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1 Characterization of infectious dose and lethal dose of 2 two strains of infectious hematopoietic necrosis virus 3 (IHNV)

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13 14 **Abstract:**

15 The ability to infect a host is a key trait of a virus, and differences in infectivity could
16 put one virus at an evolutionary advantage over another. In this study we have
17 quantified the infectivity of two strains of infectious hematopoietic necrosis virus (IHNV)
18 that are known to differ in fitness and virulence. By exposing juvenile rainbow trout
19 (*Oncorhynchus mykiss*) hosts to a wide range of virus doses, we were able to calculate
20 the infectious dose in terms of ID₅₀ values for the two genotypes. Lethal dose
21 experiments were also conducted to confirm the virulence difference between the two
22 virus genotypes, using a range of virus doses and holding fish either in isolation or in
23 batch so as to calculate LD₅₀ values. We found that infectivity is positively correlated
24 with virulence, with the more virulent genotype having higher infectivity. Additionally,
25 infectivity increases more steeply over a short range of doses compared to virulence,
26 which has a shallower increase. We also examined the data using models of virion
27 interaction and found no evidence to suggest that virions have either an antagonistic or
28 a synergistic effect on each other, supporting the independent action hypothesis in the
29 process of IHNV infection of rainbow trout.

30 **Keywords:**

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

33

34

35 **1. Introduction**

36

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

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

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

60 These LD₅₀ studies are often used to make inferences about infectivity, assuming
61 high virulence strains cause greater mortality because higher numbers of hosts become
62 infected. However, this assumed relationship between virulence and infectivity has
63 several limitations. For example, many viruses cause disease that does not result in
64 host death. Viruses can also cause sub-clinical infections, where the host becomes
65 infected but suffers no clinical disease. For example, a study of infectious pancreatic
66 necrosis virus in Atlantic salmon found that at low challenge dosages a larger
67 percentage of fish become infected than succumb to mortality (Urquhart et al., 2008).
68 Quantification of actual infection is thus critical for an accurate assessment of
69 infectivity, which is an essential component of overall viral fitness. Infectivity can be
70 quantified in much the same way as virulence. For example a range of viral exposure
71 dosages can be administered, after which hosts can be tested for infection status at a
72 specific time post-infection. The prevalence of infection at each exposure dose is then
73 used to calculate the 50% infectious dose (ID₅₀), i.e., the dose at which fifty percent of
74 exposed hosts are infected. Though the methods used to detect infection are different,
75 the ID₅₀ is determined in the same manner as the LD₅₀ (Reed & Muench, 1938). As with
76 lethal dose, minimum infectious dose, the lowest dose needed to cause an infection,
77 can also be quantified. Interpretation of virus infection studies is heavily dependent on
78 the methods used, which differ in their sensitivity and specificity for live virus, viral
79 genetic material, or host responses to infection. In this study we define infection as the
80 presence of viral RNA in the host as detected by real-time reverse transcriptase qPCR.

81 In general fewer studies have been conducted examining ID₅₀ values compared
82 to the number exploring LD₅₀ values. Among studies that determine both ID₅₀ and LD₅₀
83 values for various host:pathogen systems, the relationship between infectivity and
84 virulence is not always consistent. For example, a study of avian influenza virus in wild
85 duck and poultry found large host species effects on infectious dose, but within a host
86 species, LD₅₀ values were tightly coupled with ID₅₀ values, suggesting virulence was
87 correlated with infectivity (Swayne & Slemmons, 2008). However, in a study of Monkey

88 B virus in mice, the relationship between LD₅₀ and ID₅₀ was less consistent, with some
89 of the strains with the lowest ID₅₀ values having the highest LD₅₀ values, suggesting
90 virulence may be decoupled from infectivity (Ritchey et. al., 2005). Thus, investigation
91 of infectivity and virulence for additional pathogens is of interest, and aquatic systems
92 are ideal for experiments involving large numbers of hosts being exposed to a wide
93 range of pathogen doses.

94 Examination of infectivity and virulence across a range of virus exposure dosages
95 is a powerful method for comparing the traits of different virus strains. Such studies
96 make it possible to reveal differences in virulence and infectivity that might not be
97 apparent at single exposure dosages. This is because mortality and infectivity may
98 saturate at the same levels for different virus genotypes, but the rate of increase in
99 infection and mortality across exposure dosages may be different. In addition, the
100 relationship between infectivity and exposure dose allows for an assessment of whether
101 or not individual virions interact during the process of infection. For example, if there is
102 a linear increase in the rate of infection as dose increases, this suggests that virions do
103 not impact the infectivity of other virions. Here this is referred to as the independent
104 action model, also sometimes referred to as the mass-action principle (Regoes et al.,
105 2003; Schmid-Hempel, 2011). In contrast, if the rate of infection changes in a non-
106 linear manner as the number of virions in the exposure dose increases, this would
107 suggest that the virions interact with each other either in a synergistic or an
108 antagonistic manner, here referred to as an interaction model. If there is a synergistic
109 interaction, that could result in an invasion threshold, with a threshold dose (Regoes et
110 al., 2003; Schmid-Hempel, 2011). In this case, if the host receives less than the
111 threshold dose it will not become infected, and infection can only occur when the dose
112 meets or exceeds the threshold dose. Ultimately, whether infectivity follows the
113 independent action or interaction model can have influence epidemiological predictions
114 about disease risk and spread (Schmid-Hempel, 2011; van der Werf, 2011).

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

118 been well-studied *in vivo*, infectious hematopoietic necrosis virus (IHNV; order
119 *Mononegavirales*, family *Rhabdoviridae*, genus *Novirhabdovirus*) in rainbow trout
120 (Wargo et al., 2010; Kell et al., 2013; Peñaranda et al., 2009; Bootland & Leong, 2011;
121 Zhang & Gui, 2015). In the western United States, IHNV is endemic in salmonid fish
122 throughout a range from Alaska to California, as well as inland via rivers to Idaho
123 (Bootland & Leong, 2011). Within this range three main genogroups occur: U, M, and L,
124 each of which exhibit some host specificity (Kurath et al., 2003). Relevant to this study,
125 the M genogroup is hypothesized to have arisen in rainbow trout (Kurath et al., 2003).
126 Under certain conditions IHNV causes disease epidemics in salmonid fish, with mortality
127 due to necrosis of the hematopoietic kidney and spleen tissues (Bootland & Leong,
128 2011). Variation in virulence of IHNV strains has been reported in several studies, most
129 often tested using a single high virus exposure dose (LaPatra et al., 1993; Garver et al.,
130 2006; Wargo et al., 2010). However, infectious dose has not been previously quantified
131 for IHNV.

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

144 Five *in vivo* infection experiments were conducted using standardized one-hour
145 batch immersion challenges to assure uniform, consistent virus exposure of fish within
146 each group. Three of the experiments were independent infectious dose assays that
147 determined ID₅₀ estimates and provided a measure of the variability in those estimates.

148 In these experiments fish were separated into isolated holding tanks after challenge to
149 avoid cross-infection, and infection status was determined at 3 days post-exposure.
150 The fourth experiment was a virulence assay that determined the lethal dose of each
151 strain under the same isolation conditions used in the infectious dose assays, allowing
152 direct comparison of ID₅₀ and LD₅₀ values for the two IHNV strains. Finally, as a
153 secondary goal of this study we conducted a virulence assay using standard batch
154 holding conditions, for comparison with the results of the virulence assay with fish held
155 in isolation. This provided insight into how much of the mortality observed in standard
156 batch challenge studies is due to holding conditions or secondary fish-to-fish infection.
157 The combined data provide a comparison of the relationship between infectivity and
158 lethality of two strains of a virus of differing virulence and expand upon the previous
159 work done on the ecological parameters of various genotypes in the M genogroup of
160 IHNV (Troyer et al., 2008; Wargo et al., 2010; Wargo & Kurath, 2011; Kell et al., 2013).

161

162 **2. Materials and methods**

163

164 *2.1. Virus and host*

165

166 For this study, we used two isolates of IHNV that differ in virulence. The more
167 virulent strain is 220-90, referred to as HV for "high virulence"; the less virulent strain is
168 WRAC (alternate name, 039-82), referred to as LV for "low virulence" (Wargo et al.,
169 2010). Both strains were obtained from farmed rainbow trout in Idaho and have been
170 previously characterized for virulence (LaPatra et al., 1994; Garver et al., 2006; Wargo
171 et al., 2010). Over the glycoprotein gene of the virus there is 3.6% (58/1621
172 nucleotides) divergence between HV and LV; over the entire genome, the divergence is
173 2.8% (312/11,133 nucleotides) (Morzonov et al., 1995; Ammayappan et al., 2010). The
174 preparation of viral stocks as well as quantification of viral titer has been previously
175 described (Fijan et al., 1983; Batts & Winton, 1989; Troyer et al., 2008).

176

177 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

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

182

183 *2.2. Virus challenges to determine infectious dose*

184

185 Three experiments to determine the infectious dose (ID) of each virus genotype
186 were performed using identical methodology, differing only in the doses of virus
187 administered and number of fish in each group, as shown in Table 1. The fish in the
188 three ID experiment were from three different lots, with an average weight of 1.6 g, 1.1
189 g, and 1.1 g respectively. In each experiment, groups of fish were exposed to a range
190 of specific concentrations of virus, or mock exposed, by a 1 hour batch immersion in
191 static water (Garver et al., 2006). Water flow was then turned on and the fish were
192 washed for 1 hour. After the wash, the fish were isolated into 1 liter beakers containing
193 400 ml static water, well before detectable replication or shedding of the virus occurred,
194 to avoid cross-infection, and then held in isolation at 15 °C for three days, which is
195 when the mean viral load has previously been shown to reach maximum levels (Troyer
196 et al., 2008; Peñaranda et al., 2009). At this point each fish was euthanized, harvested
197 aseptically, and stored in an individual Whirl-pak™ at -80 °C until RNA extraction and
198 viral load quantification.

199

200 *2.3. RNA extraction and cDNA synthesis*

201

202 Total RNA was extracted from whole fish as previously described (Wargo et al.,
203 2010). Briefly, 4 ml/g fish of guanidinium thiocyanate-based denaturing solution was
204 added to each fish, and the fish was homogenized using a Seward Stomacher® 80
205 (Biomaster). RNA was extracted from 1 ml of the homogenate with phenol-chloroform,
206 precipitated, resuspended in 50 µl of water, and assessed for quality and concentration
207 by spectrophotometry. The RNA samples were then stored at -80 °C until cDNA

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

211

212 *2.4. Viral RNA quantification via qPCR*

213

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

224

225 *2.5. Virus challenge to determine lethal dose for fish held in isolation*

226

227 The lethal dose in isolation (LD-isolation) was determined by challenging fish in
228 batch as described above and then holding them in isolation for 30 days. These
229 experiments were performed on the same lot of fish as the third ID experiment,
230 approximately three months later. Groups of 20 fish with average weight 1.2 g were
231 challenged by batch immersion in 1 L of static water containing one of three specific
232 doses of HV or LV, as shown in Table 1. In addition, one group of 20 control fish was
233 mock-exposed. After the 1 hour challenge, the water was turned on for a one-hour
234 rinse, and then individual fish were netted into 1.5 L tanks in a tower rack system
235 (Aquatic Habitats). These tanks provided independent flow-through water for each fish.
236 After isolation, the fish were monitored daily for a period of 30 days at 15 °C. Each
237 treatment group had a total of 20 fish, except for LV at the 10⁴ plaque-forming units

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

241

242 *2.6. Virus challenge to determine lethal dose for fish held in batch*

243

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

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

268

269 *2.7. Statistical analyses*

270

271 The infection and mortality data were used to calculate the projected doses at
272 which fifty percent of fish were infected (ID₅₀), died in isolation (LD₅₀-isolation), or died
273 in batch (LD₅₀-batch). The calculations of ID₅₀ and LD₅₀ values were both done using
274 generalized linear models (GLM) in the statistical program R, version 3.3.1 (R Core
275 Team, 2015), using the dose.p function of the Mass package (Venables & Ripley, 2002)
276 with a quasibinomial distribution prior, as described in Breyta et al., 2014. To calculate
277 the ID₅₀ values, numbers of infected versus uninfected fish were quantified. To
278 calculate LD₅₀ values numbers of dead versus alive fish were quantified. Significant
279 differences between the suite of ID₅₀ and LD₅₀ values generated were calculated using
280 the Welch-Satterthwaite 2-tailed *t*-test in R (Breyta et al., 2014). Results from the LD
281 experiments indicated that mortality did not bracket 50% in all cases, leading to
282 uncertainty in the calculated LD₅₀ value. Therefore, using the same methods, we
283 calculated the doses at which 25% of the fish exposed to LV died (LD₂₅) and the doses
284 at which 75% of the fish exposed to HV died (LD₇₅). Differences in the kinetics of
285 mortality were assessed using Kaplan-Meier curve and log-rank test functions of the
286 Survival package of R (Therneau 2015), comparing the pooled doses of each treatment
287 against each other. Viral load data were compared using a generalized linear model
288 with response variable log-transformed viral load and explanatory factors virus
289 genotype (HV vs. LV), challenge dose, and experiment. A Tukey multiple comparison of
290 means test was used to determine differences between factors levels.

291 To determine if the relationship between proportion of fish infected and
292 challenge dose fit the independent action hypothesis (also referred to as mass-action
293 principle) the method suggested in (Regoes et. al., 2003) was utilized. To do so, the
294 challenge dose and percent fish infected data from all infectivity experiments was fit to
295 an independent action hypothesis model ($f = 1 - e^{-b*d}$) and an interaction hypothesis
296 model ($f = 1 - e^{-b*d^k}$), where f = proportion of fish infected from raw data, 1 = the
297 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 2×10^5 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 5×10^3 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 5×10^2 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 2.5×10^3 PFU/ml in experiment 2. For HV the minimum
343 infective dose was 5×10^2 PFU/ml in experiments 2 and 3 and 10^3 PFU/ml in experiment
344 1 where the 5×10^2 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 9.83×10^3 PFU/ml for LV and 1.94×10^3 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.

358

359 *3.1.3. Viral load data from infectivity experiments*

360 Overall, the viral load data for all virus-positive fish from all the ID experiments
361 were similar across dose and genotype (Figure 3). While the viral loads of individual fish
362 did vary, there were almost no significant differences between the means of the log-
363 transformed viral loads, either between doses or between genotypes ($p > 0.05$). The
364 one exception was in the first experiment, where the combined mean viral load for both
365 HV and LV at 10^4 PFU/ml was significantly lower than the combined mean viral load at
366 2×10^5 PFU/ml ($F_{2,34} = 1.87$, $p = 0.0108$).

367

368 *3.2. Virulence experiments in HV and LV*

369

370 *3.2.1. Determination of lethal dose in isolation*

371 The final mortality for LD-isolation ranged between 42-60% for HV and 20-26%
372 for LV (Figure 4A). For HV, mortality in the 10^5 PFU/ml dose was higher than the 10^3
373 and 10^4 PFU/ml doses, which were similar in final mortality. However, the kinetics for all
374 three doses of HV showed a clear dose response; the highest dose had the most rapid
375 mortality initially, and the lowest dose initially had the slowest mortality. For LV, there
376 was no strong dose response in either kinetics or final mortality. Although mortality in
377 the mock treatment group was 15%, all three fish that died were negative for IHNV via
378 plaque assay. The level of mortality in the mock treatment groups indicated that there
379 might have been elevated non-specific mortality in the virus-exposed fish as well.
380 However, all mortalities titered from the virus-exposed groups (approximately 50% of
381 all fish that died) were positive via plaque assay, with average log-transformed titers of
382 6.61 ± 0.40 standard error PFU/ml for HV and 6.50 ± 0.27 standard error PFU/ml for
383 LV. These virus titers are in the range commonly seen in fish that die after IHNV
384 exposure (Breyta et al., 2014), indicating that they died as a result of viral infection.

385

386 *3.2.2. Determination of lethal dose in batch*

387 Mortality curves for LD-batch were generated from the daily average cumulative
388 percent mortality of the three replicate tanks for each genotype and dose, with the
389 exceptions of the LV 10^4 PFU/ml dose, which had duplicate tanks, and the mock-
390 infected group, which only had one tank (Figure 4B). For HV, the average mortality
391 ranged from 60-78%. However, there was not a clear dose response for the 10^3 and
392 10^4 PFU/ml doses, both of which had similar kinetics of mortality. The mortality for the
393 three doses of LV ranged from 32-47% and followed a clearly separated dose response
394 in both kinetics and final mortality. When comparing virulence in batch to that in
395 isolation, for HV the final mortality at each dose was 15-18% higher in batch, and for
396 LV it was 12-14% higher for the 10^3 and 10^4 PFU/ml doses and 22% higher for the 10^5
397 PFU/ml in batch. Neither genotype bracketed 50% final mortality; HV mortality was all
398 above 50%, and LV mortality was all below 50%. One fish died in the mock treatment
399 group and it was negative for virus via plaque assay. Approximately 20% of the virus-
400 exposed fish that died were tested for virus and all were positive, with average log-
401 transformed titers of 6.29 ± 0.019 standard error PFU/ml for HV and 5.80 ± 0.70
402 standard error PFU/ml for LV.

403

404 *3.2.3. Analysis of mortality data*

405 Differences in virulence between HV and LV were assessed by survival analysis
406 on data from the lethal dose experiments. Log rank tests on the combined Kaplan-
407 Meier estimates for dose and genotype indicated that mortality was significantly higher
408 for HV than LV, both in isolation ($\chi^2 = 8.4$, $df = 1$, $p = 0.0037$) and in batch ($\chi^2 = 27.4$,
409 $df = 1$, $p < 0.001$). This confirms the expected phenotypic difference in virulence
410 between HV and LV. Additionally, mortality for both HV and LV was significantly higher
411 in batch than in isolation ($\chi^2 = 5.9$, $df = 1$, $p = 0.015$ and $\chi^2 = 4.7$, $df = 1$, $p = 0.031$,
412 respectively).

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

417 batch. This is likely because neither HV nor LV bracketed 50% mortality across the
418 exposure doses, with the exception of HV in isolation, and they fell on opposite sides of
419 the 50% level (Figure 4). Therefore, calculating LD₅₀ values required inferring the
420 relationship between dosage and mortality outside the range of available data, leading
421 to wide 95% confidence intervals. Furthermore, the calculated LD₅₀ above 10¹² pfu/ml
422 for LV in isolation suggested that there is no feasible dose of LV that would be able to
423 cause 50% mortality under isolation conditions. In light of this, we also calculated LD₂₅
424 and LD₇₅ values that were bracketed by the LV and HV data respectively (Figure 5).
425 This gives us the most accurate values for each genotype.

426

427 *3.3. Independent action hypothesis test*

428 Analysis of our infectivity data indicated that the relationship between virus
429 challenge dose and the proportion of fish infected was better explained by the
430 independent action hypothesis compared to the interaction hypothesis, for both
431 genotype HV and LV (Figure 6). This result was supported by a F-test comparison of the
432 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
433 as determination that there was insufficient evidence to indicate the interaction term, k
434 was significantly different from 1, for either genotype (HV: $k = 0.868 \pm 0.24$; LV: $k =$
435 1.26 ± 0.51 ; gives mean ± 1 standard error), in the formula $f = 1 - e^{-b \cdot d^k}$ (described in
436 methods). As such, the results suggest that the infection process operates under a
437 similar mechanism for HV and LV where individual virions do not inhibit or enhance the
438 infectivity of other virions. Furthermore, both genotypes were able to achieve 100% of
439 fish infected at high dosages, supporting the usage of a value of 1 for the maximum
440 proportion of fish infected in the model. However, the analysis did reveal that the
441 infectivity rate parameter (b) was 3-fold higher for HV ($b= 2.55 \times 10^{-4}$ proportion fish
442 infected/PFU virus $\pm 0.55 \times 10^{-4}$; mean ± 1 standard error) compared to LV ($b= 8.00 \times$
443 10^{-5} proportion fish infected/PFU virus $\pm 1.57 \times 10^{-5}$). This indicates that infection
444 increases more quickly as virus exposure dose increases, for HV compared to LV.

445

446 **4. Discussion**

447

448 This study was designed to provide data on how infectivity relates to virulence
449 for two well-studied IHNV genotypes that are known to differ in both in-host fitness and
450 virulence (Wargo et al., 2010; Wargo & Kurath, 2011). Overall, the results consistently
451 showed that HV had a higher infectivity than LV, which correlated with its confirmed
452 higher virulence. Furthermore, HV showed a more rapid increase in infectivity with
453 increasing exposure dosage, compared to LV, as indicated by the dose response data
454 and the independent action model. The previously documented replicative fitness
455 difference between HV and LV (Wargo 2010; 2011) also correlated with the increased
456 infectivity shown here. This confirms the finding from previous work (Wargo & Kurath,
457 2011) that HV has an advantage over LV in host entry, based on comparison of in-host
458 fitness differences after viral infection by immersion versus injection. This advantage is
459 likely to be most pronounced at an intermediate range of viral exposure doses, because
460 at very high or very low doses infection saturated at 100% or 0% for both genotypes
461 (Figure 1A). Thus neither genotype should have an advantage over the other in the
462 number of fish that become infected at extreme high or low doses, but at intermediate
463 dosage HV is predicted to infect more fish than LV, and thus have greater overall
464 fitness.

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

477 prevalence of infection (Troyer et al., 2008). This was further supported by the viral
478 load data in the present study. Here, there were no differences observed in the viral
479 load of infected fish between genotypes HV and LV. However, a previous study found
480 within-host viral loads for genotype HV were consistently higher than for LV, and a
481 larger proportion of fish were infected at the 10^4 PFU/ml challenge dose (Wargo et al.,
482 2010; Wargo & Kurath 2011). The primary difference between these studies was that in
483 the previous work, fish were exposed to virus for 12 hours, whereas they were exposed
484 for 1 hour, and viral load was quantified 12 hours earlier, in the current study. It may
485 be that the longer immersion challenge allows for infection by more virions, resulting in
486 faster viral replication kinetics that result in different viral loads, but further research is
487 needed to discern the effects of exposure time on infection and viral load.

488 In order to link infectivity and mortality data, we conducted LD experiments in
489 conjunction with the third ID experiment. The results indicated that the processes of
490 infection and virulence respond differently to variation in exposure dose. In almost all
491 cases percent infection increased with increasing dose at a different rate than percent
492 mortality, and infection prevalence was higher than mortality (Figure 7). Furthermore,
493 large increases in percent infection as dose increased were associated with relatively
494 small increases in percent mortality. The exception to this was for the increase from the
495 10^4 PFU/ml to the 10^5 PFU/ml dose of HV, where no increase in percent infection was
496 possible due to infection being at 100% at both doses, but there was an increase in
497 mortality by 15%. This implies that the exposure dose can influence mortality even
498 when all fish are infected. The calculated LD_{50} values also supported the conclusions
499 that infection does not guarantee death in that ID_{50} values were lower than the LD_{50}
500 values in nearly all cases (Figure 8). This indicates it takes more virions to kill than to
501 infect the same number of fish and that while infectivity plays a role in determining
502 virulence, it is likely not the only factor.

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

507 This implies that significant differences between LD₅₀ values may be difficult to obtain
508 for virus genotypes that cause widely different levels of mortality. Thus, while this study
509 is consistent with previous publications that virulence is correlated with previously
510 demonstrated differences in fitness for IHNV (Peñaranda et. al., 2009; Wargo et. al.,
511 2010; Wargo & Kurath, 2011), these results stress the importance of considering
512 survival kinetics when quantifying virulence.

513 The combination of batch versus isolation virulence experiments made it possible
514 to examine how holding conditions impact mortality. The difference between percent
515 mortality in the two holding conditions was consistently 12-22% higher in batch across
516 the challenge doses for both genotypes. This might be due in part to the fact that in
517 batch, infected fish are shedding virus into the water, which could be responsible for
518 multiple rounds of infection. The fact that the 10³ PFU/ml dose of LV had a higher rate
519 of mortality than infection supports this theory (Figure 6). However, the fact that the
520 10⁴ and 10⁵ PFU/ml doses of HV both had 100% infection, yet there was still an
521 increase in mortality, suggests that other factors might also be involved. Furthermore,
522 one would expect the increase in mortality due to multiple rounds of infections to be
523 greatest when the fewest number of fish were initially infected, for example at the 10³
524 PFU/ml dose, because a large number of fish are remaining for potential infection.
525 However the increase in mortality between batch and isolation was surprisingly
526 consistent, regardless of how many fish were initially infected. Constant exposure to
527 virus could force the infected fish to divert resources to fighting the exposure, which
528 allows the established infections to cause a higher incidence of mortality. It is
529 reasonable to consider that manner of exposure (i.e. through shedding or original
530 inoculum) could play a significant role in mortality rate, just as longer exposure times
531 resulted in higher infection frequencies and different viral loads in previous studies for
532 IHNV (this study compared with Wargo & Kurath, 2011). Additionally, the batch and
533 isolation treatments imposed different stressors on the fish, which could have affected
534 mortality rates differently. High fish density in hatcheries increases probability of
535 contact between infected fish and has been linked to increased stress and lower water
536 quality (Bootland & Leong, 2011). However, rainbow trout are also social animals and

537 moving individual fish to isolation may be a stressor (Øverli et al., 2002; Øverli et al.,
538 2005). Determining the role of stress, holding conditions, and multiple rounds of
539 transmission in driving IHNV induced fish mortality warrants further study.

540 In this study we have illuminated a previously unexplored relationship between
541 infectivity and virulence in an aquatic virus. We have shown that while infectivity does
542 indeed correlate with virulence, it does not appear to be the only driving factor. To our
543 knowledge, such detailed work comparing infectivity and lethality has not been done
544 with IHNV in rainbow trout or with any other fish pathogen; as such it can serve as a
545 point of comparison for future studies. Due to variation in absolute mortality levels
546 observed in IHNV virulence studies repeated in different years (Breyta et al., 2014), it is
547 valuable to have infectivity and mortality data from the same year with the same lot of
548 fish. This was also evident in the current study, as some variation in infectivity was
549 observed between experiments conducted with different lots of fish. In future work
550 similar studies with other viral strains in multiple hosts will help determine if infectious
551 dose is as variable as lethal dose across different viral strains, and if infectivity and
552 lethality vary in the same manner. Examination of the exposure dose response of
553 mortality and infection also makes it possible to characterize heterogeneity of host
554 susceptibility (Rodrigues et. al., 2009). This is essential for understanding
555 epidemiological patterns and can greatly enhance pathogen control (Gomes et. al.,
556 2014). For example, our study suggested that the relationship between infectivity and
557 exposure dose fits the independent action hypothesis. However, it is possible that virion
558 interactions do occur but this was masked by susceptibility heterogeneity (Rogeo et.
559 al., 2003; van der Werf, et. al., 2011). More importantly, this work clearly shows that
560 infectivity and virulence differences between pathogen strains may not be evident at
561 very high or very low exposure doses, and thus it is important to examine a range of
562 exposure doses to determine where fitness differences are the most important. The
563 evolutionary implications of this dose response to pathogen fitness differences warrant
564 consideration. In summary, this work has demonstrated that for IHNV in rainbow trout
565 viral infectivity is positively correlated with virulence, but the ID₅₀ values varied less
566 than the LD₅₀ values. This serves as a valuable example of the relationship between

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

569

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571

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583

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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	$10^1, 10^2, 10^3, 10^4, 2 \times 10^5$	10	3 days	1 individual
Infectious dose 2	2	$5 \times 10^2, 10^3, 2.5 \times 10^3, 5 \times 10^3,$ $7.5 \times 10^3, 10^4, 10^5$	10	3 days	1 individual
Infectious dose 3	3	$5 \times 10^2, 10^3, 2.5 \times 10^3, 5 \times 10^3,$ $7.5 \times 10^3, 10^4, 10^5$	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

711

712 Table 1: Infectious dose and lethal dose experimental designs. All experiments had a 1-hour immersion challenge in
713 batch. The "Experiment duration" and "# fish/tank" columns describe the holding conditions after the 1-hour challenge.

714 Exposure doses are given in plaque-forming units (PFU) per milliliter.

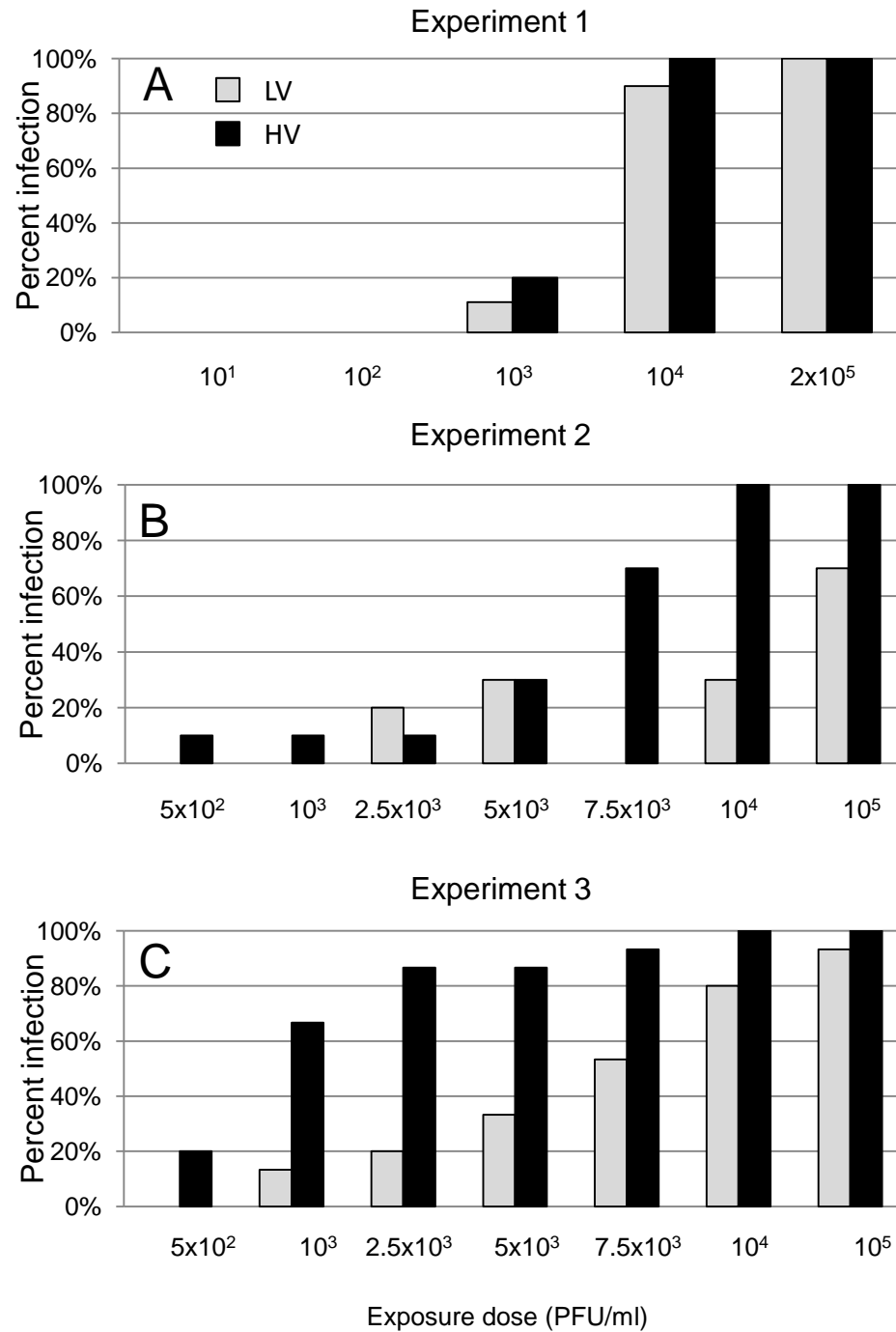


Figure 1

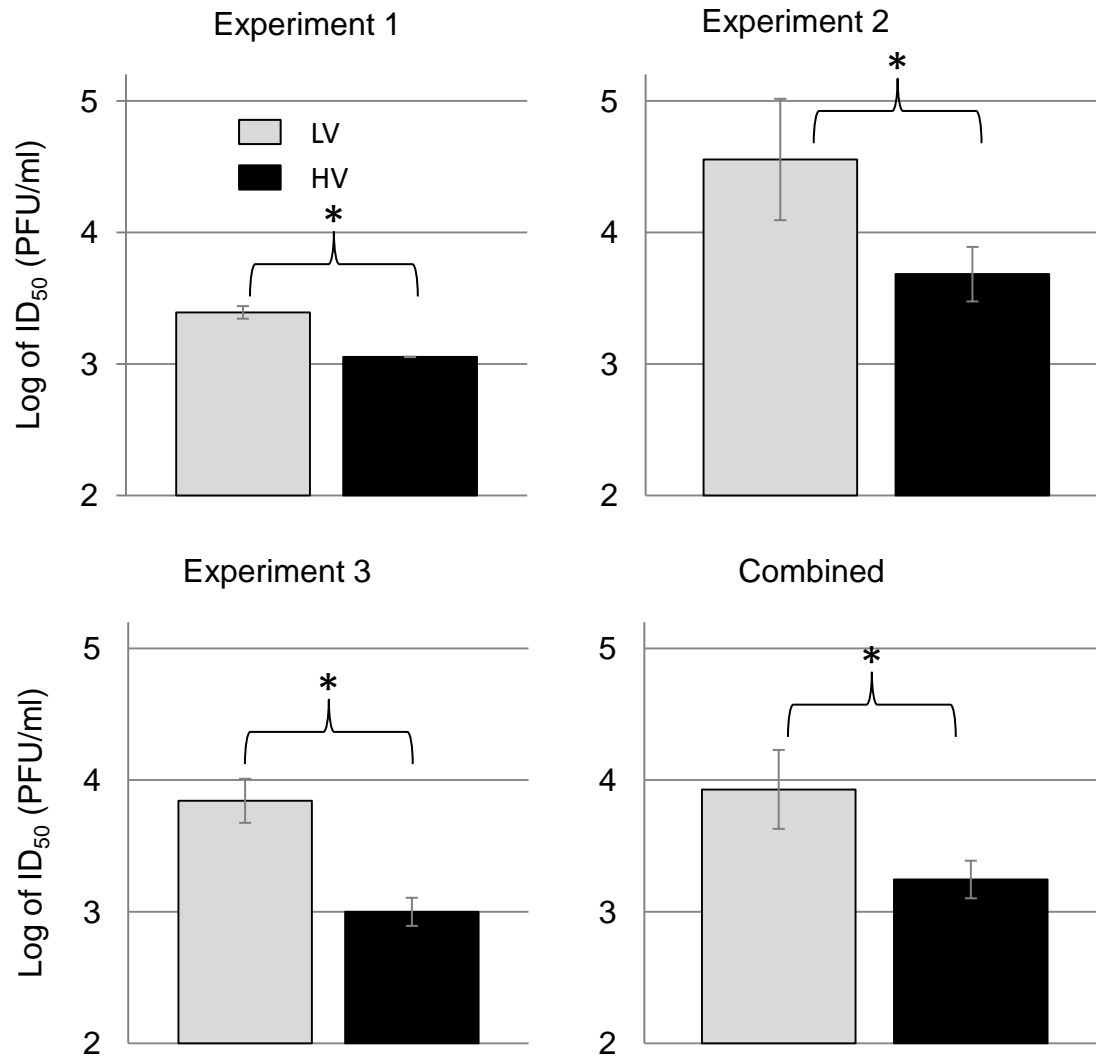


Figure 2

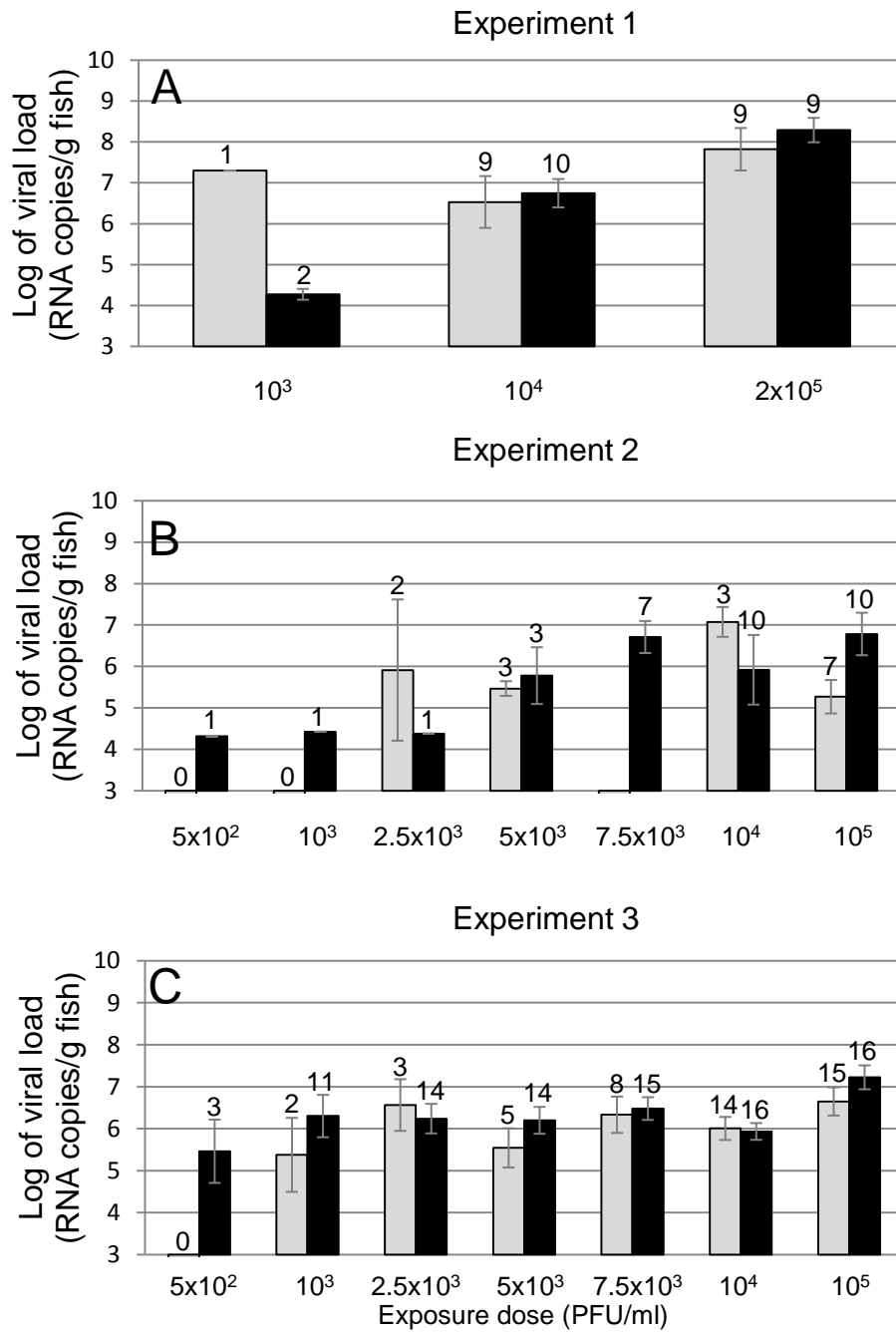


Figure 3

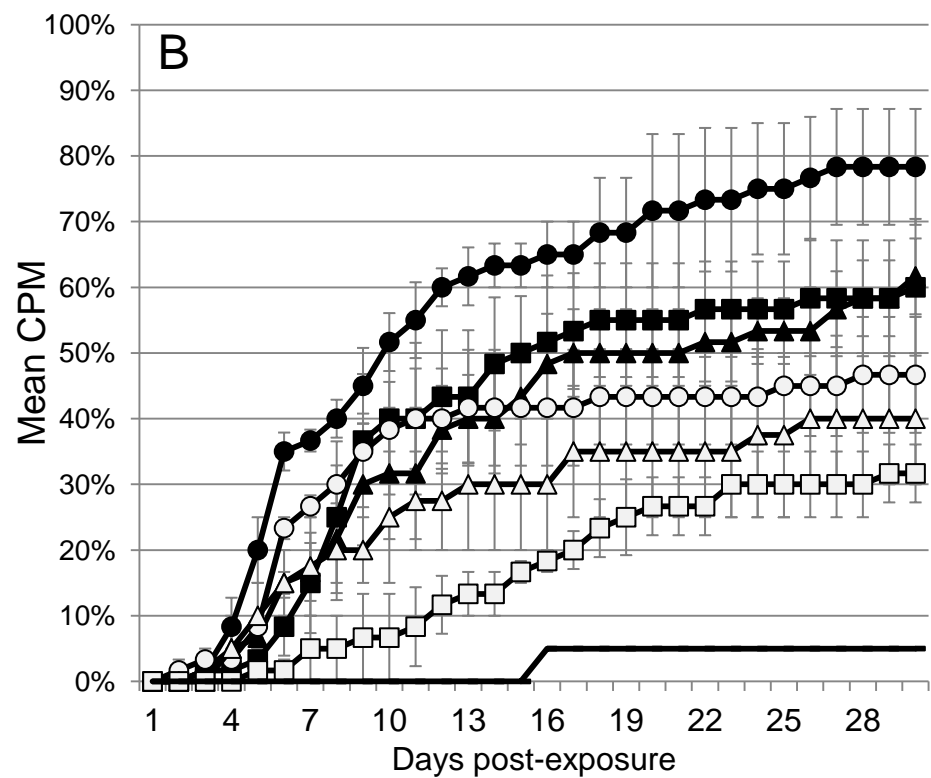
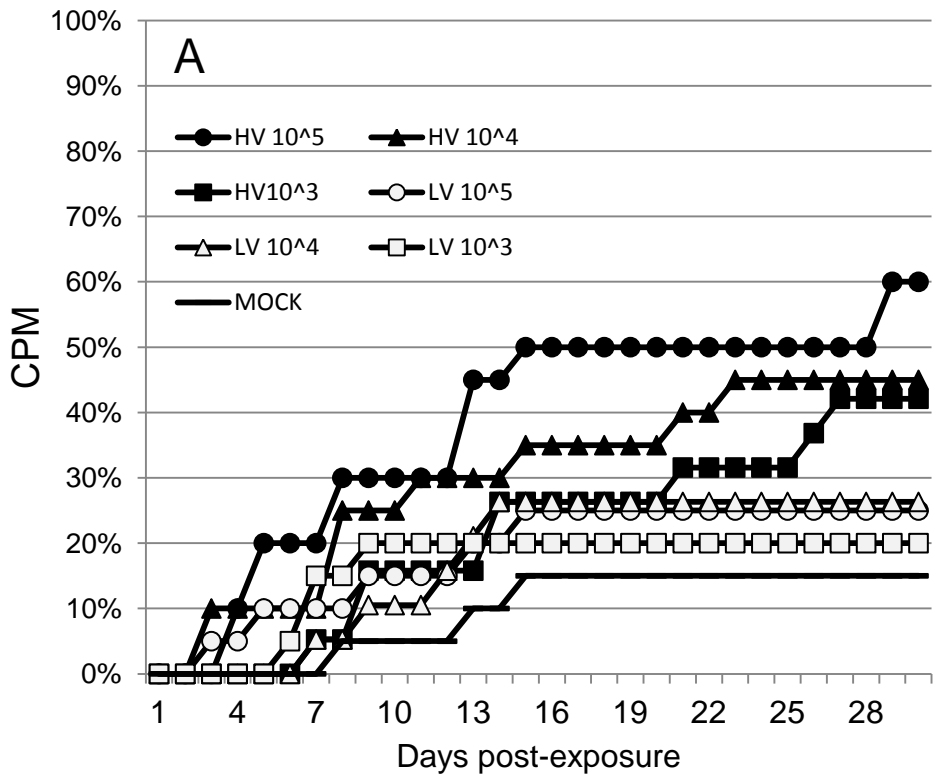


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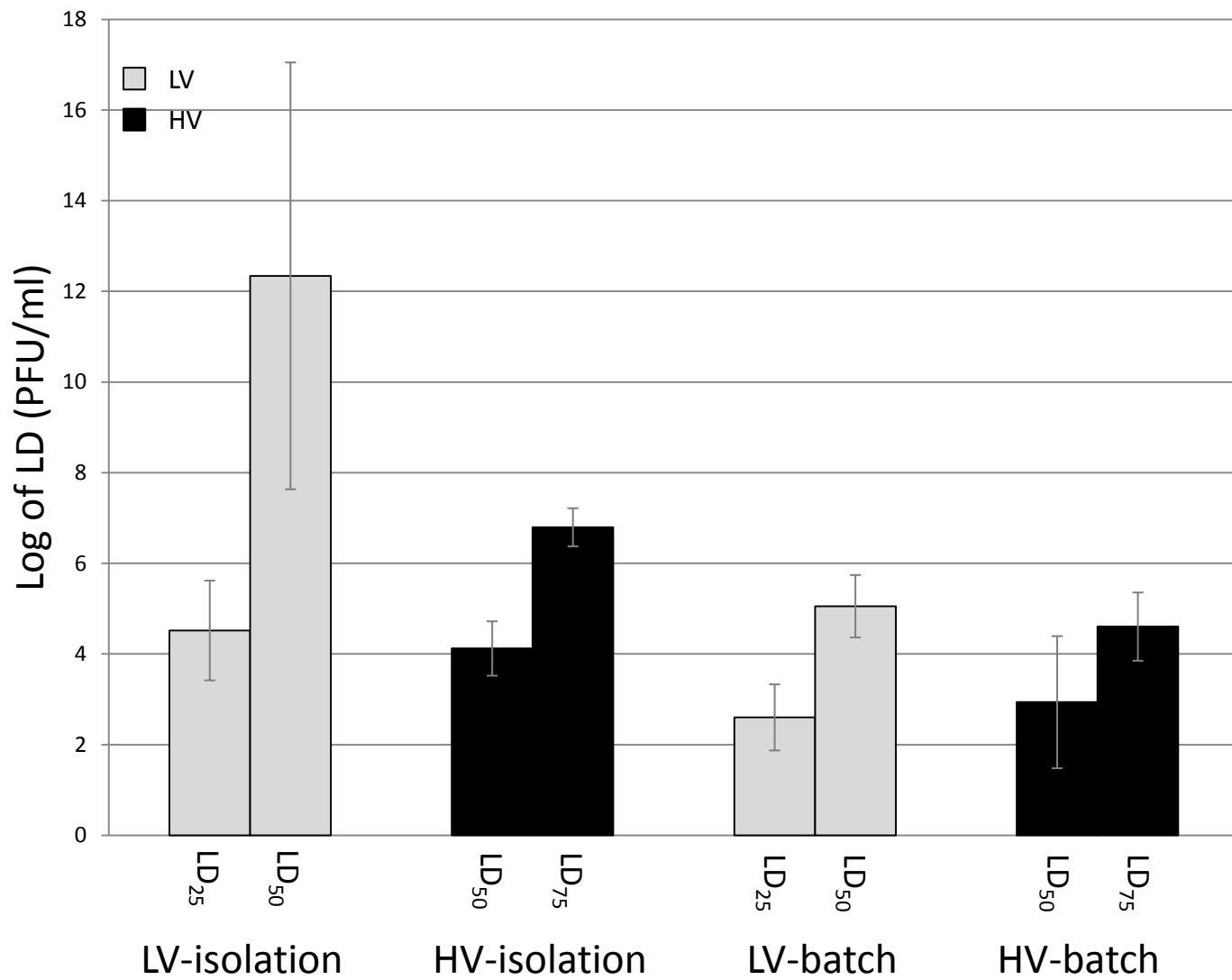


Figure 5

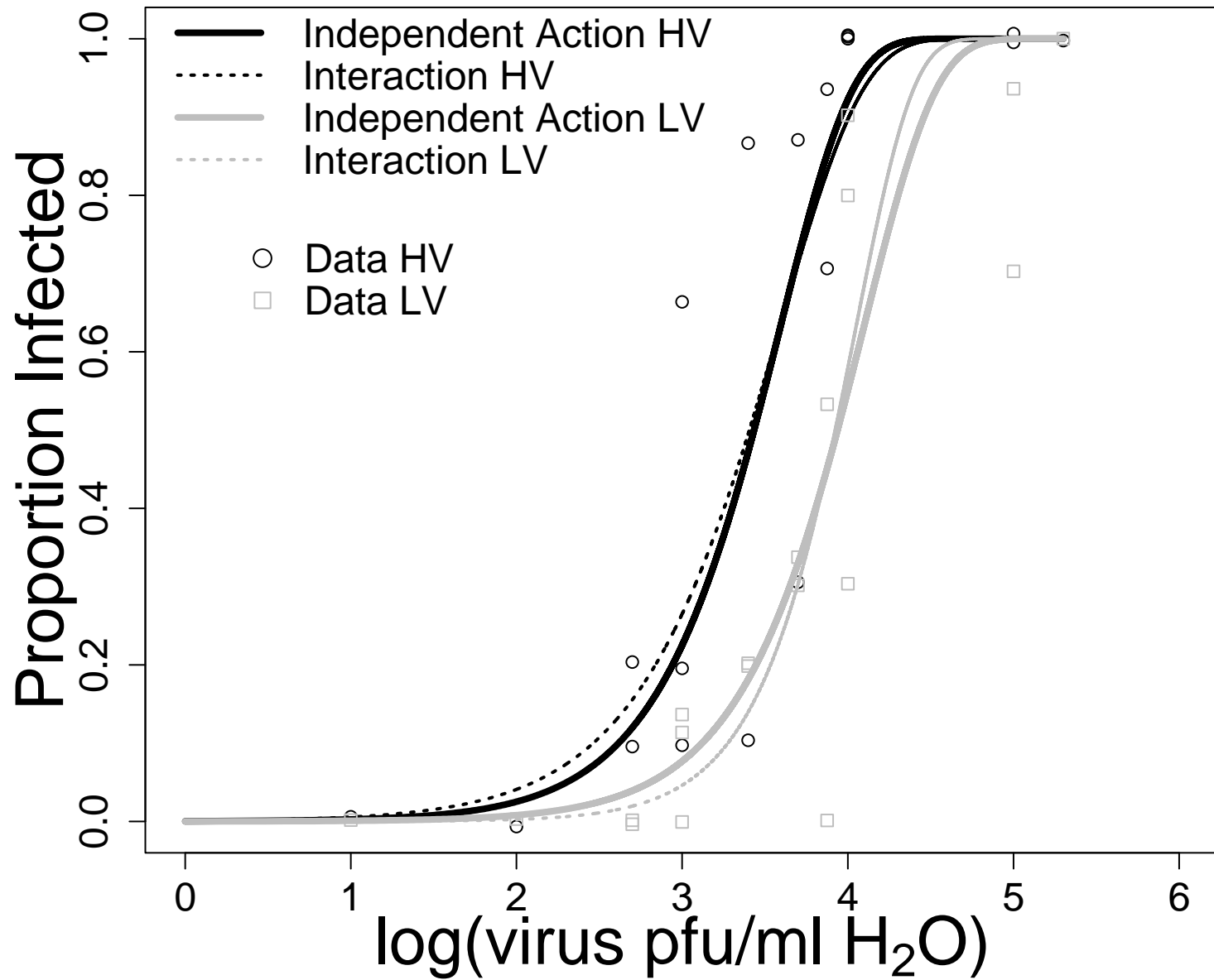


Figure 6

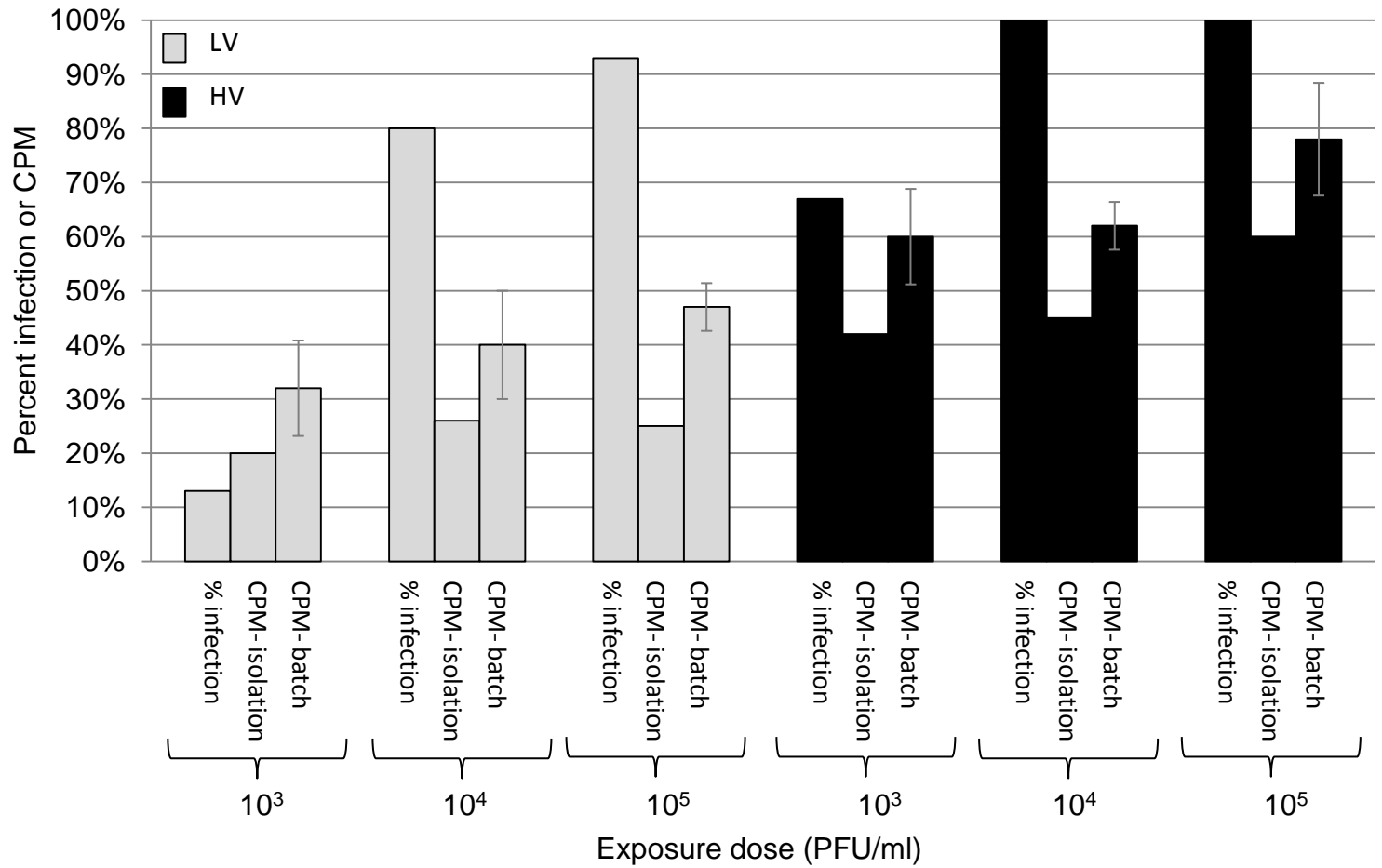


Figure 7

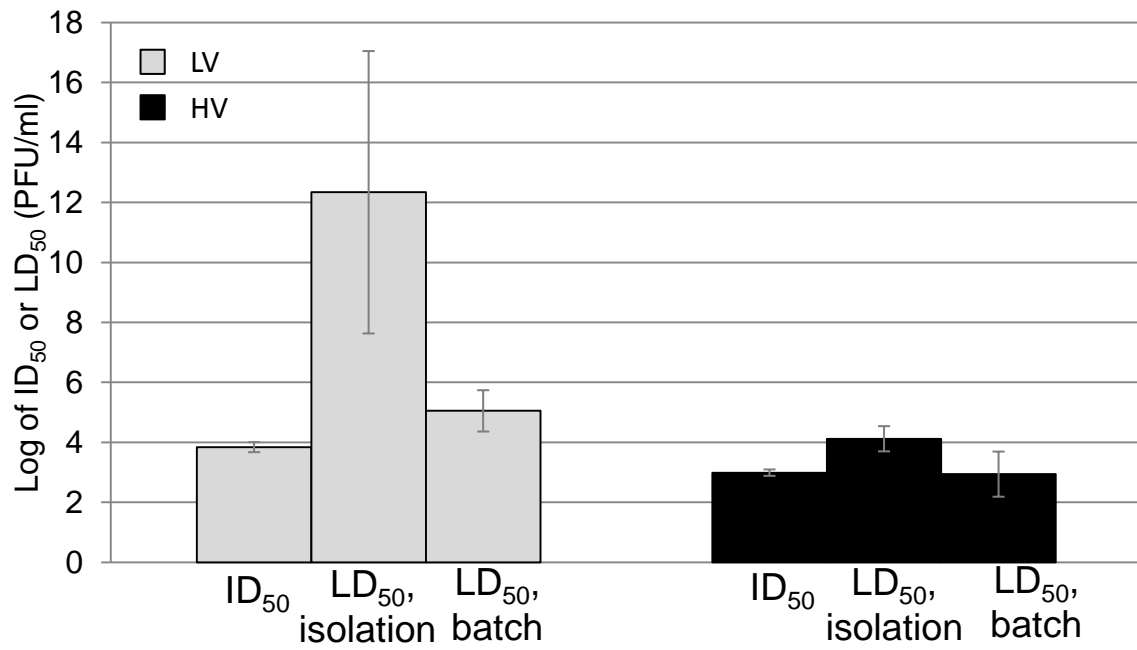


Figure 8

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 ID₅₀ and LD₅₀ values for each strain. On the left in gray is LV, and on the right in black is HV. The ID₅₀ values shown are calculated from the third experiment, which was done on the same lot of fish as the LD₅₀ 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.