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Freshwater crayfish *Astacus astacus* – a vector for infectious pancreatic necrosis virus (IPNV)

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ABSTRACT: Freshwater crayfish *Astacus astacus* were experimentally infected with infectious pancreatric necrosis virus (IPNV) strain Sp by injection, by waterbath immersion, by administration of virus in feed, and by exposure to IPNV-infected fry of rainbow trout *Salmo gairdneri*. IPNV was found in crayfish organs and haemolymph up to 1 yr after infection. Crayfish excreted IPNV into the water continuously. Fry and eggs of rainbow trout immersed into that water became infected with IPNV. IPNV particles were demonstrable by electron microscopy in the granules of crayfish haemocytes. Results indicate that the freshwater crayfish could play a role in the epizootiology of IPN.

INTRODUCTION

Infectious pancreatic necrosis (IPN), a viral disease of young salmonid fishes, causes severe losses in cultured salmonids. The disease agent, infectious pancreatic necrosis virus (IPNV), has been found in cyclostomata (1 species) and in many teleost fishes (37 species) as well as in molluscs (6 species) and crustaceans (Ahne 1985b). Crustaceans known to carry IPNV are *Carcinus maenas* (Hill 1982), *Daphnia magna* (Ahne 1984), and *Penaeus japonicus* (Bovo et al. 1984). As freshwater crayfish have become increasingly important for aquaculture, there is greater demand for the international transfer of *Astacus astacus*. Therefore, we wondered whether freshwater crayfish could play a role in the epizootiology of IPN. Although the mechanism of transmission and the spread of fish viruses are not fully understood, it is known that fish acutely or chronically infected with IPNV excrete the virus via faeces and sexual products (Wolf et al. 1963, Ahne 1983b). Horizontal transmission occurs via contaminated water or cannibalism (Ahne 1983a), whereas vertical transmission takes place via eggs (Bullock et al. 1976, Ahne & Negele 1985). Atypical host fishes and bloodsucking ectoparasites, as well as other aquatic organisms and fishery equipment, must also be regarded as sources of virus infections (Ahne 1983a). However, nothing is known about interactions between the freshwater crayfish and viruses pathogenic for fish.

In the present study we tried to answer the following questions: Does the freshwater crayfish *Astacus astacus* take up IPNV? Does the virus persist in the crayfish? Is IPNV excreted by the infected crayfish? Can rainbow trout *Salmo gairdneri* fry and eggs become infected with IPNV excreted by infected crayfish? The answers to these questions are important to an understanding of the epizootiology of IPN.

MATERIALS AND METHODS

Animals. Fifty healthy, 2 to 3 yr-old freshwater crayfish *Astacus astacus*, obtained from a local hatchery in FR Germany, were kept in 50 l tanks in running water at 10 to 12 °C. They were fed on fish, carrots, and potatoes during the experiments. Two hundred fingerlings (4 to 6 wk old) and 100 eyed eggs of rainbow trout *Salmo gairdneri*, kept in water at 10 to 12 °C, were used for the IPNV transmission experiments.

Virus and virus isolation trials. The virus used for the infection trials was IPNV-Sp grown in PG cells (Ahne 1979) Virus isolation trials from the holding water, the trout fry, and the haemolymph, gills, hepatopancreas, stomach, and muscle tissue of the freshwater crayfish were done by standard methods (Wizigman et al. 1983) using PG cells. Antennal gland, heart, gut, and gonads of the freshwater crayfish were cut into small pieces and inoculated directly on PG cell monolayers. The eggs of rainbow trout were treated as described by Ahne & Negele (1985). The viruses isolated were identified by means of neutralization tests and the indirect immunofluorescence technique using

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monospecific IPNV-Sp antiserum of rainbow trout (Kohlmeyer et al. 1986).

Electron microscopy. Antennal gland, hepatopancreas, and gills of the freshwater crayfish were prepared for electron microscopy as described by Peterson & Loizzi (1973).

Infectivity testing of IPNV in *Astacus astacus*. *Injection of virus:* Six freshwater crayfish were each injected in the haemocoel with 0.5 ml of an IPNV suspension ($10^{7.8}$ $TCID_{50}$ $0.1ml^{-1}$). Afterwards, 0.2 ml samples of haemolymph bled from the ventral sinus of the freshwater crayfish were checked for the presence of virus at 2, 4, 6, 8, 16, and 24 d after infection and then weekly.

Infection by waterbath: Nine freshwater crayfish were challenged with IPNV by the waterbath method for 1 h at 10°C using 1 l of IPNV-contaminated water ($10^{5.2}$ $TCID_{50}$ $0.1ml^{-1}$). After challenge, the crayfish were transferred to tanks supplied with running water. Their haemolymph was examined for the presence of virus at 2, 4, 6, 8, 16, and 24 d after infection and then weekly.

Infection via food: Three freshwater crayfish were fed on IPNV-contaminaned dead rainbow trout fry ($10^{4.5}$ $TCID_{50}$ $0.1g^{-1}$ fish). Their haemolymph was checked for the presence of virus at 2, 4, 6, 8, 12, and 24 d after feeding. After 34 d, the freshwater crayfish were again fed on the IPNV-infected tissue of rainbow trout. The haemolymph of the infected crayfish was examined for virus weekly. They were killed 64 d after the first feeding and their organs were checked for the presence of IPNV.

Infection by exposure to IPNV-infected rainbow trout fry: Thirty rainbow trout fingerlings were challenged with IPNV by the waterbath method (1 l water, 1 h, 10°C, $10^{5.5}$ $TCID_{50}$ $0.1ml^{-1}$ water) prior to the experiment. Two d after challenge, the running water from the aquarium containing the rainbow trout fry was diverted into an aquarium containing 3 freshwater crayfish. Eight d after infection, the fry showed a virus titer of $10^{5.2}$ $TCID_{50}$ $0.1g^{-1}$ fish. The haemolymph of the freshwater crayfish was checked for the presence of virus after 2, 4, 6, 8, 16, 24, 31, 39, and 46 d of continuous exposure. At Day 50 the freshwater crayfish were killed and their organs were checked for the presence of virus.

Infection of rainbow trout fry and eggs with IPNV excreted by *Astacus astacus*. In order to determine if rainbow trout fry and eggs could be infected with IPNV excreted by freshwater crayfish, 2 groups of 3 IPNV-inoculated freshwater crayfish were kept in 2 aquaria, each containing 1 l of water The water was checked for the presence of virus every 24 h. After 7 d, the freshwater crayfish were transferred to 2 tanks supplied with running water. Two d after the transfer, the outflowing water was directed to 2 aquaria each containing 50 fingerlings and 30 eggs of rainbow trout. The fingerlings and eggs were examined for the presence of virus at 6, 12, 24, 48, and 72 h, and at 6, 9, 12, and 21 d after exposure began. In addition, 15 rainbow trout fingerlings and 25 fish eggs were directly immersed in the remaining 1 l of water in the aquaria of the infected crayfish. After 12 h, the exposed fingerlings and eggs were transferred to 10 l tanks supplied with running water. Both eggs and fish were checked for virus after 0.25, 0.5, 1, 2, 3, 6, and 10 d following the transfer.

RESULTS

Inoculation of freshwater crayfish with IPNV

Two d after inoculation of the crayfish with IPNV, the virus could be isolated from the haemolymph (Table 1). Even 1 yr after infection, the haemolymph of 1 individual had a virus titer of $10^{5.5}$ $TCID_{50}$ $0.1ml^{-1}$ (data not shown in Table 1). In addition, the virus was detected in the organs of 4 crayfish that died during the experiment (Table 2).

Exposure of freshwater crayfish to IPNV via waterbath

The virus could be reisolated from the haemolymph of all crayfish infected using the waterbath method

Table 1. *Astacus astacus*. Detection of IPNV in the haemolymph of freshwater crayfish after intra-abdominal injection (0.5 ml) of the virus ($10^{7.8}$ $TCID_{50}$ $0.1\ ml^{-1}$). Values are infectivity (log_{10} $TCID_{50}$ $0.1ml^{-1}$ haemolymph)

	Crayfish No.						
	1	2	3	4	5	6	Control
Days after infection							
2	7.8	7.2	7.2	7.8	7.5	7.8	0.0
4	7.8	7.2	7.2	6.8	7.5	7.5	0.0
6	7.2	7.5	7.2	7.2	6.8	7.5	0.0
16	7.2	6.5	7.2	7.8	6.8	7.2	0.0
24	5.8	7.5	5.8	6.8	6.5	6.8	0.0
Weeks after infection							
5	5.8	5.5	5.8	6.2	6.5	6.2	0.0
8	d	6.8	ne	6.5	ne	6.5	0.0
10		5.5	5.8	7.5	5.8	6.8	0.0
13		5.5	5.8	7.5	5.5	6.8	0.0
18		d	d	7.2	5.5	6.8	0.0
22				d	d	6.8	0.0
26						6.8	0.0

ne: not examined; d: dead

Table 2. *Astacus astacus*. Detection of IPNV in the tissue and organs of the freshwater crayfish after intra-abdominal injection (0.5 ml) of virus ($10^{7.8}$ $TCID_{50}$ 0.1 ml^{-1}). Values are infectivity (log_{10} $TCID_{50}$ 0.1 g^{-1})

	Crayfish No.				
	1	2	3	4	Control
Time of death (d after infection):	42	93	100	150	–
Muscle	5.8	4.5	5.8	4.5	0.0
Stomach	5.5	4.5	6.2	4.5	0.0
Gills	6.8	4.5	6.2	4.5	0.0
Hepatopancreas	6.2	4.2	6.2	+	0.0
Antennal gland	+	+	+	+	0.0
Gut	+	+	+	+	0.0
Heart	+	+	+	+	0.0
Gonads	+	+	+	+	0.0

+: virus positive (without titration)

within 2 d after exposure. The virus persisted in the haemolymph for up to 14 wk (Table 3). After molting, which took place 16 wk after infection, IPNV was still present in the haemolymph of 2 individuals. Seven crayfish died during or following molting. The virus was isolated from the gills of all crayfish ($10^{1.5}$ to $10^{4.5}$ $TCID_{50}$ 0.1g^{-1}). In addition, the virus was found in different organs and tissues (hepatopancreas, stomach, gonads) of infected individuals.

Infection of freshwater crayfish with IPNV via food

The hepatopancreas and the gills of freshwater crayfish fed with IPNV-contaminated rainbow trout fry contained IPNV ($10^{1.0}$ to $10^{2.5}$ $TCID_{50}$ 0.1g^{-1} and $10^{1.0}$ $TCID_{50}$ 0.1g^{-1}, respectively) 64 d after infection.

Exposure of freshwater crayfish to IPNV-infected rainbow trout fry

The gills of the exposed freshwater crayfish showed infectivity ($10^{1.5}$ to $10^{1.8}$ $TCID_{50}$ 0.1 g^{-1}) 50 d after infection.

Infection of fry and eggs of rainbow trout with IPNV excreted by freshwater crayfish

IPNV was excreted continuously into the water by IPNV-infected freshwater crayfish. The IPNV infectivity of the water was up to $10^{2.5}$ $TCID_{50}$ 0.1ml^{-1}. Eggs of rainbow trout immersed in this water proved to be virus-positive within 12 h. Rainbow trout fry immersed in the same water exhibit titers of infectivity between $10^{1.5}$ and $10^{3.8}$ $TCID_{50}$ 0.1g^{-1} 3 d after immersion. Five of the IPNV-infected rainbow trout fingerlings kept together with 3 IPNV-free freshwater crayfish in a tank supplied with running water were killed and eaten by the crayfish within 15 d. The IPNV was detected in the gills ($10^{1.5}$ $TCID_{50}$ 0.1g^{-1}) of all 3 freshwater crayfish and in the gonads of 1 specimen 21 d after feeding on the fish. The eggs of rainbow trout kept in the effluent water of the aquaria with IPNV-infected freshwater crayfish proved to be virus-positive within 12 h.

Detection of IPNV in the tissue and haemocytes of infected freshwater crayfish by electron microscopy

The IPNV could be demonstrated in the cytoplasmatic granules of the haemocytes of the antennal gland, the gills, and the hepatopancreas (Fig. 1), as well as in the phagocytes of the connective tissue of the latter organ.

Identification of virus isolated from freshwater crayfish

All isolates were identified as IPNV strain Sp by means of neutralization tests and by the indirect immunofluorescence technique.

DISCUSSION

At present, little is known about the role of crustaceans as vectors for viruses. However, DiGirolamo et al. (1972a, b) and Hejkal & Gerba (1983) demonstrated that edible crabs may function as vectors for human pathogenic viruses (Poliovirus, Simian Rotavirus, Echovirus). These crabs were able to take up viruses from contaminated water and food. The ectoparasite of fish *Argulus foliaceus* has been identified as a vector of spring viremia of carp virus (SVCV) (Ahne 1978, 1985a). This was the first evidence of transmission of a fish pathogenic virus by crustaceans. However, our results show that the freshwater crayfish *Astacus astacus* must be considered as a mechanical vector of IPNV in aquatic systems, because we did not find any evidence for a replication of IPNV in the freshwater crayfish (Tables 1 and 3).

It is known that IPNV-infected fish excrete the virus into the water (Wolf et al. 1963). The question about uptake of IPNV by the freshwater crayfish from contaminated water has become important because rainbow trout and freshwater crayfish are often kept together in the same biotope. We have proved that the IPNV infec-

Table 3. *Astacus astacus*. Detection of IPNV in the haemolymph of freshwater crayfish after infection with the virus by the waterbath method ($10^{5.2}$ $TCID_{50}$ 0.1 ml^{-1}). Values are infectivity (log_{10} $TCID_{50}$ 0.1 ml^{-1} haemolymph)

	Crayfish No.									
	7	8	9	10	11	12	13	14	15	Control
Days after infection										
2	+	+	+	+	+	+	+	+	+	0.0
4	1.5	1.8	1.5	1.5	+	+	1.5	2.2	2.8	0.0
6	1.5	1.8	1.5	1.5	1.5	1.5	1.5	1.5	1.8	0.0
8	1.5	1.5	1.8	+	1.8	1.8	-	1.8	2.2	0.0
16	1.5	1.5	1.5	1.5	1.5	1.5	+	+	2.2	0.0
20	1.5	1.5	1.8	1.5	1.5	1.5	1.5	+	2.2	0.0
Weeks after infection										
5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	2.2	0.0
6	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	2.2	0.0
7	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	2.2	0.0
8	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.8	0.0
9	1.5	+	1.5	+	1.5	1.5	1.5	1.5	1.5	0.0
10	+	+	1.5	+	+	+	+	+	1.5	0.0
11	+	+	+	+	+	+	+	+	1.5	0.0
12	+	+	+	+	+	+	+	+	1.5	0.0
13	+	+	+	+	+	+	+	+	1.5	0.0
14	+	+	+	+	+	+	+	+	1.5	0.0
15	+	+	+	+	+	d	d	d	d	0.0
16	+	+	+	+	+					0.0
17	+	d	+	ne	ne					0.0
20	-	d	+	ne	ne					0.0
22	ne		ne	+	+					0.0
24	d		d	+	+					0.0

d: dead after molting; ne: not examined;
+: virus positive after 1 passage

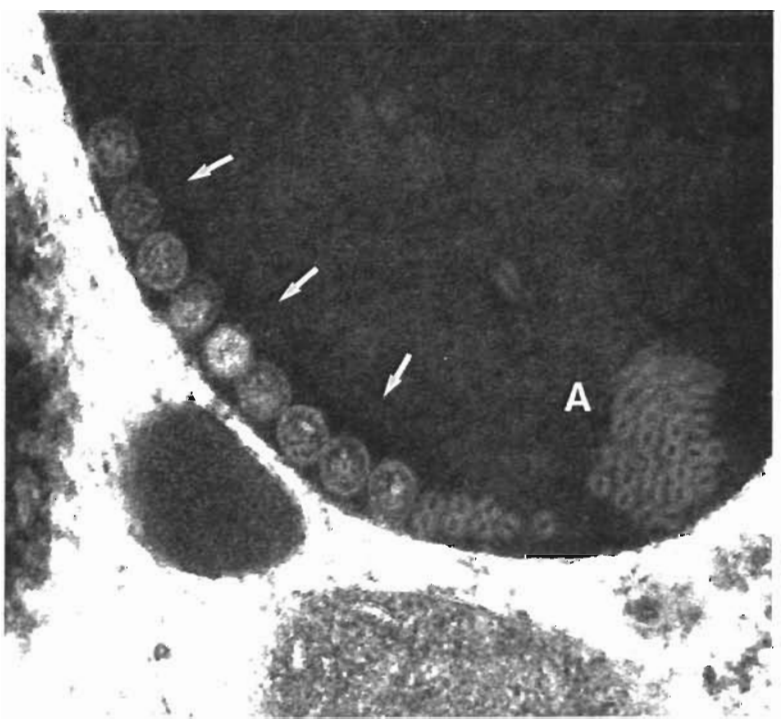

Fig. 1. *Astacus astacus*. IPNV-infected freshwater crayfish. Electron micrograph of IPNV (arrows) in a cytoplasmatic granule of a haemocyte in the hepatopancreatic tissue. A: probable subunits of the virus (150 000 ×)

tion of freshwater crayfish via contaminated water occurs by keeping freshwater crayfish together with IPNV-infected rainbow trout. Horizontal transmission of IPNV is also known to occur via cannibalism or by feeding on infected fishes (Ahne 1983a). In our experiments, the freshwater crayfish could be infected with IPNV by feeding on IPNV-contaminated rainbow trout. Under natural conditions the freshwater crayfish feeds not only on plants, insects and worms, but also on sick, weakened, and dead fish (Hofmann 1979). Our results show that *Astacus astacus* plays a role in heterologous infection cycles of IPNV. For several fish viruses, homologous infection cycles have been shown in carnivorous fishes (Ahne 1983a). Other animals such as birds and minks which feed on fish are also considered as possible vectors for IPNV (Sonstegard & McDermott 1972).

In our experiment, IPNV persisted for up to 1 yr in the organs of infected freshwater crayfish. Even after molting, the virus could be detected in the haemolymph. Considering the continuous excretion of IPNV by the freshwater crayfish, *Astacus astacus* must be regarded as a potentially important reservoir of IPNV. This fact was clearly demonstrated by the transmission of IPNV to rainbow trout fry when the fish were held in effluent from IPNV-infected freshwater crayfish.

Furthermore, it is of great epizootiological interest that eggs of rainbow trout could be infected with IPNV excreted in very low amounts (10^1 to $10^{2.5}$ $TCID_{50}$ $0.1ml^{-1}$) by the infected freshwater crayfish. The vertical transmission of IPNV through contaminated eggs has been clearly shown by Wolf et al. (1963), Bullock et al. (1976), and Ahne & Negele (1985).

The present results must be taken into consideration in programs designed to control IPN. Freshwater crayfish should be certified as free of IPNV before they are introduced into any aquaculture system. This means that freshwater crayfish should be included in the routine virological examinations as is required by law for other fishes in many countries.

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