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EFFECTS OF FISH DENSITY ON SPREAD OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS (IHNV) IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

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Abstract

Rainbow trout *(Oncorhynchus mykiss)* were held at one of seven densities (8, 4, 0.63, 0.31, 0.16, 0.08 or 0.012 fish per liter) and challenged to the infectious hematopoietic necrosis virus (IHNV) by cohabitation with a single presumably infected donor fish. The trout were exposed to the IHNV-infected fish for 11 days to determine the effect of density on occurrence, spread and prevalence of the disease. The host density and IHNV prevalence were positively associated (r^2 = 0.89, regression analysis) with no occurrence of the virus at the two lowest densities (0.08 and 0.012 fish/l). Host density, therefore, is a key factor in determining the incidence and magnitude of IHNV infection.

Introduction

Infectious hematopoietic necrosis virus (IHNV), a rhabdovirus, causes a disease (IHN) characterized by extensive necrosis of hematopoietic tissues in early life stages of economically important salmonids. It is enzootic in the northwest Pacific area of North America (Parisot et al., 1965; Amend, 1975), but has been detected in other parts of the world including Taiwan (Chen et al., 1985), Belgium (Hill, 1992), Italy (Bovo et al., 1987), France (Baudin-Laurencin, 1987) and Japan (Sano et al., 1977). IHN primarily affects mem-

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bers of the genus *Oncorhynchus*, but members of *Salmo* and *Salvelinus* also are affected (Bootland and Leong, 1999). Survivors of an IHN epizootic are thought to be carriers or latently-infected (Amend, 1975). Mulcahy et al. (1982) suggested that a 100% infection rate in susceptible individuals may result from reactivation of IHNV in life-long carriers. However, it is not clear whether survivors of an epizootic continue to carry and spread the virus or become reinfected (Drolet et al., 1994). IHNV is becoming enzootic in very many areas in an increasing rate. Key factors regarding the disease need to be quantitatively evaluated to be able to control it.

It has been suggested that fish density is a factor in disease outbreaks in various salmonids (Ahne, 1980; McCallum, 1982; Schwedler and Plumb, 1982; Mulcahy and Bauersfeld, 1983; LaPatra et al., 1996). However, no study quantitatively evaluated a wide range of densities, especially using the cohabitation method of exposure that mimics the natural method of infection. This is vital in understanding the development of the ever-spreading IHN disease in cultured and wild fish populations.

In this study, rainbow trout were exposed to IHN by cohabitation. The relationship between fish density and the spread of the infection was studied.

Materials and Methods

Fish. Rainbow trout, *Oncorhynchus mykiss,* (approximately 1.2 g), were kindly provided by the Oak Springs Fish Hatchery of the Oregon Department of Fish and Wildlife (ODFW), OR. The hatchery had an IHNV-free history. Before the experiment, the fish were held in 1-m circular tanks with 366 l dechlorinated city water replaced at a rate of 3.5-4.1 l/min. The tanks were aerated and kept at 16-17°C. The fish were fed Biodiet (Bioproducts Inc., Astoria, OR), a pelleted feed, once a day at 1% of their body weight. The fish were acclimated for about one month before use in the experiments.

*Virus propagation and cell culture***.** The IHNV isolate (193-110) was originally isolated from rainbow trout in the Hagerman Valley of Idaho (Roberti, 1987). The IHNV was prepared by passing it through a 1-2 g rainbow trout by bath exposure and then *in vitro* on an *Epithelioma papulosum cyprini* (EPC) cell line (Fijan et al., 1983). It was titered and held in liquid nitrogen until use.

Infection of donor fish. Donors (rainbow trout infected with IHNV) were obtained by exposing fish to a bath containing 10^5 virus/ml MEM for 6 h at 16°C in two aerated 7.5-l tanks (Ogut and Reno, 2004a).

Experimental design. To determine whether there was a relationship between fish density and IHNV transmission, rainbow trout were held at different densities while being exposed to a single infected donor fish (Table 1) for 11 days. Densities ranged from 0.012 to 8 fish/l by stocking either 30 or 60 fish (2.8-3.1 g) in different sized tanks (7.5, 95, 366 or 2500 l). Water temperature was 16°C. Donor fish that died during the experiment were tested for the presence of the virus. Surviving donors were killed with excessive MS-222 and tested for the presence of IHNV on EPC cell lines.

Detection of infection. The presence of IHNV was determined by plaque assay on EPC cells as described by Burke and Mulcahy (1980), modified by treatment with polyethylene glycol (PEG). Whole fish homogenates were weighed, diluted 1:5 (w/v) in Eagle's Minimum Essential Medium with Earl's salts (MEM; Sigma Chemical Co., St. Louis, MO), without serum but containing pen-strep (20 units of penicillin and 0.02 mg streptomycin/ml; Sigma Chemical Co., Louis, MO), and centrifuged (5000 rpm for 10 min) to remove debris. Two dilutions (10^{-2} and 10^{-3}) were placed into replicate wells of 24 well plates containing EPC cells. Virus homogenates were absorbed to the EPC cells by gently rocking the plates for an hour at room temperature. Exposed cells were overlaid with 0.75% methylcellulose in complete MEM and plates were incubated at 18°C. The wells were examined for plaque formation 3 and 7 days after exposure. The wells were stained with crystal violet in formalin (25% formalin, 10% ethanol, 5% acetic acid and 1% w/v crystal violet) and plaques were counted.

Table 1. Tested densities.

Tank volume (l)	*No. fish/ replicate*	*Density (fish/l)*	*No. replicates*	*Turnover rate (tank volumes/day)*
7.5	60	8	5	40.3
7.5	30	4	5	40.3
95	60	0.63	5	4.5
95	30	0.32	5	4.5
366	60	0.16	5	5.9
366	30	0.08	5	5.9
2500	30	0.012	3	2.0

Results

Fish density had a significant effect on the prevalence levels of IHNV (Fig. 1). No transfer of virus occurred at the two lowest densities (0.083 and 0.012 fish/l), although all donor fish in each of three replicate tanks (6 fish) died of IHN 3 or 4 days after exposure. Of the total 33 donor fish used in 33 treatment tanks, 26 were infected with IHNV. There were two tanks with infected recipient fish where the donors tested negative for IHNV. Four other surviving donors and one donor fish that died during the experiment also tested negative. There was a significant positive association between density and IHN prevalence (non-linear regression test, $p_{(2)} = 0.0011$). Density dependence was also examined using the method described by Varley and Gradwell (1960) that tests density dependence by plotting proportionate survival on logarithmic scales against the log of density; if a dependence exists, the S/N proportion drops sharply after a certain density (Fig. 2).

In one of the 7.5-l tank/30 fish replicates, the prevalence level was 25% while the others had very low or no prevalence (Table 2). Unexpectedly, prevalence levels were slightly lower in similar-sized tanks containing 60 rather than 30 fish. Prevalence values and density were correlated in replicates containing 60 fish (χ^2 test, $p_{(2)} = 0.004$) but there was no correlation between prevalence and density in replicates containing 30 fish due to the high variation (χ^2 test, $p_{(2)} = 0.12$) and low number of tanks that contained infected fish (6 of 18 tanks).

Discussion

There was a direct relationship between density of the recipient fish exposed to a single infectious individual and the transmission of IHNV. This demonstrates that density is a key factor in determining the level of infection after exposure to IHNV by cohabitation. In all three replicates of the lowest density, 30 fish per 2500 l tank (0.012 fish/l), the virus was not transmitted in spite of the fact that two of the three donor fish were infected, and presumably infectious. Thus, this level of fish density is likely below the threshold density for IHNV.

Rainbow trout exposed by bath and used as donors for further experiments were efficiently infected. The overall IHN related mortality in the donor fish during the experimental period was high (21 out of 25). In our study, the infection method was indirect and the “unit of infection” was the number of donor fish rather than the number of bacteria. Therefore, it was difficult to precisely evaluate the “dose”

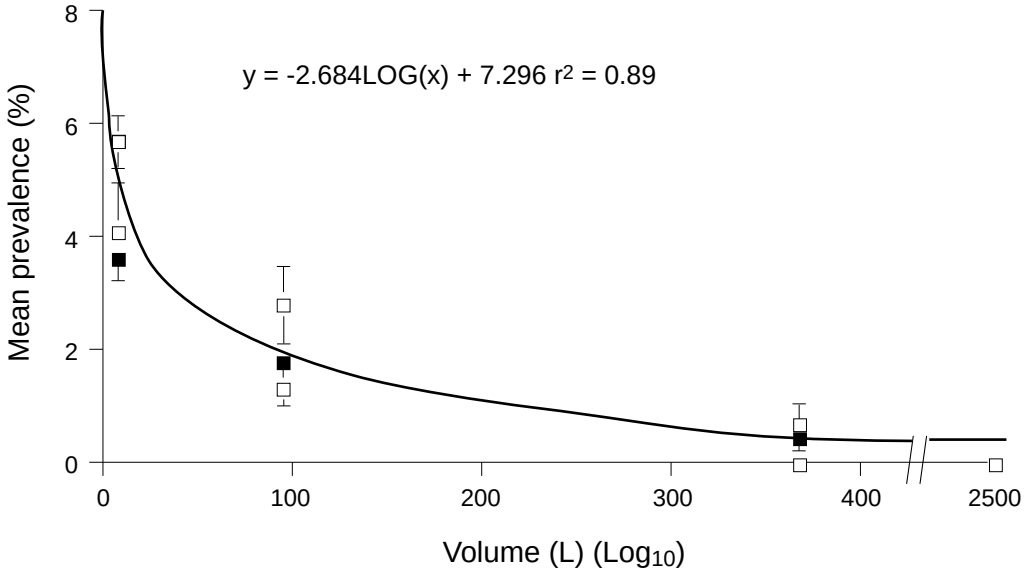

Fig. 1. Mean IHNV prevalence levels (mean±SE of 5 replicates) in four water volumes (7.5, 95, 366 and 2500 l) containing 30 (□) or 60 (■) rainbow trout, challenged by one presumably infected fish.

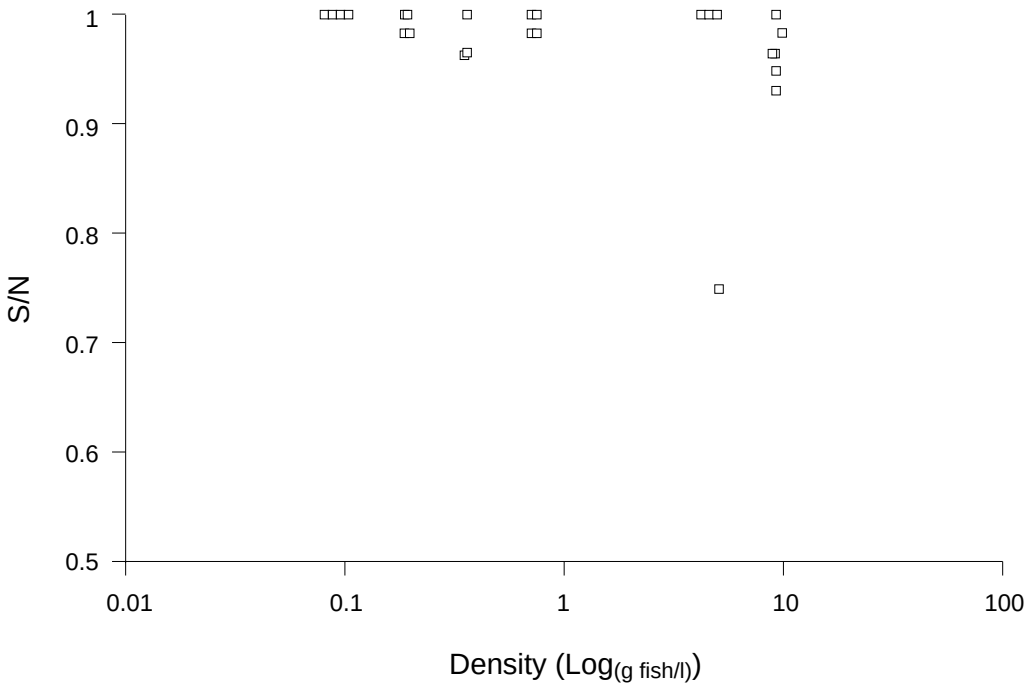

Fig. 2. Density dependence of IHNV spread in rainbow trout.

Table 2. Infection and IHN related mortalities in 7 different densities.

	7.5 l Tanks (8 fish/l)				*95 l Tanks (0.63 fish/l)*				*366 l Tanks (0.16 fish/l)*				*2500 l Tanks (0.012 fish/l)*			
Replicate	n	CM	NI	IM	n	CM	NI	IM	n	CM	NI	IM	n	CM	NI	IMR
1	**63**	11	1	0	**60**	12	1	0	61	1	0	0	**28**	4	0	0
2	**57**	10	2	1	**57**	7	1	1	58	6	0	0	25	1	0	0
3	**58**	8	2	0	**59**	9	1	1	**59**	1	0	0	**27**	3	0	0
4	**59**	9	3	2	**59**	7	1	1	**57**	3	1	1				
5	**58**	1	4	4	57	9	0	0	**59**	6	1	1				
Control	**59**	1	0	0	60	3	0	0	59	2	0	0				
	7.5 l Tanks (4 fish/l)				*95 l Tanks (0.32 fish/l)*				*366 l Tanks (0.08 fish/l)*							
1	31	6	0	0	**28**	5	1	0	28	2	0	0				
2	**32**	4	0	0	29	3	0	0	**25**	2	0	0				
3	**28**	2	1	0	**29**	6	1	0	**27**	3	0	0				
4	**27**	9	0	0	**28**	6	1	0	**32**	5	0	0				
5	**32**	10	8	1	**28**	8	1	1	**30**	6	0	0				
Control	**29**	4	0	0	29	2	0	0	30	1	0	0				

CM = Cumulative mortality
NI = Number of infected fish in tank
IM = Infected mortality (no. of deceased fish that were infected)
Bold number indicates that the donor in the tank was infected.

administered to susceptible rainbow trout. We favored cohabitation by a single infectious individual to make the exposure more natural than the more frequently utilized immersion or injection methods, which introduce bias from challenge stress into the experiment. Using a single infectious individual also provided invaluable information about individual differences of responding bath challenge. When desired, more than one infectious individual can be used for approximately the same level of exposure (central limit theorem). Additional information about the level of exposure and outcome (disease spread) could be gained by relating the level of virus in each donor fish to the prevalence in the recipient fish as in similar experiments using *A. salmonicida* (Ogut and Reno 2004b). However, in this experiment, only unit of infection was considered.

Density can affect prevalence levels in two ways. First, high density is related to deterioration of water quality that can stress animals and impair immune systems (Wedemeyer et al., 1997). Second, the probability of contact between infected animals or infectious agents increases with as density increases.

The dependence of the spread of infection on density has been reported for the golden shiner virus (Schwedler and Plumb, 1982), *Ichthyophthriasis* (McCallum, 1982), flavobacteriosis (Ahne, 1980) and, recently, furunculosis (Ogut and Reno, 2004b). Mulcahy and Bauersfeld (1983) reported that egg and alevin densities were important factors in determining the extent of mortalities caused by IHN. In incubation boxes in river water, they tested three egg densities – 9, 6.5 and 3.5 x 10^5 eggs/box – less than a 3-fold range. Mortality at the highest and medium density exceeded 90% whereas mortality at the lowest density was approximately 40%. It was suggested that the high density encountered after hatching contributed to the high mortality. It was also stated that natural epizootics of IHN were unlikely due to the low densities of eggs and alevins. Note that in our study, a much wider range of densities (more than 300-fold) was selected. LaPatra et al. (1996) did not detect any difference in prevalence in populations of 25 and 50 fish per tank. Traxler (1986) suggested that an IHNV epizootic in 2-year-old kokanee, *Oncorhynchus nerka*, may have involved increased density caused by schooling at spawning. It may be impossible to see differences in prevalence values at narrow density ranges. The restricted range of densities previously used may not have been sufficient to show an effect.

Another study to determine the effect of host density was carried out by Mulcahy et al. (1983). They concluded that fish density was a factor determining infection rates and virus titers. Samples of 50 females kept in side channels and 50 females kept in a river were taken. Prevalence in females from the side channels was 100% at a level of 4.9 x 10^4 plaque forming units (pfu)/g, whereas in females from the river 85% were infected at a level of 2.2 x 10^3 pfu/g. They placed one box with 50 yearling fish in a side channel and another 50 in a tank in the laboratory to compare virus levels. IHNV was isolated from 3 of 10 yearlings sampled from the side channel after a week. Two weeks later, the infection rate was 90% and 21 days later there was an increase of virus titers in the gills and viscera to 2.7 x 10^4 pfu/ml. No virus was detected in the fish held in the laboratory. They suggested that low water flow and high density of spawning salmon penned in the side channel caused the prevalence and virus titers.

The initial holding density for rainbow trout at the Pacific Northwest Hatcheries is 25-30 fish/l (Ras Rehis, ODFW, Nehalem Hatchery, pers. comm.), much higher than the ranges tested in this study. It is likely that the lower densities used in this study resulted in the lower prevalence. In addition, the fish were held for only 11 days in the presence of a single infectious fish. We intentionally kept the duration of the experiment short since our aim was to determine the level of infection started by a single infectious individual. As fish grow in hatcheries, the density may triple. However, the number of fish per unit at ODFW is reduced by more than half (Ras Rehis, pers. comm.). This provides more space for each fish, reducing the indirect effects of density-related pollution. Providing more space to the animals is analogous to decreasing the likeli-

hood that a fish comes into contact with an infectious fish or agent. In short, considering the mass action principle, the number of fish in a unit of volume is important. Therefore, in management, decreasing the number of fish in a pond is indirectly manipulating the interaction between infected and susceptible animals to decrease the probability of infectious contacts.

The host density has substantial effect(s) on the spread of IHNV in rainbow trout, suggesting that fish density be seriously examined when stocking fish for aquaculture or, more importantly, for stock enhancement where quality of fish is vital for survival and sustainability. Especially in places where certain diseases are a persistent problem, adjustments in stocking densities should be considered rather than trying to keep fish alive with chemotherapy.

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