

# *In Vivo* Fitness Associated with High Virulence in a Vertebrate Virus Is a Complex Trait Regulated by Host Entry, Replication, and Shedding<sup>▽</sup>

Andrew R. Wargo<sup>1,2\*</sup> and Gael Kurath<sup>1</sup>

Western Fisheries Research Center, 6505 NE 65th Street, Seattle, Washington 98115-5016,<sup>1</sup> and University of Washington, Department of Biology, 24 Kincaid Hall, Seattle, Washington 98195-1800<sup>2</sup>

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**The relationship between pathogen fitness and virulence is typically examined by quantifying only one or two pathogen fitness traits. More specifically, it is regularly assumed that within-host replication, as a precursor to transmission, is the driving force behind virulence. In reality, many traits contribute to pathogen fitness, and each trait could drive the evolution of virulence in different ways. Here, we independently quantified four viral infection cycle traits, namely, host entry, within-host replication, within-host coinfection fitness, and shedding, *in vivo*, in the vertebrate virus *Infectious hematopoietic necrosis virus* (IHNV). We examined how each of these stages of the viral infection cycle contributes to the fitness of IHNV genotypes that differ in virulence in rainbow trout. This enabled us to determine how infection cycle fitness traits are independently associated with virulence. We found that viral fitness was independently regulated by each of the traits examined, with the largest impact on fitness being provided by within-host replication. Furthermore, the more virulent of the two genotypes of IHNV we used had advantages in all of the traits quantified. Our results are thus congruent with the assumption that virulence and within-host replication are correlated but suggest that infection cycle fitness is complex and that replication is not the only trait associated with virulence.**

Despite a recent surge in research, the evolution of viral virulence remains a controversial issue (1, 7, 10, 18, 22, 37, 50, 60). Some studies have suggested a positive link between viral fitness and virulence (6, 13, 17, 39, 42, 49, 52–54, 58, 59), whereas others found no evidence of such an association (20, 24, 27) or even indicated that the converse, that virulence is negatively associated with viral fitness, is true (36, 43). Despite the fact that these studies span a wide range of virus and host taxa, a limitation of many of these studies is that viral fitness was typically estimated from only one or two virus traits, at one stage of the viral infection cycle. In reality, viral fitness is likely shaped by multiple traits at each of the viral infection stages, i.e., entry into the host, replication in the host, and shedding from the host, all of which could differentially impact selection for virulence (5).

Most estimates of viral fitness come from examinations of the within-host replication stage of the viral infection cycle. For a few systems, researchers have made an effort to provide a detailed assessment of the importance of within-host dynamics on fitness, by separately quantifying replication in single-genotype infections and the relative abilities of virus genotypes to produce infectious progeny in a coinfection environment, herein referred to as coinfection fitness (15, 39, 59). However, the connection between within-host fitness and transmission remains elusive (20, 24, 42, 52), partly due to an incomplete understanding of virus investment into shedding and entry (8, 45). These limitations are compounded in vertebrate virus systems by the fact that viral fitness is not often quantified *in vivo*, using intact, immune competent, living hosts (13, 29, 36, 42, 43,

53, 55, 59). Thus, vertebrate viral fitness has largely been examined without the complete context of host factors or viral traits likely to shape the virulence-fitness association (1, 14, 23).

We recently demonstrated that virulence is associated with viral fitness *in vivo* for two genotypes of the vertebrate virus *Infectious hematopoietic necrosis virus* (IHNV) (59). In that study, we developed the foundation and methods for determining if virulence differences between IHNV genotypes are associated with within-host viral fitness. Here we sought to determine the mechanism of these fitness differences and expand our understanding of the components of vertebrate viral fitness, by quantifying individual fitness traits associated with viral entry, within-host replication, coinfection, and viral shedding, *in vivo*. We used a traditional definition of viral fitness, the ability to replicate and produce infectious progeny in a given environment (28). Since our study goes beyond an analysis of within-host replication to assess fitness traits throughout a single viral infection cycle, we use the term “infection cycle fitness” to refer to the data we present. We examined infection cycle fitness for two IHNV genotypes previously characterized to have high (HV) and low (LV) virulence in their natural rainbow trout host (*Oncorhynchus mykiss*) (25, 59). This provided information on how viral traits at each infection stage might individually be associated with virulence, thus offering insights on possible selection on virulence in IHNV.

IHNV is a negative-sense single-stranded RNA virus in the family *Rhabdoviridae* (9). The virus is endemic in salmonid fishes in the Pacific Northwest of North America ranging from California up through Alaska. Within this range, IHNV field isolates can be divided into three genogroups, U, M, and L, with distinct geographical ranges and host specificities (31). The virus regularly causes epidemics in hatchery-reared, farm-raised, and wild fish throughout its host range, frequently re-

\* Corresponding author. Mailing address: Western Fisheries Research Center, 6505 NE 65th Street, Seattle, WA 98115-5016. Phone: (206) 526-6282. Fax: (206) 526-6654. E-mail: awargo@u.washington.edu.

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sulting in significant levels of acute disease-induced mortality due to necrosis of the host kidneys, liver, and spleen (9). Hundreds of North American field isolates of the virus have been typed, giving rise to well over a hundred unique genotypes, many of which differ in virulence (25). Longitudinal studies indicate complex movement and evolution of the virus in the field, with multiple genotypes cocirculating in some situations and new genotypes sometimes emerging and displacing resident ones (3, 56, 57).

To examine the role each stage of the viral infection cycle independently played in shaping the viral fitness-virulence association, we compared the *in vivo* performance of IHNv genogroup M, HV and LV genotypes (see below), under two different host infection regimes. To assess both virus host entry and replication as a combined trait, we infected live rainbow trout with the two IHNv genotypes by the natural route of immersion in water containing virus and then quantified viral loads in individual fish after a 3-day period of *in vivo* replication (55, 59). To independently quantify replication alone, we bypassed the host entry step of the natural infection process by administering the virus through intraperitoneal injection into the trout and subsequently quantified viral loads after a 3-day *in vivo* replication period (45). Comparison of the results from immersion and injection challenges thus provided an estimate of the contribution of host entry to fitness differences between the genotypes. In both the immersion and injection challenges, we infected fish with either a single IHNv genotype or a mixture of the two genotypes at a 1:1 ratio. The viral load for each genotype was quantified in individual fish samples by using genotype-specific reverse transcription-quantitative PCR (RT-qPCR), making it possible to compare the performance of a genotype alone with its performance in a coinfection environment, to determine the consequences of coinfection on genotype performance. Examination of the genotype composition in mixed infections also enabled us to quantify coinfection fitness. Finally, to quantify virus shedding, water was sampled at the time of fish harvest, with subsequent genotype-specific quantification of the viral loads shed by individual fish exposed to either single or mixed infections. This study expands upon previous knowledge of viral fitness by assessing infection cycle fitness in detail. As such, it provides a step toward understanding overall viral fitness in natural field infections, which is ultimately regulated by a complex set of within-host, transmission, and environmental parameters.

#### MATERIALS AND METHODS

**Virus and host.** We used two genetically distinct IHNv isolates (herein defined as genotypes) that differ in virulence in rainbow trout, herein labeled HV (high virulence) and LV (low virulence) (59), which have previously been referred to as 220-90 (HV) and WRAC/039-82 (LV) and were originally obtained from farmed rainbow trout in the field. Phylogenetic analysis previously revealed that genotypes HV and LV both belong to the M genogroup of IHNv, which has evolved host specificity for rainbow trout (31). Over the entire genome of the virus, there is 2.8% sequence divergence (312/11,133 nucleotides) between genotypes HV and LV (2, 40). The virus genotypes were maintained in the laboratory through propagation on cyprinid fish epithelioma papulosum cyprini (EPC) cells and stored at  $-80^{\circ}\text{C}$  (21, 31, 34, 59). Each genotype underwent 6 to 9 passages through EPC cells between collection from the field and use in the experiments (34). The preparation of working viral stocks and accurate quantification by plaque assay and qPCR are published elsewhere (59). The relative virulences of these viral genotypes have been characterized in controlled batch challenge experiments by several different researchers (25, 34, 59). In our hands,

TABLE 1. Total numbers of fish in each treatment group used for statistical analyses<sup>a</sup>

Infection type	No. of fish in treatment or analysis group and expt								
	Immersion <sup>c</sup>			Injection			Shedding		
	I	II	III	I	II	III	I	II	III
HV genotype alone	23	24	28	23	16	27	22	24	28
LV genotype alone	17	14	28	17	17	25	19	17	28
Mix <sup>b</sup>	22	22	26	18	21	26	22	23	21
Mock <sup>d</sup>	4	2	4	4	3	4	4	2	4

<sup>a</sup> All dead fish were excluded, because time of death could not be determined and may influence viral growth dynamics as well as viral stability. Samples that had no detectable virus were also excluded because failed RNA extraction could not be ruled out, although data for these fish are shown in Fig. 1. Ultimately, this resulted in some variation between experimental groups in the final numbers of fish used for statistical analyses. The exclusion of negative samples and dead fish as well as differences in experimental group sizes had no impact on the statistical tests or results.

<sup>b</sup> For the analyses, fish receiving a mixed infection were randomly split between HV and LV groups, so that each fish appeared only once in the analyses, to avoid pseudoreplication.

<sup>c</sup> In the immersion challenge, 8 fish were positive for virus, but their respective water samples were not, and in 9 samples, virus was detected in the water but not in the respective fish, likely due to the samples falling below the detection threshold.

<sup>d</sup> Out of 31 mock samples, 2 were positive for LV (1 each in injection groups in experiments I and III) and 1 was positive for HV (experiment III injection group) at values just above the detection threshold. This contamination is believed to have occurred during RT-qPCR and represents a quantity of virus  $\geq 2$  orders of magnitude below the average values of samples from infected fish. This low level of contamination did not significantly affect the results.

challenges of triplicate groups of 20 to 30 rainbow trout demonstrated that the HV genotype induced  $85\% \pm 6\%$  mortality and the LV genotype induced  $30\% \pm 3\%$  mortality (59).

All experiments here used research-grade, juvenile, 1- to 3-g rainbow trout (*Oncorhynchus mykiss*), obtained from Scott LaPatra of Clearsprings Foods Incorporated, and these were maintained in pathogen-free water at  $15^{\circ}\text{C}$ . Different lots of fish were utilized for each of the three experiments. All animal procedures were conducted under an approved University of Washington animal care protocol.

**Virus challenges.** Fish were challenged with virus by two methods, immersion and injection. For both challenge methods, groups of 14 to 28 fish were exposed to either the viral HV genotype alone, LV genotype alone, or a mixed infection of HV and LV at a 1:1 ratio or mock infected with no virus, as a control (Table 1). In immersion challenges, the fish were placed in static water containing  $10^4$  PFU/ml of each viral genotype for 12 h, as previously outlined (59). Thus, groups exposed to the 1:1 mixture of HV and LV had a total of  $2 \times 10^4$  PFU virus/ml in the challenge baths. After a 12-h exposure to virus in static water conditions, the immersion challenge fish were washed for 1 h using a continuous water flow and then isolated as single fish in individual tanks with 400 ml pathogen-free water. The fish were held in the individual tanks at  $15^{\circ}\text{C}$  in static water conditions for a 3-day period of within-host replication and viral shedding and then euthanized, harvested aseptically, and stored at  $-80^{\circ}\text{C}$  until processing for within-host viral load.

The three-day harvest time point was selected on the basis of *in vivo* virus growth curves. These curves were generated by infecting groups of fish with the HV or LV genotype by immersion as described above and then harvesting 5 fish from each treatment group daily for 7 days. Viral loads, quantified by qPCR as described below, indicated that both genotypes peaked in replication by 2 days after exposure to virus, after which they either stabilized or declined gradually. The choice of 3 days as the single time point for analysis was thus to ensure that each genotype had peaked in replication while avoiding the onset of mortality, at approximately day 5. This growth dynamic is consistent with previous studies of IHNv infection in rainbow trout (45, 55, 59).

To assess viral shedding, a 1-ml water sample was also taken from each immersion challenge tank at the time of fish harvest and stored at  $-80^{\circ}\text{C}$  until processing. To examine if potential differences in environmental stability of the two viral genotypes might impact the total virus quantified in the water, we held a 20-ml aliquot of the fish water from 5 fish singly infected with HV or LV (each)

in experiment II for an additional 72 h after the fish harvest on day 3, at 15°C, and then quantified the remaining viral RNA copies by qPCR.

For the injection challenge, fish were anesthetized in 60 mg tricaine methylsulfate (MS-222)/liter H<sub>2</sub>O buffered with 300 mg/liter of sodium bicarbonate. Once fish were unresponsive, 100 PFU of each virus genotype (HV, LV, or HV-LV mix, 200 PFU total) or medium without virus (mock treatment) was injected directly into the intraperitoneal (i.p.) cavity in a total injection volume of 25 µl of minimum essential medium (Gibco) buffered with 14 mM Tris-HCl (Sigma). We chose this injection challenge dose based on studies indicating transmission bottleneck sizes of 25 to 100 virions for fish exposed to virus by immersion as described above (unpublished data). In addition, a pilot study with this injection dose revealed viral loads after 3 days of within-host growth, similar to those seen in our immersion challenges (unpublished data). After injection, the fish were allowed to recover and then isolated in individual tanks containing 400 ml pathogen-free water as described above. The entire experiment (i.e., immersion, injection, and shedding) was conducted 3 independent times (experiments I to III), with different lots of rainbow trout used for each experiment. These experiments were all run independently of those presented in our previous work on IHNV virulence (59), and as such, the data presented here confirm and expand on that previous research.

**Viral load quantification.** To determine viral loads in the fish, RNA was first extracted from whole fish by using guanidinium-thiocyanate as outlined elsewhere (59). To determine the quantity of virus shedding, viral RNA was extracted from 550 µl of each of the water samples taken at the time of fish harvest, using the QIAamp MinElute virus spin kit (Qiagen) scaled up to the sample volume. At the end of the protocol, the extracted water samples were eluted in 20 µl diethyl pyrocarbonate (DEPC)-treated nuclease-free H<sub>2</sub>O (Growcells) and stored at -80°C. After viral RNA extraction, all samples underwent a general cDNA synthesis reaction using 1 to 2 µg RNA with oligo(dT), random hexamers, and Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega) in a 20-µl volume as outlined elsewhere (59). The cDNA was stored at -80°C until further use. This cDNA synthesis was nonspecific and therefore transcribed RNA from both viral genotypes and the fish host equally.

To specifically quantify the viral load for each genotype, we used two real-time quantitative PCR (qPCR) assays with genotype-specific forward primers (for HV, 5'-CCC GAT GCC AAT GGT ACA CT-3', and for LV, 5'-CCG ATG CCA ATG GTA CAC C-3'), MGB *TaqMan* probes (for HV, 5'-VIC-CCC CAA AGA GTG TTC TGA-3', and for LV, 5'-6-carboxyfluorescein [FAM]-CAA CAC AAA AGA GTG TTC TTA-FAM-3'; Applied Biosystems), and reverse primers (for HV, 5'-GTG CAT TTT CCT CCA ATA AAG TCC-3', and for LV, 5'-TTG GTA CAT TTT CCT CCA ATA AAA TCT-3') targeting the viral G gene as described elsewhere (59). To ensure that the qPCR assays were not cross-reactive and to reduce the likelihood that either genotype could mutate so as to be detected by the wrong assay, we designed the assay to target 7 bases that differed between HV and LV (1 base in the forward primer, 3 bases in the probe, and 3 bases in the reverse primer; see reference 59 for discussion). RNA from fish samples in experiments I and II were diluted 1:10, and RNA from water samples and fish from experiment III were diluted 1:5 in nuclease-free water (Growcells) before qPCR to reduce PCR inhibitors. Absolute quantification was achieved by using concentrated genotype-specific IHNV G gene transcript RNA (rHV-G and rLV-G) in an 8-step 10-fold dilution series as qPCR standards (59). Samples which were obtained from fish infected with one viral genotype were analyzed with qPCR once with the appropriate assay (for HV or LV), and samples obtained from fish infected with both genotypes (mixed infections) were analyzed twice, once with each qPCR assay, to provide accurate quantification of both viral genotypes.

**Statistics.** All analyses were carried out in R (version 2.8.0) by using general linear models (GLM). For within-host viral load analyses, the dependent variable was viral load, with the factors being genotype (HV or LV), competition (alone or mixed), exposure (immersion or injection), and experiment (I, II, or III). Full models with all higher-order interactions were first tested, and nonsignificant ( $P > 0.05$ ) terms were dropped until the minimal significant model was reached. The same analysis was conducted for shed virus populations, although the exposure factor was not included, because shed virus was measured only in the immersion challenge groups. To assess the relationship between within-host viral load and virus quantity shed from the same fish, a GLM with the dependent variable being virus quantity shed, the covariate being within-host viral load, and the factors being genotype (HV or LV), competition (alone or mixed), and experiment (I, II or III) was conducted. Both within-host viral load and virus quantity shedding were log transformed in all GLM analyses to meet test assumptions. Furthermore, for the analyses, we randomly assigned half of the mixed-infection fish to the HV group and the other half to the LV group so as not to violate the data independence requirement. The same qualitative results

were obtained when we included all fish in the analyses, and as such, figures show means of all positive samples.

To examine the proportion of HV strains in the mixed-infection populations, we utilized the proportion of HV strains in individual mixed-infection fish as the dependent variable, and the explanatory factors were exposure (immersion or injection) and experiment (I, II, or III). The proportion of HV strains was arcsine-square-root transformed to meet test assumptions. These data still violated the normality assumption after transformation, so a nonparametric Wilcoxon rank sum test was conducted, and it revealed the same results. The GLM results are shown.

Because the exact time of death could not be determined and the nature of replication and stability of virus in dead fish is unknown, we ran all statistical analyses both including and excluding dead fish (12/426 samples). The same statistical results were obtained from both types of analyses, and the results from those excluding the dead fish are shown. We also ran the analyses by both excluding and including samples that were negative for virus by qPCR (43/837 samples), and again the same statistical results were obtained by the two analyses. The data shown exclude negative samples because the possibility of failed RNA extraction in these samples could not be eliminated. This resulted in differences between experimental groups in the total number of fish used for the statistical analyses (Table 1). The statistical tests utilized were robust against differences in group sizes, and they are not believed to have influenced the statistical results.

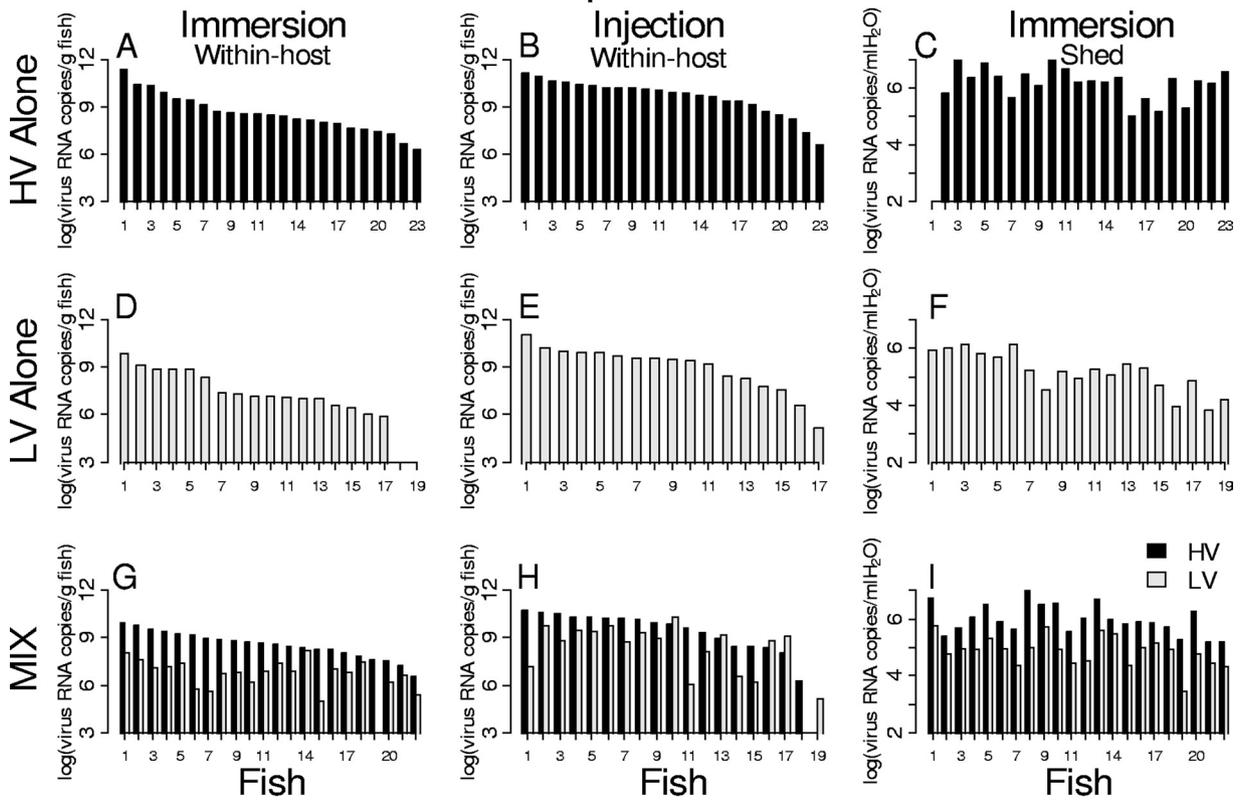
## RESULTS

**Within-host viral load.** Within-host viral loads of individual fish ranged from  $1.3 \times 10^4$  to  $2 \times 10^{11}$  viral copies/g fish in immersion challenges and  $1.3 \times 10^5$  to  $1.3 \times 10^{11}$  viral copies/g fish in injection challenges (Fig. 1). When comparing the mean within-host viral loads between treatments, we found that genotype HV consistently produced more viral copies than genotype LV in both single and mixed infections initiated by either immersion or injection challenge (for genotype effect,  $F_{1, 387} = 93$  [ $P < 0.001$ ]; Fig. 2). Comparisons of within-host viral loads in single and mixed infections revealed that there was no significant competition effect for either genotype, despite a suggestive trend that LV produced more viral copies alone than in coinfections in some groups (for competition effect,  $F_{1, 386} = 3.53$  [ $P = 0.06$ ]). In general, the viral loads in the injection challenge fish were greater than the viral loads in immersion challenge fish (for exposure effect,  $F_{1, 387} = 137$  [ $P < 0.001$ ]). The results for each of the three experiments were similar, although significantly less virus was produced in each subsequent experiment (for experiment effect,  $F_{2, 387} = 58$  [ $P < 0.001$ ]).

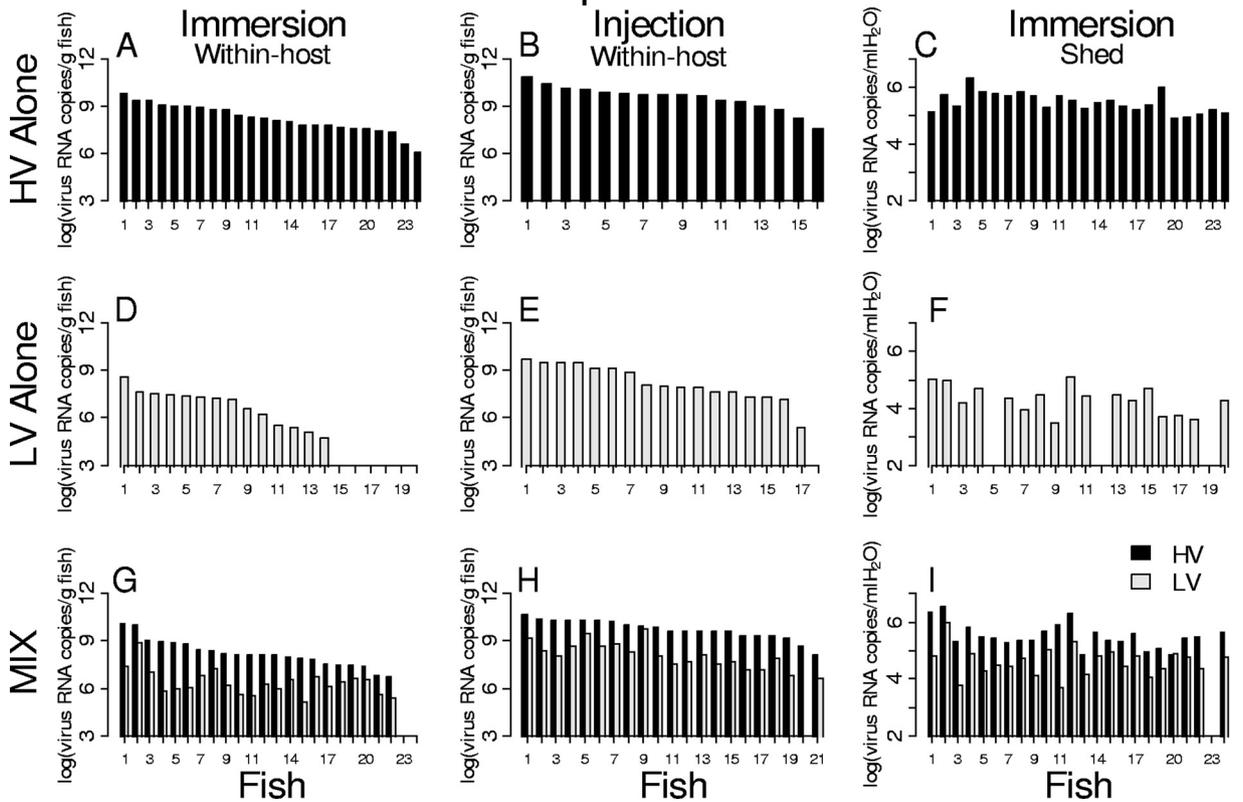
To assess if entry played a role in the within-host fitness differences between the two genotypes, we examined whether the relative performances of HV and LV differed in the immersion and injection challenges. We compared the proportions of HV in the total mixed-infection virus population in the two exposure types. The proportion of HV in the mixed infections by immersion challenge was greater than the proportion in infections by injection challenge (for exposure effect,  $F_{1, 127} = 9.6$  [ $P = 0.002$ ]; Fig. 3), with the effect being qualitatively most evident in experiments I and III and reduced in experiment II (for the exposure-by-experiment interaction,  $F_{2, 125} = 2.7$  [ $P = 0.07$ ]). Therefore, the advantage that HV had over LV appeared to be slightly diminished when the entry step was bypassed.

**Shedding.** Shed viral loads in water samples from individual immersion challenge tanks ranged from  $5 \times 10^2$  to  $3.4 \times 10^7$  viral copies/ml H<sub>2</sub>O (Fig. 1). Overall, viral shedding closely reflected the within-host viral load, in that HV produced more

### Experiment I



### Experiment II



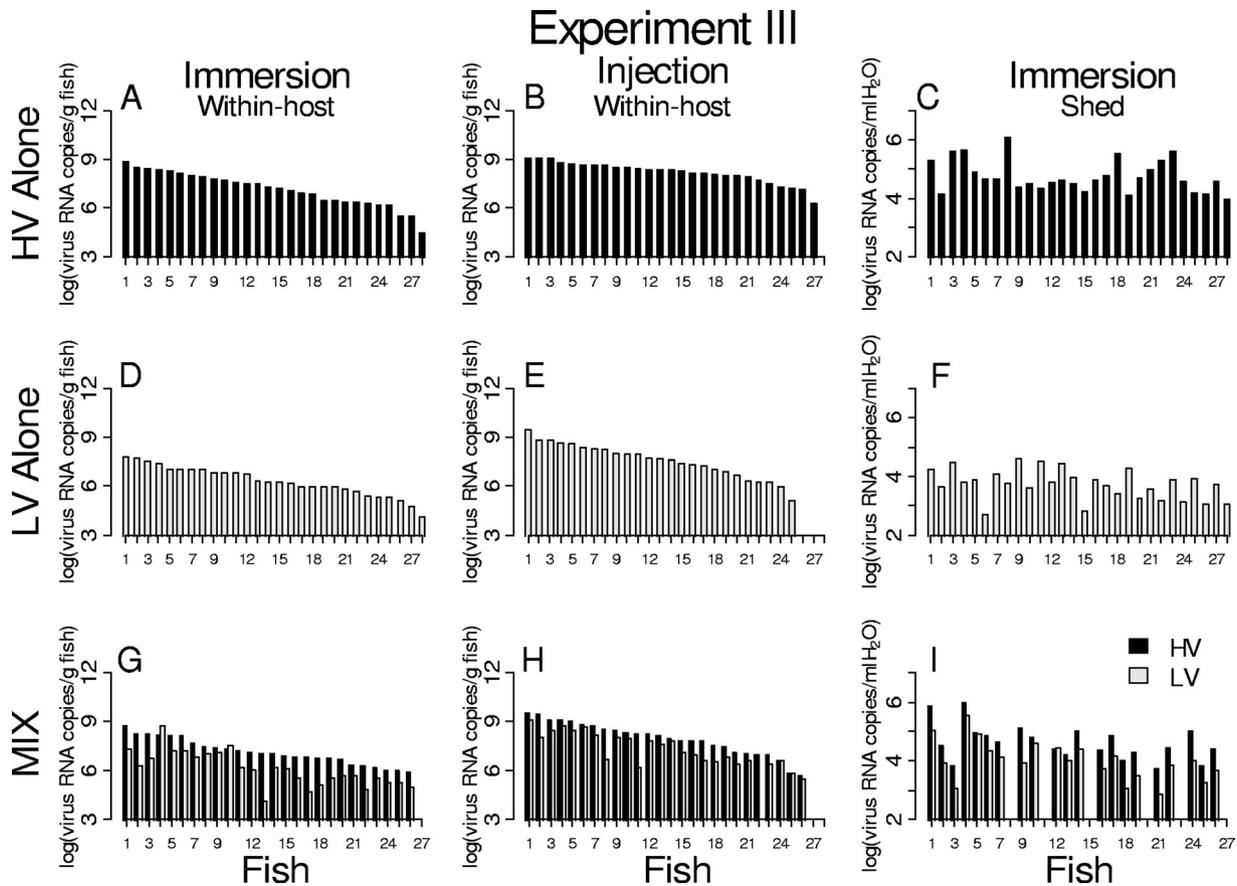


FIG. 1. Viral loads in individual fish from experiments I, II, and III. The panels show the viral RNA copies per g of fish or per ml H<sub>2</sub>O of IHNV genotypes HV and LV from within-host immersion (A, D, and G), within-host injection (B, E, and H), or shed immersion (C, F, and I) virus populations, in fish infected with HV alone (A to C), LV alone (D to F), or HV plus LV at a 1:1 ratio (G to I). Values are arranged by highest to lowest viral load in the fish. Shed virus is from immersion challenges, and so the numbers correspond to the fish as they are arranged in the within-host immersion column. For data corresponding to mixed infections, fish are arranged by viral load of HV, with corresponding viral loads of LV in the same fish given by the adjacent bar. Shed virus data from mixed infections are arranged in the same order as in the within-host immersion graph, with a different x-axis scale. All values were obtained from samples harvested at 3 days postinfection. The lower limit of the y axis represents the approximate virus detection threshold of the qPCR assays.

shed virus than LV in both single and mixed infections across all three experiments ( $F_{1, 200} = 194 [P < 0.001]$ ; Fig. 1 and 2). Also, the two genotypes performed equally well alone and in a mixed infection, suggesting the absence of a discernible competition effect ( $F_{1, 199} = 0.04 [P = 0.84]$ ; Fig. 2). Ultimately, this resulted in HV being the majority genotype in the shed virus populations from mixed infections, in all experiments (Fig. 2). To test the possibility that differential environmental stability could influence the shed virus results, a subset of water samples was held for an additional 72 h at 15°C, subsequent to fish harvest. Compared with the viral loads in the water at the time of fish harvest, 89.3% ± 3.5% of genotype HV strains and 86.4% ± 4.1% of genotype LV strains were no longer detectable after 3 days, indicating that there was no significant difference in the environmental stabilities of the two genotypes.

When we examined total virus on day 3 postinfection for individual fish, there was a significant correlation between virus quantity in the host and virus quantity shed into the water ( $F_{1, 191} = 418 [P < 0.001; R^2 = 0.76]$ ; Fig. 4). There was, however, a significant difference in the intercepts of this correlation for HV and LV, such that for a given within-host viral

load, HV-infected fish shed more virus particles into the water ( $F_{1, 191} = 42.7 [P < 0.001]$ ). The results from each experiment showed the same pattern, although the most virus shedding per within-host viral load occurred in experiment I and the least occurred in experiment III ( $F_{1, 191} = 72 [P < 0.001]$ ). We did not quantify shed virus from the injection challenge and so did not compare amounts of shedding in immersion and injection challenges.

### DISCUSSION

Breaking down the components of pathogen fitness can provide valuable insights into the evolution of pathogen life history traits, especially in regards to virulence. Here, we examined four viral infection cycle traits, namely, host entry, within-host replication, coinfection fitness, and shedding, to see how these traits contributed to infection cycle fitness differences between two IHNV genotypes which differed in virulence.

We consistently found that the more virulent genotype produced more viral copies within the host than the less virulent

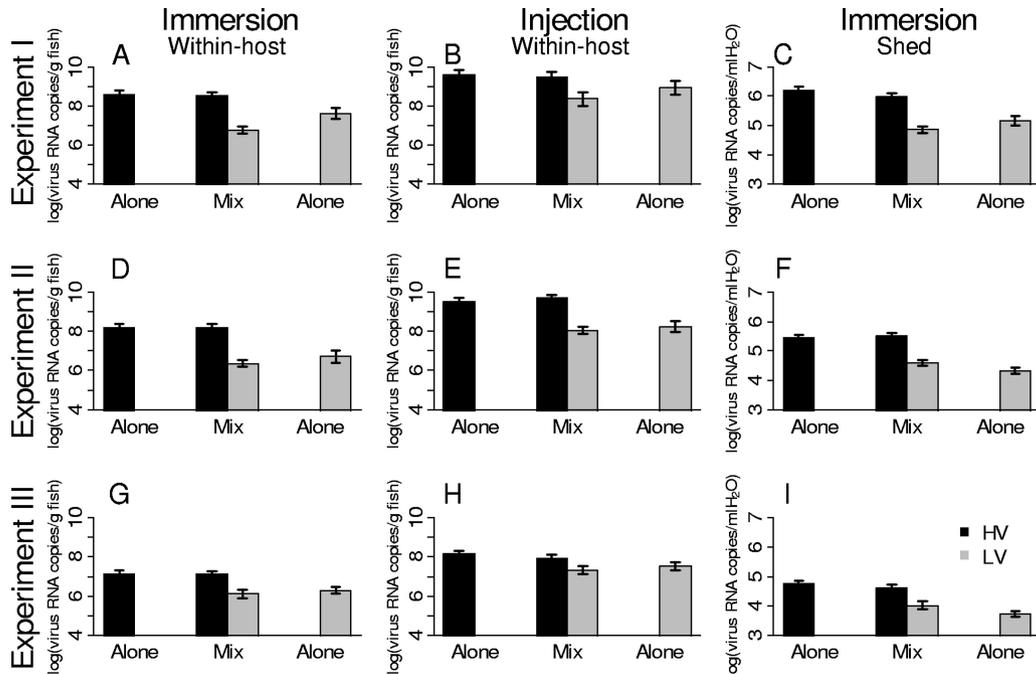


FIG. 2. Mean viral loads of fish in treatment groups in experiments I (A to C), II (D to F), and III (G to I). Panels show data for individual genotypes HV and LV from within-host immersion (A, D, and G), within-host injection (B, E, and H), or shed immersion (C, F, and I) virus populations of fish. Values are the means for all positive fish on day 3 postinfection within an experiment and treatment group, excluding fish with no detectable viral load. Error bars show 1 standard error of the mean (SEM). HV alone and LV alone come from different samples, whereas the mixed HV and LV are from the same sample. Shed virus was obtained at the time of fish harvest from the water in the immersion challenge.

genotype. This trend remained even in the injection challenges, in which host entry was bypassed, thus suggesting that the more virulent genotype had a replication advantage, independent of host entry, at the within-host replication cycle stage. The advantage of the more virulent genotype transcended to the within-host mixed-infection environment in both injection and immersion challenges, where it corresponded to a higher percentage of the total virus population and therefore had greater coinfection fitness. It is likely that the greater coinfection fitness experienced by the more viru-

lent genotype was driven primarily by its replication advantage, since the two genotypes performed equally well in a mixed infection and alone. Given that there was no consequence of competition for either genotype, there was no evidence that the genotypes were under direct competition for a limited

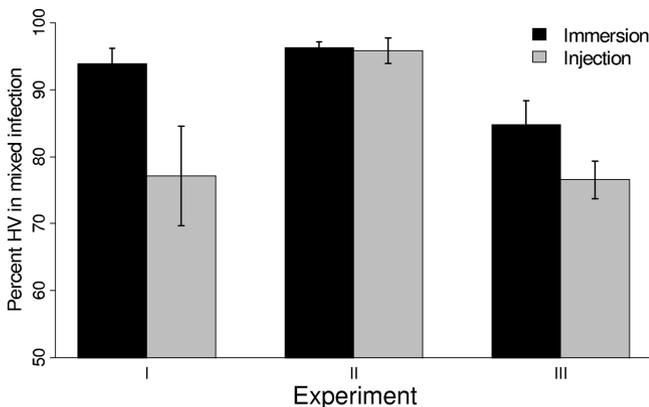


FIG. 3. Percentages of HV in the mixed infections found in within-host immersion and within-host injection samples. Values were calculated for individual fish  $\{[\text{viral load HV}/(\text{viral load HV} + \text{viral load LV})] \times 100\}$  and then averaged for all positive samples from each treatment group for each experiment. Error bars represent 1 SEM.

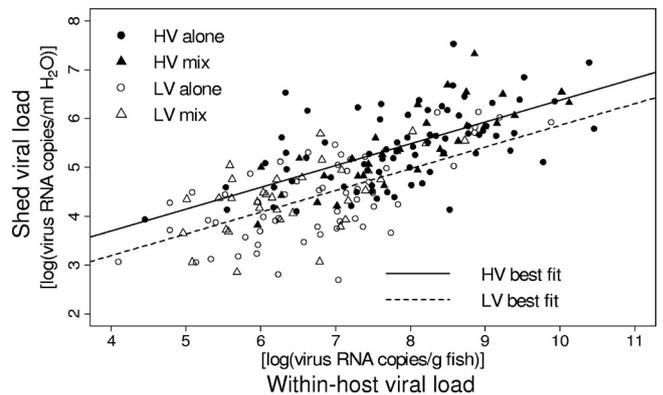


FIG. 4. Correlation of viral loads in individual fish with viral loads in the water for genotypes HV and LV at day 3 postinfection. Each point represents a single fish in experiment I, II, or III, and all samples positive for virus in both the fish and water were plotted. The best-fit lines for HV  $[\log(\text{virus copies/ml H}_2\text{O}) = \log(\text{virus copies/g fish}) \times (0.446 \pm 0.04) + (1.91 \pm 0.32)]$  and LV  $[\log(\text{virus copies/ml H}_2\text{O}) = \log(\text{virus copies/g fish}) \times (0.446 \pm 0.04) + (1.405 \pm 0.102)]$  are shown. The minimal-significance GLM model was as follows:  $\log(\text{virus copies/ml H}_2\text{O}) = \log(\text{virus copies/g fish}) + \text{genotype} + \text{experiment}$  ( $F_{1, 191} = 418 [P < 0.001; R^2 = 0.76]$ ). Competition was not significant, and mixed-infection and single-infection points were separated for graphical purposes.

resource such as host cells (see reference 59 for further discussion).

A major finding was that the degree to which HV was dominant in mixed infections was significantly reduced in injection challenges, in which the entry step was bypassed, compared to this degree in immersion challenges. This indicates that HV has an added advantage at the entry infection cycle stage, in addition to its within-host replication advantage. This demonstration of a fitness component associated specifically with host entry is a novel finding that could not be discerned in our previous immersion challenge work (59), and it would not be accessible in cell culture-based viral fitness systems.

The quantification of viral loads shed by individual fish into water added another new aspect to the *in vivo* viral fitness system that informed us about the viral infection cycle stage most relevant to transmission. In general, the viral loads shed into water correlated with viral loads in the fish, so the advantage of HV over LV observed inside the fish was reflected in the shed virus data. However, genotype HV also appeared to be more efficient at shedding, in that for a given within-host viral load, the number of genotype HV viral copies shed into the surrounding water was greater than the number of genotype LV copies. This relationship appeared to be driven by shedding kinetics rather than differences in environmental stability, because we found equivalent decay rates for HV and LV in the experimental conditions used here.

A potential complicating factor in this study is the theoretical possibility that mutations that switch the phenotype of one genotype to the other (HV to LV or vice versa) or result in the qPCR being cross-reactive may occur during these experiments. There is a substantial level of divergence (312/11,133 nucleotides) between the full genome sequences of genotypes HV and LV (2, 40). Furthermore, within the viral glycoprotein gene targeted by the qPCR assays used here, there are 55 nucleotide differences (3.5% divergence), leading to 17 amino acid changes. Although the genetic determinant of the virulence differences between these genotypes is not known, this amount of genetic divergence is sufficient that the possibility that one genotype could mutate to the other one during these experiments is negligible. We are therefore confident in the quantified phenotypic differences between the genotypes in the infection cycle fitness traits examined.

The cellular mechanisms underlying the genotype differences for each of the viral fitness traits are unknown. Studies of a different pair of IHNV genotypes from the U and M groups, which have evolved different host specificities, indicated that differential rates of viral genome replication and transcription (44), as well as immune evasion (45, 46), might be involved. Here, we observed pronounced virus fitness trait differences on day 3 postinfection, when viral density is known to have peaked in the host (45, 55). Therefore, our results support previous findings indicating that, if host immunity is involved in regulating differential growth dynamics of IHNV genotypes, it must be early innate immune functions and possibly constitutive factors which are most critical (19, 38, 45–47). In general, we have observed that the level of the host innate immune response to IHNV infection is a function of viral load rather than vice versa, and pathogenicity, in terms of histopathology, is clearly related to viral load (33, 45, 46). This pattern appears to be consistent with other rhabdoviruses, such as

vesicular stomatitis virus (VSV) and rabies virus (51). It would be interesting in the future to examine how immune regulation and the role it plays in pathogenicity might influence the long-term fitness of IHNV genotypes (14, 23).

Regardless of the mechanism, our results indicate that the more virulent genotype had an overall fitness advantage over the less virulent genotype, as defined by selection over time at the host population level. However, there is a potential fitness cost to virulence in this system, in that the more virulent genotype kills a larger percentage of the host population and thus may have a shorter transmission period (1, 11, 37, 50). It has been consistently shown that HV induces ~85% host mortality and LV induces ~30% host mortality in a 30-day period after virus exposure (25, 34, 59). The fitness consequences of this mortality difference have not been assessed here, as this would require information on total host-to-host transmission of each genotype from fish beyond the point where the mortality of HV-infected fish exceeds that of LV-infected fish. Ultimately, this would involve a quantification of long-term overall fitness for each genotype. Our goal was not to quantify overall fitness and conclusively determine if more virulent genotypes will be selected but rather to determine infection cycle fitness factors contributing to the potential fitness advantage of the more virulent IHNV genotype in terms of host entry, within-host replication, and shedding. This enabled us to examine viral infection cycle trait mechanisms by which virulence could be selected.

Clearly, additional viral traits beyond those assessed in the present study could also be positively or negatively associated with virulence. For example, we quantified coinfection fitness here by examining the relative performances of genotypes when they encountered the host at the same time. The ability of a genotype to superinfect a host already harboring an established genotype could be another important fitness parameter (38). Likewise, we compared injection and immersion challenges to independently assess the within-host replication and viral entry traits. Our assumption was that injection bypassed the host entry stage of the virus by skirting skin mucosal defenses, an important component of the fish immune system (12). This assumption is supported by findings that injection challenges typically induce higher levels of mortality than immersion challenges in rainbow trout (12, 35, 45, 48). However, the work presented here did not examine cellular entry, which is a completely separate phenomenon based largely on viral receptors. Finally, the long-term stability of the virus outside the host under changing environmental conditions is another factor which could impact the virulence-fitness association (26).

Ultimately, overall viral fitness is not a fixed parameter and varies based on a variety of complex factors, including host density, population structure, supply rate of naïve hosts, contact rates, and an array of environmental conditions, including temperature and water flow rate (4, 25, 30, 32). Here, we provided the first steps toward assessing whether virulence will be selected by determining what pathogen fitness traits are associated with virulence. A parameterization of such traits is ultimately required for the development of any population-level virulence evolution models (5). In order to better provide field-relevant parameter estimates of fitness traits, we used a genetically diverse population of trout hosts. Our data revealed

a substantial level of fish-to-fish variation, as typically observed in natural IHNV infections of fish (41, 59). The mechanisms regulating between-fish variance of IHNV are unknown and are beyond the scope of the work presented here but are currently under investigation. In the present study, to control for variation and increase our statistical power for detecting differences between treatment groups, we used large numbers of replicate hosts and repeated the entire experiment three times.

This is the third study to find results consistent with a positive relationship between IHNV virulence and viral replication fitness *in vivo*, which has now been observed in two genotype pairs and two fish host species (45, 46, 59). Additionally, studies using a third pair of IHNV genotypes, which had equal virulences, found no within-host fitness differences between the genotypes (55). Of the viral infection cycle traits examined in these studies, replication seemed to have the greatest impact on viral fitness. It remains unclear how universal these findings are for other vertebrate virus systems *in vivo*. Studies on influenza virus in ferrets and chickens have shown a positive association between virulence and replication (42, 53), whereas studies of hepatitis B virus in ducklings, foot-and-mouth disease virus (FMDV) in swine, and simian immunodeficiency virus (SIV) in primates indicate that less virulent genotypes may sometimes have the replication advantage (13, 36, 43). If replication is the main component of overall viral fitness for viruses in general, the relevance of studies of viral fitness conducted in cell culture (6, 15, 16, 27, 49, 54, 58) to infections *in vivo* would be increased. To address the general mechanisms regulating viral fitness more fully, further *in vivo* studies that consider multiple aspects of the viral infection cycle in living hosts, such as we have done here, in other systems are warranted.

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