Brood stock segregation of spring chinook salmon *Oncorhynchus tshawytscha* by use of the enzyme-linked immunosorbent assay (ELISA) and the fluorescent antibody technique (FAT) affects the prevalence and levels of *Renibacterium salmoninarum* infection in progeny

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ABSTRACT: A study of the effect of maternal *Renibacterium salmoninarum* infection levels on the prevalence and levels of bacterial kidney disease (BKD) in progeny fish was conducted at a production salmon hatchery. A total of 302 mating pairs of spring chinook salmon *Oncorhynchus tshawytscha* was screened in August 1988 for *R. salmoninarum* by an enzyme-linked immunosorbent assay (ELISA). On the basis of ELISA testing of kidney tissues from all fish and the testing of ovarian fluid samples from a subsample of the females by a direct membrane filtration fluorescent antibody technique (MF-FAT), selected egg lots were segregated into 2 groups of 30 egg lots or about 135 000 eggs each. One group contained egg lots from male and female parents that had low *R. salmoninarum* infection levels or tested negative for *R. salmoninarum* (low-BKD group), and the other group contained egg lots from female parents with relatively high *R. salmoninarum* infection levels and male parents with various infection levels (high-BKD group). The progeny groups were maintained in separate rearing units supplied with untreated river water, and were monitored for *R. salmoninarum* by the ELISA until they were released from the hatchery in April 1990. Total mortality of the juvenile fish was higher ($p = 0.0001$) in the high-BKD group (20 %) than in the low-BKD group (10 %). Mortality in the high-BKD group was highest after the fish were moved from nursery tanks to raceways, and clinical BKD became evident in this group. During the 11 mo of raceway rearing, mortality in the high-BKD group was 17 % compared with 5 % for the low-BKD group. An ELISA analysis of smolts just before release showed an *R. salmoninarum* infection rate of 85 % in the high-BKD group and 62 % in the low-BKD group. Of the positive fish, 98 % in the low-BKD group and 55 % in the high-BKD group had low infection levels, whereas 36 % in the high-BKD group and only 1 % in the low-BKD group had high infection levels. The results of this research suggest that segregation of brood stock by the ELISA and the MF-FAT can be used to reduce the prevalence and levels of BKD in hatchery-reared spring chinook salmon, even in locations with open water supplies.

INTRODUCTION

The barge or truck transportation of migrating juvenile salmonids downriver past hydroelectric dams in the Snake and Columbia rivers of the U.S. Pacific Northwest has shown substantial benefits for steelhead trout *Oncorhynchus mykiss* and the fall race of chinook salmon *O. tshawytscha*, as evidenced by greater adult

returns of transported fish compared to non-transported fish (Park 1985, Raymond 1988). However, adult returns of the spring race of chinook salmon have remained low, regardless of whether the smolts were transported or not (Raymond 1988). Because the downriver survival of spring chinook salmon smolts has improved since the implementation in the 1970's of juvenile fish transportation and other enhancement

measures designed to offset dam-related mortality, Raymond (1988) believed that problems other than smolt mortality at dams were affecting spring chinook salmon runs.

Concern is increasing that hatchery-produced spring chinook salmon smolts may be of poor physiological quality and consequently unable to survive the stresses of migration or entry into the ocean (Park 1985, Raymond 1988). Adult return rates of wild spring chinook salmon to the mid-Columbia and Snake rivers have improved since the beginning of enhancement measures at the dams (with return rates between 1.5 % and 3 % for the 1981 to 1984 out-migrants), and have consistently exceeded the return rates for hatchery-produced fish, which have remained below 0.8 % (Raymond 1988).

Park (1985) and Raymond (1988) suggested that bacterial kidney disease (BKD) caused by *Renibacterium salmoninarum* may significantly reduce survival of spring chinook salmon smolts. This is a chronic systemic disease that is transmitted vertically (Bullock et al. 1978, Evelyn et al. 1986b) and horizontally (Mitchum & Sherman 1981, Bell et al. 1984). The disease is considered to be more severe in spring chinook salmon than in many other salmonids (Bullock & Wolf 1986), and is widespread among spring chinook salmon in hatcheries in the Pacific Northwest (Raymond 1988). Bacterial kidney disease is difficult to control; there are no effective vaccines and antibiotic therapy is only partially effective (Elliott et al. 1989). Oral chemotherapy of salmonids with erythromycin reduces mortality, but it does not eliminate infections from all treated fish (Wolf & Dunbar 1959, Austin 1985). There is some evidence that juvenile anadromous salmonids infected with *R. salmoninarum* while in fresh water die of BKD upon entry into salt water (Banner et al. 1986). Researchers have suggested that the stress associated with the transition from fresh water to salt water may accelerate the mortality (Fryer & Sanders 1981, Banner et al. 1983).

In the past, the effect of BKD on anadromous salmonid populations was difficult to assess because of a lack of rapid, specific, and sensitive methods for the detection and quantification of *Renibacterium salmoninarum* in fish tissues and body fluids. The recent development of sensitive enzyme-linked immunosorbent assays (ELISA; Pascho & Mulcahy 1987, Turaga et al. 1987, Sakai et al. 1989), which detect soluble antigens of *R. salmoninarum*, have allowed the rapid analysis of large numbers of samples. A direct relationship exists between ELISA absorbance values and the *R. salmoninarum* soluble antigen concentration (Pascho & Mulcahy 1987, Turaga et al. 1987), which generally corresponds to the severity of infection (Pascho et al. 1987, Turaga et al. 1987). The test developed

by Pascho & Mulcahy (1987) has been used in combination with quantitative (Elliott & Barila 1987) and semi-quantitative (Pascho et al. 1987) versions of the direct fluorescent antibody technique (FAT; Bullock et al. 1980) to more accurately determine the prevalence and levels of BKD in salmonid populations (Pascho et al. 1987).

To understand better the effect of BKD on the downstream migration and subsequent adult returns of transported and non-transported spring chinook salmon, we began brood stock segregation studies at a fish hatchery in Idaho (USA). This brood stock segregation was based on experimental evidence that *Renibacterium salmoninarum* is transmitted vertically in association with the salmonid egg, and that the success of the transmission to the progeny is related to the infection level in the female parent (Evelyn et al. 1986b). For the first study, selected egg lots from the 1988 spring chinook salmon spawning were segregated into 2 groups. According to ELISA and FAT results, one group contained eggs from female parents that had very low *R. salmoninarum* infection levels or tested negative for *R. salmoninarum*, and the other contained eggs from female parents with relatively high *R. salmoninarum* infection levels. The progeny groups were reared separately and monitored for *R. salmoninarum* throughout hatchery rearing. Subgroups of the juvenile fish were marked with passive integrated transponder (PIT) tags for subsequent monitoring of downstream migration or coded-wire tags for monitoring adult returns.

The primary purpose of the brood stock segregation procedure was to create progeny groups of spring chinook salmon, with differing BKD profiles, that could be monitored during the out-migration and return. We realized, however, that this study could also provide information on the value of brood stock segregation as a BKD control measure. Elliott et al. (1989) suggested that the ELISA or a quantitative membrane filtration-FAT (MF-FAT) are practical methods for testing spawning fish as part of a brood stock segregation or culling program. The results of one preliminary study that used ELISA testing for brood stock segregation showed higher adult returns for progeny groups of spring chinook salmon from parents that had low *R. salmoninarum* infection levels, compared with those for progeny groups from more severely infected parents (S.L. Leek, U.S. Fish and Wildlife Service, Lower Columbia River Fish Health Center, Underwood, Washington, USA, pers. comm.).

We now report the results of testing the adult fish and subsequent monitoring of the progeny groups for *Renibacterium salmoninarum* infections in the hatchery. The findings of this study indicate that brood stock segregation by ELISA and MF-FAT screening can be a

useful tool for reducing BKD mortality during hatchery rearing of spring chinook salmon. The survival of the fish in the 2 BKD-level groups after release from the hatchery will be reported elsewhere.

MATERIALS AND METHODS

Study fish population and brood stock segregation.

Gametes were obtained from the spring chinook salmon *Oncorhynchus tshawytscha* spawned at the Dworshak National Fish Hatchery, U.S. Fish and Wildlife Service (USFWS), Ahsahka, Idaho, between August 22 and August 29, 1988. When they arrived at the collection facility, the adult fish were injected once with erythromycin phosphate (20 mg kg⁻¹ body weight). Injection occurred from about 3 mo to a few days before spawning.

Egg lots from 302 randomly selected females were held in separate incubators for possible inclusion in either the high *Renibacterium salmoninarum* infection level (high-BKD) group or the low *R. salmoninarum* infection level (low-BKD) group. The eggs from these females were fertilized with milt from 271 males (25 of these males were used to fertilize eggs from 2 females each and 3 were used to fertilize eggs from 3 females each). Unique identification numbers were assigned to each adult fish. After fertilization, each egg lot was surface-disinfected for 30 min by adding 75 ppm iodophor during water hardening, and then maintained in a separate incubator until *R. salmoninarum* testing of the adults was complete. The number of the female was used to identify each egg lot throughout the fertilization, water hardening, and incubation processes.

After the kidney tissues of each adult and the ovarian fluid from each female were tested by the ELISA, the ovarian fluid and kidneys of females that were candidates for one of the 2 BKD-level groups were further screened by the FAT. Based on the test results, 30 egg lots each were selected for inclusion in the low-BKD and high-BKD groups with about 135 000 eggs per group. Each group of egg lots was transferred to a separate 15-tray Heath incubator, where the lots were randomly assigned to trays in pairs. After hatching, the fish from the 15 trays were distributed among 6 nursery tanks and then 3 production raceways as shown in Tables 1 & 2. To prevent cross-contamination, separate equipment was used for each test group, and identical rearing conditions were maintained to the extent possible. The fry were initially fed BioDiet Starter rations (Bioproducts Inc., Warrenton, OR, USA) and then were fed Oregon Moist Pellets (Moore-Clark Co. Inc., LaConner, WA, USA) for the remainder of hatchery rearing. None of the fish in the test groups was given medicated feed, and all groups were reared on

untreated Clearwater River water. Dead fish in each nursery tank or raceway were removed daily and mortality was tabulated by hatchery personnel.

Tissue and body fluid samples from adult fish. To determine the levels of *Renibacterium salmoninarum* infection in each adult, selected tissues and body fluids were taken as the fish were spawned. Tissue lesions characteristic of those caused by *R. salmoninarum* were noted, and about 1 g each of the anterior and posterior kidney were taken aseptically from each fish and placed together in a Stomacher 80 lab bag (Tekmar, Cincinnati, OH, USA). A subsample of the ovarian fluid/egg mixture from each female was collected in a sterile paper cup as the eggs were expelled, and then the fluid portion was decanted into a plastic tube. All samples were stored frozen at -70°C prior to processing and testing by the ELISA or the FAT.

Each kidney was thawed and transferred to a clean, sterile lab bag and diluted 1:2 (w/v) in 0.01 M phosphate-buffered saline (PBS) at pH 7.4, containing 0.05% Tween 20 (PBS-T20). The suspension was homogenized for ca 1 min in a Stomacher 80 (Tekmar). A smear from each homogenate was made for the direct FAT by moistening a fine calcium alginate swab (Calgiswab Type 1; Spectrum Laboratories, Los Angeles, CA, USA) with PBS-T20, dipping it into the homogenized tissue pool, then smearing the material picked up by the swab onto both wells of a 2-well microscope slide (Cel-Line Associates Inc., Newfield, NJ, USA; 8 mm diameter wells).

For the ELISA, PBS-T20 was added to each kidney homogenate such that the total dilution was 1:4 (w/v). A portion of the diluted and homogenized kidney was then heated at 100°C for 15 min. The heated homogenates were then centrifuged at 8800 × *g* for 5 min at 22°C.

A subsample of each ovarian fluid was thawed and diluted 1:2 (v/v) in PBS-T20 and heated at 100°C for 15 min for the ELISA. The ovarian fluid was then centrifuged at 8800 × *g* for 5 min at 22°C.

The supernatants of the kidney and ovarian fluid samples were tested by the ELISA. Additional subsamples of ovarian fluid were thawed and tested directly by the MF-FAT.

Tissue samples from juvenile fish. The prevalence and levels of BKD in the test groups were monitored periodically until the fish were released from the hatchery on April 4, 1990. At each sampling time, the tissues from a minimum of 50 fish in each rearing unit were tested by the ELISA. The fish were sampled by randomly netting swimup fry in December 1988, preponding fry in April 1989, parr in July 1989, presmolts in November 1989, and smolts in March 1990 (Table 3).

The tissues sampled for *Renibacterium salmoninarum* analysis and the number of fish pooled for

Table 1. *Oncorhynchus tshawytscha*. Results of ELISA I and FAT testing for *Renibacterium salmoninarum* in female spring chinook salmon selected for the low-BKD group, and rearing unit assignments for the progeny

Female no.	Egg tray no.	Nursery tank no. ^a	Mean ELISA absorbance		FAT ^b Ovarian fluid ^e
			Kidney ^c	Ovarian fluid ^d	
750	A178	81	0.045	0.042	43
752	A178	81	0.045	0.046	0
756	A180	81	0.048	0.048	43
742	A180	81	0.049	0.042	85
806	A181	81 and 82 ^f	0.042	0.044	0
754	A181	81 and 82 ^f	0.044	0.046	0
760	A182	82	0.048	0.043	0
938	A182	82	0.046	0.045	0
820	A183	82	0.048	0.044	85
824	A183	82	0.048	0.043	0
792	A179	97	0.049	0.044	0
804	A179	97	0.043	0.043	0
932	A184	97	0.047	0.041	43
50	A184	97	0.049	0.043	0
772	A185	97 and 98 ^f	0.047	0.042	85
940	A185	97 and 98 ^f	0.044	0.045	0
790	A187	98	0.045	0.043	0
770	A187	98	0.046	0.043	0
852	A189	98	0.050	0.047	0
802	A189	98	0.050	0.046	85
830	A186	113	0.047	0.043	0
794	A186	113	0.047	0.044	43
1070	A188	113	0.050	0.043	0
1180	A188	113	0.050	0.045	0
1230	A190	113 and 114 ^f	0.049	0.045	85
1208	A190	113 and 114 ^f	0.050	0.046	43
1068	A191	114	0.047	0.044	43
778	A191	114	0.050	0.046	0
934	A192	114	0.047	0.043	43
822	A192	114	0.050	0.043	0

^a Fish in nursery tanks were combined in raceways as follows: tanks 81 and 82 (raceway B-16), tanks 97 und 98 (raceway B-17), tanks 113 and 114 (raceway B-18)

^b All kidney samples were negative for *R. salmoninarum* by the FAT

^c For kidney samples, the lowest ELISA absorbance considered positive for *R. salmoninarum* was 0.058

^d For ovarian fluid samples, the lowest ELISA absorbance considered positive for *R. salmoninarum* was 0.050

^e Calculated number of bacteria per ml of ovarian fluid

^f Fish from this egg tray were divided equally between the 2 nursery tanks, which were later combined in a single raceway

testing depended on the size of the fish; processing parameters were selected to provide sufficient sample volumes for duplicate samples in the ELISA. The swimup fry were processed as whole fish after the gastrointestinal tracts and tails had been removed. Kidneys and spleens were sampled from only the larger fish. The tissues of all fish except the smolts were processed as 3-fish or 5-fish pools (Table 3).

For the FAT, each sample was homogenized without dilution and smeared onto a 2-well microscope slide with a moistened swab as described previously.

For the ELISA, PBS-T20 was added to each sample homogenate such that the total dilution of kidney/spleen samples was 1:8 (w/v) and the total dilution of the whole fish samples was 1:4 (w/v). Sample storage and further processing were done as described previously.

For negative controls, equivalent samples of juvenile fish or tissue pools were prepared from brook trout *Salvelinus fontinalis* of the Owhi strain raised at the White Sulphur Springs National Fish Hatchery (USFWS), White Sulphur Springs, WV, USA, or at the National Fisheries Research Center-Seattle (USFWS), Seattle, WA, USA. Fish of this strain had been extensively tested and used previously as negative controls for the ELISA (Pascho et al. 1987). No *Renibacterium salmoninarum* was detected by the ELISA or the FAT in tissues from brook trout in the lots used as controls for the present study.

ELISA. Two double-antibody sandwich ELISAs were used in this study to measure the levels of *Renibacterium salmoninarum* antigen. The ELISA I was used to test the tissues and body fluids from the adult fish. When commercially prepared reagents became avail-

Table 2. *Oncorhynchus tshawytscha*. Results of ELISA I and FAT testing for *Renibacterium salmoninarum* in female spring chinook salmon selected for the high-BKD group, and rearing unit assignments for the progeny

Female no.	Egg tray no.	Nursery tank no. ^a	Mean ELISA absorbance		FAT	
			Kidney ^b	Ovarian fluid ^c	Kidney ^d	Ovarian fluid ^e
1190 ^f	A195	33	0.050	0.043	0	0
1172	A195	33	0.270	0.279	23	1 664 000
1086	A197	33	0.271	0.048	5	1 664
1216	A197	33	0.537	0.046	209	85
798	A200	33 and 34 ^g	0.398	0.047	19	2 176
998	A200	33 and 34 ^g	0.439	0.043	9	341
1058	A203	34	0.218	0.047	34	427
964	A203	34	0.186	0.042	0	7 552
138	A204	34	0.414	0.044	90	512
104 ^h	A204	34	0.749	0.709	130 800	1 024 000 000
952	A198	49	0.306	0.045	6	1 621
1052	A198	49	0.445	0.528	16	22 613 333
106	A199	49	0.463	0.072	55	247 467
1000	A199	49	0.644	0.707	TNTC ⁱ	9 002 660 000
1002	A201	49 and 50 ^g	0.471	0.047	17	104 064
746	A201	49 and 50 ^g	0.153	0.044	0	4 480
130 ^h	A205	50	0.649	0.655	TNTC	704 000 000
112	A205	50	0.559	0.465	11 480	8 917 333
102 ^h	A207	50	0.691	0.696	TNTC	13 184 000 000
832	A207	50	0.695	0.627	133 200	230 400 000
762	A194	65	0.251	0.046	0	3 115
122 ^h	A194	65	0.651	0.552	18 250	47 360 000
1196 ^h	A196	65	0.546	0.050	283	72 320
1108	A196	65	0.182	0.058	1	273 067
834	A202	65 and 66 ^g	0.149	0.047	0	2 304
1206	A202	65 and 66 ^g	0.328	0.045	1	78 336
32	A206	66	0.600	0.323	89 400	75 648
1106	A206	66	0.576	0.685	11 150	5 888 000 000
132	A208	66	0.556	0.047	691	5 077
42	A208	66	0.529	0.064	579	123 008

^a Fish in nursery tanks were combined in raceways as follows: tanks 33 and 34 (raceway B-19), tanks 49 and 50 (raceway B-20), tanks 65 and 66 (raceway B-21)

^b For kidney samples, the lowest ELISA absorbance considered positive for *R. salmoninarum* was 0.058

^c For ovarian fluid samples, the lowest ELISA absorbance considered positive for *R. salmoninarum* was 0.050

^d Number of bacteria counted or calculated per 100 microscope fields at 1000× magnification

^e Calculated number of bacteria per ml of ovarian fluid

^f Low-BKD fish inadvertently placed in the high-BKD group

^g Fish from this egg tray were divided equally between the 2 nursery tanks, which were later combined in a single raceway

^h Fish had grossly visible lesions characteristic of BKD

ⁱ Bacteria too numerous to count

able, the ELISA II was developed and used to test the tissues of the juvenile fish. In addition, the kidney and ovarian fluid samples from the 51 egg donor fish for which sufficient sample material remained (25 of the females selected for the low-BKD group and 26 of the females selected for the high-BKD group) were retested by the ELISA II, for comparison with the ELISA I results.

ELISA I: The ELISA I was performed by the final technique described by Pascho & Mulcahy (1987). The controls and the calculation of interassay correction factors were done as described by Pascho et al. (1987).

ELISA II: The ELISA II was performed by the final technique described by Pascho & Mulcahy (1987) with the following modifications: commercially prepared

reagents and buffers (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, USA) were substituted for those described in the original report. The coating solution, milk diluent/blocking solution, wash solution, ABTS peroxidase substrate system, and ABTS peroxidase stop solution were used according to the instructions of the manufacturer. Wells were coated with affinity-purified goat antibody to *Renibacterium salmoninarum* at 1 µg ml⁻¹ (w/v) in coating solution. Purified goat antibody to *R. salmoninarum* conjugated with horseradish peroxidase was used at a 1:2000 (v/v) dilution in milk diluent. *R. salmoninarum* positive control antigen at dilutions of 1:100, 1:1000, 1:2000, and 1:5000 (v/v) in PBS-T20 was included in every fifth ELISA plate. The color was allowed to develop for

Table 3. *Oncorhynchus tshawytscha*. Scheme for random sampling of the juvenile spring chinook salmon in the low-BKD and high-BKD groups for testing by the ELISA II. Equal numbers of fish were obtained from each rearing unit within a BKD-level group

Sample (Date)	Tissues sampled	Total no. of fish sampled per BKD-level group	No. of fish per pool	Total no. of pools/samples tested per group
Swimup fry (December 1988)	Whole fry; gastrointestinal tract and tail removed	300	5	60
Pre-ponding fry (April 1989)	Kidney and spleen	300	5	60
Parr (July 1989)	Kidney and spleen	540	3	180
Presmolts (November 1989)	Kidney and spleen	540	3	180
Smolts (March 1990)	Kidney and spleen	210	Individual	210

20 min at 37 °C and then stopped with 100 µl of double-strength stop solution. The absorbance was measured at 405 nm. Controls were performed and interassay correction factors were calculated as described by Pascho et al. (1987).

Designation of infection levels from ELISA absorbances. The positive-negative threshold absorbances for both ELISAs were calculated as described by Pascho et al. (1987). Each fish positive by the ELISA was assigned to a low, medium, or high *Renibacterium salmoninarum* infection level category on the basis of past studies comparing antigen levels in BKD-infected fish with concentrations of *R. salmoninarum* determined by the FAT and culture.

The ELISA I absorbance ranges for each *Renibacterium salmoninarum* infection level category were: low, 0.058 to 0.099 for the kidney tissue and 0.050 to 0.099 for the ovarian fluid; medium, 0.100 to 0.349; and high, ≥ 0.350 . Ovarian fluid samples with absorbances ≤ 0.049 , and kidney samples with absorbances ≤ 0.057 in the ELISA I were considered negative for *R. salmoninarum* infection.

Renibacterium salmoninarum-positive samples with ELISA II absorbances between the positive-negative cutoff absorbance and 0.199 were considered to have low infection levels, those with absorbances of 0.200 to 0.999 were considered to have medium infection levels, and those with absorbances ≥ 1.000 were considered to have high infection levels. Samples with the following ELISA II absorbances were considered negative for *R. salmoninarum* infection: swimup fry ≤ 0.089 , pre-ponding fry ≤ 0.069 , parr ≤ 0.071 , presmolts ≤ 0.069 , and smolts ≤ 0.073 .

Direct FAT. Purified goat antibody to *Renibacterium salmoninarum* conjugated with fluorescein isothiocyanate (FITC), obtained from the Biologics Section of the National Fish Health Research Laboratory (USFWS), Kearneysville, WV, USA, was used for each

FAT performed on samples from the adult fish. Before use, the conjugate was diluted 1:80 (v/v) in 0.01 M PBS pH 7.1, and filtered through a 0.2 µm Nuclepore polycarbonate filter. The FITC-conjugated goat anti-*R. salmoninarum* immunoglobulin used to test samples from the juvenile fish was obtained from Kirkegaard and Perry Laboratories, Inc.; the working dilution was 1:40 (v/v).

Concentration of *Renibacterium salmoninarum* from ovarian fluid by membrane filtration, FAT staining, and enumeration of the bacterium were done as described by Elliott & Barila (1987). We used their trypsin digestion method to facilitate sample filtration, and further modified the procedure as follows: nylon filters (5.0 µm, 13 mm diameter; Micron Separations, Inc., Westborough, MA, USA) were placed under the 0.2 µm polycarbonate filters to provide support during filtration, and the Eriochrome black T concentration in the counterstain was increased to 500 mg l⁻¹. From each female tested by the MF-FAT, a 0.5 ml subsample of ovarian fluid was processed and filtered, and a total of 150 microscope fields (1000 × magnification) were examined per filter.

Fixation and staining of the tissue smears were done as described by Pascho et al. (1987). On each slide, 50 microscope fields (1000 × magnification) were examined on each well, for a total of 100 fields per slide.

Bacteriological culture. Kidney tissue or ascites fluid from clinically diseased fish was cultured on KDM2 charcoal agar plates (Daly & Stevenson 1985) or selective KDM2 agar plates (Austin et al. 1983) at 15 °C for at least 60 d. Culture plates were examined at regular intervals for the presence of bacterial colonies, and material from those suspected to be *Renibacterium salmoninarum* was tested by the FAT

Statistical analyses. The chi-square test ($p < 0.05$) was used to evaluate differences in *Renibacterium sal-*

moninarum infection prevalence, and differences in the proportions of fish in various infection level categories, among the test groups.

RESULTS

ELISA I testing of spawning adults

The initial screening of the kidney samples from the 302 female spring chinook salmon by the ELISA I revealed that 111 of the fish (37 %) were positive for *Renibacterium salmoninarum* antigen (Fig. 1A). Of the fish testing positive, only 22 (20 %) had high antigen levels, and only 5 (5 %) had lesions characteristic of BKD.

A. Female Kidney (302 Fish)

B. Male Kidney (271 Fish)

Fig. 1. *Oncorhynchus tshawytscha*. Prevalence and levels of *Renibacterium salmoninarum* antigen in kidney samples of all adult spring chinook salmon tested by the ELISA I. Infection level designations are explained in the text. Number above each bar is the number of fish in that category

The ELISA I testing of kidneys from the 271 males used to fertilize the 302 egg lots indicated that 56 fish (21 %) were positive for *Renibacterium salmoninarum* antigen (Fig. 1B). Of these, 16 (29 %) had high antigen levels, and 4 (7 %) had characteristic internal lesions.

Selection of egg lots for BKD-level groups

Thirty egg lots each were assigned to the low-BKD and high-BKD groups based primarily on the kidney ELISA results for the females, and secondarily on the ovarian fluid MF-FAT results (Tables 1 & 2). The kidney ELISA results for the males were used mainly to

Table 4. *Oncorhynchus tshawytscha*. Mean ELISA I absorbance values for the kidneys of the male spring chinook salmon used to fertilize the egg lots selected for the high-BKD and low-BKD groups. Within each group, the data are arranged according to the female number. The lowest ELISA absorbance considered positive for *Renibacterium salmoninarum* was 0.058

Low-BKD group			High-BKD group		
Female no.	Male No.	Mean ELISA absorbance	Female no.	Male No.	Mean ELISA absorbance
50	645	0.046	32	633	0.041
742	683	0.043	42	841	0.047
750	207	0.048	102	47	0.046
752	649	0.041	104	65	0.047
754	487	0.041	106	99	0.052
756	97	0.047	112	895	0.051
760	771	0.044	122	493	0.054
770	459	0.041	130	725	0.041
772	197	0.049	132	273	0.083
778	579	0.045	138	963	0.047
790	653	0.041	746	729	0.043
792	261	0.041	762	867	0.043
794	575	0.043	798	427	0.086
802	699 ^a	0.041	832	35 ^a	0.041
804	699 ^a	0.041	834	35 ^a	0.041
806	709	0.041	952	365	0.046
820	129	0.047	964	543	0.043
822	783	0.041	998	789	0.047
824	937	0.045	1000	473	0.555
830	117	0.045	1002	115	0.046
852	437	0.043	1052	1557	0.046
932	749	0.045	1058	1009	0.137
934	797	0.045	1086	1109	0.042
938	743	0.049	1106	1277	0.049
940	795	0.045	1108	1327	0.043
1068	1309	0.041	1172	1687	0.044
1070	1135	0.047	1190	1635	0.041
1180	1037	0.045	1196	1945	0.045
1208	1653	0.044	1206	1883	0.043
1230	1865	0.043	1216	1063	0.045

^a Male used to fertilize 2 egg lots

eliminate any mating pairs with severely BKD-infected males from the low-BKD group (Table 4).

Because there was a limited number of females with high *Renibacterium salmoninarum* infection levels, it was necessary to extend the ELISA I kidney sample absorbance range for the high-BKD group selections down to a point where the minimum difference between females in the high-BKD and low-BKD groups was 0.099 absorbance units. The kidney ELISA absorbances for females in the low-BKD group ranged from 0.042 to 0.050 (all were BKD-negative by the ELISA I), whereas the kidney ELISA absorbances for the high-BKD group ranged from 0.149 to 0.749.

By the MF-FAT, no bacteria were detected in the ovarian fluid of 18 (60%) of the 30 fish selected for the low-BKD group, and the remaining fish had *Renibacterium salmoninarum* concentrations of 8.5×10^1 cells ml^{-1} or less. The high-BKD group contained eggs from females with *R. salmoninarum* numbers ranging from 8.5×10^1 to 1.3×10^{10} cells ml^{-1} ; 10 (33%) of the fish had concentrations greater than 1.0×10^6 cells ml^{-1} . One egg lot from a female (#1190) that was negative for *R. salmoninarum* infection by both the ELISA and the MF-FAT was inadvertently assigned to the high-BKD group.

Ovarian fluid ELISA values (Tables 1 & 2) were not used in the selection of egg donors for the 2 BKD-level groups. The ovarian fluid samples of all the females selected for the low-BKD group tested negative for *Renibacterium salmoninarum* by the ELISA even though 40% of these samples tested positive for *R. salmoninarum* cells by the MF-FAT. The ovarian fluid samples from 50% of the females in the high-BKD group tested negative for *R. salmoninarum* by the ELISA, although only one sample (fish #1190) was negative by the MF-FAT. The MF-FAT showed *R.*

salmoninarum counts as high as 1.0×10^5 cells ml^{-1} in the ELISA-negative samples from the high-BKD group.

Kidney FAT results were recorded for the egg donors (Tables 1 & 2). No *Renibacterium salmoninarum* cells were detected in the kidneys of any of the females selected for the low-BKD group. The kidneys of 25 (83%) of the females in the high-BKD group were positive for the bacterium by the FAT; counts per 100 microscope fields (1000 $\times$ magnification) ranged from 1 to too numerous to count.

By the ELISA, the kidneys of all 29 males used to fertilize the egg lots assigned to the low-BKD group, and the kidneys of 86% (25 of 29) of the males used to fertilize the egg lots in the high-BKD group, were negative for *Renibacterium salmoninarum* infection (Table 4). Only one of the males (#473) used for the high-BKD group had a high kidney antigen level.

Comparison of ELISA I and ELISA II

The retesting of kidney and ovarian fluid samples by the ELISA II showed a high degree of correlation between the results of the ELISA I and the ELISA II. The coefficients of determination (r^2) for the ELISA absorbances of the kidney and ovarian fluid samples analyzed by both tests were > 0.90 . The ELISA II gave a wider range of absorbances for both sample types. The absorbance range of the kidney samples was 0.041 to 0.709 for the ELISA I and 0.050 to 2.394 for the ELISA II, and the absorbance range of the ovarian fluid samples was 0.041 to 0.709 for the ELISA I and 0.049 to 2.606 for the ELISA II. The 2 tests differed in the number of *Renibacterium salmoninarum*-positive fish. By both tests, all of the 26 kidney samples from high-BKD-group fish tested positive for *R. salmoninarum*,

Fig. 2. *Oncorhynchus tshawytscha*. Monthly percent mortality of juvenile spring chinook salmon in the low-BKD and high-BKD groups from swimup to release from the hatchery. Mortality data are combined for all nursery tanks or raceways within a BKD-level group. Mean monthly water temperature is also shown

and all of the 25 ovarian fluid samples from low-BKD-group fish tested negative. However, although none of the kidney samples from low-BKD-group fish tested positive by the ELISA I, 36 % (9 of 25) tested positive by the ELISA II. Of the 26 high-BKD-group ovarian fluid samples tested by both ELISAs, 13 (50 %) tested positive by the ELISA I, and 11 (42 %) tested positive by the ELISA II. In all cases where *R. salmoninarum*-positive samples were detected by only one of the tests, the infection levels were low (absorbances < 0.100).

Mortality during rearing

When the swimup fry were moved from incubators to nursery tanks during November 1988, there were about 135 000 fry in the low-BKD group and about 126 000 fry in the high-BKD group. The differences in the numbers of fry in the 2 groups resulted largely from the mortality of about half of the fry in one high-BKD nursery tank (tank 34) within 10 d after the fish were placed in the tank. These deaths were believed to be related to crowding in one or more incubator trays, and these and other deaths in this time period were not included in the mortality calculations for the nursery tanks.

Mortality (Fig. 2) in the nursery tanks was more than 0.5 % mo⁻¹ in both BKD-level groups during November and December 1988. It then declined to 0.5 % mo⁻¹ or less until after the fish were placed in outside raceways in April 1989. No clinical signs of BKD were noted in the fish while they were in the nursery tanks.

A second increase in mortality in both BKD-level groups occurred during May and June 1989, when the water temperature in the raceways began to rise. Mortality during June exceeded 2 % in both groups. However, in July 1989, while mortality in the high-BKD raceways continued to exceed 2 %, that in the low-BKD group dropped to 0.2 %. During July, some of the fish in each high-BKD raceway began to show clinical signs of BKD, including swollen abdomens, exophthalmos, and lethargy. In contrast, clinical signs of BKD were rarely observed in fish in the low-BKD raceways. Viable *Renibacterium salmoninarum* was isolated in culture from a subsample of the clinically diseased fish in the high-BKD raceways.

After the monthly mortality in the low-BKD raceways decreased in July 1989, it remained below 0.3 % until the fish were released in April 1990. In contrast, mortality in the high-BKD group remained above 2 % per month through September 1990, then decreased as the water temperature declined, but continued to be at least 6 times higher than the mortality in the low-BKD group until the fish were released. Viable *Renibacterium salmoninarum* was isolated in cultures from

moribund fish. Fish in all raceways were handled for marking in December 1989, and mortality increased in both BKD-level groups during January 1990.

The total mortality from swimup to release was 20 % in the high-BKD group, significantly higher ($p = 0.0001$) than the 10 % mortality recorded for the low-BKD group. Most mortality in the high-BKD group (17 %) occurred after the fish were placed in raceways, whereas half of the mortality in the low-BKD group (5 %) occurred during that time. In contrast, the total mortality during nursery tank rearing was higher ($p = 0.0001$) in the low-BKD group (5 %) than in the high-BKD group (3 %).

Mortality differed ($p = 0.0001$) among the replicate nursery tanks and raceways within a given BKD-level group. For the low-BKD group, the total mortality was higher in nursery tank 113 (15 %) than in all other tanks, and the mortality was higher in tanks 98 and 114 (both 4 %) than in tanks 81 (2 %), 82 (3 %), and 97 (2 %). For the high-BKD group, the total mortality was higher in nursery tanks 34, 49, 50, 65, and 66 (all 3 %) than in tank 33 (2 %). The total mortality was higher in low-BKD raceway B-16 (6 %) than in raceways B-17 and B-18 (both 5 %). The overall mortality in high-BKD raceway B-20 (22 %) was higher than the mortality in raceway B-21 (15 %), which in turn was higher than that in raceway B-19 (13 %).

Prevalence and levels of *Renibacterium salmoninarum* in juvenile fish

The ELISA II results showed that the prevalence and levels of *Renibacterium salmoninarum* infections changed during rearing (Fig. 3). Most of the 5-fish pools of whole swimup fry taken from each group during December 1988 were negative for *R. salmoninarum* antigen, with 85 % of the low-BKD group samples and 93 % of the high-BKD group samples producing absorbances below the positive-negative cutoff absorbance (Fig. 3A). Infection rates did not differ between the 2 groups. All of the *R. salmoninarum*-positive pools showed low infection levels.

The April 1989 sample of pre-pending fry was taken when monthly mortality in both BKD-level groups was relatively low (0.5 %), yet the prevalence of BKD as detected by the ELISA in the kidney/spleen samples was high (Fig. 3B). All but one of the 5-fish pools from the low-BKD group and all of the pools from the high-BKD group tested positive. With the exception of 3 pools from the high-BKD group that showed high infection levels (one pool each from tanks 49, 50, and 66), all of the BKD-positive pools produced absorbances that placed them in the low-infection-level category.

The third random sample was taken in mid-July 1989

Fig. 3. *Oncorhynchus tshawytscha*. Distributions of ELISA II absorbances for random samples of juvenile spring chinook salmon in the low-BKD and high-BKD groups. The infection level designations are explained in the text. Sample sizes and processing procedures are in Table 3. Number above each bar is the number of samples (pools or individual fish) in each infection level category

when the monthly mortality rate in the high-BKD group was about 2.1 % compared with 0.2 % in the low-BKD group. At this stage clinical signs of BKD were evident in some high-BKD-group fish. However, all of the 3-fish kidney/spleen pools from both BKD-level groups tested positive by the ELISA (Fig. 3C). Most pools from the low-BKD group (62 %) and the high-BKD group (53 %) produced absorbances that placed them in the medium-infection-level category. About 8 % of the samples in each group showed high infection levels; fish in this category were detected in all raceways.

The presmolt sample was taken in November 1989 during a period of declining water temperature, when mortality had decreased in both BKD-level groups. Most 3-fish kidney/spleen pools in the low-BKD (97 %) and high-BKD (95 %) groups tested positive for *Renibacterium salmoninarum* antigen by the ELISA, but more than 90 % of the positive pools in each group showed low infection levels (Fig. 3D). None of the samples from the low-BKD group produced absorbances indicative of high infection levels, whereas 5 % of the positive samples from the high-BKD group were in this category.

The final sample was taken in March 1990, 2 wk before the fish were released from the hatchery into the Clearwater River. Analysis of the kidney/spleen samples from individual fish by the ELISA indicated that the percentage of *Renibacterium salmoninarum*-positive fish was higher ($p = 0.0001$) in the high-BKD group (85 %) than in the low-BKD group (62 %) (Fig. 3E). However, the proportions of positive and negative fish among the replicate raceways within a BKD-level group were the same. Of the positive fish, 98 % in the low-BKD group and 55 % in the high-BKD group had low infection levels, whereas 36 % in the high-BKD group and only 1 % in the low-BKD group had high infection levels.

The FAT identified *Renibacterium salmoninarum* in the smears of kidney/spleen homogenates from the pre-ponding fry, parr, presmolts, and smolts that had the highest infection levels as determined by the ELISA (absorbances ≥ 1.170). In contrast, no *R. salmoninarum* was identified by the FAT in smears from the kidney/spleen homogenates that had produced the lowest absorbances (≤ 0.096) for a given sample group. No *R. salmoninarum* was detected in the smears from whole swimup fry; all of the samples from this group had produced relatively low absorbances (≤ 0.158).

DISCUSSION

By brood stock segregation with the ELISA and MF-FAT, we created 2 groups of spring chinook salmon progeny with different patterns of *Renibacterium sal-*

moninarum infection during hatchery rearing. That distinction was evident in the mortality profiles, and in the prevalence and levels of *R. salmoninarum* antigen in the juvenile fish. These results provide further evidence for a direct relationship between the presence and level of *R. salmoninarum* in a female spring chinook salmon parent and the effect of the infection on her progeny.

The ELISA allowed the measurement of *Renibacterium salmoninarum* antigen levels in kidney tissue samples from all 573 adult salmon tested in this study, and in ovarian fluid samples from the 302 females, within the time constraints of a production hatchery schedule. We determined that 500 processed samples per day could easily be tested by the ELISA. Final placement of an egg lot in the high-BKD or low-BKD group was based on both the kidney ELISA results and results from further testing of tissue and ovarian fluid samples from each female in a relevant mating pair by the FAT. For this egg lot segregation, the kidney tissue ELISA was considered to be a crucial test because it is more sensitive than the corresponding FAT (Pascho et al. 1987, Sakai et al. 1989), and the enumeration of *R. salmoninarum* in the kidney samples by the FAT was used only as a backup to the ELISA. However, no reports have been published on the relation between *R. salmoninarum* infection levels in the kidneys of adult salmonids and the probability of vertical transmission; our laboratory is continuing investigations on this subject.

In contrast to the kidney FAT, bacterial counts in the ovarian fluid by the MF-FAT were judged more representative of infection levels in the female parent than the ELISA absorbances from the ovarian fluid samples. The ELISA consistently detected the presence of *Renibacterium salmoninarum* antigen only in samples with bacterial counts $> 1 \times 10^5 \text{ ml}^{-1}$, although the samples with the highest bacterial counts by the MF-FAT also had high ELISA absorbances. We previously observed inconsistencies between the ELISA and the MF-FAT when ovarian fluid was the test sample (data not shown). The soluble protein fraction detected by the ELISA may be absent, or at undetectable levels, in some ovarian fluid samples in which whole bacteria are observed by the MF-FAT; factors in ovarian fluid that might account for this are not yet defined. The ovarian fluid of chum salmon *Oncorhynchus keta* has an ion composition and osmolality similar to that of blood plasma, but with lower concentrations of organic constituents (Hirano et al. 1978), and at least one protein not found in plasma (Matsubara et al. 1985). Armstrong et al. (1989b) reported that ovarian fluid samples from chinook salmon showed an ability to agglutinate killed *R. salmoninarum* (killing method unspecified), and that this ability varied

among samples, but they did not further identify the agglutinin or determine its specificity. To our knowledge, the interactions between ovarian fluid and the *R. salmoninarum* soluble antigen detected by the ELISA have not been investigated.

In their report, Elliott & Barila (1987) suggested the MF-FAT is an appropriate test for evaluating management strategies that are based on the levels of *Renibacterium salmoninarum* in spawning fish. This was supported by the relative ease by which the test can be incorporated into the programs at existing diagnostic facilities, and its ability to detect *R. salmoninarum* concentrations as low as 43 organisms ml^{-1} in ovarian fluid under our test protocol. Because we did not believe that the accurate testing of several hundred samples by the MF-FAT could be accomplished because of personnel and time constraints, the initial screening of all fish by the ELISA was an ideal method to reduce the number of samples to a manageable level (90 samples were examined by the MF-FAT).

One might consider examining a smaller area of the membrane filter (we examined 150 microscope fields at 1000 $\times$ magnification, or about 5 % of the filter surface area), although this reduces the sensitivity of the test. One could also substitute a more conventional FAT in which bacteria are concentrated from ovarian fluid by centrifugation and the pellet is smeared on a microscope slide (smear FAT; Lee & Gordon 1987a, Armstrong et al. 1989b), but research results suggest that this method may lack the necessary sensitivity. In a screening test of ovarian fluid from chinook salmon, Armstrong et al. (1989b) found that 60 % of the samples were falsely negative by the smear FAT in comparison with culture, and that the FAT results obtained by 2 independent laboratories for samples from the same fish were inconsistent. Lee & Gordon (1987b) analyzed ovarian fluid samples from 190 chinook salmon by the smear FAT, an indirect MF-FAT, and culture, and reported that the proportion of samples testing positive for *Renibacterium salmoninarum* was much higher by the MF-FAT (76 %) than by the smear FAT (25 %) or by culture (4 %). Preliminary tests with the direct MF-FAT at our laboratory yielded similar results (data not shown).

We chose to quantify the numbers of *Renibacterium salmoninarum* cells in the ovarian fluid because of its direct association with the salmonid egg, and the apparent relation between the probability of vertical transmission and the presence and number of bacteria in that fluid. Intra-ovum infections in juvenile fish have been established experimentally by the exposure of salmonid eggs at 4 to 5°C for 12 to 24 h to ovarian fluid seeded with various concentrations of *R. salmoninarum* (Evelyn et al. 1986b, Lee & Evelyn 1989). Evelyn et al. (1986b) reported that infections were achieved by the

exposure of steelhead trout eggs to ovarian fluid concentrations of 1.4×10^9 *R. salmoninarum* cells ml^{-1} and by the exposure of coho salmon *Oncorhynchus kisutch* eggs to 1.3×10^{12} cells ml^{-1} , but not by exposure to lower concentrations (1.4×10^3 ml^{-1} for steelhead trout eggs and 1.3×10^6 ml^{-1} for coho salmon eggs). From later experiments, Lee & Evelyn (1989) reported that intra-ovum infections were detected in chinook salmon eggs exposed to ovarian fluid *R. salmoninarum* concentrations of 1.7×10^5 cells ml^{-1} or higher, and in smolts (195 d after hatching) that had been exposed as eggs to ovarian fluid concentrations of 1.7×10^3 cells ml^{-1} or higher. The infection rates in the smolts appeared to be higher in the groups exposed as eggs to 1.7×10^9 bacteria ml^{-1} (24 to 44 %) than in groups exposed to 1.7×10^3 bacteria ml^{-1} (8 to 10 %). Intra-ovum infections have also been detected in eggs obtained from naturally infected female coho salmon with ovarian fluid concentrations of *R. salmoninarum* as high as 10^9 to 10^{10} cells ml^{-1} (Evelyn et al. 1984a, b, 1986c). Recently, Lee & Evelyn (1989) detected *R. salmoninarum* in chinook salmon smolts obtained as eggs from naturally infected females with ovarian fluid concentrations of *R. salmoninarum* as low as 28 bacteria ml^{-1} , but not in smolts from females with no bacteria detected in the ovarian fluid.

Our study provides further circumstantial evidence for a strong relation between ovarian fluid *Renibacterium salmoninarum* concentrations and the success of vertical transmission. Although the use of untreated Clearwater River water for all rearing did not preclude the possibility of waterborne infection, and the grouping of egg lots prevented the monitoring of progeny from the individual egg lots, apparent differences in infection patterns were evident among the nursery tanks. Fish with high *R. salmoninarum* antigen concentrations by the ELISA (absorbances ≥ 1.0) were first detected (in April 1989) only in the 3 high-BKD group nursery tanks containing the progeny of the females with the highest ovarian fluid concentrations of *R. salmoninarum* by the MF-FAT (5.9×10^9 to 1.3×10^{10} cells ml^{-1}).

The results of past research suggest that the proportion of salmonid eggs that become infected by vertical transmission may be low, even when ovarian fluid concentrations of *Renibacterium salmoninarum* are high (Evelyn et al. 1984a, b, 1986b, c); subsequent amplification of the disease may occur by cross-infection among the juvenile fish in a group (Lee & Evelyn 1989). Subsamples of coho salmon egg lots from females with ovarian fluid naturally containing 10^9 to 10^{10} cells ml^{-1} have shown intra-ovum infection rates of about 5 to 15 % (Evelyn et al. 1984a, b, 1986c). We did not test eggs by the ELISA, but our results showed a low infection prevalence (7 to 15 %), and low infection

levels among the first fry samples (December 1988) compared with subsequent samples. However, the use of 5-fish pools of nearly entire fry (necessary to achieve sufficient sample volumes) rather than kidney and spleen samples as were tested from the larger fish may have significantly diluted any *R. salmoninarum* antigen present. The increases in the prevalence and severity of *R. salmoninarum* infection in both BKD-level groups through July 1989 suggest that cross-infection among the fish in a raceway, as well as infection by bacteria in the water supply, may have occurred.

Despite the strong evidence that eggs can become infected by *Renibacterium salmoninarum* in the ovarian fluid, several researchers have suggested that vertical transmission may be achieved by alternative means. Bruno & Munro (1986) showed that developing oogonia of rainbow trout could become infected when the fish were injected intraperitoneally with 2.7×10^7 *R. salmoninarum* cells each. Lee & Gordon (1987a) and Lee & Evelyn (1989) suggested that some infections detected in the progeny of females with few or no bacteria in the ovarian fluid may represent intra-ovum infections acquired from ovarian tissue before ovulation. We cannot rule out the possibility that some of the eggs in our study groups became infected before ovulation.

Although there is no clear evidence that males contribute to the vertical transmission of *Renibacterium salmoninarum* (Klontz 1983, Evelyn et al. 1986b, Lee & Gordon 1987b) we chose to test their kidneys by the ELISA primarily to eliminate egg lots fertilized by highly infected males from the low-BKD group. Furthermore, only one of the males used to fertilize eggs in the high-BKD group was highly infected (ELISA absorbance > 0.350); by chance this male was mated with one of the most highly infected females.

We did not use spermatozoa or seminal plasma as a diagnostic sample to complement the ELISA analysis of the male kidney. Daly & Stevenson (1989) demonstrated that *Renibacterium salmoninarum* agglutinates the spermatozoa of several fish species, including chinook salmon, by binding to the tails. However, the authors felt that the absence of binding to the head of the spermatozoa made this an unlikely mechanism for entry of the bacteria into the ova. In a related study, Daly & Stevenson (1990) found that the heat-stable, soluble F-antigen described by Getchell et al. (1985) was the same molecule responsible for the sperm-agglutinating property of *R. salmoninarum*. The ELISAs used in our study rely on detecting this antigen fraction in the tissues and body fluids of infected fish (Pascho & Mulcahy 1987), and spermatozoa could have contributed to false-negative reactions by adsorbing some fraction of the soluble *R. salmoninarum* antigens present in a milt sample.

The presence of *Renibacterium salmoninarum* in many of the adult chinook salmon, despite erythromycin injection, indicates that a single injection of erythromycin at 20 mg kg^{-1} body weight did not eliminate the infection. Fish with high infection levels in the kidney and ovarian fluid remained in the population, and some of these fish did not display the clinical signs of BKD that would have alerted hatchery personnel to discard or segregate their gametes. Other studies have also shown that a single injection of erythromycin at 20 mg kg^{-1} may reduce, but does not necessarily eliminate, *R. salmoninarum* infections in adult chinook salmon. Lee & Gordon (1987a) reported that 1 to 2 mo after injection, *R. salmoninarum* was detected by the FAT in the kidneys of 18% of the 317 erythromycin-injected fish and 44% of the 397 uninjected fish. In contrast, Armstrong et al. (1989b) reported that the erythromycin injection of chinook salmon broodstock did not significantly reduce the proportion of positive fish (or the proportion testing strongly positive) in a smear-FAT analysis of ovarian fluid samples from injected and uninjected fish conducted by 2 independent laboratories.

Although erythromycin injection of adult salmonids may not eliminate *Renibacterium salmoninarum* infection from these fish, evidence suggests that this practice may help to prevent or reduce infection of the progeny. Injection of erythromycin at 11 or 20 mg kg^{-1} body weight into the dorsal sinus of maturing female salmon results in the deposition of the antibiotic in the yolk of the egg (Groman & Klontz 1983, Bullock & Leek 1986, Evelyn et al. 1986a, Armstrong et al. 1989a, Brown et al. 1990). Whereas erythromycin levels in the ovarian fluid may decline to undetectable levels within 20 d after injection (Armstrong et al. 1989a), drug levels exceeding the reported in vitro minimum inhibitory concentration may persist in eggs and in fry to the late alevin stage (Bullock & Leek 1986, Evelyn et al. 1986a, Armstrong et al. 1989a, Brown et al. 1990). The timing of injections may be important. Significant drug levels have been achieved in the eggs of fish injected (20 mg kg^{-1}) as few as 9 d (Armstrong et al. 1989a) and up to 56 d (Evelyn et al. 1986a) before spawning; but not in the eggs of fish injected 70 d or more before spawning (Evelyn et al. 1986a). Even if erythromycin injections are given during the appropriate time, however, the results of Armstrong et al. (1989a) suggest that a single injection (20 mg kg^{-1}) may reduce but not eliminate vertical transmission of *R. salmoninarum* to the progeny of heavily infected females. Some of the fish in our study may have been injected with erythromycin up to about 90 d before spawning; we did not monitor erythromycin levels in the ovarian fluid, or in any eggs or fry.

Our monitoring of the prevalence and levels of

Renibacterium salmoninarum infection by the ELISA in the test fish at several points during rearing indicated a relatively slow progression of *R. salmoninarum* infection from December 1988 to the time of the parr sample (July 1989). Although a few fish with high *R. salmoninarum* infection levels were detected by the ELISA in the high-BKD group in the April pre-pending sample, clinical signs of BKD (and mortality attributed to BKD) were not evident in the group until July 1989.

Considering that the mortality was about 10 times higher in the high-BKD group than in the low-BKD group during July 1989, the similar distributions of infection levels in the random samples of fish from each group on July 19 is surprising. The percentages of highly infected samples (absorbance ≥ 1.0) were the same (8%) for both groups, although samples with extremely high absorbances (≥ 2.0 , usually associated with clinical disease) were only in the high-BKD group (4 of 180 pools). The use of 3-fish pools for ELISA testing may have masked further differences. Whether many deaths of fish in the high-BKD group were caused by something other than BKD is a tempting consideration. However, ELISA analysis revealed that all 20 moribund fish sampled from the 3 high-BKD raceways on July 20, 1989, were positive for *Renibacterium salmoninarum* infection; 19 had ELISA absorbances ≥ 1.0 , and 18 of these had ELISA absorbances ≥ 2.0 . Cultures were taken from 3 of the fish (one per high-BKD raceway), and all showed heavy growth of *R. salmoninarum*. These results indicated that BKD was the major cause of mortality in the high-BKD group at the time of sampling. (No moribund fish were found in the low-BKD raceways at this time.)

The data suggest that some unknown mechanism may have contributed to the increased survival of the fish in the low-BKD group by allowing a higher percentage of the fish to cope with medium to high *Renibacterium salmoninarum* infection levels. There is some evidence that genetic variation can influence the resistance of coho salmon to experimental injection challenges with *R. salmoninarum* (Suzumoto et al. 1977, Winter et al. 1980, Withler & Evelyn 1990), although the heritability of resistance to natural infection is not understood. Alteration of the host's immune system may possibly play an important role in the pathogenesis of BKD in young fish. For example, the exposure of fish to certain antigens early in life before the immune system is fully developed can induce a state of immunological tolerance (Manning et al. 1982). It is not known what effect the exposure of salmonid eggs to *R. salmoninarum* antigens before or during spawning may have on the subsequent response of the fry to infection by this pathogen.

The differences in mortality between the 2 BKD-level

groups continued from July 1989 until the fish were released in April 1990, even though all raceways were supplied with untreated water from a river known to harbor spring chinook salmon and other salmonid fishes. In addition, an apparent divergence existed in the distributions of BKD infection levels between the 2 BKD-level groups by the time the November 1989 sample was taken; this divergence was particularly evident when individual fish were necropsied just prior to their release.

Data from another study of brood stock segregation begun at Dworshak National Fish Hatchery in 1989 suggest that this procedure yields reproducible results when used with other year classes of spring chinook salmon. The protocol for this study was identical to that in 1988, with the exception that the ELISA II was used for all adult testing and 42 female spring chinook salmon were used as egg donors for each BKD-level group (with about 168 000 eggs per group). The ELISA II absorbances for the kidney samples from the females in the low-BKD group ranged from 0.071 to 0.140; the samples from 48% of the fish tested negative for *Renibacterium salmoninarum* by the ELISA. The ELISA II absorbances for the kidney samples from the females in the high-BKD group ranged from 0.616 to 3.046, but only 24% of the fish had gross lesions characteristic of BKD. Bacterial counts in the ovarian fluid by the MF-FAT ranged from 0 to 43 bacteria ml^{-1} for the low-BKD group females (88% had no bacteria), and 0 to 4.0×10^9 bacteria ml^{-1} for the high-BKD group females (43% had bacterial counts $\geq 1 \times 10^6$ bacteria ml^{-1}). Mortality of the juvenile fish from November 1989 through release in April 1991 was 17% for the low-BKD group and 33% for the high-BKD group (mortality after the fish were placed in raceways was 4% for the low-BKD group and 19% for the high-BKD group). A third, smaller study being conducted with another stock of spring chinook salmon at another hatchery in Idaho has also shown significantly higher mortality in high-BKD group progeny than in low-BKD group progeny (data not shown).

The results of our research suggest that segregation of brood stock by the ELISA and MF-FAT can be used to reduce the prevalence and levels of *Renibacterium salmoninarum* infection in hatchery-reared spring chinook salmon, even in locations with open water supplies. In this study, the segregation did not eliminate the disease in the low-BKD level group. However, it demonstrated that complete elimination of the disease may not be necessary to significantly increase the survival of hatchery-reared spring chinook salmon. Brood stock segregation may be a suitable alternative or supplement to chemotherapy and at most hatcheries could be done with reasonably inexpensive modifications of existing facilities.

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