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Nanoinjection as a tool to mimic vertical transmission of *Flavobacterium psychrophilum* in rainbow trout *Oncorhynchus mykiss*

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ABSTRACT: Newly fertilised eggs of rainbow trout *Oncorhynchus mykiss* were nanoinjected with *Flavobacterium psychrophilum* in order to mimic vertical transmission. Two bacterial isolates with different elastin-degrading capacity were used. All infected groups (10, 100 and 1000 colony forming units egg^{-1}) showed significantly higher cumulative mortalities than the control groups at the end of the experiment, 70 d post-hatching. The total mortalities in the control groups were below 2.5%. In the high-dose groups, 95 to 100% of the eggs died during the eyed stage. In the intermediate group infected with the elastin-negative isolate, the major mortality occurred during the eyed stage of the egg, with a total cumulative mortality of 83% at the end of the experiment. In the intermediate group infected with the elastin-positive isolate, a total mortality of 63% was recorded. In this group, diseased fry showed clinical signs of disease and morphological changes similar to those described in connection with rainbow trout fry syndrome (RTFS) shortly after the beginning of feeding. In the low dose groups, the mortality in the elastin-negative group was 14% and in the elastin-positive group 11%. The bacterium was isolated from dead eggs and fry in infected groups and demonstrated in internal organs of dead and moribund fry by immunohistochemistry. The nanoinjection method used in this study may be a useful method to study pathogens, like *F. psychrophilum*, that can be vertically transmitted.

KEY WORDS: *Flavobacterium psychrophilum* · Nanoinjection · RTFS · Rainbow trout · Eggs

INTRODUCTION

The microinjection technique, based on administration of small volumes into fish embryos, was initially developed to evaluate hepatic carcinogenicy of different chemicals (Metcalfe & Sonstegard 1984, Black et al. 1985). The method has been used in order to evaluate the impact of halogenated organic chemicals (Norrgren et al. 1993) as well as extracts from sediments and animal tissues (Metcalfe et al. 1990, Wilson & Tillitt 1996, Lundström et al. 1998) on early life-stages of fish. The microinjection assay has been refined in the nanoinjection technique by different technical improvements (Åkerman & Balk 1995, Walker et al. 1996). Significant improvements include the use of a picoinjector and a very fine glass needle, which facilitates injection volumes down to nanolitres and reduces background mortality due to the injection.

Infections with the bacterium *Flavobacterium psychrophilum* are a problem in aquaculture all over the world. Salmonids are the most common species affected (Holt et al. 1993). Early life stages are most sensitive to the bacterium and thereby are also the most seriously affected (Holt 1987, Lorenzen 1994, Madsen & Dalsgaard 1999). In rainbow trout *Oncorhynchus mykiss* fry, the disease is referred to as rainbow trout fry syndrome (RTFS), a septicaemic condition that can cause mortalities up to 70% (Lorenzen et al. 1991, Bruno 1992). The disease appears during the first 2 mo of feeding at water temperatures below 15°C

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(Lorenzen et al. 1991). Main gross pathological findings in diseased fry include an enlarged spleen, pale gills, liver and kidney, and a haemorrhagic, protruding anus (Lorenzen et al. 1991).

It is well known that some fish pathogens can be transmitted from the brood fish to the progeny, so-called vertical transmission. Vertical transmission has been reported for infectious pancreatic necrosis virus (IPNV) (Bullock et al. 1976) and infectious haematopoietic necrosis virus (IHNV) (Mulcahy & Pascho 1985). However, later studies have raised doubts concerning a vertical transmission of both IPNV and IHNV. It has been proposed that the IPNV virus is tightly adsorbed to the outer surface of the eggshell, rather than being present inside the egg (Ahne & Negele 1985). A study by Yoshimizu et al. (1989) indicated that IHNV is unlikely to survive inside the egg and experimental vertical transmission of the virus has been unsuccessful (Traxler et al. 1997).

The bacterium causing bacterial kidney disease (BKD), *Renibacterium salmoninarum*, has been shown to be vertically transmitted (Evelyn et al. 1984), and *Flavobacterium psychrophilum* has been isolated from the inside of the fertilised egg (Brown et al. 1997, Kumagai et al. 2000), as well as from internal organs and sexual products of brood fish (Holt et al. 1987, Rangdale et al. 1996, Brown et al. 1997, Ekman et al. 1999), indicating vertical transmission.

The aim of this study was to evaluate whether nanoinjection is applicable to study vertically transmitted diseases by injection of *Flavobacterium psychrophilum* in newly fertilised rainbow trout eggs.

MATERIALS AND METHODS

Bacterial isolates. Two different isolates of *Flavobacterium psychrophilum*, F9 and F169, were used in the experimental infection, both isolated from diseased rainbow trout at 2 different fish farms. The isolates were phenotypically similar, except in their abilities to degrade elastin, where F9 was able to degrade elastin and F169 was not. The isolates were stored at –80°C in tryptone and yeast extract salt (TYES) broth (Holt et al. 1993), with 17% glycerol added. Bacteria were taken from the frozen batches and incubated in TYES broth at 15°C on a shaker. After 48 h, the bacteria were harvested, washed twice in 0.9% NaCl (1500 × *g* for 10 min at 5°C) and adjusted with 0.9% NaCl to an absorption of 0.4 at 525 nm using a spectrophotometer (Shimadzu UV-1601PC). Serial dilutions were made and drop inoculation performed in triplets on TYES agar (TYES broth with 1.1% agar added) to determine the number of viable bacteria in the suspension. Further dilutions of the bacterial suspension were made with 0.9% NaCl.

Injection and sampling. Rainbow trout eggs and milt from a commercial fishfarm connected to the Swedish fish-health control program were delivered to the Institute of Applied Environmental Research (ITM), Stockholm University. The brood fish were considered to be free from IPN, IHNV and BKD (U.-P. Wichardt, Fish-health control program, Fiskhälsan FH AB, Älvkarleby, Sweden, pers. comm.). Eggs and milt originated from 1 female and 1 male respectively. After fertilisation and water hardening, 40 control eggs were randomly sampled for bacteriological examination to ensure no *Flavobacterium psychrophilum* was present in the eggs at the start of the experiment. Eggs for injection were randomly divided into 8 experimental groups, with 103 to 108 eggs per group. To immobilise the eggs during the injection and incubation they were placed in prepared holes in 1% agarose gel cast in square Petri dishes (Åkerman & Balk 1995). The eggs were kept in the agarose gel until hatching.

The eggs were injected 5 d after fertilisation. Each bacterial isolate was injected with 1 of 3 different doses: low dose (Ld) groups receiving approximately 10 colony forming units (CFU) egg^{-1}, intermediate dose (Id) groups receiving 100 CFU egg^{-1}, and high dose (Hd) groups receiving 1000 CFU egg^{-1}. One control group was injected with 0.9% NaCl and another was kept uninjected. A total volume of 50 nl was injected into the yolk of each egg. The injection procedure and equipment are described in detail by Åkerman & Balk (1995) and Walker et al. (1996). In brief, a needle of aluminium silicate glass was held in a micromanipulator (WR-87, Narishige Scientific Instrument Laboratory). The injection volume was controlled with a pico injector (PLI-100, Medical Systems) and the whole procedure was performed under a stereomicroscope (Leica MZ8, 6 to 50× magnification). After injection, the eggs were transported to the Department of Pathology, Swedish University of Agricultural Sciences, Uppsala. Eggs from each group were separately incubated in 5 l aquaria with well-aerated flow-through ground water at a temperature of 10 ± 1°C. Unfertilised eggs were removed 1 wk after injection. The fertilisation varied between 79 and 86% among groups, with a mean fertilisation rate of 83%.

The eggs were inspected and mortalities monitored daily. Dead eggs were cultured for the presence of *Flavobacterium psychrophilum*. At the eyed stage, 5 eggs from each group were sampled and cultured for the presence of *F. psychrophilum* in viable embryos.

After hatching (37 d post injection [p.i.]), the Petri dishes were removed from the aquaria. After yolk-sac absorption, the fry were fed commercial fish food (Aller Aqua AB) 3 times a day. The mortality was recorded at least twice a day. Dead or moribund fry were sampled for bacteriological examination and/or fixed for histological and immunohistochemical studies.

Five fry from the control groups were sampled at the beginning of feeding (63 d p.i.) for morphological studies. At the termination of the experiment (107 d p.i.) the remaining fish were sampled for bacteriological examination (kidney and spleen) and morphological studies.

Bacteriological examination. Bacteriological examination of control eggs sampled after fertilisation and water hardening, dead eggs and eggs sampled at the eyed stage were performed as follows: eggs were sterilised with 5% Buffodine (Evans Vanodine International) for 20 min and rinsed several times with sterile water. To be certain that the surface disinfection was successful, each egg was separately incubated in test tubes with 3 ml of TYES broth and incubated at 15°C. After 5 to 7 d, the broth was visually inspected for bacterial growth and 0.1 ml of the broth was inoculated on TYES agar. Eggs in test tubes yielding visual growth in the broth or growth on the agar plates were excluded from further studies. Eggs that proved surface-sterile were crushed with a sterile glass rod and the test tubes were incubated for another 7 d, before 0.1 ml was inoculated on TYES agar plates.

Dead and moribund fry were aseptically sampled from the brain and/or the kidney with a plastic loop and inoculated on TYES agar for 7 d. At the termination of the experiment, samples for bacteriology were taken from spleen and kidney and inoculated on TYES agar for 7 d.

Identification of reisolated *Flavobacterium psychrophilum* was performed with morphological and phenotypic characteristics including colony morphology, Gram staining, production of cytochrome oxidase (Bactidrop™Oxidase, Remel), catalase (30% H_2O_2), flexirubin-like pigment (20% KOH), reactivity in the API-zym gallery (bioMérieux) and ability to grow at 6 and 30°C in TYES broth. The results were compared with the results of Bernardet & Kerouault (1989) and with the characteristics of the type strain NCIMB 1947^T. All reisolated *F. psychrophilum* were also tested for their ability to degrade elastin on TYES agar with 0.05% elastin added (Madsen & Dalsgaard 1998).

Histology and immunohistochemistry. Fry were fixed in 10% phosphate-buffered formalin (pH 7.2 to 7.4), embedded in paraffin and sectioned and stained with haematoxylin and eosin (H&E). Between 2 and 5 sections of each fry were examined with emphasis on changes in the kidney, liver, spleen and yolk.

Immunohistochemical stainings were performed on sections mounted on SuperFrost®Plus glass (Menzel). An avidin-biotin immunoperoxidase kit, VECTASTAIN® elite ABC kit (Vector Laboratories) was used according to the manufacturer's instructions. Endogenous peroxidase activity was quenched with 3% H_2O_2 for 5 min. A commercial polyclonal antibody against *Flavobacterium psychrophilum* (RFP01, Microtek International), diluted 1:5000 in 0.05 M Tris buffer (pH 7.6), was used as primary antibody. The antibody was replaced with non-immune rabbit serum or Tris buffer in negative controls. As chromogen, 3-amino-9-ethylcarbazole (AEC) was used, and Mayer's haematoxylin as counterstain.

Statistics. Differences in mortality between the groups were analysed with Fisher's exact probability test. p-values < 0.05 were considered significant.

RESULTS

Mortality

The total cumulative mortality in the uninjected control was 2.4% (2 out of 84) and in the NaCl-injected control 1.2% (1 out of 85) (Fig. 1). All the infected groups showed a significantly higher mortality than the control groups. In the Ld groups, the total mortality in the F9 group was 10.6% (9 out of 85) and in the F169 group 13.5% (10 out of 74) (Fig. 1). There was no significant difference in mortality between the 2 Ld groups. In the Id groups, the total mortality in the F9 group was 62.7% (52 out of 83) and in the F169 group 82.9% (68 out of 82) (Fig. 1). The mortality in the F169 (elastin negative) group was significantly higher than in the F9 (elastin positive) group. In the F169 Id group, only sporadic deaths were recorded in the feeding fry, while in the F9 Id group, a mortality of 24.6% (16 out of 65 feeding fry) occurred 4 to 18 d after the start of feeding. In the Hd groups, all mortalities occurred during the egg stage, with a total mortality of 100% (80 out of 80) in the F169 group and 95.1% (78 out of 82) in the F9 group. No significant difference in mortality was recorded between the 2 Hd groups.

Bacteriology

No *Flavobacterium psychrophilum* was isolated from the eggs (n = 40) sampled after fertilisation and water hardening. The numbers of eggs and fry from which *F. psychrophilum* was isolated are shown in Table 1. The bacterium was isolated from almost all examined dead eggs and fry except in the control groups, where no *F. psychrophilum* was isolated. All the isolates from the F169-infected groups were elastin-negative, and all isolates from the F9-infected groups were elastin-positive. No *F. psychrophilum* was isolated from surviving fish in any of the groups at the end of the experiment.

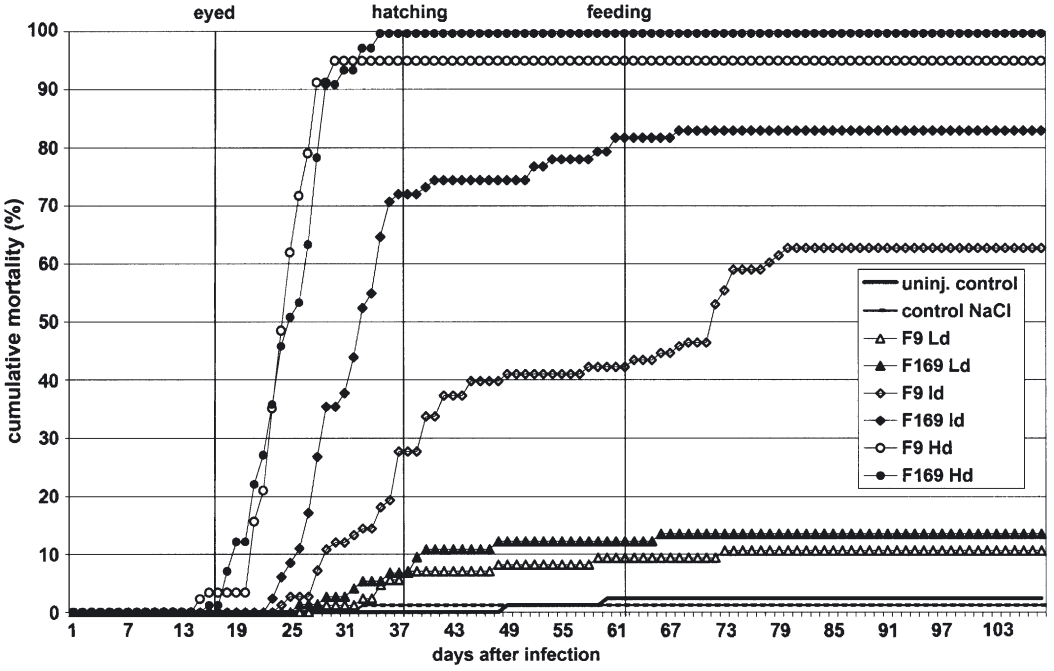


Fig. 1. *Oncorhynchus mykiss*. Cumulative mortality of rainbow trout eggs and fry after nanoinjection of 2 different *Flavobacterium psychrophilum* isolates, one able to degrade elastin (F9) and the other not (F169). Low dose (Ld) groups received 10 CFU egg^{-1}, intermediate dose (Id) groups received 100 CFU egg^{-1}, and high dose (Hd) groups received 1000 CFU egg^{-1}

Clinical signs and gross pathology

No differences in visible clinical signs or gross pathology in diseased and dead fry were recorded between the different infected groups. Diseased yolk-sac fry were lethargic and often showed precipitates in the yolk sac, sometimes accompanied by haemorrhages and a mild yolk-sac oedema (Fig. 2). In the diseased fry, prominent signs were lethargy, loss of appetite and dark pigmentation of the skin. Gross pathology findings included pale gills, pale liver and an enlarged spleen. The stomach and intestine of diseased fry were empty.

Table 1. *Oncorhynchus mykiss*. Number of rainbow trout eggs and fry from which *Flavobacterium psychrophilum* was isolated after nanoinjection of the bacterium into eggs. Total number of investigated fry and successfully surface-disinfected eggs are shown in parentheses. Two different isolates of the bacterium, F9 and F169, were used. Sampling was performed at the eyed stage, and from dead eggs and dead or moribund fry. Low dose (Ld) groups received 10 CFU egg^{-1}, intermediate dose (Id) groups received 100 CFU egg^{-1}, and high dose (Hd) groups received 1000 CFU egg^{-1}

	Control uninjected	Control NaCl	F9 Ld	F169 Ld	F9 Id	F169 Id	F9 Hd	F169 Hd
Eyed stage	0(5)	0(5)	1(5)	0(5)	5(5)	5(5)	5(5)	5(5)
Dead eggs	–	0(1)	2(2)	1(3)	9(10)	29(30)	41(41)	37(37)
Dead/moribund fry	0(2)	–	2(2)	2(3)	21(21)	8(9)	–	–

Histology and immunohistochemistry

Dead fry in the uninjected control group, control fry sampled at the beginning of feeding and fry surviving the experiment from all groups were negative in the immunohistochemical staining and no significant histopathological findings were recorded.

The morphological findings of dead or moribund fry were similar in the various infected groups. The yolk of dead or moribund yolk-sac fry contained numerous immunohistochemically positive-stained bacteria (Fig. 3). Hyperaemia and haemorrhages were sometimes seen. In all of the examined dead or moribund feeding fry, remnants of the yolk were still present with immunohistochemically positive-stained bacteria and phagocytes.

The spleen was congested. The normal architecture was destroyed and necrotic areas were often present. Large numbers of immunohistochemically positive-stained bacteria and phagocytes were dispersed in the parenchyma (Fig. 4). The distinct outlining of the spleen was often destroyed, and peritonitis with debris, positively stained phagocytes, and free bacteria were seen on the serosa (Fig. 4). In the liver, immunohisto-

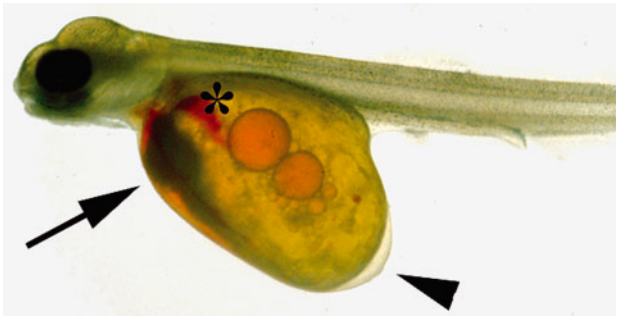
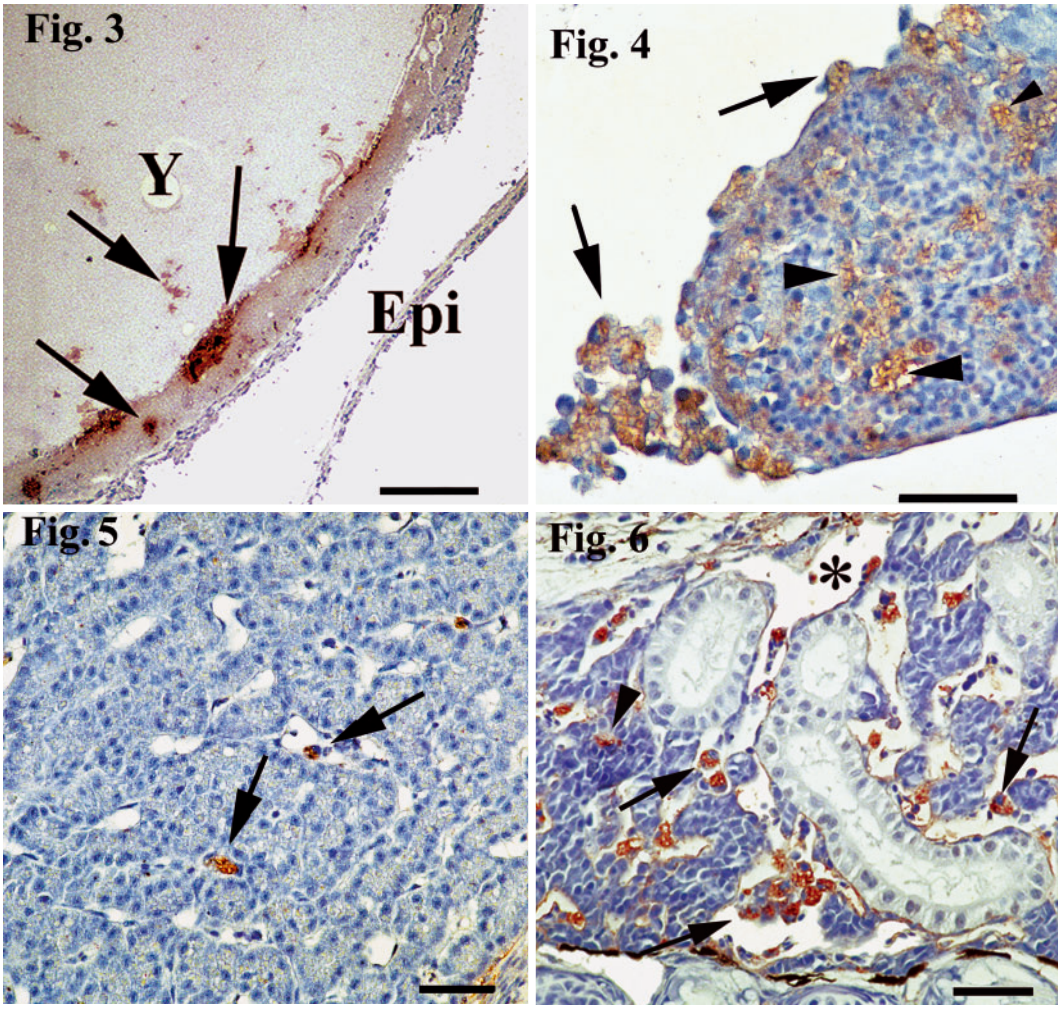


Fig. 2. *Oncorhynchus mykiss* experimentally infected with *Flavobacterium psychrophilum* using nanoinjection technique in fertilised eggs. Dead yolk-sac fry with a mild yolk-sac oedema (arrowhead), precipitate (arrow) and haemorrhages (asterisk) in the yolk

chemically positively stained phagocytes were seen in sinusoids and blood vessels (Fig. 5). In the kidney, large immunohistochemically positive-stained phagocytes were seen in the peritubular capillaries and in the haematopoietic tissue (Fig. 6). Positively stained endothelial cells were also seen (Fig. 6). Necrosis of tubular epithelium and/or haematopoietic tissue was only occasionally present. Immunohistochemically positively stained bacteria and phagocytes were also seen in the vascular system of several other organs such as the brain, gills and intestine.



Figs. 3 to 6. *Oncorhynchus mykiss* experimentally infected with *Flavobacterium psychrophilum* using nanoinjection technique in fertilised eggs. Immunohistochemical staining with a polyclonal antibody towards *F. psychrophilum* (RFP01). Avidin-biotin complex method, 3-amino-9-ethylcarbazole (AEC) as chromogen and Mayer's haematoxylin as counterstain. Fig. 3. Yolk-sac of moribund yolk-sac fry. Large amounts of bacteria are present in the yolk (arrows). Y = yolk, Epi = epidermis. Scale bar = 0.1 mm. Fig. 4. Spleen of moribund fry. Large amounts of bacteria dispersed in the parenchyma (arrowheads). Peritonitis with positively stained phagocytes on the serosa (arrows). Scale bar = 50 µm. Fig. 5. Liver of moribund fry. Positively stained phagocytes in blood vessels and sinusoids (arrows). Scale bar = 50 µm. Fig. 6. Kidney of moribund fry. Positively stained phagocytes in peritubular capillaries (arrows) and in the haematopoietic tissue (arrowheads). Positively stained endothelial cell (asterisk). Scale bar = 50 µm

DISCUSSION

Administration by nanoinjection of *Flavobacterium psychrophilum* in fertilised rainbow trout eggs resulted in significantly higher cumulative mortalities among all infected groups compared with controls. The proteolytic nature of *F. psychrophilum*, especially the elastin-degrading capacity, has been suggested to be involved in its virulence (Madsen & Dalsgaard 1998). In this experiment, significant differences between the isolates were only seen between Id groups, where the elastin-negative isolate resulted in higher mortality than the elastin-positive one. This supports the theory that other virulence factors, like serotype and/or ribotype, are involved in the virulence of *F. psychrophilum* (Lorenzen & Olesen 1997, Madsen & Dalsgaard 2000).

Flavobacterium psychrophilum could be isolated from viable eggs at the eyed stage in the Id and Hd groups but only from 1 egg in the Ld groups. Possibly the number of bacteria in the 2 Ld groups was too low to detect with ordinary culturing methods, or the bacteria did not survive. The bacterium could be cultured from a majority of the surface-disinfected, examined dead eggs and dead fry in all infected groups.

In the Hd groups and one of the Id groups (F169) a heavy mortality was recorded during the eyed stage of the eggs. *Flavobacterium psychrophilum* is not known to cause major egg mortality, but Cipriano et al. (1995) associated the bacteria with mortality among Atlantic salmon *Salmo salar* eggs at eyed stage. The bacterium has also been isolated from both live and dead lake trout *Salvelinus namaycush* eggs at eyed stage, and a possible relation to increased yolk-sac mortality was indicated (Symula et al. 1990).

The clinical signs and pathological findings of diseased and dead fry in the F9 Id group after the beginning of feeding were similar to those described in connection with RTFS (Lorenzen et al. 1991, Bruno 1992, Rangdale et al. 1999). In addition, large amounts of bacteria and phagocytes were seen in the yolk-sac remnant of the feeding fry. Baudin-Laurencin (1989) described similar changes in rainbow trout naturally infected with *Flavobacterium psychrophilum*. The presence of the bacterium in almost all internal organs, shown by immunohistochemical staining, confirms the septicaemic nature of acute RTFS (Evensen & Lorenzen 1996).

The localisation of *Flavobacterium psychrophilum* in the naturally infected egg is unclear. It is proposed that the bacteria enter the egg in connection with water hardening and are located in the perivittelin space rather than in the yolk (Kumagai et al. 2000). *F. psychrophilum* is shown not to be as susceptible to lysozyme as many other bacteria (Brown et al. 1997) and is thereby able to survive within the contents of the egg and yolk sac.

A microinjection technique has been used in studies of BKD (Brown et al. 1990a,b). Injection of *Renibacterium salmoninarum* was performed before water hardening of the eggs. The mortality in the injected control group was 28% compared with 4% in the uninjected control. In our experiment, using the nanoinjection technique, the mortality was below 2.5% in both saline injected controls and uninjected controls. Brown et al. (1990a) reported leakage of yolk material between the injection and water hardening. This might include loss of injected bacteria, with the result that the true amount of bacteria in the egg would be lower than the number injected. No leakage was observed in our experiment.

Many methods have been used in experimental infection with *Flavobacterium psychrophilum*. The most reliable methods are intramuscular (Holt 1987, Garcia et al. 2000) and intraperitoneal (Evensen & Lorenzen 1996, Madsen & Dalsgaard 1999, Rangdale et al. 1999, Garcia et al. 2000) injection. Cohabitant and bath infections have also been performed (Holt 1987, Lorenzen 1994, Madsen & Dalsgaard 1999, Garcia et al. 2000, Madetoja et al. 2000) with varying results. The nanoinjection technique used in this study might prove to be a new useful method in experimental infection with *F. psychrophilum* and possibly also for other pathogens that can be vertically transmitted.

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