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FEATURED PAPER

Impact of Vaccination and Pathogen Exposure Dosage on Shedding Kinetics of Infectious Hematopoietic Necrosis Virus (IHNV) in Rainbow Trout

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Abstract

Vaccine efficacy in preventing clinical disease has been well characterized. However, vaccine impacts on transmission under diverse field conditions, such as variable pathogen exposure dosages, are not fully understood. We evaluated the impacts of vaccination on disease-induced host mortality and shedding of infectious hematopoietic necrosis virus (IHNV) in Rainbow Trout *Oncorhynchus mykiss*. Fish, in up to three different genetic lines, were exposed to different dosages of IHNV to simulate field variability. Mortality and viral shedding of each individual fish were quantified over the course of infection. As the exposure dosage increased, mortality, number of fish shedding virus, daily virus quantity shed, and total amount of virus shed also increased. Vaccination significantly reduced mortality but had a much smaller impact on shedding, such that vaccinated fish still shed significant amounts of virus, particularly at higher viral exposure dosages. These studies demonstrate that the consideration of pathogen exposure dosage and transmission are critical for robust inference of vaccine efficacy.

Rainbow Trout *Oncorhynchus mykiss* is an important aquaculture species worldwide (Cowx 2005). The USA alone produced almost 19,900 metric tons of food-grade trout in 2019 (USDA NASS 2020). Infectious disease has been and continues to be a major problem in Rainbow Trout aquaculture (Roberts and Shepherd 1974; Meyer 1991; LaPatra et al. 1994; Lafferty et al. 2015). Vaccinations are an effective tool for managing a variety of diseases in trout aquaculture (Leong and Fryer 1993; Sommerset et al. 2005; Kurath 2008; Subasinghe 2009); however, there are many trout pathogens for which vaccines have proven ineffective or are not available. Furthermore, although many vaccines have been investigated for how well they protect against clinical disease (LaFrentz and LaPatra 2003; Adelman et al. 2008; Kurath 2008; de las Heras et al. 2010; Cain and Zinn 2011), very little is known about how they affect pathogen transmission.

Evidence from a variety of systems indicates that many vaccines provide only limited protection against transmission. For example, transmission from vaccinated hosts to unvaccinated hosts was documented in vaccinated baboons exposed to *Pertussis* (Warfel et al. 2014), in Atlantic Salmon *Salmo salar* exposed to infectious hematopoietic necrosis virus (IHNV; Long et al. 2017), and in case-control studies using live, attenuated oral polio vaccines (Sutter et al. 1991). Additionally, protection under diverse conditions commonly observed in the field, such as variable pathogen exposure dosages, is not fully understood (Langwig et al. 2019). Recent studies have indicated that vaccine efficacy is pathogen dose dependent, particularly in the context of preventing transmission (i.e., transmission blocking; Gomes et al. 2014; Langwig et al. 2017). Therefore, typical single pathogen dose vaccine trials may not accurately predict vaccine efficacy in the field. In particular, long-term or repeated pathogen exposures in the field may overwhelm

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vaccine protection against disease and transmission. If vaccinated individuals can transmit, it could result in pathogen persistence; in turn, this could allow disease to persist particularly in areas where vaccine coverage is poor. This may be a challenge to pathogen eradication (Gandon et al. 2003; Meyns et al. 2006; Van der Goot et al. 2007) and influence the potential evolutionary responses of pathogens to vaccination, such as the evolution of increased virulence (Lipsitch and Moxon 1997; Gandon et al. 2001). Ultimately, the poor understanding of vaccine impacts on transmission has greatly hindered the ability to make reliable predictions of vaccine efficacy and likely plays a role in vaccine failures observed in the field (Lavine et al. 2010).

In Rainbow Trout aquaculture, one of the leading pathogens is IHNV. This is a single-stranded, negative-sense RNA virus found in the family *Rhabdoviridae* (Bootland and Leong 1999). This pathogen is endemic to salmonid species along the Pacific coast of North America, but can now be found worldwide where trout aquaculture occurs (Sano et al. 1977; Hill 1992; OIE 2015; Dixon et al. 2016). It causes an acute disease resulting in epidemics in trout farms and hatcheries (Troyer et al. 2000) with up to 100% mortality (Bootland and Leong 1999). The virus is transmitted horizontally through the water via bodily fluids or carcass degradation, or by cohabitation with infected fish (Amend 1975; Traxler et al. 1993). High mortality due to IHNV in aquaculture settings causes extreme economic loss to the industry. As a result, vaccines against IHNV have been created to manage the spread of the pathogen. One of the most effective is a DNA vaccine developed by Corbeil et al. (1999), which has been shown to reduce salmonid mortality by up to 100% (Corbeil et al. 2000a, 2000b; Garver et al. 2005). Given the promising results of this vaccine, a similar IHNV DNA vaccine was developed and commercialized by Novartis (Basel, Switzerland), which received licensure in 2005 by the Canadian Food Inspection Agency for use in farmed Atlantic Salmon in Canada and in 2013 by the U.S. Department of Agriculture (USDA) for use in U.S. aquaculture (Salonius et al. 2007; USDA APHIS 2013; Garver and Wade 2017). Despite this vaccine's success in controlling disease in a laboratory setting, little is known regarding its efficacy in the field, particularly with regards to viral transmission. A study published by Long et al. (2017) showed that shedding and transmission of IHNV in vaccinated individuals was possible, but was reduced in Atlantic Salmon. However, Rainbow Trout have a different susceptibility to and evolutionary history with IHNV than Atlantic Salmon (LaPatra 1998). Furthermore, the viral shedding kinetics of the two fish species in the absence of vaccination are different (Garver et al. 2013; Wargo et al. 2017) and therefore likely to be differentially impacted by vaccination. Another study of a closely related pathogen, viral hemorrhagic septicemia virus, demonstrated that viral shedding from Muskellunge *Esox masquinongy* after DNA

vaccination was still possible, but only at very low levels as determined by plaque assays (Standish et al. 2016). Whether DNA vaccination will reduce viral shedding and transmission of IHNV in Rainbow Trout is unknown.

In this study, we investigated the impact of vaccination on shedding kinetics of IHNV in Rainbow Trout across a range of viral exposure dosages to simulate field variability. Host-to-host transmission through the entire infectious period is difficult to measure for IHNV and many other pathogens. However, previous studies have shown that IHNV transmission is correlated with the amount of virus shed from the host into the water (Doumayrou et al. 2019). Here, we used viral shedding that was quantified at numerous time points throughout the infection as an estimate of transmission. Although shedding kinetics have previously been characterized in this system (Wargo et al. 2017), the effects of viral exposure dosage and vaccination on these kinetics has yet to be shown. To increase the field relevance and generalizability of the study, we also investigated vaccine impacts on viral shedding kinetics in three genetically distinct trout lines used in aquaculture. Multiple fish lines are used in aquaculture, and studies have indicated that fish populations may differ immunogenetically and respond differently to vaccination (Overturf et al. 2003). This study is directed toward increasing the ability to infer IHNV vaccine transmission prevention efficacy in the field.

METHODS

Fish lines.—Three Rainbow Trout fish lines were used in the experiments, designated IHNV.R, ARS-Fp-R, and ARS-Fp-S, to reflect genetic diversity that would be found in aquaculture. Line IHNV.R was obtained from Clear Springs Foods (Buhl, Idaho) and has been selected for a variety of aquaculture production-enhancing traits such as growth and resistance to IHNV and other diseases (LaPatra and Towner 2006). Line ARS-Fp-R was bred exclusively for five generations to be genetically resistant to *Flavobacterium psychrophilum*, the causative agent of bacterial coldwater disease (BCWD), by the National Center for Cool and Cold Water Aquaculture (NCCCWA; within USDA's Agricultural Research Service) in West Virginia. Line ARS-Fp-S originated from the same ancestor as line ARS-Fp-R, but was bred for one generation to be susceptible to BCWD, and then randomly outbred to maintain susceptibility (Wiens et al. 2013). Fish lines IHNV.R, ARS-Fp-R, and ARS-Fp-S were used for experiment 1, while only IHNV.R and ARS-Fp-R were used for experiment 2.

Virus.—The pathogen used for the experiments was IHNV (*Salmonid novirhabdovirus*) isolate C (genotype mG119M; GenBank accession number AF237984). The genotype of this isolate is within the M genogroup and it was first isolated in the Idaho trout aquaculture region

(Kurath et al. 2003). For these studies, a new virus stock was propagated by passing a previous virus stock on epithelioma papulosum cyprini cells at a multiplicity of infection of 0.005 (Fijan et al. 1983). The culture was then stored at -80°C in Eagle's minimum essential media (MEM; Gibco) supplemented with 10% fetal bovine serum, as previously described (Peñaranda et al. 2011). This virus stock was enumerated by triplicate plaque assays (Batts and Winton 1989) and found to have a titer of 7.56×10^8 plaque forming units (PFU)/mL. We used a viral stock with as few passes in cell culture since its original isolation as possible (i.e., <10).

Vaccine.—The vaccine used in this study was the DNA vaccine pWg containing the glycoprotein (G) gene of IHN isolate WRAC (genotype mG010M). It was propagated using plasmid Gigaprep kits (Qiagen) from the original transfected bacteria (Corbeil et al. 1999, 2000a). This vaccine is heterologous to the virus exposure strain (IHN isolate C) used in this study. The vaccine provides highly efficient disease protection as previously characterized (Corbeil et al. 2000a; LaPatra et al. 2001; Garver et al. 2005). A total of 120 (experiment 1) or 200 (experiment 2) fish from each line were vaccinated with an intramuscular injection of 0.05 μg of the vaccine in 25 μL of phosphate buffered saline (PBS) or sham-vaccinated with 25 μL of PBS. Fish were then placed in 50-gal tanks and held in freshwater at 15°C for 30 d (450 degree days) to allow acquired immunity to develop (Corbeil et al. 2000a).

Virus exposure and shedding sampling.—At 30 d post-vaccination, fish were exposed to one of six (experiment 1) or five (experiment 2) dosages of IHN in batches grouped by treatment (10 fish per treatment in experiment 1; 20 fish per treatment in experiment 2) by placing them into 1 L of static water containing virus for 1 h. Supplemental aeration was provided during the virus challenge. Individual fish weights (mean \pm SD) were comparable between the fish lines, and averaged 2.55 ± 0.29 g and 2.46 ± 0.58 g for experiments 1 and 2, respectively. Virus exposure levels were 1×10^1 , 1×10^2 , 1×10^3 , 1×10^4 , and 1×10^5 PFU/mL water in experiment 1, and 1×10^3 , 1×10^4 , to 1×10^5 , and 1×10^6 PFU/mL water in experiment 2. These exposure levels were highly controlled and precisely quantified, and therefore we refer to them as dosages here. The method of virus exposure was exactly the same for vaccinated and unvaccinated fish. Higher dosages were used in experiment 2 because few fish were infected at the lower dosages in experiment 1, which hindered elucidation of the shedding kinetics and vaccine effects. Both experiments also contained a negative control group exposed to the cell culture medium (MEM with 10% fetal bovine serum). After exposure, fish were transferred to 6-L tanks and washed at a flow rate of 1,500–1,600 mL/min for 1 h to remove exposure virus. The fish were then individually separated into randomly assigned 0.8-L tanks in a tower rack system (Aqua-neering), with water flow set to 150 mL/min. After all fish

were distributed (approximately 2 h), water flow was turned off and a 1-mL water sample was taken from each tank (day 0) and stored in a labeled 1.5-mL polypropylene microcentrifuge tube. This day-0 sample was used to ensure that all exposure virus had been removed after the wash step. The tanks were then held static for 22 h, after which 1 mL of water was again sampled from each tank (day 1). Holding the water static allowed for an estimation of the accumulation of the total amount of virus shed over the previous 22-h period. Water flow was turned back on to 150 mL/min for a 2-h wash, which permitted 22.5 volumes of tank exchange. This flow rate and wash period ensured that all virus was removed from the tank, and was determined by a pilot study measuring virus quantity over time (Supplemental Figure 1 available in the online version of this article). This sampling and wash cycle was repeated daily until 7 d postexposure. After sampling on day 7 (which previous studies have indicated is when viral shedding declines: Wargo et al. 2017), tanks were flushed for 2 h and then flow was set to 80 mL/min. Water was then turned off to all tanks 22 h before sampling on day 10. Samples were taken until day 10 in experiment 1 and day 7 in experiment 2. Mortality was also tracked for 40 d in experiment 1 and for 30 d in experiment 2. Water samples were stored at -80°C for further processing. The sampling design allowed us to quantify the total amount of virus that each individual shed per day. Water temperature (mean \pm SD) was maintained at $15 \pm 1^{\circ}\text{C}$ and fish were provided with oxygen via air stones throughout the experiment. Fish were not fed the day before the experiment or on day 0. Fish were then fed on Mondays, Wednesdays, and Fridays during the experiment. Fish were maintained on a 12-h photoperiod. Over the experimental period, 42 fish (11.67%) died in experiment 1 and 56 fish (14%) died in experiment 2; however, dead fish were left in their tanks until completion of the study and their water was sampled as described above. At the end of the experiment, all of the remaining fish were euthanized with an overdose of 0.27 mg/mL tricaine methanesulfonate buffered with 0.09 mg/mL sodium bicarbonate. All fish experiments were approved by the William and Mary Institutional Animal Care and Use Committee under protocol number IACUC-2014-06-17-9666-arwargo.

Sample processing.—The RNA was extracted from 210 μL of the water samples using the *cador* Pathogen 96 QIAcube HT Kit (Qiagen) with a Tecan Freedom EVO 100 liquid handling robot, as per manufacturer guidelines with the following modifications. Each 210- μL water sample was transferred from the 1.5-mL microcentrifuge tube to a 96-well, 2-mL-deep lysis plate. A mixture of 84 μL VXL, 1 μL carrier RNA, and 20 μL Proteinase K was added to each sample, after which the samples were vortexed for 30 s at 6–12 \times g and allowed to incubate at room temperature for 5 min. The samples were vortexed again for 30 s at 6–12 \times g before 310 μL ACB buffer was

added. The samples were then vortexed for another 30 s at 6–12 × *g* and transferred to a nucleic acid-binding filter plate. They were pulled into the filter membrane using a vacuum manifold at 250 mbar for 180 s. Each sample was washed with 600 µL of Buffer AW1, eluted at 350 mbar for 180 s. The samples were then washed with 600 µL of Buffer AW2, eluted at 350 mbar for 60 s. They were washed again with 700 µL of 100% ethanol, eluted at 350 mbar for 30 s. Finally, the samples were dried two times (700 mbar for 30 s followed by 350 mbar for 120 s) under vacuum. A 100-µL volume of AVE buffer was then added to the filter plate and allowed to sit for 120 s. The RNA was eluted into an elution plate using the vacuum manifold at 700 mbar for 360 s. All extracted RNA was stored at –80°C until further processing.

RNA samples were transcribed to cDNA using oligo (dT) random primers and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega), with 11 µL of sample in a 20-µL reaction volume, as previously described (Wargo et al. 2010). For quantification of viral RNA, cDNA was diluted 1:2, then used in a qPCR reaction with forward and reverse primers IHNV N 796F, IHNV N 875R (Life Technologies), TaqMan probe IHNV N 818MGB (Life Technologies), and TaqMan Universal PCR Master Mix No AmpErase UNG (Life Technologies) on a QuantStudio6 qPCR machine as described elsewhere (Purcell et al. 2013). Each qPCR plate also included triplicate wells of an 8-step, 10-fold dilution series of artificial positive control (APC) DNA plasmid standard to allow for absolute quantification of viral RNA (Purcell et al. 2013). All samples fell within the linear range of detection. Our qPCR assay allowed us to distinguish between the vaccine and virus, because the qPCR assay specifically targets the IHNV N gene and the vaccine only contains the viral G gene. Therefore, only the amount of virus shed, not the vaccine, was quantified. The qPCR assay provides the number of viral RNA copies in water samples presented as virus copies per mL of water.

Statistics.—Statistical analyses were carried out in R (version 3.2.2; R Core Team, Vienna) and RStudio (version 1.1.423; RStudio Team, Boston). R code and appropriate data files are available upon request. For all analyses, assumptions of homogeneity of variance and normality were tested with residual plots, Levene's tests, and Shapiro–Wilk tests, where appropriate. Minimal models were chosen by comparing model fits using AIC values, with differences <2 considered significant. Under the assumption of parsimony, the simplest model that converged, with equal or lower AIC, was chosen. For clarity, only the statistical findings are presented in the main body of text. Details on minimal models selected can be found in the Supplemental Materials (available in the online version of this article). Of the 3,240 samples that were processed in experiment 1, only four samples tested positive out of the 900 negative controls

(fish not exposed to virus), indicating a false positive rate of 0.44%. Of the 3,200 samples that were processed in experiment 2, only eleven samples tested positive out of 1,040 negative controls, indicating a false positive rate of 1.06%. Retesting of these samples indicated that they likely became contaminated during the qPCR step. We are confident that this was not due to infection of negative control fish, because no single negative control fish was positive for two or more subsequent time steps, and the false positives were sporadically dispersed throughout the experiment. In order to correct for the false positive rate, we took an average of the negative control values and subtracted it from all of the other sample values. This was a conservative approach and likely overestimated the level of false positives. Ultimately, this correction did not change any of the models or statistical results, so the uncorrected results were presented. Unless noted otherwise, dead fish were included in the analyses of shedding data. Removal of dead fish did not significantly affect the results, likely because very few vaccinated fish died during the experiment.

Survival.—Survival was visualized using Kaplan–Meier analyses with the “survfit” function in the “survival” package in R. Escapees (12 in experiment 1 and 4 in experiment 2) were censored from the day of escape forward. Analyses for all fish in experiment 1 and unvaccinated fish in experiment 2 were done using Cox proportional hazard models in R with the “coxph” function in the “survival” package (Therneau 2015). Explanatory fixed factors in the models were vaccine, fish line, and log₁₀ (dosage), which was a continuous variable. Their interactions were also included. The baseline was set to the ARS-Fp-R unvaccinated fish treatment. Due to lack of mortality for any of the vaccinated fish in experiment 2, the coxph models were not able to converge. Therefore, a log-rank test with the “survdiff” function in R was used to compare the survival of vaccinated versus unvaccinated fish in experiment 2, pooled across all dosages and fish lines. Relative percent survival (RPS) was calculated for both experiments using the following formula: $RPS = [1 - (\text{the percent of mortality of vaccinated individuals} / \text{the percent mortality of controls})] \times 100$.

Number of fish shedding per day.—The daily number of fish that shed virus was analyzed with a logistic regression. The response variable in the model was the proportion of fish shedding per day as indicated by a positive water sample. Day 0 and the negative control dosage were not included in the model because our goal was to determine if shedding differed between treatments from the point that shedding began. The explanatory fixed factors in each of the models were vaccine, fish line, log₁₀ (dosage), and day, with interactions included. A unique identifier for each fish (fish ID) nested in day was included in the model as a random factor, because the same fish were sampled at multiple time points (repeated measures). Data were

analyzed using the “glmer” function in the “lme4” package in R (Bates et al. 2015), with a binomial data structure. The baseline model was represented by the unvaccinated, ARS-Fp-R fish treatment.

Quantity of virus shed per day.—The daily quantity of virus shed was analyzed with mixed effects models in R with the “lme” function in the “nlme” package (Pinheiro et al. 2015). The response variable in the model was $\log_{10} + 1$ transformed RNA copies of IHN per mL. Explanatory fixed factors were vaccine, fish line, \log_{10} (dosage), and day. To avoid over-parameterizing the model, only investigations of main effects and two-way interactions were possible given the number of positive replicate samples per treatment. Fish ID nested in day was included in the model as a random factor, because each fish was sampled at multiple time points (repeated measures). A maximum likelihood estimation of parameters was used due to its ability to allow for model selection and to handle sample sizes of <30 fish per treatment. Water samples with no detectable virus were dropped from the analysis because our goal was to determine if the amount of shedding differed between treatments for fish that were actively shedding virus. To ensure adequate statistical replication, this analysis was conducted for days 1–7 and dosages 10^3 , 10^4 , and 10^5 PFU/mL for experiment 1, with the addition of dosage 10^6 PFU/mL for experiment 2. Other dosages and time points were omitted because of insufficient replicate positive samples. Thus, this analysis compares the rate of shedding between fish during the peak shedding period. The baseline model was represented by the unvaccinated, ARS-Fp-R fish treatment.

Total virus shed.—Total virus shed was analyzed using a negative binomial regression with the “glm.nb” function in the “MASS” package in R (Venables and Ripley 2002). The response variable was the amount of virus shed by each individual fish per day totaled over all days of the experiment and then log transformed. Explanatory fixed factors were vaccine, fish line, and \log_{10} (dosage). Their interactions were also included. The negative control treatment was dropped from the analysis, because we were interested in comparing vaccine and dosage impacts on shedding between fish exposed to virus. However, fish exposed to virus that did not shed were included. The baseline model was represented by the unvaccinated, ARS-Fp-R fish treatment.

RESULTS

Survival

Fish mortality largely occurred between day 7 and day 25, although the kinetics differed between treatments and experiments (Figure 1). For experiment 2, vaccination reduced mortality to zero across all virus dosages and fish lines. This

reduction in mortality was significant when compared with the levels observed in unvaccinated fish ($n = 200$), after pooling across exposure dosage and fish line (log-rank test: $\chi^2 = 65$, $df = 1$, $P < 0.0001$). In both experiments, as virus exposure dosage increased, the hazard of death also significantly increased in unvaccinated fish (experiment 1: $\chi^2 = 1.63$, $df = 1$, $P < 0.05$; experiment 2: $\chi^2 = 39.29$, $df = 1$, $P < 0.001$; Supplemental Tables 1 and 2 available in the online version of this article). In vaccinated fish, this rate of increase was reduced by 34% in experiment 1 and did not occur in experiment 2 because mortality remained at zero even at high viral exposure dosages where mortality reached up to 60% in unvaccinated fish. Thus, vaccination reduced the risk of mortality in both experiments, and the effect was more pronounced at the higher virus exposure dosages compared with the lower dosages. This was largely because fish mortality was minimal at the lowest dosages even in unvaccinated fish, diminishing the possible effect of vaccination. The RPS (mean \pm SD) was $50 \pm 50\%$ at the lowest dosage in both experiment 1 and 2. The RPS at the highest dosage was $78 \pm 25\%$ in experiment 1 and $100 \pm 0\%$ in experiment 2. A fish line effect was also observed, such that the IHN.V.R fish line had a 63% and 52% lower hazard of dying in experiments 1 and 2, respectively, compared with the ARS-Fp-R line (experiment 1: $\chi^2 = 11.3$, $df = 2$, $P < 0.05$; experiment 2: $\chi^2 = 6.77$, $df = 1$, $P < 0.01$; Supplemental Tables 1 and 2). This occurred in all treatments in experiment 1 and only with unvaccinated fish in experiment 2, because mortality was zero for the vaccinated fish in both fish lines in experiment 2. None of the unexposed control fish died in experiment 2 and low-level mortality of control fish was observed in experiment 1, which did not affect the results.

Number of Fish Shedding per Day

In general, the number of fish shedding peaked on day 2 and then rapidly declined (Figure 2). The timing of shedding was highly consistent across treatments, such that more than 90% of the fish started shedding by day 2 and more than 80% stopped shedding by day 7 in both experiments. The analysis revealed that as exposure dosage increased, the probability of shedding also increased (experiment 1: $Z = 14.1$; $df = 1$, 2,231; $P < 0.001$; experiment 2: $Z = 12.6$; $df = 1$, 2,232; $P < 0.001$; Supplemental Tables 3 and 4), such that very few fish shed at the lowest dosages and all individuals shed at the highest dosages in both experiments (Figure 2). For experiment 2, this relationship differed between fish lines; the rate of increase was lower for the IHN.V.R fish line ($Z = -2.1$; $df = 1$, 2,232; $P = 0.037$; Supplemental Table 4). For experiment 1, the relationship between the probability of shedding and dosage depended on day ($Z = -7.8$; $df = 1$, 2,231; $P < 0.0001$; Supplemental Table 3). At lower dosages, the probability of shedding remained at zero through time, whereas as at the highest dosage it

decreased from nearly one to zero through time (Supplemental Figure 2a). For experiment 2, the probability of shedding the virus initially increased and then began to decrease over time ($Z = -13.0$; $df = 1, 2,232$; $P < 0.0001$; Supplemental Table 4). However, the shape of this relationship was consistent across dosages (Supplemental Figure 2b). In experiment 1, there was a general reduction in the probability of shedding across all fish lines due to vaccination, but this difference was only significant for the ARS-Fp-S fish line ($Z = -3.6$; $df = 2, 2,231$; $P < 0.001$; Supplemental Table 3; Supplemental Figure 3). There was also a greater reduction in the probability of shedding due to vaccination at early time points compared with later time points ($Z = 1.7$; $df = 1, 2,231$; $P = 0.09$; Supplemental Table 3; Supplemental Figure 4a). This is because at later time points, the probability of shedding

was zero in experiment 1 regardless of vaccine treatment, so a vaccine effect was not possible. In contrast, the probability of shedding decreased more rapidly through time in vaccinated fish compared with unvaccinated fish in experiment 2 (Supplemental Figure 4b). This resulted in no difference in the probability of shedding between vaccinated and unvaccinated fish at early time points, but a significantly lower probability in vaccinated fish at later time points ($Z = -8.1$; $df = 1, 2,232$; $P < 0.001$; Supplemental Table 4).

Quantity of Virus Shed per Day

Generally, the amount of virus shed was variable through time and depended on viral exposure dosage and vaccination (Supplemental Figure 5). In an effort to compare viral shedding rates, the daily amount of virus shed

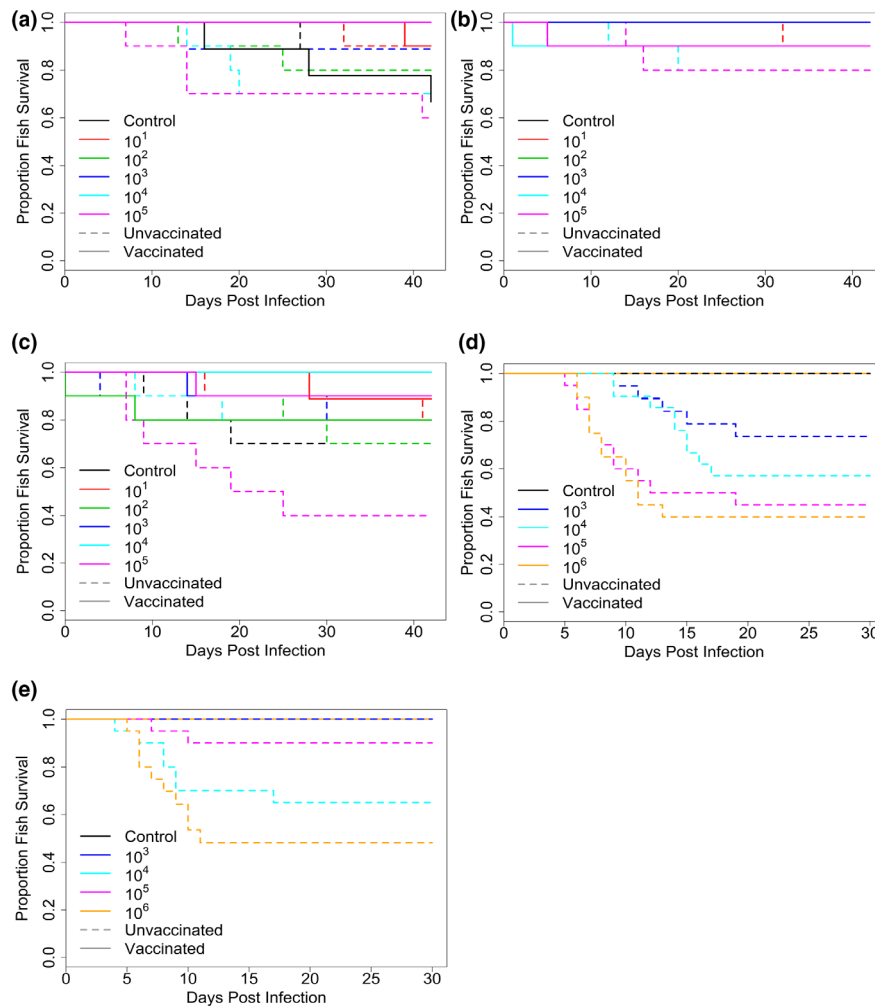


FIGURE 1. Survivorship of Rainbow Trout exposed to IHNV. Panels represent experiment 1 for fish lines (a) ARS-Fp-R, (b) IHNV.R, and (c) ARS-Fp-S, and experiment 2 for fish lines (d) ARS-Fp-R and (e) IHNV.R. Lines represent the Kaplan–Meier survivorship through time of unvaccinated (dotted line) or vaccinated (solid line) fish exposed to different dosages (PFU/mL) of virus (denoted by color). Lines that are not visible had zero mortality (proportion survival = 1) and overlap.

was analyzed for only those fish shedding detectable virus (see Methods). For both experiments, as viral exposure dosage increased, the amount of virus shed generally significantly increased (experiment 1: $F = 1,628.6$; $df = 1, 134$; $P < 0.001$; experiment 2: $F = 14.8$; $df = 1, 250$; $P < 0.001$; Supplemental Tables 5 and 6; Supplemental Figure 6). For experiment 1, the shedding rate rapidly increased until day 4 and then began to taper across all dosages (Figure 3a). In experiment 2, the shedding rate slightly increased or remained stable through time at the lowest dosages and decreased through time at the highest dosages (Figure 3b). Vaccination significantly decreased the rate of viral shedding (experiment 1: $F = 27.7$; $df = 1, 134$; $P < 0.001$; experiment 2: $F = 8.6$; $df = 1, 250$; $P = 0.004$; Supplemental Tables 5 and 6) across all treatments in both experiments (Supplemental Figure 7), but did not completely eliminate shedding. Despite suggestive trends, there was no significant difference in the amount of virus shed between the fish lines across dosages or through time (Supplemental Figure 8).

Total Virus Shed

In an effort to compare absolute viral fitness between treatments, the total amount of virus shed by each fish was analyzed. The analysis for both experiments indicated that the total amount of virus shed increased with increasing dosage (experiment 1: $Z = 17.1$; $df = 1, 295$; $P < 0.0001$; experiment 2: $Z = 7.0$; $df = 1, 314$; $P < 0.0001$; Figure 4; Supplemental Tables 7 and 8). For experiment 1, there was a significantly higher amount of virus shed by unvaccinated fish compared with vaccinated fish across all treatments ($Z = -4.5$; $df = 1, 295$; $P < 0.0001$; Figure 4a; Supplemental Table 7). For experiment 2, the amount at which vaccination reduced the total amount shed decreased as dosage increased, but remained significant across all dosages ($Z = 2.5$; $df = 1, 314$; $P = 0.012$; Figure 4b; Supplemental Table 8). Despite suggestive trends, no significant difference was observed between the fish lines in the total amount of virus shed for either experiment (Supplemental Tables 7 and 8).

DISCUSSION

This is one of the first studies to investigate vaccine impacts on host mortality and virus shedding kinetics across multiple pathogen exposure dosages (as reviewed in Langwig et al. 2019) and host lineages. The study provided an estimate of the efficacy of blocking transmission using vaccination under diverse conditions, which may be encountered in the field. In general, the IHNV DNA vaccine resulted in decreased mortality with RPS ranging from 50% to 100% across multiple genetic lines and various exposure dosages. This was even true at high pathogen exposure dosages, despite increased mortality in

unvaccinated groups. These findings are in line with other studies investigating the disease prevention efficacy of this vaccine (Corbeil et al. 2000b; Garver et al. 2005; Kurath 2008). The vaccine was much less effective at preventing viral shedding, and the majority of vaccinated fish became infected and shed virus. In addition to bolstering inference of efficacy under potential field conditions, the quantification of shedding kinetics across multiple virus exposure dosages provided insights into possible mechanisms of vaccine protection.

The reduction in mortality due to vaccination appeared to be partly driven by a significant reduction in the probability of fish shedding virus. If shedding is associated with in-host infection levels as has been previously observed (Wargo et al. 2011, 2017), this may indicate that the vaccine reduced the probability of infection. However, large numbers of vaccinated fish still shed the virus, particularly at higher viral exposure dosages. Furthermore, reductions in mortality were greater than reductions in shedding in vaccinated hosts. Although not directly measured here, these results suggest that the vaccine was only partially successful at blocking infection, and that this was not the only mechanism by which the vaccine reduced mortality.

Interestingly, vaccination also reduced the overall rate of viral shedding by infected fish through time, resulting in a lesser total amount of virus shed in vaccinated versus unvaccinated fish. The reduced shedding rate due to vaccination appears to be driven by reductions in peak shedding as well as more rapid cessation of shedding in vaccinated versus unvaccinated fish. This was most pronounced in experiment 2, likely because the lowest three dosages in experiment 1 caused very little shedding in unvaccinated fish, so a vaccine effect could not be observed. These findings are in line with others that have found that vaccination expedites pathogen clearance (De Jong and Kimman 1994; Coward et al. 2014). Again, these reductions were not absolute, in that vaccinated fish still shed a significant amount of virus. Likewise, small or insignificant reductions in shedding were often observed when mortality was reduced to zero. The bulk of this shedding occurred during days 1–4, where vaccine effects on shedding were even less pronounced. Therefore, viral load reductions can only partially explain vaccine disease protection.

Collectively, these results indicate that the mechanism by which the IHNV DNA vaccine operates involves both resistance and tolerance. Here, we define tolerance as host reductions in levels of clinical disease without reductions in pathogen load (Råberg et al. 2007). Given that the shed and within-host IHNV loads are correlated (Wargo and Kurath 2011), the vaccine appeared to make hosts more resistant by reducing the number of hosts infected, the peak viral loads within hosts, and the duration of viremia. This warrants further investigation given that within-host loads weren't measured here. Regardless, the changes in

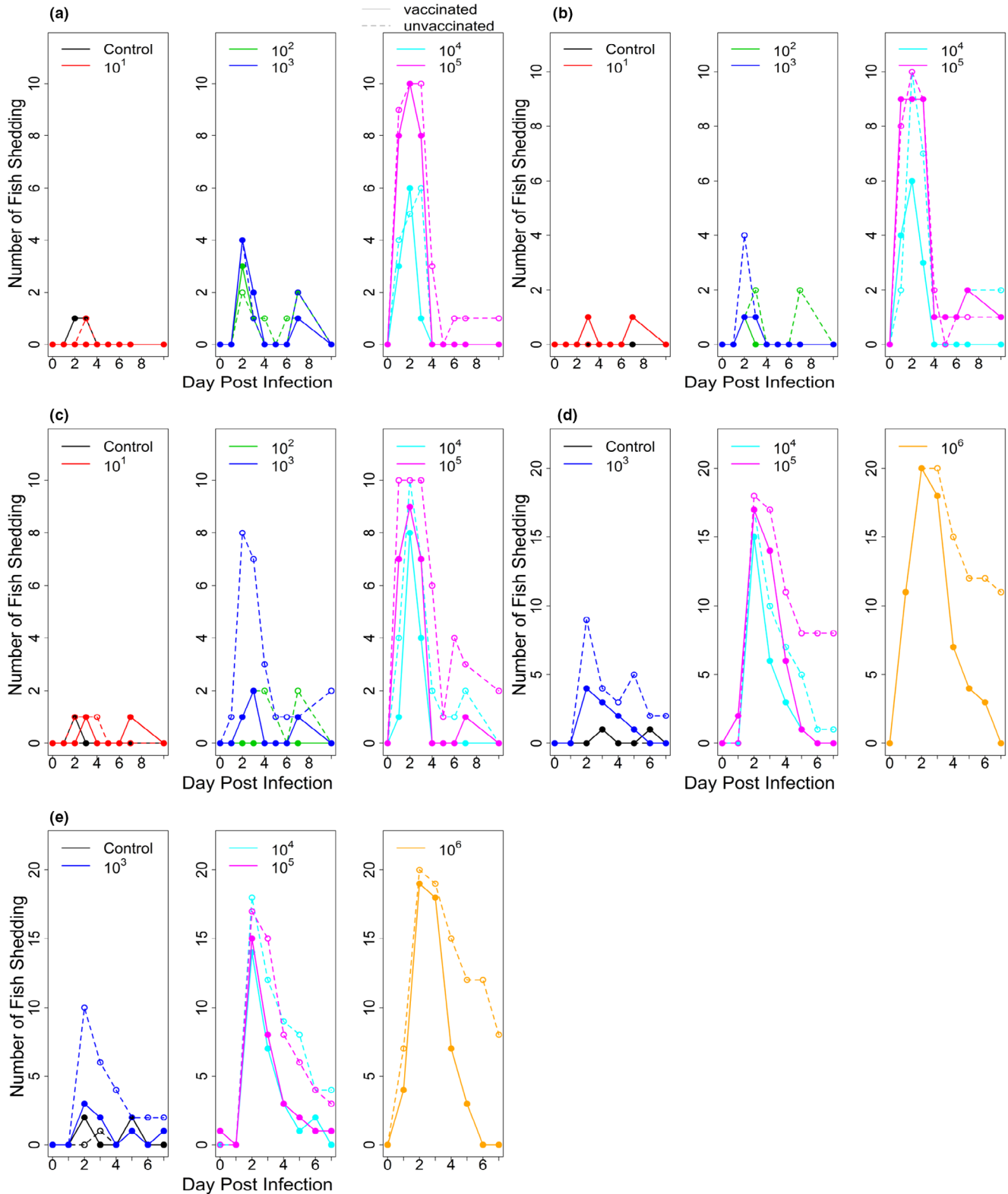


FIGURE 2. The number of fish shedding IHN virus per day. Panels represent experiment 1 for Rainbow Trout lines (a) ARS-Fp-R, (b) IHN.V.R, and (c) ARS-Fp-S and experiment 2 for fish lines (d) ARS-Fp-R and (e) IHN.V.R. Lines represent the total number of fish out of 10 (panels a-c) or 20 (panels d-e) that had detectable IHN virus shedding by qPCR through time. Unvaccinated fish are represented by the dotted line and vaccinated fish are represented by the solid line. The color in the legend represents the challenge dosage of virus in PFU/mL. Points represent when samples were taken. Lines that are not visible had zero fish shedding.

shedding (and likely infection) levels were relatively small compared with changes in survival, suggesting that a tolerance mechanism was equally (if not more) important as resistance. This tolerance mechanism allowed for increased survival of vaccinated fish, even at the same viral shedding levels as unvaccinated fish. These results are similar to a study where fish that were vaccinated against enteric redmouth disease were exposed to *Yersinia ruckeri*; although the vaccine prevented mortality, the bacterial burdens in the spleen were the same for both vaccinated and unvaccinated fish (Harun et al. 2011).

Immunologically, one protective mechanism thought to partially account for the high efficacy of the IHNV DNA vaccine is the robust stimulation of innate immune responses. When vaccinated with pWg, there is an up-regulation of *mx-1* and *vig-8* in trout (Purcell et al. 2004, 2006). After penetrating the skin defenses, this is the first line of host defense against RNA viruses and indicates that early, non-specific, type I interferon anti-viral response is important in stimulating adaptive immunity against IHNV (Verhelst et al. 2013). Peñaranda et al. (2009) showed that when trout are exposed to IHNV, *mx-1* was up-regulated on day 1, peaked on day 3, and remained high until after day 7. This generally followed the shedding kinetics described here in that shedding peaked around days 1–3 and then began to decline. Collectively, this suggests that the up-regulation of *mx-1* due to vaccination leads to more rapid viral clearance, and ultimately reduced mortality. There is also evidence that this primed interferon response provides some protection against infection and partially blocks viral replication (Park et al. 2011). However, the immune response in vaccinated fish appears to be more effective at managing disease even when viral loads remain high. This tolerance may be due to better regulation of immunopathology, similar to that of Chinook Salmon *Oncorhynchus tshawytscha* exposed to *Renibacterium salmoninarum* (Metzger et al. 2010). Given that innate immunity was not directly measured here, this warrants further investigation.

Pathogen exposure dosage had a large impact on mortality and shedding, which ultimately influenced vaccine efficacy. As dosage increased, mortality, probability of shedding, and total amount of viral shedding also increased commensurately. Vaccination appeared to attenuate this dosage effect, such that the increase in shedding with increasing dosage was reduced in vaccinated fish compared with unvaccinated fish. However, when analyzing the total amount of virus shed over the entire experiment, there was evidence that at very high dosages, prevention of shedding by vaccination (and to some extent, prevention of disease) were reduced. This suggests that at very high viral dosages, immunity provided by vaccination is diminished. These findings agree with the findings of others that increasing pathogen dosage leads to

increased infection probability and reduced vaccine efficacy, although studies are limited and the shape of the relationship varies between systems (Gomes et al. 2014; Langwig et al. 2017, 2019). A few studies have investigated whether the increase in probability of infection with pathogen exposure dosage is a function of simply more virus particles entering the host (independent action) or virus particles interacting in some way to enhance infection (mass action), such as through suppression or overwhelming of the immune system (Regoes et al. 2003; Zwart et al. 2011). Previous studies in this system indicate an independent action mechanism, in that the increased probability of infection with dosage is not greater than expected since there are simply a large number of infectious particles present (McKenney et al. 2016). However, these previous studies did not examine the duration of shedding, which could provide some insights into the nature of protection.

Our results show that the duration of viral shedding was not dosage dependent. For all dosages, peak shedding occurred between day 2 and day 4 and shedding largely stopped by day 10, as was also found by Wargo et al. (2017). Mortality, on the other hand, began around day 5, well after the majority of virus was shed; but again, the kinetics were consistent across dosages. The impact of dosage on infection kinetics in other systems is variable. Some studies suggest that lower dosages may lead to prolonged infection due to delayed immune stimulation, while others indicate that higher dosages may result in longer infection durations because they overwhelm the immune system (Li and Handel 2014). Because dosage increased the initial probability—but not duration—of shedding, this implies that immune clearance of the virus by fish operated at the same rate regardless of dosage. This suggests that increasing the dosage did not lead to suppression of the immune system and supports the independent action hypothesis of virus particles, as previously observed by McKenney et al (2016). However, the results imply that vaccine protection may operate under a threshold, wherein high viral dosages will overwhelm reductions in the probability of infection due to vaccination, and that these reductions are not absolute. This has been observed in other systems (Langwig et al. 2019) and could be a function of limits in the level of immune stimulation that vaccination can provide, such as antibody production. Examining vaccine efficacy at multiple vaccine dosages may shed light on this.

Fish line had a minor effect on shedding kinetics and vaccine protection, highlighting the importance of fish background when inferring vaccine efficacy. There was evidence that the IHNV.R fish had lower disease susceptibility, in that their mortality levels were significantly reduced. These results are not surprising given that the IHNV.R line has been selectively bred against IHNV disease. This appears to

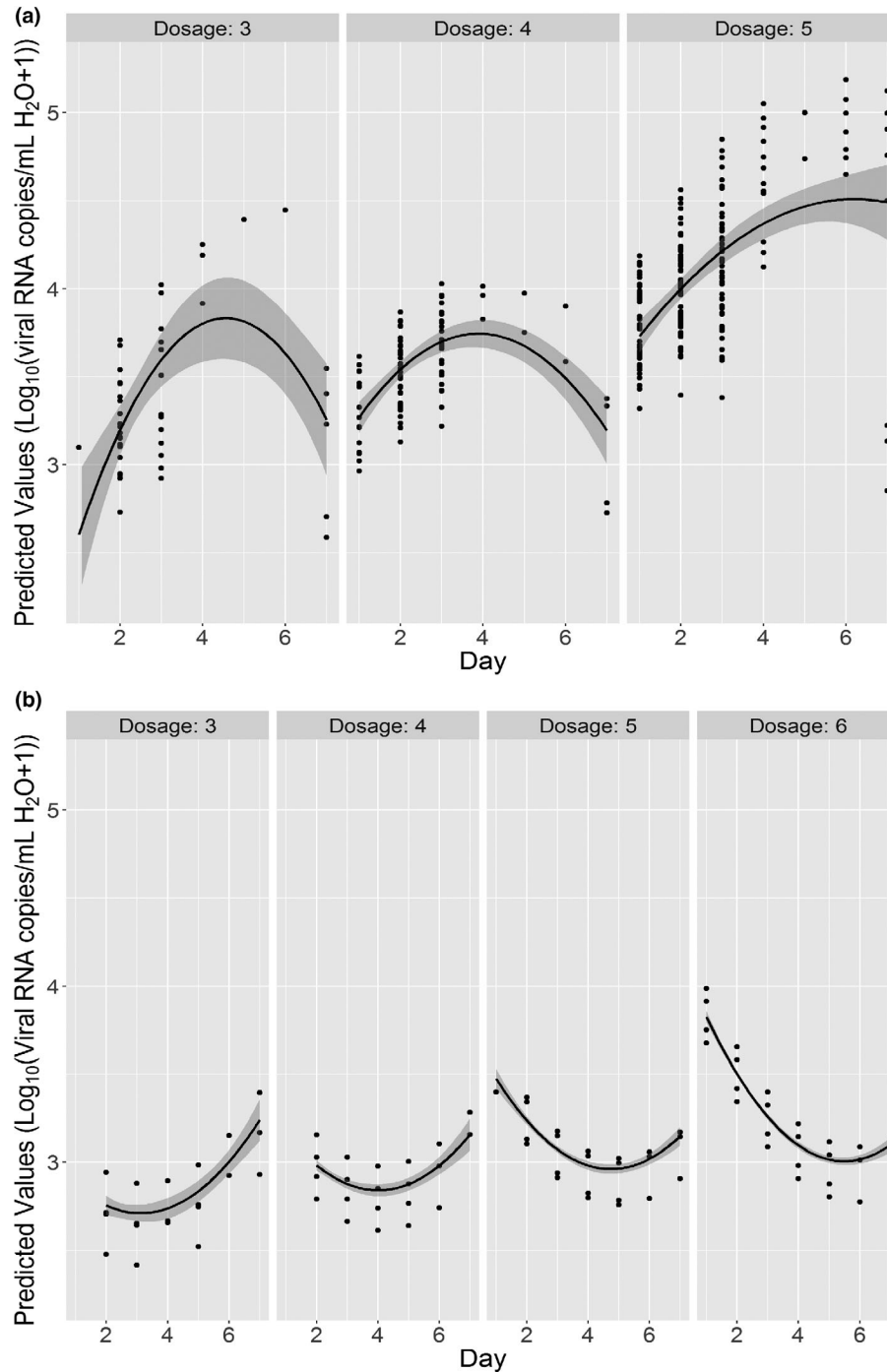


FIGURE 3. The predicted quantity of IHNV shed through time. Lines represent the best fit trend of the predicted daily amount of virus shed (\log_{10} [virus RNA copies/mL water + 1]) for various pathogen exposure dosages \log_{10} (PFU/mL) for experiment (a) 1 and (b) 2 from the statistical models described in Methods. The gray shading is the 95% CI around the model fit line. The dots are fitted values for individual fish; some individuals have the same predicted value and thus the dots overlap.

operate through both a resistance and tolerance mechanism. Resistance was evident in the finding that the probability of shedding did not increase as quickly for the IHNV.R line with virus exposure dosage compared with the other fish

lines. Yet this effect was small, and high numbers of IHNV.R fish still became infected and shed virus despite reduced levels of mortality, indicating that tolerance may be stronger than resistance. This suggests that selective

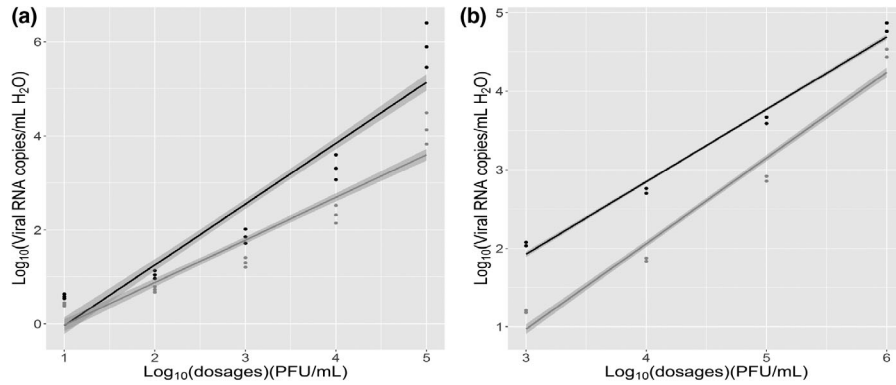


FIGURE 4. The predicted total amount of IHNV shed. Dosage is a continuous variable from (a) 10^1 to 10^5 PFU/mL for experiment 1 and (b) from 10^3 to 10^6 PFU/mL for experiment 2. Lines represent the best fit linear trend of predicted total amount of virus shed (\log_{10} [virus RNA copies/mL water + 1]) for various pathogen exposure dosages from the statistical models described in Methods. Dots represent the predicted values for each fish; some individuals have the same value and thus they overlap. Black lines represent unvaccinated hosts while gray lines represent vaccinated hosts. The gray shading is the 95% CI around the model fit line.

breeding and vaccination may operate by the same mechanism. There was also an indication that the ARS-Fp-S fish line responded better to vaccination than the other fish lines. This was largely observed when comparing viral shedding rather than fish mortality. The mechanism behind this phenomenon is unknown. Selective breeding of the IHNV.R and ARS-Fp-R lines may have reduced their immune competence compared with the ARS-Fp-S line, which has not undergone selective breeding. It has been observed that selective breeding results in reduced antibody diversity in fish (Sommer 2005; Spielman et al. 2007), and could therefore reduce vaccine competence. Host immunity to one pathogen can also have tradeoffs with susceptibility to another (Ehlinger 1977; Alcivar-Warren et al. 1997). There was some indication of this here because the ARS-Fp-R line, which has been bred for resistance to bacterial coldwater disease, suffered more mortality than the IHNV.R line. Regardless of the mechanisms, differences in fish line responses to vaccination could have major implications for disease management in aquaculture.

In summary, our results indicated that IHNV vaccine efficacy is dependent on pathogen exposure dosage and fish line. Overall, vaccination reduced pathogen fitness by an order of magnitude or less (i.e., resulted in reduction in total shedding). If viral shedding is correlated with transmission as other studies have suggested (Wargo and Kurath 2012; Doumayrou et al. 2019), this would imply that vaccinated hosts are still able to transmit the virus, despite experiencing little clinical disease. Epidemiologically, the ability of vaccinated individuals to transmit the disease could greatly hinder management and eradication of disease, particularly if there is transmission between vaccinated and unvaccinated fish (e.g., between wild and cultured populations; Troyer and Kurath 2003). There are also potential long-term evolutionary consequences, such

as the selection for increased pathogen virulence (Gandon et al. 2001; Mackinnon et al. 2008). Evidence of vaccine-induced virulence has been observed in Marek's disease virus (Davison and Nair 2004; Nair 2005; Atkins et al. 2013; Read et al. 2015). Whether this could occur in the IHNV system warrants further investigation. However, vaccines such as the one used in the present study, which reduce clinical disease but allow transmission, are of particular concern.

This study illustrates the importance of considering exposure dosage, infection, and transmission when evaluating vaccine efficacy. The development of vaccines that prevent transmission would be beneficial. This could improve disease management in vaccinated and unvaccinated host populations by increasing herd immunity (Metcalfe et al. 2015). A transmission-blocking vaccine would alleviate the potential risk of vaccine-induced selection for virulence (Gandon et al. 2001). A variety of IHNV vaccines and other fish pathogen vaccines are available or currently in development (Collins et al. 2019). However, the transmission prevention efficacy of these vaccines under common field conditions, such as variable pathogen dosages or fish lines, is largely unknown. Our results were consistent with the one other study examining IHNV transmission after DNA vaccination (Long et al. 2017). In that study, the authors reported that transmission from vaccinated to unvaccinated Atlantic Salmon via cohabitation occurred in 50% of the population. It should be noted that Long et al. (2017) used different host infection routes, species, housing conditions, virus strains, and detection methods. How these factors affect viral transmission success warrants further investigation. Despite these differences, both studies indicate that transmission from vaccinated fish can occur and is likely not a rare event. Many other fish vaccines, particularly those delivered

through non-injection routes (e.g., immersion or with feed) have been shown to have lower disease prevention efficacy than the DNA vaccine studied here (Kurath 2008; Plant and LaPatra 2011). Whether this translates to lower transmission-blocking efficacy is unclear; however, if that is the case, it is likely to heavily impact their utility for disease management. Our results indicate that selective breeding against IHNV may also act via a similar tolerance mechanism as vaccination, wherein disease (but not infection levels or transmission) is reduced. This would result in the same potential concerns about pathogen persistence and virulence evolution associated with vaccines also being applicable to selective breeding. Overall, this and future studies into vaccines that block transmission are likely to facilitate the development, evaluation, and efficacy of disease management programs.

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SUPPORTING INFORMATION

Additional supplemental material may be found online in the Supporting Information section at the end of the article.