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Abstract:

The ability to infect a host is a key trait of a virus, and differences in infectivity could put one virus at an evolutionary advantage over another. In this study we have quantified the infectivity of two strains of infectious hematopoietic necrosis virus (IHNV) that are known to differ in fitness and virulence. By exposing juvenile rainbow trout (*Oncorhynchus mykiss*) hosts to a wide range of virus doses, we were able to calculate the infectious dose in terms of ID_{50} values for the two genotypes. Lethal dose experiments were also conducted to confirm the virulence difference between the two virus genotypes, using a range of virus doses and holding fish either in isolation or in batch so as to calculate LD_{50} values. We found that infectivity is positively correlated with virulence, with the more virulent genotype having higher infectivity. Additionally, infectivity increases more steeply over a short range of doses compared to virulence, which has a shallower increase. We also examined the data using models of virion interaction and found no evidence to suggest that virions have either an antagonistic or a synergistic effect on each other, supporting the independent action hypothesis in the process of IHNV infection of rainbow trout.

Keywords:

Infectivity, Virulence, Infectious hematopoietic necrosis virus, Infectious dose, Lethal dose, Independent action hypothesis

1. Introduction

The ability to infect a host is necessary for a virus to propagate, and if one virus strain can do so better, it will likely have a competitive advantage over other strains. Certainly, a variety of other parameters also contribute to the absolute fitness of a virus, such as replication, shedding, and duration of infection, all of which are shaped by virus and host factors (Wargo & Kurath, 2012). However, without the important first step of host entry and initiation of infection these other parameters cannot be realized and viral fitness is diminished to zero. In this paper, we are concerned with infectivity, defined here as the ability of a pathogen to enter a host and begin replication, and virulence, defined here as the ability of a pathogen to kill its host.

A long-standing question has been the relationship between virus infectivity and virulence. However, few studies have examined how the relationship between virulence and infectivity might depend on virus exposure dosage. The paucity of such studies is surprising considering that it is well known that for viruses, infection and mortality are heavily shaped by exposure dose. In fact, because of the strong effect of exposure dose on disease outcome, viral virulence has often been characterized across a range of dosages. Such studies often calculate the 50% lethal dose (LD_{50}), i.e., the virus dose at which fifty percent of exposed hosts die (Reed & Muench, 1938; Knittel, 1981; Engelking & Leong, 1989; LaPatra et al., 1993; Kim & Faisal, 2010). The LD_{50} is typically determined in a controlled experiment in which a range of exposure doses are administered to equivalent groups of hosts, and the resulting mortality at each dose is used to generate a dose-response curve and calculate the LD_{50} value. Such studies also

make it possible to quantify the minimum lethal dose, the lowest dose at which mortality is observed (Kothary & Babu, 2001; Ward et al., 1986).

These LD_{50} studies are often used to make inferences about infectivity, assuming high virulence strains cause greater mortality because higher numbers of hosts become infected. However, this assumed relationship between virulence and infectivity has several limitations. For example, many viruses cause disease that does not result in host death. Viruses can also cause sub-clinical infections, where the host becomes infected but suffers no clinical disease. For example, a study of infectious pancreatic necrosis virus in Atlantic salmon found that at low challenge dosages a larger percentage of fish become infected than succumb to mortality (Urquhart et al., 2008). Quantification of actual infection is thus critical for an accurate assessment of infectivity, which is an essential component of overall viral fitness. Infectivity can be quantified in much the same way as virulence. For example a range of viral exposure dosages can be administered, after which hosts can be tested for infection status at a specific time post-infection. The prevalence of infection at each exposure dose is then used to calculate the 50% infectious dose (ID_{50}), i.e., the dose at which fifty percent of exposed hosts are infected. Though the methods used to detect infection are different, the ID_{50} is determined in the same manner as the LD_{50} (Reed & Muench, 1938). As with lethal dose, minimum infectious dose, the lowest dose needed to cause an infection, can also be quantified. Interpretation of virus infection studies is heavily dependent on the methods used, which differ in their sensitivity and specificity for live virus, viral genetic material, or host responses to infection. In this study we define infection as the presence of viral RNA in the host as detected by real-time reverse transcriptase qPCR.

In general fewer studies have been conducted examining ID_{50} values compared to the number exploring LD_{50} values. Among studies that determine both ID_{50} and LD_{50} values for various host:pathogen systems, the relationship between infectivity and virulence is not always consistent. For example, a study of avian influenza virus in wild duck and poultry found large host species effects on infectious dose, but within a host species, LD_{50} values were tightly coupled with ID_{50} values, suggesting virulence was correlated with infectivity (Swayne & Slemmons, 2008). However, in a study of Monkey

B virus in mice, the relationship between LD_{50} and ID_{50} was less consistent, with some of the strains with the lowest ID_{50} values having the highest LD_{50} values, suggesting virulence may be decoupled from infectivity (Ritchey et. al., 2005). Thus, investigation of infectivity and virulence for additional pathogens is of interest, and aquatic systems are ideal for experiments involving large numbers of hosts being exposed to a wide range of pathogen doses.

Examination of infectivity and virulence across a range of virus exposure dosages is a powerful method for comparing the traits of different virus strains. Such studies make it possible to reveal differences in virulence and infectivity that might not be apparent at single exposure dosages. This is because mortality and infectivity may saturate at the same levels for different virus genotypes, but the rate of increase in infection and mortality across exposure dosages may be different. In addition, the relationship between infectivity and exposure dose allows for an assessment of whether or not individual virions interact during the process of infection. For example, if there is a linear increase in the rate of infection as dose increases, this suggests that virions do not impact the infectivity of other virions. Here this is referred to as the independent action model, also sometimes referred to as the mass-action principle (Regoes et al., 2003; Schmid-Hempel, 2011). In contrast, if the rate of infection changes in a non-linear manner as the number of virions in the exposure dose increases, this would suggest that the virions interact with each other either in a synergistic or an antagonistic manner, here referred to as an interaction model. If there is a synergistic interaction, that could result in an invasion threshold, with a threshold dose (Regoes et al., 2003; Schmid-Hempel, 2011). In this case, if the host receives less than the threshold dose it will not become infected, and infection can only occur when the dose meets or exceeds the threshold dose. Ultimately, whether infectivity follows the independent action or interaction model can have influence epidemiological predictions about disease risk and spread (Schmid-Hempel, 2011; van der Werf, 2011).

Here we examined and compared prevalence of infection and mortality over a range of virus exposure doses to characterize the relationship between infectivity and virulence for an aquatic virus in fish hosts. We utilized a virus-host system that has

been well-studied *in vivo*, infectious hematopoietic necrosis virus (IHNV; order *Mononegavirales*, family *Rhabdoviridae*, genus *Novirhabdovirus*) in rainbow trout (Wargo et al., 2010; Kell et al., 2013; Peñaranda et al., 2009; Bootland & Leong, 2011; Zhang & Gui, 2015). In the western United States, IHNV is endemic in salmonid fish throughout a range from Alaska to California, as well as inland via rivers to Idaho (Bootland & Leong, 2011). Within this range three main genogroups occur: U, M, and L, each of which exhibit some host specificity (Kurath et al., 2003). Relevant to this study, the M genogroup is hypothesized to have arisen in rainbow trout (Kurath et al., 2003). Under certain conditions IHNV causes disease epidemics in salmonid fish, with mortality due to necrosis of the hematopoietic kidney and spleen tissues (Bootland & Leong, 2011). Variation in virulence of IHNV strains has been reported in several studies, most often tested using a single high virus exposure dose (LaPatra et al., 1993; Garver et al., 2006; Wargo et al., 2010). However, infectious dose has not been previously quantified for IHNV.

We compared the infectious dose and lethal dose for two virus strains within the M genogroup of IHNV, previously characterized as having high virulence and low virulence in rainbow trout based on mortality caused to the host due to infection at a single, high challenge dose (Wargo et al., 2010). The rainbow trout used here were from an aquaculture stock that is not inbred, and thus provided a host background for testing viral traits that is relevant to field conditions. The two virus strains have been previously studied, and their virulence correlates positively with in-host viral replicative fitness, as well as host entry and shedding (Wargo et al., 2010; Wargo & Kurath, 2011). Here we exposed groups of juvenile rainbow trout to a range of doses of each genotype and then measured the infection prevalence, infection intensity, and daily mortality in order to quantify infectivity and analyze the relationship between exposure dose and both infection and mortality.

Five *in vivo* infection experiments were conducted using standardized one-hour batch immersion challenges to assure uniform, consistent virus exposure of fish within each group. Three of the experiments were independent infectious dose assays that determined ID_{50} estimates and provided a measure of the variability in those estimates.

In these experiments fish were separated into isolated holding tanks after challenge to avoid cross-infection, and infection status was determined at 3 days post-exposure. The fourth experiment was a virulence assay that determined the lethal dose of each strain under the same isolation conditions used in the infectious dose assays, allowing direct comparison of ID_{50} and LD_{50} values for the two IHNV strains. Finally, as a secondary goal of this study we conducted a virulence assay using standard batch holding conditions, for comparison with the results of the virulence assay with fish held in isolation. This provided insight into how much of the mortality observed in standard batch challenge studies is due to holding conditions or secondary fish-to-fish infection. The combined data provide a comparison of the relationship between infectivity and lethality of two strains of a virus of differing virulence and expand upon the previous work done on the ecological parameters of various genotypes in the M genogroup of IHNV (Troyer et al., 2008; Wargo et al., 2010; Wargo & Kurath, 2011; Kell et al., 2013).

2. Materials and methods

2.1. Virus and host

For this study, we used two isolates of IHNV that differ in virulence. The more virulent strain is 220-90, referred to as HV for "high virulence"; the less virulent strain is WRAC (alternate name, 039-82), referred to as LV for "low virulence" (Wargo et al., 2010). Both strains were obtained from farmed rainbow trout in Idaho and have been previously characterized for virulence (LaPatra et al., 1994; Garver et al., 2006; Wargo et al., 2010). Over the glycoprotein gene of the virus there is 3.6% (58/1621 nucleotides) divergence between HV and LV; over the entire genome, the divergence is 2.8% (312/11,133 nucleotides) (Morzonov et al., 1995; Ammayappan et al., 2010). The preparation of viral stocks as well as quantification of viral titer has been previously described (Fijan et al., 1983; Batts & Winton, 1989; Troyer et al., 2008).

The fish were research-grade, juvenile, 1-3 g rainbow trout, provided by Dr. Scott LaPatra of Clear Springs Foods, Incorporated. The experiments were performed

on three different lots of fish from this source. Stock fish were maintained in flow-through freshwater that had been sand filtered and UV irradiated. All experiments were conducted at 15 °C. All animal procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

2.2. Virus challenges to determine infectious dose

Three experiments to determine the infectious dose (ID) of each virus genotype were performed using identical methodology, differing only in the doses of virus administered and number of fish in each group, as shown in Table 1. The fish in the three ID experiment were from three different lots, with an average weight of 1.6 g, 1.1 g, and 1.1 g respectively. In each experiment, groups of fish were exposed to a range of specific concentrations of virus, or mock exposed, by a 1 hour batch immersion in static water (Garver et al., 2006). Water flow was then turned on and the fish were washed for 1 hour. After the wash, the fish were isolated into 1 liter beakers containing 400 ml static water, well before detectable replication or shedding of the virus occurred, to avoid cross-infection, and then held in isolation at 15 °C for three days, which is when the mean viral load has previously been shown to reach maximum levels (Troyer et al., 2008; Peñaranda et al., 2009). At this point each fish was euthanized, harvested aseptically, and stored in an individual Whirl-pak™ at -80 °C until RNA extraction and viral load quantification.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from whole fish as previously described (Wargo et al., 2010). Briefly, 4 ml/g fish of guanidinium thiocyanate-based denaturing solution was added to each fish, and the fish was homogenized using a Seward Stomacher® 80 (Biomaster). RNA was extracted from 1 ml of the homogenate with phenol-chloroform, precipitated, resuspended in 50 µl of water, and assessed for quality and concentration by spectrophotometry. The RNA samples were then stored at -80 °C until cDNA

synthesis using M-MLV reverse transcriptase with random heximer primers, as previously described (Wargo et al., 2010). A standard amount of 5 µL of RNA was used in each cDNA reaction and the final 20 µl of cDNA was diluted 1:10 in 180 µl of water.

2.4. Viral RNA quantification via qPCR

Viral load of HV or LV in each fish was quantified using genotype-specific qPCR assays as previously described (Wargo et al., 2010). Briefly, 5 µl of each diluted cDNA sample was combined with forward and reverse primer and Taqman probe specific for either HV or LV and then amplified on a 7900HT ABI Prism machine. Since each fish was exposed to only one genotype, each cDNA sample was tested only for the genotype expected. Transcript RNA standards specific to each virus genotype were used for determining absolute viral RNA copy number. Verification of parity between the two genotype-specific assays has been reported previously (Wargo et al., 2010). These assays detect both genomic and messenger RNA (Purcell et al., 2006), and this combined quantity will be referred to as viral load per gram of host tissue.

2.5. Virus challenge to determine lethal dose for fish held in isolation

The lethal dose in isolation (LD-isolation) was determined by challenging fish in batch as described above and then holding them in isolation for 30 days. These experiments were performed on the same lot of fish as the third ID experiment, approximately three months later. Groups of 20 fish with average weight 1.2 g were challenged by batch immersion in 1 L of static water containing one of three specific doses of HV or LV, as shown in Table 1. In addition, one group of 20 control fish was mock-exposed. After the 1 hour challenge, the water was turned on for a one-hour rinse, and then individual fish were netted into 1.5 L tanks in a tower rack system (Aquatic Habitats). These tanks provided independent flow-through water for each fish. After isolation, the fish were monitored daily for a period of 30 days at 15 °C. Each treatment group had a total of 20 fish, except for LV at the 10^4 plaque-forming units

(PFU)/ml dose and HV at the 10^3 PFU/ml dose, which both had 19 fish. To confirm virus as cause of death, plaque assays were performed on approximately 50% of the fish that died during the experiment (Burke & Mulcahy, 1980; Batts & Winton, 1989).

2.6. Virus challenge to determine lethal dose for fish held in batch

Fish pathogen mortality experiments are traditionally done in batch with replicate groups of fish held together for the duration of the experiment. While batch conditions are believed to most closely mimic natural conditions, they differ from the isolation conditions used to determine infectivity, and these differences could potentially impact virulence. For example, while the initial doses for batch and isolation treatments are the same, over the course of the experiment the fish held in batch have the potential to transmit virus to each other. As such, fish in the batch conditions may receive further exposure to virus that is not possible in the isolation conditions. Batch conditions also potentially have different stressors for the fish than isolation conditions. Therefore, in order to address questions about the differences in mortality assessed in different holding conditions and to more directly compare results with previous data (Troyer et al., 2008; Wargo et al., 2010; Wargo & Kurath, 2011; Kell et al., 2013), a lethal dose experiment in which fish were held in replicate batch groups was conducted in addition to the one in which they were held in isolation conditions.

The batch lethal dose experiment (LD-batch) was performed simultaneously with the LD-isolation experiment described above. The procedure is similar to that described in Wargo et al., 2010 and Breyta et al., 2014. Triplicate groups of 20 fish were challenged along with the LD-isolation fish, in addition to one group of 20 control fish that were mock-exposed. After 1 hour of exposure, the water was turned on and allowed to flow for the duration of the experiment. Fish were held in groups of 20 and daily monitoring for mortality proceeded for 30 days at 15 °C, as in the LD-isolation experiment. One of the tanks of LV at 10^4 PFU/ml had a malfunction and was lost, so this treatment had duplicate tanks. Approximately 20% of the fish that died were titered for virus by plaque assay as above.

2.7. Statistical analyses

The infection and mortality data were used to calculate the projected doses at which fifty percent of fish were infected (ID_{50}), died in isolation (LD_{50}-isolation), or died in batch (LD_{50}-batch). The calculations of ID_{50} and LD_{50} values were both done using generalized linear models (GLM) in the statistical program R, version 3.3.1 (R Core Team, 2015), using the dose.p function of the Mass package (Venables & Ripley, 2002) with a quasibinomial distribution prior, as described in Breyta et al., 2014. To calculate the ID_{50} values, numbers of infected versus uninfected fish were quantified. To calculate LD_{50} values numbers of dead versus alive fish were quantified. Significant differences between the suite of ID_{50} and LD_{50} values generated were calculated using the Welch-Satterthwaite 2-tailed *t*-test in R (Breyta et al., 2014). Results from the LD experiments indicated that mortality did not bracket 50% in all cases, leading to uncertainty in the calculated LD_{50} value. Therefore, using the same methods, we calculated the doses at which 25% of the fish exposed to LV died (LD_{25}) and the doses at which 75% of the fish exposed to HV died (LD_{75}). Differences in the kinetics of mortality were assessed using Kaplan-Meir curve and log-rank test functions of the Survival package of R (Therneau 2015), comparing the pooled doses of each treatment against each other. Viral load data were compared using a generalized linear model with response variable log-transformed viral load and explanatory factors virus genotype (HV vs. LV), challenge dose, and experiment. A Tukey multiple comparison of means test was used to determine differences between factors levels.

To determine if the relationship between proportion of fish infected and challenge dose fit the independent action hypothesis (also referred to as mass-action principle) the method suggested in (Regoes et. al., 2003) was utilized. To do so, the challenge dose and percent fish infected data from all infectivity experiments was fit to an independent action hypothesis model ($f = 1 - e^{-b*d}$) and an interaction hypothesis model ($f = 1 - e^{-b*d^k}$), where f = proportion of fish infected from raw data, 1 = the maximum proportion of fish that can become infected, b = infection rate determined

298 from model fit, *d* = challenge dose, and *k* = interaction term determined from model fit.
299 If *k* = 1 this indicates virions act independently of each other, and thus supports the
300 independent action hypothesis. If *k*<1 this suggests there is an antagonistic interaction
301 between virions such that as more virions are added they each have a harder time
302 infecting the host. If *k*>1 this indicates there is a synergistic interaction between virions
303 such that as more virions are added they each have an easier time infecting the host.
304 The models were fit to the data using the function "nls" in R version 3.2.0, to calculate
305 values for *b* and *k*. Whether or not the data had a significantly better fit to the
306 interaction model or the independent action model was then determined using an F-test
307 with the "anova" function in R. This was further evaluated by determining if 1.96X the
308 standard error of *k* overlapped with 1. These analyses were conducted for genotypes
309 HV and LV separately, to determine if the relationship between challenge dose and
310 proportion of fish infected was different for the two genotypes.
311
312 **3. Results**
313
314 *3.1. Determination of infectious dose for high and low virulence genotypes, HV and LV*
315
316 *3.1.1. Infection prevalence in three infectious dose (ID) experiments*
317 The first ID experiment tested a broad range of challenge doses from 10^1 to
318 $2x10^5$ PFU/ml. The results indicated that HV and LV functioned similarly with respect to
319 the percent of fish infected at each dose (Figure 1A), such that for both genotypes no
320 fish were infected at the lowest dose and there was 100% infection at the highest dose.
321 At the 10^3 and 10^4 PFU/ml doses, although LV had a lower frequency of infection than
322 HV, the differences corresponded to only one fish and were not significant ($p > 0.05$).
323 In the second experiment we tested additional doses in the 10^3 to 10^4 PFU/ml range
324 (Figure 1B). HV infectivity was reproducible for the two doses tested in both
325 experiments, 10^3 and 10^4 PFU/ml. The additional doses in between 10^3 and 10^4 PFU/ml
326 resulted in a regular increase in percent infection with HV. For LV, however, the percent
327 of fish infected in the second experiment was lower overall than in the first experiment,

328 and there was no dose response between $5x10^3$ and 10^4 PFU/ml. In the third
329 experiment, the overall frequencies of infection were higher for both HV and LV than in
330 the previous two experiments (Figure 1C). For HV the percent infection increased more
331 rapidly between the $5x10^2$ and 10^3 PFU/ml doses, compared with the first and second
332 experiments, and then slowly climbed as the dose increased, reaching 100% infection
333 at 10^4 PFU/ml as before. LV, in contrast with the second experiment, exhibited a more
334 regular stepwise increase in infection rate along the entire dose range, similar to the
335 manner of increase seen for HV in the three experiments, though at lower infection
336 frequencies. Overall, the percent of fish infected with LV was lower than HV at all doses
337 in experiment 3, and LV never reached 100% infection, even at 10^5 PFU/ml.
338
339 *3.1.2. Analysis of infectivity data*
340 The results of all three ID experiments were used to determine the minimum
341 infectious dose observed. For LV, the lowest dose that caused infection was 10^3 PFU/ml
342 in experiments 1 and 3, and $2.5x10^3$ PFU/ml in experiment 2. For HV the minimum
343 infective dose was $5x10^2$ PFU/ml in experiments 2 and 3 and 10^3 PFU/ml in experiment
344 1 where the $5x10^2$ PFU/ml dose was not tested. This data also gives an estimate of the
345 probability of infection at the minimum dose where infection was observed. For both LV
346 and HV, this was 10-20%. However, because of the number of fish used in each
347 experiment, differences in probability less than 10% in experiments 1 and 2 and
348 differences less than about 7% in experiment 3 cannot be resolved.
349 The percent infection data from each experiment was then used to calculate
350 50% infectious dose (ID_{50}) values for both genotypes (Figure 2). In each experiment,
351 the ID_{50} for LV was significantly higher than for HV (experiment 1, $t = 13.8$, $df = 3.01$,
352 $p = 0.001$; experiment 2, $t = 3.38$, $df = 6.94$, $p = 0.012$; experiment 3, $t = 8.33$, $df =$
353 8.50, $p < 0.001$). Therefore, a lower dose is needed to infect 50% of fish with HV than
354 with LV. We also calculated the mean of the ID_{50} values from the three experiments as
355 $9.83x10^3$ PFU/ml for LV and $1.94x10^3$ PFU/ml for HV (Figure 2). The significant
356 difference between the genotypes remained ($t = 4.76$, $df = 33.5$, $p < 0.001$). By these
357 measures, HV is approximately 5-fold more infectious than LV.

3.1.3. Viral load data from infectivity experiments

Overall, the viral load data for all virus-positive fish from all the ID experiments were similar across dose and genotype (Figure 3). While the viral loads of individual fish did vary, there were almost no significant differences between the means of the log-transformed viral loads, either between doses or between genotypes ($p > 0.05$). The one exception was in the first experiment, where the combined mean viral load for both HV and LV at 10^4 PFU/ml was significantly lower than the combined mean viral load at $2x10^5$ PFU/ml ($F_{2,34} = 1.87$, $p = 0.0108$).

3.2. Virulence experiments in HV and LV

3.2.1. Determination of lethal dose in isolation

The final mortality for LD-isolation ranged between 42-60% for HV and 20-26% for LV (Figure 4A). For HV, mortality in the 10^5 PFU/ml dose was higher than the 10^3 and 10^4 PFU/ml doses, which were similar in final mortality. However, the kinetics for all three doses of HV showed a clear dose response; the highest dose had the most rapid mortality initially, and the lowest dose initially had the slowest mortality. For LV, there was no strong dose response in either kinetics or final mortality. Although mortality in the mock treatment group was 15%, all three fish that died were negative for IHNV via plaque assay. The level of mortality in the mock treatment groups indicated that there might have been elevated non-specific mortality in the virus-exposed fish as well. However, all mortalities titered from the virus-exposed groups (approximately 50% of all fish that died) were positive via plaque assay, with average log-transformed titers of 6.61 ± 0.40 standard error PFU/ml for HV and 6.50 ± 0.27 standard error PFU/ml for LV. These virus titers are in the range commonly seen in fish that die after IHNV exposure (Breyta et al., 2014), indicating that they died as a result of viral infection.

3.2.2. Determination of lethal dose in batch

Mortality curves for LD-batch were generated from the daily average cumulative percent mortality of the three replicate tanks for each genotype and dose, with the exceptions of the LV 10^4 PFU/ml dose, which had duplicate tanks, and the mock-infected group, which only had one tank (Figure 4B). For HV, the average mortality ranged from 60-78%. However, there was not a clear dose response for the 10^3 and 10^4 PFU/ml doses, both of which had similar kinetics of mortality. The mortality for the three doses of LV ranged from 32-47% and followed a clearly separated dose response in both kinetics and final mortality. When comparing virulence in batch to that in isolation, for HV the final mortality at each dose was 15-18% higher in batch, and for LV it was 12-14% higher for the 10^3 and 10^4 PFU/ml doses and 22% higher for the 10^5 PFU/ml in batch. Neither genotype bracketed 50% final mortality; HV mortality was all above 50%, and LV mortality was all below 50%. One fish died in the mock treatment group and it was negative for virus via plaque assay. Approximately 20% of the virus-exposed fish that died were tested for virus and all were positive, with average log-transformed titers of 6.29 ± 0.019 standard error PFU/ml for HV and 5.80 ± 0.70 standard error PFU/ml for LV.

3.2.3. Analysis of mortality data

Differences in virulence between HV and LV were assessed by survival analysis on data from the lethal dose experiments. Log rank tests on the combined Kaplan-Meier estimates for dose and genotype indicated that mortality was significantly higher for HV than LV, both in isolation ($\chi^2 = 8.4$, df = 1, $p = 0.0037$) and in batch ($\chi^2 = 27.4$, df = 1, $p < 0.001$). This confirms the expected phenotypic difference in virulence between HV and LV. Additionally, mortality for both HV and LV was significantly higher in batch than in isolation ($\chi^2 = 5.9$, df = 1, $p = 0.015$ and $\chi^2 = 4.7$, df = 1, $p = 0.031$, respectively).

The 50% lethal dose (LD_{50}) values were calculated in the same manner as the ID_{50} values (Figure 5). For both lethal dose experiments the calculated LD50 values for genotypes HV and LV did not differ significantly ($P>0.05$), despite differing by more than 8 orders of magnitude for LD_{50}-isolation, and 2 orders of magnitude for LD_{50}-

batch. This is likely because neither HV nor LV bracketed 50% mortality across the exposure doses, with the exception of HV in isolation, and they fell on opposite sides of the 50% level (Figure 4). Therefore, calculating LD_{50} values required inferring the relationship between dosage and mortality outside the range of available data, leading to wide 95% confidence intervals. Furthermore, the calculated LD50 above 10^{12} pfu/ml for LV in isolation suggested that there is no feasible dose of LV that would be able to cause 50% mortality under isolation conditions. In light of this, we also calculated LD_{25} and LD_{75} values that were bracketed by the LV and HV data respectively (Figure 5). This gives us the most accurate values for each genotype.

3.3. Independent action hypothesis test

Analysis of our infectivity data indicated that the relationship between virus challenge dose and the proportion of fish infected was better explained by the independent action hypothesis compared to the interaction hypothesis, for both genotype HV and LV (Figure 6). This result was supported by a F-test comparison of the data fit to the two models (Anova; HV: $F_{1,17}$=0.28, p=0.6; LV: $F_{1,17}$=0.26, p=0.6), as well as determination that there was insufficient evidence to indicate the interaction term, k was significantly different from 1, for either genotype (HV: k = 0.868 ± 0.24; LV: k = 1.26 ± 0.51; gives mean ± 1 standard error), in the formula $f = 1 - e^{-b*d^k}$ (described in methods). As such, the results suggest that the infection process operates under a similar mechanism for HV and LV where individual virions do not inhibit or enhance the infectivity of other virions. Furthermore, both genotypes were able to achieve 100% of fish infected at high dosages, supporting the usage of a value of 1 for the maximum proportion of fish infected in the model. However, the analysis did reveal that the infectivity rate parameter (b) was 3-fold higher for HV (b= 2.55 x10^{-4} proportion fish infected/PFU virus ± 0.55 x 10^{-4}; mean ± 1 standard error) compared to LV (b= 8.00 x 10^{-5} proportion fish infected/PFU virus ± 1.57 x 10^{-5}). This indicates that infection increases more quickly as virus exposure dose increases, for HV compared to LV.

4. Discussion

This study was designed to provide data on how infectivity relates to virulence for two well-studied IHNV genotypes that are known to differ in both in-host fitness and virulence (Wargo et al., 2010; Wargo & Kurath, 2011). Overall, the results consistently showed that HV had a higher infectivity than LV, which correlated with its confirmed higher virulence. Furthermore, HV showed a more rapid increase in infectivity with increasing exposure dosage, compared to LV, as indicated by the dose response data and the independent action model. The previously documented replicative fitness difference between HV and LV (Wargo 2010; 2011) also correlated with the increased infectivity shown here. This confirms the finding from previous work (Wargo & Kurath, 2011) that HV has an advantage over LV in host entry, based on comparison of in-host fitness differences after viral infection by immersion versus injection. This advantage is likely to be most pronounced at an intermediate range of viral exposure doses, because at very high or very low doses infection saturated at 100% or 0% for both genotypes (Figure 1A). Thus neither genotype should have an advantage over the other in the number of fish that become infected at extreme high or low doses, but at intermediate dosage HV is predicted to infect more fish than LV, and thus have greater overall fitness.

A previous investigation of IHNV in fish farms found that virus titers in water prior to an epizootic range from undetectable to 0.07 PFU/ml, and in the early stages of an epizootic titers were measured at around 50 PFU/ml (Zhang & Congleton, 1994). At these low levels, our data suggests infection is unlikely to occur with either genotype, so fitness differences would not be realized. However, because our sample sizes were between 10-15 fish, our data cannot resolve differences less than 7-10%, and it may be that even at the low doses tested there are quantitatively small but biologically relevant differences in infectivity, especially when fish population sizes are large. Additionally, the duration of exposure in the current experiment was one hour, and exposure times in the field are likely much longer, perhaps measured in days or weeks. Although the relative ability to infect given longer exposure times has not been well characterized, we have observed that for IHNV a longer immersion exposure does result in a higher

prevalence of infection (Troyer et al., 2008). This was further supported by the viral load data in the present study. Here, there were no differences observed in the viral load of infected fish between genotypes HV and LV. However, a previous study found within-host viral loads for genotype HV were consistently higher than for LV, and a larger proportion of fish were infected at the 10^4 PFU/ml challenge dose (Wargo et al., 2010; Wargo & Kurath 2011). The primary difference between these studies was that in the previous work, fish were exposed to virus for 12 hours, whereas they were exposed for 1 hour, and viral load was quantified 12 hours earlier, in the current study. It may be that the longer immersion challenge allows for infection by more virions, resulting in faster viral replication kinetics that result in different viral loads, but further research is needed to discern the effects of exposure time on infection and viral load.

In order to link infectivity and mortality data, we conducted LD experiments in conjunction with the third ID experiment. The results indicated that the processes of infection and virulence respond differently to variation in exposure dose. In almost all cases percent infection increased with increasing dose at a different rate than percent mortality, and infection prevalence was higher than mortality (Figure 7). Furthermore, large increases in percent infection as dose increased were associated with relatively small increases in percent mortality. The exception to this was for the increase from the 10^4 PFU/ml to the 10^5 PFU/ml dose of HV, where no increase in percent infection was possible due to infection being at 100% at both doses, but there was an increase in mortality by 15%. This implies that the exposure dose can influence mortality even when all fish are infected. The calculated LD_{50} values also supported the conclusions that infection does not guarantee death in that ID_{50} values were lower than the LD_{50} values in nearly all cases (Figure 8). This indicates it takes more virions to kill than to infect the same number of fish and that while infectivity plays a role in determining virulence, it is likely not the only factor.

It was interesting that despite the significant differences in virulence between HV and LV by survival analyses, the calculated LD_{50} estimates did not differ significantly for either lethal dose experiment. This was largely because mortality did not bracket 50% for either genotype and thus uncertainly around the calculated LD50 values was large.

This implies that significant differences between LD_{50} values may be difficult to obtain for virus genotypes that cause widely different levels of mortality. Thus, while this study is consistent with previous publications that virulence is correlated with previously demonstrated differences in fitness for IHNV (Peñaranda et. al., 2009; Wargo et. al., 2010; Wargo & Kurath, 2011), these results stress the importance of considering survival kinetics when quantifying virulence.

The combination of batch versus isolation virulence experiments made it possible to examine how holding conditions impact mortality. The difference between percent mortality in the two holding conditions was consistently 12-22% higher in batch across the challenge doses for both genotypes. This might be due in part to the fact that in batch, infected fish are shedding virus into the water, which could be responsible for multiple rounds of infection. The fact that the 10^3 PFU/ml dose of LV had a higher rate of mortality than infection supports this theory (Figure 6). However, the fact that the 10^4 and 10^5 PFU/ml doses of HV both had 100% infection, yet there was still an increase in mortality, suggests that other factors might also be involved. Furthermore, one would expect the increase in mortality due to multiple rounds of infections to be greatest when the fewest number of fish were initially infected, for example at the 10^3 PFU/ml dose, because a large number of fish are remaining for potential infection. However the increase in mortality between batch and isolation was surprisingly consistent, regardless of how may fish were initially infected. Constant exposure to virus could force the infected fish to divert resources to fighting the exposure, which allows the established infections to cause a higher incidence of mortality. It is reasonable to consider that manner of exposure (i.e. through shedding or original inoculum) could play a significant role in mortality rate, just as longer exposure times resulted in higher infection frequencies and different viral loads in previous studies for IHNV (this study compared with Wargo & Kurath, 2011). Additionally, the batch and isolation treatments imposed different stressors on the fish, which could have affected mortality rates differently. High fish density in hatcheries increases probability of contact between infected fish and has been linked to increased stress and lower water quality (Bootland & Leong, 2011). However, rainbow trout are also social animals and

moving individual fish to isolation may be a stressor (Øverli et al., 2002; Øverli et al., 2005). Determining the role of stress, holding conditions, and multiple rounds of transmission in driving IHNV induced fish mortality warrants further study.

In this study we have illuminated a previously unexplored relationship between infectivity and virulence in an aquatic virus. We have shown that while infectivity does indeed correlate with virulence, it does not appear to be the only driving factor. To our knowledge, such detailed work comparing infectivity and lethality has not been done with IHNV in rainbow trout or with any other fish pathogen; as such it can serve as a point of comparison for future studies. Due to variation in absolute mortality levels observed in IHNV virulence studies repeated in different years (Breyta et al., 2014), it is valuable to have infectivity and mortality data from the same year with the same lot of fish. This was also evident in the current study, as some variation in infectivity was observed between experiments conducted with different lots of fish. In future work similar studies with other viral strains in multiple hosts will help determine if infectious dose is as variable as lethal dose across different viral strains, and if infectivity and lethality vary in the same manner. Examination of the exposure dose response of mortality and infection also makes it possible to characterize heterogeneity of host susceptibility (Rodrigues et. al., 2009). This is essential for understanding epidemiological patterns and can greatly enhance pathogen control (Gomes et. al., 2014). For example, our study suggested that the relationship between infectivity and exposure dose fits the independent action hypothesis. However, it is possible that virion interactions do occur but this was masked by susceptibility heterogeneity (Rogeos et. al., 2003; van der Werf, et. al., 2011). More importantly, this work clearly shows that infectivity and virulence differences between pathogen strains may not be evident at very high or very low exposure doses, and thus it is important to examine a range of exposure doses to determine where fitness differences are the most important. The evolutionary implications of this dose response to pathogen fitness differences warrant consideration. In summary, this work has demonstrated that for IHNV in rainbow trout viral infectivity is positively correlated with virulence, but the ID_{50} values varied less than the LD_{50} values. This serves as a valuable example of the relationship between

viral infectivity and virulence in a naturally co-evolved vertebrate host-pathogen association.

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Experiment	Fish lot	Exposure doses (PFU/ml)	# fish/dose/strain (at initial batch challenge)	Experiment duration	# fish/tank (for holding)
Infectious dose 1	1	10^1, 10^2, 10^3, 10^4, $2x10^5$	10	3 days	1 individual
Infectious dose 2	2	$5x10^2$, 10^3, $2.5x10^3$, $5x10^3$, $7.5x10^3$, 10^4, 10^5	10	3 days	1 individual
Infectious dose 3	3	$5x10^2$, 10^3, $2.5x10^3$, $5x10^3$, $7.5x10^3$, 10^4, 10^5	15	3 days	1 individual
Lethal dose, isolation	3	10^3, 10^4, 10^5	20	30 days	1 individual
Lethal dose, batch	3	10^3, 10^4, 10^5	3 groups of 20	30 days	20 grouped

Table 1: Infectious dose and lethal dose experimental designs. All experiments had a 1-hour immersion challenge in batch. The "Experiment duration" and "# fish/tank" columns describe the holding conditions after the 1-hour challenge. Exposure doses are given in plaque-forming units (PFU) per milliliter.

Figures

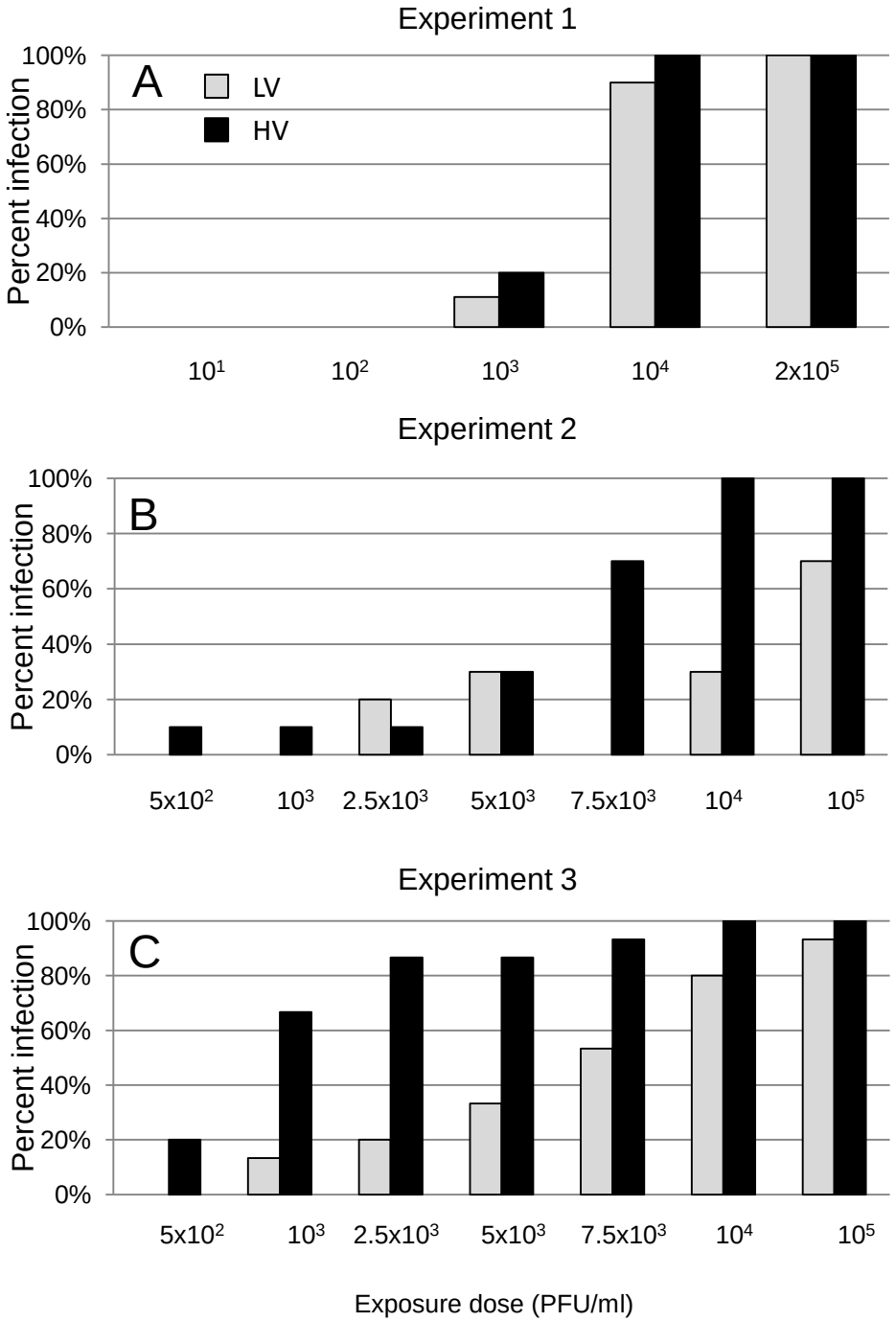


Figure 1

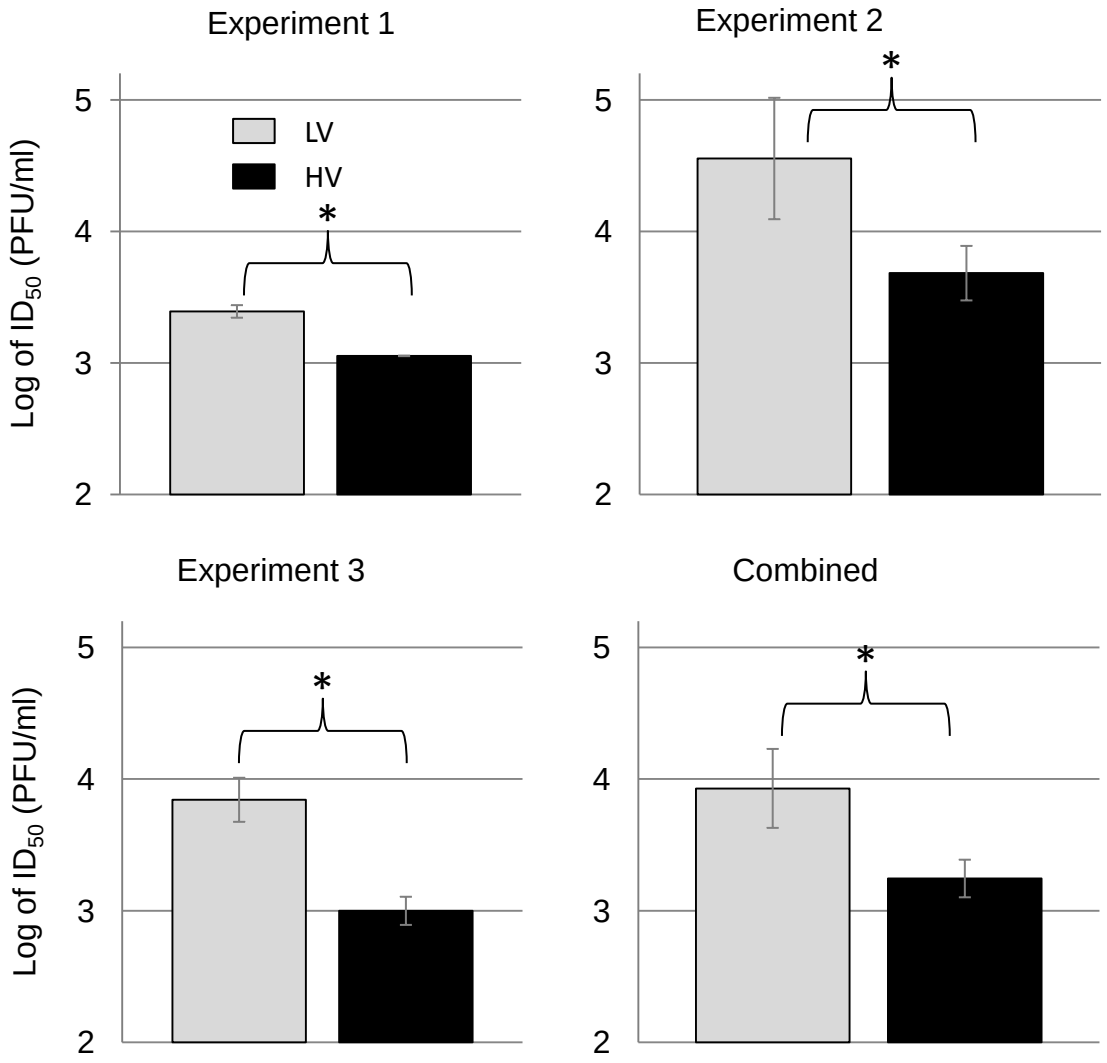

Experiment 1
Experiment 2
Experiment 3
Combined
LV
HV
Log of ID50 (PFU/ml)
5
4
3
2
*

Figure 2

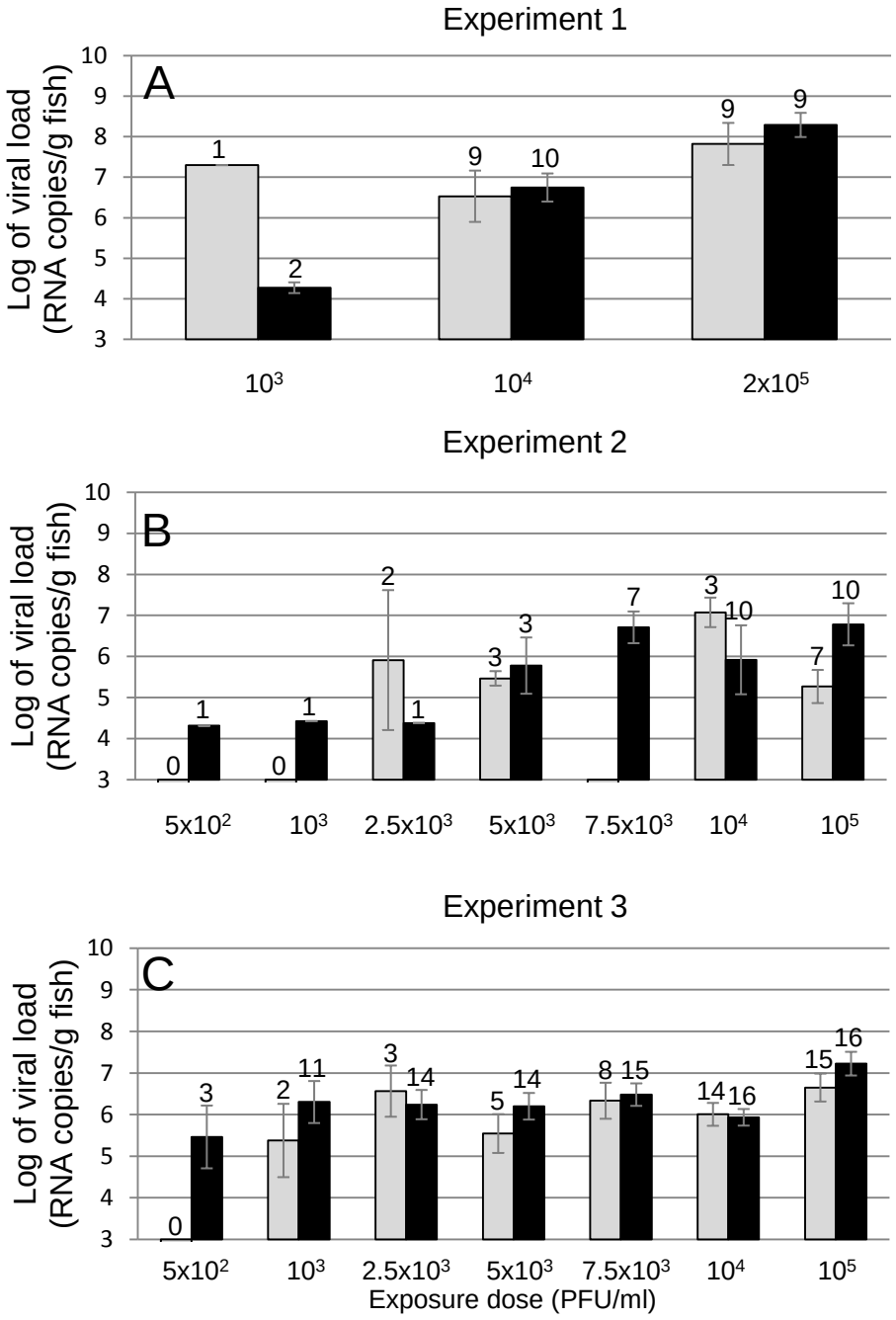
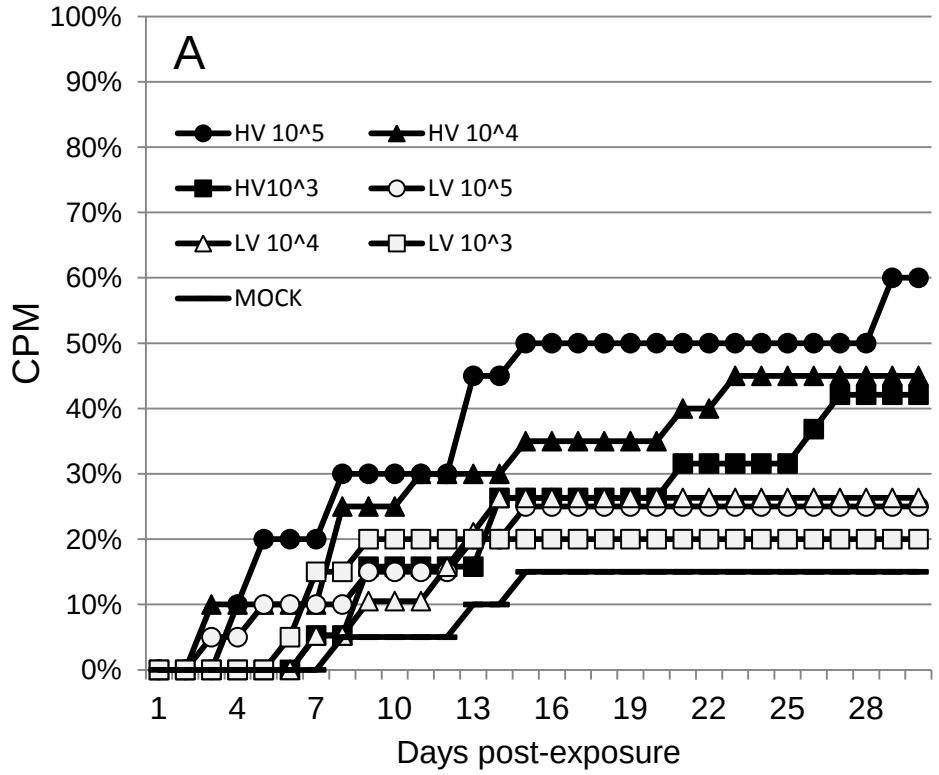

Experiment 1
A
Log of viral load (RNA copies/g fish)
10
9
8
7
6
5
4
3
1
2
9
10
9
9
10³
10⁴
2x10⁵
Experiment 2
B
Log of viral load (RNA copies/g fish)
0
1
0
1
2
1
3
3
7
3
10
7
10
5x10²
10³
2.5x10³
5x10³
7.5x10³
10⁴
10⁵
Experiment 3
C
Log of viral load (RNA copies/g fish)
0
3
2
11
3
14
5
14
8
15
14
16
15
16
5x10²
10³
2.5x10³
5x10³
7.5x10³
10⁴
10⁵
Exposure dose (PFU/ml)

Figure 3


A
HV 10^5
HV 10^4
HV10^3
LV 10^5
LV 10^4
LV 10^3
MOCK
CPM
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1
4
7
10
13
16
19
22
25
28
Days post-exposure

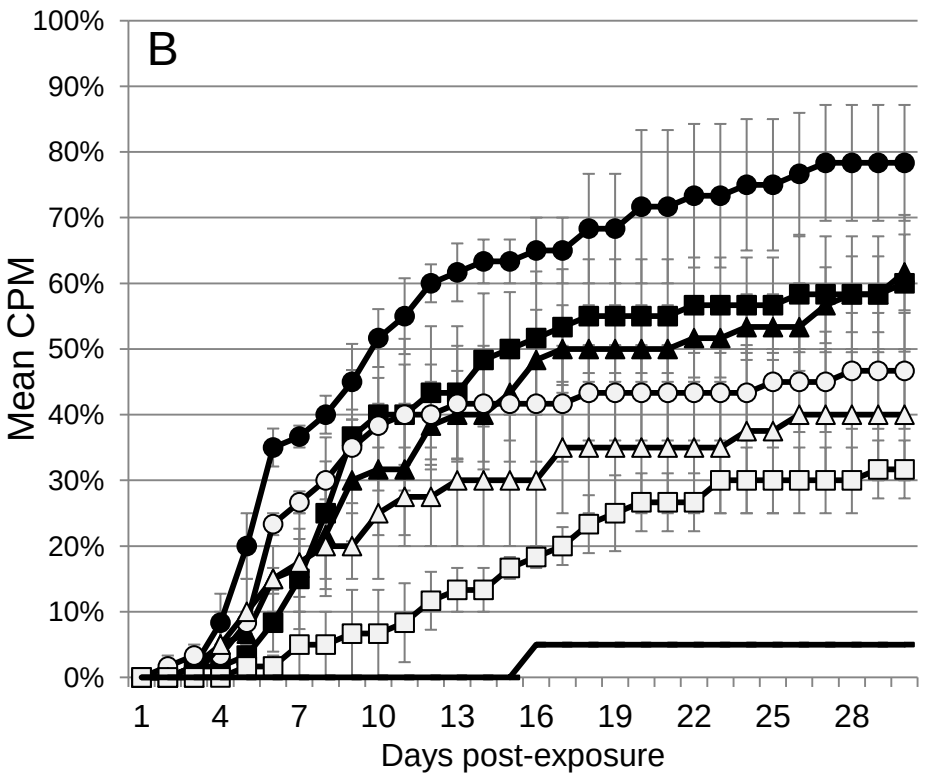

B
Mean CPM
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1
4
7
10
13
16
19
22
25
28
Days post-exposure

Figure 4

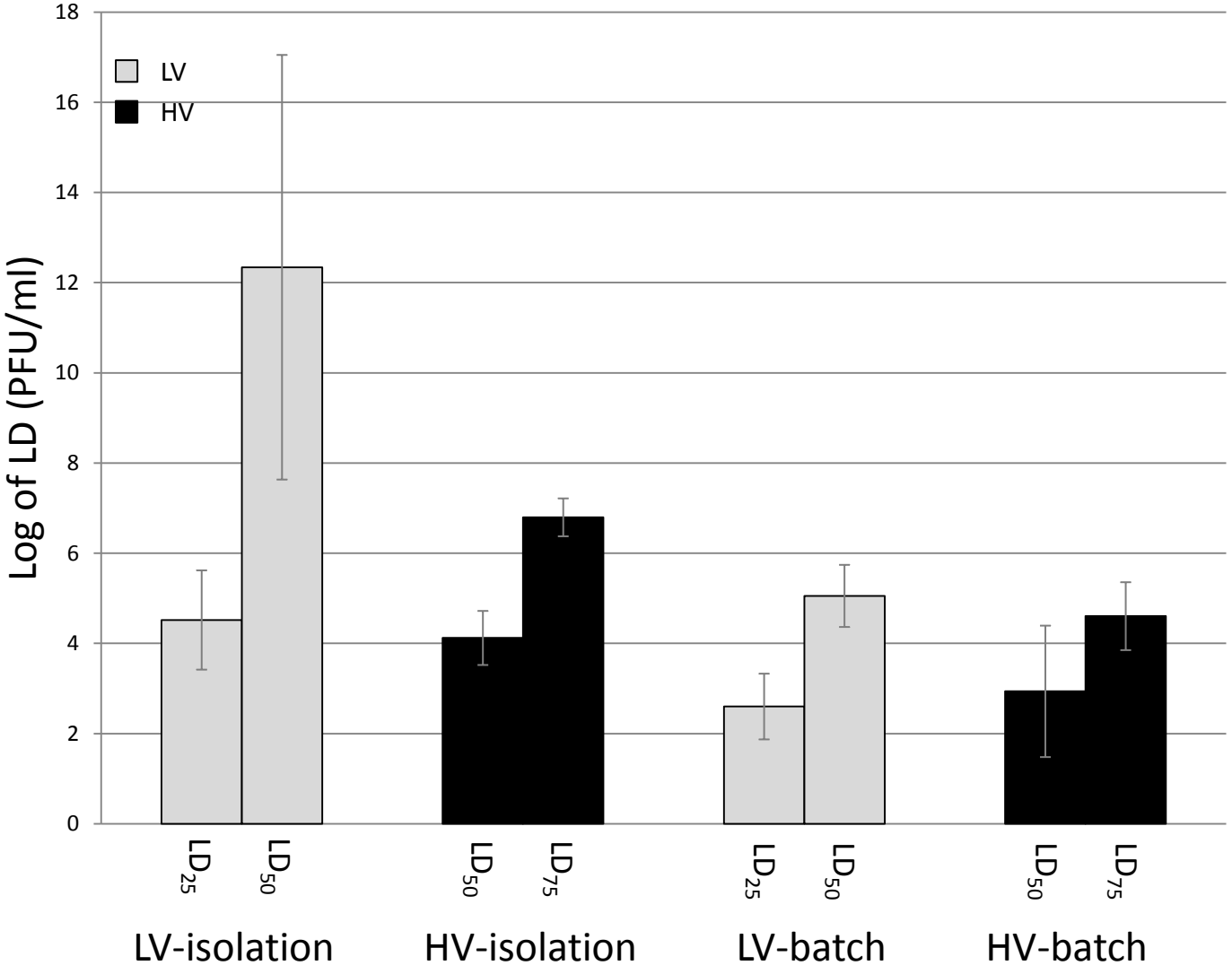

LV
HV
Log of LD (PFU/ml)
0
2
4
6
8
10
12
14
16
18
LD25
LD50
LD50
LD75
LD25
LD50
LD50
LD75
LV-isolation
HV-isolation
LV-batch
HV-batch

Figure 5

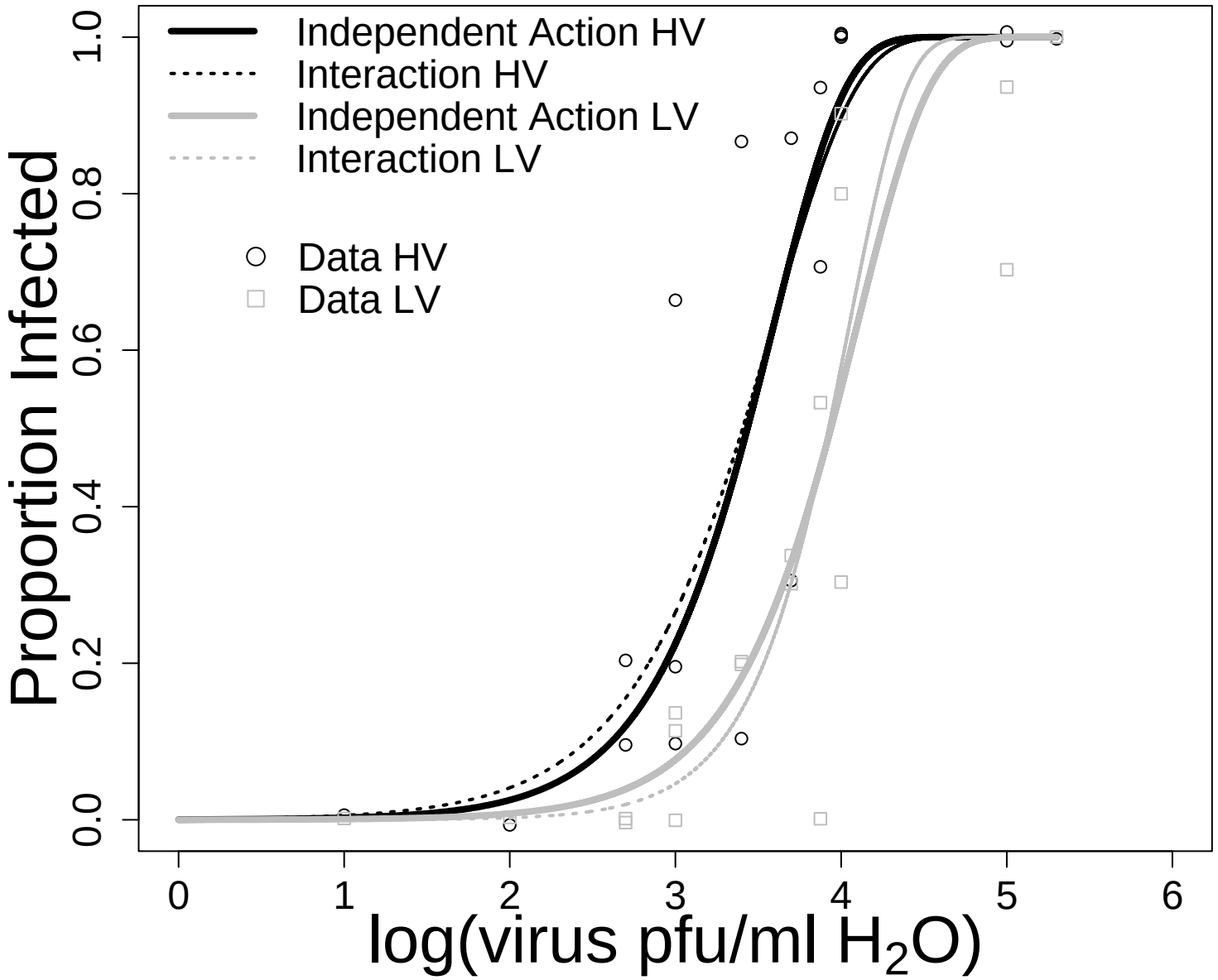

Independent Action HV
Interaction HV
Independent Action LV
Interaction LV
Data HV
Data LV
Proportion Infected
0.0
0.2
0.4
0.6
0.8
1.0
0
1
2
3
4
5
6
log(virus pfu/ml H_2O)

Figure 6

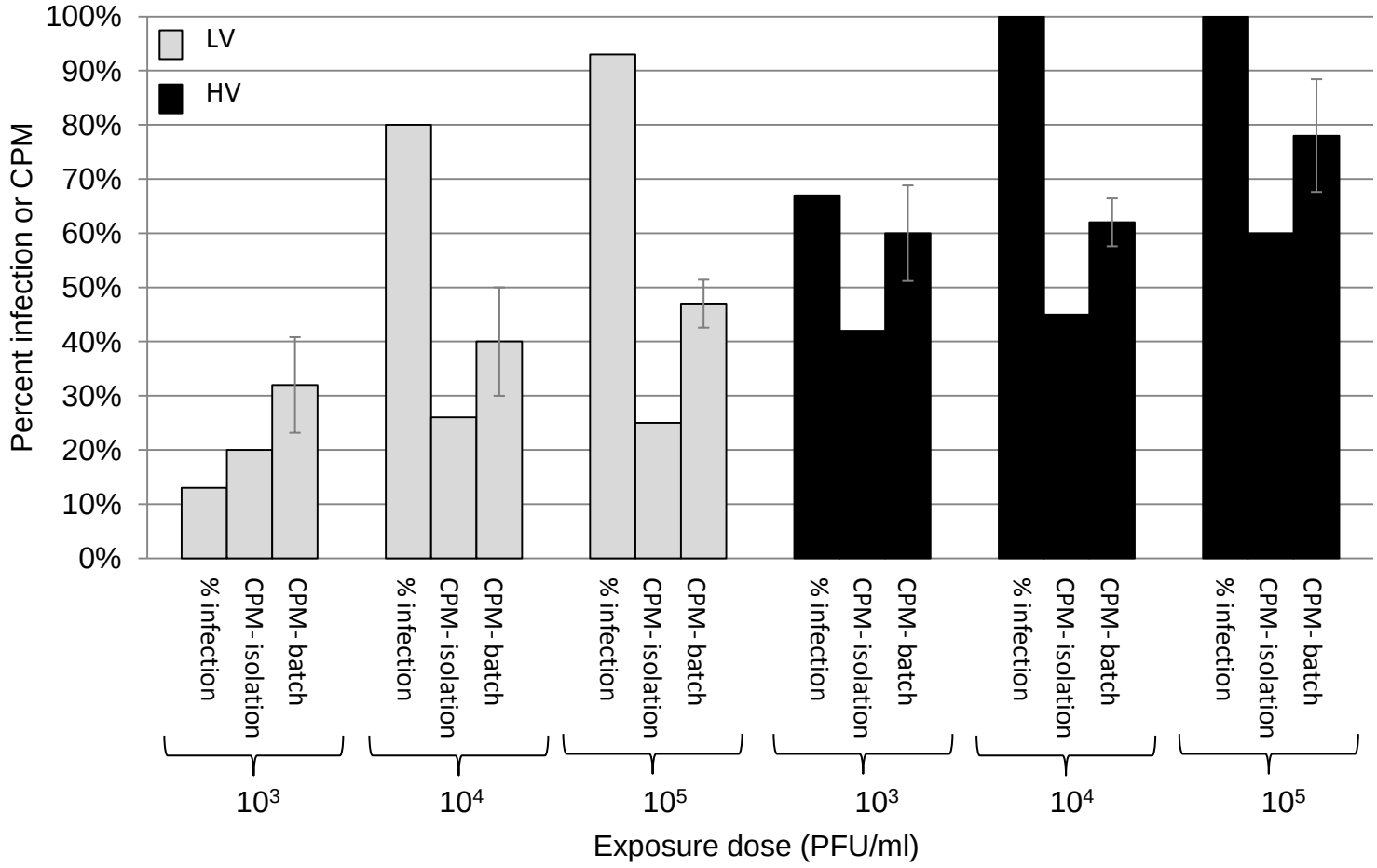

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Percent infection or CPM
LV
HV
% infection
CPM- isolation
CPM- batch
10³
10⁴
10⁵
Exposure dose (PFU/ml)

Figure 7

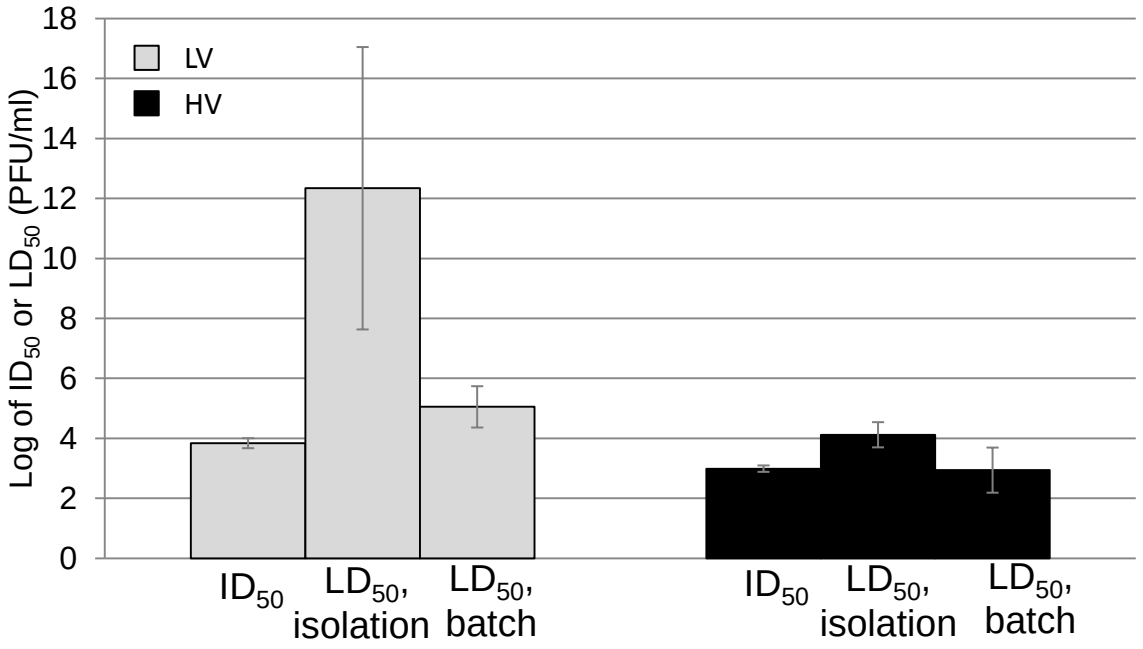

18
16
14
12
10
8
6
4
2
0
LV
HV
Log of ID50 or LD50 (PFU/ml)
ID50
LD50, isolation
LD50, batch
ID50
LD50, isolation
LD50, batch

Figure 8

Figure legends for paper

Figure 1: Percent infection data from infectious dose experiments. In all panels, LV is gray and HV is black. In A and B, n=10; in C, n=15. Note that the *x*-axis dose values in A are different than those of B and C.

Figure 2: 50% infectious dose (ID_{50}) values. The combined ID_{50} represents the mean of log base 10 transformed ID_{50} values from the first three experiments. The error bars show the 95% confidence interval. In each experiment, the difference between the LV ID_{50} and HV ID_{50} was statistically significant ($p < 0.05$).

Figure 3: Viral load data from infectious dose experiments. In all panels, LV is gray and HV is black. In A and B, n=10; in C, n=15. Data presented as mean of log base 10 transformed viral load (+/- 1 standard error), at various exposure doses. Only virus-positive fish are included in the mean, the number of which is indicated by the values above the bars.

Figure 4: Mortality curves from the lethal dose experiments. A: Mortality from the LD-isolation experiment with fish in individual tanks. B: Mortality from the LD-batch experiment. For B, data points show the average mortality of three tanks of 20 fish for each dose and genotype, with the exception of the mock group, which had only a single tank, and the LV 10^4 PFU/ml dose, which had two tanks. Error bars show the standard error.

Figure 5: Comparison of LD_{25}, LD_{50}, and LD_{75} values from both isolation and batch virulence experiments in units of log of PFU/ml. Values from LD-isolation are on the left; values from LD-batch are on the right. For LV, the two bars indicate the LD_{25} and LD_{50} values, and for HV the two bars indicate the LD_{50} and LD_{75} values. The error bars show the 95% confidence interval.

Figure 6: Comparisons of percent infected and percent mortality values at the three challenge doses used in the ID-3, LD-isolation and LD-batch experiments. Percent infected data is from experiment 3, which was performed on the same lot of fish as the

LD-isolation and LD-batch experiments. LV is on the left, in gray, and HV is on the right in black. The batch mortality values represent the mean of triplicate tanks (+/- 1 standard error).

Figure 7: Comparisons between ID_{50} and LD_{50} values for each strain. On the left in gray is LV, and on the right in black is HV. The ID_{50} values shown are calculated from the third experiment, which was done on the same lot of fish as the LD_{50} experiments. The error bars indicate the 95% confidence interval.

Figure 8: Test of Independent Action Hypothesis. Data points show relationship between challenge dose (x-axis) and proportion of fish infected (y-axis), for genotypes HV (black circles) and LV (grey squares). Thick solid line is the independent action model ($f = 1 - e^{-b*d}$) fit to the data for HV (black) and LV (grey). Thin dotted line is the interaction model fit ($f = 1 - e^{-b*d^k}$) to the data for HV (black) and LV (grey). Where *f* = proportion of fish infected from raw data, 1= the maximum proportion of fish that can become infected, *b*=infection rate determined from model fit, *d*=challenge dose, and *k* = interaction term determined from model fit. *k* = 1 indicates independent action, *k*<1 indicates antagonistic interaction, and *k*>1 indicates synergistic interaction. For the independent action model $b = 2.55 \times 10^{-4} \pm 0.55 \times 10^{-4}$ and $8.00 \times 10^{-5} \pm 1.57 \times 10^{-5}$ proportion fish infected/PFU virus, for HV and LV respectively (value ± 1 standard error). For the interaction model $b = 7.61 \times 10^{-4} \pm 15.20 \times 10^{-4}$ and $7.71 \times 10^{-6} \pm 35.47 \times 10^{-5}$ proportion fish infected/PFU virus; and $k = 0.868 \pm 0.240$ and 1.26 ± 0.51, for HV and LV respectively (value ± 1 standard error). As such, *k* overlapped with 1 for both HV and LV, supporting independent action model. There was no significant difference in model fit between independent action and interaction models by anova (HV: $F_{1,17}=0.28$, $p=0.6$; LV: $F_{1,17}=0.26$, $p=0.6$), so null hypothesis of independent action could not be rejected. Data was fit to models using “nls” function in the R programming language.