifocal branchial hyperplastic lesions involving the fusion of gill lamellae and a mucous cell hyperplasia (Adams & Nowak 2001, Roberts & Powell 2003), clinical AGD results in few respiratory disturbances (Powell et al. 2000, Powell & Nowak 2003). The assessment of AGD in salmon is by examination of raised white mucoid patches on the gills, which requires the separation of the gill arches for visual examination. This exposes the fish to branchial trauma and abrasion.

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Tenacibaculum maritimum (formerly *Flexibacter maritimus*) affects a wide variety of species including red seabream *Pagrus major*, black seabream *Acanthopagrus schegeli*, rock bream *Oplegnathus fasciatus*, Japanese flounder *Paralichthys olivaceus* (Baxa et al. 1986, Wakabayashi et al. 1986), Dover sole *Solea solea* (Bernardet et al. 1990), turbot *Scophthalmus maximus* (Alsina & Blanch 1993), Atlantic salmon *Salmo salar*, rainbow trout *Onchorynchus mykiss*, striped trumpeter *Latris lineata* and greenback flounder *Rhombosolea taprina* (Handlinger et al. 1997). Although primarily a skin infection causing ulcerative dermatitis (Handlinger et al. 1997), gill infections where a necrotizing branchitis occurs are not uncommon (Handlinger et al. 1997). Gill abrasion has been reported to enhance the susceptibility of catfish *Ictalurus punctatus* to infection with *Flavobacterium columnare* (Bader et al. 2003), and skin abrasion has been used to enhance infections of salmonids to *T. maritimum* (J. Carson pers. comm.). Therefore, the aim of this study was to investigate the respiratory effects of gill abrasion, acute branchitis caused by an experimentally induced *T. maritimum* infection, and their interaction in Atlantic salmon exhibiting sub-clinical amoebic gill disease.

MATERIALS AND METHODS

Preparation of bacterial cultures. A culture of *Tenacibaculum maritimum* was isolated in 2000 by the Department of Primary Industry Water and Environment from the skin of salt water farmed rainbow trout exhibiting cutaneous erosion in Tasmania, Australia. The culture was designated 00/3280. The bacteria were isolated on the medium of Anacker & Ordal (1959) formulated with seawater. Isolates were identified using a 16S rRNA PCR primer set specific for *T. maritimum* (Carson 1998). Cultures were stored frozen at −80°C on MicroBank (Pro-Lab Diagnostics) beads until required.

Cultures for infection trials were prepared by inoculating 200 ml of Shieh's medium (Song et al. 1988) formulated with seawater mineral salts buffer (MSB) in a 1 l conical flask and incubated with gentle agitation (30 cycles min^{-1}) at 20 to 22°C for 48 h. The cell suspension was harvested by centrifugation at 2500 × *g* RCF for 20 min and the pellet washed twice with sterile seawater. Harvested cells were resuspended in 15 ml sterile seawater. Cell numbers were determined using serial dilution.

Fish, surgical procedures and inoculation. Atlantic salmon *Salmo salar* (mean mass ± SE: 579.3 ± 27.6 g; fork length ± SE: 37.8 ± 0.4 cm) were originally obtained from Springfield Fisheries, Scottsdale, Tasmania. Fish were maintained in the laboratory for at least 1 yr in full strength seawater (35 ppt) at 15 to 17°C and fed a commercial pelleted diet to satiation daily. During the acclimation/holding period the salmon naturally acquired a low level of amoebic gill disease (not readily determined by gross pathology). Fish were anaesthetised with 0.03 ml l^{-1} clove oil in aerated seawater then transferred to a surgical table where they were maintained under anaesthesia with 0.015 ml l^{-1} clove oil in chilled aerated seawater flowing over the gills in a retrograde direction. Fish were cannulated via the dorsal aorta as described for trout by Soivio et al. (1975) with a PE50 (Clay Adams) cannula filled with Cortland's marine fish saline (Wolf 1963, Milligan et al. 1991).

Following surgery, while still under anaesthesia, the gill filaments were separated by gently stroking (abrasion) using a blunt sterile spatula on both sides. Fish were directly inoculated by pipetting 1 ml of seawater *Tenacibaculum maritimum* suspension (~2×10^{12} CFU ml^{-1}) onto each side of the gills (2 ml total per fish) (Group 1, n = 10, Table 1) after Powell et al. (2004). Control fish were abraded but not inoculated with bacteria (Group 2, n = 13, Table 1). Fish were then placed in a black acrylic box supplied with flowing aerated seawater (16 to 17°C). To assess the effect of abrasion and the interaction with *T. maritimum* infection, fish were inoculated with bacteria without prior abrasion (Group 3, n = 7, Table 1) and controls were left unabraded and uninoculated (Group 4, n = 9, Table 1).

Blood sampling and analysis. At intervals during the recovery period (6, 12, 24, 30, 36 and 48 h) a 500 µl blood sample was anaerobically withdrawn from the cannula and replaced with an equal volume of heparinised Cortland's marine saline (100 IU lithium heparin, Sigma-Aldrich). Approximately 100 µl was injected into oxygen and pH electrodes (Microelectrodes) connected to a Cameron Instrument Company blood gas meter. Twelve microlitres of whole blood was used for determination of whole blood oxygen content using an Oxy-Con (S. Nicol, Department of Physiology, University of Tasmania) calibrated against room air. Duplicate microhaematocrit samples were made in 20 µl haematocrit tubes and centrifuged at

Table 1. *Salmo salar*. Number of fish per group, and gill abrasion and bacterial inoculation characteristics of salmon used in the experiment

Group	No. of fish	Gill abrasion	Inoculation
1	10	Yes	Yes
2	13	Yes	No
3	7	No	Yes
4	9	No	No

10 000 × *g* for 5 min (Statspin RIII, Statspin Technologies) and a 20 µl sample of whole blood was used for haemoglobin determination using a commercial haemoglobin assay (Sigma Diagnostics, Procedure 525).

The ratio of oxygen specifically bound to haemoglobin was calculated according to:

$$O_2 : Hb = \frac{C_aO_2 - (\alpha \times P_aO_2)}{[Hb]_{wb}}$$

where C_aO_2 is the arterial whole blood oxygen content (ml 100 ml^{-1}), P_aO_2 is the arterial oxygen tension (mm Hg), α is the oxygen solubility coefficient (from Cameron 1986) and $[Hb]_{wb}$ is the whole blood haemoglobin concentration (g 100 ml^{-1}). Mean cellular haemoglobin concentration (MCHC) was calculated according to:

$$MCHC = \frac{[Hb]_{wb}}{Hct}$$

where Hct is the packed cell volume (haematocrit) (%).

Pathological assessment of gills. Fish surviving 48 h were euthanised with an overdose of clove oil (>0.03 ml l^{-1}). Mucus was swabbed from the gills of all dead and euthanised fish and streaked onto Sheih's marine agar plates (Song et al. 1988). The gills were then removed and fixed in 10% seawater buffered formalin for histology. Agar plates were incubated at 18°C for up to 5 d and were checked daily for the appearance of colonies typical of *Tenacibaculum maritimum*.

Formalin fixed gills were embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin. The proportion of filaments with characteristic multifocal hyperplastic AGD-like lesions (Adams & Nowak 2001) were determined (Powell et al. 1995, Speare et al. 1997).

Statistical analysis. Different mortality rates between the different treatments lead to an unbalanced design, so treatments were analysed using analysis of variance with time as a factor. Where significant differences occurred, a Student-Newman-Keuls post-hoc analysis was used to determine differences between sample points. p-values of less than 0.05 were considered to be significant.

RESULTS

Both *Tenacibaculum maritimum* inoculated-abraded (Group 1) and uninoculated-abraded fish (Group 2) had high initial mortality rates with a plateau in cumulative mortality at 30 h post-surgery/inoculation and then rising sharply again 30 to 48 h post-surgery/inoculation (Fig. 1). Fish that were inoculated with *T. maritimum* but with unabraded gills (Group 3) showed a similar pattern of mortality to those in the abraded groups but the level of mortality was lower up to 30 h post-surgery/inoculation. However, between 36 and 48 h the rate of mortality increased rapidly to a final level of mortality similar to that of the abraded groups (Fig. 1). Unabraded and uninoculated control fish (Group 4) showed a low rate of mortality reaching a maximum of 22% at 48 h (Fig. 1). Bacterial colonies with characteristics typical of *T. maritimum* (pale yellow, rough, ragged-edged, adherent colonies) were isolated from fish inoculated with bacteria. No *T. maritimum*-like bacterial colonies were recovered from fish that were not inoculated.

There were no significant effects between treatments or over time for P_aO_2 (Fig. 2). However, there were significant decreases in C_aO_2 for fish with abraded gills, both inoculated (Group 4, $F_{4,14} = 22.9$, $p < 0.0001$) and uninoculated (Group 2, $F_{5,30} = 12.5$, $p < 0.0001$) with *Tenacibaculum maritimum* (Fig. 2). There were no significant differences for unabraded inoculated (Group 3, $F_{5,25} = 0.335$, $p = 0.8866$) or controls (Group 4, $F_{5,20} = 1.01$, $p = 0.4367$) gills (Fig. 2). Abrasion alone (Group 2, noinfection with *T. maritimum*) and inoculation alone (Group 3) resulted in a significant initial decrease in pH_a at 12 h post-surgery, an increase at 24 h, and then a decrease at 36 h post-surgery ($F_{5,37} = 7.21$, $p < 0.0001$, $F_{5,25} = 28.1$, $p < 0.0001$ respectively. Overall, the Group 3 showed a greater drop in pH_a. Control (Group 4) fish showed a similar significant decrease in pH_a at 12 h followed by a significant increase at 24 h ($F_{5,31} = 6.07$, $p = 0.0005$) that was

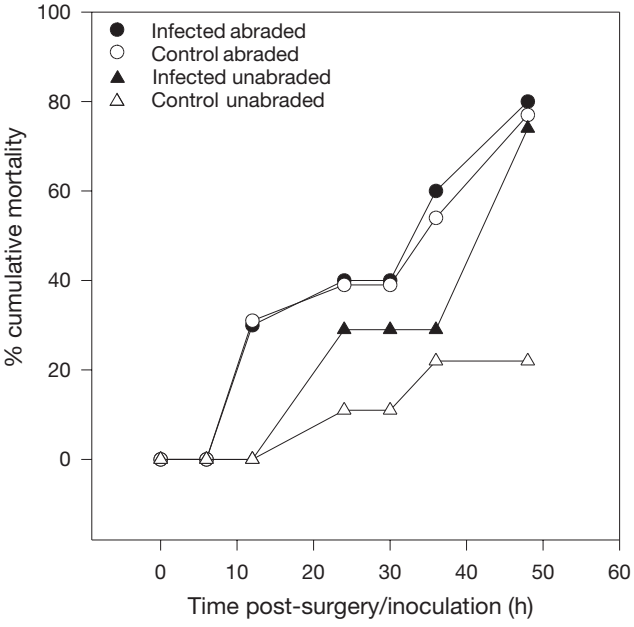

Fig. 1. *Salmo salar*. Percent cumulative mortality for cannulated Atlantic salmon with abraded (Groups 1 and 2, circles) and unabraded (Groups 3 and 4, triangles) gills infected (solid symbols) and uninfected controls (open symbols) with *Tenacibaculum maritimum*

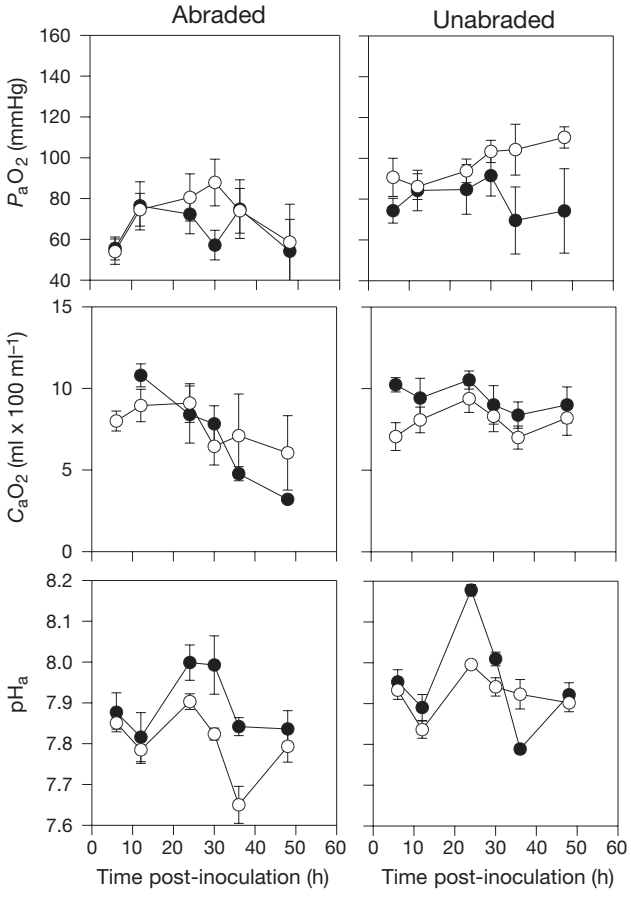

Fig. 2. *Salmo salar*. Mean (±SE) arterial oxygen tension (P_aO_2), whole blood oxygen content (C_aO_2) and pH (pH_a) of AGD-affected Atlantic salmon that had their gills abraded or unabraded and inoculated (Groups 1 and 3, ●) or non-inoculated (Groups 2 and 4, ○) with *Tenacibaculum maritimum*

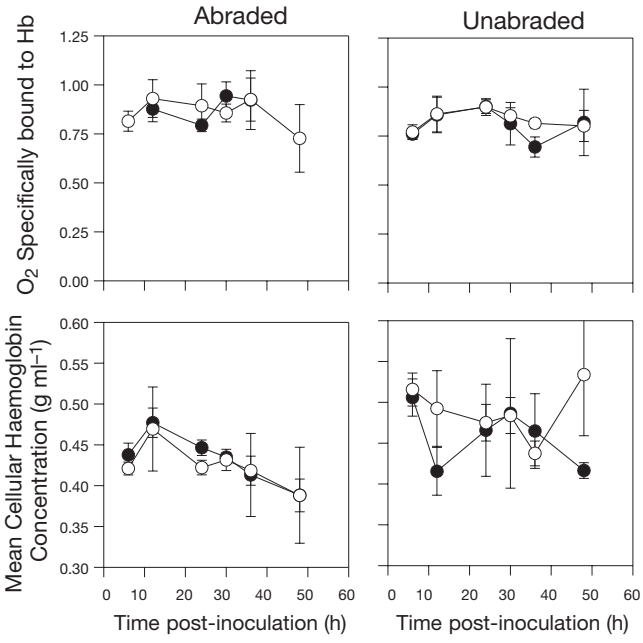

Fig. 3. *Salmo salar*. Mean (±SE) ratio of oxygen specifically bound to haemoglobin and mean cellular haemoglobin concentration of affected by amoebic gil disease (AGD) Atlantic salmon that had their gills abraded or unabraded and inoculated (Groups 1 and 3, ●) or non-inoculated (Groups 2 and 4, ○) with *Tenacibaculum maritimum*

maintained until 48 h post-surgery (Fig. 2). The pH_a of abraded-inoculated fish (Group 1) was unaffected ($F_{5,23} = 1.82$, $p = 0.1476$). There were no significant differences in the amount of oxygen specifically bound to haemoglobin for any of the treatments (Fig. 3). Although variable, the mean cellular haemoglobin concentrations did not differ statistically between groups of change over time (Fig. 3).

All of the fish examined in this study had AGD-like lesions prior to gill abrasion or inoculation with *Tenacibaculum maritimum* as evidenced by the presence of AGD-like lesions even on the unabraded, uninoculated fish. Histological examination on completion of the experiment showed 17.7 ± 2.6 % (mean ± SE) of the gill filaments had AGD-type lesions (Fig. 4) with no difference between treatments. The gills from fish that had been abraded (Groups 1 and 2) showed signs of telangiectasis and congestion (Fig. 4). Fish that had been inoculated with *T. maritimum* (Groups 1 and 3) showed localised regions of close association of filamentous bacteria with the respiratory epithelium (Fig. 4). Bacteria associated filamental necrosis appeared to progress proximally from the distal part of the filament.

DISCUSSION

Survival of AGD-compromised salmon following anaesthesia and surgery was comparable to rates seen in other studies (Powell et al. 2000, Powell & Nowak 2003). Non-AGD-affected salmon usually recover from surgery readily (Powell et al. 2000). In the current study, gill abrasion further decreased the survival of salmon post-surgery irrespective of whether fish were inoculated with *Tenacibaculum maritimum*.

Gill abrasion leads to extensive telangiectasis (Powell et al. 2004) and this further develops into hyperplastic lesions similar to those seen in fish with AGD (Fig. 4B) and that shown by Adams & Nowak (2004). It is difficult to visually determine the extent of AGD on fish prior to use, since gill abrasion also leads to AGD-like lesions. However, gill abrasion lesions can be differentiated using histology by the absence of parasome-containing amoebae associated with the lesion (Fig. 4D,E). Histological examination was performed on samples from fish that were moribund or

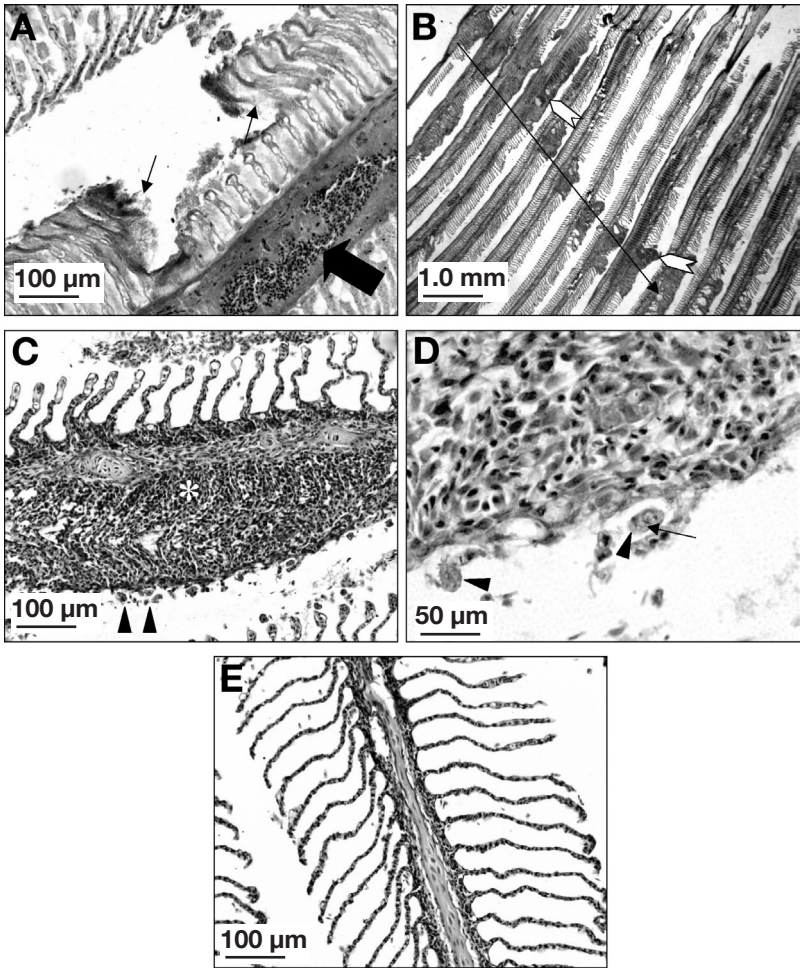

Fig. 4. *Salmo salar* gills. (A) Focal branchial lamellar necrosis associated with filamentous bacterial mats (small arrows) and congestion in the central venous sinus (large arrow) on the gills of Atlantic salmon inoculated with *Tenacibaculum maritimum* (H&E). (B) Hyperplastic lesions associated with gill abrasion (open arrows) and the direction of abrade (long arrow) in the gills of Atlantic salmon (haemotoxylin and eosin, H&E). (C) A characteristic amoebic gill disease (AGD) lesion with parenchymal hyperplasic tissue (asterisk) and peripherally associated amoebae (arrowheads) (H&E). (D) Amoebae associated with a hyperplastic AGD-type gill lesion (arrowheads) showing the characteristic parasome of *Neoparamoeba* sp. (arrow) (H&E). (E) Non-AGD affected area of the gills of control (uninoculated) Atlantic salmon (H&E)

48 h post-surgery, therefore, the degree of AGD may be greater than that pre-surgery.

Salmon with abraded gills appeared to have a lower arterial PO_2 (although not statistically significant) and showed a continuous decline in whole blood oxygen content (Fig. 3) regardless of inoculation with *Tenacibaculum maritimum*. However, fish with unabraded gills appeared to maintain blood oxygen content and P_aO_2 (Fig. 3). Interestingly, none of the treatments reduced the ratio of oxygen specifically bound to haemoglobin, and this ratio remained high. This suggests a rapid recovery from surgery-associated hypoxaemia and that both abrasion and inoculation had little effect on the saturation of haemoglobin with oxygen, i.e. the oxygen haemoglobin carrying capacity and affinity were unaffected. The apparent differences in blood oxygen (tension and content) between abraded and non-abraded fish is likely explained in terms of diffusional limitation of gas transfer across the compromised gill (Ultsch & Gros 1979, Powell & Perry 1999). Indeed, fish with significant structural compromise have severely restricted respiratory performance (Hughes & Nyholm 1979).

The arterial pH of all groups returned to normal levels rapidly following surgery with pH_a values occurring within the expected ranges for salmonids. Interestingly, although the fish had underlying AGD, blood pH was not compromised with a characteristic acidosis reported previously (Powell et al. 2000, 2001, Powell & Nowak 2003). The reason for this probably lies in the fact that the respiratory acidosis associated with AGD occurs 3 to 4 d post-inoculation (M. D. Powell, J. O. Harris & M. Leef unpubl. data). There was a significant increase in pH_a in all fish groups between 12 and 24 h post-surgery/inoculation, with the most pronounced rise in pH seen in fish inoculated with *Tenacibaculum maritimum* (Group 3, Fig. 2). Many parasitic infections, as well as chemical irritation, will result in gill mucus production that inhibits carbon dioxide excretion and causes respiratory acidosis (Powell & Perry 1996, Powell et al. 1998, 2000). Byrne et al. (1991) demonstrated that brook trout *Salvelinus fontinalis* challenged with *Flavobacterium branchiophilum* showed no significant change in blood pH 1 and 4 d post-challenge. In contrast, rainbow trout (Byrne et al. 1995) showed a significant decrease in blood pH 24 h post-challenge. Fish challenged with *Neoparamoeba pemaquidensis* (putative agent of AGD) show an acute respiratory alkalosis initially (1 to 3 d post-challenge). This is followed by the onset of a respiratory acidosis at 3 to 4 d post-challenge (M. D. Powell, J. O. Harris & M. Leef unpubl. data) similar to that seen clinically for AGD (Powell et al. 2000). Therefore, in the present study it is likely that the rise in pH_a with *T. maritimum*-inoculated fish was indicative of the onset of gill infection as this was not seen in uninoculated fish (Fig. 3). The decline in pH_a

of *T. maritimum*-inoculated fish between 24 and 36 h post-surgery/inoculation may be equivalent to the acidosis observed in clinically AGD-affected fish. Mortality rates escalated between 36 and 48 h for inoculated fish, which corresponds with the onset of the acidosis seen in the abraded Fish Groups 1 and 2.

In conclusion, it would appear that gill abrasion causes profound respiratory disturbances that most likely result in reduced post-surgical survival. Infection of the gills with *Tenacibaculum maritimum* likely caused respiratory disturbances, but only after 30 h. These effects were likely to be also confounded by branchial abrasion. There was no evidence that gill abrasion and *T. maritimum* inoculation had an additive effect upon mortality. The underlying infection of fish with AGD appeared to have little effect upon the outcome of whether gills were abraded or infected with *T. maritimum*, suggesting that underlying AGD does not predispose fish to *T. maritimum* infections and vice versa.

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