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DETECTION AND TRANSMISSION OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS IN RAINBOW TROUT

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Abstract: Detection and transmission of Infectious Hematopoietic Necrosis Virus in rainbow trout (*Salmo gairdneri*) was studied at a commercial trout hatchery. Transmission of virus was demonstrated via water, feed and contaminated eggs. If eggs from carrier females were incubated several weeks in virus-free water, the resulting fry did not become infected. However, if fry subsequently became infected they were lifetime carriers. Infectious virus was readily detectable in most tissues of moribund fish; in carriers it was detected in sex products of spawning fish, and in samples from the intestine of post-spawning fish, but not in samples from blood, feces, kidney, or liver. The carrier rate was not significantly different between sexes. It was concluded that adult carriers are the reservoir of infection and that transmission occurs primarily when carriers shed virus and expose susceptible fish or eggs.

INTRODUCTION

Infectious Hematopoietic Necrosis Virus (IHNV) was recognized in sockeye salmon (*Oncorhynchus nerka*) and chinook salmon (*O. tschawytcha*) in the 1950's, but was not recognized in rainbow trout (*Salmo gairdneri*) until 1967 at a fish farm near Chilliwack, British Columbia.[3,4] By 1973 outbreaks of IHNV in rainbow trout had occurred in Minnesota, South Dakota, Montana, Idaho, Washington, West Virginia, and Colorado.[4,9,15] All outbreaks in trout, including the first, apparently originated from fry or egg shipments from a single source.

This study was initiated at the hatchery from which the virus originated to determine how the disease was being transmitted and to develop methods for prevention or control. Certain aspects of detection and transmission of IHNV in rainbow trout are reported here.

MATERIALS AND METHODS

Fathead minnow (FHM) cell cultures were used for viral isolation. Eagle's minimum essential medium (Earle's Balanced Salt Solution) and 10% agamma newborn calf serum were used in all cell cultures, at pH 7.6. Penicillin (100 IU/ml) and streptomycin (100 μg/ml) were routinely added and nystatin (40 IU/ml) was added only when primary cultures of embryonic rainbow trout were prepared. Virus isolations were performed as described by Amend[1] and samples were decontaminated with antibiotics.[2] Virological examinations were made according to Wolf[13] and primary monolayer cultures were prepared according to Fryer, *et al.*[5] Virus was quantified by the microtiter technique with the 50% endpoint ($TCID_{50}$) calculated by the method of Reed and Muench.[10]

RESULTS AND DISCUSSION

The Carrier Status

Inasmuch as IHN epizootics occurred in progeny hatched from eggs from the test hatchery, adult brook stock was circumstantially suspected as the reservoir of infection. In the first test, seminal fluid and ovarian fluid from 3- and 4-year-old rainbow trout spawning stock were tested for virus. IHNV was detected only in the ovarian fluid, but later studies indicated there was no significant

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difference ($P = .05$) by chi square analysis in the prevalence of IHNV carriers between sexes (Table 1). This finding is in sharp contrast with sockeye salmon and chinook salmon where female carriers far outnumbered male carriers[11] (Grischkowsky and Amend, manuscript in preparation).

Although virus could be detected in carrier males, females were used during most of the study because more females than males were available. Over a nine month period (September through May), when spawning activities were in progress, examination of 619 five-fish pools with about 20 pools taken weekly yielded an average IHNV detection rate of 26.2% (Table 2). No stock of fish was free of virus. The carrier rate was lowest in fall spawners, but no difference was detected between winter and spring spawners. Although there appeared to be some seasonal variation in the incidence of carriers, this is not considered to be of any practical significance.

Although IHNV in diseased fry or mature fish was easily demonstrated, a test for carriers in prespawning fish when sex products are not available would be advantageous for hatchery biologists. Consequently, I examined other tissues from two known carrier females to attempt to detect virus. Kidney, liver, spleen, pyloric caeca, intestine, serum, feces and ovarian fluid were processed separately and diluted 1:100 (except for ovarian fluid, which was not diluted) before quantitating virus. No virus was isolated from kidney, liver, spleen, feces, or serum, but virus was isolated from pyloric caeca, intestine and ovarian fluid. The average concentration of virus was about $10^{6.5}TCID_{50}$/gm in both the intestines and caeca and $10^{4.5}TCID_{50}$/ml in ovarian fluid. For comparison, eight other

TABLE 1. Prevalence of IHNV Carriers in Male and Female Rainbow Trout.

	Females			Males		
	Number of Fish	Number with Virus	%	Number of Fish	Number with Virus	%
Sample 1	30	5	17	30	0	0
Sample 2	60	5	8	27	3	11
Sample 3	6	3	50	21	6	35
Total	96	13	14	78	9	12

TABLE 2. Incidence of IHNV in female Rainbow Trout. Approximately 20 five-fish pools of ovarian fluid were collected weekly during the course of one spawning season and tested for IHNV.

	No. 5-fish Pools	Positive for IHNV	%
Fall spawners (Sept.-Nov.)	280	42	15.0
Winter spawners (Dec.-Feb.)	210	76	36.2
Spring spawners (March-May)	129	44	34.1
Total	619	162	26.2

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fish had a virus concentration in ovarian fluid ranging from $10^{1.0}$ to $10^{6.5}TCID_{50}$/ml, but other tissues were not tested.

The above results indicated that intestine or caeca were likely sources for detecting prespawning carriers. To test this possibility, a group of 3-year-old female rainbow trout, which were approaching first maturity, were sorted into spawners (eggs could be expressed) and prespawners (eggs could not be expressed). Tests of ovarian fluid from the spawners revealed that 9 of 30 fish (30%) were carriers. However, IHNV was isolated from only one of 20 caeca samples (5%) from prespawners. The difference between spawners and prespawners was significant ($P = .05$) by chi square analysis. Later, feces and viscera from prespawning female carriers were tested on several occasions and virus rarely was detected. Because caeca samples must be homogenized and diluted to prevent toxicity to cell cultures,[13] IHNV may have been present in low levels but undetected due to the dilution factor. Ovarian fluid can be placed on cell cultures undiluted; therefore testing of sexually mature fish appears to be the most reliable procedure at present to detect IHNV in carrier female rainbow trout.

IHNV could easily be detected in spawning fish, but it was not known if the fish recently had been infected or were carriers which shed virus during or after the stress of spawning. To determine if fish surviving an infection would become carriers, I held about 300 6-month-old rainbow trout, undergoing a natural epizootic, in dechlorinated municipal water known to be free of virus. Mortalities stopped within three weeks and thereafter no virus was isolated from nearly 20 fish sacrificed and tested over the next two years. Only 27 fish survived to maturity at three years of age. Of the 27, nine (33%) were positive IHNV carriers: 3 of 6 females and 6 of 21 males. This observation demonstrated that rainbow trout exposed to IHNV at an early age can carry the virus for several years and shed it during spawning. However, the question of latency, continued or intermittent shedding of virus, in prespawning carriers was not resolved.

During the course of this study, I noticed that groups of rainbow trout had a higher incidence of virus carriers several days after eggs had been removed (spawned out) than did groups of fish from which eggs were being removed for the first time. (Usually fluid can be expressed from spawned-out females for up to a week or more.) On one occasion, a group of 18 mature females which had survived an IHN epizootic were being held in virus-free water at 10 C. At the first spawning no virus was detected in the ovarian fluid; eight days later however, two fish were shedding virus, and after 26 days three fish were shedding virus. The fish were not completely spawned-out when they were tested, which accounts for the prolonged time ovarian fluid was obtained. These results indicate that more fish shed virus following the stress of spawning than during the first spawning act.

Transovarian Transmission

Epizootics of IHN have occurred in new areas from fish transported as eggs, and virus has been demonstrated in sex products of spawning fish; therefore, it appeared likely the virus could be transmitted with eggs. To determine if progeny of naturally infected carrier females would contract the disease, I artificially fertilized about 400 eggs from each of about 100 females with milt from 100 separate males. Ovarian fluid was collected and tested for virus before adding sperm. Males were not tested for virus because this experiment was performed early in the study when preliminary evidence indicated (Sample 1, Table 1) that males were not carriers. This belief later proved to be false, but test results were not affected by this omission of information. IHNV was isolated from 21 females, then eggs from the 21 infected fish and eggs from 16 unaffected fish were transported to a virus-free water

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supply. Eggs from the other 63 uninfected fish were not needed for the test and therefore discarded.

Four groups of eggs from infected females and four from uninfected females were hatched and reared at 10 C at an independent laboratory. At the Western Fish Disease Laboratory, where the incubation temperature was 10 C, eggs hatched 42 days after arrival. After hatching, nine groups from infected fish and eight groups from uninfected fish were reared at 10 C, and seven infected and five uninfected groups were reared at 14 C. All groups of fry were periodically tested for virus. The experiments were terminated after 100 days at the independent laboratory and after 150 days at the Western Fish Disease Laboratory.

No signs of disease were observed or virus isolated from any group of fish (Table 3). Inasmuch as signs usually appear in natural epizootics within 21 to 35 days after hatching, the fish were held for longer than required for disease to occur if virus had been present. To further test for presence of virus, I stressed some fish by handling them in a net for 30 seconds. Disease did not occur and virus could not be isolated from these fish. To determine if these fish were resistant, I exposed 200 fry in the water to about $10^{4.0}TCID_{50}$/ml of IHNV for 30 min. After 30 days at 10 C, 196 (98.0%) fish were dead and virus was reisolated from moribund fish (Table 3). No mortality occurred in the control group, indicating that if virus had been present in the original group of eggs from infected females, the progeny should have died.

From the above tests I surmised that virus was not present in these fish, even though they had come from infected parents. A possible explanation is that virus is not transmitted within the egg but rather as an external contaminant. In these tests, if virus was an external contaminant and eggs were placed in a virus-free water supply, the virus could have been washed away during incubation because about 42 days elapsed after fish were placed in the virus-free water supply before they hatched. Another possible explanation is that a latent stage exists within the embryo, but if so either the frequency of virus expression was too low to detect by the sample size tested or the right set of circumstances did not exist at either laboratory to induce the disease.

Some latent viruses are expressed when infected tissues are processed to obtain primary cell cultures. Physiological changes occurring in cells during harvesting and planting apparently induce a change in the virus-host relationship in some latently infected cells so that viral replication occurs and the cells become diseased.[12] To determine if a latent stage of IHNV could be induced in embryos from carrier females, I opened 10-eyed eggs from each of four known carrier fish and prepared primary cell cultures. These primary cell cultures developed into normal appearing monolayers, and no abnormalities were observed through 35 subcultures. When no cytopathology occurred in the initial cultures, media from these cells was placed on FHM cells, a cell line known to be susceptible to IHNV. No virus was detected. A subculture of primary cells was then challenged with IHNV at 10^2TCID_{50}/ml to determine if they were susceptible to the cytopathic effects of virus. Within five days at 15 C, cytopathology typical of IHNV was evident and a titer of $10^{6.7}TCID_{50}$/ml was obtained. Thus virus probably would have been detected if present in the original primary culture. Although these data are inconclusive because the sample size was small, no other data have been reported indicating the presence of a latent stage of virus in rainbow trout embryos from known carriers of IHNV.

If virus is transmitted as an external contaminant on eggs, it must be present at or shortly after the time of hatching to infect fry. The ability of virus to infect fish via eggs was demonstrated by dipping 200 advanced-eyed eggs into ovarian fluid containing about 10^5TCID_{50}/ml IHNV for 5 min. Eggs were then hatched in 9 C flowing water. A control

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TABLE 3. Incidence of IHNV in progeny of naturally infected and uninfected Rainbow Trout broodfish reared at two different laboratories and the susceptibility of fry from infected parents to experimental challenge.

	Water Temperature	No. of Adults Tested	Approximate No. of fry	Total Mortality	% Mortality	IHNV Isolated
Laboratory 1 (WFDL)①						
eggs from infected adults	10 C	9	3600	47	1.3	No
	14 C	7	2800	92	3.3	No
eggs from uninfected adults	10 C	8	3200	65	2.3	No
	14 C	5	2000	70	3.5	No
Laboratory 2						
eggs from infected adults	10 C	4	1600	not recorded	est. 10%	No
eggs from uninfected adults	10 C	4	1600	not recorded	est. 10%	No
Experimental challenge of② fry from infected adults	10 C	—	200	196	98	Yes
Unchallenged controls	10 C	—	200	0	0	No

① fry were reared for 150 days at laboratory 1 and 100 days at laboratory 2.

② exposed to $10^{4.0}$ $TCID_{50}$/ml of IHNV in water.

group of eggs was treated similarly, except that no virus was present in the ovarian fluid. Dead fish were collected daily and each week tested for virus.

All eggs hatched 3 to 5 days after the test started; 97% of the control group and 93% of the test group produced viable fry. No virus was detected the first week, but IHNV was isolated from the test group by the end of the second week, and throughout the next three weeks, although mortalities were relatively low. Mortality averaged less than 10% per week for the first five weeks, but 60% of the fish died with typical signs of IHNV during the sixth week (Figure 1). When the test ended after the eighth week, 86.1% of the experimental fish and 3.1% of the control fish had died. Virus was not isolated from the control group. It was not possible to determine if all fish that died became infected at hatching or if only a few more were infected and the virus later spread to the others. This test has been repeated on three different occasions with similar results. The disease, therefore, can be transmitted on advanced eggs exposed to ovarian fluid contaminated with IHNV.

Viral Hemorrhagic Septicemia (VHS), a disease of rainbow trout caused by the Egtved virus and enzootic to Europe, is similar to IHN, but IHNV is serologically and culturally distinct from the Egtved

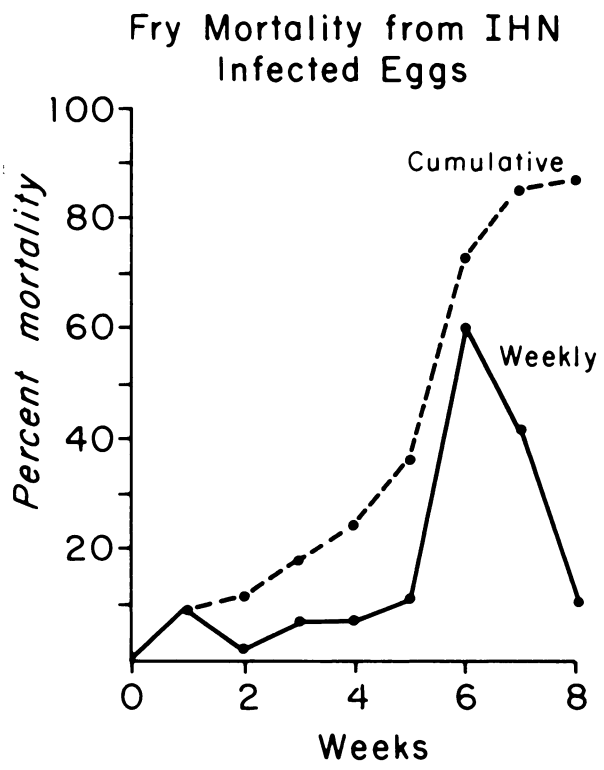

FIGURE 1. IHN mortality resulting from egg contamination. Rainbow trout eggs were experimentally exposed to infected ovarian fluid for 5 min, 3 to 5 days before hatching in 8-10 C water.

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virus.[8,14] Jorgensen[7] gave evidence that VHS was not transmitted from infected brood fish to fry through the egg, but that fry became infected when placed in water containing infected brood fish. He surmised that if eggs were reared in a virus-free water supply, the virus would wash away before the eggs hatched. The virus appeared to be transmitted only as an external contaminant. His results are similar to those presented here for IHNV but differ from those of Wingfield and Chan[11] who studied IHN in chinook salmon (*O. tshawytcha*) and concluded that IHNV was within the egg.

Fish-to-Fish Transmission

Transmission of IHNV by adding infectious cell culture fluid to water, by placing infected fish with noninfected fish, and by injecting the virus into fish has been reported.[3] Transmission through the water was further demonstrated by placing 40 infected fish at the head of a 76-liter trough and 25 noninfected fish at the other end of the trough. The two groups were separated 30 cm by screens; the water flow was about 1 liter/min. and the temperature 10 C. Within two weeks the disease spread to fish at the lower end of the trough. More than one-half of the exposed fish died and virus was reisolated from fish at each end of the trough. No fish died in an adjacent trough containing unexposed fish under similar conditions. This observation confirmed fish-to-fish transmission through water.

Dietary transmission was demonstrated by feeding infected fry to noninfected fry. Infected fry were ground and daily portions were kept frozen (—20 C) until fed. The diet contained $10^{6.2}TCID_{50}/g$ and was fed for five consecutive days to two separate groups of 500 rainbow trout in flowing water at 10 C. An equal number of fish fed a similar diet of uninfected fish served as a control. Mortality in groups receiving the infected diet occurred within two weeks and peak mortality occurred between the second and third week. The average cumulative mortality after six weeks, when mortality ceased, was 81.5% compared with 5.2% for the control group. Moribund fish in the infected groups showed typical signs of the disease, and virus was isolated from them. Mortalities in the control group were considered incidental because they did not exhibit signs typical of IHN and no virus was isolated. Transmission of IHNV in the feed was also reported for sockeye salmon.[6]

CONCLUSIONS

1. Once fish became infected with IHNV, they apparently remained carriers throughout life. Both sexes carried virus and shed it during spawning. Physiological changes associated with spawning appeared to augment shedding of virus.

2. IHNV was easily detected in moribund fish and from sexually mature fish, but virus could only rarely be detected in asymptomatic prespawning carriers. Therefore, testing mature fish is the most reliable means of detecting IHNV carriers. Gonadal fluids are perhaps the best source for isolation of virus from carrier fish because they are not diluted and fish do not have to be killed to obtain a sample.

3. IHNV could not be detected in embryos or progeny of carrier fish, but recently contaminated fry or mature eggs were readily infected. This observation indicates that the virus may not be transmitted inside the egg.

4. Adult carriers appear to be the reservoir of the infection. Transmission occurs (a) by the shedding of virus, thereby exposing susceptible fish or eggs, (b) by the consumption of contaminated food, and (c) by artificial methods.

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