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Evidence for a carrier state of infectious hematopoietic necrosis virus in chinook salmon *Oncorhynchus tshawytscha*

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ABSTRACT: In British Columbia, Canada, infectious hematopoietic necrosis virus (IHNV) is prevalent in wild sockeye salmon *Oncorhynchus nerka* and has caused disease in seawater net-pen reared Atlantic salmon *Salmo salar*. In this study, chinook salmon *Oncorhynchus tshawytscha* experimentally exposed to an isolate of IHNV found in British Columbia became carriers of the virus. When Atlantic salmon were cohabited with these virus-exposed chinook salmon, IHNV was isolated from the Atlantic salmon. Identification of chinook salmon populations that have been exposed to IHNV may be difficult, as virus isolation was successful only in fish that were concurrently infected with either *Renibacterium salmoninarum* or *Piscirickettisia salmonis*. Also, IHNV-specific antibodies were detected in only 2 of the 70 fish experimentally exposed to the virus. Two samples collected from chinook salmon exposed to IHNV while at a salt water net-pen site had a seroprevalence of 19 and 22%; however, the inconsistencies between our laboratory and field data suggest that further research is required before we can rely on serological analysis for identifying potential carrier populations. Because of the difficulty in determining the exposure status of populations of chinook salmon, especially if there is no concurrent disease, it may be prudent not to cohabit Atlantic salmon with chinook salmon on a farm if there is any possibility that the latter have been exposed to the virus.

KEY WORDS: Infectious hematopoietic necrosis virus · Chinook salmon · Carrier state · Cohabitation study · Serology

INTRODUCTION

The viral disease infectious hematopoietic necrosis (IHN) has been reported in a number of salmonid species (Bootland & Leong 1999). In 1992, IHN was reported for the first time in salt water net-pen reared Atlantic salmon *Salmo salar* off the coast of Vancouver Island, British Columbia, Canada (Armstrong et al. 1993). After this report, the disease was reported on several other farms near the first case. In 1996, an industry action plan was implemented to mitigate the impact of this viral disease. As part of this plan several farms were fallowed simultaneously. Some sites then re-introduced Atlantic salmon while others introduced chinook salmon *Oncorhynchus tshawytscha*.

Producers considered raising chinook salmon because they are more resistant to IHN virus (IHNV) than Atlantic salmon (Traxler et al. 1993). Because chinook salmon do not succumb to disease when exposed to the IHNV isolate found in Atlantic salmon in British Columbia, they were assumed not to be infected with the virus. However, it was unknown whether chinook salmon could become carriers of the virus and transmit it to susceptible fish. This question became important once producers wanted to re-introduce Atlantic salmon to previously affected sites that housed chinook salmon.

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Persistent infections with IHNV have been reported in rainbow trout *Oncorhynchus mykiss* (Amend 1975, Drolet et al. 1995, Kim et al. 1999); however, this state is usually reported in fish that have survived an IHN epizootic. There has been 1 report where adult rainbow trout seroconverted but did not exhibit clinical signs of disease, and IHNV was detected in these fish at spawning (Hattenberger-Baudouy et al. 1995). Some studies have also indicated that adult fish previously exposed to IHNV could transmit the virus to other susceptible salmonids (Mulcahy et al. 1983, LaPatra et al. 1989).

The objective of this study was to determine whether chinook salmon exposed to IHNV could transmit the virus to other fish and, if so, whether the virus or IHNV-specific antibodies could be used to identify potential carrier populations.

MATERIALS AND METHODS

All cohabitation studies were conducted at the Pacific Biological Station using a single pass ambient salt water flow through system. The water temperature ranged from 9 to 13°C during the course of the study.

IHNV exposure of stock population. One hundred and seventy post-smolt[1] chinook salmon (average ± SD weight 121.8 ± 17.8 g, n = 10) and 15 post-smolt Atlantic salmon (~50 g) were exposed together to IHNV in a static bath of salt water (380 l) for 1 h at a concentration of 1.6×10^4 plaque forming units ml^{-1} of water. The IHN virus used in this study was isolated from Atlantic salmon in British Columbia in 1992. The virus was grown in epithelioma papillosum cyprini (EPC) cells and passed 3 times in cell culture before the fish were challenged. After the exposure period, water flow (approximately 10 l min^{-1}) was returned to the 760 l tank (Tank A).

After exposure to IHNV, the fish were monitored for 276 d. Dead fish were removed daily and tested for IHNV by viral isolation as described in Traxler et al. (1997) with the exception that 1% methylcellulose was not used in the overlay step. A viral assay was considered positive if cytopathic effects (rounded-up refractory cells on the cell monolayer) were observed within 7 d of incubation at 15°C. A nested reverse transcriptase polymerase chain reaction (RT-PCR) test for IHNV was used as a confirmatory test for all positive viral assays. The protocol used was provided by William Batts at the Western Fisheries Research Center, United States Geological Survey, Biological Resources Division, Seattle, WA, USA (1997) and is described in St-Hilaire (2001). The presence of *Renibacterium salmoninarum* and *Piscirickettsia salmonis* infections were determined using Gram-stained imprints of suspect organs. *R. salmoninarum* was identified by the presence of small, Gram-positive bacilli, whereas *P. salmonis* was diagnosed by observing multiple (often in doublets), small, Gram-negative coccoid organisms within phagocytes or hepatocytes (Almendras & Fuentealba 1997).

Cohabitation studies. ***Cohabitation Trial 1:*** Eleven asymptomatic chinook from Tank A were transferred into a 200 l tank (Tank B) 22 d after exposure to IHNV. Seven days after the transfer, 11 naïve Atlantic salmon were added to the tank with the chinook salmon and the fish were monitored for mortality. The fish (~300) that remained in the tank where the 11 Atlantic salmon originated served as negative controls and were kept on the same ambient salt water supply as Tank B. One week before transfer, 20 Atlantic salmon were killed from this population and tested for IHNV-specific antibodies.

All fish that died in the experimental tank were tested for IHNV, and positive viral assays were tested with a nested RT-PCR for virus confirmation. The experiment was terminated 60 d after the introduction of Atlantic salmon into Tank B, and all remaining Atlantic and chinook salmon were tested for IHNV. Ten fish from the Atlantic salmon control tank were also tested for virus at the end of the study.

Water-borne exposure: Ten chinook salmon were removed from Tank A 22 d post-exposure and placed into a 200 l tank (Tank C). Seven days after the transfer of the chinook, 7 naïve Atlantic salmon from the same source as those used in the cohabitation Trial 1 were introduced to another tank (Tank D), which received its water from Tank C. Fish in both tanks C and D were monitored for mortality for 60 d and all dead fish were tested for virus. This experiment and cohabitation Trial 1 were run simultaneously, and the same control tank was used for both experiments. The experiment was terminated after 60 days, and all remaining fish were tested for virus.

Cohabitation Trial 2: Five months after the IHNV exposure in Tank A, the last remaining 'sentinel' Atlantic salmon was removed from that tank and tested for IHNV. Forty of the remaining chinook salmon were transferred into another tank and monitored. Six chinook salmon were left in Tank A. At this time a group of 16 Atlantic salmon (~50 g), held in dechlorinated Nanaimo city water, were transferred to a salt water tank (Tank E) (40 l). Eight days later, 7 of these Atlantic salmon were introduced into Tank A with the 6 chinook salmon. Fish in this tank and the 9 remaining Atlantic salmon in Tank E, in the same water supply as Tank A, were monitored for mortality over a 3 mo period. All fish that died were tested for virus. The experiment was terminated after 102 d and all remaining fish were tested for virus.

[1]This term refers to fish that have adapted to salt water

IHNV-specific antibody profile in chinook salmon exposed to IHNV. Between Days 3 and 64 post-exposure to IHNV, 7 samples of 10 chinook salmon each were taken from Tank A. Fish were killed and blood was collected from the caudal vein for antibody analysis by the 50% plaque reduction technique described by LaPatra et al. (1993b). Two modifications were made to the protocol: the cell monolayer was not pretreated with 7% polyethelene glycol, and the cell culture plates were incubated at 15°C instead of 18°C. The killed fish were also tested for virus using the same technique described earlier for fish that died during the course of this study.

The prevalence of seropositive fish was calculated by summing the total number of seropositive fish and dividing the sum by the number of fish tested. A fish was considered positive if it had an antibody titer ≥20. Antibody titers were reported as the inverse of the highest serum dilution that resulted in a 50% reduction in the average number of plaques found in the negative control. The lowest dilution tested was 1:20 and the highest was 1:160.

Field study. Blood samples were collected from chinook salmon exposed to IHNV while at a salt water net-pen site in British Columbia, and the serum was tested for IHNV-specific antibodies. Chinook salmon were tested for IHNV-specific antibody twice while clinically affected Atlantic salmon were at the site (approximately 1 mo after the initial diagnosis was made) and at 2 other times — 9 and 18 mo — after the last Atlantic salmon were removed from the site.

Blood was collected from the chinook salmon on the processing line during harvests, via the heart, after the fish were stunned with carbon dioxide gas. Fish were sampled by a convenience sampling method: as one fish was completed the next fish in line was bled. The number of fish sampled varied from 18 to 58, depending on the time available. Three milliliter vacutainer tubes with no additive (Becton Dickinson, Franklin Lakes, NJ, USA), and 1½", 22 gauge needles (Becton Dickinson) were used to collect the blood. Samples were kept on ice until they were brought to the laboratory where they were allowed to clot at 4°C. Within 24 h of collection the blood was centrifuged at 1500 × *g* for 10 min at 4°C. Serum was removed and stored at –20°C until it could be evaluated for antibodies using the same technique described for the laboratory experiment.

The prevalence of seropositive fish in each harvest was calculated by dividing the number of fish that had IHNV-antibody titers greater than 20 by the number of fish tested (×100). A 95% CI was calculated using the exact binomial method (Daniel 1987) and the computer program EpiInfo 6.0 (Centers for Disease Control and Prevention, Atlanta, GA, USA).

RESULTS

Laboratory IHNV exposure

Fourteen of the 15 Atlantic salmon in Tank A died within 15 wk of exposure, and 11 of these deaths occurred within 6 wk of exposure (Table 1). Eight of these 11 fish (73%) tested positive for the virus. Granuloma-like lesions (i.e., multifocal whitish lesions) were observed in 2 fish, and *Piscirickettsia salmonis* was identified. The only surviving Atlantic salmon was removed from Tank A 8 d before cohabitation Trial 2 and was positive for IHNV.

Twenty-seven chinook salmon in Tank A died during the course of this study (Table 1), but only 2 died during the first 15 wk (105 d) post-exposure to IHNV. Only 1 of these 2 fish was positive for the virus. The fish that was positive for IHNV also had multifocal white lesions throughout the liver, suggestive of a co-infection with *Piscirickettsia*. This pathogen had been identified in the Atlantic salmon in the same tank as the chinook salmon, and *Piscirickettsia* has been reported in other fish held in salt water in this facility at this time of the year.

Six of the other 25 chinook salmon that died from the stock tank were positive for IHNV, but 4 of these 6 fish also had signs consistent with bacterial kidney disease (BKD) (Table 1). Kidney imprints were made from 2 of the fish and large numbers of small Gram-positive bacilli were detected.

Several other chinook salmon, besides the 4 that were positive for IHNV, also had signs of BKD (Table 1). Fourteen chinook salmon that died between 105 and 276 d post-exposure to IHNV had no gross lesions on post-mortem examination and were negative for virus. No chinook salmon that died 26 wk after the initial exposure to IHNV was positive for the virus. The cause of death was undetermined.

Co-habitation experiments

The 20 Atlantic salmon tested from the control tank before cohabitation Trials 1 and 2 were all negative for IHNV-specific antibodies. IHNV was confirmed in Atlantic salmon from all cohabitation experiments (Table 2).

Cohabitation Trial 1

Within 2 mo of introducing Atlantic salmon to Tank B with IHNV exposed chinook salmon, 64% (7 of 11) of the Atlantic salmon died. Four of the 7 fish tested positive for IHNV, and the other 3 had post-mortem

Table 1. Summary of when fish died in Tank A after exposure to infectious hematopoietic necrosis virus (IHNV), whether IHNV was isolated from the fish, whether fish exhibited multiple granuloma-like lesions and whether *Renibacterium salmoninarum* or *Piscirickettsia salmonis* was detected

Day of death post-exposure to IHNV	IHNV isolated	Granuloma-like lesions	Other pathogens
Atlantic salmon			
7	+	–	
8[a]	+	–	
9[a]	+	–	
9	–	–	
11[a]	+	–	
14	–	+	*P. salmonis*
26	+	–	
39	–	–	
54	–	–	
93	–	–	
97	+	+	*P. salmonis*
Chinook salmon			
60	+	+	*P. salmonis*
87	–	–	
116	–	–	
136	–	–	
136	–	–	
142	+	+	*R. salmoninarum*
142	–	+	*R. salmoninarum*
144	+	–	
144	–	+	Not examined microscopically
149	+	+	Not examined microscopically
153	+	+	Not examined microscopically
159	+	+	Not examined microscopically
164	–	+	Not examined microscopically
174	–	+	Not examined microscopically
179	+	–	
179	–	–	
203	–	–	
210	–	–	
212	–	–	
213	–	+	Not examined microscopically
213	–	–	
225	–	–	
239	–	+	Not examined microscopically
245	–	–	
247	–	–	
251	–	+	Not examined microscopically
274	–	–	

[a]Two fish died on these days and both were positive for IHNV

lesions consistent with IHNV (petechial haemorrhage throughout the visceral cavity), but no virus was isolated. None of the chinook salmon died during the course of the study, and all remaining fish (chinook and Atlantic salmon) were negative for the virus at the end of the study.

Water-borne exposure

Within 2 mo of introducing Atlantic salmon into Tank D, 4 of the 7 Atlantic salmon died. Two of these fish tested positive by viral assay, but only 1 fish was positive for IHNV by RT-PCR. Two chinook salmon died during the course of the experiment and both fish were negative for IHNV. At the end of the study 1 of the 8 remaining chinook salmon tested positive for IHNV. This fish also had granuloma-like lesions in its kidney suggestive of a BKD. The remaining 3 Atlantic salmon were negative for the virus.

Ten Atlantic salmon in the same ambient salt water supply but not exposed to chinook salmon were examined at the end of these trials and were all negative for IHNV.

Cohabitation Trial 2

Within 1 mo of introducing Atlantic salmon to Tank A containing the IHNV-exposed chinook salmon, 1 Atlantic and 1 chinook salmon died. The Atlantic salmon was positive for IHNV, but the chinook salmon was not. The remainder of the fish did not

Table 2. Summary of the viral status of dead and killed fish from the laboratory cohabitation experiments with IHNV-exposed chinook salmon and naïve Atlantic salmon

Expt	No. of infected Atlantic salmon[a]/ total Atlantic salmon		No. of infected chinook salmon[a]/ total chinook salmon		No. of infected control fish/ total
	Dead	Killed	Dead	Killed	
Cohabitation Trial 1	4/7	0/4	0	0/11	0/10
Water-borne exposure	1/4	0/3	0/2	1/8[b]	Same fish as in cohabitation Trial 1
Cohabitation Trial 2	1/1	0/6	0/1	0/5	0/9

[a]As determined by virus isolation and RT-PCR
[b]IHNV-positive fish also had gross clinical signs consistent with bacterial kidney disease

die and were negative for virus at the end of the study. The Atlantic salmon in the control tank that died (4 of 9) during the study were negative for IHNV and had large dermal erosions consistent with a marine *Cytophaga* spp. infection (Kent et al. 1988).

IHNV antibody profile laboratory exposure

In total, 70 asymptomatic chinook salmon were killed and tested for virus and IHNV-specific antibodies within the first 64 d post-exposure (Table 3). Of these fish, none tested positive for the virus and only 2 had IHNV-specific antibody titers (Table 3). The first seropositive fish was detected 14 d after exposure to IHNV, and the second seropositive fish was detected 64 d post-exposure. Both of these fish had titers ≥160.

Field study

All chinook salmon tested while Atlantic salmon were dying of IHN were negative for IHNV-specific antibodies (n = 18 and 24). Samples collected from chinook salmon 9 and 18 mo after the Atlantic salmon were removed from the site had a seropositive prevalence of 22% (13 of 58; 95% CI 12.5 to 35.3) and 19% (9 of 47; 95% CI 9.1 to 33.3), respectively. Seven of the 13 fish with IHNV antibodies in the samples collected 9 mo after the Atlantic salmon were removed from the site had titers ≥160. The other 6 fish with antibodies in this sample had titers between 20 and 80. All fish with IHNV antibodies in the sample collected 18 mo after the Atlantic salmon had been removed from the site had antibody titers ≥160.

DISCUSSION

The findings from this study indicate that some chinook salmon exposed to IHNV may become carriers of the virus, and these carriers may transmit the virus to susceptible fish if cohabited for a period of time. This study also indicates that identification of carrier chinook salmon, or populations of chinook salmon that may contain carrier fish, is difficult using standard virus isolation techniques, unless fish have concurrent infections. It may also be difficult to identify populations of chinook salmon that have been exposed to the virus, using serological analysis, because the prevalence of fish that seroconvert is low. This may be problematic if fish with an unknown history of exposure are cohabited with fish that are susceptible to IHNV, such as Atlantic salmon.

Table 3. Summary of the prevalence of IHNV and IHNV-specific antibodies in the chinook salmon killed from Tank A after their exposure to a 1 h static bath with 10^4 plaque forming units of infectious hematopoietic necrosis virus ml^{-1} of water

Day post-exposure to IHNV	Virus assay (+ve/total fish tested)	IHNV antibody (+ve/total fish tested)
0	0/3	0/3
3	0/10	0/10
8	0/10	0/10
14	0/10	1/10
17	0/10	0/10
22	0/10	0/10
29	0/10	0/10
64	0/10	1/10

High losses associated with IHNV were not observed in chinook salmon exposed to IHNV in this study. This finding was consistent with anecdotal reports given to us by producers raising chinook salmon in salt water net-pen sites with IHNV infected Atlantic salmon in British Columbia and agrees with other laboratory studies that exposed chinook salmon to strains of IHNV found in British Columbia or a similar virus electropherotype (Rucker 1953, LaPatra et al. 1993a, Traxler et al. 1993). On the basis of the ribonuclease protection assay data, the IHNV isolate used in this study was genetically similar to other IHNV isolates found in British Columbia from sockeye salmon (E. Anderson, Department of Microbiology, University of Maine, Orono, Maine, USA, 1999, pers. comm.), which historically have been classified as electropherotype 1 (Hsu et al. 1986).

We were unable to isolate IHNV from any of the chinook salmon killed from Tank A. Although cell culture has a detection level of approximately 10^2 particle forming units (Bootland & Leong 1999), only a relatively small amount of tissue is processed and evaluated. Therefore, the sensitivity of this test may not be adequate to detect virus in fish that have low titers or focal infections. Drolet et al. (1995) and Kim et al. (1999) were able to detect defective interfering virus particles in rainbow trout that had survived an epizootic of IHN; however, they were not able to conclude whether IHN survivors could transmit the virus to susceptible fish. To assess whether asymptomatic chinook salmon in this study were infected and shedding virus, we cohabited them with susceptible fish. Atlantic salmon were used as the susceptible species as they are extremely sensitive to IHNV (Traxler et al. 1993).

Despite detection of IHNV in only 1 chinook salmon used in the transmission experiments, the virus was confirmed in the Atlantic salmon after cohabitation with the chinook salmon for varying periods of time in all 3 ex-

periments. Furthermore, our study showed that transmission from asymptomatic chinook salmon through water without direct fish to fish contact is possible.

It was unlikely that the source of IHNV for the Atlantic salmon was anything other than the chinook salmon as the control Atlantic salmon, maintained on the same water supply, never became infected. Although some salmonid pathogens (e.g, *Renibacterium salmoninarum*, *Piscirickettsia salmonis*) occur in the salt water supply, we have never seen IHN, or detected the virus or IHNV-specific antibodies in fish maintained at the Pacific Biological Station that were not experimentally exposed to the virus. Furthermore, fish were maintained in salt water, and although IHNV has been shown to survive in salt water for a few weeks (Toranzo & Hetrick 1982), it is rarely detected in fish during their salt water migration period (Bootland & Leong 1999). It is possible in cohabitation Trial 2 that virus was held over in the tank from the last Atlantic salmon, as this fish was removed only 1 wk before the introduction of the naïve fish; however, in the first 2 trials completely new tanks were used, and some Atlantic salmon still succumbed to the virus. Therefore, we conclude that the most likely source of the virus for Atlantic salmon in our cohabitation studies was the virus-exposed chinook salmon.

It was only after the chinook salmon were diagnosed with piscirickettsia and BKD, 60 and 142 d after exposure to IHNV, respectively, that we were able to isolate virus from some of the fish, and almost exclusively in fish that had signs of concurrent infections (6 of 8) (Tables 1 & 2). Perhaps immunosupression from the co-infections was sufficient to permit the expression of IHNV in carrier fish. *Renibacterium salmoninarum*, the causative agent of BKD, has been shown to cause immunosupression by reducing the respiratory burst activity of phagocytes (Kaattari et al. 1988) and production of antibodies in fish (Brown et al. 1996). In other words, had there not been concurrent infections with other pathogens during the course of the study, we may not have detected the virus in the chinook salmon population. As it was, our findings help confirm that chinook salmon can become infected with IHNV despite not showing clinical signs of disease, and the virus may persist in some fish for a long time.

How many chinook salmon become carriers, how long these fish remain infected with IHNV and whether they have to be stressed to shed virus are issues that require further investigation. However, given that in most natural or aquaculture settings exposure to multiple pathogens and other stressors is inevitable, it may be prudent not to cohabit chinook salmon that have been previously exposed to IHNV with a species of fish, such as Atlantic salmon, that is known to be very susceptible to the virus.

Identification of populations of chinook salmon that have been exposed to IHNV may be difficult as fish do not show signs of disease (mortality), and the virus could not be isolated from asymptomatic fish. Serology has been used by other researchers to identify previous exposure to IHNV in rainbow trout (Hattenberger-Baudouy et al. 1989, LaPatra et al. 1993b), chinook salmon (Engelking & LaPatra 1996, LaPatra 1998), sockeye salmon *Oncorhynchus nerka* (Traxler et al. 1997) and Atlantic salmon (LaPatra 1998, St-Hilaire 2001). In many of these studies IHNV-specific antibodies were detectable in fish for a longer time than the virus, and the prevalence of seropositive fish in a population was high.

Our laboratory data suggest that very few chinook salmon (2 of 70) exposed to an isolate of IHNV found in British Columbia had a detectable antibody response, using the 50% plaque reduction technique. The low prevalence of seropositive fish in the population suggests that a large sample size would be required to use serology as a tool for determining the exposure status of a population with any certainty.

These findings were not consistent with those of Engelking & LaPatra (1996) and LaPatra (1998). The seroprevalence in chinook salmon populations in those studies was greater than 13%. This discrepancy may be due to the variable pathogenicity of IHNV in different geographic regions to different species of fish (LaPatra et al. 1993a). Perhaps if we had used a strain of IHNV that was more pathogenic to chinook salmon, such as IHNV electropherotype 2 or 3, as was the case in the 2 earlier studies, instead of IHNV electropherotype 1, typically found in British Columbia (Hsu et al. 1986, LaPatra 1993, LaPatra et al. 1993a), we would have observed a different immune response. The discrepancy may also have been due, in part, to the difference between a controlled laboratory exposure of IHNV and natural exposure to the virus. Both Engelking & LaPatra (1996) and LaPatra (1998) studied the antibody response in hatchery or wild chinook salmon populations.

In our field survey, antibodies were detected in blood samples collected from chinook held for over 5 mo in salt water net-pens adjacent to IHNV infected Atlantic salmon. The seroprevalence on 2 separate occasions was 22 and 19%. These figures were more consistent with those found by Engelking & LaPatra (1996) and LaPatra (1998), suggesting that more chronic exposure may be required to induce detectable antibody titers. The lack of consistency in the antibody response for fish held in different conditions suggests that more research is required before serology can be used to determine the IHNV exposure status of chinook salmon populations in British Columbia.

In conclusion, we showed that chinook salmon with undetectable IHNV infections can serve as reservoirs for the virus and can transmit the virus to Atlantic salmon, a highly susceptible species. Because of the difficulty in determining the exposure status of populations of chinook salmon, we recommend that Atlantic salmon not be maintained at the same net-pen farm as chinook salmon if there is any possibility that the latter have been exposed to the virus.

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