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Experimental induction of gill disease in Atlantic salmon *Salmo salar* smolts with *Tenacibaculum maritimum*

Mark Powell[1,*], Jeremy Carson[2], Rebecca van Gelderen[1]

[1]School of Aquaculture, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania, Locked Bag 1370 Launceston, Tasmania 7250, Australia

[2]Fish Health Unit, Tasmanian Aquaculture and Fisheries Institute, Department of Primary Industries, Water and Environment, PO Box 46, Kings Meadows, Tasmania 7249, Australia

ABSTRACT: An experimentally induced bacterial infection of marine Atlantic salmon *Salmo salar* smolt gills was developed using strains of *Tenacibaculum maritimum* originally isolated from disease outbreaks in Tasmania. The gills of salmon were inoculated with a high concentration of bacteria (4×10^{11} cells per fish) of either strain 00/3280 or 89/4747 *T. maritimum*. Gentle abrasion of the gills was used to enhance the progression of gill disease. One strain (00/3280) was highly pathogenic, causing morbidity and mortality within 24 h post-inoculation, and produced acute focal branchial necrosis associated with significant increases in plasma osmolality and lactate concentration compared with controls (non-inoculated) or strain 89/4747-inoculated fish. There were no differences in the whole body net ammonium flux between control (non-inoculated) and strain 00/3820-inoculated fish. Gill abrasion resulted in acute telangiectasis and focal lamellar hyperplasia in all fish regardless of bacterial inoculation. This work provides the basis of a challenge model suitable for investigating the pathophysiological processes associated with acute branchial necrosis in marine fish, suggesting that osmoregulatory and possibly respiratory dysfunction are the primary consequences of infection.

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

INTRODUCTION

Bacterial diseases of freshwater fish epithelia have been extensively studied with the primary focus upon bacteria from the Cytophagales, such as bacterial gill disease caused by *Flavobacterium branchiophilum* (Ostland et al. 1994, 1995), and necrotizing skin diseases such as columnaris disease caused by *F. columnare* (Thomas-Jinu & Goodwin 2004) and bacterial cold-water disease caused by *F. psychrophilum* (Cipriano et al. 1996 and review by Nematollahi et al. 2003). However, in marine fishes relatively few bacterial skin and gill diseases have been characterised. *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*) has been reported to cause significant skin and gill pathology in 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 tapirina* (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).

The aim of this research was to investigate whether experimental inoculation of the gills of Atlantic salmon with *Tenacibaculum maritimum* could cause acute gill disease and to identify potential processes that may be responsible for mortality.

*Email: mark.powell@utas.edu.au

MATERIALS AND METHODS

Preparation of bacterial cultures. Cultures of *Tenacibaculum maritimum* were isolated by the Department of Primary Industry, Water and Environment from the skin of farmed salmon from Tasmania, Australia, with clinical cases of cutaneous erosion disease. The cultures were designated 89/4747 (Atlantic salmon) and 00/3280 (rainbow trout) and were isolated in 1989 and 2000 respectively. The bacteria were isolated on the medium of Anacker & Ordal (1959), formulated with seawater. Isolates were identified using a 16S ribosomal RNA (rRNA) PCR primer set specific for *T. maritimum* (Carson 1998). Cultures were stored frozen at –80°C on MicroBank beads (Pro-Lab Diagnostics) 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 µg RCF (relative centrifugal force) for 20 min and the pellet washed twice with sterile seawater. Harvested cells were resuspended in 15 ml of sterile seawater.

Experimental series 1: Infection study. Atlantic salmon smolts of mean mass (±SE) 77.0 ± 2.9 g were acclimated to full strength seawater (35 ppt) over a period of 10 d, then allocated to triplicate tanks (4 fish per tank, n = 12 per treatment) and allowed to habituate for 24 h prior to anaesthetization with AQUI-S (0.04 ml l^{-1}) or clove oil (0.03 ml l^{-1}). The gill arches were then separated and all of the filaments abraded by stroking gently with a blunt spatula. Aliquots (0.5 ml) of *Tenacibaculum maritimum* culture in sterile, filtered seawater were applied to the gill arches on each side of the fish (total 1 ml *T. maritimum* culture, ~4×10^{11} cells per fish, n = 12 per treatment). Controls consisted of fish that were inoculated with filtered seawater only (n = 12, repeated twice). An additional group of fish was inoculated with 1 ml of *T. maritimum* (strain 00/3280) culture onto the gills without prior filament separation or abrasion. Fish were allowed to recover in their respective tanks and monitored over the subsequent 5 d. Moribund fish were removed and cultures made from the gills on Shieh's agar (Song et al. 1988) formulated with seawater mineral salts agar (MSA), and colonies were confirmed as *T. maritimum* by colony colour and shape, as well as indirect fluorescent antibody test (IFAT) using a rabbit antibody prepared with a whole cell formalin-inactivated cell suspension of *T. maritimum* 89/0235 (rainbow trout).

Experimental series 2: Comparative pathology of *Tenacibaculum maritimum* strains. In Experimental series 1 there was an apparent difference in the pathogenicity of the 2 strains of *T. maritimum* used. Thus, it was decided to further examine the effects of acute exposure of smolts to the 2 different strains.

Seawater-acclimated Atlantic salmon smolts were allocated to 30 l round tanks (6 fish per tank, 3 tanks per treatment) following anaesthesia, gill abrasion and inoculation with either strain 89/4747 or 00/3280 of *Tenacibaculum maritimum*. Controls consisted of fish that had the gill filaments separated but were inoculated with seawater. Fish were allowed to recover and maintained in static aerated seawater at 17.8 to 18.4°C (Fig. 1A), 79 to 95% O_2 saturation (Fig. 1B) for 24 h post-inoculation. Total ammonium concentrations were measured using the method of Verdouw et al. (1978). During the exposure, mori-

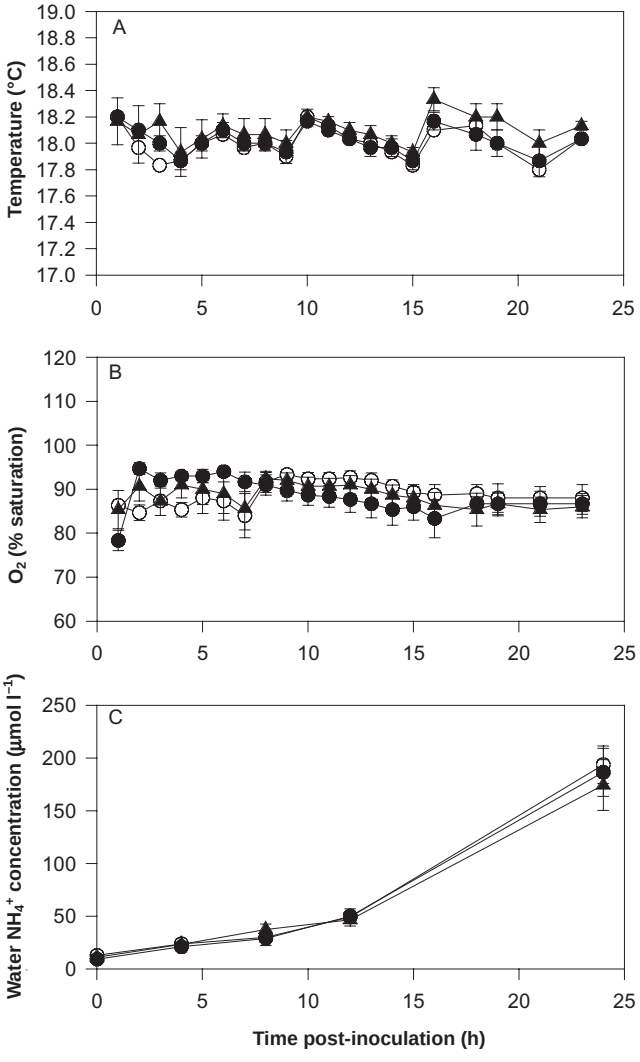

Fig. 1. (A) Mean (±SE) temperature, (B) oxygen saturation and (C) water ammonium concentration for tanks containing Atlantic salmon *Salmo salar* smolts experimentally inoculated with *Tenacibaculum maritimum* (●) strain 00/3280, (▲) strain 89/4747 or (○) controls over a 24 h static challenge

bund fish (unresponsive to touch) were removed, anaesthetised with 0.04 ml l^{-1} clove oil and bled from the caudal vein. Heparinised blood (100 IU ammonium heparin, Sigma-Aldrich) was then centrifuged at 8000 × *g*, the plasma decanted and frozen at –20°C for further analysis. The gills were swabbed onto MSA for confirmation of the presence of *T. maritimum* using colony morphology and IFAT. The gills were then removed and fixed in seawater Davidson's solution for histological examination. After 24 h of exposure, surviving fish were killed by anaesthetic overdose (clove oil >0.03 ml l^{-1}) and sampled as for moribund fish.

Plasma osmolality was determined using a Vapro 5520 vapour pressure osmometer (Wescor). Plasma chloride was determined according to the method of Zall et al. (1956) and plasma lactate concentrations were determined using a commercial lactate assay kit (Sigma Diagnostics, protocol 735). Gills were embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin.

Experimental series 3: Effects of *Tenacibaculum maritimum* infection on ammonium flux. In Experimental series 2, despite the removal of moribund fish inoculated with *T. maritimum* strain 00/3280, the total ammonium concentrations in the water remained equivalent to that of fully stocked tanks that experienced no morbidity (*T. maritimum* strain 89/4747 and control tanks). Therefore, the net ammonium flux was measured for fish inoculated with *T. maritimum* strain 00/3280 and controls.

The gills of seawater-acclimated smolts were abraded and inoculated as described above with either strain 00/3280 or seawater (controls), and placed into individual black acrylic flux chambers (internal volume of 1 l) supplied with flowing seawater and constant aeration (1 fish per chamber, 9 fish per treatment). Following 24 h of recovery, the flow to the flux chambers was stopped and a water sample (5 ml) removed and immediately frozen (–20°C) for later analysis. Following a 3 h period, a second water sample was removed and similarly frozen, after which the water flow to each flux chamber was reinstated. Fish were then removed, killed by anaesthetic overdose (clove oil >0.03 ml l^{-1}) and the gills swabbed onto MSA for subsequent confirmation of the presence/absence of *Tenacibaculum maritimum* using colony morphology and IFAT.

Plasma osmolality, chloride concentrations and lactate concentrations were compared statistically between strains (00/3280 and 89/4747) and controls using analysis of variance with a Bonferroni corrected *t*-test planned contrast relative to controls. p values of less than 0.05 were considered to be significant.

Water samples were analysed for total ammonium concentration using the method of Verdouw et al. (1978). Whole body net ammonium flux was then calculated according to:

$$J_{net}\,NH_4^+ = \frac{[NH_4^+]_{t=1} - [NH_4^+]_{t=0} * V}{M * T}$$

Where $[NH_4^+]_{t=x}$ is the concentration of total ammonium (mmol l^{-1}) in the water sample at the start or end of the flux period, V is the volume of the flux chamber, M is the mass of the fish (g) and T is the duration of the flux period (h). Net whole body ammonium fluxes were compared with zero and between inoculated and control fish using a *t*-test. p values of less than 0.05 were considered to be significant.

RESULTS

Experimental series 1: Infection study

The highest rate of mortality (70%) occurred within 48 h post-inoculation in fish that had received gill abrasion and infection with strain 00/3280 (Fig. 2). Fish with non-abraded gills inoculated with strain 00/3280 had a maximum mortality rate of 50% at 96 h, whereas fish inoculated with strain 89/4747 and controls had

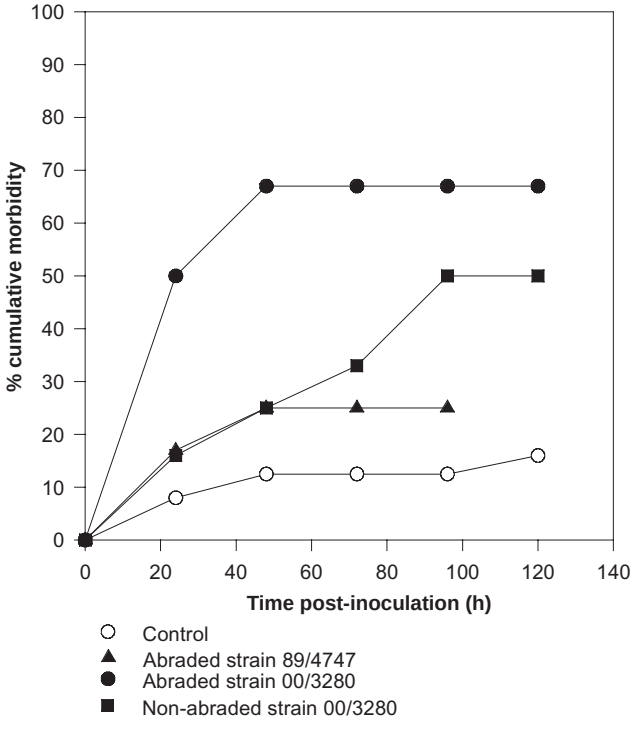

Fig. 2. *Salmo salar* infected with *Tenacibaculum maritimum*. Percent cumulative mortality of Atlantic salmon smolts experimentally inoculated with (●) strain 00/3280 following a light abrasion, or (■) no abrasion of the gills, (▲) strain 89/4747 following light abrasion or (○) controls (light abrasion, no bacteria)

maximum mortality rates of 25 and 11% at 48 h respectively (Fig. 2). Bacterial colonies with characteristics typical of *Tenacibaculum maritimum* were isolated from all fish within those groups inoculated with bacteria. The 00/3280-inoculated fish returned heavy bacterial growth from gill smears compared with the light bacterial growth from gill smears of control (non-inoculated) fish (Table 1).

Experimental series 2 and 3: Comparative pathology and ammonium flux experiments

Fish inoculated with strain 00/3280 showed a higher rate of morbidity than fish treated with strain 89/4747 or controls over the 24 h challenge experiment (Fig. 3A). There was heavy bacterial growth from gill smears of fish inoculated with *Tenacibaculum maritimum* bacteria (both strains 00/3280 and 89/4747) and poor growth from smears taken from control fish (Table 1). Bacteria isolated from fish inoculated with strain 00/3280 returned a positive result when tested with IFAT, while bacterial smears from fish inoculated with strain 89/4747 and control fish were negative (Table 1). Water ammonium concentrations increased in all tanks over the exposure duration; however, levels remained below 200 µmol l^{-1} (Fig. 1C). Moribund fish from the strain 00/3280 groups had significantly higher plasma osmolality than fish that had been inoculated with strain 89/4747 or control animals at 24 h post-inoculation ($F_{2,19}$ = 8.61, p = 0.0022) (Fig. 3B). However, there were no significant differences in the plasma Cl^- concentration between the groups ($F_{2,19}$ = 2.99, p = 0.0742) (Fig. 3C). There were significantly elevated plasma lactate concentrations in moribund fish inoculated with strain 00/3280 compared with either strain 89/4747-inoculated or control fish after 24 h of exposure ($F_{2,19}$ = 6.24, p = 0.0082) (Fig. 3D). There was a significant negative net whole body ammonium flux in both control and strain 00/3280-inoculated fish. However, there was no significant difference in the magnitude of the net ammonium flux between control and strain 00/3280-inoculated fish (t_{31}= 1.47, p = 0.1508) (Fig. 3E). Smears from fish inoculated with strain 00/3280 showed heavy growth of bacteria typical of *T. maritimum*, whereas control (non-inoculated) fish showed no bacterial growth (Table 1).

Table 1. *Tenacibaculum maritimum* infecting *Salmo salar*. Recovery of bacteria from gills following experimental inoculation and indirect fluorescent antibody test (IFAT) confirmation. ND = not determined; – = no growth/negative; +/– = inconclusive; + = poor growth/positive; ++ = moderate growth; +++ = heavy growth

T. maritimum strain	00/3280 Scraped	00/3280 Non-Scraped	89/4747 Scraped	Control
Expt 1: Infection study				
Culture	+++	+++	+++	+
Expt 2: Comparative pathology				
Culture	+++	ND	+++	+
iFAT	+	ND	–	+/–
Expt 3: Ammonium flux	+++	ND	ND	–

Histopathology

Morbid salmon inoculated with strain 00/3280 but not 89/4747 exhibited gills with yellowish mucoid aggregations at the filament tips. Histologically, there was focal epithelial necrosis with extensive bacterial mats overlaying necrotic tissue (Fig. 4A,B). Bacterial associated necrosis of the filaments occurred in what appeared to be a distal to proximal direction. There was very limited (only seen in 1 fish out of 6 examined) evidence of bacterial associated necrosis with strain 89/4747 and no necrosis in control (non-inoculated) gills (Fig. 4C). Abraded gills often showed signs of telangiectasis and congestion, irrespective of whether fish were inoculated with *Tenacibaculum maritimum* (Fig. 4E,F).

DISCUSSION

Gill abrasion enhances the susceptibility of catfish *Ictalurus punctatus* to infection with *Flavobacterium columnare* (Bader et al. 2003) and has been used to enhance the reliability of *Tenacibaculum maritimum* challenge in salmonids (J. Carson pers. comm.). In our study, abrasion enhanced the severity and rate at which the disease progressed leading to mortality. There were, however, clear differences in the susceptibility of fish to different strains of *T. maritimum*. Strain 00/3280 was pathogenic, whereas strain 89/4747 was not. Indeed, strain 89/4747 failed to be detectable with IFAT. Failure or ambiguity of IFAT techniques have been reported with *F. psychrophilum* (Vatos et al. 2002). Both this species and its warm water counter-part *F. columnare* have a highly labile glycocalyx responsible for adhesion (Nematollahi et al. 2003) and *F. columnare* is also associated with chondroitinase (AC lyase) activity (Stringer-Roth et al. 2002).

Apart from the glycocalix, *F. columnare* adherence to gill tissue is also dependent upon the presence of D-glucose or N-acetyl-D-glucosamine (Decostere et al. 1999). It is possible that the requirements of strain 89/4747 *T. maritimum* for adherence to gills were not fully met because of the cellular microclimate of the salmon gill. Failure to detect strain 89/4747 *T. maritimum* using IFAT may also reflect a difference in the bacterial glycocalyx of this strain compared with the pathogenic strain 00/3280.

The branchial epithelial necrosis caused by strain 00/3280, and less so with strain 89/4747 *Tenacibaculum maritimum* infection was similar to that reported by Handlinger et al. (1997) for naturally infected Atlantic salmon and rainbow trout. However, in our study, telangiectasis and focal hyperplasia of the branchial epithelium due to gill abrasion was common in control fish (non-inoculated) and fish inoculated with both strains of *T. maritimum*. Interestingly, the hyperplastic lesions on the gills were similar to those seen with amoebic gill disease, but with the difference that gill abrasion lesions were associated with telangiectasis, not parasome-containing amoebal trophozoites (Adams & Nowak 2001, 2003). It appears that the branchial necrosis causes a strong osmoregulatory disturbance in Atlantic salmon with significant increases in plasma osmolality. This osmoregulatory disturbance probably involves ionoregulatory dysfunction. Although the mean plasma chloride concentrations were elevated in moribund salmon inoculated with strain 00/3280, this was not statistically different from controls. In other situations where extensive branchial necrosis occurs, such as oxidative injury, similar osmoregulatory disturbances may be present without any clear changes in plasma chloride levels (Powell & Harris 2004). Similarly, reductions in plasma osmolality have been reported with experimental *Flavobacterium branchiophilum* infections of freshwater rainbow trout, although these were in association with a reduction in plasma electrolytes including chloride (Byrne et al. 1995). Plasma lactate concentrations were clearly elevated in moribund salmon suggesting that acute respiratory failure may occur pre-mortem. It is not clear whether the probable limited oxygen transfer was due to diffusive limitations in the gill or reduced perfusion of the gill. Byrne et al (1991, 1995) did not demonstrate any reduction in blood pO_2 in freshwater brook trout *Salvelinus fontinalis* and rainbow trout experimentally infected with *F. branchiophilum*. However, Foscarini (1989) demonstrated a transient bradycardia and an acute branchial hyperplasia and oedema in *F.*

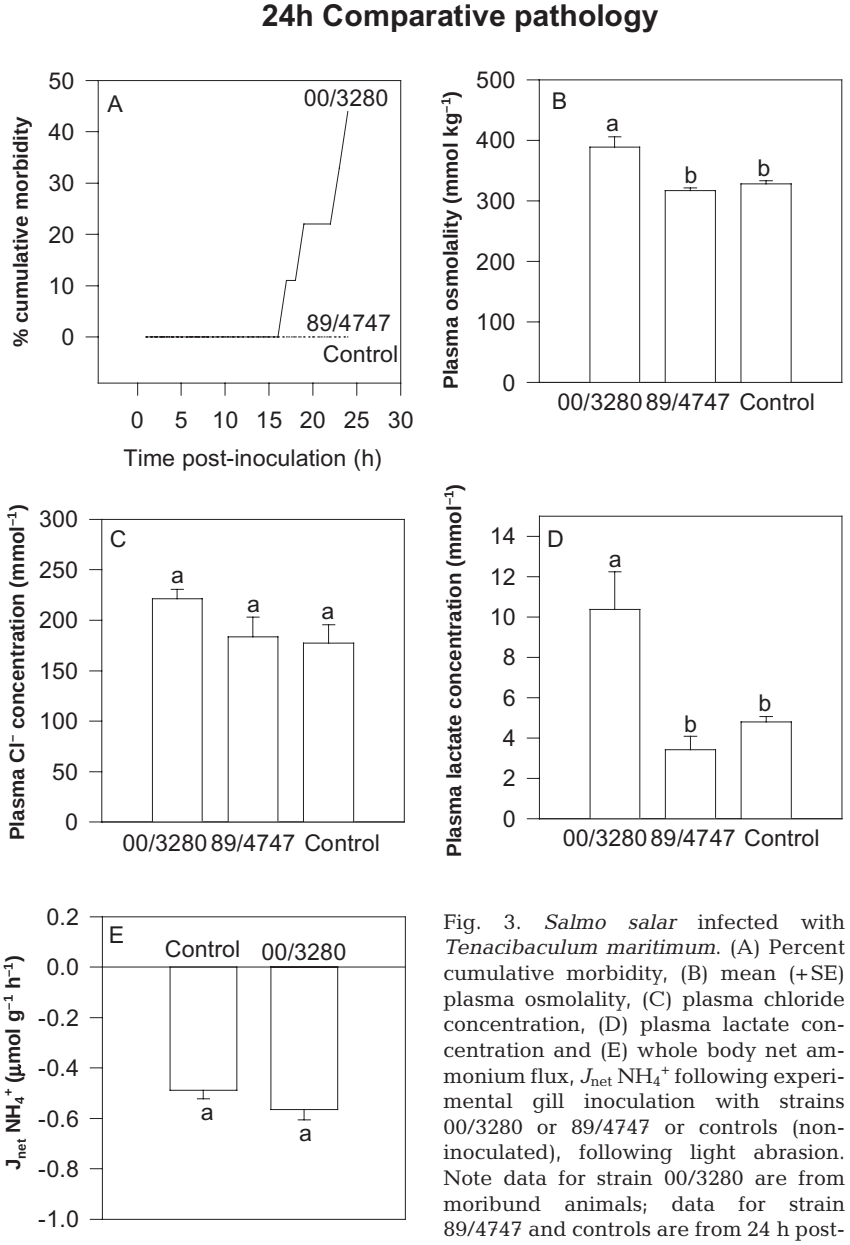

Fig. 3. *Salmo salar* infected with *Tenacibaculum maritimum*. (A) Percent cumulative morbidity, (B) mean (+SE) plasma osmolality, (C) plasma chloride concentration, (D) plasma lactate concentration and (E) whole body net ammonium flux, J_{net} NH_4^+ following experimental gill inoculation with strains 00/3280 or 89/4747 or controls (non-inoculated), following light abrasion. Note data for strain 00/3280 are from moribund animals; data for strain 89/4747 and controls are from 24 h post-inoculation. Different letters indicate statistical significance

columnaris (now *F. columnare*)-exposed eel *Anguilla japonica*. It is, therefore, not possible to exclude either diffusive or perfusive limitations to gas exchange as a cause of plasma lactate elevations in *T. maritimum*-infected salmon.

Despite the apparent deterioration in water quality, even with declining fish density, in the 24 h comparative pathology trial of fish inoculated with strain 00/3280, there were no significant differences in the rate of ammonium efflux. Increased ammonium effluxes have been reported in response to acute freshwater exposures of Atlantic salmon affected by amoebic gill disease (Roberts & Powell 2003). Similarly rainbow trout experimentally infected with *Flavobacterium branchiophilum* had elevated plasma ammonium tensions (Byrne et al. 1995). It would appear that in acute *Tenacibaculum maritimum* infection of salmon, ammonium excretion rates are not significantly affected.

In conclusion, we have demonstrated that direct inoculation of high concentrations of *Tenacibaculum maritimum* directly onto the gills of Atlantic salmon smolts will induce a necrotic branchitis which appears to be enhanced by prior abrasion of the gill epithelium. Progression of the infection results in an acute and ultimately lethal osmoregulatory and potentially respiratory dysfunction.

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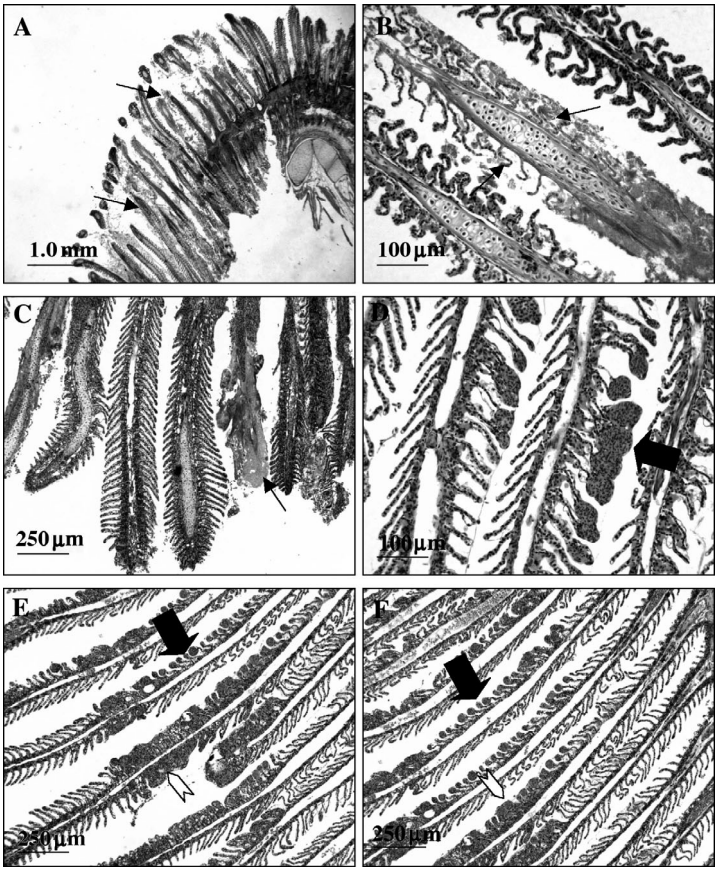

Fig. 4. *Salmo salar* infected with *Tenacibaculum maritimum*. (A,B) Extensive branchial necrosis associated with filamentous bacterial mats (arrows) on the gills of Atlantic salmon smolts inoculated with strain 00/3280. (C) Filamental necrosis associated with filamentous bacteria on the gills of salmon inoculated with *T. maritimum* strain 89/4747. (D) Telangiectasis (large black arrow) associated with the gills of control, (E) strain 00/3280-inoculated and (F) strain 89/4747-inoculated fish. Note the hyperplastic lesions also associated with gill abrasion (white arrow)

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