

**EARLY LIFE-STAGE OUTBREAKS OF SYSTEMIC BACTERIAL COLD-WATER
DISEASE - IS THE CAUSAL AGENT *FLEXIBACTER PSYCHROPHILUS*
VERTICALLY TRANSMITTED IN SALMONIDS?**

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Abstract

Anecdotal data and early lab tests indicated that *Flexibacter psychrophilus* was responsible for high losses in early life stages of steelhead trout in a California hatchery and further, that the pathogen may be vertically transmitted. To determine the source of infections, eggs and embryos from 17 steelhead trout were taken at selected developmental stages. Some of the broodstock steelhead trout used for this study had been injected with erythromycin prior to spawning. Others had been injected with oxytetracycline, others had not been injected. The eggs/embryos were incubated in TYE broth and determined to be surface-disinfected when no bacterial growth was isolated from the broth after 72 h incubation at 17 °C. Surface-disinfected eggs/embryos were then homogenized and cultured in TYE and growth was identified as *F. psychrophilus* by biochemical and immunological assays. A source of surface *F. psychrophilus* contamination was the hatchery water, infecting the surface of 29% of eggs/embryos. *F. psychrophilus* was also detected within the ovarian fluid samples of 10% of the broodstock trout. *F. psychrophilus* was isolated from the contents of 13% of newly spawned eggs, as well as from 7% of eyed eggs and from 4% of newly hatched alevins. There was no difference observed in the proportion of eggs infected with *F. psychrophilus* from antibiotic-injected versus non-injected fish. However, the mortalities were monitored in the remaining progeny of the experimental fish and the progeny if the broodstock injected with erythromycin experienced significantly lower mortalities due to coldwater disease. *In vitro* experiments indicate that *F. psychrophilus* is somewhat resistant to lysozyme concentrations of up to 2 mg ml⁻¹ (greater than concentrations found within a salmonid egg). The results of this study indicate that *F. psychrophilus* may be transmitted both horizontally and vertically within salmonid hatcheries.

Introduction

High mortalities due to systemic bacterial cold-water disease (BCWD) were documented in steelhead trout (*Oncorhynchus mykiss*) at a hatchery in Northern California. Losses up to 85% during the first two months of rearing were observed in some lots of fish. All steelhead lots developed systemic BCWD within 1 to 4 weeks of button-up. The eggs and young fish had been

incubated in sand filtered spring water, which is fish-free. Yellow colonies typical of *Flexibacter psychrophilus*, causal agent of BCWD, were isolated on medium inoculated with surface disinfected steelhead egg homogenates. In the light of these anecdotal data and early tests, the study documented here was initiated, the purpose of which was to determine if *F. psychrophilus* could be transmitted within steelhead eggs, and if this was the route of the observed infections due to *F. psychrophilus*. Another objective of this study was to determine if injecting antibiotics into broodstock female steelhead would reduce the prevalence of intra-ovum infections (if any) due to *F. psychrophilus*.

Materials and Methods

Two stocks of steelhead trout were examined. Five female fish from the Scott Creek (SC) stock were injected with erythromycin (20 mg kg⁻¹ fish weight) upon receipt and at 30 d intervals thereafter. Seven females from the San Lorenzo (SL) stock were injected with tetracycline (20 mg kg⁻¹ fish weight), following the same regime. Five more SL females were left uninjected. At spawning samples of eggs were taken from each fish and transported to the laboratory in their own coelomic fluid. Additional eggs were sampled from each fish after the eggs had been fertilized, surface disinfected and water-hardened with 100 ppm povidone/iodine for 1h, and then rinsed in the hatchery water. Additional samples were taken from each fish at the eyed and hatch stage.

To examine the eggs/embryos for intra-ovum infections due to *F. psychrophilus* we followed a modified protocol of Evelyn *et al.* (1984). In the laboratory, 5 unfertilized, non water-hardened eggs were blotted on sterile filter paper and then placed in individual tubes containing 3 ml of tryptone-yeast extract broth that had been supplemented with 0.5% (v/v) newborn calf serum (TYE). In the case of the surface disinfected eggs, eyed eggs, and newly hatched sac fry, 5 eggs/embryos were treated as above, 5 additional eggs/embryos from each sample were surface disinfected again with 400 ppm povidone/iodine for 15 minutes, after which they were rinsed 5 times with sterile distilled water. The disinfected eggs/embryos were placed in TYE broth as above. All TYE tubes were incubated at 17 °C for 72h and then examined for turbidity. The TYE broth from each tube was streaked onto TYE plates and those plates were then incubated at 17 °C for 72h.

In order to determine if *F. psychrophilus* was transmitted within the steelhead eggs, the following was done. If the TYE broth in a given tube was clear and free of turbidity, the egg/embryo was crushed and homogenized with a sterile glass rod, and the homogenate was then incubated at 17 °C for an additional 72h, after which the homogenate was streaked onto TYE plates and the plate incubated at 17 °C for an additional 72h.

To examine the possibility that *F. psychrophilus* could survive inside salmonid eggs, the contents of 50 steelhead eggs from the uninjected SL group were taken aseptically with 1 ml syringes and disposable 18 ga. needles. The contents of 5 eggs were pooled into sterile Eppendorf tubes, so that there were 10 pooled samples in total. The egg contents were then spiked with *ca.* 10 *F. psychrophilus* cells. The tubes were then incubated at 17 °C for 7d. The egg contents were then streaked onto TYE plates and the plates were then incubated at 17 °C for 72h. Any growth was confirmed as being due to *F. psychrophilus* according to the criteria outlined above.

All growth on all plates was examined to determine if it was due to *F. psychrophilus*. Growth was deemed to be positive if it met the following criteria: Gram negative short rods, producing yellow pigment on TYE medium, that the yellow pigment turned red when streaked onto filter paper soaked in 1N NaOH (indicative of flexirubin, the pigment produced by *Flexibacter spp.*),

growth at 17 °C, but not at 30 °C, no growth when the TYE broth (before homogenization) was streaked onto TYE plates, and a positive slide agglutination result. The slide agglutination was done with a saline suspension of the growth to be examined, and antisera raised against *F. psychrophilus* in rabbits. The antisera was kindly supplied by Dr. R. Hedrick (Department of Veterinary Medicine, University of California at Davis).

To determine whether *F. psychrophilus* is resistant or susceptible to lysozyme, which is found in salmonid eggs and is probably responsible for passive defense of the developing embryos against bacterial pathogens, we followed a modification of the procedure described by Yousif *et al.* (1994). A suspension of *F. psychrophilus* cells was made in phosphate-buffered saline (PBS) and adjusted to an absorbance of *ca.* 10.0 at 540 nm. We also suspended *Aeromonas salmonicida* (an A+ strain) cells in the same way, as a positive control for lysozyme activity. The bacteria (both *F. psychrophilus* and *A. salmonicida*) were then diluted 1/10 in solutions of hen egg white lysozyme in PBS (pH 6.2), at concentrations of 0, 0.1, 1.0, and 2.0 mg ml⁻¹. Samples of bacteria in each lysozyme concentration were taken at 0, 30, 60, and 90 minutes. The cells were washed once in PBS and then serially diluted 100-fold to 10⁻⁶. 25 µl of each diluted sample were dropped, in triplicate, onto TYE plates. The plates were then incubated at 17 °C for 48h, after which colonies were counted. Results are expressed as the percentage reduction in cell number, using the colony counts from the control tubes (0 mg ml⁻¹ lysozyme) as the standard (0%) reduction.

At the outset of this study it became apparent that *F. psychrophilus* was contaminating the surface of some of the eggs/embryos (see **Results and Discussion**), despite iodine disinfection procedures at the hatchery. In order to determine the susceptibility of *F. psychrophilus* to povidone the above experiment was repeated, except that the bacterial suspensions (both *F. psychrophilus* and *A. salmonicida*) were exposed to povidone/iodine concentrations of 0, 10, 100, and 500 ppm in sterile, distilled water. The procedure was as above, except that samples were taken at 0, 30 and 60 minutes only.

The remaining progeny of the 17 experimental fish were reared in the hatchery according to standard hatchery practice and mortalities were monitored. Any mortalities were determined to be due to BCWD by culture and immunoassays, in addition to noting characteristic pathological signs and direct observation of typical *Flexibacter spp.* cells from spleen squashes examined at 600 - 1000x by phase microscopy.

Results and Discussion

A source of infection due to *F. psychrophilus* was the hatchery water. Thirty percent of all of the newly spawned and fertilized eggs, eyed eggs, and yolk sac fry were surface contaminated with *F. psychrophilus* (Table 1). This was despite the fact that the newly spawned eggs had been surface disinfected with povidone/iodine at the hatchery. This may be a problem unique to this particular hatchery, i.e., the bacteria in the water infect the surface of eggs/embryos after the initial disinfection. We also isolated *F. psychrophilus* from the surface of 10% of newly spawned eggs that had only been in contact with ovarian fluid from the spawning female, indicating that the females themselves were the source of infection in these cases. Therefore, complete surface disinfection is essential, especially in hatcheries with pathogen-free water. The iodine/povidone experiment indicated that *F. psychrophilus* is not resistant to iodine at 100 ppm (Table 2).

Table 1. Percentage (%) of eggs or embryos positive for *Flexibacter psychrophilus*. SC = Scott creek, SL = San Lorenzo, Erythro. = erythromycin, Oxytet. = oxytetracycline, Not - not injected

Fish #	Stock	Injected	Surface	Ovarian	Egg	Eyed	Alevin
1	SC	Erythro.	47	15	15	0	0
2	SC	Erythro.	27	0	n.d.	20	0
3	SC	Erythro.	20	10	50	20	40
4	SC	Erythro.	9	0	10	0	0
5	SC	Erythro.	14	20	0	20	0
Average			23	9	15	12	8
6	SL	Oxytet.	53	0	10	0	0
7	SL	Oxytet.	47	0	10	0	0
8	SL	Oxytet.	47	0	10	15	10
9	SL	Oxytet.	33	30	10	0	0
10	SL	Oxytet.	10	0	0	10	n.d.
11	SL	Oxytet.	40	20	30	10	n.d.
12	SL	Oxytet.	0	30	10	20	0
Average			33	12	12	8	2
13	SL	Not	20	10	0	0	0
14	SL	Not	0	10	15	0	0
15	SL	Not	53	10	30	n.d.	10
16	SL	Not	33	10	0	0	0
17	SL	Not	33	10	10	0	0
Average			28	10	11	0	2
Overall average			29	10	13	7	4

Table 2. Susceptibility of *Flexibacter psychrophilus* and *Aeromonas salmonicida* to povidone/iodine

Bacterial species	Iodine concentration (ppm)	% Reduction of cfu after exposure of t=		
		0 min	30 min	60 min
<i>Aeromonas salmonicida</i>				
	0	0	0	0
	10	0	100	100
	100	0	100	100
	500	0	100	100
<i>Flexibacter psychrophilus</i>				
	0	0	0	0
	10	0	50	100
	100	0	98	100
	500	0	100	100

F. psychrophilus was isolated from the contents of 13% of newly spawned eggs, as well as from 7% of eyed eggs and from 4% of newly hatched alevins (Table 1). Previously it was thought that *Renibacterium salmoninarum*, causal agent of bacterial kidney disease, may be the only bacterial pathogen of salmonids that could survive within salmonid eggs (Evelyn *et al.* 1984, Barker *et al.* 1991, Yousif *et al.* 1994). Most other bacterial salmonid pathogens are Gram negative, including *F. psychrophilus*. Susceptibility to lysozyme is a characteristic of many fish pathogens (Grinde 1989), and Yousif *et al.* (1994) have shown that a number of Gram negative fish pathogens are susceptible to lysozyme purified from coho salmon (*O. kisutch*) eggs. However, those authors did not test *F. psychrophilus* for lysozyme susceptibility. Our *in vitro* experiments for lysozyme susceptibility indicate that *F. psychrophilus* is somewhat resistant to lysozyme. Exposure of *F. psychrophilus* to 2 mg ml⁻¹ for 90 minutes resulted in only a 44% reduction in the number of viable cells, as compared to a 99% reduction in *Aeromonas salmonicida* viability when *A. salmonicida* was exposed under the same conditions (Table 3). These data were supported by the fact that *F. psychrophilus* was isolated from 100% of the samples of egg contents that had been spiked with *F. psychrophilus* cells. It would seem that *F. psychrophilus* is somewhat resistant to the defense systems that are present within salmonid eggs.

Table 3. Susceptibility of *Flexibacter psychrophilus* and *Aeromonas salmonicida* to hen egg white lysozyme

Bacterial species	Lysozyme concentration (mg ml ⁻¹)	% Reduction of cfu after exposure of t=			
		0 min	30 min	60 min	90 min
<i>Aeromonas salmonicida</i>					
	0.0	0	0	0	0
	0.1	1	56	74	53
	1.0	2	56	78	67
	2.0	1	99	98	99
<i>Flexibacter psychrophilus</i>					
	0.0	0	0	0	0
	0.1	0	20	0	31
	1.0	0	10	60	46
	2.0	0	30	55	44

There was no significant difference in the prevalence of intra-ovum infection due to *F. psychrophilus* within eggs and embryos from antibiotic injected, versus non-injected broodstock (Table 1). This was the case with both the Scott Creek (injected with erythromycin) and San Lorenzo (approximately half injected with tetracycline) stocks. Nor was there any significant difference in the prevalence of surface contamination due to *F. psychrophilus* within the ovarian fluid of antibiotic injected versus non-injected female broodstock (Table 1). However, there were significantly fewer mortalities due to BCWD within the Scott Creek stock than the San Lorenzo stock (Table 4). The Scott Creek broodstock had been injected with erythromycin before spawning.

Table 4. Mortalities due to BCWD within progeny of antibiotic-injected versus non-injected salmonids, SH = Steelhead, C = Coho, SC = Scott Creek, SLR = San Lorenzo River, E = Erythromycin, O = Oxytetracycline, N = Not injected

- Average Daily Mortality (%) -							
Stock	n	Inj.	Week 1	Week 2	Week 3	Week 4	Proj. Ann. Mort. (%)
SH-SC	7629	E	0.01	0.10	0.07	0.13	28.3
SH-SC	8729	E	0.16	0.06	0.05	0.08	31.9
SH-SLR	8648	O	0.13	0.54	1.05	1.56	299.3
SH-SLR	8818	O	0.12	0.44	1.00	1.75	302.0
SH-SLR	9630	N	0.11	0.26	0.87	2.21	314.8
SH-SLR	6348	N	0.13	0.28	0.28	0.45	104.0
C-SC	8184	E	0.07	0.07	0.04	0.03	19.2
C-SC	8188	E	0.05	0.06	0.10	0.03	21.9
C-SC	7795	E	0.03	0.07	0.05	0.04	17.3

It should be pointed out that the *F. psychrophilus* isolated from the contents of the newly spawned eggs, and eyed eggs (Table 1) may have been located within the perivitelline space of the eggs, rather than within the egg proper, i.e., the yolk itself. It is not possible to determine this within the limits of this experimental design. However, in light of the data obtained from the *in vitro* lysozyme experiment (Table 3), and from the spiked egg content experiment, it seems that *F. psychrophilus* can survive within salmonid egg contents.

Surface contamination due to *F. psychrophilus* within the water is a serious concern for the hatchery in question, and this may well be the case for other hatcheries. This hatchery has a fish-free water source. However, amphibians, insects, snails, and possibly other animals may be reservoirs of infection, releasing *F. psychrophilus* into the water. Additionally, *F. psychrophilus* from the water source may be different from the strains found in ovarian fluid, within eggs, or infecting small fish. Tests to clarify these uncertainties are in progress. It also seems likely that the pathogen is transmitted through female ovarian fluid and therefore surface disinfection is critical. It seems likely that there are multiple routes of infection due to *F. psychrophilus* in juvenile salmonids and vertical transmission may be included in these routes. Further work needs to be done to examine the efficacy of broodstock injection with antibiotics.

References

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