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Method for serial passage of infectious hematopoietic necrosis virus (IHNV) in rainbow trout

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ABSTRACT: Transmission is a fundamental component of pathogen fitness. A better understanding of pathogen transmission can greatly improve disease management. In particular, controlled studies of multiple rounds of natural transmission (i.e. serial passage) can provide powerful epidemiological and evolutionary inferences. However, such studies are possible in only a few systems because of the challenges in successfully initiating and maintaining transmission in the laboratory. Here we developed an efficient and reproducible cohabitation method for conducting controlled experiments investigating the effects of serial passage on infectious hematopoietic necrosis virus (IHNV) in rainbow trout. This method was used to investigate the transmission efficiency and kinetics of viral shedding of IHNV over 3 serial passages. Transmission efficiency decreased from 100 to 62.5% over the passage steps and was associated with a decrease in virus shedding into water. A shift in the peak of viral shedding was also observed, from Day 2 post immersion for passage 0 to at least 24 h later for all subsequent passages. Finally, the characterization of viruses after 1 round of transmission and propagation on cells showed no change in glycoprotein (G gene) sequences or viral virulence compared to the ancestral virus stock. The methods developed provide valuable tools for reproducible population-level studies of IHNV epidemiology and evolution.

KEY WORDS: Transmission · Evolution · Viral shedding · Virulence · Salmonid

1. INTRODUCTION

Understanding pathogen transmission dynamics can provide powerful insights into infectious disease management. Ultimately, transmission dynamics determine the rate at which pathogens spread and how many hosts become infected (Anderson & May 1992, Nelson & Williams 2013). Despite their epidemiological importance, pathogen transmission dynamics are difficult to study, are rarely quantified, and frequently warrant further investigation. When transmission has been studied, it is typically inferred either from disease dynamics at a host population level, from *in vitro* studies, or under artificial transmission routes (Wargo & Kurath 2012). Although these studies can be highly informative, they are often limited in their ability to accurately predict transmission on an individual host basis or how these dynamics change as the pathogen undergoes multiple rounds of transmission from one host to the next. The ability to make such predictions can be greatly facilitated by controlled *in vivo* laboratory studies using multiple rounds of natural host-to-host transmission (e.g. Ebert 1998, Mackinnon & Read 2004, Yourth & Schmid-Hempel 2006, Chapuis et al. 2011). These serial passage experiments (SPEs) can yield critical epidemiological information (Ebert 1998). Such a method for transmission and epidemiological inference would be particularly beneficial for management of the prevalent fish pathogen infectious hematopoietic necrosis virus (IHNV), which despite various control efforts remains highly problematic in the culture of salmonids.

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IHNV is a single-stranded negative-sense 11.1 kb RNA virus (species *Salmonid novirhabdovirus*) in the genus *Novirhabdovirus* and family *Rhabdoviridae* (Schütze et al. 1995). The virus typically causes an acute virulent disease in many species of salmon and trout and is of global concern for aquaculture, fisheries, and conservation (Kim et al. 2005, Bootland & Leong 2011, OIE 2017). Juvenile salmonids are the most susceptible to disease caused by the virus, but infection and symptoms can also occur in adult fish (Bootland & Leong 2011). IHNV was first identified in the Pacific Northwest region of North America, where it is believed to have originated, but it has since spread and become one of the most prominent salmonid pathogens around the world, particularly in rainbow trout *Oncorhynchus mykiss* aquaculture (Nichol et al. 1995, Kurath et al. 2003). Because of its management importance, the virus has been extensively studied (reviewed by Dixon et al. 2016), with a growing body of literature on viral fitness and emergence, which is believed to be driven by transmission (Wargo et al. 2012, 2017, Wargo & Kurath 2012, Kell et al. 2013, 2014, Breyta et al. 2014, 2016, McKenney et al. 2016). However, few studies have directly quantified the transmission dynamics of IHNV, particularly through multiple rounds of transmission (Foreman et al. 2015, Dixon et al. 2016).

IHNV is transmitted via fish bodily fluids such as urine, feces, milt, mucus, or decay and ingestion of carcasses (Mulcahy et al. 1983, Nishimura et al. 1988, LaPatra et al. 1989, Bootland & Leong 2011). This can result in direct horizontal transmission through contaminated water, feed, or aquaculture supplies (Amend 1975). Eggs can also become externally contaminated resulting in fish becoming infected upon hatching, often referred to as pseudo-vertical transmission, which is prevented in aquaculture with iodine treatment (Mulcahy et al. 1983). Because juvenile fish typically suffer more clinical disease, they were originally believed to be the primary source of transmission, but recent epidemiological studies suggest adult fish may play an important role (Breyta et al. 2016, 2017, Ferguson et al. 2018). Transmission within and between aquaculture and wild fish occurs and is complex (Troyer & Kurath 2003, Saksida 2006); thus, a better understanding of transmission dynamics at the individual fish level could help to disentangle this complexity.

Previous experimental studies of IHNV transmission typically used a cohabitation design where large groups of infected and naïve fish were held together for 11 to 35 d. Infection status of naïve fish (transmission) was then assessed through disease symptoms or mortality and confirmed in a subset of fish using traditional virological methods such as plaque assays (Amend 1975, Mulcahy et al. 1983, Traxler et al. 1993, Ogut & Reno 2004a). These studies provided valuable insights into the process of disease dynamics at a population scale, revealing that these dynamics are acute and influenced by environmental parameters such as fish density (e.g. Ogut & Reno 2004a). However, these studies were limited in their ability to track transmission on an individual fish basis, determine how many rounds of transmission occurred, classify the source of infection (donor fish versus recently infected naïve fish), and accurately quantify transmission in the absence of clinical disease. The number of biological replicates possible with this large cohabitation study design is also limited. Ultimately, these limitations made it difficult to estimate important epidemiological parameters (Anderson & May 1992, van den Driessche & Watmough 2002).

More recently, viral shedding has been used as a proxy for transmission. These studies suggest that there is a significant degree of individual host variation, in the amount of viral shedding, which is influenced by a variety of factors such as within-host viral load, host genetics, virus entry, infectious dose, environmental conditions, and exposure dose (Wargo & Kurath 2011, Wargo et al. 2012, 2017, Garver et al. 2013, Langwig et al. 2017). However, these studies assume that transmission is proportional to the quantity of viral shedding, which has not been fully validated. These previous studies also investigated shedding from fish infected after waterborne immersion with viruses previously grown *in vitro* on cells, rather than directly shed from fish. It is unknown whether the infectivity and transmission dynamics differs between cultured and naturally shed virus.

In this study, we developed a quick, efficient, and reproducible procedure for studying transmission of IHNV in serial passage using a natural cohabitation method under laboratory conditions. This method was designed to allow precise quantification of the level of transmission after each round of transmission and incorporate a large number of biological replicates. These studies made it possible to elucidate the relationship between viral shedding and transmission, determine how the kinetics of shedding change after each round of transmission, and investigate how host density affects transmission of the virus. We also characterized the virus for its glycoprotein (G) gene sequence and virulence after 1 round of transmission to investigate potential effects of serial passage on viral selection and evolution.

2. MATERIALS AND METHODS

2.1. Fish-to-fish viral transmission

Several experiments were conducted to evaluate the efficiency of fish-to-fish transmission of IHNV in rainbow trout *Oncorhynchus mykiss* and develop a serial passage method. A specific pathogen free aquaculture rainbow trout line, provided by Clear Springs Foods, was used in these experiments. Fish weighed approximately 1 to 7 g and were maintained in specific pathogen free, UV-irradiated fresh water at 15°C. These experiments were approved by William & Mary's Institutional Animal Care and Use Committee (IACUC) under IACUC protocol 2014-11-17-12311-arwargo. The virus used in these experiments was IHNV (species *Salmonid novirhabdovirus*) isolate LR80 (genotype mG007M; GenBank accession no. L40878), which is from the M genogroup believed to be adapted to *O. mykiss* at 15°C (Nichol et al. 1995, Kurath et al. 2003, Garver et al. 2006). The virus was propagated and titered on an *Epithelioma papulosum cyprini* (EPC) fish cell line (Fijan et al. 1983) prior to experiments, then stored as virus stocks at −80°C as previously described (Batts & Winton 1989). The virus dose was not reconfirmed at the time of exposure in these experiments but Batts & Winton (1989) indicate titers of viral stocks are stable.

Six different transmission experiments were conducted (Fig. 1; labelled Expts 1–6). To generate passage zero (P_0), groups of rainbow trout (10–30 fish) were initially infected by immersing them in static water with supplemental aeration containing IHNV from virus stocks at a dose of 1×10^6 plaque-forming units (PFU) ml^{-1} for 1 h in 6 l tanks. A 1 h wash (flow at 1500 ml min^{-1}) to remove all residual virus was immediately done after the 1 h exposure. Then, 1 (Expts 1 and 2) or 3 (Expts 3–6) fish were transferred into individual 0.8 l tanks, with aeration. In Expt 1, fish were kept in these tanks in static water for 72 h (Fig. 1A). At this point, P_0 fish were removed and a new naïve fish (referred to as P_1, i.e. passage 1) was added to each tank. The P_1 fish were immersed for 24 h, transferred into new tanks containing virus-free water, rinsed for 1 h (water flow at 250–300 ml min^{-1}), and held in static conditions with supplemental aeration at 15°C for 3 d. We consider the initial immersion time of the naïve fish with the virus or donor fish as Day 0 in this and all other experiments. In Expts 2 to 6, each individual (Expt 2; Fig. 1B) or group (Expts 3–6; Fig. 1C) of P_0 fish was left to shed the virus in static water for 24 h. At this point, either 1 (Expt 2) or 3 (Expts 3–6) new naïve P_1 fish were placed in a nylon bag and added to each tank. Fish were held in these tanks for 24 h. This allowed for water and virus flow between the P_0 and P_1 fish but kept the fish separated so the viral passage could be differentiated. The P_1 fish were then transferred into new tanks, released from the nylon bag, rinsed, and held in tanks for 2 (Expts 5 and 6) to 3 (Expts 2–4) days in the same conditions as for Expt 1. For Expts 5 and 6, an additional 1 (Expt 5) or 2 (Expt 6) rounds of serial passages, named passage 2 (P_2) and passage 3 (P_3), were performed, following the same procedure as for P_0 fish, with cohabitation beginning 2 d after initial exposure (Fig. 1). The tanks were kept under continuous aeration, which also vigorously mixed the water. A volume of 800 µl of water was collected daily from each individual tank at the same time each day, on the day of fish addition to the tank and subsequently for 2 to 4 d. Water samples were stored at −80°C daily and later processed to evaluate transmission success and the cumulative kinetics of viral shedding of each passage step (Expts 1–3, 5, and 6). Water samples were only collected at 1 time point (Days 2 and 4 for P_0 and P_1 fish, respectively) in Expt 4. The fish were then euthanized by adding 0.27 mg ml^{-1} Tricaine-S (MS 222; Western Chemical) buffered with 0.09 mg ml^{-1} sodium bicarbonate directly into each tank. All the fish from 1 tank within a passage were collected into a single Whirl-pak® bag (Nasco) and stored at −80°C. None of the fish died during any of the transmission experiments.

2.2. Viral quantification in water

Total RNA was extracted from water samples with a Tecan Freedom EVO® 100 liquid handler using the *cador* Pathogen 96 QIAcube HT Kit (Qiagen), according to the manufacturer's protocol. A volume of 105 µl of VXL mix (84 µl buffer VXL with additional 20 µl Proteinase K and 1 µg carrier RNA) was added to each 210 µl water sample, vortexed at 1000 rpm for 30 s, and incubated for 5 min at room temperature. A volume of 367.5 µl of buffer ACB was added, the sample was vortexed (1000 rpm for 30 s), and then 685 µl of the sample was transferred to the capture plate and subjected to a 3 min vacuum at 25 kPa. Each sample was washed using 600 µl of buffers AW1 and AW2 and eluted with a 1 min vacuum at 35 kPa, then rinsed with 600 µl absolute ethanol (vacuum for 30 s at 35 kPa). Subsequently, the capture plate was dried for 1 min at 70 kPa, then for 2 min at

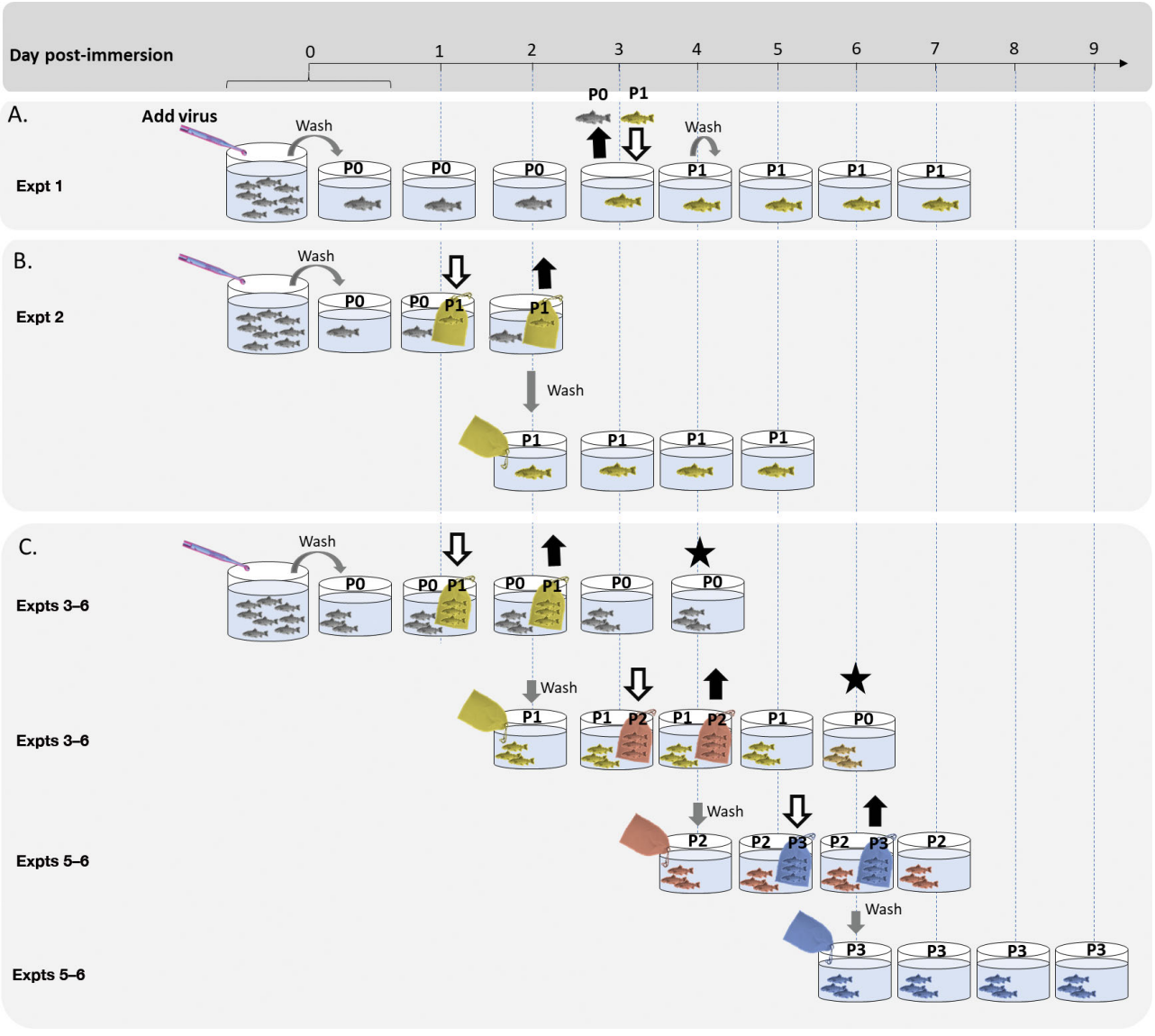

Fig. 1. *In vivo* serial passage designs for experiments on infectious hematopoietic necrosis virus in rainbow trout. (A) Expt 1, (B) Expt 2, and (C) Expts 3 to 6, as described in Section 2. (⇨): add fish to tank; (➡): remove fish from tank; (➡): fish transfer or 1 h wash. Black stars indicate fish held in static conditions in tanks for 3 d only in Expts 3 and 4; otherwise, they were held static for 4 d as described in Section 2. All donor and recipient tanks were sampled in these experiments. P: passage

35 kPa. Total RNA was eluted by incubating 100 µl of buffer AVE on the column for 2 min, then applying a 70 kPa vacuum for 6 min. All extracted RNA was stored at –80°C until further processing. RNA samples were converted to cDNA using 11 µl of extracted RNA sample and Moloney murine leukemia virus reverse transcriptase (Promega), random hexamers, and oligo dT in a total reaction volume of 20 µl as described in Wargo et al. (2010). The cDNA was stored at –80°C until future use.

For virus quantification, cDNA samples were diluted 1:2 with RNase/DNase-free water (Fisher Scientific) and then underwent quantitative PCR (qPCR), using TaqMan probe IHNV N 818 MGB, forward primer IHNV N 796 F, and reverse primer IHNV N 875R, targeting the viral nucleocapsid (N) gene, in a 12 µl reaction as previously described (Purcell et al. 2013). Viral quantification was performed in 384-well optical plates (Applied Biosystems) with the APC viral N gene plasmid standard (Purcell et al. 2013). The qPCR quantified the number of viral RNA copies, presented here as virus copies per milliliter of water. A $\log_{10}(x + 1)$ transformation was applied to the viral load data for figures and analyses.

2.3. Virus propagation from fish

The P_1 fish from the 3 replicate tanks in Expt 4 were thawed on ice for approximately 3 h and weighed. This experiment was chosen at random to isolate and characterize the virus after 1 round of passage (P_1). A volume of 2 ml g^{-1} of fish of minimum essential medium (MEM) supplemented with 100 units ml^{-1} of penicillin (Gibco), 100 µg ml^{-1} streptomycin (Gibco), 20 µg ml^{-1} gentamycin (Gibco), 2.5 µg ml^{-1} Amphotericin B (Gibco), and 7 ml 1 M Tris-HCl (Sigma) (media named MEM-0) was added to each Whirl-pak® (Nasco) bag of 3 fish. Using the flat bottom of a small glass beaker, pressure was applied to the fish through the bag to homogenize their contents. Once all the fish tissue was dislodged from the skin and no large pieces remained, the contents of each bag were transferred into separate 50 ml sterile centrifuge tubes and stored on ice. A second aliquot of 2 ml MEM-0 g^{-1} of fish was used to rinse the remaining fish tissue into the centrifuge tube. The 3 centrifuge tubes were then centrifuged at 1000 × *g* for 10 min at 4°C, and the resulting supernatant was removed and stored at −80°C. The pellet was discarded.

Each of the 3 virus isolates (P1fish_1 to P1fish_3; Expt 3) were propagated on 2 d old confluent monolayers of EPC cells (Fijan et al. 1983) in 75 cm^2 flasks containing 1×10^8 cells at confluence. The cells were maintained in MEM-0 supplemented with 10% fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco), and 10 ml 7.5% sodium bicarbonate substituted for the Tris-HCl (media named MEM-10). A 500 µl volume of each fish supernatant virus isolate (P1fish_1 to P1fish_3), positive control (LR80 ancestor stock), and negative control (MEM-10) was added to their respective flask, manually rocked back and forth gently for 10 s, and incubated at 15°C for 7 to 9 d. Each day, the flasks were checked for cytopathic effect, which was quantified as percent of cell monolayer lysed. Once approximately 80% of the cell monolayer had lysed, the contents of the flask were transferred into a 50 ml centrifuge tube and centrifuged at 1000 × *g* for 10 min at 10°C. The supernatants were then collected and stored at −80°C for future use. These viral isolates were labelled P1cell_1, P1cell_2, and P1cell_3.

Viruses collected directly from fish supernatants (P1fish_1 to P1fish_3) and from propagation on fish cells (P1cell_1 to P1cell_3) were titered on EPC cells using plaque assays. Serial 10-fold dilutions of each sample were prepared in MEM-0, and 200 µl of each dilution (10^{-1}–10^{-4} for viruses from fish supernatants and 10^{-4}–10^{-7} dilutions for viruses propagated on cells) was inoculated into the wells containing 1×10^6 cells pretreated with a final concentration of 7% (w/v) polyethylene glycol (PEG 20 000; Sigma) in triplicate as described in Batts & Winton (1989). A positive control (LR80 ancestor stock with a mean titer of 2.22×10^9 PFU ml^{-1}) and a negative control (MEM-10) were additionally titered. The multiplicity of infection (MOI) was estimated for virus used to seed the P1cell_1 to P1cell_3 cultures, using the viral titer determined for the P1fish_1 to P1fish_3 isolates that initiated these cultures. The MOI was 0.022, 0.0094, and 0.006 for P1cell_1, P1cell_2, and P1cell_3, respectively.

2.4. Sequencing of the G gene

Viral RNA was extracted from 200 µl of virus isolates P1fish_1 to P1fish_3, P1cell_1 to P1cell_3, and a negative control (water) using the QIAamp *cador* Pathogen Mini Kit (Qiagen) according to the supplier's procedure. The extracted samples were eluted in 100 µl of elution buffer and stored at −80°C. RNA samples were converted to cDNA as described in Section 2.2.

The full open reading frame encoding region of the G gene was amplified by PCR for each cDNA using primers For3 (5′-CAC TTT TGT GCT TTT AGA CAG-3′; Thermo Fisher Scientific) and Rev1617.C (5′-TTC TGT CTG GTG GGG AGG A-3′). The PCR reaction was performed in a 0.2 ml nuclease-free PCR tube containing 1× buffer, 1.5 mM $MgCl_2$, 800 µM dNTPs (200 µM each), 400 nM both primers, 2 units of Platinum® *Taq* DNA polymerase (Invitrogen; Thermo Fisher Scientific), and 2 µl of cDNA diluted 1:2 in water, with a volume totaling 25 µl. The tubes were held for 2 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C. They were then held for 10 min at 72°C and stored at −80°C. Amplification of DNA during PCR was verified through gel electrophoresis before purification.

The PCR products were purified with a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's protocol. DNA was eluted in 25 µl of 1× low TE buffer (pH 8.0). The DNA was quantified using a Qubit® dsDNA BR Assay Kit (Invitrogen), and the purified DNA was sequenced using BigDye Terminator technology (PE Applied Biosystems). For sequencing, 25 ng of purified DNA was incubated in a 5 µl reaction volume containing 0.875 µl buffer, 0.25 µl of BigDye reagent, and 640 nM of primer at 96°C for 1 min followed by 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Six primers were used to

obtain the full G gene sequence (3 forward primers: For3, For714 [5′-CAC CTC TTT GTT GAT AAA ATC-3′], and For1142 [5′-GAG GTA TCC AAG GAC AGG-3′]; 3 reverse primers: Rev1617.C, Rev707 [5′-ATC TCT TGG CTG GAA TCA-3′], and Rev972 [5′-GAG GCC TTC ACC AGA TC-3′]). The sequencing products were precipitated with 95% ethanol and 3 M sodium acetate (pH 5.2), centrifuged at 2500 × *g* for 45 min, and resuspended in 20 µl of Hi-Di Formamide. They were then denatured for 2 min at 95°C and loaded onto the ABI 3130XL sequencer. Geneious 10.2.3 was used to analyze chromatograms, trim the sequences, and obtain the consensus sequence of the LR80 ancestor stock and the 6 virus isolates.

2.5. Virulence challenge

The virulence of the LR80 ancestor stock and the 3 virus isolates from P_1 fish that were propagated on fish cells was assessed by challenging rainbow trout through waterborne immersion exposure (Garver et al. 2006). Viral isolates directly isolated from fish were not tested because their titers were not high enough to achieve the desired challenge dose. Fish were exposed to virus isolates in triplicate tanks (n = 20 fish tank^{-1}; mean weight = 2.74 g). Fish were placed in aerated individual 6 l tanks containing 995 ml of pathogen-free 15°C water. A volume of 5 ml of the corresponding virus isolate diluted in MEM-10, to achieve a final dose of 2×10^5 PFU ml^{-1}, was then added to each tank and the water held static for 1 h. Three additional control tanks were set up the same way but had 5 ml of MEM-10 added in place of virus. After a 1 h exposure period, water flow was resumed at a rate of 100 ml min^{-1} and maintained for 35 d. Mortalities in each of the 15 tanks were recorded and removed daily for 35 d post challenge. At the end of the experiment, all surviving fish were euthanized as described in Section 2.1. For the duration of the experiment, fish were fed 1% body weight every other day (#2 Crumble; Zeigler).

2.6. Statistical analysis

The $\log_{10}$-transformed cumulative amounts of virus shed after each serial passage in Expts 3 to 6 were analyzed using multi-factor ANOVA. Cumulative virus was measured on Day 2 for P_0 and on Day 3 for all subsequent passages. The dependent variable viral load was $\log_{10}$ transformed so as to adhere to the assumptions of variance homogeneity and normality, which were validated using Levene's test and the normal probability plot of residuals. The explanatory fixed factors included experiment (Expts 3–6) and/or passage (P_0–P_3). Comparisons between passages steps were only made in the experiments where they occurred. This resulted in 3 separate analyses, P_0 vs. P_1, P_1 vs. P_2, and P_2 vs. P_3, in which data from some experiments were used more than once. To avoid an inflated Type I error, a Bonferroni correction was applied to account for multiple testing, where alpha was divided by 3, such that results were considered significant if $p < 0.017$. Tukey's post hoc comparisons were used to identify significant differences between factor levels. Virulence data were analyzed using survivorship analysis with a Cox proportional hazards regression from the coxph() function in the package survival in R (Therneau & Grambsch 2000). The response variable was day of death, with censoring of live fish on the last day of the experiment. The explanatory factor was virus isolate. The proportional hazards assumption of the model was validated using the cox.zph function, ensuring that the parameter rho was not significantly different from zero. The inclusion of tank as a random effect was also evaluated using the Cox proportional hazards mixed-effects model with the coxme() function from the coxme package (Therneau 2015). We compared the 2 model fits using a likelihood ratio test and Akaike's information criterion (AIC) value comparison, with differences in AIC value >2 considered significant. Statistical analyses were carried out using R version 3.3.3 software (R Core Team 2013).

3. RESULTS

3.1. Number of fish shedding through passages

The efficiency of fish-to-fish transmission was investigated by quantifying the number of fish shedding at each of up to 3 passages of IHNV in rainbow trout. The unit of replication in these studies was the tank, so the numbers of fish represent the numbers of pools of 1 (Expts 1 and 2) or 3 (Expts 3–6) fish contained in each tank. All the P_0 fish exposed to virus previously propagated in cell culture became infected and shed, except in Expt 1, where 90% of the P_0 fish shed (Table 1 and Fig. 2A). After the first passage, only 10% of the P_1 fish shed detectable virus in Expt 1 without cohabitation (Table 1). In Expt 2, where a single P_0 fish was placed in cohabitation with a single P_1 fish, no transmission occurred (Table 1). Because of the lack of transmission be-

Table 1. Transmission efficiency across 6 experiments. Virus quantity = cumulative amount of virus shed by P_0 fish at Day 2 in $\log_{10}$(virus RNA copies ml^{-1} H_2O + 1) (±SE). Transmission efficiency ratio was calculated 2 d after the start of exposure to virus for P_0 and 3 d after the start of cohabitation for P_1 to P_3. P: passage

Expt	No. of replicates	No. of fish replicate^{-1}	Virus quantity	Transmission efficiency (positive/total) P_0	P_1	P_2	P_3
1	10	1	4.24 ± 0.51	9/10	1/9	NA	NA
2	5	1	4.95 ± 0.23	5/5	0/5	NA	NA
3	5	3	6.32 ± 0.17	5/5	5/5	NA	NA
4	3	3	4.43 ± 0.15	3/3	3/3	NA	NA
5	10	3	5.13 ± 0.07	10/10	10/10	9/10	NA
6	10	3	4.58 ± 0.10	10/10	10/10	8/10	5/8

tween single fish, we evaluated the efficacy of transmission using groups of 3 fish at each passage step for all subsequent experiments. In Expts 3 to 6, where groups of fish were held in cohabitation with groups of 3 P_0 fish, all of the P_1 fish became infected and shed virus (Table 1), indicating 100% transmission. Further serial passages investigated in Expts 5 and 6, using groups of 3 donor and 3 recipient fish, indicated that transmission success steadily decreased with number of passages. Only 90 or 80% of fish (Expts 5 and 6, respectively; Table 1) became infected and shed virus after the second passage after the end of cohabitation and 62.5% after the third passage (Expt 6; Table 1).

3.2. Kinetics of viral shedding through passages

The kinetics of cumulative viral shedding at each of up to 3 passage steps were examined. At passage 0, the fish in Expts 1 and 2 showed a similar pattern of no detectable shedding at Days 0 and 1, followed by a 4 to 5 order of magnitude increase in cumulative shedding from Day 1 to Day 2 (Fig. 2). The cumulative amount of virus then tended to decrease after Day 2, although the change was not statistically significant, with a slightly larger decrease for Expt 1 (mean ± SE; Day 2: 4.25 ± 1.00, Day 4: 3.23 ± 1.45; Fig. 2A) compared to Expt 2 (Day 2: 4.95 ± 0.45, Day 3: 4.88 ± 0.52; Fig. 2B). Because mean shedding peaked at Day 2 (Fig. 2), the shedding of P_0 was only quantified at Day 2 in the other experiments (Expts 3–6).

In contrast, the majority of P_1 fish began shedding 48 h after the first exposure to virus, with only 4 of the 25 fish not shedding until 72 h after first exposure (Expts 3, 5, and 6; Fig. 3). Shedding from the P_1 fish continued to increase until Day 3 in all experiments where it was quantified. After Day 3, cumulative shedding began to decrease in Expt 3 and was not quantified in the other experiments (Fig. 3A).

The pattern in shedding kinetics for P_2 fish was more variable than the P_0 and P_1 fish (Fig. 4). Of the 20 fish in Expts 5 and 6 where the kinetics of cumulative shedding were quantified, 11 fish began shedding 48 h after first exposure to virus, 6 fish after 72 h, and 2 fish after 96 h; the remaining fish did not shed virus (Fig. 4). When averaging the virus shedding across all replicates, peak shedding occurred on Day 3 and then began to slightly decline in both experiments (Fig. 4). However, at the individual fish level, peak shedding was dispersed in the 2 independent experiments, with 47.4% of the fish reaching peak shedding on Day 3 (9 of 19), 26.3% on Day 2 (5 of 19), and 26.3% on Day 4 (5 of 19) (Expts 5 and 6; Fig. 4).

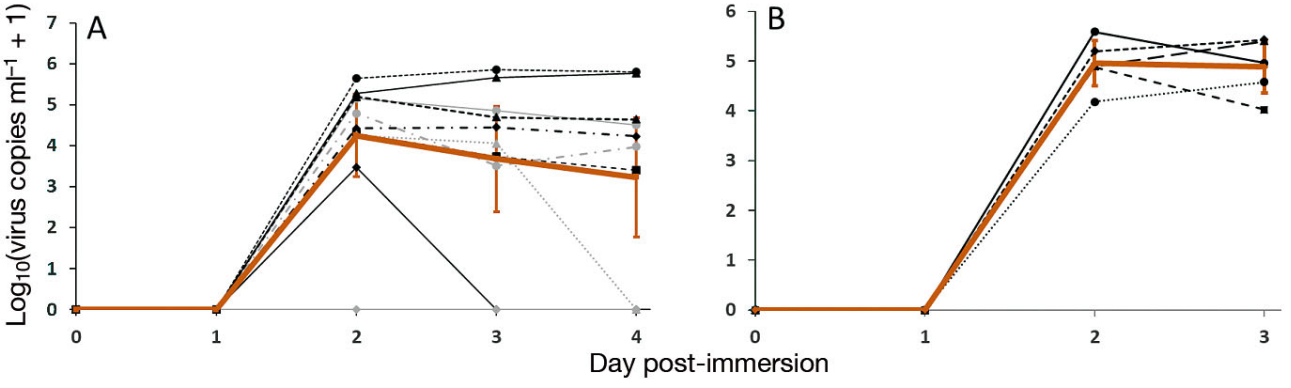

Fig. 2. Cumulative viral shedding of individual P_0 fish in static water. (A) Expt 1 (n = 10) and (B) Expt 2 (n = 5). Each line corresponds to the cumulative $\log_{10}$(virus RNA copies ml^{-1} H_2O + 1) shed through time of individual fish in a tank. The bold red line represents the mean virus shed through time ± SE (vertical bars). P: passage

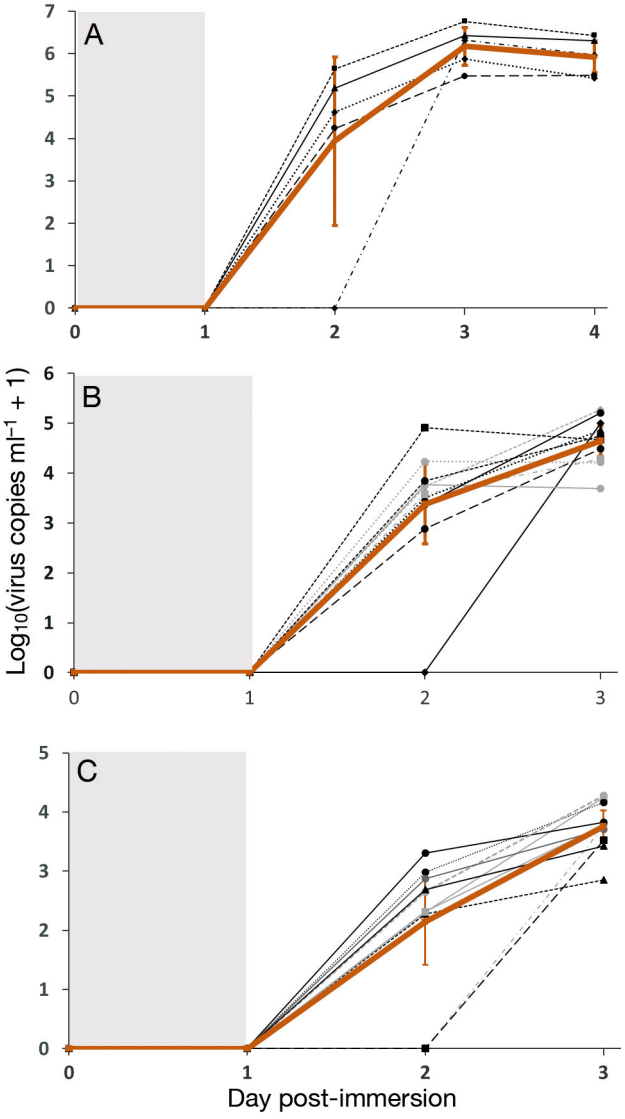

Fig. 3. Viral shedding of P_1 fish after cohabitation with P_0 fish in static water. (A) Expt 3 (n = 5), (B) Expt 5 (n = 10), and (C) Expt 6 (n = 10). Each line corresponds to the cumulative $\log_{10}$(virus RNA copies ml^{-1} H_2O + 1) shed through time of the 3 fish in an individual tank. The bold red line represents the mean virus shed through time ± SE (vertical bars). The grey box corresponds to the time windows for the P_0/P_1 fish cohabitation. P: passage

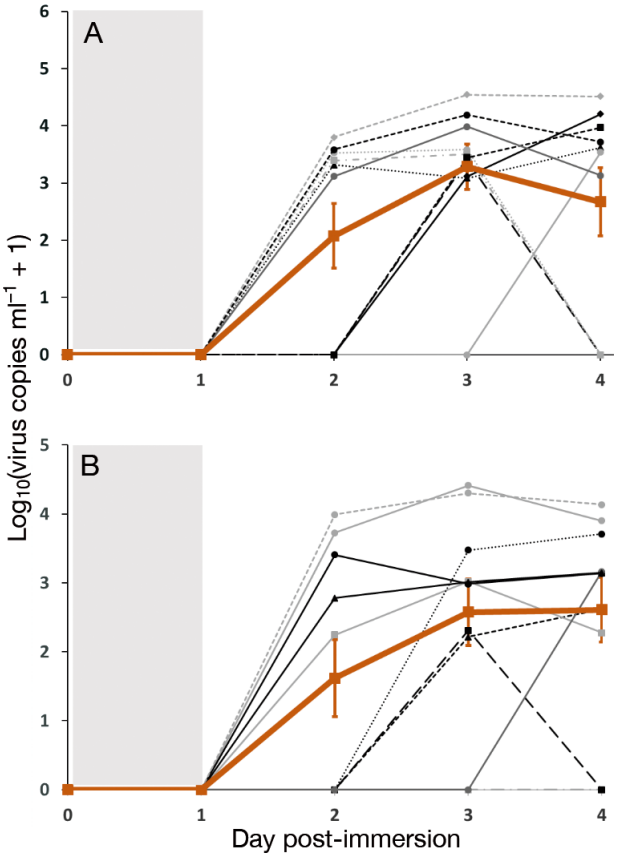

Fig. 4. Viral shedding of P_2 fish after cohabitation with P_1 in static water. (A) Expt 5 (n = 10) and (B) Expt 6 (n = 10). Each line corresponds to the cumulative $\log_{10}$(virus RNA copies ml^{-1} H_2O + 1) shed through time of the 3 fish in an individual tank. The bold red line represents the mean virus shed through time ± SE (vertical bars). The grey boxes correspond to the time windows for the P_1/P_2 fish cohabitation. P: passage

Similar to the P_2 fish, the pattern in shedding kinetics for the P_3 fish was also dispersed over time. On average, the P_3 fish shed the highest amount of virus into the water on Day 4; however, the mean shedding between Days 2, 3, and 4 was not statistically different (Expt 6; Fig. 5). At the individual level, peak shedding was also dispersed, with 50.0% of the fish reaching peak shedding on Day 4 (3 of 6), 16.7% on Day 2 (1 of 6), and 33.3% on Day 3 (2 of 6) (Expt 6; Fig. 5).

Finally, we compared the cumulative amount of virus shed at each of the passage steps. When comparing passages 0 and 1, no significant interaction between experiment and passage was found (Fig. 6; $F_{3,48} = 3.18$, $p = 0.032$, $\alpha = 0.017$; Expts 3–6). This indicated that the overall pattern of shedding between P_0 and P_1 was not significantly different for each experiment despite variable suggestive trends (Fig. 6 and Fig. A1A in the Appendix). In general, the shedding of virus in the P_1 fish was significantly lower than that in the P_0 fish ($F_{1,48} = 22.55$, $p < 0.0001$). A significant main effect of experiment was also observed ($F_{3,48} = 68.46$, $p < 0.0001$), such that more virus was shed overall in Expt 3 than in the other experiments (post hoc Tukey's HSD test, $p < 0.001$). When comparing passages 1 and 2, no significant interaction between experiment and passage was also found (Fig. 6; $F_{1,33} = 1.50$, $p = 0.22$, $\alpha = 0.025$; Expts 5 and 6). Over-

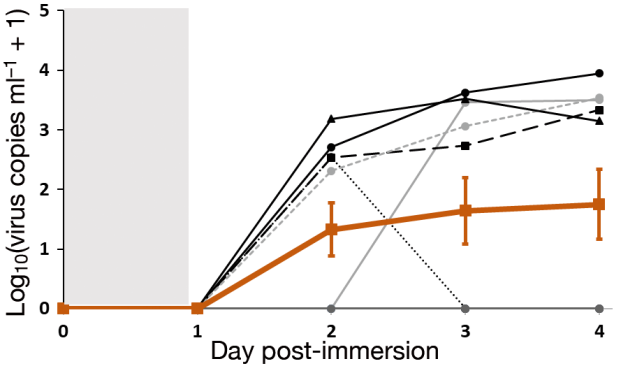

Fig. 5. Viral shedding of P_3 fish after cohabitation with P_2 fish in static water. Each line corresponds to the cumulative $\log_{10}$(virus RNA copies ml^{-1} H_2O + 1) shed through time of the 3 fish in an individual tank (Expt 6). The bold red line represents the mean virus shed through time ± SE (vertical bars). The grey boxes correspond to the time windows for the P_2/P_3 fish cohabitations. P: passage

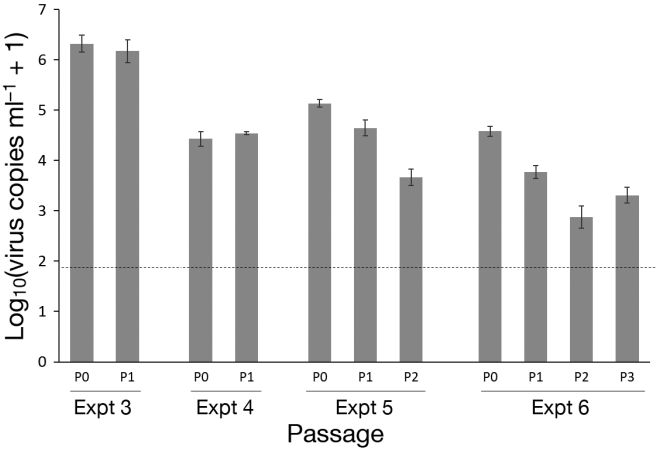

Fig. 6. Mean (±SE) cumulative virus shed after 24 h cohabitation at each passage step (P_0 to P_3). Shedding data of P_0 fish were at Day 2 post immersion and at Day 3 for P_1 to P_3. Dashed line indicates the virus detection threshold determined using the N gene plasmid standard as described in Purcell et al. (2013). P: passage

all, the shedding of virus in the P_2 fish was significantly lower than that in the P_1 fish in all experiments where examined (Fig. 6 and Fig. A1B; $F_{1,33}$ = 17.60, $p < 0.001$; Expts 5 and 6). No significant differences were observed in viral shedding between P_2 and P_3 (Fig. 6; $F_{1,11}$ = 0.03, p = 0.88; Expt 6).

3.3. Quantity of virus isolated from fish

The virus was harvested, propagated, and quantified from the pools of 3 fish from each tank of passage 1 in Expt 4. Two of the virus isolates (P1fish_1 and P1fish_2) harvested directly from fish lysed approximately 90 to 95% of the cells by 4 d post infection, whereas the third virus isolate directly from fish (P1fish_3) only lysed 80% by Day 6. The LR80 positive control isolate lysed approximately 80% of cells by 6 d post infection, and none of the cells were lysed in the negative control culture. The concentration of the 3 viral isolates harvested directly from fish was 4.36×10^6 (P1fish_1), 1.88×10^6 (P1fish_2), and 1.25×10^5 PFU ml^{-1} (P1fish_3). After 1 round of passage of the virus isolates from the fish supernatants through EPC cells, the virus concentrations were 2.28×10^8 (P1cell_1), 1.72×10^8 (P1cell_2), and 3.19×10^8 PFU ml^{-1} (P1cell_3). Their titers were similar to the LR80 positive control (3.04×10^8 PFU ml^{-1}).

3.4. Genetics of virus after transmission

The coding region of the G gene for each IHNV virus isolate directly harvested from fish and after 1 round of propagation on EPC cells was determined using Sanger sequencing. We obtained the complete G gene sequences of the 3 viral isolates extracted from fish (P1fish_1 to P1fish_3) and from propagation on cells (P1cell_1 to P1cell_3) as well as the original sequence of the virus stock. Comparative analysis between sequences revealed no genetic differences between the virus isolates and the virus stock.

3.5. Virulence of virus after transmission

The virulence of 3 IHNV isolates, which had undergone 1 round of transmission through rainbow trout (P_1) followed by culture on cells, was examined and compared to that of the original isolate of the ancestor virus. The mean cumulative mortality of rainbow trout was quantified through 35 d post challenge (Fig. 7). Rainbow trout infected with LR80 ancestor stock had a cumulative mortality of 42% (±1% SE) (Fig. 7), the majority of which occurred between 5 and 14 d post virus exposure. The 3 passaged P_1 viruses caused cumulative mortalities ranging between 42 ± 4 and 51 ± 12% (Fig. 7), with similar kinetics as the LR80 ancestor. The negative control had 2% mortality. Survival analysis revealed that the inclusion of tank as a random effect did not improve model fit (AIC_{coxph} without random effect = 1139.4 vs. AIC_{coxme} with random effect = 1137.4, ΔAIC = 1.91). Thus, tank as a random effect was dropped from the analysis. Cox proportional hazards analysis revealed that infection by all isolates significantly increased

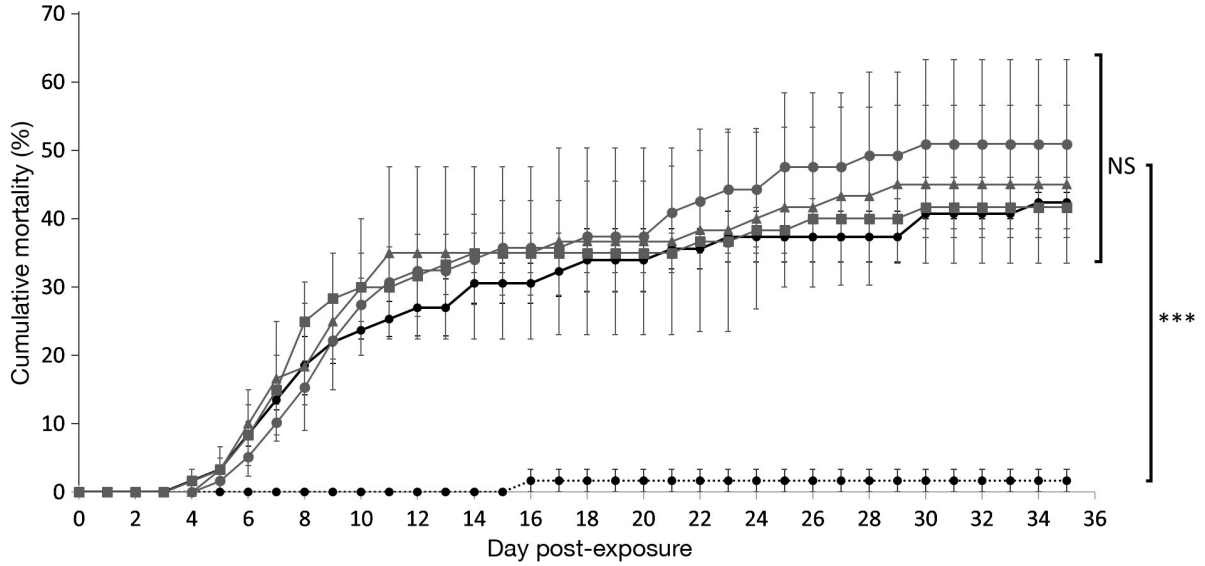

Fig. 7. Mean (±SE) cumulative mortality of rainbow trout exposed to virus isolates after 1 round of transmission through rainbow trout (P1cell_1: —●—; P1cell_2: —▲—; and P1cell_3: —■—). The LR80 virus isolate ancestor stock (—●—) and negative control (···●···) are also included. Lines correspond to mean (±SE) of triplicate tanks of 20 fish treatment^{-1}. Vertical brackets show non-significant effect (NS) and significant effect (***$p < 0.0001$). P: passage

fish mortality relative to the negative control (proportional hazards model comparing all isolates to negative control; df = 4, $\chi^2 = 52.89$, $p < 0.0001$; Fig. 7). However, mortality in all passaged virus treatments was not significantly different from the LR80 ancestor stock (proportional hazards model comparing all isolates to each other; df = 3, $\chi^2 = 0.77$, $p = 0.86$; Fig. 7).

4. DISCUSSION

We successfully developed a method to investigate fish-to-fish transmission of IHNV in rainbow trout and maintain transmission for up to 3 rounds of serial passage with an *in vivo* cohabitation design. Using our method, we were able to evaluate viral transmission rate and shedding kinetics throughout serial passage. We found that transmission success was 100% at the first passage and steadily decreased with each passage step to a minimum value of 62.5% at passage 3. The mechanism driving this decrease in transmission success between serial passages is unknown, but it is likely a compounding effect of reductions in viral dosages. Infection probability of fish by IHNV is dose dependent, meaning fewer fish become infected as dose decreases (Ogut & Reno 2004a, Wargo & Kurath 2012, McKenney et al. 2016, Langwig et al. 2017). We intentionally chose the window of peak shedding to maximize probability of transmission in this study. However, it is also possible that shedding quantity is dose dependent, such that fish exposed to less virus shed a lower quantity of virus (Urquhart et al. 2008, Hershberger et al. 2010, 2011). In general, we observed that peak shedding was reduced at each serial passage step, likely resulting in fewer fish becoming infected and fish shedding a lower amount of virus at the subsequent passage steps, in a compounding fashion. Whether transmission continues to decline or plateau with additional passages warrants further exploration.

We also observed that infections became more asynchronous with increasing serial passage, i.e. the day and amount of peak shedding was more dispersed in P_2 and P_3 fish than in P_0 fish. This may have also resulted in the window of peak transmission becoming more dispersed. To address this, we used pools of 3 fish at each passage rather than a single fish to provide a more complete representation of the between-fish variation in amount and timing of peak viral shedding. The mechanisms driving the observed shedding asynchrony are again unknown but likely to be a result of decreased and more variable viral exposure dosage with increasing passages, as follows. The P_0 fish were exposed to a very high and uniform dosage of virus (1×10^6 PFU ml^{-1}) because they were infected directly from a virus stock. In contrast, in subsequent passages, fish were exposed to virus shed by fish from the previous passage step. Because there are high levels of individual host variation in the duration and quantity of shedding (Wargo et al. 2017), the P_1–P_3 fish were likely exposed to more variable and generally lower doses (10^4–10^6 RNA copies ml^{-1}, roughly equal to 10^2–10^3 PFU ml^{-1}; Purcell et al. 2006)

compared to the P_0 fish. Empirical and theoretical studies in other systems have suggested that viral infection kinetics and synchrony can be dose dependent (Cummings et al. 2012, Littwitz-Salomon et al. 2017). Viruses may take longer to initiate infection and reach peak values at lower dosages compared to higher dosages (Chu & Volety 1997, Cummings et al. 2012, Abdoli et al. 2013, Jarungsriapisit et al. 2016). This is further supported by our finding that peak shedding shifted from 48 h post immersion in fish challenged with virus grown in cell culture (P_0) to 72 h in fish exposed to virus shed from other fish (P_1–P_3). In addition, host clearance may be faster at lower virus exposure doses (Jarungsriapisit et al. 2016).

Our results indicated that transmission success was notably lower when fish were placed alone in cohabitation with a single fish than when 3 donor fish were in cohabitation with 3 recipient fish. Although viral concentration in the water was sometimes greater in tanks with 3 fish (Expts 3 and 4) than tanks with 1 donor fish (Expts 1 and 2), there were just as many cases where viral concentration was equally as high when only 1 fish was present (Expts 5 and 6). However, higher densities always resulted in greater transmission, suggesting transmission success is driven by density effects in addition to viral exposure dose. Density effects on IHNV transmission have been observed by others (Ogut & Reno 2004b). A variety of mechanisms could drive these density effects. Fish density produces a wide range of stressors in various fish species such as deterioration in water quality, overcrowding, or adverse social interactions (Pickering & Pottinger 1987, Ellis et al. 2002). Some studies reported that a high fish density changes the susceptibility to diseases by down-regulating the immune system in rainbow trout (Cnaani 2006, Yarahmadi et al. 2016). Thus, physiological stress from high densities might be an important contributing factor in fish disease and mortality (Davis et al. 2002, Mateus et al. 2017). Such density effects could result in greater transmission rates in high-density host settings such as aquaculture (Olson & Thomas 1994, Noble & Summerfelt 1996, Lafferty et al. 2015) and have important evolutionary consequences (Kennedy et al. 2016). From an epidemiological perspective, models used to infer the dynamics of directly transmitted waterborne pathogens, such as IHNV, typically assume an absence of density effects (Anderson & May 1992). Given our results, and those of others using this system, this assumption needs further evaluation to produce more accurate epidemiological inference (Dwyer et al. 1997, Fenton et al. 2002).

Another potential factor that could influence transmission probability is the duration of fish cohabitation. In this study, we chose the longest cohabitation duration possible to allow transmission but prevent recipient fish from reinfecting donor fish. This and previous studies indicated that IHNV shedding begins between 25 and 48 h after the initiation of fish exposure to virus (Ogut & Reno 2004a, Wargo et al. 2017). Therefore, by cohabitating fish for only 24 h, recipient fish were not provided with enough time to begin shedding virus to reinfect recipient fish. This allows for powerful evolutionary studies because virus isolates can be phenotyped and genotyped at each passage step.

There was no change in genetics or virulence among the ancestral viral stock and the passaged isolates as measured by mortality or G gene sequence. Our goal was not to determine if serial passage results in small evolutionary changes in IHNV. Rather, our method was intended to quantify if initial movement of virus from cell culture to fish results in rapid changes in virulence. In SPEs under controlled laboratory conditions, the number of passages required to drive evolution of pathogens is highly variable (e.g. Brugh & Perdue 1991, Ebert 1998, Mackinnon & Read 2004, Chapuis et al. 2011, Barclay et al. 2012, Guidot et al. 2014, Valero-Jiménez et al. 2017). It is therefore possible that more than the 1 passage would be required to drive IHNV evolution. Likewise, although the cumulative mortality observed was similar to that of previous studies with this ancestral viral stock (Breyta et al. 2016), it is one of many phenotypic traits of the virus that could undergo evolution (Wargo & Kurath 2012), and we cannot rule out the possibility of evolution in other traits. It should also be noted that the Sanger sequencing of the G gene used can only characterize the consensus sequence of the virus and will not reveal minority sequence variants (e.g. Wright et al. 2011, Iyer et al. 2015). The absence of changes in the G gene also does not eliminate the possibility of changes in other parts of the viral genome. Our results are consistent with previous studies that passage of the virus through a few generations of cell culture does not typically result in virus evolution (Gonzalez et al. 1991, Chen et al. 2003, Bellec et al. 2014). Additional studies examining whether or not multiple rounds of serial passage through fish results in evolution may be enlightening.

In conclusion, we developed a powerful tool to evaluate transmission efficiency using a cohabitation method of exposure that mimics the natural mode of IHNV transmission in rainbow trout. Although previous groups have investigated IHNV transmission during cohabitation, our method differed in several important ways. Perhaps the most notable difference is that previous studies have used a design in which

large groups of fish are cohabitated for long periods of time (Amend 1975, Mulcahy et al. 1983, Traxler et al. 1993, Ogut & Reno 2004b, Balmer et al. 2017). Given the acute nature of IHNV transmission, numerous rounds of transmission back and forth between donor and recipient fish likely occurred in these previous studies, making it impossible to determine the original source of transmission. In our study, small groups of fish were housed together for a short period of time, such that only 1 round of transmission was possible from donor to recipient fish. Furthermore, mortality has typically been used as the metric to verify transmission success, whereas we quantified the load of shed virus. Previous studies have indicated that many trout which become infected and shed virus do not die; thus, mortality likely underestimates transmission (Wargo et al. 2017). Our study design thus allows for detailed investigations of IHNV evolution, epidemiology, and disease dynamics under various environmental conditions, such as temperature, which may influence these dynamics. For example, this method could be used for calculation of important epidemiological parameters such as transmission rate (β) and the basic reproduction number (R_0) (van den Driessche & Watmough 2002). To date, efforts to model IHNV dynamics have largely been reliant on viral shedding, field, and disease prevalence data to quantify transmission rates because of a lack of experimental data on fish-to-fish transmission (Foreman et al. 2015, Ferguson et al. 2018). A better understanding of transmission will likely allow disease managers, particularly of IHNV in rainbow trout, to better predict the risk, timing, duration, and magnitude of epidemics.

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Appendix

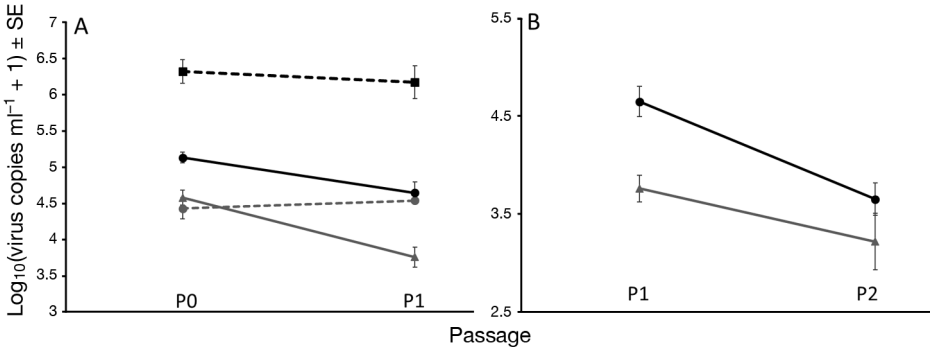

Fig. A1. Mean (±SE) cumulative virus shed after 24 h cohabitation between passages (P) at each experiment (Expt 3: —■—; Expt 4: --●--; Expt 5: —●—; and Expt 6: —▲—). Comparison in viral shedding (A) between P_0 and P_1 and (B) between P_1 and P_2. Shedding data of P_0 fish were at Day 2 post immersion and at Day 3 for P_1 to P_3

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