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Ian Bricknell

*University of Maine - Main*, [ian.bricknell@umit.maine.edu](mailto:ian.bricknell@umit.maine.edu)

Sally D. Molloy

Michael R. Pietrak

Deborah A. Bouchard

[deborah.bouchard@maine.edu](mailto:deborah.bouchard@maine.edu)

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# Experimental Transmission of Infectious Pancreatic Necrosis Virus from the Blue Mussel, *Mytilus edulis*, to Cohabiting Atlantic Salmon (*Salmo salar*) Smolts

Sally D. Molloy,<sup>a</sup> Michael R. Pietrak,<sup>b</sup> Ian Bricknell,<sup>b</sup> Deborah A. Bouchard<sup>c</sup>

Department of Molecular and Biomedical Sciences, Aquaculture Research Institute, University of Maine, Orono, Maine, USA<sup>a</sup>; School of Marine Sciences, Aquaculture Research Institute, University of Maine, Orono, Maine, USA<sup>b</sup>; University of Maine Cooperative Extension, Aquaculture Research Institute, University of Maine, Orono, Maine, USA<sup>c</sup>

**Integrated multitrophic aquaculture (IMTA) reduces the environmental impacts of commercial aquaculture systems by combining the cultivation of fed species with extractive species. Shellfish play a critical role in IMTA systems by filter-feeding particulate-bound organic nutrients. As bioaccumulating organisms, shellfish may also increase disease risk on farms by serving as reservoirs for important finfish pathogens such as infectious pancreatic necrosis virus (IPNV). The ability of the blue mussel (*Mytilus edulis*) to bioaccumulate and transmit IPNV to naive Atlantic salmon (*Salmo salar*) smolts was investigated. To determine the ability of mussels to filter and accumulate viable IPNV, mussels were held in water containing log 4.6 50% tissue culture infective dose(s) (TCID<sub>50</sub>) of the West Buxton strain of IPNV ml<sup>-1</sup>. Viable IPNV was detected in the digestive glands (DGs) of IPNV-exposed mussels as early as 2 h postexposure. The viral load in mussel DG tissue significantly increased with time and reached log 5.35 ± 0.25 TCID<sub>50</sub> g of DG tissue<sup>-1</sup> after 120 h of exposure. IPNV titers never reached levels that were significantly greater than that in the water. Viable IPNV was detected in mussel feces out to 7 days postdeuration, and the virus persisted in DG tissues for at least 18 days of depuration. To determine whether IPNV can be transmitted from mussels to Atlantic salmon, IPNV-exposed mussels were cohabited with naive Atlantic salmon smolts. Transmission of IPNV did occur from mussels to smolts at a low frequency. The results demonstrate that a nonenveloped virus, such as IPNV, can accumulate in mussels and be transferred to naive fish.**

Globally, Atlantic salmon (*Salmo salar*) growers are currently integrating blue mussel (*Mytilus edulis*) crops on salmon farms to diversify crops and increase the environmental sustainability of farms. This is an evolving aquaculture technique called integrated multitrophic aquaculture (IMTA), which reduces potential environmental impacts of commercial aquaculture systems by combining the cultivation of fed aquaculture species (finfish) with extractive aquaculture species (e.g., shellfish and seaweed) (1–3). The shellfish extractive component removes organic particulate wastes, such as uneaten fish food, and the seaweed removes dissolved inorganic nutrients (3, 4). There are environmental and economic benefits to integrating shellfish on fish farms; however, shellfish may also filter finfish pathogens in the environment and influence pathogen dynamics on the farm.

Mussels cultured adjacent to Atlantic salmon cages could serve as reservoirs or as sinks for important finfish pathogens. Bivalves bioaccumulate both viral and bacterial finfish pathogens (5–10). The physiology of the pathogen influences whether the pathogen remains viable in shellfish tissues and is shed back into the environment. Mussels are capable of concentrating the bacterial pathogen *Vibrio anguillarum* and releasing viable pathogen at high concentrations in feces (9). In contrast, the enveloped viral pathogen, infectious salmon anemia virus, and the sea louse, *Lepeophtheirus salmonis*, are taken up by mussels and inactivated (5, 10–12). The nonenveloped viral fish pathogen, infectious pancreatic necrosis virus (IPNV), can persist in scallop tissues; however, the fate of IPNV has not yet been determined in mussels (7).

IPNV, a member of the *Aquabirnavirus* genus within the family *Birnaviridae*, is a nonenveloped virus with a bisegmented double-stranded RNA genome (13, 14). The virus is extremely stable in

fresh, estuarine, and marine waters and has the most persistent infectivity of any fish virus (15, 16). IPNV is the etiological agent of infectious pancreatic necrosis (IPN), which causes high mortalities in cultured salmonids, particularly in fry and fingerling and during smolt transfer (17). Survivors of viral outbreaks continue to carry virus in the viscera for the remainder of their life in the absence of disease symptoms. These asymptomatic fish serve as reservoirs of IPNV, shedding virus in feces and urine and in reproductive products (18). In the United States, IPNV is considered endemic to Maine and Canadian maritime waters. Globally, with increasing production of Atlantic salmon, it has also become a significant pathogen in the marine environment (19) and is now the most important viral disease in the European salmon industry (19). In Norway and the Shetland Islands, IPN has been associated with high mortalities in Atlantic salmon post-smolts about 8 weeks after transfer to seawater (20, 21), with 70% of Atlantic salmon marine farm sites infected (22).

IPNV carrier fish may be the most important reservoir for spreading the disease; however, there are many other potential reservoirs. In addition to asymptomatic farmed fish, infectious IPNV has been isolated from cohabiting farmed and wild fish,

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Address correspondence to Sally D. Molloy, sally.dixon@umit.maine.edu, or Deborah A. Bouchard, deborah.bouchard@maine.edu.

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shellfish, sediments below the net pens, and birds (7, 23, 24). IPNV has been isolated from wild marine fish (including but not limited to saithe, Atlantic cod, pollock, and hake) species in the vicinity of marine salmon farms but has also been detected in wild salmonid fish that have had no contact with hatchery-reared fish (25). In the case of IMTA, it is of great importance to determine whether the vastly increased number of mussels, e.g., approximately 25 tons for an average-sized pen, on an IMTA farm have the potential to accumulate IPNV and shed virus at a rate and quantity that would impact Atlantic salmon.

The ability of mussels to accumulate viable IPNV in tissues and to transmit IPNV to naive Atlantic salmon after smoltification was investigated. In order to determine the ability of mussels to filter and accumulate viable IPNV, mussels were exposed to known concentrations of IPNV and virus levels in the digestive gland (DG) tissues were determined using viral culture methods. DG tissues and feces from IPNV-exposed mussels were analyzed post-depuration to determine whether IPNV persisted in mussels and whether viable virus was shed from mussels. Finally, a cohabitation challenge was performed to determine whether IPNV-exposed mussels could transmit virus to naive Atlantic salmon.

## MATERIALS AND METHODS

**Mussel and fish maintenance.** Market-sized mussels were obtained from a commercial mussel grower and were maintained in static systems at 10°C in artificial seawater (ASW; Crystal Seas, Baltimore, MD). Mussels were fed a diet of mixed-species algal paste (Innovative Aquaculture, Skerry Bay, British Columbia, Canada). Mussels were maintained in static systems containing 0.5 liter of ASW per mussel at 10°C in both trials.

Atlantic salmon S0 smolts (mean  $\pm$  standard error [SE], 55.64  $\pm$  0.7 g) were obtained from a commercial fish hatchery in New Brunswick, Canada with a 10-year screening history of testing IPNV-free. Fish were maintained at 10  $\pm$  2°C in a recirculation system with artificial seawater. Fish were fed a commercial pellet (BioOregon; Bio-Olympic, Westbrook, ME) at 1% of their body weight per day. Prior to all experiments, 5% of salmon and mussel populations were screened for IPNV via culture and quantitative reverse transcription-PCR (qRT-PCR) analyses, as described below.

**Cell culture maintenance and virus propagation.** CHSE-214 cells were maintained in minimum essential medium (MEM; Invitrogen, Carlsbad, CA) with Earle's salts supplemented with 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY) at 15°C. For virus isolation assays, CHSE-214 cells were transferred to 24- or 96-well culture plates. Cells were allowed to attach and acclimate for 24 h at 15°C in order to achieve 75 to 80% confluence.

The West Buxton (WB) isolate of IPNV was passaged through juvenile brook trout and propagated a single time in CHSE-214 cells grown at 15°C in MEM containing 5% FBS and gentamicin (50  $\mu$ g ml<sup>-1</sup>). When the cells demonstrated a 75% cytopathic effect (CPE), the cells and supernatant were collected, and virus was stored at -80°C. Prior to all experiments, a virus stock was thawed and filtered through a 0.45- $\mu$ m-pore-size filter to remove cell clumps. The titer of the stock was determined by 50% tissue culture infectious dose (TCID<sub>50</sub>) analysis in CHSE-214 cells.

**Culture analysis of tissue, fecal, and water samples.** IPNV was quantified in mussel DG tissues, mussel fecal matter, pooled salmon kidney, and spleen tissues, and in water samples, by performing TCID<sub>50</sub> analysis in CHSE-214 cells. Water samples were filtered through 0.45- $\mu$ m-pore-size filters. Tissue samples were diluted 5-fold (wt/vol) in sterile phosphate-buffered saline (PBS) and homogenized before diluting 10-fold in MEM with Earle's salts containing gentamicin (50  $\mu$ g ml<sup>-1</sup>; MEM-G). Fecal pellets were diluted 1:10 (wt/vol) with MEM-G and homogenized in a TissueLyser (Qiagen) for 10 s at a frequency of 15 s<sup>-1</sup>. Tissue and fecal homogenates were filtered through 0.45- $\mu$ m-pore-size filters before seri-

ally diluting them. Negative control filtrates were not diluted before applying them to cells.

To quantify virus, each dilution was added in 100- $\mu$ l volumes to four wells of a 96-well plate containing CHSE-214 cells. Some samples, such as the negative control samples that were not expected to have virus, were tested for the presence or absence of IPNV by inoculating 100- $\mu$ l volumes into duplicate wells of 24-well plates seeded with CHSE-214 cells. After a 1-h viral adsorption period, the inoculum from wells receiving DG homogenate 10<sup>-1</sup> filtrate dilutions and from wells receiving negative control samples were removed to prevent cell cytotoxicity before the addition of 1.0 ml of the appropriate fresh medium containing 5% FBS and gentamicin (5). Plates were incubated at 15°C with 5% CO<sub>2</sub> and observed daily for visible CPE for 7 days. The TCID<sub>50</sub> was calculated according to the method of Reed and Muench (26). For mussel DG samples that were below the detection limit of the assay, titers were reported as less than the detection limit of log 2.7 TCID<sub>50</sub> ml<sup>-1</sup>. Salmon kidney and spleen samples that were below the detection limit were reported as less than the detection limit of log 2.3 TCID<sub>50</sub> ml<sup>-1</sup>.

**RNA isolations and qRT-PCR.** Each salmon kidney or mussel DG sample was placed in a 2.0-ml tube containing 600  $\mu$ l of RLT buffer (Qiagen, Inc., Valencia, CA) and a 5-mm stainless steel bead (Qiagen). Samples were further processed in the TissueLyser (Qiagen) twice for 2 min at a frequency of 28/s. RNA extractions were carried out with an RNeasy minikit (Qiagen) with a QiaShredder (Qiagen) and DNase treatment (Qiagen) on the column in the Qiacube automated work station (Qiagen). The yield and quality of the RNA was assessed using an RNA 6000 Nano LabChip kit (Agilent Technologies, Santa Clara, CA) and an Agilent 2100 Bioanalyzer (Agilent Technologies).

cDNA was synthesized from 2.0  $\mu$ g of RNA in 20- $\mu$ l reactions containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl<sub>2</sub>, 4 mM concentrations (each) of deoxynucleoside triphosphates (Applied Biosystems, Carlsbad, CA), random hexamer primers at 5 mM (Applied Biosystems), 20 U of recombinant RNase inhibitor (Applied Biosystems), and 50 U of MultiScribe reverse transcriptase (Applied Biosystems). Reactions were incubated at 25°C for 10 min and at 37°C for 2 h, and they were finally heat inactivated at 85°C for 5 s.

Real-time PCR assays were performed using the MX4000 Multiplex Quantitative PCR system (Stratagene, Santa Clara, CA). Reactions were carried out in 25- $\mu$ l volumes. Using Primer3 software, a primer-probe set was designed to amplify a 100-bp sequence in the WB IPNV major capsid gene, VP2 (accession no. AF342727) (Table 1). Quantitative PCR (qPCR) analysis was performed on Atlantic salmon kidney cDNA samples to confirm the culture results and not to quantify IPNV RNA. However, an endogenous control gene, the elongation factor 1 $\alpha$  (ELF-1 $\alpha$ ) gene, was included in the assay for quality control purposes (27, 28) (Table 1). The ELF-1 $\alpha$  housekeeping gene was used to assess the starting amount of RNA in mussel DG tissues and to normalize the gene-specific product data (Table 1) (5). Samples were considered positive if all three qPCR replicate reactions generated amplification curves and threshold cycle ( $C_T$ ) values of  $\leq$  39. Each sample was analyzed by qPCR in triplicate. The change in the abundance of IPNV VP2 in mussel DG tissue was normalized to mussel ELF-1 $\alpha$  RNA and calculated using the 2<sup>- $\Delta\Delta C_T$</sup>  method (29). Positive and no-template controls in each of the processes—RNA extraction, cDNA synthesis, and real-time PCR—were carried out through real-time PCR analysis.

To validate the IPNV qRT-PCR assay using the mussel ELF-1 $\alpha$  gene as a housekeeping gene, the relative efficiencies of IPNV VP2 (99%) and mussel ELF-1 $\alpha$  (98.2%) primer-probe sets were compared. cDNA was synthesized from RNA isolated from mussel DG homogenates after inoculation with stock IPNV. Triplicate qPCRs targeting both IPNV VP2 and mussel ELF-1 $\alpha$  were performed on serial 10-fold dilutions of the cDNA. The  $\Delta C_T$  ( $C_{T\text{IPNV}} - C_{T\text{ELF-1}\alpha}$ ) was plotted against the log RNA input to create a semilog regression line. The slope of the line was <0.1 (0.047), indicating that the amplification efficiencies of the IPNV VP2 primer-probe set and of the ELF-1 $\alpha$  primer-probe set are approximately equal.

TABLE 1 Primers and probes used for qRT-PCR analysis

| Gene                                     | Primer or probe | Sequence (5'–3')                  | Reference |
|--|-----------------|-----------------------------------|-----------|
| IPNV VP2                                 | Forward         | GAAGTCTTTCTGAGGTGGAGAG            |           |
|  | Reverse         | ATTCCTTTGGTCACTAGTTGGT            |           |
|  | TaqMan probe    | FAM-TAACAGCTTGATGTCCCTGACAACA-MGB |           |
| ELF-1 $\alpha$ gene ( <i>M. edulis</i> ) | Forward         | CGGAGTCAACAAGATGGACA              | 5         |
|  | Reverse         | AACTGCTGACTTCCTTCTGGA             | 5         |
|  | TaqMan probe    | FAM-CAGTGAAGCCCGATTATGGA-MGB      | 5         |
| ELF-1 $\alpha$ gene ( <i>S. salar</i> )  | Forward         | CCCCTCCAGGACGTTTACAAA             | 28        |
|  | Reverse         | CACACGGCCACAGGTACA                | 28        |
|  | TaqMan probe    | FAM-ATCGGTGGTATTGGAAC-MGB         | 28        |

For each triplicate set of qPCRs targeting IPNV VP2, the cycle number plotted against the dilution factor resulted in a linear plots with a slope of  $-3.2$ , which indicates efficient amplification in the IPNV assay.

**Detection limit of TCID<sub>50</sub> endpoint dilution assay and real-time RT-PCR in IPNV-inoculated mussel DG homogenates.** The DGs from eight mussels were harvested, pooled, weighed, and diluted 2-fold in sterile PBS. A uniform homogenate of the DG tissue was divided equally into nine 900- $\mu$ l samples. Serial 10-fold dilutions of stock IPNV, ranging in titer from log 7.5 to log 0.5 TCID<sub>50</sub> ml<sup>-1</sup>, were prepared in MEM cell culture medium. Each virus dilution was added in 100- $\mu$ l volumes to eight of the nine homogenate samples and thoroughly mixed to achieve predicted titers ranging from log 6.5 to log <1 TCID<sub>50</sub> ml<sup>-1</sup>. MEM was added to the ninth homogenate sample, which served as a negative control for the TCID<sub>50</sub> and real-time PCR assays. RNA was isolated from duplicate 75-mg samples taken from each of the nine homogenates. The remaining homogenates were processed for TCID<sub>50</sub> analysis in CHSE-214. DG homogenate samples were diluted 1:9 (wt/vol) in MEM-G and filtered on 0.45- $\mu$ m-pore-size filters. In addition to the original 2-fold dilution of tissues, a 2.5-fold dilution was carried out before preparing serial 10-fold dilutions to 10<sup>-10</sup> in unsupplemented MEM-G. TCID<sub>50</sub> assays were carried out as described above.

**IPNV mussel exposure trials.** In trial 1, mussels were randomly assigned to four tanks containing 7.5 liters of ASW until each tank contained 15 mussels. IPNV stock (log 9.6 TCID<sub>50</sub> ml<sup>-1</sup>) was added to triplicate tanks containing mussels to a final concentration of log 4.6  $\pm$  0.04 (standard error) TCID<sub>50</sub> ml<sup>-1</sup>. An equivalent amount of MEM was added to a control tank containing mussels and to a tank containing water only. A sixth system, containing water only, received the IPNV inoculum. All tanks received a dose of mixed-species algal paste (Innovative Aquaculture) to a final concentration of 10<sup>5</sup> cells ml<sup>-1</sup>. The water in each tank was mixed thoroughly, and 5-ml water samples were taken from each of the six tank systems for culture analysis. Water and random triplicate mussel samples were taken at 2, 24, 48, 72, and 120 h postexposure (hpe). The shell of each mussel was surface disinfected with a 5% sodium hypochlorite solution, followed by a swabbing with 70% ethanol. The shell length of each mussel was recorded, and DG (hepatopancreas) tissue was removed for culture and molecular analyses. DG tissues for molecular analysis were stored in RNAlater (Ambion, Austin, TX) for 24 h at 4°C before the RNAlater was removed and further storage at  $-80^{\circ}\text{C}$ . Water samples were processed for culture analysis only. Culture analysis of mussel and water samples was carried out in CHSE-214 cells.

In trial 2, duplicate tanks with the three following treatments were set up: virus only, virus and mussels, and mussels only. Tanks contained the same ratio of ASW per mussel (0.5 liter per mussel), received the same dose of algae paste (10<sup>5</sup> cells ml<sup>-1</sup>), and received the same target IPNV dose. IPNV stock or MEM was added to appropriate tanks and mixed thoroughly. Water samples were taken from each tank immediately after mixing. The average initial concentration of IPNV in tanks treated with virus was log 4.2  $\pm$  0.2 TCID<sub>50</sub> ml<sup>-1</sup>. Tanks were treated daily with alga

paste, and water samples were taken daily at 4 days postexposure (dpe) for culture analysis. At 4 dpe, two mussels from IPNV-exposed tanks and control tanks were processed for culture analysis as described above. The remaining control and IPNV-exposed mussels were used in the IPNV shedding experiment.

**Mussel IPNV shedding trial.** Eight mussels exposed to IPNV for 4 days were disinfected as described above and rinsed in fresh ASW. To remove IPNV-laden water from the buccal cavity, mussels were placed into individual tanks containing 1 liter of ASW for 30 min. Mussels were visually inspected to ensure that the mussels had opened during the 30 min, suggesting a water exchange in the buccal cavity. Shells were again disinfected and then rinsed in ASW, and each mussel was placed in individual tanks containing clean 0.5 liter of ASW and algae. At 24-h intervals of depuration, feces and pseudofeces (collectively referred to as fecal matter) and 5-ml water samples were collected from each tank, and the mussels were placed in tanks containing fresh 0.5 liter of ASW with algae. Fecal matter pellets were weighed after centrifugation at 1,000  $\times$  g for 10 min and processed for virus isolation by culture analysis. Fecal pellets were diluted 1:10 (wt/vol) with MEM and homogenized in the TissueLyser (Qiagen) for 10 s at a frequency of 15 s<sup>-1</sup>. The titer of IPNV per g of fecal matter was determined by TCID<sub>50</sub> analysis, as described above.

**Mussel salmon cohobitation trial.** The cohobitation trial was conducted in two identical independent recirculation systems, each with nine 75-liter tanks containing aerated ASW. Each system possessed independent mechanical filtration (BBF4; International Filter Solutions), biofiltration, and UV sterilization (130 W; Emperor Aquatics). The UV sterilization systems were designed to provide 440 mW of disinfection power per cm<sup>2</sup>, which is slightly less than twice the reported amount required to inactivate IPNV (30).

For each of the duplicate systems, four treatments were randomly assigned to the nine tanks and tanks were labeled accordingly. Per system, treatments included (i) a single tank containing 24 untreated salmon to act as sentinels for system contamination, (ii) duplicate tanks containing 12 naive salmon and 12 IPNV-injected salmon, (iii) triplicate tanks containing 24 salmon and a mesh sock of 36 control mussels, and (iv) triplicate tanks containing 24 salmon and a mesh sock of 36 IPNV-exposed mussels. Salmon were then randomly distributed between the 18 tanks, with 24 salmon per tank.

At 9 days prior to beginning the cohobitation trial, 500 mussels were distributed between two tanks containing 40 liters of 10°C aerated ASW. The mussel tanks were isolated from the wet lab in which the fish cohobitation trial was being conducted. Eight days before starting the cohobitation trial, 40 ml of MEM was added to the tank containing the control mussels. The second tank was treated with 40 ml of filtered IPNV stock. The water in the tanks was mixed vigorously before taking 5-ml water samples. TCID<sub>50</sub> analysis was performed on both the water samples and the original IPNV stock.

At 8 dpe and time zero for the cohobitation trial, the average viral load was determined for 20 IPNV-exposed mussels and 20 control mussels to

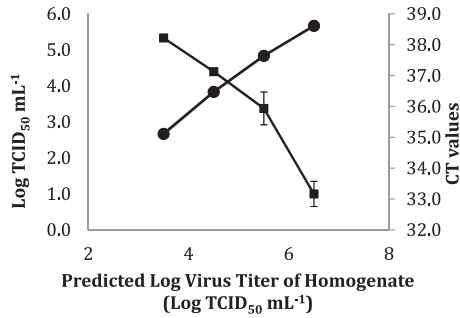


FIG 1 Log TCID<sub>50</sub> (●) of IPNV-inoculated mussel digestive gland homogenates determined in CHSE-214 cells and average  $C_T$  values (■) as measured with TaqMan qRT-PCR using primers specific for IPNV VP2. The  $C_T$  values represent averages  $\pm$  the standard error of the mean ( $n = 2$ ).

make sure that they were IPNV negative. DG tissues from IPNV-exposed and control mussels were processed for TCID<sub>50</sub> analysis or the presence or absence of IPNV via culture, respectively, as previously described. The remaining mussels were disinfected with 5% bleach and 70% ethanol before being placed in clean ASW for 30 min to remove IPNV-laden water from the buccal cavity. Mussels were distributed into plastic mesh socks with 36 mussels per sock. One control mussel sock or one IPNV-exposed mussel sock was secured in each of the 12 mussel-salmon cohabitation tanks containing 12 IPNV-naive salmon. After 12 days of cohabitation, the mussels were removed from all of the tanks, and the viral load was determined for three mussels per sock by culture analysis.

Four replicate salmon-salmon cohabitation IPNV challenges were initiated by anesthetizing all 24 fish in a replicate simultaneously in aerated ASW containing 100 mg of MS-222 (Western Chemical, Ferndale, WA) liter<sup>-1</sup> (20, 31). Using a random-number table, 12 fish were selected for intraperitoneal (i.p.) injection with 100  $\mu$ l of diluted IPNV stock ( $3.2 \times 10^7$  TCID<sub>50</sub> ml<sup>-1</sup>). The adipose fins of i.p.-injected fish were clipped before returning fish to tanks to recover with the noninjected fish.

At 8, 16, and 21 days after IPNV exposure (dpe), 6 randomly selected salmon were sampled from each of the 18 tanks. On the final day of sampling at 26 dpe, all of the remaining fish in each tank were euthanized and sampled. Fish were euthanized in ASW containing 200 mg of MS-222 liter<sup>-1</sup>. Kidney samples were taken and processed for qRT-PCR analysis. Kidney and spleen samples were aseptically removed from each fish, weighed, and processed for virus isolation by culture analysis (described above) in order to detect the presence or absence of IPNV. Diluted tissue homogenate filtrates were stored at  $-80^\circ\text{C}$  and later analyzed by TCID<sub>50</sub> assays to determine the IPNV titer.

**Statistics.** For qRT-PCR data, a one-way analysis of variance (ANOVA) was performed on  $\Delta\Delta C_T$  values with alpha set at 0.05. For culture data from mussel DG samples, the detection limit for the culture assay, log 2.7 TCID<sub>50</sub> ml<sup>-1</sup>, was used for the data points that originated from negative culture results. ANOVAs were performed on log-transformed TCID<sub>50</sub> data. Studentized residuals were tested for normality and equal variance within treatments using the Shapiro-Wilks test ( $\alpha = 0.05$ ) and Levene's test for equal variance ( $\alpha = 0.05$ ), respectively. If an ANOVA resulted in a significant F test for treatment effects, a Fisher protected least-significant-difference (LSD) procedure was performed to determine significant differences between means, with alpha set at 0.05. For data exhibiting heteroscedasticity, a Welch's ANOVA was performed.

A Pearson chi-squared test for independence was used to compare the percentages of IPNV-positive fish in the two recirculation systems. The percentage of IPNV-positive fish was also compared between fish cohabitating with i.p.-injected fish and fish cohabitating with IPNV-exposed mussels. The results were considered significant if  $P \leq 0.05$ .

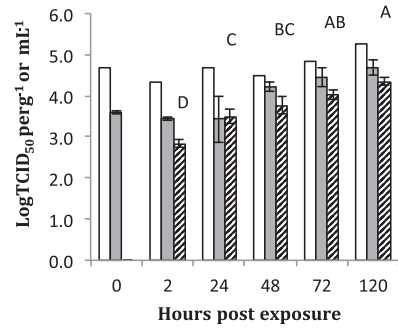


FIG 2 Log TCID<sub>50</sub> of IPNV per ml of water in tanks containing mussels (▨) or lacking mussels (□) or per g of mussel digestive gland tissue (▧) over time. Graphs represent the average log TCID<sub>50</sub> g of tissue<sup>-1</sup>  $\pm$  the standard error of the mean with  $n = 9$  mussels and the average log TCID<sub>50</sub> ml of water<sup>-1</sup>  $\pm$  the standard error of the mean with  $n = 3$  tanks. Means represented by different capital letters are significantly different (Fisher LSD,  $\alpha = 0.05$ ).

## RESULTS

**IPNV detection limits of qRT-PCR and culture analyses in mussel DG tissues.** The detection limits of the IPNV qRT-PCR assay and the culture assays were compared in IPNV-inoculated mussel DG homogenates. qRT-PCR detected IPNV RNA in mussel homogenates with predicted titers of log 6.5 to 3.5 TCID<sub>50</sub> ml<sup>-1</sup>, with an increase in  $C_T$  value as the predicted log IPNV titer decreased (Fig. 1). The reliable detection limit for the qRT-PCR assay was measured at log 3.8 TCID<sub>50</sub> ml<sup>-1</sup>, although the predicted titer for that sample was log 4.5 TCID<sub>50</sub> ml<sup>-1</sup>. IPNV was detected in homogenates with predicted titers of log 3.5 TCID<sub>50</sub> ml<sup>-1</sup>; however, only one of the two replicate homogenates had positive  $C_T$  values. Further, within that positive homogenate sample, only two of the three replicate qRT-PCRs had positive  $C_T$  values.

The reliable detection limit for viable IPNV isolation by culture analysis was log 2.7 TCID<sub>50</sub> ml<sup>-1</sup>. Viable IPNV was detected by culture analyses in DG homogenates with predicted titers of log 6.5 to 3.5 (Fig. 1). The titers determined in CHSE-214 cells decreased in a linear fashion as the predicted titers decreased ( $R^2 = 0.99$ ); however, the determined titers were lower than the predicted titers by an average of log  $0.8 \pm 0.05$ . The most dilute sample in which virus was detected had a predicted titer of log 3.5 TCID<sub>50</sub> ml<sup>-1</sup>; however, the measured titer was log 2.7 TCID<sub>50</sub> ml<sup>-1</sup>. For samples at predicted titers of log 2.5 TCID<sub>50</sub> ml<sup>-1</sup> and lower, no virus was detected by culture and qRT-PCR assays were negative.

**IPNV uptake by mussels.** Mussels accumulate viable IPNV in their DG tissues as early as 2 hpe (Fig. 2). Only five of the nine replicate mussels were positive by virus isolation at 2 hpe, with an average titer of log  $2.8 \pm 0.1$  TCID<sub>50</sub> g<sup>-1</sup> ( $n = 9$ ). For all of the other time points in the trial, all of the mussels were positive for virus. In mussel exposure trial 1, there was no significant tank effect on the mean viable IPNV titer in mussel DG tissues ( $F = 0.9842$ ;  $P = 0.3859$ ). Time did have a significant effect on the IPNV titer in mussel DG tissues ( $F = 12.0460$ ;  $P = 0.0001$ ). The mean log TCID<sub>50</sub> of IPNV g of DG tissue<sup>-1</sup> was significantly greater at 120 hpe ( $4.4 \pm 0.1$  TCID<sub>50</sub> g<sup>-1</sup>) compared to that at 2 hpe ( $t = 6.36$ ;  $P = 0.0001$ ), 24 hpe ( $3.5 \pm 0.2$  TCID<sub>50</sub> g<sup>-1</sup>) ( $t = 3.59$ ;  $P = 0.0006$ ), and 48 hpe ( $3.8 \pm 0.2$  TCID<sub>50</sub> g<sup>-1</sup>) ( $t = 2.46$ ;  $P = 0.01$ ) (Fig. 2).

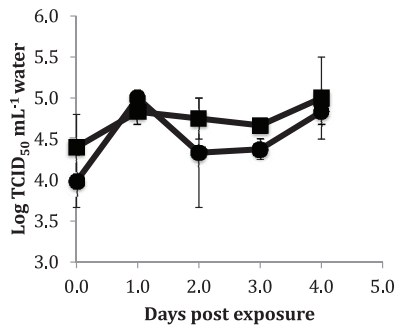


FIG 3 Log TCID<sub>50</sub> of IPNV per ml of water in tanks containing mussels (●) or lacking mussels (■) over time. Graphs represent the average log TCID<sub>50</sub> ml of water<sup>-1</sup> ± the standard error of the mean with *n* = 2 tanks.

In mussel exposure trial 1, there was no significant difference in viable IPNV titer between the DG tissues and the water of tanks containing mussels over all of the time points ( $F = 3.222$ ;  $P = 0.078$ ) (Fig. 2). The average IPNV titer in water of tanks containing mussels did differ significantly with time ( $F = 11.76$ ;  $P = 0.0126$ ), with an increase in average log TCID<sub>50</sub> ml of water<sup>-1</sup> by log 1.3 in 120 h (Fig. 2). In mussel exposure trial 2, however, there was no significant difference in viable IPNV titer over time in water from tanks containing mussels ( $F = 1.7998$ ;  $P = 0.2415$ ) (Fig. 3).

The accumulation of IPNV in mussel DG tissues was confirmed by qRT-PCR analysis (Fig. 4). IPNV segment A RNA levels peaked at 24 hpe and were significantly higher than IPNV RNA levels at 2 hpe ( $t = 4.93$ ;  $P = 0.0006$ ) and at 120 hpe ( $t = -2.61$ ;  $P = 0.0157$ ). At 120 hpe, the IPNV RNA levels remained significantly higher than the levels at 2 hpe ( $t = 2.32$ ;  $P = 0.0244$ ).

**IPNV shedding by mussels.** The average IPNV titer in DG tissues of mussels exposed to IPNV for 5 days was log 5.35 ± 0.25 TCID<sub>50</sub> g of DG tissue<sup>-1</sup>. With depuration of 1 to 7 days, IPNV-exposed mussels released viable IPNV in the fecal matter (Table 2). Viable IPNV was detected in mussel feces as early as 1 day postdepuration (dpd) and out to 7 dpd. Of the eight replicate mussels, only replicate 6 continuously released detectable levels of IPNV in the fecal material from 3 to 7 dpd. For replicate 6, the peak mussel feces IPNV titer of log 4.5 TCID<sub>50</sub> g of feces<sup>-1</sup> occurred at 5 dpd (Table 2). While other replicate mussels released

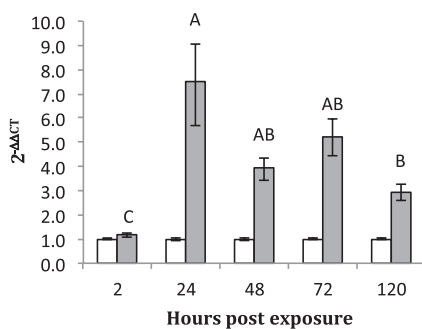


FIG 4 Average relative abundance of IPNV VP2 RNA in mussel digestive glands at 2, 24, 48, 72, and 120 h after exposure to MEM (□) or after exposure to IPNV (■), as measured with a TaqMan qRT-PCR in trial 1. Graphs represent averages ± the standard errors of the mean with *n* = 3 and *n* = 9 for MEM-treated and IPNV-exposed mussels, respectively. Means with different capital letters are significantly different (Fisher LSD,  $\alpha = 0.05$ ).

TABLE 2 CPE observations in mussel feces for mussel replicates 1 to 8

| Mussel replicate | CPE (log TCID <sub>50</sub> ) after various times (days) of depuration <sup>a</sup> |        |        |        |        |        |        |
|------------------|---|--------|--------|--------|--------|--------|--------|
|                  | 1 day   | 2 days | 3 days | 4 days | 5 days | 6 days | 7 days |
| 1                | No CPE  | No CPE | ND     | ND     | ND     | ND     | ND     |
| 2                | 3.7   | No CPE | ND     | ND     | ND     | ND     | ND     |
| 3                | No CPE  | No CPE | No CPE | No CPE | 4.7    | No CPE | 2.9    |
| 4                | No CPE  | No CPE | No CPE | No CPE | No CPE | No CPE | 2.9    |
| 5                | No CPE  | No CPE | 1.7    | No CPE | 4.7    | No CPE | 2.8    |
| 6                | No CPE  | No CPE | 1.7    | 1.7    | 4.2    | 3.2    | 2.1    |
| 7                | No CPE  | No CPE | No CPE | No CPE | ND     | ND     | ND     |
| 8                | No CPE  | No CPE | No CPE | No CPE | 3.3    | No CPE | ND     |

<sup>a</sup> CPE, cytopathic effect; ND, not determined (sample not taken after mussel death). The CPE is expressed as the log TCID<sub>50</sub> of IPNV per g of mussel feces.

detectable levels of IPNV in fecal matter one to three times, the IPNV loads in the fecal material were comparable to those of replicate 6. IPNV was not detected in the fecal matter of negative control mussels.

Viable IPNV was detected at very low levels (log 1.7 to 2.7 TCID<sub>50</sub> ml of water<sup>-1</sup>) in the water only in the first few days of mussel depuration (Table 3). The IPNV titer in the DGs was determined for mussel replicates 1 and 2 that died 1 dpd (log 3.8 and log 4.6 TCID<sub>50</sub> g of DG tissue<sup>-1</sup>) and replicates 7 and 8 that died 4 and 6 dpd (log 5.3 and log 5.1 TCID<sub>50</sub> g of DG tissue<sup>-1</sup>), respectively (Table 3). By 21 dpd, IPNV was not detected in the DG tissues of the remaining replicates. IPNV was not detected in water samples from tanks containing negative control mussels, nor was IPNV detected in the tissues of negative control mussels.

#### Transmission of IPNV from mussels to Atlantic salmon.

Prior to the cohabitation trial, mussels were exposed to water inoculated with IPNV stock (log 5.2 ± 0.2 TCID<sub>50</sub> of IPNV ml of water<sup>-1</sup>) or to water treated with equivalent volumes of MEM for 8 days. After 8 days of exposure to IPNV, all of the mussels sampled were positive for virus, and the mean log TCID<sub>50</sub> of IPNV g of DG tissue<sup>-1</sup> was log 5.2 ± 0.2 (*n* = 19). IPNV was not detected in any of the control mussels treated with MEM (*n* = 20).

Mussels were cohabitated for 18 days with naive Atlantic salmon. All mussels were then removed from tanks, and a subset was analyzed for virus isolation by culture for quantity or the presence or absence of viable IPNV. IPNV was not detected in any of the control mussels (*n* = 18). After 18 days in the cohabitation tanks, all IPNV-exposed mussels analyzed were positive for virus. The mean IPNV titer in DG tissue (log 3.1 ± 0.04 TCID<sub>50</sub> of IPNV g of tissue<sup>-1</sup>; *n* = 18) decreased significantly by 2 orders of magnitude compared to that of the mussels sampled prior to the cohabitation trial ( $F = 165.94$ ;  $P = 0.0001$ ).

There were no salmon mortalities during the cohabitation trial in any of the treatments. However, fish were monitored weekly for the presence of IPNV for 4 weeks by randomly selecting 6 fish for lethal sampling from each tank. All sentinel salmon and salmon cohabitating with control mussels tested negative for IPNV via culture and qRT-PCR analysis. In the salmon/salmon cohabitation treatment group, every salmon i.p. injected with IPNV tested positive for viable IPNV via culture (Tables 4 and 5). IPNV was detected via culture in 1 of 12 salmon cohabitating with the i.p.-injected salmon at 8 dpe in replicate 1 and at 21 dpe in replicate 2 (Tables 4 and 5). In the IPNV-exposed mussel treatment group, the mean number of IPNV-positive cohabitating salmon was 1.0 ± 0.26 (*n* = 6) of 24 (Table 4). All of the IPNV-positive salmon cohabitating with IPNV-exposed mussels were detected at 8 and 16 dpe (Table 5).

TABLE 3 CPE observations in mussel digestive gland tissue for mussel replicates 1 to 8

| Mussel replicate | CPE (log TCID <sub>50</sub> ) after various times (days) of depuration <sup>a</sup> |        |                  |        |                  |        |                  |        |                  |        |                  |        |                  |        |
|------------------|---|--------|------------------|--------|------------------|--------|------------------|--------|------------------|--------|------------------|--------|------------------|--------|
|                  | 1 day   |        | 2 days           |        | 3 days           |        | 4 days           |        | 5 days           |        | 6 days           |        | 7 days           |        |
|                  | H <sub>2</sub> O  | Tissue | H <sub>2</sub> O | Tissue | H <sub>2</sub> O | Tissue | H <sub>2</sub> O | Tissue | H <sub>2</sub> O | Tissue | H <sub>2</sub> O | Tissue | H <sub>2</sub> O | Tissue |
| 1                | No CPE  |        | 1.7              | 3.8    |                  |        |                  |        |                  |        |                  |        |                  |        |
| 2                | 2.7   |        | NA               | 4.6    |                  |        |                  |        |                  |        |                  |        |                  |        |
| 3                | 1.8   |        | No CPE           |        | No CPE           |        | No CPE           |        | No CPE           |        | No CPE           |        | No CPE           |        |
| 4                | 1.7   |        | No CPE           |        | No CPE           |        | No CPE           |        | No CPE           |        | No CPE           |        | No CPE           |        |
| 5                | 1.7   |        | No CPE           |        | No CPE           |        | No CPE           |        | No CPE           |        | No CPE           |        | No CPE           |        |
| 6                | No CPE  |        | No CPE           |        | 1.7              |        | No CPE           |        | No CPE           |        | No CPE           |        | No CPE           |        |
| 7                | 1.7   |        | No CPE           |        | No CPE           |        | No CPE           | 5.3    | No CPE           |        | No CPE           |        | No CPE           |        |
| 8                | 1.7   |        | No CPE           |        | No CPE           |        | No CPE           |        | No CPE           |        | No CPE           | 5.1    | No CPE           |        |

<sup>a</sup> CPE is expressed as the log TCID<sub>50</sub> of IPNV per g of mussel feces. CPE, cytopathic effect; NA, not applicable.

The cohobitation trial was carried out in two identical saltwater recirculation systems, each with identical sets of treatment groups randomly assigned to the nine tanks in each system. The IPNV infection status (positive or negative) in Atlantic salmon was statistically independent of the systems ( $\chi = 0$ ;  $P = 1.0$ ). The IPNV infection status was also independent of cohobitation treatment, i.e., cohobitation with i.p.-injected salmon versus cohobitation with IPNV-exposed mussels ( $\chi = 0.788$ ;  $P = 0.3749$ ).

The viral load in each of the salmon kidney/spleen samples that originally tested positive for IPNV was determined by TCID<sub>50</sub> analysis. qRT-PCR analysis was also performed on RNA isolated from kidney tissues of 5% the total fish in the cohobitation trial, as well as on RNA isolated from kidney tissues from fish that tested positive for IPNV by culture. Overall, the viral load, determined by culture, was very low, ranging from log 2.3 to 4.6 TCID<sub>50</sub> g of tissue<sup>-1</sup> (Table 5). There was no significant difference in IPNV titer between fish harvested at different time points ( $F = 1.3097$ ;  $P = 0.2916$ ). qRT-PCR analysis performed on RNA from these same fish generated very high  $C_T$  values (36.5–39) or no  $C_T$  value at all (Table 5). In most cases only 1 or 2 out of the triplicate qPCRs generated  $C_T$  values (Table 5). The majority of the IPNV-positive fish were those that had been injected with IPNV.

At the end of the trial the viral load (log 4.1 TCID<sub>50</sub> g of tissue<sup>-1</sup>) was measurable for only one of the two salmon cohobitating with i.p.-injected salmon since the levels of IPNV were below the detection limits of the TCID<sub>50</sub> and qRT-PCR assays in the second salmon (Table 5). The viral load was determined by culture (log 3.1 TCID<sub>50</sub> g of tissue<sup>-1</sup>) for only one of the six IPNV-positive salmon that cohobitated with IPNV-exposed mussels (Table 5). The levels of IPNV in all six of these fish were below the detection limit of the qRT-PCR assay.

TABLE 4 Number of IPNV-positive salmon in replicate groups of salmon injected intraperitoneally with IPNV, cohobitants of intraperitoneally injected salmon, and cohobitants of IPNV-exposed mussels

| Treatment group                      | No. of IPNV-positive salmon/no. of salmon tested for replicate: |       |      |      |      |      |
|--------------------------------------|---|-------|------|------|------|------|
|                                      | 1   | 2     | 3    | 4    | 5    | 6    |
| Intraperitoneally injected with IPNV | 12/12   | 12/12 |      |      |      |      |
| Salmon cohobitants                   | 1/12  | 1/12  |      |      |      |      |
| IPNV <sup>+</sup> mussel cohobitants | 1/24  | 2/24  | 0/24 | 1/24 | 1/24 | 1/24 |

TABLE 5 IPNV titers (TCID<sub>50</sub>) and qRT-PCR values ( $C_T$ ) generated from salmon kidney samples that originally tested positive for IPNV via culture

| Days postexposure | Treatment <sup>a</sup>      | IPNV titer (log TCID <sub>50</sub> g of kidney tissue <sup>-1</sup> ) | $C_T$ <sup>b</sup> |
|-------------------|-----------------------------|---|--------------------|
| 8                 | IPNV mussel cohob-recipient | <2.3  | No $C_T$           |
|                   | IPNV mussel cohob-recipient | <2.3  | No $C_T$           |
|                   | Salmon cohob-recipient      | 4.1   | 39.7*              |
|                   | Salmon cohob-i.p.           | <2.3  | 39.8*              |
|                   | Salmon cohob-i.p.           | 3.6   | No $C_T$           |
|                   | Salmon cohob-i.p.           | 3.3   | 39.5*              |
|                   | Salmon cohob-i.p.           | 3.6   | 39.7*              |
|                   | Salmon cohob-i.p.           | 3.3   | 39.6*              |
|                   | Salmon cohob-i.p.           | 3.9   | No $C_T$ †         |
|                   | Salmon cohob-i.p.           | 4.6   | 37.8**             |
| 16                | IPNV mussel cohob-recipient | <2.3  | No $C_T$           |
|                   | IPNV mussel cohob-recipient | 3.1   | No $C_T$           |
|                   | IPNV mussel cohob-recipient | <2.3  | No $C_T$           |
|                   | IPNV mussel cohob-recipient | <2.3  | No $C_T$           |
|                   | Salmon cohob-i.p.           | 3.1   | No $C_T$           |
|                   | Salmon cohob-i.p.           | 3.1   | 38.5**             |
|                   | Salmon cohob-i.p.           | 3.3   | No $C_T$           |
|                   | Salmon cohob-i.p.           | 4.1   | 38.2*              |
|                   | Salmon cohob-i.p.           | 4.1   | No $C_T$           |
|                   | Salmon cohob-i.p.           | 4.3   | 36.5***            |
| 21                | Salmon cohob-recipient      | <2.3  | No $C_T$           |
|                   | Salmon cohob-i.p.           | 2.3   | No $C_T$           |
|                   | Salmon cohob-i.p.           | 2.8   | 39.7*              |
|                   | Salmon cohob-i.p.           | <2.3  | No $C_T$           |
|                   | Salmon cohob-i.p.           | 3.3   | 39.8**             |
|                   | Salmon cohob-i.p.           | 3.3   | No $C_T$           |
|                   | Salmon cohob-i.p.           | 2.9   | 39**               |
|                   | Salmon cohob-i.p.           | 2.9   | 39**               |
| 26                | Salmon cohob-i.p.           | <2.3  | 39.4*†             |
|                   | Salmon cohob-i.p.           | 3.2   | 39.3*              |
|                   | Salmon cohob-i.p.           | 3.3   | 39.9*              |
|                   | Salmon cohob-i.p.           | NA  | No $C_T$           |
|                   | Salmon cohob-i.p.           | <2.3  | No $C_T$           |
|                   | Salmon cohob-i.p.           | NA  | NA                 |

<sup>a</sup> The recipients were fish cohobitating (cohob) with IPNV-intraperitoneally injected (“i.p.”) salmon or IPNV-exposed mussels.

<sup>b</sup> \*, one of three reactions produced  $C_T < 40$ ; \*\*, two of three reactions produced  $C_T < 40$ ; \*\*\*, three of three reactions produced  $C_T < 40$ ; †, some RNA degradation; NA, not applicable.

## DISCUSSION

Shellfish play a critical role in an IMTA system by extracting particulate bound organic nutrients; however, as bioaccumulating organisms, they may also influence pathogen dynamics by serving as a reservoir or as a sink for important finfish pathogens. Fish farmers applying IMTA need to have a clear understanding of how the culturing of filter-feeding organisms in close proximity to finfish cages will impact possible disease transmission at their farms. The potential for shellfish to accumulate and shed viable pathogen from their tissues depends largely on the physiology of the pathogen. Mussels are capable of bioaccumulating and shedding bacterial pathogens, such as *V. anguillarum*, and yet appear to inactivate the enveloped viral pathogen infectious salmon anemia virus (5, 9, 10). The potential for mussels to bioaccumulate and transmit the nonenveloped viral pathogen, IPNV, to Atlantic salmon has now been assessed.

Biosecurity is essential in controlling diseases such as IPN. Therefore, it is critical to understand the risks associated with transferring Atlantic salmon smolts to seawater net pens that are in the vicinity of mussels that have had the opportunity to accumulate IPNV. Mussels may increase both the risk of infection with IPNV in the post-smolts in addition to increase the risk of IPNV disease outbreak. The prevalence of IPNV in Atlantic salmon is increasing in countries with intensive Atlantic salmon farming such as Scotland and Norway (22, 23). Although IPN outbreaks in Atlantic salmon have not been a recent problem in North America, there are endemic strains of IPNV in the region (32, 33). Further, IPNV can persist in seawater and sediments and has been detected in many bivalve species (23, 34, 35). Therefore, Atlantic salmon may be at risk for becoming infected with IPNV during the stress of saltwater acclimation.

To determine whether mussels could act as an IPNV reservoir, we used viable virus isolation by culture assays and molecular techniques to measure viral loads in digestive gland tissues of IPNV-exposed mussels. Fish pathogens, including IPNV, are known to persist in the digestive gland (hepatopancreas) tissues of shellfish (7, 9). Culture-based and molecular detection of pathogens in mussel digestive gland tissues, however, is difficult due to cell cytotoxicity and PCR inhibitors present in the digestive gland tissues (5). It was therefore important to optimize and determine the detection limits of the assays used to measure viral load in these tissues.

Previously, virus isolation techniques from mussel digestive gland tissues were optimized (5). To better interpret data for viral load in mussel digestive gland tissue, the detection limits of the IPNV qRT-PCR assay and the culture assays were compared in IPNV-inoculated mussel DG homogenates (Fig. 1). The IPNV culture assay was determined to be more sensitive than qRT-PCR detection of IPNV in mussel tissue homogenates. While qRT-PCR detected IPNV RNA in mussel homogenates with a predicted titer of  $\log 3.5 \text{ TCID}_{50} \text{ ml}^{-1}$ , the assay only detected RNA in one of the duplicate samples and in only out of three of the triplicate reactions performed on that tissue sample. Therefore, the qRT-PCR assay only reliably detected virus in the mussel digestive gland homogenate with a predicted titer of  $\log 4.5 \text{ TCID}_{50} \text{ ml}^{-1}$ . The reliable IPNV detection limit for this assay is  $\log 3.8 \text{ TCID}_{50} \text{ ml}^{-1}$ , the actual titer determined by  $\text{TCID}_{50}$  analysis (Fig. 1). The inconsistencies of virus detection among homogenate replicates and among reaction replicates is likely due to the low amounts of virus

in the samples and the sensitivity of the assays. These inconsistencies in virus detection in samples with low levels of virus have been observed in other studies comparing real-time RT-PCR and culture-based assays (36). Orpetveit et al. demonstrated comparable to greater sensitivity in an IPNV qRT-PCR assay compared to virus isolation from kidney tissue from IPNV carrier Atlantic salmon, although the detection limit of the qRT-PCR assay was not determined in the fish tissues. Fish kidney homogenates in the Orpetveit study, however, were applied to CHSE-214 cells at a higher dilution that likely decreased the sensitivity of their culture assay. Further, the sensitivity of virus isolation by cell culture can differ dramatically between laboratories due to differences in cell line maintenance.

The lower sensitivity of the qRT-PCR assay compared to the culture assay may be due to PCR inhibitors present in the mussel digestive gland tissues. The detection limit of the IPNV qRT-PCR assay in fish kidney homogenates was not determined; however, a decrease in sensitivities when performed on mussel digestive gland tissues compared to Atlantic salmon kidney tissues (5, 37) was observed for other culture and qRT-PCR assays. This suggests that PCR inhibition in mussel digestive gland tissues is greater than that in fish kidney samples.

Mussels significantly accumulate viable IPNV in their digestive gland tissues over time (Fig. 2). Viral loads in mussel digestive gland tissues increased significantly with time, peaking at 72 to 120 hpe. The level of IPNV in mussel tissues was not, however, significantly greater than IPNV levels in the water, indicating that mussels do not efficiently remove IPNV particles from the water column. The small particle size of IPNV (60 nm) may contribute to the inefficiency of particle uptake by the mussel; however, mussels can concentrate hepatitis A particles in their tissues 100 times above the viral concentrations in the water despite their small particle size of 27 nm (16, 38). Viral adsorption by shellfish can differ drastically for two viral particles of the same size, indicating that there are other factors that contribute to virus uptake (39). Although particle size is important in virus uptake by shellfish, the main mechanism for virus uptake is by entrapment in mucus, which is dependent upon ionic bonding between the viral particle and anionic moieties in the mucus (40). Factors such as temperature, salinity, particle charge, and mucus production by the shellfish drastically affect virus uptake and may be contributing factors to the inefficient uptake of IPNV by mussels (41).

The presence of IPNV in mussel digestive glands was confirmed by qRT-PCR (Fig. 4). The level of IPNV RNA peaked at 24 to 72 hpe, earlier than peak levels of viable IPNV, which occurred at 72 to 120 hpe (Fig. 2 and 4). This observation is difficult to explain. It is possible that the difference in timing of the peak IPNV levels between the two assays is due to the detection of nonviable and viable IPNV particles by the qRT-PCR assay. Unlike culture-based assays, qRT-PCR cannot distinguish between viable and nonviable viral particles. If nonviable or immature IPNV particles were present in the stock and if these particles were less stable than viable particles in the mussel DG tissue, then viral RNA in DG tissue would decrease over time. This might mask the accumulation of viable particles, which was observed in the culture data. In both analyses, the levels of IPNV were significantly greater at 120 hpe than at 2 hpe, demonstrating that levels of IPNV accumulate over time in mussel digestive gland tissues.

In the first mussel exposure, IPNV titers significantly increased over time in the water of tanks containing mussels; however, this



increase in virus titer was not repeatable in a second mussel exposure trial (Fig. 2 and 3). If the IPNV levels in the water were truly increasing over time, this would indicate that IPNV is replicating in the mussel tissues. IPNV has been isolated from adult moribund scallops; however, replication of IPNV in shellfish tissues has not yet been demonstrated (35). Our evidence does not support nor disprove viral replication in mussels. The fact that the increasing titer effect was not repeatable suggests that the detection of a significant effect was likely due to a type I error. The inconsistencies in IPNV titer in water over time could be due to the small volume of water analyzed. Further, at early time points the IPNV titer in the water may have been lower due to viral aggregates. Disaggregation of the virus over time could be responsible for the higher titers observed at the later time points.

In IPNV-exposed mussels, viable IPNV persists in mussel digestive gland tissues for at least 18 days of depuration. Over this period of time, the titer of IPNV in digestive gland tissues decreased by 2 orders of magnitude. This suggests that mussels are either inactivating viable particles in their tissues and/or releasing IPNV particles into the environment. This study demonstrated that IPNV-exposed mussels do release viable IPNV via their feces (Table 2). IPNV was detected in feces out to 7 days of depuration with titers as high as  $\log 4.5$  TCID<sub>50</sub> g<sup>-1</sup> after 5 days of depuration. Further, IPNV was detected in Atlantic salmon that cohabitated with these mussels (Table 4). Although all 19 mussels analyzed for IPNV titer after cohabitating for 18 days with Atlantic salmon were positive for IPNV, IPNV was detected in digestive glands of mussels in the shedding trial only up to 7 days of depuration. IPNV was not detected after 21 days in the shedding trial; however, only two mussels were tested. The mussels in the shedding trial and the cohabitation trial were maintained in different tank systems, and it is possible that the different environments affected shedding of IPNV particles.

Mortensen et al. were not able to demonstrate replication of IPNV serotype N1 in scallops challenged by injection or bath immersion; however, these researchers did detect viable IPNV in the digestive gland tissues of scallops 50 days after bath exposure to IPNV (7). Further, Mortensen et al. observed viable IPNV in the digestive gland, adductor muscle, gonads, and rectum tissues at 333 dpe in scallops challenged by injection of IPNV into the adductor muscle (7). This supports the data demonstrating that IPNV can persist in mussel digestive gland tissues for at least 18 days. Although Mortensen did not detect IPNV in scallop feces, the presence of IPNV in the scallop rectum out to 333 days of depuration indicates that IPNV can persist in shellfish tissues for long periods of time and is at least periodically shed via the rectum. The persistence of IPNV in these tissues for such long durations does not rule out the possibility of low-level replication of the virus in shellfish tissues.

The fact that IPNV persists in IPNV-exposed mussels and is shed via the feces puts susceptible cohabitating fish at risk to exposure to IPNV. The IPNV titer in mussels that cohabitated with Atlantic salmon decreased by 2 orders of magnitude over 18 days of cohabitation. It is likely that some of the virus was released in mussel feces, exposing cohabitating Atlantic salmon in the tank.

Transmission of IPNV did occur from mussels to fish at a low frequency. The frequency of transmission, however, was comparable to the frequency of transmission from IPNV-injected salmon to naive salmon used here (Table 4). This demonstrates that a nonenveloped virus can accumulate in mussels and be

transferred to naive fish. The frequency of transmission, and thus the risk on IMTA farms, may increase if a more virulent strain of virus or a more susceptible salmon population were in question (21, 43).

The WB strain of IPNV (VR299) is the North American type strain and the endemic strain in Maine (33). Although virulent in brook trout (*Salvelinus fontinalis*), no outbreaks of WB IPNV in Atlantic salmon have been reported. It was therefore not surprising that no mortalities in the present study were due to IPNV in IPNV-positive fish. At the end of the trial, the viral loads in fish injected with IPNV overall were low, with TCID<sub>50</sub> g<sup>-1</sup> values ranging from  $\log 2.3$  to 4.6 and  $C_T$  values of 36.5 to no  $C_T$  value at all (Table 5). The viral load in recipient fish cohabitating with IPNV-injected fish or IPNV-exposed mussels was also very low and often below the detectable level of the TCID<sub>50</sub> assay ( $10^{2.3}$  TCID<sub>50</sub> ml<sup>-1</sup>) and below the detection limit of the qRT-PCR assay. The viral loads in these fish were too low to cause clinical IPNV.

Despite its low virulence in Atlantic salmon, the WB strain of IPNV was used in the present study because it is a relevant IPNV strain in Maine. Atlantic salmon farmers in Maine are integrating blue mussel crops on their marine salmon sites, and it is important to determine the risk of transmission of the endemic IPNV strain on IMTA farms in Maine. It is possible that the risk of transmission of IPNV isolates, for example, the Scottish or Norwegian isolates, may be much higher (21, 43). Therefore, to rule out IPNV transmission on mussel/salmon farms in other regions, cohabitation experiments with Atlantic salmon and IPNV-exposed mussels should be carried out with relevant endemic strains. In addition, IPNV-free mussels should be cohabitated with Atlantic salmon carrying and shedding a virulent strain of IPNV to determine whether mussels can accumulate sufficient loads of virus to act as a reservoir to naive Atlantic salmon.

In conclusion, transmission of viable virus from IPNV-exposed mussels to naive Atlantic salmon is possible. The low frequency of transmission of the WB strain of IPNV to Atlantic salmon suggests a low risk of transmission occurring on an IMTA farm in Maine. Although the risk is low, it is still notable, given that IPNV is a reportable pathogen, and any report of virus on a Maine salmon farm, even in the absence of disease, would result in the culling of fish (42). Further, the risk of IPNV transmission may be greater on mussel/Atlantic salmon farms in other regions, such as Norway and Scotland, where endemic strains of IPNV are more virulent in Atlantic salmon. Cohabitation experiments with shellfish, fish, and relevant nonenveloped viral pathogens of the region should be performed to determine the disease risks of IMTA for a specific location.

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