BACTERIAL COLDWATER DISEASE IN WESTSLOPE CUTTHROAT TROUT:

HATCHERY EPIDEMIOLOGY AND CONTROL

by

Eileen K. N. Ryce and Alexander V. Zale

Montana Cooperative Fishery Research Unit, USGS
Department of Ecology, Montana State University
Bozeman, Montana 59717

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Abstract

Bacterial coldwater disease, caused by the gram-negative bacterium *Flavobacterium psychrophilum*, is responsible for significant losses of hatchery-reared salmonids worldwide. We used Washoe Park State Fish Hatchery in Anaconda, Montana, as a case study to enhance understanding of the disease in a hatchery setting and to develop practical hatchery-management strategies to better control the pathogen and the disease. Washoe Park typically loses 30 to 45% of its westslope cutthroat trout *Oncorhynchus clarki lewisi* production to the disease annually. Our objectives were to determine where the pathogen was located in the hatchery system, how it was transmitted, and what factors caused disease outbreaks. We found the bacterium in the warm-spring water source, in the degassing water tower, and in production and broodstock fish. It was transmitted both horizontally and vertically, with both male and female parents passing the pathogen on to their offspring. Transmission from females was vertical only, but both horizontal and vertical transmission from males occurred. Iodine surface-disinfection post-fertilization eliminated the pathogen from egg surfaces, thereby limiting horizontal transmission. Chronic and mild acute stress did not result in disease outbreaks, but a combination of acute stress events associated with moving juvenile production fish from indoor to outdoor raceways did. These fish harbored the pathogen, primarily in cranial tissues, prior to the outbreak. Measures resulting from our findings implemented at Washoe Park to reduce horizontal transmission included cleaning and sterilization of hatchery structures and iodine surface-disinfection of eggs post-fertilization. Eradication of the pathogen from the hatchery is unlikely, but efforts to reduce the frequency and intensity of stress events should reduce the frequency of disease outbreaks; management to reduce the number of fish carrying the pathogen may minimize losses during outbreaks.

Introduction

Bacterial coldwater disease, caused by the gram-negative bacterium *Flavobacterium psychrophilum*, is a septicemic infection that originated in the northwestern U.S. and is responsible for significant losses of hatchery-reared salmonids worldwide. It is especially problematic at hatcheries rearing fish for native species restoration, because the wild, undomesticated strains used for restoration are prone to stress in the hatchery environment and stress induces the disease. Currently, bacterial coldwater disease is the only fish disease found in State fish hatcheries in Montana. The Washoe Park State Fish Hatchery in Anaconda, Montana, is the only facility in the state that produces westslope cutthroat trout *Oncorhynchus clarki lewisi* suitable for restoration programs. However, the severity of bacterial coldwater disease at this hatchery limits fish production and therefore implementation of the westslope cutthroat trout restoration program. Losses typically occur in three episodes among young fish from shortly after swim-up until they reach a length of about 75 mm. The first outbreak occurs in indoor raceways at first feeding, the second about 3 weeks later also indoors, and the third about 3 weeks after the juvenile fish are moved to outdoor raceways. In aggregate, the three outbreaks can achieve losses of about 30 to 45% annually. Successful control of bacterial coldwater disease at Washoe Park State Fish Hatchery would therefore facilitate restoration of native westslope cutthroat trout in Montana.

Typical clinical signs of bacterial coldwater disease include lethargy, dorsal skin erosion, ascites (accumulation of fluid in the peritoneal cavity), bilateral exopthalmia, pale gills, and hemorrhagic vent with trailing mucoid casts. It often causes death if untreated. Concurrent problems, such as infestation with parasites, are common (Branson 1998). Internally, the most obvious finding is splenomegaly, where the spleen can be 2 to 5 times its normal size and is usually friable in consistency. The surrounding peritoneum and fat are usually red and the kidneys may be pale and slightly swollen. Intestines often contain yellow or white mucoid discharge and the terminal gut may be congested. Damage to the spleen can be seen histologically and the bacteria are present within the damaged tissue (Branson 1998). Signs associated with neurological disruptions can also occur, including whirling behavior around the longitudinal axis, post-cephalic protrusion of the cranium, spinal deformities, and loss of melanocyte control in the posterior body, which is also known as black-tail (Kent et al. 1989; Meyers 1989). Microscopic features of the neurologic lesions include fibrous inflammation of the vertebral canal at the junction of the spinal cord and the medulla oblongata. The inflammation causes an upward compression of the anterior position of the spinal cord. Erosion and necrosis of vertebral bone and cartilage are also apparent (Meyers 1989). Many of the clinical signs associated with bacterial coldwater disease are also symptomatic of other fish diseases; therefore, a positive identification of the bacterium is necessary for confirmation of the disease.

Incorporation of the antibiotic oxytetracycline into fish food was traditionally the most common form of treatment for bacterial coldwater disease. The drug is usually effective in hatcheries experiencing the condition for the first time, but resistance towards this drug is developing (Branson 1995). Nevertheless, oxytetracycline remains the treatment of choice in North America. Amoxycillin is now commonly used in Europe, but resistance to it is also increasing (Branson 1998). Increasing incidence of resistant bacteria and recurrent outbreaks of disease shortly after a treatment continue to be problems, and no alternative treatments are currently available (Dalsgaard and Madsen 2000). A vaccine has been developed using the immunogenic outer membrane fraction of the *F. psychrophilum* bacterium (Rahman et al. 2002), but it is not yet commercially available and its viability and suitability for commercial production require further investigation. At present, the most effective form of disease control is to prevent outbreaks from occurring by reducing stress on the fish.

Objectives

Our goal was to better understand the ecology of *F. psychrophilum* in hatcheries and thereby facilitate development and testing of control measures, eventually leading to increased and enhanced restoration efforts. Although Washoe Park State Fish Hatchery was the model for our research, our findings are applicable to hatcheries throughout Montana and the Pacific Northwest. Specifically, our objectives were:

1. To determine where the pathogen occurred in the hatchery.

The pathogen was known to occur in juvenile westslope cutthroat trout in the hatchery, but whether it was found in the hatchery separate from fish, in the water supply, or in the broodstock was unknown. Such information would allow for targeted management actions to reduce abundance and occurrence of the pathogen.

2. To determine avenues of transmission within the hatchery.

Whereas horizontal transmission (from adults to external egg surfaces) was assumed to occur at Washoe Park, vertical transmission (from adults to the insides of eggs, including via sperm) had not been documented in westslope cutthroat trout. An understanding of all avenues of transmission is necessary to break the cycle of transmission.

3. To determine what factors cause disease outbreaks.

An understanding of the circumstances under which disease outbreaks occur can lead to strategies designed to minimize the frequency of such circumstances. Because bacterial coldwater disease is known to be induced by stress, we

investigated levels and types of stress associated with hatchery operations that elicited disease outbreaks.

Methods

Identification of the bacterium

Samples of *F. psychrophilum* were positively identified using a 3-step process. In the first step, samples were cultured on Shieh's agar at 18 °C for at least 72 hours (Shieh 1980). Colonies identified as possible *F. psychrophilum* based on gross morphology were isolated. These colonies were stained with a Gram stain (Thoesen 1994) in the second step. Gram-negative colonies in which bacteria were 2 to 7 μm long and rod-shaped were considered possible *F. psychrophilum*. An agglutination test was performed on these using a *F. psychrophilum*-specific antibody developed by the Idaho Fish Health Center, USFWS, Orofino, Idaho (Thoesen 1994). Colonies testing positive were definitively considered to be *F. psychrophilum*.

Location of the pathogen

Three samples were collected from plastic Koch rings in the hatchery water tower on 31 January 2003. Water is distributed from the tower to indoor and outdoor raceways throughout the hatchery. The Koch rings provide surface area to facilitate degassing and oxygenation of the spring water as it enters the hatchery. Three samples were collected from the inside surface of the overflow pipe from the warm-spring (13 °C) water supply to the hatchery on 16 April 2003.

Kidney, spleen, and cranial tissues of four hatchery-resident female broodstock (3 and 4 years old; 227 to 405 mm FL) were sampled on 31 January 2003. Testes of 10 male hatchery broodstock (age 3 years) were sampled on 24 June 2003. These broodstock were examined for both internal and external clinical signs of bacterial coldwater disease at the time of sampling.

The hatchery reinvigorates the genetic makeup of its broodstock by fertilizing some eggs produced at the hatchery with milt from wild-caught males. The resulting offspring are used as broodstock. Kidney, spleen, and cranial tissues of wild males used for this purpose from Rogers Lake (2 June 2003), Deep Creek (29 May 2003), and Quintonkin (29 May 2003) were sampled. Ten males from each location were tested.

Avenues of transmission

We followed the protocol outlined by Brown et al. (1997) to determine if the pathogen was transmitted from adult broodstock to their offspring vertically (i.e., to the inside of the egg). We first determined if the bacterium was transmitted

horizontally and present on the external surface of each egg; if not, we tested the internal contents of the egg. Thirty eggs were collected from each of 10 broodstock females on 29 May 2003; ovarian fluid of each was tested for the presence of *F. psychrophilum*. The 30 eggs from each female were separated into three groups of 10; 10 eggs were left unfertilized, 10 were fertilized with milt from hatchery broodstock males and activated with hatchery water, and 10 were fertilized, activated with hatchery water, and disinfected with iodine (100 ppm Iodophore for 30 minutes post-fertilization). Each egg was individually washed in sterilized de-ionized water and incubated individually in TYE broth at 18 °C for 72 hours. Shieh's agar was inoculated with the broth as described above. Eggs that tested negative on their surface by this procedure were crushed and cultured to test for internal presence of *F. psychrophilum*.

To determine if eggs which tested surface-positive following fertilization were becoming infected from milt or hatchery water, eggs from five females were mixed together and split into 10 groups of 10 eggs, each group of which was fertilized with milt from a separate male on 24 June 2003. The 10 eggs fertilized by each male were split into two groups of 5, activated with either hatchery water or sterilized water, and tested for the presence of *F. psychrophilum* on their surface. If hatchery water was a contributing factor, we expected that fertilized eggs activated in it would exhibit a higher frequency of infection than those activated in sterilized water. The milt of the 10 males was tested for the presence of *F. psychrophilum*.

To determine if the bacterium found in male milt was originating in the testes or was a product of contamination from other bodily fluids, testes from 10 broodstock males were removed on 24 June 2003. The testes were externally sterilized with ethanol, macerated, and tested for the presence of *F. psychrophilum*.

The effectiveness of varying durations of iodine surface disinfection was tested by exposing replicate lots (3) of fertilized eggs to 100 ppm Iodophore treatments of 0, 30, 60, or 90 minutes. Three subsamples of 5 eggs were collected from each lot and tested for external presence of *F. psychrophilum*. We also assessed the effects of these treatments on egg survival by recording survival to eye-up.

Factors eliciting disease outbreaks

To determine types and severity of stress required to elicit bacterial coldwater disease outbreaks at hatcheries, we experimentally exposed fish to chronic and acute stresses in a controlled, factorial experiment. Chronic stress was induced by maintaining age-0 juvenile westslope cutthroat trout at the hatchery in uncovered indoor raceways. We expected fish in these raceways to be periodically stressed by the presence of hatchery personnel engaged in routine activities. Equivalent fish kept in covered raceways were assumed to be free of

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such chronic stress. Acute stress was induced by exposing 50 fish from each group (chronic stress and chronic-stress free) to a simulated stocking event on 8 September 2003, in which fish were loaded into a hatchery truck, transported by road for 2 hours, and released back into indoor hatchery raceways; 50 fish from each group were not subjected to the simulated stocking event. Accordingly, groups of 50 fish were exposed to either neither stress, chronic stress only, acute stress only, or both stress types. Mean length of fish at the time of the acute stress event was 70 mm FL. The fish were monitored daily for signs of disease and mortality for one month following the simulated acute-stress event.

Three weeks after moving 114,000 age-0 production fish from indoor to outdoor raceways as part of normal hatchery operations during autumn 2003, a severe bacterial coldwater disease outbreak occurred that resulted in a 10% loss of annual production. The outbreak was attributed by hatchery personnel to stress during the transfer caused perhaps by reduced water flow rates, a possible brief reduction in water quality, change in diet, or the change in ambient physical conditions experienced by the fish. We tested tissues (cranium, kidney, and spleen) of 25 surviving fish for presence of *F. psychrophilum*. Concurrently, we examined the same tissues in our four experimental-stress groups (25 fish from each). Fish in these groups were from the same stock as those that experienced the outbreak, but had never been moved to outside raceways. We were interested in determining if these "healthy" juvenile fish were already carrying the pathogen, and could thereby be expected to experience an outbreak if exposed to sufficient stress.

Results

Location of the pathogen

The causative agent of bacterial coldwater disease, *F. psychrophilum*, was isolated in the hatchery water tower where the incoming spring water is degassed and oxygenated. All three sample cultures were positive. Degassing and oxygenation of the water in the tower is aided by plastic Koch rings, which inadvertently provide a large surface area for bacteria to grow on.
The bacterium was also found in the warm-spring head that is a water source for the hatchery. All three sample cultures were positive.

Only one of the four hatchery-resident female broodstock tested positive for *F. psychrophilum*. The pathogen was found in the kidney, spleen, and cranium of this fish. In addition, it exhibited internal clinical signs of bacterial coldwater disease, including swollen organs covered in white film and coelomic fluid.
Testes of 7 of 10 male hatchery-resident broodstock tested positive. None of the hatchery broodstock exhibited external clinical signs of the disease.

Wild-caught broodstock males tested positive from Rogers Lake (6 of 10), Deep Creek (3 of 10), and Quintonkin (2 of 10). Only cranial tissues were positive in the three Deep Creek and two Quintonkin fish, but the pathogen was found in kidney (4 of 6), spleen (3 of 6), and cranial (5 of 6) tissues of the six Rogers Lake fish.

Avenues of transmission

The ovarian fluid of none of the 10 females tested positive for the presence of *F. psychrophilum*, and only 2 of the 100 unfertilized eggs were surface-positive for the bacterium (Table 1); these two positives likely resulted from contamination or leaching from inside the eggs. Female broodstock are therefore likely not responsible for significant horizontal transmission of the pathogen during fertilization. However, the bacterium was present on the surfaces of 60 of the fertilized eggs (Table 1), suggesting that the bacterium largely originated either from the milt or the hatchery water used to activate the sperm or both. Only 17 fertilized eggs treated with iodine were surface-positive (Table 1).

Of the 98 unfertilized surface-negative eggs, 14 (14.3%) were positive internally, indicating vertical transmission from female broodstock (Table 2). The internal-positive rate among fertilized surface-negative eggs was about twice as high (27.5%), inferring vertical transmission from male broodstock as well. Iodine disinfection likely had no effect on vertical transmission, as the internal-positive rate of treated fertilized eggs was only slightly lower (22.9%; Table 2).

Aggregate surface-positive rates of fertilized eggs activated with either hatchery water or sterilized water were both 74% (Table 3). Milt of 8 of 10 males was positive for *F. psychrophilum* (Table 3). Bacteria present in the hatchery water supply therefore likely confer little if any additional infection during fertilization; bacteria in the milt are responsible for the presence of bacteria on the surfaces of fertilized eggs. Externally-sterilized testes of 7 of 10 male broodstock tested positive for *F. psychrophilum*, demonstrating that the bacterium found in milt was not a product of contamination from other bodily fluids. Male broodstock are therefore responsible for the observed horizontal transmission of the pathogen to external egg surfaces during fertilization.

Iodine surface-disinfection was equally effective at all treatment durations. None of the subsamples from any of the three treatment durations was surface-positive for *F. psychrophilum*, but 8 of 9 control subsamples were. Survival to eye-up was about 80% and was not significantly different among treatments ($P = 0.1340$; analysis of variance).

Factors eliciting disease outbreaks

No external clinical signs of disease, no disease outbreaks, no mortality, and no differences in growth existed among the four groups of 50 juvenile fish exposed

to the four stress combinations during the month after the induced acute stress event. Apparently, these stresses were insufficient to elicit a disease outbreak, as many of these fish (62%) harbored the pathogen, especially in cranial tissues (Table 4). Multiple tissues were positive in only 16% of these fish. Surviving fish from the unscheduled stress event associated with transfer to outdoor raceways were also infected, but the pathogen was present in multiple tissues in 56% of these (Table 4).

Discussion

Flavobacterium psychrophilum, the pathogen that causes bacterial coldwater disease, was ubiquitous throughout Washoe Park State Fish Hatchery. It was found in the warm-spring water supply, in the degassing water tower, in the female broodstock, in both the hatchery-resident and wild-caught male broodstock, and in the juvenile fish produced. Juvenile fish exhibiting no clinical signs of disease harbored the pathogen, primarily in the cranium, and became diseased following a severe, acute stress event.

Female broodstock likely did not contribute significantly to horizontal transmission during fertilization, but male broodstock clearly did. Fortunately, post-fertilization iodine treatment to surface-disinfect eggs was effective and did not affect egg viability. However, iodine treatment had no effect on bacteria transmitted vertically to the insides of eggs by both male and female broodstock. At present, no management strategies exist to reduce vertical transmission, with the possible exception of minimizing horizontal transmission such that fewer broodstock will be carrying the pathogen (and hence be less able to transmit the pathogen vertically). Hatchery personnel are currently (2004) attempting to reduce prevalence of the pathogen in male broodstock through oxytetracycline and fluorofenicol injections and oxytetracycline-coated feed. These actions are aimed at reducing vertical transmission. Sperm washing techniques, as commonly used in animal husbandry of domestic mammals to eliminate pathogens, may warrant future investigation also.

Eradication of the pathogen from the hatchery is unlikely, but reducing its abundance on structures may limit its transfer to fish. Reduced incidence among the hatchery fish stock would be expected to limit mortalities during stress events that elicit the disease. Of course, limiting the number and severity of stress events would be helpful as well. As our failed stress experiment suggested, stress events must be acute and severe to elicit disease, and limiting stress severity and frequency should therefore enhance production.

Bacterial coldwater disease was less of a problem at Washoe Park State Fish Hatchery in 2003 than ever before, presumably because of implementation of management strategies to reduce the numbers of bacteria entering and residing in the hatchery and to reduce the probability of horizontal transmission. These

included cleaning and sterilization of incoming pipes from the spring sources, removal of the Koch rings from the degassing tower, and iodine surface-disinfection of all eggs after fertilization. Typically, three disease outbreaks, cumulatively resulting in about 30 to 45% mortality, are experienced while age-0 fish are in the hatchery building or shortly after transfer to outdoor raceways. No such outbreaks of disease occurred within the hatchery building in 2003. Absence thereof was attributed by hatchery personnel to management actions taken based on our findings.

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Table 1. Numbers of eggs surface-positive for *Flavobacterium psychrophilum*, by treatment.

Female	Treatment		
	Unfertilized	Fertilized	Fertilized and disinfected
1	0/10	10/10	9/10
2	0/10	10/10	4/10
3	0/10	1/10	0/10
4	0/10	0/10	0/10
5	1/10	10/10	0/10
6	0/10	10/10	1/10
7	0/10	8/10	2/10
8	0/10	3/10	0/10
9	1/10	3/10	1/10
10	0/10	5/10	0/10
Total	2/100	60/100	17/100

Table 2. Numbers of surface-negative eggs internally positive for *Flavobacterium psychrophilum*, by treatment.

Female	Treatment		
	Unfertilized	Fertilized	Fertilized and disinfected
1	2/10	--	1/1
2	0/10	--	0/6
3	0/10	0/9	3/10
4	0/10	5/10	2/10
5	1/9	--	1/10
6	1/10	--	4/9
7	3/10	0/2	2/8
8	3/10	2/7	1/10
9	2/9	3/7	3/9
10	2/10	1/5	2/10
Total	14/98	11/40	19/83

Table 3. Numbers of eggs surface-positive for *Flavobacterium psychrophilum*, by treatment.

Male	Milt	Treatment	
		Hatchery water	Sterilized water
1	+	2/5	3/5
2	+	2/5	2/5
3	+	5/5	4/5
4	-	5/5	3/5
5	+	5/5	3/5
6	+	3/5	4/5
7	+	3/5	5/5
8	+	4/5	4/5
9	-	3/5	4/5
10	+	5/5	5/5
Total	8/10	37/50	37/50

Table 4. Numbers of individuals infected with *Flavobacterium psychrophilum*, by tissue, in the stress experiment.

F. psychrophilum	No stress	Treatment			
		Acute stress only	Chronic stress only	Chronic and acute stress	Anaconda disease outbreak
Negative	7	10	8	13	7
Cranium only	10	7	7	6	3
Kidney only	1	2	3	3	1
Spleen only	2	2	3	0	0
Cranium and kidney	2	3	1	3	4
Cranium and spleen	1	0	1	0	4
Kidney and spleen	2	1	2	0	3
All three tissues	0	0	0	0	3
Total	25	25	25	25	25