

Persistence of infectious pancreatic necrosis virus (IPNV) in scallops *Pecten maximus*

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ABSTRACT: Infectious pancreatic necrosis virus (IPNV), serotype N1 isolated from scallops *Pecten maximus* in Norway, was propagated and used in both inoculation and bath challenge experiments with scallops *in vivo*. Although virus titers measured in scallop tissues decreased, depuration of virus was not complete during the experimental periods. IPNV was still detectable 11 mo after injection. The highest virus titer was found in the hepatopancreas, but virus was also detectable in other tissues, as well as in the hemolymph. After a bath challenge, uptake of IPNV was shown. Virus was present in hepatopancreas, gonad, kidney, mantle, gill, rectum and in the hemolymph 1 d after the uptake. The titer was highest in the hepatopancreas where virus was detectable at the end of the experiment, 50 d after challenge. Virus levels in the rectum decreased below detectable levels after Day 30. Titers decreased rapidly in the hemolymph where no virus could be detected after Day 8. Challenges did not result in increased mortality or in clear pathological changes in the scallops. No evidence of viral replication within the scallops was found.

INTRODUCTION

In July 1988, mortalities affected both spat and adult scallops *Pecten maximus* at a shellfish hatchery near Bergen, western Norway. Infectious pancreatic necrosis virus (IPNV) serotype N1 (Christie et al. 1988) was isolated from moribund adult scallops (Mortensen et al. 1990).

IPNV belongs to the virus family 'Birnaviridae' (Dobos et al. 1979, Dobos & Roberts 1983, Brown 1984), and viruses from this family have been isolated from several species of marine invertebrates (Hill 1976, Bovo et al. 1984, Lo et al. 1988).

It is still unclear whether the aquatic birnaviruses might act as pathogens for bivalve molluscs, although Hill & Alderman (1979) reported moderate pathological changes in oysters infected with 2 aquatic birnaviruses isolated from the clam *Tellina tenuis* and the oyster *Ostrea edulis*.

It has been shown that isolates from *Tellina tenuis* and from oysters are biochemically and serologically different from the major aquatic birnavirus serotypes (Hill 1976, Underwood et al. 1977). The aquatic birnaviruses have thus been divided into 2 serogroups, with

the IPNV serotypes in serogroup I, and the above-mentioned shellfish isolates, together with a few isolates from fish (Hill 1982, Olesen et al. 1988), in serogroup II. The recent isolation of an aquatic birnavirus serogroup II from an epizootic of salmonid fish (Ahne et al. 1989), and the facts that IPNV has been isolated from marine invertebrates and that shellfish isolates of IPNV or IPNV-like viruses may induce typical signs of infectious pancreatic necrosis in rainbow trout fry (Hill 1982), might indicate that viruses from the 2 serogroups are not strictly specific for fish and shellfish respectively.

As IPNV may be transmitted via faeces and sexual products from infected fish (Wolf et al. 1963) and with decaying infected fish, the virus might subsequently be associated with bivalve molluscs which filter and accumulate particles from the environment. The hepatopancreas is the major organ involved in depuration of digested matter, and a finding of virus in the hepatopancreas might thus indicate a contamination without pathological significance. However, the total depuration of foreign particles in bivalve molluscs is known to be slow (Stauber 1950, Hay & Scotti 1986), and the bivalve molluscs are considered to serve as

vectors and reservoirs of various viruses (Mason & MacLean 1962, Metcalf & Stiles 1965, Feng 1966, Canzonier 1971, Hay & Scotti 1986) including aquatic birnaviruses (Hill et al. 1984).

Our aim was to study the possible role of scallops as vectors for aquatic birnaviruses, and a series of experiments was performed in order to answer some of the questions regarding the virus pathogenicity, persistence, uptake and distribution in scallops.

MATERIALS AND METHODS

Animals. All experiments were performed with adult scallops *Pecten maximus*, acclimatized to laboratory conditions. IPNV was not detected in hepatopancreas samples from control scallops prior to the infection experiments.

The first injection experiment was performed on scallops with an average shell height of approximately 10 cm, originating from the bay of St. Brieuc, northern Brittany, France. The scallops were kept at 11 °C in a 60 l plastic tank with recirculating seawater of salinity 28 to 32 ‰ and fed a suspension of *Chaetoceros calcitrans*.

Scallops used in the other injection and bath experiments were collected by divers near the island of Sotra, western Norway. The scallops had an average shell height of approximately 12 cm. The scallops were kept at 10 to 12 °C in 250 l aquaria with running seawater of salinity 34 to 35 ‰, and fed a suspension of *Skeletonema costatum* occasionally supplemented with *Isochrysis galbana* and *Tetraselmis suecica*.

Virus. The virus used was IPNV, serotype N1 isolated from Norwegian scallops *Pecten maximus* (Mortensen et al. 1990).

Cell culture. The rainbow trout gonad (RTG-2) cell line (Wolf & Quimby 1962) was used in virus propagations, detections and titrations. Cells were cultured at 20 °C in Earle's modification of minimum essential medium (EMEM) (Flow) supplied with 10 % foetal bovine serum (Flow), 1 % non-essential amino acids (Flow), 10 ml L-glutamine (200 mM) and 10 ml Gentamicin solution (10 mg ml⁻¹) per liter. As confluent cell layers of the RTG-2 cell line are known to produce interferon (Okamoto et al. 1983) all plates were prepared the day before use, adding 25 to 30 000 cells per well (ca 9 × 10⁴ cm⁻²).

Virus titrations. Virus titrations were performed by end-point dilutions on RTG-2 cell layers in 96-well Nunclon microtiterplates using 12 wells per dilution. Infected cell layers were identified by the cytopathic effect 6 d after inoculation. Virus titers were calculated as TCID₅₀ ml⁻¹ or TCID₅₀ g⁻¹ tissue according to the method of Reed & Muench (1938). In cases of doubt,

50 µl supernatants from the wells were inoculated onto fresh RTG-2 cell cultures and incubated another 6 d.

Virus detections. For virus detections, 500 µl filtrate of 1:49 dilutions of tissues in EMEM cell culture medium were inoculated onto RTG-2 cell cultures in 25 cm² Nunclon tissue culture flasks. Supernatants (50 µl) from the flasks were transferred to new cell cultures twice (Expts 3 & 4).

Histology. Tissue samples were fixed in buffered 4 % formol, embedded in paraffin, sectioned, stained with Hematoxylin-Erythrosin-Saffron and observed at 40 to 400 × magnification under a light microscope.

Virus challenges. Four virus challenges were performed; 3 by injection (Expts 1 to 3) and 1 by bath (Expt 4).

Expt 1: This experiment was performed to determine the changes in the total virus content in the scallops after injection. Ten scallops were each inoculated with 1 ml of a viral suspension diluted in EMEM having a titer of 10^{7.0} TCID₅₀ ml⁻¹. Equal portions of the viral suspension were injected into the branchial vein, the hepatopancreas and the adductor muscle. One scallop was sacrificed 20 h after inoculation, and 3 scallops at each of Days 7, 14 and 21 after inoculation. The adductor muscle was removed, and the rest of the tissue was homogenized in 100 ml sterile seawater with an Ultra-turrax homogenizer. The homogenate was centrifuged twice for 30 min at 5500 × g at 10 °C in a Beckman L8-60M ultracentrifuge. Virus titers of the homogenates were determined as described above.

The virus stock suspension used for inoculations was diluted 1:99 with sterile seawater and incubated at 11 °C. Titrations were performed after 24 h and at Days 4, 7, 14 and 18 as described above.

Expt 2: To determine the distribution of virus in different organs, 17 scallops were each injected with 1 ml of virus suspension with a titer of 10^{7.3} TCID₅₀ ml⁻¹ as described above. Two weeks after inoculation the 16 surviving scallops were sacrificed (one died during the experiment). Tissue samples of approximately equal size were taken from hepatopancreas, kidney, the tip of the gonad (ovary) posterior to the lobe of the intestine, mantle, adductor muscle, and gill of each scallop. The pieces were pooled, diluted in EMEM cell culture medium, pounded in a Stomacher Lab-Blender 80, and filtered through 0.2 µm disc filters. In addition 500 µl hemolymph was drawn from the branchial vein of each specimen, pooled, and filtered. All samples were titrated as described above.

Expt 3: A long-term experiment was performed using 90 scallops, each injected with 1 ml of virus suspension with a titer of 10^{7.3} TCID₅₀ ml⁻¹ as described above. Each of 30 control scallops was injected with 1 ml of EMEM cell culture medium.

Five scallops were sacrificed at each of 17 samplings until Day 193 after inoculation. One scallop was sacrificed at Days 234, 262, 296 and 333. Hepatopancreas was removed from each scallop, kept separate, and virus titrations were performed as described above. Pieces of hepatopancreas were sampled for histological examination at Days 11, 25, 39, 52, 66, 84, 193, 234, 262, 296 and 333. Hemolymph was sampled at Days 5, 15, 25, 51, 126, 158, 193, 234, 262, 296, 333, and virus titrations were performed as described above (see Table 1). From Day 66 pooled samples from different organs (as described above + rectum) were prepared at each sampling. Faeces samples were collected from the tanks 3 times between Days 120 and 140 after inoculation.

Control scallops were sacrificed at the end of the experiment, and pieces of hepatopancreas were dissected for virus detections as described above.

The suspension of IPNV in EMEM used to inoculate the scallops was stored in a refrigerator at 5 °C and titrated 11 times during the experimental period.

Virus recovered from hemolymph, intestine, gill and mantle samples collected at Day 126 was sent to Norbio a/s, Bergen, Norway, for serotype verification by ELISA.

Expt 4: A bath challenge was performed by exposing 60 scallops to IPNV in a 250 l aquarium containing 100 l seawater to which had been added a virus suspension to a final titer of $10^{4.5}$ TCID₅₀ ml⁻¹. A further 100 l of seawater was added after 3 h, a slow flow (ca 1 l min⁻¹) was started after 6 h, and a normal flow (ca 3 l min⁻¹) was started after 12 h. The temperature was kept at 11 °C throughout the experiment.

Five scallops were sacrificed at each of 12 samplings until Day 50 after exposure (see Fig. 4, Table 2). Before dissections the scallops were kept in a tank with clean, running seawater for 2 h, flushed twice with phosphate-buffered saline containing Tween 80 (0.05 %) and thereafter twice with sterile seawater. Samples from hepatopancreas were kept separate. Samples from other organs and from hemolymph were pooled. Pieces of hepatopancreas, kidney and gonad were fixed for histological examinations at each sampling. Virus titrations and detections were performed as described above.

RESULTS

Expt 1

The virus titer present in the scallops decreased from $10^{7.0}$ TCID₅₀ ml⁻¹ at the time of inoculation to approximately $10^{5.1}$ TCID₅₀ ml⁻¹ after 3 wk (Fig. 1). The titer of the virus stock suspension diluted 1:99 in sterile seawater and incubated at 11 °C showed a slight decrease. No scallops died during the experimental period.

Expt 2

Virus titers of pooled samples from different organs (hepatopancreas, kidney, the tip of the gonad, mantle, adductor muscle, gill and hemolymph) of 16 inoculated scallops are shown in Fig. 2. The highest titer ($10^{5.8}$

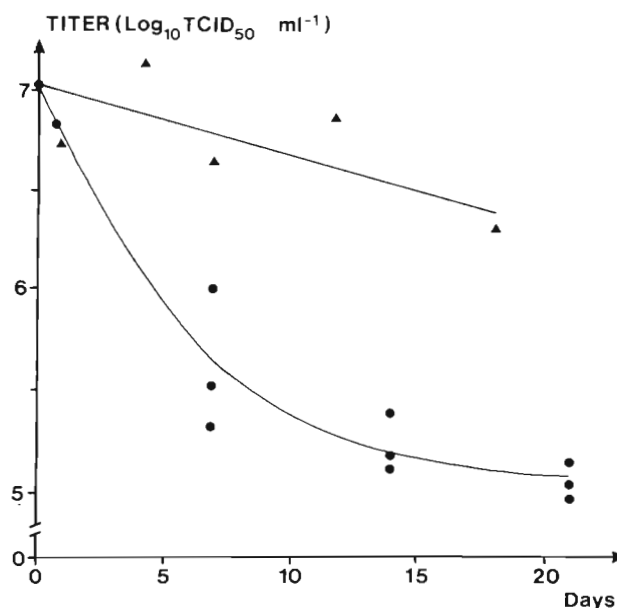


Fig. 1. *Pecten maximus*. Titer (\log_{10} TCID₅₀ ml⁻¹) of IPNV in whole scallops (●) and in seawater at 11 °C (▲). Each point represents the virus titer from one individual scallop kept in a recirculating water system

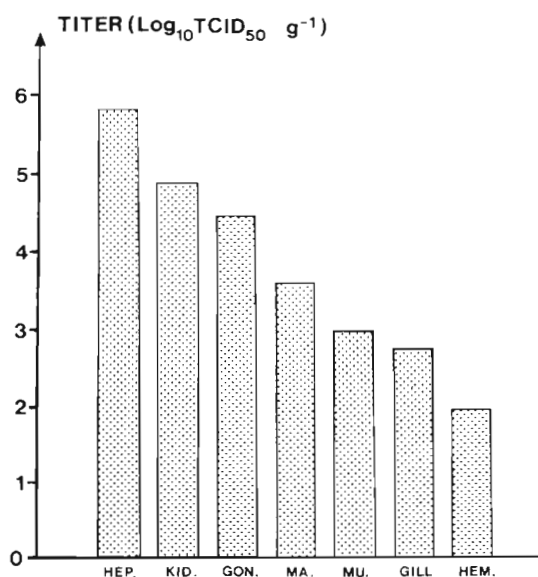


Fig. 2. *Pecten maximus*. Titer of IPNV (\log_{10} TCID₅₀ g⁻¹ tissue) in scallops 14 d after virus injection. Each value represents the virus titer in a pooled sample from tissue samples of 16 scallops. Samples were homogenates of hepatopancreas (HEP), kidney (KID), the tip of the gonad (GON), mantle (MA), muscle (MU), gill and hemolymph (HEM)

TCID₅₀ g⁻¹ tissue) was detected in the hepatopancreas sample, and the lowest (10^{2.0} TCID₅₀ ml⁻¹) in the hemolymph. One scallop died during the experimental period.

Expt 3

The virus titers of hepatopancreas tissue seemed to stay at a relatively stable level (ca 10^{5.7} TCID₅₀ g⁻¹ tissue) until 3 wk after inoculations (Fig. 3). A rapid decline from ca 10^{5.7} to 10^{3.6} TCID₅₀ g⁻¹ tissue occurred from Day 20 to Day 39. The titers varied between 10^{4.5} and 10^{3.0} TCID₅₀ g⁻¹ tissue during the rest of the experimental period, but showed a slight general decline (Fig. 3). At Day 333 the virus concentration was below titratable level (ca 10^{2.5} TCID₅₀ g⁻¹).

Virus was detected in different organs throughout the experimental period. As shown in Table 1, virus titers were determined in filtered hemolymph at Days 5, 15, 25 and 51. Later virus was detected in the hemolymph at Days 126 and 234, but not at Days 158, 262, 296 and 333. Virus reisolated at Day 126 was verified as IPNV serotype N1. Virus could not be detected in the 3 faeces samples.

Virus was not detected in hepatopancreas samples from control scallops.

Histological examinations of hepatopancreas tissue did not reveal any morphological changes. Two virus-inoculated scallops and one control scallop died during the experimental period.

The titer of the IPNV suspension used in the inoculations and kept refrigerated declined from 10^{7.6} to 10^{3.3} TCID₅₀ ml⁻¹ during the 11 mo experimental period.

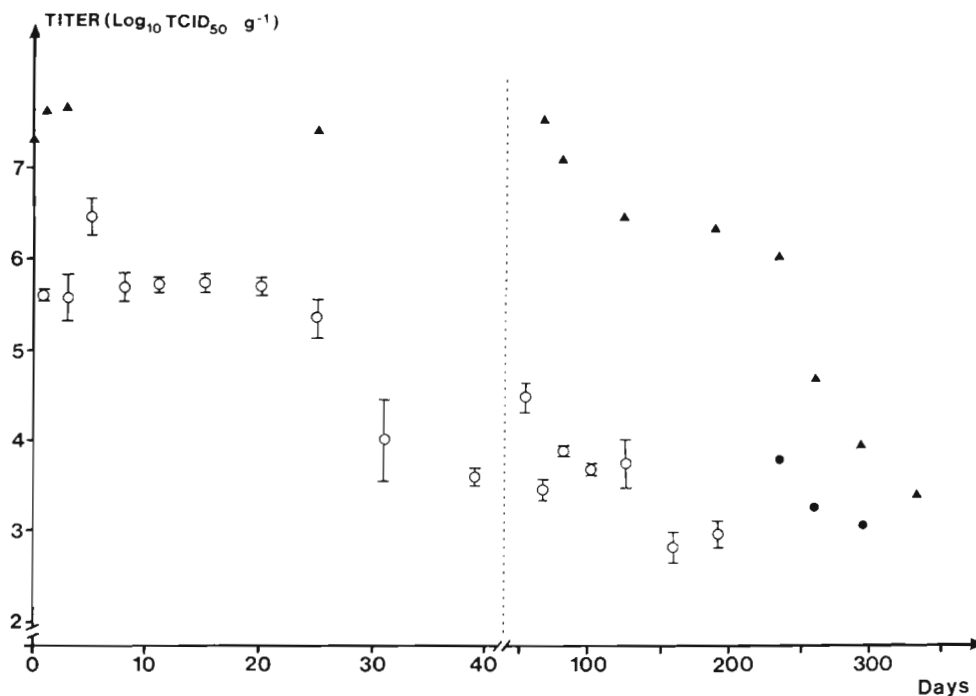


Fig. 3. *Pecten maximus*. Titer of IPNV (\log_{10} TCID₅₀ g⁻¹ hepatopancreas tissue) in scallops after the injection of virus. (o) Mean values from 5 individuals. Single values (●) are marked at Days 158, 262 and 296. No value is marked at Day 333, as the titer was below the level of countability by end point dilution (ca 10^{2.5} TCID₅₀ g⁻¹). Vertical bars indicate standard error of mean. (▲) Titers of the virus suspension, kept refrigerated during the experimental period.

Table 1. *Pecten maximus*. Titers of IPNV (\log_{10} TCID₅₀ g⁻¹ tissue) in different organs of scallops after virus inoculation by injection: kidney (Kid.), gonad (Gon.), mantle (Ma.), adductor muscle (Mu.), gill, rectum (Rect.) and hemolymph (Hem.) (\log TCID₅₀ ml⁻¹). Each value represents a pooled sample from 5 scallops, except for the last 4 samplings (1 scallop each). +: Sample containing virus below the level of countability by end point dilution (ca 10^{2.5} TCID₅₀ g⁻¹); -: sample where virus was not detected

Day	Kid.	Gon.	Ma.	Mu.	Gill	Rect.	Hem.
1							
3							
5							1.6
15							1.4
25							1.5
51							1.8
66		3.0		-	+	3.0	
84	+	3.5	+	+	+	3.0	
99	2.9	3.0	+	-	+	3.4	
126	3.0	2.9	+	-	+	3.7	+
158	-	+	+	+	+	+	-
193	+	+	+	+	-	+	-
234	+	3.5	+	-	+	+	+
262	+	+	+	+	+	+	-
296	+	+	+	+	-	+	-
333	+	+	-	+	+	+	-

Expt 4

After the bath challenge virus titers of hepatopancreas tissue declined rapidly, from an average of 10^{5.1} to approximately 10^{3.5} TCID₅₀ g⁻¹ tissue during the first week (Fig. 4). Virus was detected in the hepatopancreas from all individuals, except from one scallop on the last day of sampling (Day 50). Hepato-

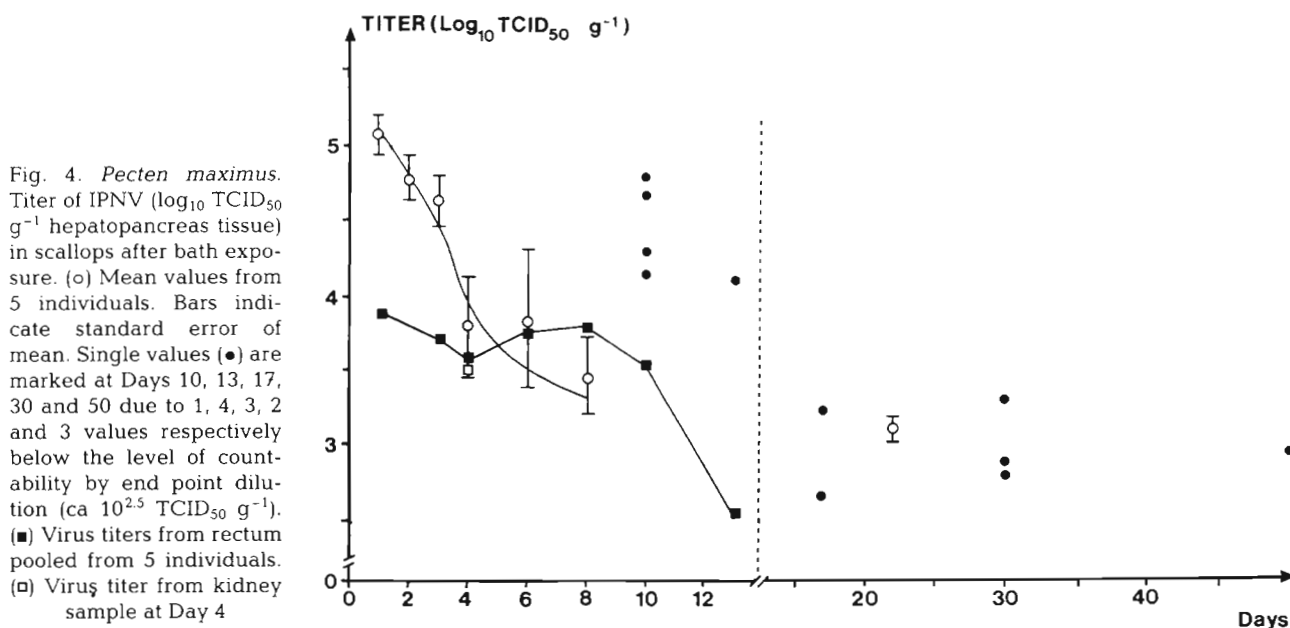


Fig. 4. *Pecten maximus*. Titer of IPNV (\log_{10} TCID₅₀ g⁻¹ hepatopancreas tissue) in scallops after bath exposure. (○) Mean values from 5 individuals. Bars indicate standard error of mean. Single values (●) are marked at Days 10, 13, 17, 30 and 50 due to 1, 4, 3, 2 and 3 values respectively below the level of countability by end point dilution (ca $10^{2.5}$ TCID₅₀ g⁻¹). (■) Virus titers from rectum pooled from 5 individuals. (□) Virus titer from kidney sample at Day 4

pancreas sampled during the last 6 wk of the experimental period showed large individual variations, and some titer values were below the level of countability. Virus titers of samples from rectum showed a sharp decline after Day 10 and decreased to below titratable level after Day 13 (Fig. 4). Virus was detected until Day 30 and was not detected at Day 50.

Virus was detected in the kidney 1 d after the challenge but not at Days 2 and 3. At Day 4, a titer of $10^{3.5}$ TCID₅₀ g⁻¹ tissue was demonstrated, but no virus could be detected in kidney tissue during the rest of the experimental period.

Table 2. *Pecten maximus*. Titers of IPNV in different organs of scallops after bath exposure: gonad (Gon.), mantle (Ma.), adductor muscle (Mu.), gill, and hemolymph (Hem.) (\log_{10} TCID₅₀ ml⁻¹). Each symbol represents a pooled sample from 5 scallops. +: Sample containing virus below the level of countability by end point dilution (ca $10^{2.5}$ TCID₅₀ g⁻¹); -: sample where virus was not detected

Day	Gon.	Ma.	Mu.	Gill	Hem.
1	+	+	-	+	+
2	-	-	-	+	1.8
3	-	-	-	+	+
4	-	+	-	+	-
6	-	+	-	+	-
8	+	+	-	+	+
10	-	+	-	+	-
13	-	-	-	-	-
17	-	+	-	-	-
22	-	-	-	-	-
30	-	-	-	+	-
50	-	+	-	-	-

As shown in Table 2, virus was occasionally detected in the gonad, mantle, gill, and in filtered hemolymph. Virus could not be detected in the adductor muscle.

Histological examinations of hepatopancreas, kidney and gonad tissues revealed no clear pathological changes. Highly vacuolized cells and a diffuse organization of the epithelia were observed in the digestive tubules during the first 4 d of the experiment. The number of hemocytes in the hepatopancreas seemed normal. No scallops died during the experimental period.

DISCUSSION

The results shown in Fig. 1 indicate that IPNV injected into scallops was either rapidly inactivated or excreted. The decrease in virus titers of hepatopancreas tissue shown in Fig. 3 occurred approximately 3 wk after inoculation, and the reduction in virus titers in whole scallops seemed thus more rapid than the reduction of virus in the hepatopancreas tissue alone. As reviewed by Sminia & Van der Knaap (1986, 1987) the molluscan hemolymph contains a number of components which might neutralize virus infectivity, such as humoral factors of both enzymatic and non-enzymatic character. A T3 coliphage neutralizing activity in oyster (*Crassostrea gigas*) hemolymph was recently shown by Bachère et al. (1990). Destruction of IPNV might also occur intracellularly in circulating hemocytes, as it has been shown that oyster hemocytes may take up virus particles *in vitro* (Fries & Tripp 1970).

The reduction of virus titers might be due to excretion, and our findings of virus in the hepatopancreas and rectum samples in Expts 3 and 4 indicate that both injected and ingested virus could move through the alimentary tract. The highest titers in hepatopancreas in Expts 2, 3 and 4 suggest that the hepatopancreas was the main organ involved in the depuration processes, and may be in accordance with the results of Metcalf & Stiles (1965) who found that the concentration of ingested enteric viruses increased in oyster hepatopancreas at the same time as the virus concentration decreased in other tissues.

Detections of virus in kidney samples indicated that an excretion of virions maybe also could occur via excretory products. Our results might thus be in accordance with Halder & Ahne (1988) who found that freshwater crayfish *Astacus astacus* infected with IPNV excreted infective virus particles continuously into the water.

In bivalves, ingested virus may persist sequestered in tissues, and thus appear protected from neutralization and depuration processes (Canzonier 1971, Metcalf & Stiles 1965, Hay & Scotti 1986). It was shown in Expt 4 that virus ingested by filtration of contaminated water was present in the hemolymph. In Expt 3 injected virus was present in hemolymph samples as long as 234 d after injection. A comparison of results from Expt 2, 3 and 4 indicates that both injected and filtered virus probably circulated with the hemolymph and could reach and persist in different tissues. The fact that the virus titers were highest in the hepatopancreas is in agreement with the results obtained by Canzonier (1971) who showed that most of the Coliphage S-13 accumulated by clams filtering virus-containing water was sequestered in the digestive gland.

The persistence of IPNV in the ovaries of scallops may indicate that the eggs represent a favourable environment for virus. In salmonid fish, vertical transmission of IPNV via the eggs is known (Wolf et al. 1963) and transmission can occur despite iodine treatment of eggs (Bullock et al. 1976). Ahne & Negele (1985) suggested the virus might be protected in lobes and pores of the egg shell.

In contrast to the results observed after injections, after the bath challenge virus detection in organs other than hepatopancreas and rectum was scarce. Mantle and gill tissues contain numerous mucus-secreting cells, and the occasional virus detections in mantle and gill might be a result of trapping of virus from the passing water. Di Girolamo et al. (1977) suggested that trapping of virus particles by ion bonding in the mucus of the digestive tract is the principle way of introduction.

Comparing results from these experiments, it seems likely that the scallops were able to inactivate and/or eliminate a virus challenge administered by bath more efficiently than one administered by injection. The histological changes observed in sections from hepatopancreas tissue sampled during the first days of the bath challenge experiment probably indicate increased activity in the digestive tubules due to the virus exposure. Lack of obvious histological changes indicates that the isolated IPNV did not act as a pathogen for the scallops.

Throughout the experiments no increase in virus titers was observed, and thus no viral replication was proven. However, in view of the persistence of the virus in different organs, the possibility of a slow rate of replication occurring simultaneously with inactivation and/or excretion processes cannot be excluded.

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