SAN FRANCISCO Estuary & Watershed SCIENCE

Infectious Hematopoietic Necrosis Virus Transmission and Disease Among Juvenile Chinook Salmon Exposed in Culture Compared to Environmentally Relevant Conditions

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ABSTRACT

The dynamics of IHNV infection and disease were followed in a juvenile Chinook salmon population both during hatchery rearing and for two weeks post-release. Cumulative weekly mortality increased from 0.03%–3.5% as the prevalence of viral infection increased from 2%–22% over the same four-week period. The majority of the infected salmon was asymptomatic. Salmon demonstrating clinical signs of infection shed 1000 pfu mL^{-1} of virus into the water during a 1 min observation period and had a mean concentration of 10^6 pfu mL^{-1} in their mucus. The high virus concentration detected in mucus suggests that it could act as an avenue of transmission in high density situations where dominance behavior results in nipping. Infected smolts that had migrated 295 km down river were collected at least two weeks after their release. The majority of the virus positive smolts was asymptomatic. A series of transmission experiments was conducted using oral application of the virus to simulate nipping, brief low dose waterborne challenges, and cohabitation with different ratios of infected to naïve fish. These studies showed that asymptomatic infections will occur when a salmon is exposed for as little as 1 min to $>10^2$ pfu mL^{-1}, yet progression to clinical disease is infrequent unless the challenge dose is $>10^4$ pfu mL^{-1}. Asymptomatic infections were detected up to 39 d post-challenge. No virus was detected by tissue culture in natural Chinook juveniles cohabitated with experimentally IHNV-infected hatchery Chinook at ratios of 1:1, 1:10, and 1:20 for either 5 min or 24 h. Horizontal transmission of the Sacramento River strain of IHNV from infected juvenile hatchery fish to wild cohorts would appear to be a low ecological risk. The study results demonstrate key differences between IHNV infections as present in a hatchery and the natural environment. These differences should be considered during risk assessments of the impact of IHNV infections on wild salmon and trout populations.

KEYWORDS

Chinook salmon, infectious hematopoietic necrosis virus, Sacramento River, hatchery impacts on natural fish, asymptomatic viral infection

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INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV) has been a significant viral pathogen of Chinook salmon (*Oncorhynchus tshawytscha*) at the Coleman National Fish Hatchery (CNFH) since it began operation in 1942 (Ross and others 1960). Epizootics were common among juvenile fall-run Chinook salmon with high mortality and the subsequent release of large numbers of IHNV-exposed juveniles. In 2000, an ozone water treatment plant began operation and virus has not been detected since in any production fish at the CNFH. Periodic hatchery epizootics due to IHNV continue to be observed in other California hatcheries, the most recent being the serious losses of Chinook salmon in 1998, 2000, and 2001 at the California Department of Fish and Game (CDFG) Feather River Hatchery.

Strains of IHNV found in the upper Sacramento River are related to a larger group of isolates, referred to as the L clade, obtained from the southern coastal areas of Oregon and anadromous fish waters of northern California (Kurath and others 2003). These viruses are one of three clades of IHNV. The U clade is found in Alaska, British Columbia, Washington, Oregon and the M clade is present in the southern Idaho commercial rainbow trout (*O. mykiss*) industry. Virus isolates in the L clade share similar electrophoretic profiles for their structural proteins and when grouped by this method are considered electropherotype 3 (Hsu and others 1986). Isolates in the L clade also demonstrate a degree of host specificity with a tendency to be more virulent for Chinook salmon then rainbow trout (G. Kelley, pers. comm.; LaPatra and others 1990). In California, IHNV is most commonly detected in sexually mature Chinook salmon and steelhead that show no clinical signs of disease but potentially contain high concentrations of virus in the ovarian fluid or semen at the time of spawning. The mechanisms by which the virus is sustained in anadromous salmonid populations is currently unknown, but adult-to-adult transmission is proposed as one potential means for the annual recurrence of virus among salmon returning to Central Valley hatcheries. The upper Sacramento River is unique for Chinook salmon systems as it has four distinct runs of salmon (fall, late-fall, winter, and spring) that overlap to some degree with respect to upriver migration or holding or spawning periods (Healy 1991).

Transmission of viral and other pathogens from cultured to naturally-spawned (natural) or wild (no hatchery influence) fish, or vice versa, is one element amid the controversy over the impact of aquaculture on natural systems (Hedrick 1998; Schramm and Piper 1995). There are fewer reports describing viral epizootics in wild fish compared to those for hatchery or farmed salmonid populations (Williams and Amend 1976; Burke and Grischkowsky 1984; Traxler and Rankin 1989; Olivier 2002). However, viruses and a number of other potential microbial pathogens are clearly natural parts of aquatic ecosystems supporting salmonid populations.

To examine some of the important questions regarding the epidemiology of IHNV infections in Central Valley Chinook salmon, we conducted a series of experimental studies. The studies focused on the transmission of IHNV by following a group of naturally infected juvenile Chinook salmon at CNFH and the outcomes of experimental exposures of juvenile Chinook salmon to environmentally relevant concentrations of virus.

METHODS

Sample Collection and Viral Assay

Juvenile salmon samples were produced from a triangular whole fish cross-section comprised of gill, anterior kidney, liver, muscle and skin. A 0.1-mL decontaminated sample at various dilutions (2x to 40x) was inoculated onto replicate wells of a plate containing drained epithelioma papulosum cyprini (EPC) cells pretreated with 7% polyethylene glycol solution (Batts and Winton 1989). The cell cultures were incubated at 15°C for a minimum of 18 d. Virus identification was performed by dot blot using rabbit antisera to IHNV or immunohistochemistry on fixed cell sheets using monoclonal antibody (Drolet and others 1993; Arnzen and others; catalog no. 3-MAB-UC-IHNV-14D DiagXotics, Wilton, Conn.).

Infection in Hatchery Juveniles Before and After Release

In 1996 and 1997, CNFH juvenile fall-run Chinook Salmon were assayed for IHNV both prior to release and up to 15 d post-release (Foott and Williamson 1996; Free and Foott 1998). Post release fish were collected in a rotary screw trap near Knights Landing (rkm 145) 295 km from the release site (Figure 1). Sampling occurred on three separate release groups in 1996 and two groups in 1997. Approximately 8% of the fish in each release group were marked by an adipose fin clip and coded wire tag. In 1997, one raceway (#22) in the early stage of an IHNV outbreak was selected for intensive weekly monitoring. A total of 92 fish was collected weekly by cast net from the top, middle, and lower sections of the 490-m raceway and 46 two-fish pools were assayed for virus. Mortality for each 7-d period was recorded to calculate weekly percent mortality.

Virus Shedding from Moribund Juveniles

During an April 1996 IHN epizootic, eight juvenile Chinook salmon showing clinical signs of the disease (exophthalmia, weak condition, pale gills, dark in color) were netted from affected hatchery raceways and placed into aerated 100-mL containers of 13°C sterile water. A 10-mL water sample was removed from the aquaria after 1, 10, and 30 min, and mixed with 10 mL of a 14% (w/v) solution of polyethylene glycol (MW 20,000) in MEM with 500 μg/mL gentamycin; 1,000 IU/mL penicillin and 1000 μg/mL streptomycin; 25 μg/mL amphotericin B; 0.06% $NaHCO_3$; and 5% fetal bovine serum (PEG14). After the 30-min water sample was taken, fish were euthanized with an overdose of MS 222 and both mucus and kidney-spleen samples collected from each fish. The mucus was scraped off both sides of the body using a sterile scalpel blade and added to a similar volume of PEG14. The kidney-spleen sample was processed for a 100x inoculum in a 50% solution of PEG14. A plaque assay was performed on the centrifuged (3000 x g, 10 min, 4°C) samples inoculated onto EPC cultures overlaid with a MEM 10 with 1% methylcellulose solution.

Waterborne and Oral Challenges

In April and June 1997, challenges were performed on juvenile Chinook salmon in the Fish Disease Containment Laboratory at the University of California, Davis. An IHNV isolate from moribund CNFH Chinook salmon fry collected in March 1997, was grown in multiple 150 cm^3 flasks of EPC cells and harvested when cell lysis was complete. The supernatant was clarified by centrifugation (500 x g, 10 min, 4°C), pooled, and used within 4 h for the challenges. The April experiment used replicate 25 fish groups (CNFH fall-run Chinook from a test negative raceway, mean wt 1.37 g) given either waterborne or oral challenges. Waterborne challenges were performed in aerated water baths containing

5.7×10^3, 5.7×10^4, and 5.7×10^5 pfu mL^{-1} virus. The fish were held for 1, 10, and 30 min in their respective viral bath, transferred to separate 10-L aquaria, and reared in pathogen-free well water at 11 to 12°C for 19 d. Mortality was recorded daily and dead fish were frozen at –80°C for later viral assays. Additional 25 fish replicates were lightly anesthetized and a 25-µL drop of the IHNV supernatant solution diluted 2x with 14% PEG (concentrations as above plus 10^6 pfu mL^{-1} virus) placed in their mouth. The PEG carrier was chosen to represent mucus shed from an infected fish. The oral challenges were to determine if nipping behavior could be a mode of viral transmission. Four or five fish pooled viral samples were assayed from all 19 days post-exposure (dpe) survivors.

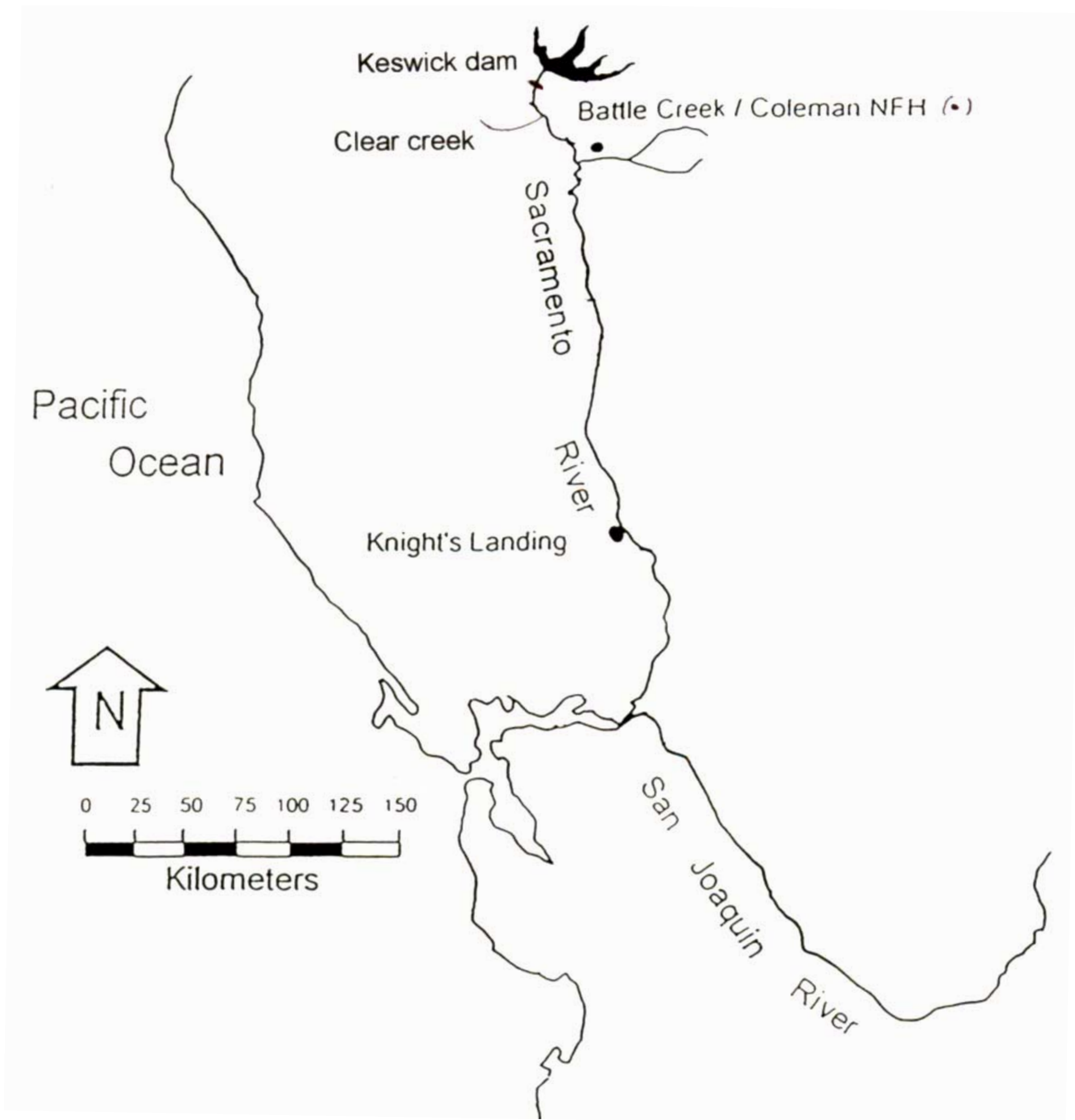

Figure 1. Map of juvenile Chinook collection sites.

In June, replicate groups of 25 CNFH late-fall run Chinook salmon juveniles (from a test negative raceway, mean 3.6 g) were challenged with the same 1997 virus isolate at 1.1 x 10^2 and 1.1 x 10^3 pfu mL^{-1} virus in a 1-min or 30 min static bath. Additional replicates received a 50 μL drop of a 2.2 x 10^4 pfu mL^{-1} virus solution in a methyl cellulose carrier placed in their mouth. Replicate groups were held at either 11°C or 15°C for up to 25 d. Frozen dead fish and 25 dpe survivors were assayed for virus. Each of the above challenge groups had appropriate negative controls challenged with MEM.

Transmission from Infected Cultured to Natural Juveniles

Three separate experiments were conducted with natural juvenile Chinook salmon from the upper Sacramento River collected by the U.S. Fish and Wildlife Service (USFWS) Northern Central Valley Fish and Wildlife Office (NCV FWO) and juvenile hatchery Chinook salmon from CNFH (Foott and others 2000). The natural juveniles were captured in rotary screw traps below Red Bluff Diversion Dam (RBDD, rkm 391) and transported to the California-Nevada Fish Health Center's wet laboratory. Laboratory effluent was treated with 4 mg L^{-1} chlorine for 12 min, de-chlorinated with activated charcoal filters, and discharged into a gravel leach field. Within 3 h of arrival at the laboratory, four to five natural fish were placed into tanks for a 1-h acclimation period prior to the introduction of IHNV-infected hatchery fish. Meanwhile, a similar number of naturals was held within a 4,000 cm^3 cage inside the exposure tank. The caged group was included to distinguish waterborne from physical contact exposures as nipping behavior could be a route of viral transmission from infected mucus (LaPatra and others 1989). Unexposed natural fish (control) were also tested for virus in each experiment. The ratio of cohabitated natural salmon to infected hatchery salmon was 1:1 for experiment 1, 1:10 for experiment 2, and 1:20 for experiment 3. The 1:20 ratio was based on the maximum natural to hatchery fall-run Chinook smolt ratio estimated from rotary screw trap collections at RBDD in April to May of 1999 (P. Gaines, NCV FWO, pers. comm.).

Replicate exposures occurred in 1-m diameter circular tanks supplied with 17 L min^{-1} flow of 10 to 13°C water. This flow was determined to be the minimum that produced an upstream swimming orientation. The center of the tanks contained a 25-cm circular divider to encourage circular swimming. Total volume for fish interaction was 177 L. Hatchery fish were given a 0.1-mL intra-peritoneal injection of supernatant harvested from EPC cultures infected with an isolate from a 1997 IHNV epizootic. Five to ten days post-injection, infected hatchery fish were marked in a 40-min bath of 0.02 g L^{-1} Bismark brown dye. Infected fish were used for the exposures within 4 d of the marking operation. Co-habitation exposures of 5 min (4 replicates per experiment) or 24 h (2 replicates per experiment) were chosen to simulate both a brief and a "worst-case" river interaction, respectively. After each exposure, all fish were captured and the unmarked naturals moved to 15-L flow-through aquaria supplied with aerated 12 to 14°C water. Three to five minutes were required to net and sort the exposure groups. Half of each natural exposure group was stressed by holding them within a net for 30 s each day for the first 3 d of captivity. The naturals were sampled for virus 7 dpe and the hatchery fish directly after the exposure. In addition to the standard 18 d primary incubation, a blind pass culture was started 4 to 6 d post-inoculation for each natural fish sample. In experiments 2 and 3, two fish

pools of injected hatchery salmon tissues were assayed for virus.

Single High Dose and Repeated Low Dose Exposures

Juvenile Chinook salmon were exposed to IHNV at high or low doses, or by repeated low doses of virus. Mortality and presence of IHNV in the tissues of exposed fish was examined over time. Chinook salmon (Iron Gate Hatchery origin, 4.0 g) held in 12°C well water were exposed to IHNV (fCLChn–n6 = CNFH fall-run adult 2002 isolate) at a concentration of 7.0×10^4 pfu mL^{-1} for 1 h. After virus exposure, flow of well water to replicate aquaria was resumed. In a second set of trials, juvenile Chinook salmon (Nimbus Hatchery origin, 0.47 g) were exposed to repeated low doses (1.90×10^0, 10^1,10^2, and 10^3 pfu mL^{-1}) of IHNV for 30 min on each of five consecutive days. The presence of IHNV in tissues of healthy appearing or dead fish was evaluated by isolation in cell cultures. Concentrations of virus in selected tissues were estimated by titration on these cell cultures to establish the pfu g^{-1}.

Results

Infection in Hatchery Juveniles Before and After Release

In 1996 and 1997, CNFH juvenile Chinook salmon were monitored for viral infection throughout their rearing cycle and for up to 15 d post-release (Table 1). IHNV was diagnosed in four of five release groups approximately two weeks prior to their release from the hatchery. Virus was isolated from smolts collected at the Knights Landing trap located 295 rkm from their release point and up to 15 d post-release (Table 1). We believe the majority of smolts collected at the trap were of CNFH origin based on the presence of coded-wire tagged fish in the sample and their large size. Post-release incidence of infection ranged from 0% to 20%. The first release group in 1997 demonstrated a relatively steady prevalence of IHNV infection from release through 15 d post-release. Over 70% of the virus positive smolts were asymptomatic. The prevalence of IHNV infection in the 1997 surveyed production raceway (#22) ranged from 2% to 22% with weekly mortality rates of 0.3% to 3.5% (Table 2). Most of the samples were from asymptomatic fish. This raceway had 158.5 m^3 of rearing volume with approximately 44,000 fish (278 fish m^{-3}) and an 8 h exchange rate.

Table 1. Incidence of IHNV infection detected in two-fish pool samples of CNFH Chinook smolts prior to release and 6 to 15 d post-release at Knights Landing trap. Sampling occurred on 3 separate release groups in 1996 and 2 groups in 1997. Data recorded as number of samples positive / total samples (%) and number of days post-release (d)

Release Group	*Pre-Release*	*Knights Landing Trap*		
		7d	*10d*	*15d*
1996				
1	0 / 45 (0)	4 /20 (20)	NT[a]	NT
2	15 / 55 (27)	2 / 16 (13)	NT	NT
3	13 / 30 (43)	3 / 22 (14)	NT	NT
1997				
1	9 / 45 (20)	7 / 45 (16)	4 / 45 (9)	7 / 46 (15)
2	9 / 45 (20)	NT	0 / 45 (0)	NT

a. Not tested.

Table 2. Weekly percent mortality and prevalence of IHNV infection (POI) in two-fish pool samples collected from raceway 22 in March and April 1997

	March 14–20	*March 21–27*	*March 28–April 3*	*April 4–8*
Cumulative Mortality	0.26%	0.40%	1.48%	3.52%
POI IHNV	1/46 (2%)	4/46 (9%)	7/46 (15%)	10/46 (22%)

Virus Shedding from Moribund Juveniles

Virus was rapidly shed from moribund salmon with 50 to 2,500 pfu mL^{-1} isolated after a period of only 1 min of contact with the water. Mean concentrations of approximately 1000 pfu mL^{-1} were also seen in samples collected after 10 and 30 min of contact (Figure 2). Mucus contained between 6.0×10^4 and 2.0×10^7 pfu mL^{-1}. Difficulty in volume measurement and the influence of surface water makes the mucus virus concentration a conservative estimate. On average, kidney-spleen samples contained only a log more IHNV than mucus.

Waterborne and Oral Challenges

Fish in the bath and oral challenge groups in the April 1997 experiment incurred relatively low mortality: 7.6% and 13.8%, respectively. While salmon exposed in a 1-min virus bath showed a positive dose-mortality relationship, this trend was not observed in fish exposed for 10 or 30 min (Figure 3). Pooled samples from challenge survivors showed that many of the fish in each bath exposure group were infected with IHNV. The prevalence of infection in the survivors ranged from 30% to 89% with the 10^3 pfu mL^{-1} dose of exposure groups having the lowest values (Figure 3). Significantly, a 1 min immersion in 5.7×10^3 pfu mL^{-1} resulted in a 30% prevalence of infection. By contrast, IHNV was isolated from fewer oral challenge survivors (0% to 30%). Highest mortality (30.6%) occurred among fish given the 5.7×10^5 pfu mL^{-1} oral challenge. However, no mortality trend was associated with virus concentration in the oral challenges (Figure 4).

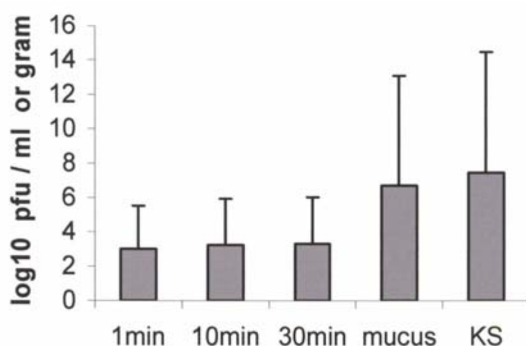

Figure 2. Average IHNV titer with standard error of the mean bars (expressed as log10 pfu mL^{-1} or g^{-1}) shed into water by clinically diseased Chinook juveniles after 1, 10 and 30 min of transfer into sterile water, and contained in the skin mucus and the kidney and spleen (KS) of the fish. The volume of the mucus samples was approximated by drop measurement.

In the June experiment, juvenile Chinook salmon exposed by bath to IHNV and then held at 11°C had a 63% cumulative mortality compared with 37% for the 15°C groups (Table 3). Mortality was influenced by *Flavobacterium columnare* and *Aeromonas hydrophila* infections. These bacterial infections were detected in 25% of the survivors sampled at 25 dpe. We believe that the salmon were sub-clinically infected by bacteria at time of transfer to the wet lab facility. The concurrent bacterial infection limited evaluations of the IHNV challenge effects on mortality but virus transmission after brief exposures to relatively low virus concentrations was again demonstrated in this study. In particular, IHNV was detected in salmon exposed to 10^2 pfu mL^{-1} for only 1 min (Table 3). Overall, virus was detected in 96% of both the 11°C survivor and dead fish samples. The detection rate in the 15°C group was 68% in 25 dpe survivors and 35% among dead fish. All control groups were negative for virus indicating that experimental groups were unexposed to IHNV prior to start of both April and June studies.

Transmission from Infected Cultured to Natural Juveniles

Natural fall-run Chinook salmon used in the first two experiments were approximately 25% to 30% smaller in fork length (mean, 38 mm and 41 mm, respectively) than the infected hatchery fish (mean, 55 mm). We observed that smaller, natural fish tended to stay near the surface and center of the tanks.

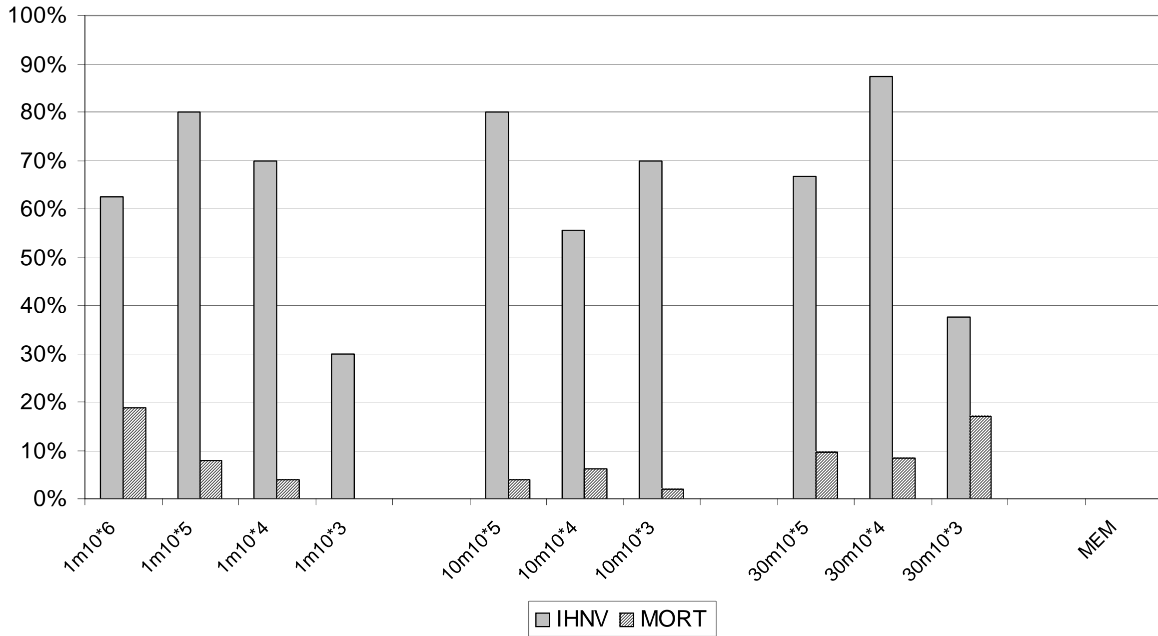

Figure 3. April 1997 IHNV bath (1, 10, and 30 min {m}) at 5.7 x 10^6, 10^5, 10^4, and10^3 pfu mL^{-1}. Prevalence of IHNV infection (IHNV) in four to five fish pools of 19 dpe survivors and cumulative percent mortality (MORT) for each challenge group and negative controls (MEM).

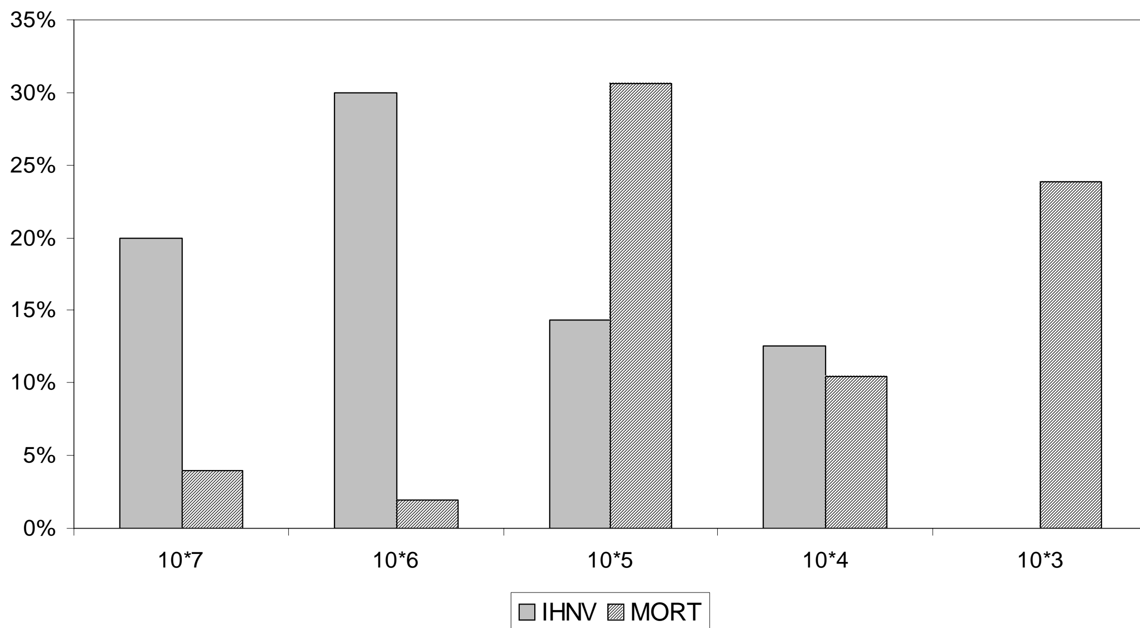

Figure 4. April 1997 IHNV oral challenge (25-µ L drop of PEG viral suspension) at 5.7 x 10^7,10^6, 10^5, 10^4, and10^3 pfu mL^{-1}. Prevalence of IHNV infection (IHNV) in five fish pools of 19 dpe survivors and cumulative percent mortality (MORT) for each challenge group.

Table 3. Cumulative percent mortality (% mort) for replicates A and B and combined (CUM). IHNV infection incidence (test positive / total sample in two-fish pools) of 25 dpe survivor and individual mortalities of juvenile Chinook challenged by a 1 and 30 min bath immersion in 1.1 x 10^2 and 1.1 x 10^3 pfu mL^{-1} virus as well as an oral application of 2.2 x 10^4 pfu mL^{-1} virus in methyl cellulose (MC). Negative control fish receive a 30-min bath challenge with MEM or oral application of MC. Fish were held at 11°C and 15°C for 25 d and assayed for virus. Mortalities were frozen and later assayed for virus.

Treatment	*11°C % mort*	*11°C IHNV survivors*	*11°C IHNV mortality*	*15°C % mort*	*15°C IHNV survivors*	*15°C IHNV mortality*
1 min 10^2						
A	64	3 / 3	3 / 3	36	4 / 4	1 / 2
B	68	2 / 2	4 / 4	8	3 / 4	0 / 1
CUM	66	100%	100%	22	88%	33%
1 min 10^3						
A	76	2 / 2	3 / 4	80	1 / 1	1 / 4
B	76	1 / 1	4 / 4	40	3 / 3	0 / 2
CUM	76	100%	88%	60	100%	17%
30 min 10^2						
A	28	5 / 5	2 / 2	20	2 / 2	0 / 1
B	24	3 / 3	2 / 2	36	2 / 3	1 / 2
CUM	26	100%	100%	28	80%	33%
30 min 10^3						
A	48	3 / 3	2 / 2	32	0 / 4	2 / 2
B	64	2 / 3	4 / 4	12	2 / 5	1 / 1
CUM	56	83%	100%	22	22%	100%
Oral 10^4						
A	33	2 / 2	1 / 1	50	0 / 1	0 / 2
B	33	2 / 2	1 / 1	0	0 / 3	0 / 2
CUM	33	100%	100%	25	0%	0%
MC	60[a]	0 / 1	0 / 1	0	0 / 3	0 / 1
MEM	64[a]	0 / 2	0 / 4	52[a]	0 / 3	0 / 3

a. *Flavobacterium columnare* and *Aeromonas hydrophila* infections.

The injected hatchery fish used for experiment 1 were asymptomatic, however, each fish was determined to be infected with IHNV (Table 4). The mean virus concentration in their pooled tissue samples was 1.2 x 10^4 pfu g^{-1}. Many of the virus-injected hatchery salmon used in experiment 2 demonstrated IHNV clinical signs and all tested virus positive (Table 4). Separate gill and kidney-liver samples, from a sub-sample of eight hatchery fish from experiment 2, were assayed for virus. Mean virus concentration was 2.8 x 10^4 pfu g^{-1} in gill and 1.4 x 10^4 pfu g^{-1} in the kidney and liver tissue. Natural Chinook, resembling out-migrant smolts (increased silvering, >60 mm fork length), were selected for experiment 3. They were similar in size to the infected hatchery salmon (means 69 and 63 mm, respectively). Most of the infected hatchery fish in experiment 3 showed clinical signs of infection and approximately 30% of the 24-h exposure group died prior to the end of the cohabitation period. All virus-injected hatchery fish from experiment 3 tested positive for virus (Table 4). No virus was isolated from any of 269 natural Chinook salmon used in all 3 experiments. Two natural salmon died in both experiments 2 and 3. Columnaris lesions were seen in a number of experiment 3 natural fish after 7 d of captivity.

Table 4. Prevalence of IHNV infection in juvenile natural and virus-injected hatchery Chinook salmon. Natural salmon were cohabitated or held in cages with injected hatchery fish for either 5 min or 24 h. Half of each natural cohabitation group was subjected to netting stress for the first 3 d of the 7 d post-exposure period. Data reported as number positive / total number of samples per exposure group.

Treatment	*1:1*	*1:10*	*1: 20*
5 min			
Cohabitated + stress	0 / 8	0 / 8	0 / 8
Cohabitated	0 / 8	0 / 8	0 / 8
Caged	0 / 6	0 / 8	0 / 8
24 h			
Cohabitated + stress	0 / 4	0 / 5	0 / 4
Cohabitated	0 / 4	0 / 5	0 / 3
Caged	0 / 4	0 / 4	0 / 8
No exposure control	0 / 1	0 / 34	0 / 17
Injected hatchery fish	16 / 16	80 / 80[a]	98 / 98[a]

a. Two-fish pool samples.

Single High Dose Virus Exposure

Fish showed no signs of disease upon first sampling at 3 dpe (Table 5) but virus was present as detected in the gill, skin and a pool of the kidney and spleen tissues. Virus concentrations were approximately 10^3 pfu g^{-1}. Surprisingly, virus was then not detected among healthy appearing Chinook salmon on days 5 and 8. Virus was again detected at 16 and 22 dpe in 1 of 3 fish with concentrations of virus reaching 10^6 pfu g^{-1}. No virus was detected from the final sample at 100 dpe.

Repeated Low Dose Virus Exposures

Repeated low dose exposures resulted in IHNV infections only when concentrations of virus were 1.90 x 10^2 pfu mL^{-1} or greater at each of the 5 exposures (Table 6). There was no mortality experienced by any of the virus-exposed Chinook salmon at any of the doses

Table 5. Detection of IHNV among juvenile Chinook salmon following exposure to a single, high dose (7.0 x 10^4 pfu mL^{-1} for 1 h) of the virus in water.

		IHNV Presence in Tissues (pfu g^{-1})		
DPE	*No. Fish +[a] / No. Fish*	*Gill*	*Skin*	*Kidney and Spleen*
3	2 / 3	2 / 3 (10^3)	2 / 3 (10^3)	2 / 3 (10^3)
5	0 / 3			
8	0 / 3			
16	1 / 3	1 / 3 (10^4)	1 / 3 (10^3)	1 / 3 (10^5)
22	1 / 3	1 / 3 (10^6)	1 /3 (10^4)	1 / 3 (10^6)
79	0 / 3			
100	0 / 3			

a. Three control fish sampled at each time point were negative for IHNV.

but virus was detected beginning at 11 dpe in the highest dose (1.9 x 10^3 pfu mL^{-1}) in the gill, skin, and kidney-spleen with concentrations up to 10^5 pfu g^{-1}. Virus was again detected in fish examined at 23 dpe in both the 1.9 x 10^2 and the 1.9 x 10^3 pfu mL^{-1} doses with most virus (10^5 pfu g^{-1}) present in the gill compared to the other tissues sampled. Virus was detected in only one fish sampled at 39 dpe in the gill and skin tissues and only from the highest dose (1.9 x 10^2 pfu mL^{-1}) and the concentrations of the virus (10^3 pfu g^{-1}) were lower than at earlier time points. No infected fish were detected at sample times of 79 and 100 dpe.

Discussion

The transmission of IHNV infections among juvenile Chinook salmon was examined in a series of field and laboratory experiments. Subclinical infections were the most common outcome of virus exposure both among naturally and experimentally infected salmon. The presence of subclinical infections among certain hatchery-reared salmonids and efficient adult to adult transmission of IHNV

Table 6. Detection of infectious hematopoietic necrosis virus (IHNV) among juvenile Chinook salmon following multiple low dose exposures to virus in the water.

		Tissue: Positive / Total Sample (Titer in pfu g^{-1})			
Days	*Dose*	*Fish + / Fish Sampled*	*Gill*	*Skin*	*Kidney and Spleen*
8	All doses	0 / 3			
11	1.9	0 / 3			
	1.9 x 10^1	0 / 3			
	1.9 x 10^2	0 / 3			
	1.9 x 10^3	2 / 3	1 / 3 (10^4)	2 / 3 (10^5)	2 / 3 (10^4 and 10^5)
23	1.9	0 / 3			
	1.9 x 10^1	0 / 3			
	1.9 x 10^2	1 / 3	1 / 3 (10^5)	1 / 3 (10^4)	1 / 3 (10^4)
	1.9 x 10^3	2 / 3	2 / 3 (10^3)	0 / 3	0 / 3
39	1.9	0 / 3			
	1.9 x 10^1	0 / 3			
	1.9 x 10^2	0 / 3			
	1.9 x 10^3	1 / 3	1 / 3 (10^3)	1 / 3 (10^3)	0 / 3
79 and 100	All doses	0 / 3			

may be the principal means by which the virus persists in Chinook salmon populations in the waters of California supporting anadromous salmonids.

In eight of the ten years prior to the operation of CNFH's ozone water disinfection system, IHNV epizootics occurred in juvenile fall-run Chinook salmon reared at the hatchery. Cumulative mortality of individual raceways could be in excess of 15%, with large numbers of asymptomatic infected fish eventually released into the Sacramento River. The hatchery practice of using mortality rate as an indicator for incidence of infection does not appear to be valid. We observed that the incidence of asymptomatic infections in an affected raceway population was variable and 6 to 23 times higher than weekly percent mortality. The majority of infected CNFH smolts collected after their release were also asymptomatic. In 1996 and 1997, smolts with asymptomatic infections were detected up to 15 d post-release and after traveling 295 rkm.

We cannot accurately predict the outcome of these infections as both external tissues (gill and skin) and internal organs were pooled for viral samples. Given the low virulence observed in laboratory challenges, it is possible that epidermal infections may not always lead to systemic disease. Other researchers have documented that epidermal cells of the skin, gill, and esophagus are the initial sites of viral replication following waterborne challenges (Mulcahy and others 1983; Yamamoto and others 1992; Drolet and others 1994; Helmick and others 1995). Laboratory trials by Yamamoto and others (1992) demonstrated an early involvement of gill and epidermal tissues following waterborne exposures of rainbow trout to higher dose

challenges of IHNV. They concluded that virus may undergo some replication in these tissues as early as 1 dpe, then spread to internal organs which begin to show positive for the virus at 3 dpe and thereafter. LaPatra and others (1989) reported that virus concentrations in the mucus increased from 24 to 48 h post-challenge suggesting replication was occurring in the epidermis. In our studies, gill tissues were positive in fish up to 39 d, a time point well past the sampling dates in the studies by Yamamoto and others (1992). While the gills and skin were viewed as more initial and transient locations for virus replication in rainbow trout, we speculate that in Chinook salmon juveniles these tissues may remain infected for longer periods of time and perhaps support virus growth in the absence of an extended involvement of internal organs.

There was a distinct difference between the mortality observed during hatchery epizootics and our experimental viral challenges. We demonstrated that environmentally relevant challenges (1 min at 10^2 and 10^3 pfu mL^{-1}) incurred a high incidence of asymptomatic infections yet relatively low mortality (<9% in 19 dpe). Extended exposure at high virus concentrations (>10^4 pfu mL^{-1}) and high densities of hosts may be required for disease amplification as the Sacramento River strain of IHNV shows relatively low virulence. In the 1997 laboratory challenge studies, we showed that 30 min static bath challenges with 5.7×10^3 pfu mL^{-1} produced only 10% cumulative mortality over 19 d. This challenge concentration of 1,000 pfu mL^{-1} may at times be environmentally relevant, particularly in high fish density situations, as this viral load was shown to be released into the water by clinically infected smolts.

Simulation of virus transmission by nipping infected smolts (oral application of methyl cellulose and PEG virus suspensions) showed that challenge dosage was somewhat related to incidence of infection but not mortality. The high virus concentration (mean of 10^6 pfu mL^{-1}) observed in mucus from clinically ill smolts suggests that this avenue of transmission may be important in high density situations where dominance behavior results in nipping. Water temperature may influence the observed host-pathogen relationship as salmon reared at 11°C had 100% infection 25 d after an oral challenge; however no virus was detected in similarly challenged salmon reared at 15°C. Heightened immune response could explain this observation. LaPatra and others (1993) reported that rainbow trout survivors do produce neutralizing immunoglobulin after IHNV challenge. This antibody response was protective in passively immunized cohorts. Type 1 interferon gene expression in spleen cells, following exposure to IHNV, has also been demonstrated in rainbow trout (Purcell and others 2004). Kidney cells from IHNV infected salmonids have been shown to produce interferon-like cytokines and Mx protein (Congleton and Sun 1996; Trobridge and others 1997). A rapid interferon response with increased temperature may play a role in the clearance of the virus. The Mx protein is an anti-viral protein induced by type 1 interferon and has been detected in juvenile Atlantic salmon injected with poly I:C (Salinas and others 2004). These authors reported that fish held at 14°C responded within 1 d post-injection at levels that took 6 d in fish held at 6°C. Amend (1970) reported that the CNFH strain of IHNV was temperature sensitive and that raising virus-exposed fry at 14°C or higher could minimize losses due to IHNV. CNFH isolates in the last 10 years appear to be less sensitive to elevated water temperatures as demonstrated by *in vitro* culture at 18°C as well as chronic outbreaks in juvenile salmon at water temperatures in excess of 17°C.

Ogut and Reno (2004) report that no IHNV mortality occurred to groups of rainbow trout exposed to infected donor fish despite the detection of virus in 7% to 20% of the 1.2 g trout sampled as whole fish homogenates at 2 to 4 dpe. Viral infection and limited replication in the skin and gills without subsequent systemic infection and disease could explain this observation. These authors state that no virus was detected in the exposed populations from 5 to 8 dpe. A similar pattern of virus detection also occurred in our high dose challenge where IHNV was detected at 3 dpe but not at 5 or 8 dpe, but then again was present at 16 and 22 dpe. The basis of this observation is unknown but it indicates that virus infections may not be a steady progressive event but fluctuate from below to above the detection limits of current diagnostic methods. Alternatively, small sample size ($n = 3$) could have influenced the lack of virus detection at 5 and 8 dpe in our study. Unfortunately, sample sizes and time points after 39 d were few and thus additional investigations of Chinook salmon exposed in this manner may be necessary to reveal if the virus is retained for longer periods in essentially a silent or subclinical infection.

Transmission of IHNV from infected cohorts to naïve fish is infrequent when the infectious load (virus concentration and duration of exposure) is limited. In our study, natural Chinook salmon juveniles were cohabitated with experimentally IHNV-infected hatchery Chinook salmon at ratios of 1:1, 1:10, and 1:20 for either 5 min or 24 h. No virus was detected by tissue culture in any of the exposed natural salmon sampled at 7 dpe. We did not test serum from the natural Chinook for antibody titer to IHNV. It is possible that the observed resistance, in some portion of the 111 natural salmon held with virus-infected donors, could have been due to immunity from previous virus exposure (LaPatra and others 1993). Our exposure system should have detected IHNV transmission if the concentration of waterborne virus was 10^3 pfu mL^{-1} or greater. Based on a pilot experiment, IHNV could be isolated from gill-liver-kidney tissue pools of hatchery juveniles 4 dpe after a 5 min exposure to 2.5×10^3 pfu mL^{-1}. Smith and others (2000) reported that infectious pancreatic necrosis virus (IPNV) epizootics of young trout are point-source rather than propagative in nature. These authors observed that a minimum virus concentration (number of infected fish added) was necessary to establish infection in susceptible cohorts. Once this threshold was met, all susceptible fish became infected at a similar time point.

Brief pulses of virus at low concentrations and at low host densities would likely be the conditions of transmission of IHNV among wild juvenile salmon. In comparison, cultured fish are reared at significantly higher densities and in confined environments. These conditions influence viral disease as they allow for rapid horizontal transmission. Reno (1998) describes the importance of frequent contact of a susceptible host with an infectious source (along with the transmission coefficient (β) of the infectious agent) in determining whether an epizootic will occur. The positive relationship between the density of susceptible hosts and a low-level point source of infection was confirmed for infectious pancreatic necrosis virus infection among juvenile trout by Bebak-Williams and others (2002). When pathogen concentration was high, the effect of host density on death rate and time of peak death rate was reduced. LaPatra and others (1996) also described the positive relationship between cultured sturgeon density and white sturgeon iridovirus infection rate.

Given our data on transmission under environmentally relevant conditions, we believe that the potential of horizontal transmission of the Sacramento River strain of

IHNV from infected hatchery fish to naturally occurring juvenile salmon is quite low. Furthermore, the ecological outcome of IHNV transmission (mortality) to natural salmon juveniles in the Sacramento River would be minimal. Differences found among naturally acquired IHNV infections or experimental infections induced by environmentally relevant doses and those induced by artificially severe challenges need to be considered when undertaking risk assessments of the transmission of IHNV in natural systems.

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