The Role of Virulence in *In Vivo* Superinfection Fitness of the Vertebrate RNA Virus Infectious Hematopoietic Necrosis Virus

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We have developed a novel *in vivo* superinfection fitness assay to examine superinfection dynamics and the role of virulence in superinfection fitness. This assay involves controlled, sequential infections of a natural vertebrate host, *Oncorhynchus mykiss* (rainbow trout), with variants of a coevolved viral pathogen, infectious hematopoietic necrosis virus (IHNV). Intervals between infections ranged from 12 h to 7 days, and both frequency of superinfection and viral replication levels were examined. Using virus genotype pairs of equal and unequal virulence, we observed that superinfection generally occurred with decreasing frequency as the interval between exposures to each genotype increased. For both the equal-virulence and unequal-virulence genotype pairs, the frequency of superinfection in most cases was the same regardless of which genotype was used in the primary exposure. The ability to replicate in the context of superinfection also did not differ between the genotypes of equal or unequal virulence tested here. For both genotype pairs, the mean viral load of the secondary virus was significantly reduced in superinfection while primary virus replication was unaffected. Our results demonstrate, for the two genotype pairs examined, that superinfection restriction does occur for IHNV and that higher virulence did not correlate with a significant difference in superinfection fitness. To our knowledge, this is the first assay to examine the role of virulence of an RNA virus in determining superinfection fitness dynamics within a natural vertebrate host.

Measurements of relative viral fitness are often derived from competition experiments performed *in vitro*, wherein a mixture of two viral variants is used to simultaneously infect cultured cells (coinfection) and the variant with the highest titer at various sampling times is declared more fit than the other (1, 2). According to this convention, viral fitness is defined as the ability to produce infectious progeny in a given environment (3, 4). These experiments are useful to directly compare two variants and can demonstrate subtle fitness differences between two variants that may not have been evident by comparing single-infection kinetics. Recently, there have been a number of coinfection fitness studies using *in vivo* models for vertebrate viruses (2, 5–21). For example, competition experiments have been performed using many species of arbovirus in their mosquito vectors and have determined correlations between viral fitness and epidemiologic observations or recorded disease severity in humans (11, 14). Through such studies, a variety of viral traits have been associated with viral fitness, including replication rate, fidelity of genome replication, rate of viral assembly, entry, shedding, and virulence (7, 20, 22–25). However, such studies exploring fitness of vertebrate viruses *in vivo* are limited in number.

Coinfection studies are essential for measuring the differences between viral variants in their ability to enter and replicate within the host because the two variants in question compete simultaneously in an identical host environment and thus must overcome similar challenges by the host immune response. In contrast, superinfection studies, defined here as infection of a single host with multiple, genetically distinct virus variants of the same species resulting from sequential exposures to each virus, investigate the ability of a variant to compete with an established viral population and overcome the host immune response triggered as a result of prior, and continuous, infection. Thus, the environment entered by a superinfecting virus is potentially very different than that entered by the primary infecting virus. A deeper understanding of the viral traits associated with superinfection fitness may provide

insight into the mechanisms required to overcome a hostile host environment. Furthermore, it is plausible that concurrent infections with multiple viral variants in the field are more likely to occur as a result of superinfection rather than simultaneous infection. In fact, viral superinfection in the field has been reported for numerous human RNA viruses, including hepatitis C virus, dengue virus, and human immunodeficiency virus (26–28). While the importance of understanding the factors involved in viral superinfection in these systems is clear, controlled experimental infection studies performed in natural hosts face many challenges.

Numerous controlled superinfection experiments using plant virus systems have demonstrated that primary viral infection can either inhibit or enhance subsequent infection and prevent or speed the onset of disease symptoms (29–31). Interestingly, these outcomes appear to be both virus and plant species specific, with few commonalities between systems. In contrast, few reports describe experimental superinfection of vertebrates. In one example, researchers performed superinfection of domestic chickens with two strains of infectious bursal disease virus (IBDV), a double-stranded RNA virus, with the aim of determining the effect of superinfection on pathogenesis (5). They observed that infection of young chickens with a mild disease-causing strain of the virus prior to infection with a more pathogenic strain resulted in suppression of disease compared to that in chickens infected with only the pathogenic strain or chickens infected simultaneously

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with both strains. In another important study, Yeh et al. measured viral replication following sequential challenges with heterologous simian immunodeficiency virus (SIV) variants in rhesus monkeys and found that the superinfecting variant replicated to low levels (32). The time between primary and secondary virus challenge was not a controlled factor in this study; therefore, conclusions could not be made about the importance of time between infections on viral replication in superinfection. In addition to these, many studies have investigated the effect of concurrent multiple infection with viruses of different species in natural and model hosts (4, 29, 31, 33–35). Thus, the dynamics of *in vivo* superinfection with variants of the same viral species have yet to be extensively defined for a vertebrate RNA virus and may provide insight into the mechanisms behind many of the field observations of superinfection for other important vertebrate viruses.

Infection with infectious hematopoietic necrosis virus (IHNV) of the natural hosts *Oncorhynchus mykiss* (rainbow trout) and *Oncorhynchus nerka* (sockeye salmon) has been used as a tractable model system for studying viral fitness in coinfection *in vivo* (16, 17, 19, 20, 36). IHNV is a significant pathogen of salmonid species which originated in the North American Pacific Northwest and has since spread to Europe and Asia (37). We previously demonstrated that natural genetic viral variants collected from the field (hereafter referred to as genotypes) vary significantly in virulence in rainbow trout (20, 38, 39). Virulence of IHNV variants is defined here as mortality caused to a host as a result of viral infection. *In vivo* coinfection competition between two virus genotypes of equal relative virulence (genotype B and genotype C) revealed that these viruses were also of equal relative fitness (17, 36). Interestingly, *in vivo* coinfection competition experiments with variants displaying high virulence (genotype HV) and low virulence (genotype LV) demonstrated that observed differences in virulence correlated with significant differences in in-host replication as well as host entry and virus shedding (19, 20). Importantly, higher virulence was consistently associated with greater viral fitness in multiple traits associated with coinfection. Therefore, the HV and LV strains used in the present work have high and low coinfection fitness, respectively, as well as high and low virulence, in rainbow trout.

To expand our understanding of viral fitness to include fitness in superinfection, we have developed an *in vivo* superinfection assay using IHNV infection of rainbow trout. This assay allowed us to examine superinfection dynamics and investigate the effect of time between exposures to each genotype on the relative ability of IHNV genotypes to establish secondary infection and replicate during superinfection. We performed duplicate superinfection experiments using this assay with 5 time intervals between exposures using two virus genotype pairs: one pair of equal relative virulence and one pair of unequal relative virulence. This work comprises a detailed investigation into superinfection fitness and virulence using controlled *in vivo* assays in a natural vertebrate host-virus system.

MATERIALS AND METHODS

Ethics statement. All animal procedures were completed in accordance with recommendations by the American Association for Laboratory Animal Science and approved under a University of Washington IACUC protocol (UW 3042-12-3042-14). All effort was taken to minimize suffering.

FIG 1 *In vivo* superinfection assay design. (A) Diagram describing the *in vivo* superinfection assay. (B) Treatment groups for each superinfection experiment, illustrated for assays using genotypes B and C. Assays with genotypes HV and LV were performed with the same design. gt, genotype; n, number of individual fish used in each group.

Virus and host. Four genetically distinct isolates (genotypes) of IHNV were used in this study, herein labeled B, C, HV, and LV. All genotypes were originally obtained from farm-cultured rainbow trout in the field and subsequently characterized for virulence (17, 19, 40). All four genotypes belong to the M genogroup of IHNV and thus have evolved host specificity for rainbow trout (41). Genotypes HV and LV were previously referred to as 220-90 and WRAC/039-82, respectively (42). Genotypes B and C were previously described as FF020-91/MB20 and FF030-91/MC30, respectively (17). All virus genotypes were maintained through propagation, between 4 and 9 passages, on cyprinid fish epithelioma papulosum cyprini (EPC) cells and stored at -80°C as described elsewhere (19). Preparation of working stocks of virus and quantification by plaque assay were performed as previously described (19). The stability of these genotypes in static water has been assessed previously, and the genotype pairs used here were found to have equal stability for the duration of 12-h challenge conditions (data not shown). Genotype HV has consistently been shown to have greater virulence than genotype LV and typically induces approximately 80% mortality in exposed fish, whereas LV induces approximately 20% mortality at the same challenge dosage (19). Genotypes B and C have consistently been shown to have equal virulence, both inducing about 65% mortality in exposed fish (39).

All experiments were performed using juvenile, 1- to 2-g, specific-pathogen-free, research-grade rainbow trout (*Oncorhynchus mykiss*) obtained from Scott LaPatra, Clear Springs Foods, Inc. Fish were maintained in pathogen-free water at 15°C . Multiple lots of fish were used for the experiments described here. Each lot of juvenile rainbow trout used for experimentation was held in a single tank as one population prior to use.

***In vivo* superinfection assay.** A summary schematic of the assay can be found in Fig. 1A. For each experiment, 79 individuals were randomly selected from a single lot of fish (one population) and netted into individual 1.5-liter tanks in a stand-alone tower system (Aquatic Habitats). Each treatment group was randomly assigned 9 to 15 fish, with the exception of the mock control group, which was assigned 4 fish (Fig. 1B). Tanks were arranged in the tower system in a blocked design so that each row contained individuals from all treatments to control for row effects. Water temperature was held at a constant 15°C for the entirety of the experiment. Each tank received 200 ml water prior to introduction of the fish. Chal-

challenge was initiated by adding 1 ml of water containing 4×10^7 PFU/ml of a single genotype to each individual tank (final concentration, 2×10^5 PFU/ml). Fish were then held in static challenge for 12 h. Fish undergoing a mock challenge were exposed to 1 ml water containing minimum essential medium (Flow Laboratories, Rockville, MD) in place of virus. Following 12 h of static immersion challenge, water lines were connected to each tank and water flow was initiated and allowed to flow for at least 1 h, which has been shown to remove all detectable challenge inoculum from the tank (data not shown). Previous experiments have demonstrated that 1 h of water flow through the tanks is sufficient to remove any virus detectable by quantitative reverse transcription-PCR (qRT-PCR) from the water in the tanks (20). Fish were held in flowthrough water conditions for the desired interval time before secondary challenge. Interval time was calculated to be the time between the starts of each challenge. For example, for the 24-h challenge interval, fish were held for 12 h in static water with the primary virus genotype and 12 h in flowthrough conditions and then challenged for 12 h under static conditions with the secondary virus genotype (equals a total of 24 h between initiation of primary challenge and initiation of secondary challenge). All fish were held in flowing water conditions for 72 h following the completion of secondary challenge, and then all the fish in the experiment were euthanized by adding an overdose of MS-222 solution buffered with sodium bicarbonate directly to each tank. Each individual fish was placed into an individual bag and stored at -80°C until RNA extraction, described below.

Two independent experiments were performed with each time interval between exposures for each genotype pair. The two experiments with the same time interval and the same genotype pair were performed with a different lot of rainbow trout to ensure independence.

In-host viral replication and Mx-1 gene expression. For each experiment, 114 individual fish were randomly selected from a single lot of fish (one population) and netted into individual 1.5-liter tanks in a stand-alone tower system (Aquatic Habitats) as described above. Each treatment group was randomly assigned 45 fish, with the exception of the mock control group, which was assigned 24 fish. Challenge or mock treatment was initiated as described above, and fish were then held in static challenge for 12 h. Water flow was then initiated and continued for the entirety of the experiment. At sampling time points of 0, 13, 24, 48, 72, 96, 120, 144, and 168 h after initiation of challenge, 5 fish from each virus-challenged group and 3 fish from the mock-challenged group were euthanized and stored at -80°C as described above.

Viral load quantification. Total RNA was extracted from individual whole fish using guanidine-thiocyanate as described previously (19). All samples then underwent a general cDNA synthesis reaction using random hexamer and oligo(dT) primers that transcribed all viral genotypes and host RNA equally (19). For quantification of viral load, cDNA was diluted 1/10 and then subjected to quantitative PCR using genotype-specific assays targeting the virus glycoprotein gene as previously described (19). Briefly, the qPCRs consisted of 5 μl diluted cDNA, 200 nM genotype-specific minor groove binder (MGB) probe, and 900 nM each genotype-specific primer in a 12- μl total reaction volume as described elsewhere (36). A control reaction mixture containing RNA transcripts of the glycoprotein genes of each genotype were included in each set of cDNA synthesis reactions and then utilized in each qPCR run to generate an 8-step, 10-fold dilution series standard curve for each assay (19). Since the qRT-PCR methodology quantifies RNA copies, it provides a measure of total viral RNA (both genome and message) of each genotype and not a direct measure of infectious particles (43). Therefore, we use the term "viral load" to refer to total viral genome copies plus viral mRNA per gram of host tissue at the time of harvest. A consistent relationship between viable virus quantities determined by plaque assay and qPCR viral load has been reported for IHNV in rainbow trout (43).

Measurement of Mx-1 induction. cDNA derived from individual whole fish used for the viral growth kinetic experiments was also used for qPCR analysis of rainbow trout *Mx-1* transcription in IHNV-infected versus mock-infected fish. The primer and probe sequences for this assay

have been previously described (44). The expression level of *Mx-1* was normalized against the expression level of a housekeeping gene, acidic ribosomal phosphoprotein P0 (ARP) (44). Analysis and calculation of fold change relative to mock-infected control groups have also been previously described (44).

Statistical analysis. All statistical tests were carried out in R (version 2.11.1).

Superinfection frequency data were analyzed in two ways, exact multinomial goodness-of-fit and Fisher exact tests. To examine whether the ability of a virus genotype to infect a fish was influenced by the presence of another virus genotype in the host, we conducted a multinomial goodness-of-fit analysis. This analysis tests whether or not the frequency of falling into each of the infection status categories (superinfected, primary virus only, secondary virus only, uninfected) is significantly different than the frequency expected based on the single-exposure treatments. In other words, it is testing whether or not the observed frequency of fish in the four infection categories is independent of exposure type (superinfected versus single). This test utilizes the observed frequencies in single exposure to calculate expected frequencies in superinfection exposure by the following formula: expected = (ratio of infected to uninfected for relevant primary single-infection control $\times$ ratio of infected to uninfected relevant secondary single-infection control) $\times$ the number of fish in the superinfection group. For example, the expected number of fish to be superinfected when B is the primary virus is calculated as follows: observed ratio of infected to uninfected in B $\rightarrow$ mock single-infection group $\times$ observed ratio of infected to uninfected in mock $\rightarrow$ C single-infection group $\times$ total number of fish challenged in superinfection group B $\rightarrow$ C. This analysis was conducted for each experiment at each interval and each superinfection group (B first and C second, etc.). This resulted in 20 total tests per genotype pair. To control for inflated type I error due to multiple tests, we utilized Bonferroni's adjustment ($\alpha = 0.05/20 = 0.0025$) (45). We used this correct α value to determine statistical significance at a P value of <0.0025 .

To examine if the ability of a genotype to infect a fish was dependent on the order of exposure in superinfection, Fisher exact tests were utilized. This test compares the frequencies of fish falling into the four infection categories (superinfected, primary only, secondary only, uninfected) between the two superinfection exposure treatments (genotype X first and Y second, genotype Y first and X second) in each experiment. The null hypothesis is that the distribution of fish among the four categories is independent of which genotype was used for the primary exposure. We carried out this test for each experiment and genotype pair (HV versus LV, B versus C). This resulted in 10 tests total. To correct for inflated type I error due to multiple tests, we utilized Bonferroni's adjustment ($\alpha = 0.05/10 = 0.005$) (45). We used this correct α value to determine statistical significance at a P value of <0.005 .

To determine if there were statistical differences in viral load between treatment groups, general linear models (GLM) were conducted. The dependent variable was viral load, with the independent factors being genotype (B or C and HV or LV), competition (superinfection or single infection), order (first or second), and interval period (12, 24, 48, 96, or 168 h). We initially included experiment as a factor (levels = 1 and 2), but it was not found to be significant so it was dropped from all further analyses. The experiments were designed to control for variation in viral load due to timing of exposure, i.e., single infections were conducted at both the primary and secondary time points (Fig. 1B). This made it possible to compare the viral load attributed to a genotype in the superinfection treatment group to its corresponding single-infection control (i.e., secondary superinfection to secondary single infection) and control for variation which might be induced by exposure timing, thus avoiding non-biologically relevant comparisons (single primary to superinfection secondary and vice versa). As such, the GLM analysis was split into four tests per genotype pair to minimize these non-biologically relevant comparisons. Test 1 was competition by genotype by interval for virus administered at the primary infection period. Test 2 was competition by genotype by interval

for virus used at the secondary infection period. Test 3 was order by competition by interval for a single genotype in the pair of interest. Test 4 was order by competition by interval for the other genotype in the pair of interest. This resulted in 8 separate tests. To control for inflated type I error due to multiple tests, we utilized Bonferroni's adjustment ($\alpha = 0.05/8 = 0.00625$). For interaction terms that were found significant by GLM, Tukey's honestly significant difference (HSD) tests were conducted to determine the precise treatment groups (levels of factors) that differed. Here we focused on biologically relevant comparisons. Viral load data were log transformed to meet test assumptions of normality and homogeneity of variances. The GLM analysis includes only fish positive by qPCR and, in the case of superinfection treatments, those which were positive for both genotypes. The analysis was also conducted setting all negative fish to the minimum detection threshold of qPCR (100 copies/g fish). This alternative analysis did not change the overall conclusions for either genotype pair.

RESULTS

Superinfection assay design. To investigate superinfection *in vivo*, we have developed a novel assay, illustrated in Fig. 1A. For this assay, we defined 8 treatment groups as shown in Fig. 1B. Groups 1 and 2 are designated reciprocal superinfection groups that were sequentially exposed to both viral genotypes. Groups 3 and 4 were exposed to just one genotype during the primary exposure period and then subsequently exposed to virus-free medium (mock exposed) during the secondary exposure period. Groups 5 and 6 were exposed to virus-free medium during the primary exposure period and then subsequently exposed to only one of the genotypes during the secondary exposure period. Groups 3 to 6 served as single-exposure controls and were used to inform the interpretation of observations from the superinfection groups. For most experiments, we included a coinfection group exposed to a 1:1 ratio of both genotypes during the secondary exposure period. Finally, for all experiments, we included a group exposed to virus-free medium during each exposure period to serve as a mock control.

Superinfection using equal-virulence genotype pair B and C.

(i) Frequency of superinfection. To assess the frequency with which superinfection occurred for the equal-virulence genotype pair B and C (17), individual fish from the superinfection groups were analyzed for the presence or absence of each virus genotype. Superinfection was concluded to have occurred when a fish sample was found to be positive for the presence of both viral genotypes at the time of harvest. Figure 2 shows the mean percentage of fish from superinfection groups infected with detectable amounts of both viral genotypes at the time of harvest for two independent experiments performed for each time interval. In experiments with 12- and 24-h intervals between exposures, the mean percentage of fish that were superinfected ranged from 60 to 73%. For interval periods from 24 to 96 h, as the time interval between exposures to each genotype increased, the percentage of fish found to be superinfected decreased. However, with an interval of 168 h between exposures to each viral genotype, the percentages of fish infected with both genotypes increased compared to the 96-h interval. For all intervals, the majority of fish that were not superinfected at the time of harvest were infected with only the virus used for the primary infection.

A central goal of this work was to determine whether restriction of secondary infection occurs in rainbow trout already infected with IHNV. Thus, our null hypothesis was that if the restriction of secondary infection does not occur, the probability of

FIG 2 Frequency of superinfection in groups exposed to genotypes B and C (equal-virulence pair) with increasing time intervals between exposures. Black bars represent the percentages of superinfection in groups of fish exposed to genotype C before genotype B. White bars represent the percentages of superinfection in groups of fish exposed to genotype B before genotype C. The gray bar represents the percentage of fish infected with both genotypes following exposure to both genotypes at a 1:1 ratio during the secondary exposure period (coinfection control). Percentages are an average of two independent experiments ($\pm$ the standard error of the mean [SEM]).

observing superinfection in individual fish sequentially exposed to both genotypes would be equal to the probability of observing single infections in individual fish exposed only to one genotype at the relevant exposure times. To test this hypothesis, we used the observed infection frequencies in the single-infection control groups to determine the expected numbers of fish from the superinfection treatment groups that should fall into each of four infection categories: superinfected, infected with primary genotype, infected with secondary genotype, and uninfected. These data for genotypes B and C are shown in Table 1. These observed and expected frequencies were then used for analysis in an exact multinomial goodness-of-fit test to determine the probability of observing a given distribution among infection categories for fish in the superinfection groups. For each of two experiments performed at each time interval, we found that the distribution of individual fish observed within the infection categories differed significantly from what would have been expected under the null hypothesis (exact multinomial goodness of fit; $P < 0.05$; Bonferroni adjusted) (Table 1). We found that superinfection in this system occurred less often than would be expected, and single infection with only the genotype used to establish the primary infection occurred more often than expected under the null hypothesis. This result suggests that primary infection significantly restricts subsequent infection at all intervals tested, beginning at 12 h postinfection.

In addition, we were interested in whether the distribution of individual fish into the four infection status categories was dependent on which genotype was used first (i.e., B or C). For all experiments with all intervals examined, the distribution of fish across the four infection categories was not found to depend on the order of exposure to each genotype (Fisher exact test; $P < 0.05$; Bonferroni adjusted) (Table 1). This result suggests that the observed restriction of secondary infection did not depend on the genotype used for primary infection and that genotypes B and C of equal relative virulence have equal abilities to restrict, or establish, secondary infection.

(ii) Impact of superinfection on viral load. To determine the

TABLE 1 Distribution of fish in infection status categories for genotypes B and C

Interval (expt no.)	Genotype exposure ^a		No. of fish with each observed infection status (no. of fish expected to have each infection status) ^b				Adjusted <i>P</i> value by ^c :	
	1°	2°	Superinfection	1° only	2° only	Uninfected	Goodness of fit ^d	Fisher exact test ^e
12 h (1)	B	C	4 (10)	4 (0)	1 (0)	1 (0)	0.000	1.000
	C	B	5 (8)	3 (0)	0 (0)	0 (0)	0.000	
	B	Mock		6	0	0		
	C	Mock		3	0	0		
	Mock	B		0	6	0		
	Mock	C		0	6	0		
12 h (2)	B	C	13 (15)	0 (0)	2 (0)	0 (0)	0.000	0.367
	C	B	9 (13.3)	5 (1.7)	1 (0)	0 (0)	0.000	
	B	Mock		9	0	0		
	C	Mock		9	0	0		
	Mock	B		0	8	1		
	Mock	C		0	9	0		
24 h (1)	B	C	10 (15)	5 (0)	0 (0)	0 (0)	0.000	1.000
	C	B	9 (13.3)	5 (1.7)	1 (0)	0 (0)	0.000	
	B	Mock		9	0	0		
	C	Mock		9	0	0		
	Mock	B		0	8	1		
	Mock	C		0	9	0		
24 h (2)	B	C	12 (13.3)	3 (0)	0 (1.7)	0 (0)	0.000	0.769
	C	B	7 (11.8)	8 (1.5)	0 (1.5)	0 (0.18)	0.006	
	B	Mock		8	0	1		
	C	Mock		8	0	1		
	Mock	B		0	8	1		
	Mock	C		0	9	0		
48 h (1)	B	C	7 (10.5)	4 (0)	4 (4.5)	0 (0)	0.000	0.315
	C	B	2 (15)	10 (0)	1 (0)	2 (0)	0.000	
	B	Mock		7	0	3		
	C	Mock		9	0	0		
	Mock	B		0	8	0		
	Mock	C		0	9	0		
48 h (2)	B	C	3 (11.7)	10 (0)	1 (3.3)	1 (0)	0.000	1.000
	C	B	0 (11.7)	15 (0)	0 (3.3)	0 (0)	0.000	
	B	Mock		8	0	1		
	C	Mock		3	0	6		
	Mock	B		0	8	1		
	Mock	C		0	9	0		
96 h (1)	B	C	7 (13.3)	6 (0)	0 (1.7)	2 (0)	0.000	0.388
	C	B	8 (11.7)	4 (3.3)	2 (0)	1 (0)	0.000	
	B	Mock		7	0	2		
	C	Mock		7	0	2		
	Mock	B		0	9	0		
	Mock	C		0	9	0		
96 h (2)	B	C	0 (10)	9 (0)	4 (5)	2 (0)	0.000	0.367
	C	B	0 (11.7)	14 (3.3)	0 (0)	1 (0)	0.000	
	B	Mock		8	0	1		
	C	Mock		9	0	0		
	Mock	B		0	7	2		
	Mock	C		0	9	0		
7 days (1)	B	C	2 (15)	9 (0)	1 (0)	2 (0)	0.000	0.950
	C	B	0 (13.3)	14 (1.7)	0 (0)	0 (0)	0.000	
	B	Mock		6	0	3		
	C	Mock		7	0	2		
	Mock	B		0	9	0		
	Mock	C		0	9	0		

(Continued on following page)

TABLE 1 (Continued)

Interval (expt no.)	Genotype exposure ^a		No. of fish with each observed infection status (no. of fish expected to have each infection status) ^b				Adjusted <i>P</i> value by ^c :	
	1°	2°	Superinfection	1° only	2° only	Uninfected	Goodness of fit ^d	Fisher exact test ^e
7 days (2)	B	C	4 (9.1)	6 (2.6)	3 (2.6)	2 (0.7)	0.000	1.000
	C	B	3 (11.8)	11 (1.5)	0 (1.5)	1 (0.2)	0.000	
	B	Mock		9	0	0		
	C	Mock		9	0	0		
	Mock	B		0	8	1		
	Mock	C		0	9	0		

^a 1°, primary infection; 2°, secondary infection.
^b Expected infection status is calculated as the proportion of fish likely to be in each category based on the observed infection frequencies of single-infection groups multiplied by the total number of fish in the superinfection group.
^c *P* values following Bonferroni's adjustment.
^d An exact multinomial test was used to determine whether the observed distribution of fish in infection status categories is equal to the expected distribution based on single-infection controls (null hypothesis). An analysis was performed for each superinfection group for each experiment. A *P* value of <0.05 indicates support to reject the null hypothesis, i.e., the observed distribution does not equal the expected distribution.
^e The Fisher exact test was used to determine whether the distribution of fish in infection status categories was independent of the genotype used for primary infection (null hypothesis). A *P* value of <0.05 indicates support to reject the null hypothesis, i.e., distribution is not independent of the genotype used first.

impact of superinfection on the ability of each genotype to replicate within the host, we compared the mean viral load of each genotype in superinfected fish with the mean viral load in single-infection group fish exposed to that same genotype during the corresponding exposure period (Fig. 3). Experiments performed with a 96-h interval between exposures were not included in this analysis because no fish were found to be superinfected in groups exposed to genotype C before genotype B in either experiment

FIG 3 Impact of superinfection on viral load for all experiments performed with genotypes B and C, where B is used for primary infection (A) or C is used for primary infection (B). Viral loads attributed to genotype C (black squares) and genotype B (white diamonds) in individual fish from each treatment group (x axis) are shown for replicate experiments for each time interval between exposures as indicated. Horizontal dotted lines indicate detection levels for the genotype-specific qRT-PCR assays. Differences between groups were analyzed by analysis of variance (ANOVA) with Bonferroni's correction.

FIG 4 Viral growth kinetics and *Mx-1* induction in single infections with genotypes B and C. (A) Mean log viral load in 5 fish sampled at each time point, excluding virus-negative samples, in rainbow trout following single exposure using our superinfection protocol conditions with genotype B (white diamonds) or genotype C (black squares) ($\pm$ SEM). The horizontal dotted line indicates detection levels for the genotype-specific qRT-PCR assays. "Num pos/total" indicates the prevalence of virus-positive fish out of total fish sampled at each time point for each genotype. (B) Mean normalized *Mx-1* transcription fold changes, relative to the mock control group, in the same fish shown in the viral load curves (A). Results of fish exposed to genotype B (white diamonds) and genotype C (black squares) are shown.

performed. We used general linear models (GLM) to determine the statistical significance of these trends.

For genotype B, there was a significant effect of superinfection on viral load that was dependent on the order of exposures to each genotype (competition*order interaction; $F_{1,223} = 13.56$, $P = 0.0046$). Using a *post hoc* Tukey Honestly significant difference (HSD) test, we determined that this significance was likely driven by a decrease in the viral load of genotype B in superinfected fish compared to that in fish singly infected with genotype B during the secondary exposure period. This decrease in viral load during superinfection was not observed when genotype B was used to establish the primary infection. When this same analysis was performed for genotype C, we found that the observed greater replication of the primary infection was statistically significant prior to Bonferroni's adjustment for multiple tests but was not significant following adjustment (competition*order interaction; $F_{1,228} = 5.78$, unadjusted $P = 0.0169$, adjusted $P = 0.204$). Based on this difference, we performed a separate GLM analysis to test whether the replication of each genotype might be differently affected by superinfection competition. However, this analysis determined no significant dependence of the superinfection effect on genotype during the secondary exposure period (competition*genotype interaction; $F_{1,222} = 0.065$, $P = 1$). This suggests that the genotypes do not differ significantly in their ability to replicate in the presence of another genotype within the same

FIG 5 Frequency of superinfection in groups exposed to genotypes HV and LV (unequal-virulence pair) with increasing intervals between exposures. Black bars represent the percentages of superinfection in groups of fish exposed to genotype HV before genotype LV. White bars represent the percentages of superinfection in groups of fish exposed to genotype LV before genotype HV. The gray bar represents the percentage of fish infected with both genotypes following exposure to both genotypes at a 1:1 ratio during the secondary exposure period. Percentages are an average of two independent experiments ($\pm$ SEM).

host. However, the secondary virus demonstrated a significant replication disadvantage in superinfection compared to its performance in single infection whereas the primary virus did not experience this disadvantage. This pattern was consistently observed across all delay intervals between exposures, indicating that time between exposures did not significantly influence the impact of superinfection on viral replication.

(iii) *Mx-1* expression does not correlate with timing of superinfection restriction. As a preliminary investigation into the mechanism of the observed superinfection restriction, we performed single infections with either genotype B or genotype C and measured both viral load and *Mx-1* gene expression in five individual whole fish at each time point corresponding to the timing of secondary exposures (13, 24, 48, 96, and 168 h). As an alpha interferon-stimulated gene, *Mx-1* gene expression is known to be induced, along with many other interferon-stimulated genes, as part of a strong innate immune response following IHNV infection in rainbow trout (44, 46, 47). Mean viral loads of fish infected with either genotype B or genotype C sampled at points up to 7 days postinfection are shown in Fig. 4A. These viral growth curves demonstrated that these two genotypes had similar replication kinetics within the host. As Fig. 4B demonstrates, *Mx-1* expression was upregulated beginning at 72 h following exposure to each genotype and continued to increase as the infection progressed. However, superinfection restriction was observed beginning at 12 h after exposure to the primary genotype, and therefore, the kinetics of measurable *Mx-1* response did not correlate with the onset of restriction.

Superinfection using the unequal-virulence genotype pair HV and LV. (i) Frequency of superinfection. The mean percentage of fish found to be superinfected following exposure using the low- and high-virulence IHNV genotypes (HV and LV, respectively) with variable intervals between exposures is shown in Fig. 5. As seen with the equal-virulence genotype pair, superinfection frequency decreased as time between exposures increased. Additionally, we determined that for groups with greater than 12 h between exposures, the frequency of superinfection observed

differed significantly from the frequency that would have been expected based on single-infection control groups, indicating that superinfection restriction occurs with this genotype pair (exact multinomial goodness of fit; $P < 0.05$; Bonferroni adjusted). Infection status data from these experiments are shown in Table 2.

To test whether high virulence was associated with a greater ability to establish a secondary infection for these genotypes, we compared the frequency of superinfected fish in groups exposed to genotype LV prior to genotype HV with the frequency of superinfection when exposed to HV prior to LV. For some experiments, there was a significant difference in the distribution of fish into the four infection categories when comparing the reciprocal superinfection groups (Fisher exact test; $P < 0.05$; Bonferroni adjusted) (Table 2). The frequency of superinfection in groups of fish exposed to LV followed by HV was significantly greater than that in the reciprocal group for one of two experiments performed at intervals of 24 h, 48 h, and 7 days between exposures. These findings were reversed for one of two experiments with a 96-h interval between exposures (Fig. 5).

(ii) Impact of superinfection on viral load. To test the ability of HV and LV to replicate during superinfection, we again compared the viral load of each genotype in single infections to the viral load of the same genotype in the context of a superinfection (Fig. 6). As was observed for the equal-virulence pair, there was a significant superinfection effect that was dependent upon the order of exposures to each genotype (competition*order interaction; $F_{1,247} = 31.23$, $P = 0.001$, and $F_{1,228} = 24.19$, $P < 0.001$, for HV and LV, respectively). As with the equal-virulence pair, while the primary virus appeared unaffected, the virus genotype used during the secondary exposure period in superinfection groups replicated to significantly lower levels than in groups exposed to single genotypes during the secondary exposure period (Fig. 6).

To address the question of whether virulence was associated with the ability to replicate in superinfection for genotypes HV and LV, we compared the impacts of superinfection on viral load of the two genotypes and found no significant difference (competition/genotype interaction; $F_{1,236} = 0.0043$, $P = 1$). Thus, measured viral loads of both genotypes were equally impacted by superinfection and differences in virulence did not correlate with viral replication in superinfection. As seen for the equal-virulence pair, similar patterns were observed across intervals and, as such, the interval period did not significantly influence the impact of superinfection on viral replication.

(iii) *Mx-1* expression does not correlate with timing of superinfection restriction. We performed single infections with either genotype HV or genotype LV and measured both viral load and *Mx-1* gene expression in five individual whole fish at each time point corresponding to the timing of secondary exposures. In addition to the time points assayed for genotypes B and C, fish were sampled at time zero, which corresponds to the beginning of the challenge period. As observed for the genotype pair B and C, the kinetics of replication of HV and LV were very similar (Fig. 7A). Expression of *Mx-1* was significantly induced beginning 48 h after infection (Fig. 7B). Thus, we again observed a lag in *Mx-1* upregulation relative to superinfection restriction, which began at 24 h following primary infection (Fig. 5).

DISCUSSION

This study was designed for two purposes, to characterize the *in vivo* dynamics of superinfection and to assess the superinfection

fitness of two pairs of IHN virus genotypes in rainbow trout. While the superinfection dynamics were quantified by measuring the overall frequency of superinfection and the effect of superinfection on viral load at different delay times between pathogen exposures, the superinfection fitness of each genotype was measured by comparing the relative abilities of each genotype to establish secondary infection or to replicate within a superinfected host. By using genotype pairs of equal and unequal virulence, we were also able to provide a preliminary assessment of the importance of virulence for *in vivo* superinfection fitness. The results presented here apply only to the specific virus genotypes tested here and do not support a generalized conclusion regarding the role of virulence in superinfection fitness. However, our results with genotypes HV and LV suggest that higher virulence should not be assumed to correlate with significantly greater superinfection fitness.

Superinfection was observed in most groups at each time interval between exposures, but with decreasing frequency as the time between exposures increased. The observed restriction of superinfection within a single host suggests that competition, either direct or apparent, occurs between the two genotypes. Direct competition may be responsible for restriction if primary infection results in saturation of sufficient target cells within the host such that the secondary, superinfecting virus does not have access to susceptible cells and thus cannot establish a productive, detectable infection. Biacchesi et al. demonstrated that superinfection of virus-susceptible EPC cells with IHN virus and a closely related rhabdovirus species, viral hemorrhagic septicemia virus, is restricted (48). This refractory state is hypothesized to be a result of either competition for host entry receptors or competition for endocytotic machinery (48). The latter mechanism has been reported for cellular superinfection exclusion by another rhabdovirus, vesicular stomatitis virus, in cultured mammalian cells (49).

However, direct competition within the host seems unlikely in our system when considering results from *in vivo* coinfection studies previously performed using these same IHN virus genotype pairs (17, 19, 20, 36). None of the IHN virus genotypes used for coinfection studies demonstrated a significant reduction in replication in simultaneous coinfection competition compared to single-infection controls (19, 36). This was observed even though fish in coinfection groups were initially challenged with twice the total virus compared to fish in single-infection groups, and total measured viral load was, at times, doubled in coinfecting fish compared to that in singly infected fish (19, 20). These results suggest that host tissues and target cells for replication were not limited in coinfection. However, this possibility cannot be ruled out for the case of superinfection. Target cells required for entry into the host may be saturated or killed during the primary infection, inhibiting a secondary virus from entering. Similarly, as a primary infection progresses within the target hematopoietic tissues of an individual fish, viral spread and necrosis may leave very few naive, susceptible cells for a secondary incoming virus to establish a productive and detectable infection. However, if this were true, one might expect to see a continual decrease in viral load of the secondary virus as the interval between primary and secondary infection increases, a result which was not observed in our system.

Apparent competition has been described as a refractory state resulting from immune stimulation following primary viral infection (50). For IHN virus infection in rainbow trout, it has been thoroughly documented that, within the first few days of infection, the host response is dominated by a strong induction of numerous

TABLE 2 Distribution of fish in infection status categories for genotypes HV and LV

Interval (expt no.)	Genotype exposure ^a		No. of fish with each observed infection status (no. of fish expected to have each infection status) ^b				Corrected <i>P</i> value by ^c :	
	1°	2°	Superinfected	1° only	2° only	Uninfected	Goodness of fit ^d	Fisher exact test ^e
12 h (1)	HV	LV	10 (15)	4 (0)	1 (0)	0 (0)	0.000	1.000
	LV	HV	13 (15)	2 (0)	0 (0)	0 (0)	0.000	
	HV	Mock	0	9	0	0		
	LV	Mock	0	9	0	0		
	Mock	HV	0	0	9	0		
	Mock	LV	0	0	9	0		
12 h (2)	HV	LV	7 (6.7)	6 (8.3)	0 (0)	2 (0)	0.000	1.000
	LV	HV	10 (10)	5 (0)	0 (5)	0 (0)	0.000	
	HV	Mock	0	9	0	0		
	LV	Mock	0	6	0	3		
	Mock	HV	0	0	9	0		
	Mock	LV	0	0	4	5		
24 h (1)	HV	LV	6 (10)	9 (5)	0 (0)	0 (0)	1.000	1.000
	LV	HV	10 (15)	5 (0)	0 (0)	0 (0)	0.000	
	HV	Mock	0	9	0	0		
	LV	Mock	0	9	0	0		
	Mock	HV	0	0	9	0		
	Mock	LV	0	0	6	3		
24 h (2)	HV	LV	2 (15)	13 (0)	0 (0)	0 (0)	0.000	0.000
	LV	HV	12 (15)	1 (0)	1 (0)	0 (0)	0.000	
	HV	Mock	0	9	0	0		
	LV	Mock	0	9	0	0		
	Mock	HV	0	0	9	0		
	Mock	LV	0	0	9	0		
48 h (1)	HV	LV	1 (13.3)	12 (0)	0 (1.7)	2 (0)	0.000	1.000
	LV	HV	3 (15)	11 (0)	1 (0)	0 (0)	0.000	
	HV	Mock	0	8	0	1		
	LV	Mock	0	9	0	0		
	Mock	HV	0	0	9	0		
	Mock	LV	0	0	9	0		
48 h (2)	HV	LV	0 (13.3)	15 (1.7)	0 (0)	0 (0)	0.000	0.000
	LV	HV	6 (15)	2 (0)	5 (0)	2 (0)	0.000	
	HV	Mock	0	9	0	0		
	LV	Mock	0	9	0	0		
	Mock	HV	0	0	9	0		
	Mock	LV	0	0	8	1		
96 h (1)	HV	LV	1 (13.3)	8 (1.7)	2 (0)	3 (0)	0.000	1.000
	LV	HV	1 (8)	3 (1)	5 (5.3)	5 (0.7)	0.000	
	HV	Mock	0	8	0	0		
	LV	Mock	0	6	0	4		
	Mock	HV	0	0	8	1		
	Mock	LV	0	0	8	1		
96 h (2)	HV	LV	7 (9.1)	7 (2.6)	0 (2.6)	1 (0.7)	0.308	0.043
	LV	HV	0 (10)	13 (0)	0 (5)	2 (0)	0.000	
	HV	Mock	0	7	0	2		
	LV	Mock	0	6	0	3		
	Mock	HV	0	0	9	0		
	Mock	LV	0	0	7	2		
7 days (1)	HV	LV	1 (13.3)	11 (0)	0 (1.7)	2 (0)	0.000	1.000
	LV	HV	1 (8.3)	9 (0)	1 (6.7)	4 (0)	0.000	
	HV	Mock	0	8	0	1		
	LV	Mock	0	5	0	4		
	Mock	HV	0	0	9	0		
	Mock	LV	0	0	9	0		

(Continued on following page)

TABLE 2 (Continued)

Interval (expt no.)	Genotype exposure ^a		No. of fish with each observed infection status (no. of fish expected to have each infection status) ^b				Corrected <i>P</i> value by ^c :	
	1°	2°	Superinfected	1° only	2° only	Uninfected	Goodness of fit ^d	Fisher exact test ^e
7 days (2)	HV	LV	0 (13.3)	15 (1.7)	0 (0)	0 (0)	0.000	0.008
	LV	HV	3 (15)	6 (0)	5 (0)	1 (0)	0.000	
	HV	Mock	0	9	0	0		
	LV	Mock	0	9	0	0		
	Mock	HV	0	0	9	0		
	Mock	LV	0	0	8	1		

^a 1°, primary infection; 2°, secondary infection.
^b Expected infection status is calculated as the proportion of fish likely to be in each category based on the observed infection frequencies of single-infection groups multiplied by the total number of fish in the superinfection group.
^c *P* values following Bonferroni's adjustment.
^d An exact multinomial test was used to determine whether the observed distribution of fish in infection status categories is equal to the expected distribution based on single-infection controls (null hypothesis). Analysis was performed for each superinfection group for each experiment. A *P* value of <0.05 indicates support to reject the null hypothesis, i.e., the observed distribution does not equal the expected distribution.
^e The Fisher exact test was used to determine whether the distribution of fish in infection status categories was independent of the genotype used for primary infection (null hypothesis). A *P* value of <0.05 indicates support to reject the null hypothesis, i.e., the distribution is not independent of the genotype used first.

interferon-stimulated genes, including *Mx-1* (51). In addition, pretreatment of rainbow trout with an injection of poly(I:C), a TLR-3 agonist, offers significant protection from infection and mortality following challenge with IHNV (34). We therefore con-

ducted a preliminary study to determine whether innate immune stimulation following primary infection might correlate with restriction of secondary infection. However, we found that the timing of systemic *Mx-1* transcription upregulation following infec-

FIG 6 Impact of superinfection on viral load for all experiments performed with genotypes HV and LV, where LV is used for primary infection (A) or HV is used for primary infection (B). Viral loads attributed to genotype HV (black squares) and genotype LV (white diamonds) in individual fish from each treatment group (*x* axis) are shown for replicate experiments for each time interval between exposures as indicated. Horizontal dotted lines indicate detection levels for the genotype-specific qRT-PCR assays. Differences between groups were analyzed by ANOVA with Bonferroni's correction.

FIG 7 Viral growth kinetics and *Mx-1* induction in single infections with genotypes HV and LV. (A) Mean log viral load in 5 fish sampled at each time point, excluding virus-negative samples, in rainbow trout following single exposure using our superinfection protocol conditions with genotype LV (white diamonds) or genotype HV (black squares) ($\pm$ SEM). The horizontal dotted line indicates detection levels for the genotype-specific qRT-PCR assays. "Num pos/total" indicates the prevalence of virus-positive fish out of total fish sampled at each time point for each genotype. (B) Mean normalized *Mx-1* transcription fold changes, relative to the mock control group, in the same fish shown in the viral load curves (A). Results of fish exposed to genotype LV (white diamonds) and genotype HV (black squares) are shown.

tion with each IHN virus genotype lagged behind the first time point at which we see significant superinfection restriction. Although this suggests a mechanism other than apparent competition, it does not exclude the possibility that innate immune signaling may play a significant role in the restriction observed. Our observations are based on the analysis of *Mx-1* transcripts from whole fish and not specifically from target tissues, such as kidney and spleen, or from potential entry sites, such as gills, skin, or intestine. Therefore, it is possible that biologically relevant, local induction of *Mx-1* occurs earlier in these tissues but is not yet detectable by our methods until a strong systemic response has been elicited. Additionally, many interferon-induced genes other than *Mx-1* are up-regulated in response to IHN virus infection and may play a role in defense against secondary infection (44, 46, 47, 52, 53). Ultimately, the mechanism underlying superinfection restriction in our system is of great interest, and future work will be directed at identifying and characterizing the mechanism(s) in rainbow trout.

An important aspect of the development of this system was the selection of appropriate statistical analyses for determining the significance of the results obtained. One concern regarding the analysis of the superinfection frequency data is that the frequency of infection observed in the single-infection groups was not uniform among all experiments, ranging from 57 to 100% (mean, 92%) infection with no detectable pattern based on interval. Therefore, in addition to the analysis presented in Results, we performed a more conservative exact multinomial test using a single probability of infection based on the lowest probabilities observed for each genotype to account for variability in our infection efficiency. Using this more conservative analysis, we found

that, for both genotype pairs, the observed superinfection frequencies differed significantly from the expected frequencies only when exposures were separated by 96 h and 7 days. This second, more conservative analysis demonstrated that even in the case of the minimum infection efficiency observed in our system, the restriction of superinfection observed at these later intervals was significant and likely biologically relevant.

Another aim for this project was to investigate whether the level of restriction was dependent on which genotype was used for the primary exposure. In other words, do the genotypes differ in their ability to establish, or restrict the establishment of, secondary infection? Our analyses determined that for the genotypes of equal relative virulence, B and C, there were no significant differences in the frequencies of superinfection between groups exposed to genotype B before C or genotype C before B for any experiments or any intervals between exposures. Although similar results were obtained for most experiments with genotypes HV and LV, the superinfection frequency was significantly different in some experiments at some intervals. The frequency of superinfection was significantly higher in groups exposed to genotype LV before genotype HV than in the reciprocal group for one of two experiments in three of the five intervals tested. Interestingly, for the 96-h interval experiments, one experiment demonstrated the opposite effect. While we conclude from these experiments that virulence alone does not determine overall superinfection fitness, based on the three experiments that did find a significant difference in superinfection fitness, we cannot exclude the possibility that HV may have some advantage over LV in this system.

A strength of this *in vivo* superinfection assay is the ability to quantify the viral load of each genotype in individual superinfected hosts and compare the level of viral replication to that found in individuals from groups exposed to just one of the genotypes at the same exposure period. This analysis allows us to determine the ability of each genotype to replicate in a superinfection context compared to a single infection and to determine whether there is a cost to viral replication in superinfection. We observed a clear disadvantage for the superinfecting, or secondary, virus with regard to viral replication, while the primary infecting virus was unaffected by subsequent infection. These results support the hypothesis put forward by Ashraf et al. that reduced secondary viral replication drove the observed reduction in pathogenesis of IBDV superinfection in juvenile chickens when first infected with a mild strain and then challenged with a virulent strain of the virus (5). In addition, superinfection studies using dengue virus in mammalian and mosquito cell cultures demonstrated a reduction in the viral titer of the superinfecting virus compared to that in single-infection controls (54). However, we see no significant increase in the replication disadvantage of the secondary virus with increasing time between exposures, while Pepin et al. observed that increased time between incubation of mosquito cells with the primary virus and exposure to a superinfecting variant was correlated with a decrease in the titer of the secondary virus (54). Additionally, significant differences were observed between dengue virus strains with regard to replication during superinfection in a cell culture system, whereas no differences in superinfection replication fitness between IHN virus genotypes were observed in this *in vivo* system.

One of the central questions driving this work involved the role of virulence in determining fitness in superinfection. In our previous studies with coinfection of genotypes HV and LV, we ob-

served a clear correlation of higher virulence with higher coinfection fitness. Therefore, we hypothesized that increased virulence would be associated with an increased ability to establish, or prevent the establishment of, secondary infection and an increased ability to replicate within a superinfected host. While we did observe a trend of increased frequency of secondary infection with genotype HV relative to LV with most intervals, this was not evident in all experiments, and we did not detect any advantage associated with virulence when we examined the viral loads in superinfected fish. This suggests that high virulence may be associated with some advantage in entry and/or establishment of secondary infection but that, once infection is established, there is no replication advantage for any genotype examined. A replication disadvantage was seen for the secondary virus with all genotypes tested, suggesting a common mechanism for superinfection restriction that none of these viruses were able to overcome.

Although we found that virulence was not associated with an advantage in replication, as we originally hypothesized, the trend toward increased capability to establish superinfection or prevent establishment of a secondary infection may endow a high-virulence variant with a discrete competitive advantage in a region in which multiple variants cocirculate and infection rates are high. Such an advantage may result in a selection for increased virulence within a viral population and may have profound impacts on the host population, particularly when this advantage is combined with the entry, replication, and shedding advantages previously documented for more-virulent genotypes of IHNV (20). It is important to note that our observation that virulence plays a very limited role, if any, in superinfection fitness is based on the investigation of only one pair of IHNV variants of high or low virulence, and investigations with different variants may yield different results. Although additional studies will be necessary to make conclusions about the full role of virulence, our observations do suggest that virulence alone does not determine superinfection fitness in this system.

The superinfection assay we have developed here has provided us with novel insight into the *in vivo* dynamics of viral superinfection in a vertebrate host and the importance of virulence in superinfection fitness, using numbers of individual hosts sufficient to achieve high statistical power. Further studies of superinfection dynamics using this IHNV rainbow trout system and other vertebrate virus systems will be important for deeper understanding of determinants and mechanisms of viral superinfection in the field and what role this might play in virulence evolution.

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Mention of trade names does not imply U.S. Government endorsement.

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