

Epidemiology and Control of Infectious Diseases  
of Salmonids in the Columbia River Basin

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by

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## ABSTRACT

The Department of Microbiology at Oregon State University with funding from the Bonneville Power Administration has conducted a study since 1983 relating to the epidemiology and control of three diseases of salmonids in the Columbia River Basin. These diseases are ceratomyxosis, caused by the protozoan parasite Ceratomyxa Shasta, bacterial kidney disease, the etiological agent of which is Renibacterium salmoninarum and infectious hematopoietic necrosis which is caused by a rhabdovirus. Each of these diseases is difficult or impossible to treat with antimicrobial agents.

The presence of the infectious stage of C. shasta was again detected at Little Goose Dam on the Snake River. The prevalence of ceratomyxosis increased from 1.1% in 1984 to 10% in 1985. None of the susceptible rainbow trout exposed in the Yakima and Umatilla Rivers died of this disease. Ceratomyxosis in resistant chinook salmon smolts seined from the Columbia River just above the estuary seems dependent on whether or not they are held after capture in fresh or salt water. In fresh water the disease incidence ranged from 7-19%, whereas in salt water it ranged from 0-3%. These results which suggest that recovery from ceratomyxosis may occur after the smolts enter salt water are different from those obtained with susceptible Alsea steelhead trout where experimental groups in salt water have died at the same rate as those in fresh water.

Comparing data from groups of Columbia River chinook smolts held after capture in either fresh or salt water, R. salmoninarum is a much more effective pathogen in the salt water environment. After four years of sampling smolts in the open ocean, numbers of this microorganism sufficient to cause death have been detected in chinook (7%) and, coho salmon (2%) and steelhead trout (1%). Results from three years of sampling have consistently

indicated that additional fish infected with R. salmoninarum will be detected if egg washings are included in the procedures for monitoring bacterial kidney disease in adults.

Antigenic differences among strains of R. salmoninarum and common antigens present on both R. salmoninarum and other Gram positive bacteria have been demonstrated for the first time using monoclonal antibodies. All of the monoclonal antibodies belong to the murine IgG1, IgG3 or TgG2a class and subclass.

Field studies at Round Butte Hatchery with the molecular filtration apparatus detected IHNV in effluent water from the adult holding pond and in water from a tank containing steelhead trout fry infected with IHN disease. The concentrations of IHNV detected in these samples suggested that in the order of  $10^{10}$  virions are being released each day into the Deschutes River at the peak of steelhead trout spawning at Round Butte Hatchery. Isolation of IHNV from dead eggs suggested that virus replication during incubation may be a possible cause of egg mortality. Two possible reasons for inconsistencies in the data from the IHNV transmission studies at Round Butte Hatchery are: 1) UV treatment does not completely sterilize the water and 2) vertical transmission occurs but under, as yet, undescribed conditions. Constant IHNV production over a prolonged period has been recorded in unfiltered ovarian fluid samples. Filtration eliminates this virus production. These observations suggest that cellular components in ovarian fluid are responsible for producing the delayed appearance of IHNV after storage at 4°C for 8 to 16 days.

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Cooperators in this study are: the Portland General Electric Company, through their representative Mr. Don Ratliff, which owns Round Butte Hatchery and purchased the ultraviolet sterilization equipment; the personnel of the Oregon Department of Fish and Wildlife (ODFW) who operate Round Butte Hatchery, the ODFW also supplied the different stocks of fish and coordinated livebox placement; Dr. Warren Groberg, virologist of the ODFW who assisted in experimental design and sampling at Round Butte Hatchery; Mr. Craig Banner of the ODFW who helped collect samples from the ocean; the United States Fish and Wildlife Service, Seattle Fisheries Research Center whose staff, directed by Dr. Dan Mulcahy, jointly sampled the steelhead trout at Round Butte Hatchery; the National Marine Fisheries Service personnel who helped in the collection of smolts from their Jones Beach seining facility; Dr. William Pearcy of the Oregon State University School of Oceanography and his research team who were responsible for the collection of juvenile salmonids from the ocean; and personnel of the Army Corps of Engineers and the Grant County Public Utility district who helped coordinate the placing of liveboxes at selected Columbia River and Snake River Dams.

## INTRODUCTION

Successful propagation and enhancement of fisheries resources requires control of fish pathogens. This study, which has been funded by the Bonneville Power Administration since 1983, focuses on three of these disease agents: Ceratomyxa Shasta, Penibacterium salmoninarum and infectious hematopoietic necrosis virus.

### Ceratomyxa shasta

Prior to this project Ceratomyxa shasta was known to exist in the mainstream Columbia River to its confluence with the Deschutes River. Our livebox studies designed to more precisely define the range of the infective stage of C. shasta within the Columbia River Basin, have detected this parasite upriver in the mainstream Columbia River to the confluence with the Snake River. The parasite has also been detected for the first time in the Snake River at Little Goose Dam. These results mean upriver salmonids are exposed to C. shasta for a much longer period than previously believed. Results obtained during 1983 by collecting upriver smolts just prior to entering the estuary indicated that approximately 10% of these fish are infected with ceratomyxosis after migration down the Columbia River.

The nature of the infectious stage of C. Shasta, like most other myxosporidan parasites, is unknown. Recent research with Plyxosoma cerebralis, another myxosporidan infecting salmonid fish, has suggested the oligochaete tubifex acts as an intermediate host in its life cycle. However, our attempts to transmit ceratomyxosis to susceptible fish by exposing them to tubifex incubated with infected viscera and spores have been unsuccessful. Other experiments we are continuing are attempting to visualize the infective stage of c. shasta by using fluorescently labelled antibodies directed against

spores of this parasite and to transmit this disease in the laboratory by exposing susceptible rainbow trout to fish that have died of certaomyxosis in tanks containing mud from areas where C. shsta is endemic and sterilized mud seeded with tubificid worms.

## Materials and Methods

### Experimental Animals

Ceratomyxa Shasta-susceptible rainbow trout (Salmo gairdneri) were obtained from Oak Springs Hatchery and held at the Oregon State University, Fish Disease Laboratory (OSU-FDL). Salmonid stocks to be tested for resistance to this parasite were obtained from Oak Springs Hatchery and the OSC Fish Toxicology Laboratory. Steelhead trout (Salmo gairdneri) and chinook salmon (Oncorhynchus tshawytscha) used in studying the effects of salt water on the progress of infection were obtained from Rock Creek Hatchery and the OSU-FDL. In 1984 salmonid smolts exposed to C. shasta during their migration down the Columbia River were obtained by beach seine at Jones Beach (Rkm 75) on the Columbia River.

### Exposure to Ceratomyxa shasta

Procedures for exposure to, and detection of, C. shasta are described by Fryer (1984). All groups of fish used in the stock resistance portion of this study were exposed for five days to the infectious stage of C. shasta in the Willamette River near Corvallis, Oregon. The five day exposure period was chosen because this length of time has been shown to be sufficient to cause a high incidence of infection in control fish when the number of infectious units was high (Zinn et al., 1977). After exposure these fish were returned to the OSU-FDL and held for 100 days.

For fish used in distribution studies the exposure period at each of the selected sites was 14 days. After exposure all fish were transported to Round Butte Hatchery Isolation Facilities (RBH-IF) and held until termination 120 days later.

Ceratomyxa shasta susceptible steelhead trout and resistant chinook salmon which were used to determine the effects of salt water on the progress of infection were exposed to the infective stage for three days in the Willamette River near Corvallis, Oregon. The three day exposure period was chosen because our goal was to achieve a low level of infection in the susceptible steelhead trout. After exposure, the groups were divided, half going to fresh water facilities at the OSU-FDL and half going to salt water facilities at the Oregon State University, Marine Science Center (OSU-MSU). All exposed fish were killed after 100 days and examined for C. shasta. Salmonid smolts collected by beach seine were also divided with half of the fish going to the RBH-IF and half to the OSU-MSU. These fish were held for 150 days. In all experiments, fish that died within 10 days after arrival at each facility were considered handling mortalities and were not included in the results.

#### Spore Purification and Antisera Production

Spore purification and antisera production and labelling have been previously described (Fryer 1984). In 1985, antisera was produced not only to broken spores, but also to whole spores and to prespore stages by repeated intravenous injections (8) into the ear veins of New Zealand white rabbits.

#### Concentration of Ceratomyxa shasta Infective Stage

Four, 100 liter samples of Willamette River collected at Corvallis, Oregon (Rkm 212) from a location known to contain the infective stage of C. shasta were differentially filtered using a molecular filtration unit (Pellicon Cassette System, Millipore Corp.) with a pore size of 0.5  $\mu\text{m}$ . Materials concentrated by this procedure were injected intraperitoneally into susceptible rainbow trout and smears were made for examination by bright light microscopy and fluorescent antibody techniques.

#### Transmission of Ceratomyxa shasta Infective Stage

Rainbow trout infected with ceratomyxosis were placed with unexposed, susceptible rainbow trout in tanks containing several different substrates. These substrates consisted of mud from areas where C. shasta is endemic (La Camas Lake and the Willamette River), mud sterilized by autoclaving and sterilized mud seeded with tubificid worms. Control tanks contained these substrates and unexposed fish only. All infected fish were fin clipped and allowed to decompose in the tanks after death from ceratomyxosis. As they died, all unclipped fish were examined for spores or prespore stages of the parasite.

### Results and Discussion

#### Geographic Distribution

In 1983 and 1984, susceptible fish were exposed at selected sites (Bonneville, Dalles, John Day, McNary, Priest Rapids, Ice Harbor and Little Goose Dam) in the Columbia River Basin. Results from these exposures (Fryer 1984) extended the range of the infectious stage by about 200 river miles, up the mainstream of the Columbia River to, but not above, its confluence with the Snake River, thence into the Snake River to Little Goose Dam.

In 1985, fish were exposed for 14 days in May and July. In this first set of exposures at Little Goose Dam, the level of ceratomyxosis increased from 1.1% in 1984 to 10% in 1985. No infections developed in fish held at any exposure site during May in the Columbia River (Table 1). Fish were also exposed in the Umatilla and Yakima Rivers to determine if the infective stage of c. shasta was present in these Columbia River tributaries. None of the animals held at these sites during the May exposure period died of ceratomyxosis. The presence of ceratomyxosis in rainbow trout exposed at McNary Dam in 1983 and 1984 and at Little Goose Dam in 1984 and 1985 extends the range of this parasite about 200 miles upriver, into the Snake River drainage. In this survey the incidence of infection was dependent on the location of the exposure site and time of exposure. The importance of the exposure location was illustrated by Johnson (1975) who found that infection incidence varied by as much as 71% in fish exposed simultaneously at sites within a 160 meter distance in the Willamette River.

Although the geographic range of C. shasta has been extended into the Snake River, liveboxes containing susceptible rainbow trout have not been placed in the mainstream Snake River above Little Goose Dam. The infections at Little Goose Dam indicate the infective stage of C. shasta occurs further upstream in the Snake River and possibly into its tributaries. At present the only tributaries of the Snake River that have been examined using livebox techniques are in Oregon. These are the Imnaha River and the Grande Ronde and two of its tributaries (Wallowa River and Lookingglass Creek) (Fryer 1984). Infected salmonids return to these Snake River tributaries, and although spores are released into these waters the presence of the infective stage was not demonstrated. This phenomenon has been reported by other investigators (Johnson 1975; Sanders et al. 1970) and further indicates that

Table 1. Incidence of Ceratomyxa shasta in susceptible rainbow trout (Salmo gairdneri) exposed at selected dams in the Columbia River Basin during May 1985.

Location	Number of fish recovered	Number of fish infected with <u>Ceratomyxa shasta</u> <sup>2</sup>	Percent of fish infected with <u>Ceratomyxa shasta</u>
Columbia River			
Dalles Dam	68	0	0
McNary Dam	78	0	0
Priest Rapids Dam	67	0	0
Snake River			
Little Goose Dam			
Lower site <sup>3</sup>	50	6	12
Upper site <sup>4</sup>	53	4	7.5

<sup>1</sup>Number of fish exposed minus handling mortalities.

<sup>2</sup>All experimental groups were examined 120 days after initial exposure.

<sup>3</sup>Exposed in downstream migrant collection facility.

<sup>4</sup>Exposed in forebay of dam.

the infectious process requires some unknown factor(s) not present in many tributaries.

#### Resistance of Salmonid Strains

In 1985 we tested Umatilla River steelhead trout and Shasta and Oak Springs rainbow trout for their resistance to infection by C. Shasta. Oak Springs rainbow trout are highly susceptible to infection by C. shasta and served to confirm the presence of the pathogen and allowed comparison of infection levels among groups of exposed fish. The strain of steelhead trout from the Umatilla River was resistant to infection (0% level) by C. shasta. The Shasta rainbow trout from Mt. Shasta Hatchery in northern California were susceptible to infection, having an infection incidence of 57%, while the incidence of infection among the Oak Springs rainbow trout was 33%.

The resistance of Umatilla steelhead trout and the other upriver salmonid stocks previously tested (Carson, Bonneville, Cowlitz, Oxbow, Innaha, upriver brights, and Lookingglass chinook and Sandy coho salmon and Skamania, Deschutes, Clearwater, Umatilla, Wallowa and Innaha steelhead trout) (Zinn et al. 1977; Buchanan et al. 1983; Fryer 1984) indicates that the presence of the infectious stage of C. shasta acts as a selective factor on salmonid populations. Although all upriver salmonids tested have proved resistant to infection, these conclusions were drawn from experiments in which periods of exposure were only five days. As we reported previously, longer exposure periods result in a higher prevalence of infection (Fryer 1984). Continual exposures to the infective stage of this parasite in a water supply has caused serious fish losses at the Cowlitz Trout Hatchery throughout its history (Tipping and Kral 1984). Extension of the infective stage of C. shasta to

Little Goose Dam some 200 river miles above the Deschutes River means that upriver salmonids are exposed to this parasite for a much longer time than previously recognized.

#### Effects of Salt Water on Fish Infected with Ceratomyxa shasta.

The effects of salt water on the progress of infection was studied using C. shasta susceptible steelhead trout, and resistant coho (Oncorhynchus kisutch) and chinook salmon strains. In 1984, Alsea steelhead trout and Big Creek coho salmon (Fryer 1984), and in 1985, Alsea steelhead trout and Round Butte chinook salmon were exposed to the infectious stage of C. shasta in the Willamette River. Half of each group were then transferred to salt water at the OSU-MSU and half were held in fresh water at the OSU-FDL. In both years, all of the steelhead trout held in fresh water died of ceratomyxosis while 88% (in 1984) and 100% of those in salt water died of the disease (Table 2). None of the C. shasta resistant Big Creek coho (Fryer, 1984) or Round Butte chinook salmon (Table 2) transferred to salt water developed the disease. In fresh water the only infection observed in either group was in one coho salmon which developed a muscle lesion.

Results from these two years show that in C. shasta susceptible strains, such as Alsea steelhead trout, the disease process in both fresh and salt water continues at the same rate. Resistant strains will remain highly refractive to ceratomyxosis when exposed to the infective stage immediately prior to transfer to salt water.

As suggested previously, prolonged exposure to the infective stage of C. shasta should cause a higher infection rate even among resistant stocks. Indeed, in both 1983 and 1984, 14% of the chinook salmon captured by beach seine from July to September in the lower Columbia River were infected with

Table 2. Effects of salt water on steelhead trout (*Salmo gairdneri*) and chinook salmon (*Oncorhynchus tshawytscha*) exposed to the infectious stage of *Ceratomyxa shasta* during 1985.

Salmonid	Exposure period (days)	Fresh water			Salt water		
		No. of fish recovered'	No. of fish infected	Percent infected	No. of fish recovered'	No. of fish infected	Percent infected
Alsea steelhead trout	3	24	24	100	37	37	100
	Control <sup>2</sup>	25	0	0	38	0	0
Round Butte chinook salmon	3	27	0	0	30	0	0
	Control <sup>2</sup>	25	0	0	27	0	0

<sup>1</sup>Number of fish exposed minus number of fish which died before spores were detected.

<sup>2</sup>Control fish were not exposed to the infectious stage of *C. Shasta*.

ceratomyxosis. This rate of infection is higher than found in any of the stock susceptibility experiments in which exposures were seven days or less.

The prevalence of ceratomyxosis in chinook salmon smolts seined from the Columbia River just before entering the estuary seems dependent on whether or not the fish are held after capture in fresh or salt water. In fresh water the disease prevalence of ceratomyxosis ranged from 7-19% (Table 3); in contrast, in the groups held in salt water the incidence of ceratomyxosis was greatly reduced (0-3%) suggesting recovery from the disease agent (Table 4). All Columbia River salmonid stocks tested have proved resistant to ceratomyxosis and this data unlike that obtained with susceptible Alsea steelhead trout, suggests that resistant fish infected while in fresh water may recover after entry into salt water. These observations suggest that Columbia River smolts migrating rapidly through areas containing the infective stage of C. shasta will suffer few losses as a result of ceratomyxosis. However, those that do not migrate quickly and remain in fresh water will suffer a considerably higher mortality than recognized by previous stock susceptibility studies.

#### Investigation into the Nature of Ceratomyxa shasta Infective Stage

Samples of Willamette River water containing the infective stage of C. shasta were differentially filtered and all materials in each sample larger than 0.45  $\mu\text{m}$  were injected into susceptible rainbow trout. These fish are being observed for development of ceratomyxosis. These concentrates are also being examined by light and fluorescent microscopy; however, no spores or definite prespore stages have been identified.

Infected fish have been allowed to decompose in the tanks containing a variety of substrates. All fish known to be uninfected when placed in the tanks are removed upon death and examined for spores or prespore stages. No

Table 3. Prevalence of Ceratomyxa shasta in chinook salmon (Oncorhynchus tshawytscha smolts beach-seined from the Columbia River (Rkm 75) and held in fresh water for 150 days at Round Butte Hatchery Isolation Facility.

Date collected 1984	Number of fish collected <sup>1</sup>	Fish that died	Mortalities infected with <u>C. shasta</u>	Percent of fish collected infected with <u>C. shasta</u> <sup>2</sup>
July 5	88	76	6	7
26	75	22	12	16
31	84	31	14	17
Aug. 16	82	34	11	13
23	87	74	13	15
Sept. 20	47	29	9	19
Totals	463	266	65	14

<sup>1</sup>Number of fish collected minus handling mortalities.

<sup>2</sup>All fish alive at termination were examined and none were infected with C. shasta

Table 4. Prevalence of Ceratomyxa shasta in chinook salmon (Oncorhynchus tshawytscha) smolts beach-seined from the Columbia River (Rkm 75) and held in salt water for 150 days at Oregon State University Marine Science Center.

Date collected 1984		Number of fish collected <sup>1</sup>	Fish that died	Mortalities infected with <u>C. shasta</u>	Percent of fish collected infected with <u>C. shasta</u> <sup>2</sup>
July	5	93	86	0	0
	26	76	76	0	0
	31	75	70	2	3
Aug.	16	57	56	1	2
	23	89	80	3	3
Sept.	20	70	68	0	0
Totals		460	436	6	1.3

<sup>1</sup>Number of fish collected minus handling mortalities.

<sup>2</sup>All fish alive at termination were examined and none were infected with C. Shasta.

ceratomyxosis has been detected in these control fish; however, the disease cycle may require some aging period in the substrate.

#### Renibacterium salmoninarum

Renibacterium salmoninarum, the causative agent of bacterial kidney disease (BKD) is recognized as one of the major bacterial infections of salmon and problems caused by it extend throughout Columbia River Basin fish hatcheries. Renibacterium salmoninarum was detected, during 1983, in 13% of the chinook salmon smolts seined from the Columbia River just before entering the estuary. These observations serve to further delineate the continued economic impact of this pathogen after smolts are released from hatcheries. Limited data in the literature also suggests that BKD continues to cause mortality after salmonid smolts enter salt water. Salmonids caught in the open ocean off the coast of Oregon and Washington have contained BKD lesions and harbored the organism. Since sampling began in 1981, R. salmoninarum has been found by the fluorescent antibody test in 11% of the chinook salmon with lesions in 2.5% of these fish. The presence of lesions is an especially important observation indicating an ongoing open ocean mortality.

Examination by Cram stain and fluorescent antibodies of cryostat-sectioned fertilized eggs collected after one month of incubation in a fish pathogen-free water supply have revealed the presence of bacteria morphologically identical to R. salmoninarum on or in the egg wall. These observations extend those of Evelyn et al. (1984) and further suggest vertical transmission of this disease.

Knowledge of the serology and antigenic composition of R. salmoninarum is important for the development of reliable serological methods for detection of BKD infections. Although it has been generally accepted there is only one

antigenic type of this bacterium (Bullock et al., 1974), there has been limited experimentation done to serologically compare isolates (Getchell et al., 1985). Recent observations indicate there may be more than one antigenic type and that cross-reactions may occur with bacteria from other genera (Austin and Rogers, 1980; Austin et al., 1985). The purpose of developing monoclonal antibodies against isolates of R. salmoninarum was to produce a reagent to a antigen unique to R. salmoninarum that can be used for immunodiagnosis to eliminate false positive reactions.

#### Materials and Methods

##### Experimental Animals and Detection of Renibacterium salmoninarum

Salmonids of different year classes were purse seined from the ocean off the coast of Oregon and Washington and examined for the presence of R. salmoninarum by the fluorescent antibody test (FAT) as described by Fryer (1984). The same salmonids collected from the Columbia River at Jones Beach and transported to RBH-IF or OSU-FDL as part of the C. shasta studies were also examined for R. salmoninarum infections. All fish that died within 10 days of arrival at either facility were considered handling deaths and not included in the data.

Eggs from spawning chinook salmon at Round Butte Hatchery were incubated and hatched at the OSU-FDL. Smears made from the kidney, spleen and egg washings were examined by FAT for R. salmoninarum. These eggs and resulting fry were cultured for R. salmoninarum on KDM-2 and charcoal agar (Daly and Stevenson, 1985). Egg washings were prepared by collecting approximately 100 eggs at spawning into a beaker containing 10 ml of phosphate buffered saline (pH 7.2). The eggs and PBS were stirred briefly and the liquid decanted. The liquid was centrifuged at 2010 x g for 20 min, the supernatant discarded and

the pellet smeared on a slide and examined by FAT for the presence of fluorescing bacteria characteristic of R. salmoninarum.

#### Production of Monoclonal Antibodies

Monoclonal antibody technology makes it possible to analyze strain specific and cross-reacting antigens among a particular bacterial species. This type of data is necessary for the most effective vaccine production and for the detection of carrier fish.

A modification of the method described by Oi and Herzenberg (1980) was used to produce hybridomas secreting antibody against three strains of R. salmoninarum (Fryer 1984). Strains used were Lea-1-74 (ATCC 33209) (LB) isolated from chinook salmon at Leaburg Hatchery, RB-1-73 (RB) from chinook salmon at Round Butte Hatchery and K50 from Atlantic salmon (Salmo salar) in Norway. Lymphocytes harvested from mice immunized with R. salmoninarum were fused with SP2 mouse myeloma cells in medium containing 50% polyethylene glycol. After incubation in hypoxanthine, aminoptein, thymidine selective medium, each well of the tissue culture plate containing visible hybridomas was tested by the indirect enzyme-linked immunosorbent assay (ELISA) for production of anti-R. salmoninarum antibody. Hybridoma cultures producing the desired antibody were cloned and assayed twice. Selected hybridomas were grown to be frozen in storage or for in vitro assay.

The ELISA used to screen for hybridomas producing anti-R. salmoninarum antibody was modified as described below to increase sensitivity and obtain consistent results. Different concentrations of R. salmoninarum cells were attached to 96-well polystyrene microtiter plates (Immulon) which were previously washed with poly-L-lysine. Test serum or supernatant from cultures containing a hybridoma was added to the wells then incubated with peroxidase-

conjugated anti-mouse antibody. A positive culture was detected by a visible color reaction following addition of 0-phenylene diamine substrate. The reaction was quantified by determination of optical density using an automated microplate reader (Biotek EL310). The ELISA was used to detect positive hybridomas and for cross reactivity assays.

## Results and Discussion

### Prevalence of BKD in Ocean and Columbia River Salmonids

Additional kidney smears taken from salmonids captured in the open ocean off the coast of Oregon and Washington during 1984 were examined nonquantitatively for R. salmoninarum. Table 5 shows the cumulative data since seining began in 1981. Consistently, chinook salmon have had the highest incidence of the bacterium and lesions associated with the disease. Lesions of BKD and kidney smears containing >100 R. salmoninarum bacteria per microscope field at 400X magnification have also been detected in coho salmon and one steelhead trout. This level of R. salmoninarum in a kidney smear is readily demonstratable by Gram stain and represents, in our opinion, an ongoing active BKD infection which will shortly result in death of the animal.

Chinook salmon seined from fresh water in the Columbia River and then held in salt water at the OSU-MSC for 150 days had extremely high levels (12-68%) of R. salmoninarum as compared to groups captured at the same time but held in fresh water at the RBH-IF (1-122) (Tables 6 and 7). These results are similar to Banner et al. (1983) who found the incidence of R. salmoninarum, detected by FAT, in smolts sampled while still in fresh water could not be used to forecast the prevalence of BKD after moving fish to salt water. In three groups in which R. salmoninarum was not detected, approximately 10% of the animals had died from BKD after 100 days in salt water. Three additional

Table 5. Prevalence of Renibacterium salmoninarum in juvenile salmonids captured in the ocean off the coast of Oregon and Washington from 1981 through 1984<sup>1</sup>.

Salmonid species	Numbers examined	Percent positive by FAT for <u>R. salmoninarum</u> '	Percent positive with >100 bacteria per microscope field <sup>2</sup>
Chinook salmon	878	11	7
Coho salmon	2276	4	2
Chum salmon	197	3	0
Pink salmon	85	6	0
Steelhead trout	99	3	1
Cutthroat trout	104	1	0

<sup>1</sup>All fish were examined for R. salmoninarum by the fluorescent antibody test.

<sup>2</sup>Represents, in our opinion, fish with ongoing active BKD infections.

Table 6. Prevalence of Renibacterium salmoninarum in chinook salmon (Oncorhynchus tshawytscha) smolts beach-seined from the Columbia River (Rkm75) and held 150 days in salt water at Marine Science Center Fish Disease Laboratory.

Date collected 1984	Number collected	Holding mortalities	Mortalities infected with <u>R. salmoninarum</u>	Percent of mortalities infected with <u>R. salmoninarum</u>	Percent of fish collected infected with <u>R. salmoninarum</u> <sup>1</sup>
July 5	93	86	43	50	54
26	76	76	41	54	54
31	75	70	42	60	61
Aug. 16	57	56	7	13	12
23	89	80	34	43	34
Sept. 20	70	68	29	43	68
Totals	460	436	196	45	45

<sup>1</sup>All fish were examined by the fluorescent antibody test for R. salmoninarum.

Table 7. Prevalence of Renibacterium salmoninarum in chinook salmon (Oncorhynchus tshawytscha) smolts beach-seined from the Columbia River (Rkm75) and held 150 days in fresh water at Round Butte Hatchery Isolation Facility.

Date collected 1984	Number collected	Holding Mortalities	Mortalities infected with <u>R. salmoninarum</u>	Percent of mortalities infected with <u>R. salmoninarum</u>	Percent of fish collected infected with <u>R. salmoninarum</u> <sup>1</sup>
July 5	88	76	2	3	3
26	75	22	1	5	12
31	84	31	4	13	7
Aug. 16	82	34	5	15	6
23	87	74	1	1	1
Sept. 20	47	27	2	7	4
Totals	463	266	15	17	6

<sup>1</sup>All fish were examined by the fluorescent antibody test for R. salmoninarum.

groups, in which the microorganism was detected, suffered losses ranging from 17-49% (Banner et al. 1983). To determine whether or not R. salmoninarum was detectable in smolts immediately after seining from the Columbia River, the handling mortalities that died within 10 days after arrival at our holding facilities were examined by FAT. This procedure detected R. salmoninarum in 13% (24/181) of the fish examined. Comparing these data with the results from the groups held in fresh and salt water suggests that R. salmoninarum is a much more effective pathogen in the salt water environment. The recurrent finding of R. salmoninarum and lesions characteristic of BKD in juvenile salmonids seined from the open ocean further supports this hypothesis. Additionally, we have recently completed horizontal transmission experiments in salt water at the OSU-MSU which show that >80% of chum salmon (Oncorhynchus keta) smolts exposed to  $10^6$  cells/ml of R. salmoninarum for 30 min will die within 90 days, often with characteristic lesions of BKD.

#### Transmission of Renibacterium salmoninarum

Egg washings and kidney smears have been collected from individual spawning adult spring chinook salmon at Round Butte Hatchery during 1983, 1984 (Fryer, 1984) and again in 1985. Results from 1985 as in the previous years indicate that additional fish infected with R. salmoninarum will be detected if egg washings and smears from the spleen are examined for the BKD microorganism. In 1984 12 of the 20 (60%) spleens collected were infected with BKD (Fryer 1984) and in 1985 three of the 30 (10%) spleens collected from the fish spawned were infected; several of these infections were not detected in either the kidney or egg washing samples. The results reported by us in 1984 and again in 1985 have consistently demonstrated that the examination of only kidney samples for BKD will underestimate the incidence of this disease

and that the spleen and egg washings should be considered when examining for BKD carrier fish.

Our previous work (Fryer, 1984) has indicated that fluorescing bacteria typical of R. salmoninarum can still be found in fertilized eggs examined just prior to hatching (after one month of incubation). Attempts to culture the microorganism from these eggs have been unsuccessful. These efforts are continuing in 1985 by sampling Round Butte Hatchery chinook salmon eggs and fry at frequent intervals during incubation and after hatching.

#### Monoclonal Antibodies

Four to five stable antibody producing hybridoma clones were selected for serological comparisons of R. salmoninarum. Isoelectric focusing procedures further confirmed, by the presence of a single family of protein bands on Servalyt precoated gels, that the antibodies produced by these hybridomas were monoclonal. The antibodies produced by these clones were characterized by class and subclass of immunoglobulin. All were classified as murine IgG1 or IgG3, with the exception of one in the IgG2a class and subclass. Murine IgG subclasses have distinct biological properties that are important in determining applicability and methods of purification (Goding 1983). IgG1 and IgG2a are major subclasses in the mouse and can be purified by binding to Staphylococcal protein A. Eight of the 13 monoclonal antibodies selected for this study belong to these two subclasses and can be purified and labelled for use as FAT reagents. Glycolipids of cell membranes are considered highly immunogenic (Goding 1983) and Collins (1982) reported the presence of an unusually large number of glycolipids in R. salmoninarum. Monoclonal antibodies K23/15, K8/9, RG8/6, R.F8/9 and LE3/5 were all classified as IgG3 and may be associated with cell membrane glycolipids.

Cross reactivity of the monoclonal antibodies was tested by ELISA (Table 8). All of the K50 monoclonal antibodies, with the exception of K18/4, reacted only with the K50 and LB strains. All of the LB and RB monoclonal antibodies reacted only with the LB and RB strains. There was no cross reactivity between K50 and RB. The specificity of these reactions was confirmed by cross adsorptions. The monoclonal antibodies produced so far indicate the LB and K50 strains have a common antigenic determinant and that the LB and RB strains have a common antigenic determinant.

The reactivity of the monoclonal antibodies was then tested against other strains of R. salmoninarum (Table 9). With the exception of K18/4, none of the K50 monoclonal antibodies reacted with any of the other strains tested. The LB and RB monoclonal antibodies reacted only with the Jones Beach strain. Reactivity was confirmed by cross adsorptions. There was no difference in reactivity when any of these antigens were heat treated, indicating the monoclonal antibodies are directed against heat stable antigens. Strains of R. salmoninarum from different geographic locations are being collected for further cross reactivity testing. Fusions using different strains of R. salmoninarum will be done pending results of these assays.

The specificity of the monoclonal antibodies was tested by ELISA against other Gram positive bacteria. Only K18/4 showed reactivity, which appears to be against a common antigen shared by these Gram positive bacteria (Table 10). The demonstration of this common antigen increases evidence for cross reactions with bacteria from other genera when polyclonal serum is used as a diagnostic reagent.

Fluorescent antibody tests have been routinely used for the detection of BKD infections. It was therefore desirable to test the reactivity of the monoclonal antibodies by FAT. In contrast to ELISA reactivity, the immunofluorescent reactivity of the K50 monoclonal antibodies showed less

Table 8. Reactivity of monoclonal antibodies against Renibacterium salmoninarum as tested by enzyme-linked immunosorbent assay.

<u>R. salmoninarum</u> strain used for fusion	Monoclonal antibody	<u>R. salmoninarum</u> antigen used		
		K50	RB	LB
K50 <sup>1</sup>	K34/4	+		+
	K8/9	+		+
	K23/15	+		+
	K11/12	+		+
	K18/4	+	+	+
RB <sup>2</sup>	RC10/1	-	+	+
	RC7 /2	-	+	+
	RF8/9	-	+	+
	RG8/6	-	+	+
LB <sup>3</sup>	LB11/11	-	+	+
	LB11/9	-	+	+
	LB9/8	-	+	+
	LE3/5	-	+	+

<sup>1</sup>Isolated from Atlantic salmon in Norway.

<sup>2</sup>Isolated from chinook salmon at Round Butte Hatchery, Oregon.

<sup>3</sup>Isolated from chinook salmon at Leaburg Hatchery, Oregon.

Table 9. Reactivity of monoclonal antibodies with Renibacterium salmoninarum isolates tested by enzyme-linked immunosorbent assay.

Monoclonal antibody	<u>R. salmoninarum</u> antigen used					
	K70 <sup>1</sup>	SS <sup>2</sup>	McK <sup>3</sup>	CR <sup>4</sup>	JB <sup>5</sup>	SIL <sup>6</sup>
K50						ND <sup>7</sup>
K18/4	+	+	+	+	+	+
RB			ND	-	+	
LB			ND	-	+	

<sup>1</sup> Isolated from Atlantic salmon in Great Britian.

<sup>2</sup> Isolated from chinook salmon at South Santiam Hatchery, Oregon.

<sup>3</sup> Isolated from chinook salmon at McKenzie Hatchery, Oregon.

<sup>4</sup> Isolated from chinook salmon at Cole Rivers Hatchery, Oregon.

<sup>5</sup> Isolated from chinook salmon at Jones Beach (Rkm75) on the Columbia River, Oregon.

<sup>6</sup> Isolated from coho salmon at Siletz Hatchery, Oregon.

<sup>7</sup> ND - not done.

Table 10. Reactivity of monoclonal antibodies against Renibacterium salmoninarum with other Gram positive bacteria tested by enzyme-linked immunosorbent assay.

Monoclonal Antibody	Bacterial antigen used		
	<u>Lactobacillus</u> <u>piscicola</u>	<u>Bacillus</u> <u>subtilis</u>	<u>Streptococcus</u> <u>lactis</u>
K50			
K18/4	+	+	+
RB			
LB			

specificity (Table 11). The reactivity of the LB and RB monoclonal antibodies by FAT correlated with results obtained with ELISA.

Antigenic differences among strains of R. salmoninarum have been demonstrated using monoclonal antibodies. This information will be important in the development of certain vaccines and standard reagents for immunodiagnosis. The specific antigenic determinant against which these monoclonal antibodies are directed will be analyzed by immunological detection of proteins on nitrocellulose (western blot). The monoclonal antibodies produced so far cannot be used individually as standard reagents for detection of R. salmoninarum by immunofluorescent assay or ELISA but it may be possible to produce a reagent for immunodiagnosis of BKD by combining two or more well-defined, specific monoclonal antibodies.

#### Infectious Hematopoietic Necrosis Virus

Infectious hematopoietic necrosis virus (IHNV) has recently become more widespread in the Columbia River Basin and has caused severe losses among chinook salmon and steelhead trout at several Columbia River Basin fish hatcheries. No anti-IHNV drugs are known; therefore, management techniques to avoid the virus, especially during the egg incubation and fry stages, are being tested. Since 1983 at Round Butte Hatchery, we have used W-treated water for rearing of fish and have also selected eggs and sperm from virus-free adults.

During the spawning of steelhead trout in 1984 at Round Butte Hatchery a unique and potentially very important observation was made. On three separate occasions IHNV was detected in ovarian fluid samples after storage for 6-9 days at 4°C. No virus had been detected in these samples when collected at spawning. Routine sampling for IHNV requires only the processing of tissues

Table 11. Reactivity of monoclonal antibodies against strains of Renibacterium salmoninarum tested by the indirect fluorescent antibody test.

R. <u>salmoninarum</u> a n t i g e n	Origin of R. <u>salmoninarum</u> strains	Monoclonal antibody			
		K50	RB	LB	
K50	Saltwater pen culture facility, Norway, Atlantic salmon		+	-	-
RB	Round Butte Hatchery, Oregon, chinook salmon	+/-	+		+
LB	Leaburg Hatchery, Oregon, chinook salmon	+	+		+
K70	England, Atlantic salmon	+/-	-		-
SS	South Santiam Hatchery, Oregon, chinook salmon	ND	-		-
McK	McKenzie River Hatchery, Oregon, chinook salmon	ND	-		-
CR	Cole Rivers Hatchery, Oregon, chinook salmon	+/-	-		-
JB	Jones Beach, Columbia River Oregon, chinook salmon	+/-	+		+
SIL	Siletz River Hatchery, Oregon, coho salmon	+/-	-		-

ND = not done

+ = >10 cells/field fluorescing

+/- = 1 - 5 cells/field fluorescing

= 0 cells/field fluorescing

and sex fluids taken at spawning; however, this delayed appearance of virus indicates that sampling only at spawning may yield false negatives. These observations raise the possibility that IHNV is more widespread among salmonid populations than previously considered. Further, the production of IHNV by constituent(s), probably cellular, in ovarian fluid represents a novel method for studying the biology of IHNV.

## Materials and Methods

### Virus Propagation and Detection

Procedures for virus propagation have been described previously (Fryer 1984). Chinook salmon embryo (CHSE-214) and epithelioma papillosum cyprini (EPC) cell lines were continuously cultured in Eagle's minimum essential medium (MEM) supplemented with fetal calf serum (10%),  $\text{NaHCO}_3$  (0.075%), penicillin (100 iu/ml), streptomycin (100 ug/ml) and glutamine (1.0%). The EPC MEM growth medium was buffered with Tris hydrochloride instead of  $\text{NaHCO}_3$ . Growth temperatures were 16°C for CHSE-214 cells and 22°C for EPC cells.

Plaque assay procedures as described previously (Fryer 1984) were similar to Burke and Mulcahy (1980). Briefly, assays were performed using confluent EPC monolayers grown in multi-well tissue culture plates. Samples were diluted in Hank's balanced salt solution (HBSS). Replicate 0.1 ml samples ( $10^0$ - $10^{-4}$ ) were inoculated onto monolayers in individual wells and allowed to adsorb for 60 min. Sample inoculum was removed and 1% methyl-cellulose dissolved in double strength MEM plus 5% fetal calf serum overlay medium (MEM-5) was added. Following 10 days of incubation at 16°C cells were fixed with formalin and stained with 1% crystal violet solution. Plaques were counted

and plaque forming units per ml (PFU/ml) were determined in replicate wells containing 10-300 plaques.

Ovarian and seminal fluids were collected and processed as previously described (Fryer 1984) except supernatant fluids were mixed 1:1 with an antibiotic solution (McDaniel 1979) before inoculation onto cells. Ovarian fluid samples were also passed through 0.22  $\mu$ m acrodisc filters (Gelman) to obtain cell-free preparations. Tissue cells from ovarian fluid samples were cultured in 75  $\text{cm}^2$  tissue culture flasks in a media consisting of  $\text{NaHCO}_3$  buffered MEM plus 10% fetal calf serum with antibiotics (McDaniel 1979).

#### Fish Egg Inoculations

To determine whether survival and/or replication of IHNV occurred during incubation coho salmon (Cole Rivers Hatchery) and steelhead trout (Leaburg Hatchery) eggs were fertilized, water hardened for 1 h and injected into the yolk using a 30 gauge needle with 0.01 ml of a IHNV suspension containing  $1.53 \times 10^6$  and  $5.25 \times 10^5$  PFU/ml, respectively. Eggs in the control groups were injected with MEM only and handled in the same manner as those in the experimental groups. The eggs were held in fish pathogen-free well water at the OSU-FDL until hatching. Five eggs were collected and individually assayed for IHNV survival at five day intervals. The eggs were allowed to hatch and fry that died were cultured for IHNV. Dead steelhead trout eggs were sampled beginning on day 10 of embryonic development.

#### Concentration of IHNV from Water

Laboratory experiments with the tangential flow filtration apparatus were continued during 1985 and field applications of this process conducted at Round Butte Hatchery. The Pellicon cassette system used had a membrane

exclusion size of 100,000 molecular weight. Based on data gained from our laboratory experiments with virus stabilizing agents, fetal bovine serum (FBS) (0.1%) was added to each water sample to improve virus recovery.

Water samples from Round Butte Hatchery were collected at selected times from March through May, 1985. During this period, nine samples of the hatchery water supply, three samples of effluent water from the steelhead trout holding pond and one sample of effluent water from a 6 ft circular tank containing steelhead trout fry infected with IHNV were collected. Adult steelhead trout in the holding pond were also infected with IHNV.

Fifty liters of each sample was collected in a sterile carboy, supplemented with 0.1% FBS, and either filtered at the hatchery or transported to our laboratory in Corvallis. Maximum storage of samples before filtering was three days at 4°C. One hundred milliliters of each sample was also collected in sterile bottles and supplemented with 0.1% FBS. Each sample was inoculated directly onto monolayers of EPC cells to determine whether virus detection was possible without employing molecular filtration. The 100 ml samples and the retentate and backflush solutions collected from each 50 liter sample were concentrated further by ultracentrifugation. The pellet from this step was resuspended in a small volume of tissue culture fluid and inoculated onto EPC cells.

#### Design of IHNV Transmission Studies

The purpose of this two-part study at Round Butte Hatchery was to determine, on a production scale, if IHNV is horizontally transmitted via the water supply or is transmitted vertically via infected gametes from carrier adults or if both routes of transmission occur. The experimental design of the studies in 1985 is the same as in 1984 and have been described in detail previously (Fryer 1984) (Appendix A).

In 1985 there was continuous monitoring of the **W** treatment system to insure that the radiation output was always within the manufacturer's specifications remaining continuously above 68% on the W monitor. In addition, total bacterial counts of the water immediately before and after UV exposure were performed at selected intervals. Spread plates of 1.0, 0.25 and 0.1 ml water samples in triplicate were made using trypticase soy agar (Difco) and cytophaga agar (Anacker and Ordal 1959). Plates were incubated for 5-7 days at 16°C and the total bacterial counts showed the water treatment system to be killing >90% of the bacteria in the water supply. Turbidity measurements of hatchery water were recorded daily to determine if siltation could be affecting the W treatment system. Readings were taken using a Spectronic 20 at 520 pm using nanopure water as the control. No siltation was detected in any of the samples.

## Results and Discussion

### Recovery of IHN and IPNV by Molecular Filtration

Previously we have reported a 67% recovery of IHN by molecular filtration of seeded OSU-FDL water supplemented with 1% fetal calf serum (Fryer 1984). During 1984 we continued experiments with this filtration procedure by seeding IHN into deionized and Round Butte Hatchery water. Describing results using deionized water allows other researchers to evaluate and compare their data with ours.

Filtration of unsupplemented water continued to result in a low virus recovery, again indicating the need for a virus stabilizer (Table 12). Several concentrations, 0.01, 0.1 and 1.0%, of FBS were used to supplement the deionized water during filtration. Virus recoveries of 95 and 96% resulted

when 1.0 and 0.1% FBS were used, respectively, and decreased to 45% with the use of 0.01% FBS (Table 12).

Supplementation with beef extract resulted in virus recoveries only slightly lower than those with FBS. With the addition of 0.03 and 0.3% beef extract, 80 and 61%, respectively, of the seed virus was recovered. Addition of either 0.01 or 0.1% glycine did not enhance the recovery of IHNV and gave recoveries of <1.0%; lower values than obtained from unsupplemented water (Table 12).

The potential for recovery by molecular filtration of another fish virus, infectious pancreatic necrosis virus (IPNV), was also evaluated (Table 13). An average of 29% recovery was obtained when seeded deionized water was filtered; adding 0.1% FBS to the water before filtration increased the recovery to 68%, a value similar to that obtained for IHNV.

Field applications of the molecular filtration system were to be at Round Butte Hatchery. Therefore, laboratory trials were continued using hatchery water (Table 14). Using 1.0% FBS as a supplement, IHNV recoveries were 100 and 59% from two filtrations. Reducing the concentration of FBS resulted in recoveries averaging 68% from two runs with an initial volume of 10 liters and 71% from three runs of 50 liters each. Pretreatment to prevent virus absorption of the filter membranes by recirculation of 1.0% FBS failed to improve virus recovery, suggesting that low recoveries of IHNV and possibly IPNV resulted from virus inactivation rather than absorption to the membrane.

The data from these tests indicated that molecular filtration procedures can effectively concentrate IHNV and IPNV from water, provided the water sample is supplemented with a virus stabilizing agent. A concentration of 0.1% FBS appears optimum for IHNV recovery. At the lowest concentration of FBS tested (0.01%), virus recovery was reduced about 20%. At the higher

Table 12. Recovery of virus by tangential flow filtration from deionized water seeded with known concentrations of infectious hematopoietic necrosis virus.

Filtrate run and handling	Initial volume of water (liters)	Initial virus concentration (PFU/ml)	Retentate volume (mLs)	Infective particles in retentate (PFU/ml)	Percent virus recovery	
					Retentate	Filtrate
Deionized water	10	75	245	$1.0 \times 10^5$	13	<1
0.01% FBS <sup>1</sup> added	10	130	197	$5.8 \times 10^5$	45	<1
0.1% FBS added	10	79	186	$7.6 \times 10^5$	96	<1
1.0% FBS added	10	19	343	$1.8 \times 10^6$	95	<1
0.01% glycine added	10	44	134	<10	<1	<1
0.1% glycine added	10	76	103	10	<1	<1
0.03% beef extract added	10	21	252	$1.7 \times 10^5$	80	<1
0.3% beef extract added	10	46	250	$2.8 \times 10^5$	61	<1

<sup>1</sup>Fetal bovine serum.

Table 13. Recovery of virus by tangential flow filtration from deionized water seeded with known concentrations of infectious pancreatic necrosis virus.

Filtrate run and handling	Initial volume of water (liters)	Initial virus concentration (PFU/ml)	Retentate volume (mls)	Infective particles in retentate (PFU/ml)	Percent virus recovery	
					Retentate	Filtrate
Deionized water	10	$1.4 \times 10^3$	247	$4.4 \times 10^6$	31	<1
Deionized water	10	$1.9 \times 10^3$	358	$5.0 \times 10^6$	26	<1
0.1% FBS <sup>1</sup> added	10	$1.3 \times 10^3$	345	$7.4 \times 10^6$	57	<1
0.1% FBS added	10	$1.4 \times 10^3$	482	$1.1 \times 10^7$	79	<1

<sup>1</sup>Fetal bovine serum.

Table 14. Recovery of virus by tangential flow filtrates from Round Butte Hatchery water seeded with known concentrations of infectious hematopoietic necrosis virus.

Filtrate run and handling	Initial volume of water (liters)	Initial virus concentration (PFL!/ml)	Retentate volume (mls)	Infective particles in retentate (PFU/ml)	Percent virus recovery	
					Retentate	Filtrate
1.0% FBS <sup>1</sup> added	10	120	167	1.2x10 <sup>6</sup>	100	<1
1.0% FBS added	10	130	179	7.6x10 <sup>5</sup>	59	<1
0.1% FBS added	10	110	282	6.0x10 <sup>5</sup>	55	<1
0.1% FBS added	10	120	275	9.6x10 <sup>3</sup>	80	<1
0.1% FBS added	50	19	270	5.9x10 <sup>5</sup>	63	<1
0.1% FBS added	50	14	280	6.3x10 <sup>5</sup>	88	<1
0.1% FBS added then water transported to OSU	50	24	303	7.8x10 <sup>5</sup>	63	<1
Pretreatment of filter with 1.0% FBS	10	190	179	3.5x10 <sup>5</sup>	18	<1

<sup>1</sup>Fetal bovine serum.

concentration (1.0%), although viral recovery was the same, the cost of adding FBS becomes a factor, and the time of filtration was greatly prolonged due to increased viscosity of the retentate. Fetal bovine serum also stabilizes IHNV for a considerable period of time, with one sample after the virus seed and FBS were added at Round Butte Hatchery the water was transported to OSU, about 5 h, and then held overnight at 4°C. Recovery under these conditions was 63% (Table 14), comparable to other runs in which filtration began immediately after water collection and virus addition.

Field studies using molecular filtration to detect wild type IHNV were conducted at selected time intervals during March through May, 1985 at Round Butte Hatchery. Throughout this period, nine samples of the hatchery water supply were filtered to determine whether the virus was entering the hatchery system through the water supply. To optimize the chances of virus isolation from water, samples of water where fish were diagnosed positive for IHNV were also taken; three samples of effluent from the adult steelhead trout holding pond and one from a 6 ft circular tank where steelhead trout fry were dying of IHN disease were filtered.

No IHNV was detected in any of the nine samples of the hatchery water supply tested (Table 15). The inability to isolate virus from these samples results from either the low concentration or perhaps the absence of virus in the sample collected. Infectious hematopoietic necrosis virus was isolated (and confirmed by serum neutralization) in two samples of effluent water from the adult holding pond at concentrations of approximately 1 PFU per 5 ml of water filtered. These two water samples were taken when approximately 40 adult steelhead trout were present. A later sample when 15 adults remained in the pond did not yield virus. The virus was also detected and confirmed by serum neutralization at a level of approximately 1 PFU per 50 ml in effluent water from the tank containing steelhead trout fry infected with IHN disease.

Table 15. Recovery of infectious hematopoietic necrosis virus by tangential flow filtration from Round Butte Hatchery water.

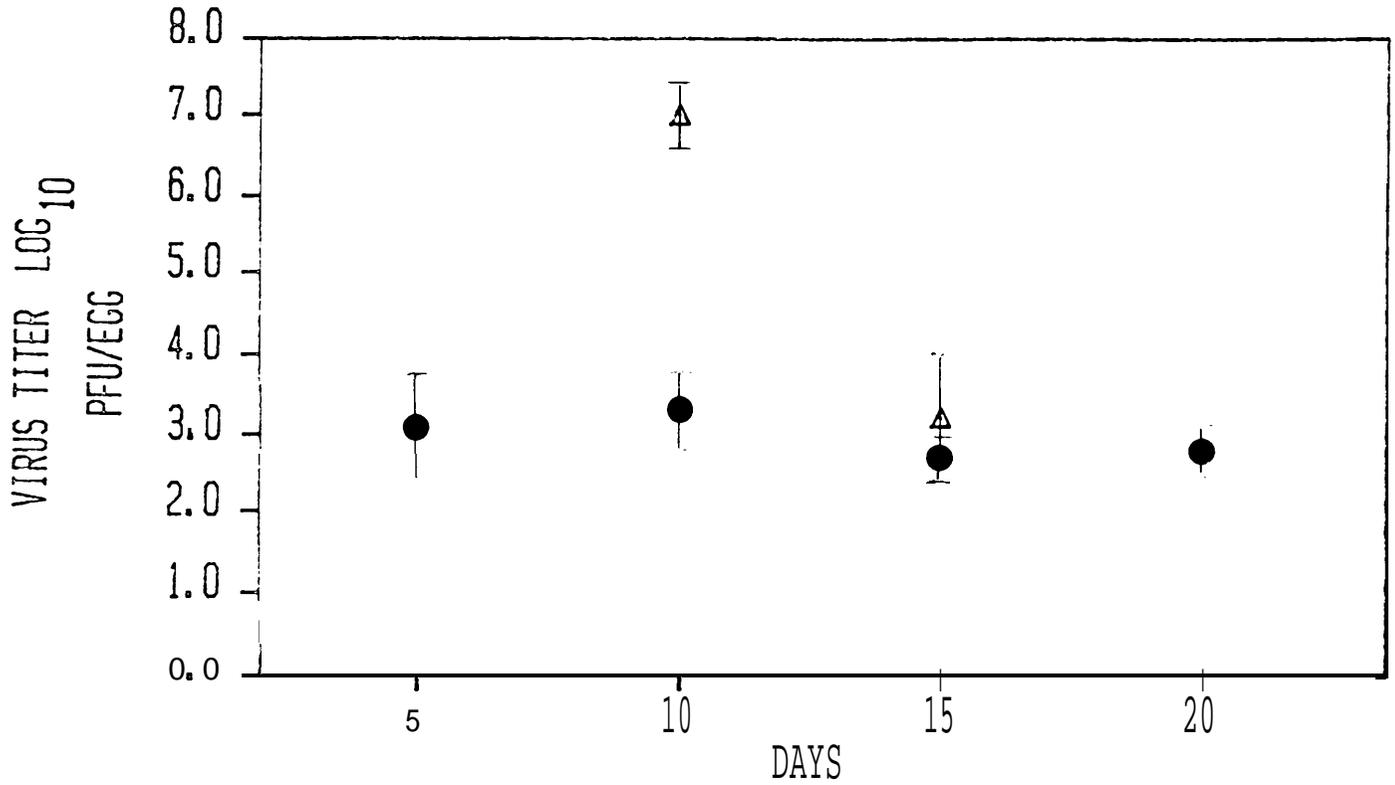
Water source	Number of samples	Virus recovery			
		Retentate	Untracentrifuged retentate	Unfiltered water	Ultracentrifuged water
Hatchery water supply before entering hatchery	9	No virus	No virus	No virus	No virus
Effluent water from adult holding pond		IHNV 2/3 samples	IHNV 2/3 samples	Not done	Not done
Effluent water steelhead trout fry	1	IHNV	IHNV	No virus	IHNV after blind passage

The effluent from the adult holding pond and the tank containing steelhead fry is released directly into the Deschutes River at Lake Simtustus. We estimated that approximately  $5 \times 10^8$  IHN virus particles were released in the effluent water during the 24 hour period when water was collected from the adult holding pond. At this time only 40 fish were present; however, at the peak of spawning approximately half of the 2,000 adults present were infected with IHN. This suggests IHN levels in the order of  $10^{10}$  were being released each day into the Deschutes River. Results from data obtained by Leong and Turner (1979) at Round Butte Hatchery also indicated that similar levels of virus were released each day in effluent water from egg trays containing infected fry. Virus particles released from these fish are a possible source of infection for downstream fish populations because IHN can survive for approximately seven weeks in soft and hard lake water at 10°C (Wedemeyer et al. 1978) and can be easily transmitted from fish to fish (Wingfield and Chan 1970).

#### Survival of IHN in Fish Eggs

Previously we reported survival of IHN with a slow decrease in titer for 18 days in unfertilized rainbow trout eggs and not only survival but replication after injection of eyed steelhead trout eggs (Fryer 1984). Further experiments were conducted with steelhead trout eggs which were infected 1 h post fertilization. Virus survival was monitored throughout the developmental period (24 days) and titers remained relatively constant (Fig. 1). Replication of IHN was detected in dead eggs sampled on day 10, indicating that the virus may have been the cause of death; however, no virus was detected in the fry that were sampled. Mulcahy and Pascho (1985) have also recently reported the isolation of IHN from dead sockeye salmon

Figure 1. Virus recovered from fertilized steelhead trout (Salmo gairdneri) eggs injected with known concentrations ( $\log_{10} = 3.7$ ) of infectious hematopoietic necrosis virus.



Mean + SD of virus recovered from five viable eggs (O) and from five dead eggs (Δ) individually sampled and assayed. No virus detected (<400 pfu/egg) in dead eggs sampled on day 20.

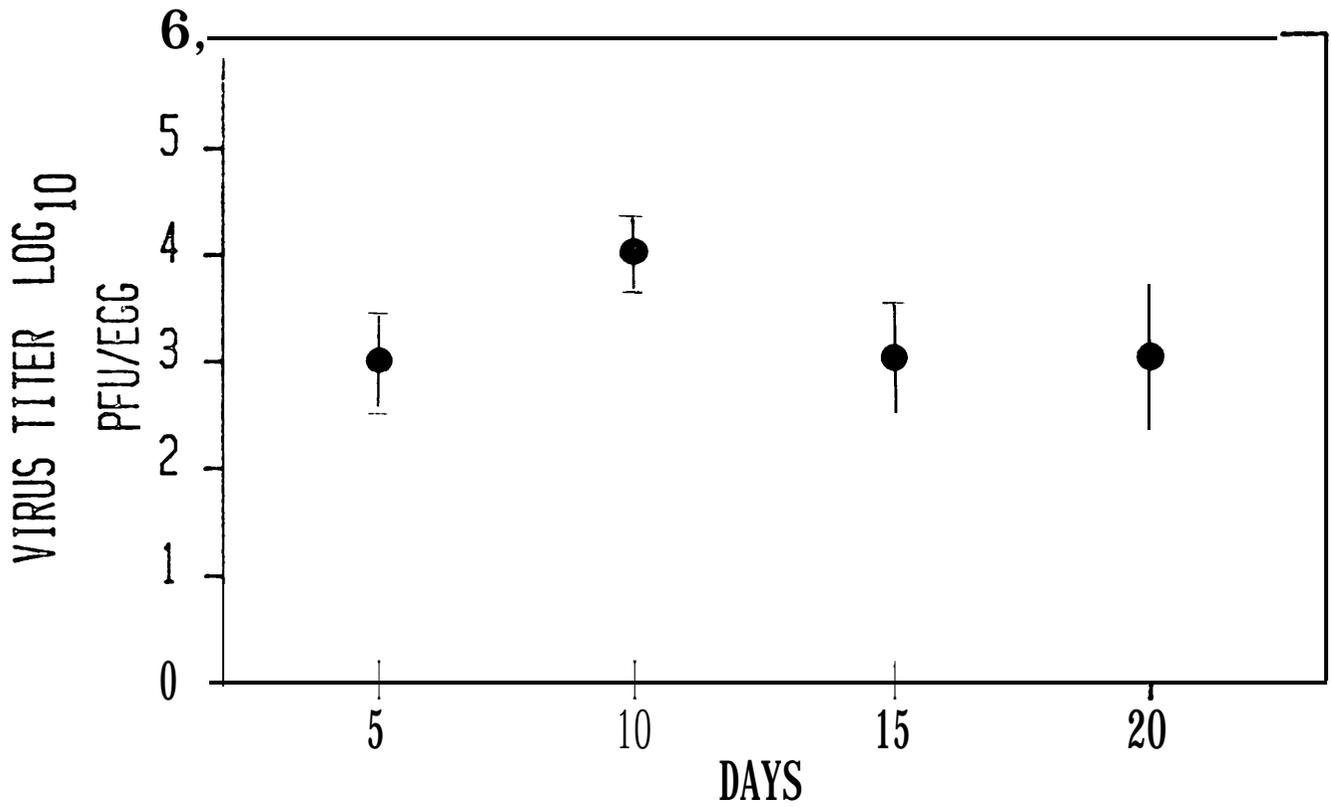
(Oncorhynchus nerka) eggs and fry. Egg survival to hatching seemed to correlate with the amount of virus injected, only 6% of the eggs injected with  $10^6$  PFU/ml hatched compared to 15% of the eggs injected with  $10^4$  PFU/ml.

Coho salmon eggs injected with IHNV 1 h post fertilization had about a two log decrease in virus survival during the 30 day period of embryonic development (Fig. 2). This species appeared more refractive to IHNV with approximately 35% of the injected eggs hatching. The resulting fry were observed and IHNV was isolated from several mortalities.

These laboratory experiments demonstrated survival of IHNV in unfertilized eggs and virus replication in developing embryos and hatched fry. Although the virus was artificially introduced into these eggs the prolonged survival and replication that occurred suggests vertical transmission of this disease is possible. Isolation of virus from dead eggs suggests that IHNV replication during incubation may be a possible cause of egg mortality.

Selected lots of steelhead trout eggs from the Round Butte Hatchery vertical transmission experiments were incubated at the OSU-FDL and sampled daily for IHNV. In 1984 only viable eggs were sampled and one egg collected on day 16 from the group with high-titer IHNV parents was positive for the virus. We concluded that the results from egg inoculations plus the detection of IHNV associated with an egg from known positive parents suggests vertical transmission; however, the finding of only one PFU (>400 virus particles) from a single egg indicates it is an infrequent event. Recently Mulcahy and Pascho (1985) reported isolation of the virus from dead eggs. This year's sampling at Round Butte Hatchery included dead eggs and fry; no IHNV was isolated from these samples.

Figure 2. Virus recovered from fertilized coho salmon (Oncorhynchus kisutch) eggs injected with a known concentration ( $\log_{10} = 4.2$ ) of infectious hematopoietic necrosis virus.



Mean + SD of virus recovered from five eggs individually sampled and assayed. No virus detected (<400 pfu/egg) by plaque assay on days 25 and 30.

## Transmission of THNV at Round Butte Hatchery

Adult steelhead trout used in the horizontal transmission experiment at Round Butte Hatchery had a IHN carrier rate of 63% in the females and 13% in the males (Table 16, Appendix A). In this experiment half of the eggs divided into 8 subgroups were incubated and resulting fry reared in UV treated water. Eight replicate subgroups were held in untreated hatchery water and fish in two of these developed IHN disease (groups 4 and 5). The IHN incidence in adults of these two groups was not significantly different from that of the overall carrier rate; however, they did have higher titers of virus in their 18 fish milt pools. This empirical observation could support the postulated mechanism of vertical transmission of IHN which involves virus binding to sperm and subsequently being carried into the egg (Mulcahy and Pascho 1984).

Untreated water was used in the incubation and rearing of group 5 and UV treated water was supplied to group 4. Although IHN occurred in both, the course of the disease was different. The fish reared in untreated water experienced an explosive, high mortality which reached approximately 90%. The disease in fish held in UV treated water was less severe and the mortality did not exceed 5%. Similar results have been obtained at Dworshak National Fish Hatchery where ozone was used to treat water supplies (Joe Lientz, personal communication). Fish in untreated water experienced high losses; mortality was low in the populations in ozone treated water.

The production experiments to test vertical transmission involved adult steelhead trout which had an overall IHN prevalence of 71% in the females and a carrier rate of 16% in males (Appendix A). The gametes from these fish were separated into groups which had high titers of virus and those which had no apparent virus. A total of 200,000 fry resulted from eight group (four with

Table 16. Infectious hematopoietic necrosis virus titers in sex fluids from production groups of steelhead trout (Salmo gairdneri) spawned at Round Butte Hatchery.

Date of spawning 1985	Production Group Number <sup>1</sup>	IHNV Titer (PFU/ml)	
		Pooled Ovarian Fluids <sup>2</sup>	Pooled Seminal Fluids <sup>2</sup>
Jan. 29	1	9.7x10 <sup>3</sup>	5x10 <sup>0</sup>
	2	1.5x10 <sup>4</sup>	
	3	9.5x10 <sup>4</sup>	
	4	7.5x10 <sup>4</sup>	
	5	2.3x10 <sup>5</sup>	
March 7	6	2.9x10 <sup>6</sup>	1.0x10 <sup>1</sup>
	7	3.4x10 <sup>6</sup>	
	8	4.4x10 <sup>6</sup>	

<sup>1</sup>Each production group consisted of sex fluids from 18 males and 18 females. After fertilization each group was divided into two equal subgroups.

<sup>2</sup>Assays of individual sex fluid samples showed 18/144 (13%) of the males positive for IHNV and 91/144 (63%) of the females positive for IHNV.

high titer gametes and four with no virus), all of which were incubated and reared in UV treated water. None of these groups developed IHN disease.

Results of vertical transmission studies which have been conducted at Round Butte Hatchery during 1984 and 1985 are consistent. In both years, there has been no IHNV outbreaks in either fry from parents with high virus titers or those from parents with no apparent virus. But, there is inconsistency upon comparison of these results with those from the production groups used in the horizontal transmission experiments. Considering results from the vertical transmission experiments, it would be expected that no group of fish reared in UV treated water would develop IHN disease. However, during the two year duration of these studies, there have been groups of fish in UV treated water which have experienced this disease. There are several potential reasons for this. Among them are the following: 1) the UV treatment does not completely sterilize the water; therefore, virus in decreased numbers and/or virulence escape to infect fish and 2) vertical transmission occurs, but under, as yet, undescribed conditions.

#### Delayed Appearance of IHNV in Ovarian Fluids

The delayed appearance of IHNV in steelhead trout eggs stored at 4°C was first observed in 1984 while conducting vertical transmission experiments at Round Butte Hatchery. Detection of the virus was made from ovarian fluid of egg pools sampled eight days post-spawning in parents which had ovarian fluid determined to be THNV negative at spawning. In 1985 we repeated this observation and were also able to demonstrate this phenomenon in stored eggs from individual female fish. The data not only showed that some groups of stored gametes change from negative to positive (e.g. 0 to  $10^4$  PFU/ml) but also that in fluids of some initially positive gametes the titer increased during the storage period (e.g.  $10^2$  PFU/ml to  $10^4$  PFU/ml).

Experiments were initiated to determine the mechanism responsible for the delayed appearance of IHNV and to develop a diagnostic technique to detect it. Studies to determine the mechanism of this phenomenon were conducted on individual ovarian fluid samples from adult steelhead trout females. These included: 1) comparison of filtered (0.22  $\mu$ m membrane filters) and non filtered ovarian fluid collected at the time of spawning with ovarian fluid taken from stored gametes 8 or 16 days post-spawning, 2) seeding of large tissue culture flasks with ovarian fluid taken at the time of spawning, removal of this fluid after 24 h, addition of cell culture medium and determination of IHNV titers from this medium after selected intervals of incubation, and 3) seeding of multi-well tissue culture plates with ovarian fluid taken at the time of spawning and incubation of these samples with different media treatments. Samples were taken from these plates 8 or 16 days post-spawning and IHNV titers determined.

The results showed: 1) there was no difference in the virus titers of filtered ovarian fluid sampled at spawning and after the 8 or 16 day storage period, 2) there were increased IHNV titers in unfiltered ovarian fluid samples stored alone or with eggs for either 8 or 16 days, and 3) eleven of 24 ovarian fluid samples incubated in tissue culture flasks with media changes exhibited a constant production of virions. With the results obtained thus far, inoculation of a 24-well tissue culture plate with 0.5 ml of ovarian fluid at spawning appears to provide the most reliable method for detecting the delayed appearance of IHNV. After incubation for 24 hours the ovarian fluid sample was removed and 0.5 ml of a 1% methyl-cellulose MEM-5 overlay added to each well. Infectious hematopoietic necrosis virus titers were then calculated from samples taken from this plate 8 or 16 days post-spawning.

## SUMMARY AND CONCLUSIONS

During fiscal year 1983, Bonneville Power Administration funded a study concerning the epidemiology and control of three infectious diseases of salmonids in the Columbia River Basin. These serious fish pathogens are: Ceratomyxa Shasta, the causative agent of ceratomyxosis, Renibacterium salmoninarum, the causative agent of bacterial kidney disease and the viral disease agent infectious hematopoietic necrosis virus.

### Ceratomyxa shasta

The presence of the infectious stage of C. shasta at Little Goose Dam, first detected during the 1984 exposures, was reconfirmed during 1985. The level of ceratomyxosis detected increased from 1.1% in 1984 to 10% in 1985. None of the susceptible rainbow trout exposed during May 1985 in the Yakima and Umatilla Rivers died of ceratomyxosis. An upriver strain of steelhead trout from the Umatilla River was resistant to infection.

Results from two years of experiments show that in C. Shasta-susceptible strains of salmonids, ceratomyxosis progresses at the same rate in both fresh and salt water. Resistant strains remain highly refractive to ceratomyxosis when exposed to the infective stage immediately prior to transfer to salt water. The prevalence of ceratomyxosis in chinook salmon smolts seined from the Columbia River immediately prior to entering the estuary seems dependent on whether they are held in fresh or salt water after capture. In fresh water the disease prevalence ranged from 7-19%; in contrast, in the groups held in salt water the incidence of ceratomyxosis was greatly reduced (0-3%).

### Renibacterium salmoninarum

Additional kidney smears taken from salmonids captured in the ocean off the coast of Oregon and Washington during 1984 were examined for R. salmoninarum. Lesions of bacterial kidney disease and kidney smears containing >100 R. salmoninarum bacteria per microscopic field have been detected in chinook salmon (7%), coho salmon (2%) and steelhead trout (1%). These levels of bacteria are readily demonstratable by Gram stain and represents an ongoing infection that will shortly result in death of the animal. Comparing data from groups of Columbia River smolts held in either fresh or salt water suggests that R. salmoninarum is a much more effective pathogen in the salt water environment. Results from three years of sampling indicate that the presence of R. salmoninarum will be detected more frequently if egg washings are examined for this microorganism. Antigenic differences among strains of R. salmoninarum have been demonstrated for the first time using monoclonal antibodies. The demonstration of common antigens present on both R. salmoninarum and other Gram positive bacteria indicates that cross reactions (false positive results) may occur with bacteria from other genera when polyclonal serum is used as a diagnostic reagent.

### Infectious Hematopoietic Necrosis Virus

Continued testing indicated that molecular filtration procedures can effectively concentrate IHNV and also another fish virus infectious pancreatic necrosis virus from water, provided the water is supplemented with a stabilizing agent such as fetal bovine serum. A concentration of 0.1% fetal bovine serum appears optimum for virus recovery. Field studies at Round Butte Hatchery with this apparatus detected wild type IHNV in effluent water from the adult holding pond and in water from a tank containing steelhead trout

infected with IHN disease. The levels of IHNV detected in these samples suggested in the order to  $10^{L0}$  virions were being released each day into the Deschutes River at the peak of spawning.

Titers of IHNV in steelhead trout eggs injected one hour post fertilization remained relatively constant throughout the developmental period. Titers in coho salmon eggs similarly injected, decrease about two logs during the 30 day incubation period. Isolation of virus from dead eggs suggests that IHNV replication during incubation may be a possible cause of egg mortality.

In the vertical transmission experiments at Round Butte Hatchery no outbreaks of IHNV disease have occurred in fry from parents with high IHNV titers or no detectable IHNV titers. Inconsistencies develop when comparing these results to those from the horizontal transmission experiments. Two possible reasons are: 1) UV treatment does not completely sterilize the water and 2) vertical transmission occurs, but under, as yet, undescribed conditions. During 1985 the fish reared in untreated water experienced a high mortality which exceeded 90%; whereas, those in UV treated water suffered a mortality which did not exceed 5%. These results are similar to those obtained at Dworshak National Fish Hatchery where ozone was used to treat water supplies.

Changes in virus titer have not been observed with 0.2  $\mu$ m membrane filtered ovarian fluid sampled at spawning or after a 8 or 16 day storage period. In contrast, IHNV production has occurred for a prolonged period in unfiltered ovarian fluid samples cultured using tissue culture techniques and media. These observations suggest that cellular components in ovarian fluid are responsible for the delayed appearance of IHNV.

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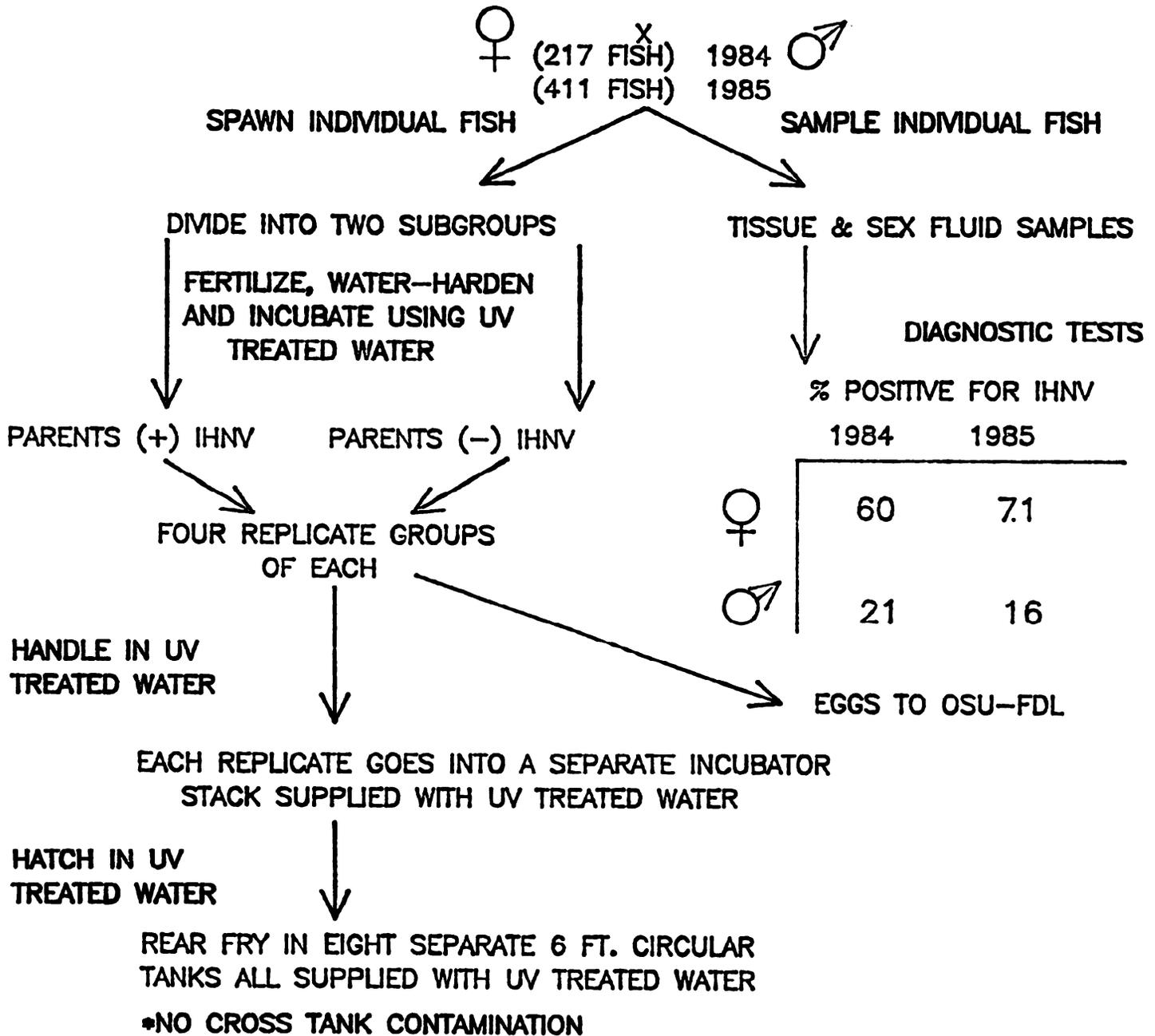
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APPENDIX A  
FLOW SHEET OF ROUND BUTTE HATCHERY  
IHNV EXPERIMENTS

# "CULL-OUT" CROSSES/VERTICAL TRANSMISSION

\* VERTICAL TRANSMISSION = INFECTION VIA ADULT TO PROGENY



\*EXPECTED BENEFIT: DETERMINATION OF EXISTENCE OF VERTICAL TRANSMISSION