Type of contribution: *Research Aritcle*

Co-exposure to multiple *Ranavirus* types enhances viral infectivity and replication in a larval amphibian system

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Abstract

Multiple pathogens commonly co-occur in animal populations, yet few studies demonstrate how co-exposure of individual hosts scales up to affect transmission. Although viruses in the genus *Ranavirus* are globally widespread and multiple virus species or strains likely co-occur in nature, no studies have examined how co-exposure affects infection dynamics in larval amphibians. We exposed individual *Rana aurora* (Northern red-legged frog) larvae to *Ambystoma tigrinum* virus (ATV), frog virus 3 (FV3), or an FV3-like strain isolated from a frog-culturing facility in Georgia, USA (RCV-Z2). We compared single-virus to pairwise co-exposures, while experimentally accounting for dosage. Co-exposure to ATV and FV3-like strains resulted in almost twice as many infected individuals compared to single-virus exposures, suggesting an effect of co-exposure on viral infectivity. The viral load in infected individuals exposed to ATV and FV3 was also higher than the single-dose FV3 treatment, suggesting an effect of co-exposure on viral replication. In a follow-up experiment, we examined how the co-occurrence of ATV and FV3 affected epizootics in mesocosm populations of larval *Pseudacris triseriata* (Western chorus frog). Although ATV did not generally establish within host populations (<4% prevalence), when ATV and FV3 were both present, this co-exposure resulted in a larger epizootic of FV3. Our results emphasize the importance of multi-pathogen interactions in epizootic dynamics and have management implications for natural and commercial amphibian populations.

Introduction

Classic theory and empirical research on infectious disease, in both wildlife and humans, has predominantly focused on the interaction between a single host and a single pathogen (Anderson et al. 1992, Hudson et al. 2002, Keeling & Rohani 2008, Rigaud et al. 2010, Tompkins et al. 2010). While substantial biological insights have been derived from such studies, multiple pathogens often co-occur (Petney & Andrews 1998, Pedersen & Fenton 2006, Balmer & Tanner 2011, Knowles et al. 2013, Griffiths et al. 2014, Stutz et al. 2018) and can result in transmission dynamics that deviate from classical expectations (Alizon et al. 2013a, Johnson et al. 2015, Seabloom et al. 2015), especially if host individuals become simultaneously or sequentially infected with different pathogens (i.e. co-infected or super-infected, respectively).

Research in a variety of systems has shown that ecological interactions among pathogens within a host, such as priority effects, competition, and facilitation, alter pathogen replication rates, probability of infection, clearance rates, and host survival (de Roode et al. 2005, Pedersen & Fenton 2006, Johnson & Hoverman 2012, Johnson, Preston, Hoverman, & LaFonte 2013, Nunn et al. 2014, Seabloom et al. 2015). While modeling studies have demonstrated how these within-host dynamics can scale up to affect transmission dynamics within host populations (Mideo et al. 2008, Alizon 2013), empirical studies linking scales in natural systems are limited. A notable exception involves de-worming experiments in wild buffalo populations, which show that co-infection with nematodes and the bacterial agent of tuberculosis increases host mortality (Jolles et al. 2008, Ezenwa & Jolles 2015). In an epidemiological model of the system, increased removal of hosts due to co-infection limited tuberculosis transmission in a manner consistent with large-scale epidemiological patterns in the field (Jolles et al. 2008, Ezenwa & Jolles 2015). Understanding how pathogen co-exposure affects pathology and transmission requires more

43 studies that explore the impacts of pathogen co-exposure across multiple biological scales
44 (Mihaljevic 2012, Gog et al. 2014, Buhnerkempe et al. 2015, Johnson et al. 2015).
45 Viruses of the genus *Ranavirus* (family: Iridoviridae) provide a tractable and relevant
46 model system for exploring the effects of multiple pathogens at both the within- and among-host
47 spatial scales. Ranaviruses infect amphibian communities globally and can cause massive die-off
48 events (up to 100% mortality), constituting a major threat to wild and commercially maintained
49 amphibian populations (Gray et al. 2009b, Lesbarrères et al. 2012, Gray & Chinchar 2015).
50 There are several reasons to suspect that co-exposure to multiple *Ranavirus* types (e.g. viral
51 species or strains) could be common in nature and influence transmission dynamics. First, this
52 viral genus is genetically and ecologically diverse, with different type species and strains that
53 show variability in epidemiological traits. For instance, two species of the genus common to
54 North America – *Ambystoma tigrinum virus* (ATV) and *Frog virus 3* (FV3) – can easily be
55 differentiated based on genomic characteristics, but also by their variability in infectivity, with
56 ATV being more host-specific to salamanders (urodeles) and FV3 being more host-generalist,
57 capable of infecting amphibians, reptiles, and some fish (Chinchar et al. 2009, 2011, 2017).
58 Furthermore, unique strains of ATV and FV3 differ in the rates at which they cause host
59 mortality, which we refer to as virulence (Brunner & Collins 2009, Hoverman et al. 2010).
60 Finally, both ATV and FV3 can be highly prevalent across the landscape, and their spatial
61 distributions broadly overlap (Tornabene et al., Gray et al. 2007, Ridenhour & Storfer 2008,
62 Greer et al. 2009, Brunner et al. 2011, Hoverman, Mihaljevic, et al. 2012, Gray & Chinchar
63 2015), suggesting a high potential for co-occurrence. To date, however, no studies have
64 considered the effect of multiple *Ranavirus* species or strains on disease outcomes or epizootics.
65 While ATV has high infectivity in salamanders (Picco et al. 2007, Brunner & Collins

2009), there is mixed evidence that strains of ATV are able to infect anuran (frog and toad) larvae (Jancovich et al. 2001, Schock et al. 2008). For example, of the three frog species experimentally exposed to ATV by Schock et al. (2008), all three species showed susceptibility to ATV infection, and a small proportion of individuals died of ATV-induced disease. However, Jancovich et al. (2001) exposed two frog species to ATV – including a different population of one species also studied by Schock et al. (2008) – and found no signs of infection. Together, these data suggest that ATV infections in anurans are possible, though the probability of infection likely varies among species and populations, and possibly among ATV strains. FV3, in contrast, shows high infectivity among diverse host species, and infection often leads to mortality in both anuran and salamander larvae (Brunner et al. 2005, Picco et al. 2007, Schock et al. 2008, Hoverman et al. 2010, 2011). Given that anurans and salamanders often co-occur as larvae (Hoverman, Gray, et al. 2012, Johnson, Preston, Hoverman, & Richgels 2013) and that ranaviruses infect multiple host species, it is likely that co-exposure and subsequent within-host interactions between virus types is relatively common in nature.

Here, we examined the effects of co-exposure to ATV and FV3 on mortality and transmission dynamics in larval amphibians. We conducted two experiments to assess how the effects of co-exposure scale-up from within-host outcomes to between-host transmission, ultimately affecting epizootics. Theory suggests that the effect on co-exposure on transmission will depend on disease outcomes and pathogen replication within hosts (Pedersen & Fenton 2006, Jolles et al. 2008, Mideo et al. 2008, Ezenwa & Jolles 2011). For instance, if within-host interactions lead to more rapid host death, then transmission of co-occurring pathogens could be dampened, leading to smaller epizootics. However, if co-exposure facilitates the invasion of pathogens, enhances within-host replication, or increases host tolerance to infection,

89 transmission could be enhanced, leading to larger epizootics (Mideo et al. 2008, Ezenwa & Jolles
90 2011, Sofonea et al. 2015). *A priori* we expected that, due to the generally high infectivity and
91 virulence of both ATV and FV3, co-exposure would increase host mortality and therefore limit
92 epizootic size. To test these expectations, we performed experimental infections at two scales.
93 We first exposed larval frogs individually to one or two virus types to determine how co-
94 exposure affected mortality rate, the probability of infection, and within-host viral replication.
95 We then conducted an experiment using replicate populations of larval frogs. Here, we pre-
96 exposed larvae to either FV3 or ATV, and we added these individuals to small populations of
97 susceptible larval frogs to explore how co-exposure affected the proportion of individuals
98 infected and the average viral load. Our results indicated that co-exposure enhances viral
99 infectivity and viral replication, illustrating the need to further explore how *Ranavirus* types are
100 distributed across the landscape and how this might affect epizootics.
101
102 **Materials and Methods**
103 *Viruses and culturing*. Aliquots of *Ambystoma tigrinum* virus (ATV; Regina ranavirus
104 (RRV) #11800) and frog virus 3 (FV3; #061405) were generously provided by V. Gregory
105 Chinchar. The RRV strain of ATV was originally isolated in 1997 from *Ambystoma tigrinum* in
106 Regina, Saskatchewan, Canada (Bollinger et al. 1999), and the FV3 strain is also a wild-type
107 strain isolated from *Rana pipiens* populations of the Midwestern United States in the 1960's
108 (Granoff et al. 1965). An aliquot of the *Rana catesbeiana* virus (RCV-Z2) strain of FV3
109 (hereafter referred to as R-FV3) isolated from a ranaculture facility in Georgia in 2006, was
110 generously provided by Matthew Gray and Debra Miller (GenBank accession no. EF101698;
111 Miller and Rajeev 2007, Claytor et al. 2017). This strain was the cause of a die-off event in the

facility's bullfrog population, and is twice as virulent as wild-type FV3 in some amphibian species (Hoverman et al. 2010). We propagated the three viruses through immortalized fathead minnow (FHM) cells fed with Eagle's minimum essential medium (MEM) with Hank's salts, containing 5% fetal calf serum. Titer of the resulting viral stocks was determined by plaque assays using serial dilutions of the stock, resulting in titers represented in plaque forming units (PFU). It is important to note that we were unable to obtain an accurate titer of the R-FV3 stock before the start of the first experiment, which likely explains the observed lower-than-expected infectivity.

Experiment 1: Individual-level. This experiment assessed the individual, host-level effects of co-exposure to ATV and FV3. Egg masses of *Rana aurora* were field-collected from wetlands in Oregon in spring 2012 and shipped to the University of Colorado at Boulder. Egg masses were first washed with sterile deionized water to remove any possible residual virions and were then placed into plastic containers for rearing. Larvae were reared at 20°C with a 12:12 hour day:night photoperiod and fed ground TetraMin® fish flakes (Tetra) *ad libitum* until reaching Gosner stage 30 (Gosner 1960). At this time, larvae were randomly placed into individual, covered plastic containers (with drilled air holes) filled with 1-L of carbon-filtered, UV-sterilized water and allowed to acclimate for 24h. A subset of 15 larvae were euthanized by immersion in 1% buffered MS-222 and tested for infection to verify that none of the larvae harbored latent infections prior to experimentation (see quantitative PCR methods below). None of these individuals tested positive for ranaviruses.

Twenty-five larvae were assigned to each of 10 experimental treatment groups: a no-virus control, single dose of each virus alone (n=3 treatments), double dose of each virus alone (n=3 treatments), and each pairwise combination of the three viruses (i.e. a single dose of each of two

viruses; n=3 treatments). Using this experimental design, we were able to account for additive and substitutive effects (e.g. dosage effects vs. effects of multiple strains). The control treatment consisted of a sham exposure to a 60µL aliquot of virus-free MEM. On 22 May 2012, a single dose (~$1x10^6$ PFU) or double dose (~$2x10^6$ PFU) of the respective virus or viruses was added to each larva's container via sterile pipette tip. Thus, larvae were passively exposed to each virus inoculate, which likely better mimics natural transmission conditions relative to injection-based methods.

After virus addition, individuals were fed *ad libitum* every other day for the extent of the experiment. Complete water changes were conducted with carbon-filtered, UV-sterilized water every 4 days post-exposure (dpe) to ensure adequate water quality for the larvae. Standard protocols to avoid cross contamination between containers involved sterilizing dip nets with a 10% bleach solution for 10 minutes, followed by rinsing with sterile water to remove any residual bleach. Container and experimental room surfaces were cleaned with a 2% solution of Nolvasan between each container's water changes, allowed to sit for 10 minutes, and then rinsed with sterile water.

The experiment ran for 21 d and mortality of larvae was monitored daily. If an individual died, the individual was extracted from its container, rinsed thoroughly with de-ionized water to remove any non-infecting virions that may have adhered to the individual's skin, and then the entire individual was placed into a microcentrifuge tube and stored at -20°C for later processing. After 21d, all surviving larvae were euthanized in 1% buffered MS-222. These individuals were then washed thoroughly with de-ionized water, placed into individual microcentrifuge tubes, and stored at -20°C for later processing.

Experiment 2: Population-level. A follow-up experiment tested how co-occurrence of

158 ATV and FV3 in a larval amphibian population would affect transmission dynamics. Because the
159 first, individual-level experiment showed qualitatively similar effects of co-exposure in the
160 ATV+FV3 and ATV+R-FV3 treatments (see Results below), only ATV and FV3 were used for
161 this experiment. In the spring of 2013, we were unable to obtain more *R. aurora* egg masses;
162 instead, we collected egg masses of *Pseudacris triseriata* from local sites in Colorado, washed
163 them with sterile deionized water, and reared them in plastic containers at 20°C with a 12:12
164 hour day:night photoperiod. Hatching larvae were fed ground TetraMin® fish flakes (Tetra) *ad*
165 *libitum* until reaching Gosner stage 30 (Gosner 1960).
166 The overall design of the experiment was to establish replicate populations of 10
167 uninfected larvae and then introduce 2 previously virus-exposed larvae into each population to
168 track the spread of virus and determine if co-occurrence of ATV and FV3 alters the rate of
169 spread and overall epizootic size. We used this method of transmission, instead of using passive
170 exposure to MEM-suspended virus, because we wanted to assure that the behavior of infected
171 hosts was allowed to affect transmission. To generate infected hosts for addition to the
172 experimental populations, we randomly assigned a subset of the larvae to one of three exposure
173 groups: FV3-exposure, ATV-exposure, and sham-exposure. Larvae were housed in 50-L covered
174 plastic tubs (with drilled air holes) at densities no greater than 1 larva per liter of water. On 20
175 June 2013, larvae were passively batch-exposed to a dosage of $5x10^6$ PFU L^{-1} water of the
176 respective virus or a sham exposure with an equivalent volume of virus-free MEM, using batches
177 of 50 larvae. Larvae were held in these containers for 4d to initiate infection. In order to later
178 identify which individuals were previously exposed, before adding the exposed individuals to the
179 susceptible populations, we sedated each exposed individual and used a pair of micro-scissors to
180 create a notch on the posterior, dorsal end of the tail. Unfortunately, these notches had healed by

181 the end of the experiment and it was exceedingly difficult to identify which individuals were
182 previously exposed.
183 Uninfected (i.e. susceptible) experimental populations were also established on 20 June
184 2013. We randomly selected 10 unexposed larvae and placed them in 15-L covered plastic tubs
185 (with drilled air holes) filled with 12-L of carbon-filtered, UV-sterilized water. After the 4d
186 batch-exposure, on 24 June 2013, each uninfected population received one of the following
187 combinations of exposed larvae: (1) two sham-exposed larvae, (2) two FV3-exposed larvae, (3)
188 two ATV-exposed larvae, or (4) one FV3-exposed larvae and one ATV-exposed larvae. Thus,
189 each microcosm population contained 12 total *P. triseriata* larvae (10 susceptible and 2 exposed)
190 for a total density 1 larva per liter of water. Each of the four treatments was replicated 6 times for
191 a total of 24 experimental units.
192 We destructively sampled three replicates four days after the addition of the two exposed
193 tadpoles (4dpe). This sample allowed us to establish an early epizootic time-point for comparison
194 to late-stage epizootics. Larvae were extracted from each tub and individually euthanized in 1%
195 buffered MS-222. As above, larvae were rinsed, placed into individual microcentrifuge tubes,
196 and stored at -20°C for later processing. Starting at 5dpe, 80% water changes were implemented
197 every 4 days for each remaining replicate. Mortality was continually monitored, and any
198 deceased individuals were extracted from tubs, rinsed, and stored, as above. At 21dpe, the
199 experiment was terminated and individuals processed as described above.
200
201 *Tissue processing and DNA extraction*. Frozen samples were allowed to thaw to room
202 temperature and 500µL of MEM was added to each microcentrifuge tube. The samples were then
203 manually homogenized using a motorized homogenizer. This tissue homogenate was then

centrifuged at 3000g for 1min. A 500µL aliquot of the resulting supernatant solution was placed into a new sterile microcentrifuge tube and used for DNA extraction. Qiagen™ DNeasy Blood and Tissue extraction kits and standard protocols were used to extract 250µL of buffered DNA suspension from each supernatant aliquot. DNA samples were stored at -20°C for later processing.

Quantitative PCR amplification of viral DNA. The viral load of each DNA extract (in viral copy number equivalents) was evaluated using quantitative polymerase chain reaction (qPCR), estimated by comparison to a dilution series of standard DNA. We created a synthetic double-stranded DNA standard by synthesizing a 250bp fragment of the major capsid protein (MCP) gene (gBlocks® Gene Fragments; Integrated DNA Technologies™), which is conserved among *Ranavirus* species (e.g. ~97% sequence similarity between ATV and FV3 strains). We used a 10-fold dilution series from $2x10^8$ gene copies down to $2x10^1$ gene copies of standard DNA. Standards and samples were run in duplicate.

The qPCR protocol amplifies a ~70bp region of the MCP, allowing the protocol to identify many *Ranavirus* species. However, importantly, the protocol cannot distinguish between virus species within a sample (Forson & Storfer 2006, Picco et al. 2007). Thus, we were unable to assess the simultaneous presence of virus types (i.e. co-infection). To test each sample for ranavirus infection, a 2.5µL volume of sample DNA was added to a reaction volume of 17.5µL containing the following reagents: 10µL TaqMan® 2X Universal PCR Master Mix (No AmpErase UNG), 0.06µL forward primer (for a final concentration of 0.1µM; 5' ACA CCA CCG CCC AAA AGT AC 3'), 0.18µL reverse primer (for a final concentration of 0.1µM; 5' CCG TTC ATG ATG CGG ATA ATG 3'), 0.05µL fluorescent TaqMan® probe (with a starting

227 concentration of 100pmol μL^{-1}; 5' FAM-CCT CAT CGT TCT GGC CAT CAA CCA C-TAM
228 3'), and 7.21µL molecular grade water (Forson & Storfer 2006, Picco et al. 2007). All custom
229 primers and probes were ordered through Life Technologies™. Samples were run in 96-well
230 plates on an Applied Biosciences® machine for 40 cycles: 95°C denaturing (20s), 54°C
231 annealing (20s), and 72°C extension (30s). Two positive ATV and FV3 controls and two
232 negative controls were run on each plate.
233 After qPCR analysis, the starting sample DNA concentrations of all virus-positive
234 samples were estimated using a Quant-iT™ PicoGreen® dsDNA Assay Kit (Life
235 Technologies™). All viral loads were standardized to viral copy number per ng of sample DNA.
236 We also quantified the DNA concentration of a random subset of non-infected samples in order
237 to verify that viral detection was not dependent on a high concentration of initial sample DNA.
238
239 *Viral DNA sequencing of infected samples*. We attempted to sequence a small region of the viral
240 genome from all infected samples in order to verify the identity of the infecting virus(es).
241 However, this method is not reliable at determining if multiple virus types are coinfecting an
242 individual, especially if there are rare variants. Therefore, this analysis determined only the
243 identity of the virus with the most DNA present in the sample (i.e. the most abundant virus in a
244 given individual). Still, this sequencing helped narrow down the mechanisms driving the effects
245 of co-exposure. We amplified a ~350bp fragment of the MCP gene using a hemi-nested PCR
246 protocol (Kattenbelt et al. 2000). The amplicon from each infected sample, along with a custom
247 sequencing primer (5' ACT ATG CCA CCT CCA TC 3'), was sent to Quintara Biosciences™
248 for Sanger sequencing. We also amplified and sequenced the same MCP gene fragment from the
249 three *Ranavirus* strains used in the study (ATV, FV3, and R-FV3). We compared the sequencing

data from each infected sample to that of the original viral strains.

Statistical analyses: Experiment 1. All statistical analyses were conducted in the open-source software, *R* (R Core Development Team 2013). From the first experiment, we had three types of data for each of the 10 viral exposure treatments: survival, proportion of individuals becoming infected, and viral load per infected larva. We compared survival rates with a Mantel-Haenszel test, using the '*survival'* package. We first compared among single- and double-dose single-virus treatments, with dosage as the predictor variable. Because we found no difference in the survival rate between single- and double-dosages for the single-virus exposures (χ_1^2=2.6; P=0.11), we then compared the double-dose, single-virus treatments to the co-exposure treatments and control. A Cox proportional hazards model yielded the same qualitative results.

We conducted our analyses of proportion infected and viral load using the Bayesian statistical programming language, *Stan*, interfacing through *R* via the package '*rstan*'. We use this method because of the language's flexibility in specifying the model structures for our analyses. In all cases, we used broad, vague priors for model parameters. We have made our code publicly available at the following link: https://bitbucket.org/jrmihalj/ranavirus_coexposure. To quantify the effects of virus identity, dosage, and co-exposure on the proportion of individuals that became infected, we conducted a logistic regression. Note that we conducted a similar analysis in the '*brglm*' package in *R*, which uses a frequentist approach, and the results were qualitatively the same (not shown). The model that was fit in *Stan*, however, showed a more precise match between data and model predictions.

We structured our model as follows (using *R* linear model syntax for ease of interpretation): Number_Infected ~ 0 + ATV + FV3 + RFV3 + Double:ATV + Double:FV3 + Double:RFV3 + FV3:RFV3 + ATV:RFV3 + ATV:FV3, where Number_Infected follows a

273 binomial distribution, with $k = 25$ (i.e. the number of individuals exposed in each treatment).
274 Thus, our model estimated a baseline effect of each virus type (i.e. a single-dose effect), a virus-
275 specific effect of double dosage, and then the interactions between each virus type. These
276 interactions represent the effect of co-exposure, which is an effect above and beyond the effect of
277 a double dosage. Each one of model effects is compared to an intercept of zero, which on the
278 logit scale equals 50% prevalence. Thus, for instance, a strong negative effect of ATV would
279 mean that far less than 50% of individuals became infected with a single dose of ATV. In
280 general, we were interested in whether the co-exposure effects are larger than all of the double-
281 dose effects, indicating significant synergy between the two co-inoculating viruses.
282 Finally, to compare viral loads among treatments, we followed a similar approach as our
283 treatment of the data on proportion infected. This model only used viral load data from the
284 individuals that became infected. We therefore constructed a linear model predicting the natural
285 log-transformed average viral copy number per ng DNA for each infected individual (averaged
286 over the duplicate qPCR runs). We used similar model structure as above, except in this case we
287 estimated an intercept representing the average infection intensity, and we did not include the
288 single or double-dose ATV treatments, due to lack of any infections. We also added a term
289 accounting for whether or not the individual died during the experiment. The model structure
290 was therefore: Viral_Load ~ Intercept + FV3 + RFV3 + Double:FV3 + Double:RFV3 +
291 FV3:RFV3 + ATV:RFV3 + ATV:FV3 + Died. In this case, then, we were interested to see if the
292 co-exposure groups had higher than average infection intensities, which again would indicate a
293 synergistic effect of co-exposure.
294 *Statistical Analyses: Experiment 2*. Similar to the first experiment, the second experiment
295 had three response variables: survival rate, infection prevalence, and average viral load.

296 However, this time, the response variables were population-specific, with 3 replicate populations
297 per time-point (4dpe and 21dpe) and per treatment (FV3, ATV, FV3+ATV, Control). To
298 compare survival among treatments, we used a Cox proportional hazards model with replicate
299 population as a frailty term (i.e. analogous to random intercept term). We compared infection
300 prevalence between the FV3 and FV3+ATV treatments by creating a generalized linear mixed
301 effects model in *Stan* with prevalence explained by treatment, time (early vs. late), and their
302 interaction. We excluded the ATV-only treatments due to the small number of infections and to
303 simplify the analysis. The model was therefore of the form: Number_Infected ~ Treatment +
304 Time + Treatment*Time, where Number_Infected follows a binomial distribution with $k = 12$,
305 the number of individuals in each replicate.
306 We similarly compared the viral load between the FV3 and FV3+ATV treatments by
307 creating a linear mixed effects model with viral load (transformed as in the first experiment)
308 explained by a treatment-by-time interaction, a fixed effect for whether the individual died or
309 not, a fixed effect for day of death, and a random effect for replicate population. This model was
310 of the form: Viral_Load ~ Intercept + Treatment + Time + Treatment*Time + Died +
311 Random(Replicate).
312
313 **Results**
314 *Experiment 1*
315 In the first experiment with *Rana aurora*, larvae experienced mortality throughout all
316 treatments, including controls (Fig. A1). However, survival did not differ among treatments (χ^2_6
317 = 6.2; p = 0.40; Fig. A1). Infection prevalence in *R. aurora* ranged from 0-76%, with no
318 individuals becoming infected in the sham control and no individuals becoming infected in the

319 ATV-only treatments (including single- and double-doses of ATV).

320 Importantly, co-exposure to ATV and either FV3 or RFV3 caused a synergistic effect,
321 enhancing overall infectivity compared to the double-dose treatments of single virus types (i.e.
322 ATV, FV3, and RFV3 alone; Fig. 1a). Thus, the infection prevalence in co-exposure groups was
323 nearly twice as high as single-virus exposures. However, co-exposure to both FV3-like strains
324 (i.e. FV3+RFV3) did not cause such an effect (Fig. 1a). Indeed in the statistical model, the co-
325 exposure effects of ATV:FV3 and ATV:RFV3 were larger than all other effects (Table 1),
326 demonstrating a synergistic effect of co-exposure on viral infectivity.

327 Infected individuals that died during the experiment had, on average, higher viral loads
328 compared to infected individuals that survived to the end of the experiment (Table 2; Fig. A2).
329 We also found that for RFV3, a double dose exposure led to a detectably higher average viral
330 load compared to the single dose exposure, and there was a similar trend for FV3 (Fig 1b; Table
331 2). The viral loads of the ATV+FV3 co-exposed individuals were higher than the loads of
332 individuals exposed to a single dose of FV3, but there was no difference in viral load in the
333 ATV+FV3 treatment compared to exposure to a double dose of FV3 (Fig. 1b; Table 2). The
334 other co-exposure treatments (ATV+RFV3 and FV3+RFV3) had similar viral loads to the single-
335 dose exposure treatments of FV3 and RFV3 (Fig. 1b; Table 2).

336 We successfully sequenced viral DNA from all but three of the infected individuals. The
337 three individuals from which we did not successfully sequence had the three lowest viral loads.
338 Sequencing results revealed that infected individuals were predominantly infected by the FV3-
339 like strains. However, because FV3 and R-FV3 are indistinguishable based on this sequencing
340 method, we could not reliably determine whether coinfection occurred (i.e. simultaneous
341 presence of multiple virus types). Interestingly, four sequences from the R-FV3 single-virus

342 exposures and the ATV + R-FV3 exposures showed 100% sequence identity to one another but
343 did not perfectly match the sequences of the three viruses used in this experiment. We searched
344 for similar sequences on GenBank® via BLAST, which revealed a 100% match to an isolate of
345 FV3 discovered in lungless salamanders of the Great Smokey National Park, TN (Gray et al.
346 2009a). The source of this contamination – whether two viruses were co-isolated from the bull
347 frog culturing facility, or whether the original R-FV3 stock was contaminated post-culturing – is
348 unclear, but it is unlikely to have affected our results.
349
350 *Experiment 2*
351 No *Pseudacris triseriata* individuals died in the control group. Across all treatments, no
352 individuals had died by 4 days post-exposure (dpe). Overall, only four individuals died in the
353 ATV-only treatment (5.5% of all replicate individuals), and 12 (16%) and 14 (19%) individuals
354 died in the FV3+ATV and FV3-only treatment replicates, respectively. When testing for an
355 effect of co-exposure on survival rates, we found no overall difference between the survival rates
356 in the FV3-only and FV3+ATV treatments (χ^2_1, = 0.16, p = 0.68), although there was significant
357 variation in survival rates among replicates ($\chi^2_{1.42}$ = 5.3, p = 0.038).
358 No individuals in the control treatment became infected; however, unlike *R. aurora*,
359 which showed no infectivity with ATV, three *P. triseriata* individuals in the ATV-only treatment
360 became infected, which were detected in the three different replicates (one per replicate) at
361 21dpe. Treatment and time post-exposure interacted to drive infection prevalence (Fig. 2; Table
362 3); for the FV3-only treatment, the proportion of infected individuals increased more consistently
363 and substantially over time compared to the FV3+ATV treatment (Fig. 2). All three replicates of
364 the FV3-only treatment at 21dpe had the same proportion of individuals infected (8/12, 66%).

The FV3+ATV treatment replicates had more variable prevalence. In one of the FV3+ATV treatments at 4dpe, 9/12 (75%) of individuals were infected, which was a substantially larger proportion compared to all other 4dpe replicates, and the highest prevalence in the experiment overall (Fig. 2).

Individuals that died in the experiment had, on average, higher viral loads compared to infected individuals that were sampled prior to mortality (Fig. 3; Table 4). Because all of the individuals that died were in the 21dpe treatments, the average viral load increased between the 4dpe and 21dpe treatments (Fig. 3). There were no overall effects of co-exposure on viral load in this experiment (Table 4).

We amplified and sequenced viral DNA from 85% of the infected individuals (n = 45 / 53). Of the three individuals that tested positive for infection in the ATV-only treatments, two DNA samples amplified, and their sequence data matched that of ATV, verifying that *P. triseriata* can become infected with ATV. All sequences from the FV3-only treatment matched FV3 DNA. Only one individual from the FV3+ATV treatment was infected with ATV, which was also the host with the highest observed viral load (4.76×10^6 viral DNA copies ng^{-1} DNA) and came from the replicate population with the highest infection prevalence (75% in the FV3+ATV, 4dpe treatment).

Discussion

We conducted experiments to identify the effects of co-exposure to multiple ranaviruses at the scale of both individual hosts and experimental populations. For individual hosts, co-exposure to *Ambystoma tigrinum* virus (ATV) and frog virus 3 (FV3) increased the infection success of FV3. However, this same effect did not hold for co-exposure to two more related

388 strains (FV3 and *Rana catesbeiana* virus (RCV-Z2), herein R-FV3), indicating that viral identity
389 and viral relatedness may be important for predicting the outcome of co-exposure. At the host
390 population-scale, we found some evidence that, when ATV co-occurs with FV3, co-exposure can
391 lead to higher infection prevalence in the population. By conducting experiments at both the
392 individual- and population-level scales, results of this study indicate that the co-occurrence of
393 *Ranavirus* species has the potential to alter epizootic dynamics in natural amphibian populations.
394 In our first experiment, in which we exposed individual *Rana aurora* to multiple
395 ranaviruses, we demonstrated several expected results that help validate our methods and
396 experimental design. First, we found higher infection prevalence in the double-dose FV3
397 treatment, compared to the single-dose FV3 treatment, showing that our chosen differences in
398 dose led to measurable differences in infectivity. We also found higher viral loads in individuals
399 that died compared to surviving individuals. This result is intuitive, especially considering
400 evidence that the virulence of ranaviruses is at least partially associated with within-host viral
401 replication (Brunner & Collins 2009). We also show that ATV is not highly infectious in larval
402 frogs, corroborating previous findings (Jancovich et al. 2001, Schock et al. 2008); however, we
403 demonstrate that *Pseudacris triseriata* is susceptible to this virus in our second experiment.
404 Although mortality was high in our first experiment, the mortality patterns were consistent across
405 treatments, and we believe that the basic results outlined above show that our methods were
406 unbiased.
407 Critically, our first experiment provides evidence for an effect of co-exposure on viral
408 infectivity and viral replication. The data suggest that co-exposure to ATV and FV3
409 synergistically increased the host's probability of infection with FV3. This effect of co-exposure
410 with ATV was seen with two FV3-like strains, wild-type FV3 and R-FV3. Notably, we saw this

411 co-exposure effect with ATV+R-FV3, even though we were unable to accurately quantify the
412 titer of the R-FV3 stock, which demonstrates a robust effect of co-exposure on prevalence. We
413 also saw that co-exposure to ATV and FV3 led to higher average viral load compared to the
414 single-dose FV3 treatment. Given that no individuals became infected with ATV alone, and that
415 the ATV+FV3 co-exposure constitutes a single dose of FV3, this latter result suggests a
416 synergistic effect of co-exposure on viral replication within a host. We believe our results imply
417 that ATV and FV3 likely either coinfected or superinfected the hosts and that exposure to ATV
418 facilitated the invasion and subsequent proliferation of FV3 within larvae (discussed more
419 below).

420 Our second experiment, in which we exposed experimental populations of *P. triseriata* to
421 multiple ranaviruses, provides some additional evidence for the effect of co-exposure on
422 epizootics. Although we detected ATV infection in only one individual in the ATV+FV3
423 treatment group, from one replicate population, this individual exhibited the highest overall viral
424 load in our experiment, even after only 4dpe. This individual also was sampled from the replicate
425 population with the largest epizootic in the experiment (75%). This evidence, in combination
426 with the result that ATV only rarely infects these frogs, suggests that when ATV is able to
427 establish infections in a population concurrent with FV3, there is the potential for larger
428 epizootics.

429 We suspect that the effect of co-exposure was not as strong in the second experiment
430 because of the difference in viral delivery and dosage, or perhaps host species identity.
431 Specifically, ATV was only able to establish in one replicate co-exposure population (as
432 evidenced by our sequencing methods), and we thus only saw one FV3+ATV co-exposure
433 population with an effect. There are several plausible explanations for this outcome. First, the

majority of pre-exposed individuals may not have become infected, and therefore the susceptible, replicate populations were never exposed to ATV. Second, the pre-exposed individuals were infected, but cleared the infection prior to the population phase of the experiment. Or third, the pre-exposed individuals were infected with ATV but cleared the infection during the population phase of the experiment before ATV could infect other susceptible individuals. Because we were not successful in marking pre-exposed individuals, we cannot distinguish between these scenarios.

In the first experiment, each co-exposed individual was passively exposed to ~$1x10^6$ PFU of ATV in solution. However, in the second experiment, the susceptible larvae in the replicate populations could only become exposed to ATV if the pre-exposed individuals were infectious. Thus, it is likely that if we had passively exposed the replicate populations to ATV in a way similar to our first experiment, a larger effect of co-exposure would be seen. The differences in effects between the two experiments could also be due to differences in the effects of co-exposure among amphibian species. It is known that variability in FV3 infectivity among amphibian species has phylogenetic and ecological correlates (Hoverman et al. 2010, 2011).

Somewhat surprisingly, we did not see an effect of co-exposure on overall survival rates in our experiments. We ran our experiments for 21d, which in previous studies has been long enough to see 20-100% mortality due to ranavirus infection in other species of frogs and salamanders (Brunner et al. 2005, Hoverman et al. 2010). Given that case-mortality rates tend to be high (> 90%) for ranaviruses, it is likely that more individuals, especially in the co-exposure treatments with higher viral loads, would have died due to infection if we carried out the experiments for a longer time period.

Based upon the evidence from the first experiment, we propose two hypotheses for the

457 observed increase in infectivity and viral replication following host co-exposure to ATV and
458 FV3. First, exposure to these two distinct virus types could lead to non-overlapping immune
459 responses in the amphibian larvae, which leads to a trade-off that decreases the efficacy of the
460 host's response to FV3, facilitating invasion. While there is ample evidence for resource
461 competition in multi-strain infections (Read & Taylor 2001, Mideo et al. 2008, Alizon et al.
462 2013b), few studies have documented the possible immune trade-offs imposed by multi-strain
463 infections (Balmer & Tanner 2011). In the *Ranavirus* system, along with complex innate
464 immune responses, *Xenopus laevis* adults produce long-lasting anti-FV3 IgY antibodies, and
465 larvae produce less effective innate and adaptive responses (Chinchar et al. 2011, Chen & Robert
466 2011). However, it is unknown if exposure to ATV elicits overlapping innate and adaptive
467 responses with FV3. Future experiments that determine the degree of antibody specificity
468 between ATV and FV3 and that alter the timing of exposure between FV3 and ATV may help to
469 further test this hypothesis of the effect of co-exposure.
470 A second, alternative hypothesis for the effect of co-exposure is viral recombination. It is
471 possible that, if ATV and FV3 coinfect the same host cells, recombination could occur to
472 produce a novel, more infectious virus. Genomic evidence from multiple *Ranavirus* species
473 suggests high recombination frequency and shows that these viruses are prone to host-shifts due
474 to gene acquisition and subsequent adaptation (Jancovich et al. 2003, 2010, Abrams et al. 2013).
475 Recombination has been employed to explain the collinearity and the one inversion between the
476 ATV and FV3 genomes (Eaton et al. 2007). Furthermore, it was recently discovered that the R-
477 FV3 strain we used here (RCV-Z2) is the product of a recombination event between an FV3-like
478 strain and a common midwife toad virus (CMTV)-like strain from Europe, and this
479 recombination is likely the cause of the high virus-induced mortality rate of this strain (Claytor et

480 al. 2017). This hypothesis of recombination could be tested by isolating many viruses from the
481 co-exposure group via plaque assay, growing the viruses in culture, and conducting full genome
482 sequencing and alignment to both FV3 and ATV.

483 Our results illustrate that in natural amphibian populations, co-occurrence of ATV and
484 FV3 could alter epizootic dynamics. Specifically, if ATV can establish in a larval frog
485 population, co-occurrence with FV3 could result in more infected individuals and subsequently
486 higher mortality rates in the long run. This effect seems particularly relevant for wetlands in
487 which salamanders and frogs cohabitate. If ATV is present and infects the local salamanders and
488 FV3 establishes in the anuran populations, spillover of ATV from the salamanders could enhance
489 FV3 epizootics in the frogs. Also, because FV3 is adept at infecting salamanders as well (Schock
490 et al. 2008), it is likely that such a scenario would increase infection prevalence and intensity in
491 the urodele population. Thus, our results illustrate the need to consider co-exposure and co-
492 infection in the amphibian-*Ranavirus* system and emphasize the need for field data on ATV and
493 FV3 co-occurrence at both the wetland- and host individual-levels.

494 This study adds to a growing body of literature that illustrates the important
495 consequences of multi-pathogen interactions in mediating pathology and transmission.
496 Furthermore, our results emphasize the importance of multi-scale experiments for understanding
497 how interactions among pathogens influence transmission. In general, the impact of co-exposure
498 on transmission will depend on how pathogen interactions within hosts feedback on between-
499 host dynamics. In the *Ranavirus* system, co-exposure increased pathogen infection success and
500 viral replication within hosts but did not result in more rapid host death, ultimately leading to
501 increased transmission when both pathogens co-occurred. Research that integrates multi-scale
502 experiments across a variety of systems will help us better understand the conditions under

503 which co-exposure will significantly impact epidemics and epizootics.

504

505 **Acknowledgements**

506 We would like to thank the Johnson Laboratory at CU-Boulder, particularly MB Joseph, for their
507 helpful comments on a previous version of this manuscript. We are especially grateful to the hard
508 work of two laboratory technicians, C. Ramsay and B. Camenga. This research was supported by
509 the NSF Graduate Research Fellowship Program (DGE 1144083), the NSF (DEB-1149308) and
510 NIH (R01GM109499), a Beverly Sears Graduate Student Research Grant, CU EBIO
511 Departmental Research Grants, and the CU Natural History Museum Grant Program.

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659

660 **Table Legends and Tables**

661

662 **Table 1.** Effects of co-exposure on the proportion of *Rana aurora* hosts that became infected.
663 The median and 95% credible interval (CI; the Bayesian analog to the 95% confidence interval)
664 are shown for the coefficients in the logistic model. In general, effects whose 95% CI do not
665 overlap zero are considered biologically meaningful. Co-exposure effects whose 95% CI do not
666 overlap zero demonstrate an effect of co-exposure over and above any effects of dosage. These
667 meaningful co-exposure effects are bolded.

Effect		**Median Coefficient**	**95% CI**
Baseline effects of each virus	ATV	-8.72	(-20.03, -3.28)
	FV3	-1.44	(-2.59, -0.50)
	RFV3	-0.78	(-1.68, 0.08)
Effects of double-dose, compared to baseline	Double:ATV	1.60	(-7.53, 12.80)
	Double:FV3	1.22	(-0.01, 2.61)
	Double:RFV3	-0.17	(-1.47, 1.03)
Co-exposure effects, above and beyond effects of dosage	FV3:RFV3	0.40	(-1.04, 1.84)
	ATV:RFV3	**8.52**	**(4.16, 16.75)**
	ATV:FV3	**8.64**	**(4.32, 16.94)**

668

669 **Table 2.** Effects of co-exposure on the log-transformed viral concentration (viral DNA copy
670 number per total ng of DNA in the sample) of infected *Rana aurora* hosts. We refer to viral
671 concentration as viral load for clarity. The median and 95% credible interval are shown for the
672 coefficients in the linear model. Because there is no baseline effect of ATV included in these
673 models due to zero ATV-infected individuals, the effects of co-exposure are compared to the
674 single dose cases. Thus, the meaningful effect of the ATV:FV3 co-exposure shows that this
675 treatment led to higher viral loads compared to the single-dose FV3 treatment. [a]The effect of
676 double dose on FV3 viral load was marginal, whereby the 94.4% CI does not overlap zero (i.e.
677 an $\alpha = 0.056$ in frequentist statistics).
678

Effect		**Median Coefficient**	**95% CI**
Baseline viral load (i.e. average across all cases)	Intercept	0.680	(-4.30, 5.76)
Effect of mortality	**Died**	**5.55**	**(3.89, 7.16)**
Effect of each virus, compared to baseline	FV3	-2.18	(-8.03, 2.43)
	RFV3	-2.78	(-7.74, 3.40)
Effects of double dosage, compared to single dosage	*Double:FV3*	*3.23*	*(-0.08, 6.59)*[a]
	Double:RFV3	**1.78**	**(0.01, 6.2)**
Effects of co-exposure, compared to single dosage	FV3:RFV3	-0.706	(-6.17, 4.8)
	ATV:RFV3	0.166	(-2.48, 2.96)
	ATV:FV3	**3.39**	**(1.01, 5.78)**

679

680 **Table 3.** Effects of time and co-exposure on the prevalence of infection in experimental
681 *Pseudacris triseriata* populations. As in Table 1, the median and 95% credible interval are
682 shown for the coefficients in the logistic model. The meaningful interaction was driven by a
683 more substantial increase in prevalence over time in the FV3-only treatment group (Fig. 2).
684

Effect		**Median Coefficient**	**95% CI**
Time effects (factors)	4dpe	-0.329	(-10.26, 9.56)
	21dpe	3.88	(-6.24, 14.12)
Treatment effects	FV3	0.643	(-9.25, 10.62)
	FV3+ATV	3.23	(-7.18, 13.68)
Interaction effect	**Time x Treatment**	**-1.90**	**(-3.43, -0.45)**

685
686

687 **Table 4.** Effects of time and co-exposure on the prevalence of the viral concentration (viral load)
688 in infected *Pseudacris triseriata*. The median and 95% credible interval are shown for the
689 coefficients in the linear model. In this model we included a random effect of replicate
690 population on the intercept, and therefore the random effect standard deviation and residual
691 standard deviation are shown.

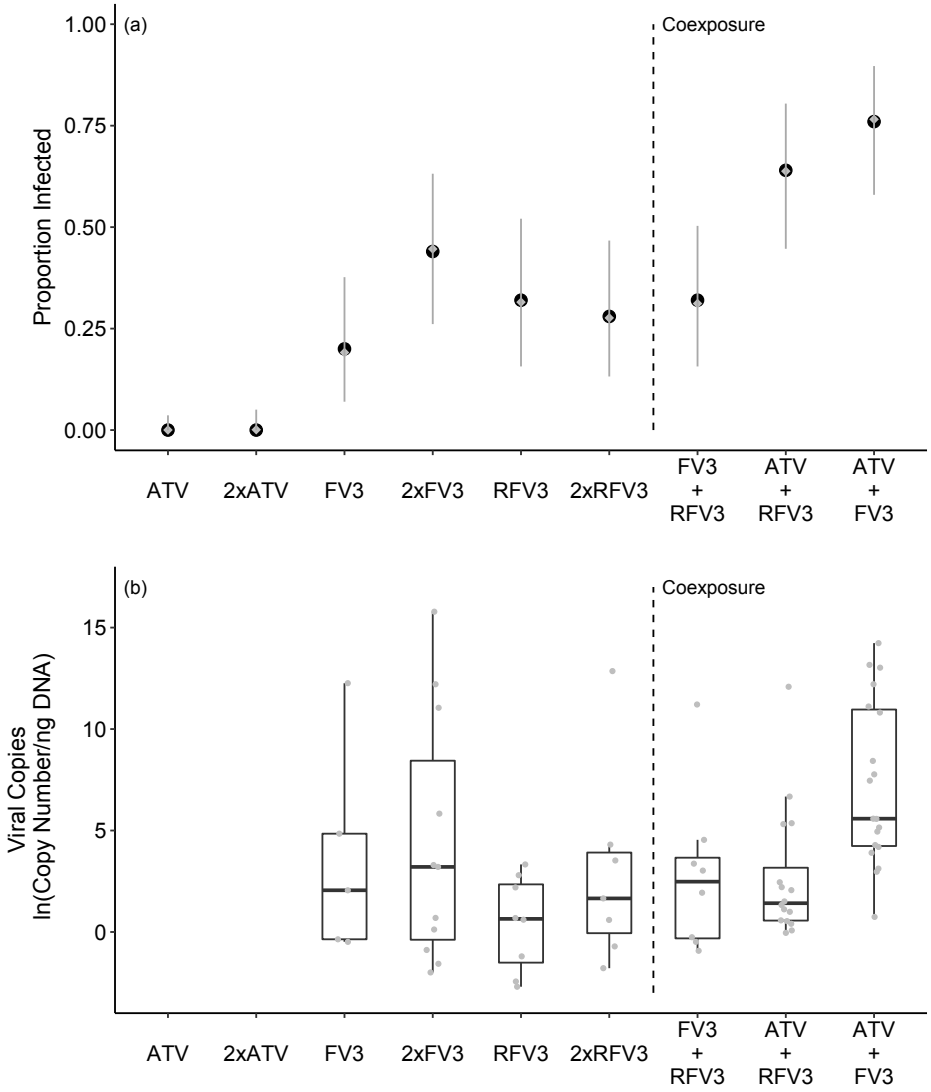
Effect		**Median Coefficient**	**95% CI**
Baseline viral load (i.e. average across all cases)	Intercept	4.35	(-3.61, 12.42)
Effect of mortality	**Died**	**3.01**	**(0.17, 5.82)**
Time effects (factor)	4dpe	-1.69	(-7.84, 4.5)
	21dpe	0.716	(-6.66, 8.3)
Treatment effects	FV3	1.33	(-5.11, 7.71)
	FV3+ATV	-2.16	(-9.6, 5.77)
Interaction	Time x Treatment	0.724	(-2.92, 4.2)
Standard deviation among replicates	$\sigma_{replicate}$	1.40	(0.15, 3.45)
Residual standard deviation	$\sigma_{residual}$	3.88	(3.2, 4.86)

692

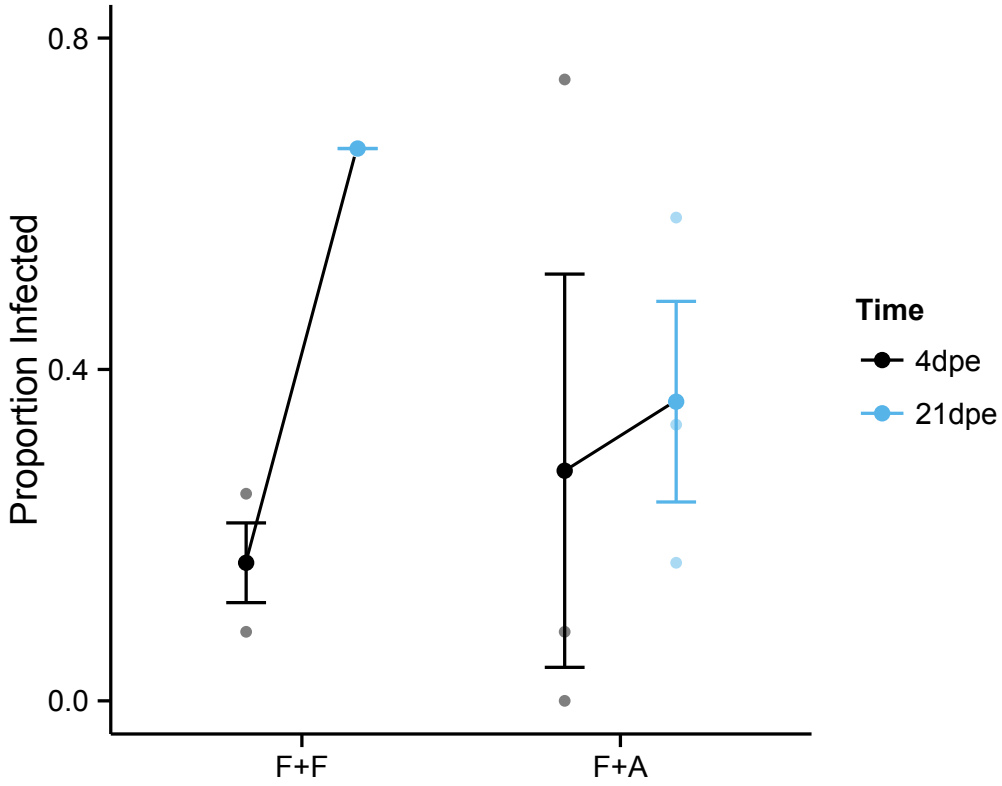
693

Figure Legends and Figures

Figure 1. (a) Proportion of *Rana auora* individuals infected in all experimental groups from experiment 1. Gray points are the median predictions from the statistical model, and gray bars represent the estimated 95% credible intervals to show error in our estimates. (b) Boxplots of viral copy number per treatment group, with gray points representing the viral load of each measured host. The box represents the inter-quartile range (IQR; between first and third quartiles), and the center line marks the median value. The whiskers extend from the box to the highest or lowest value that is within 1.5 x IQR.



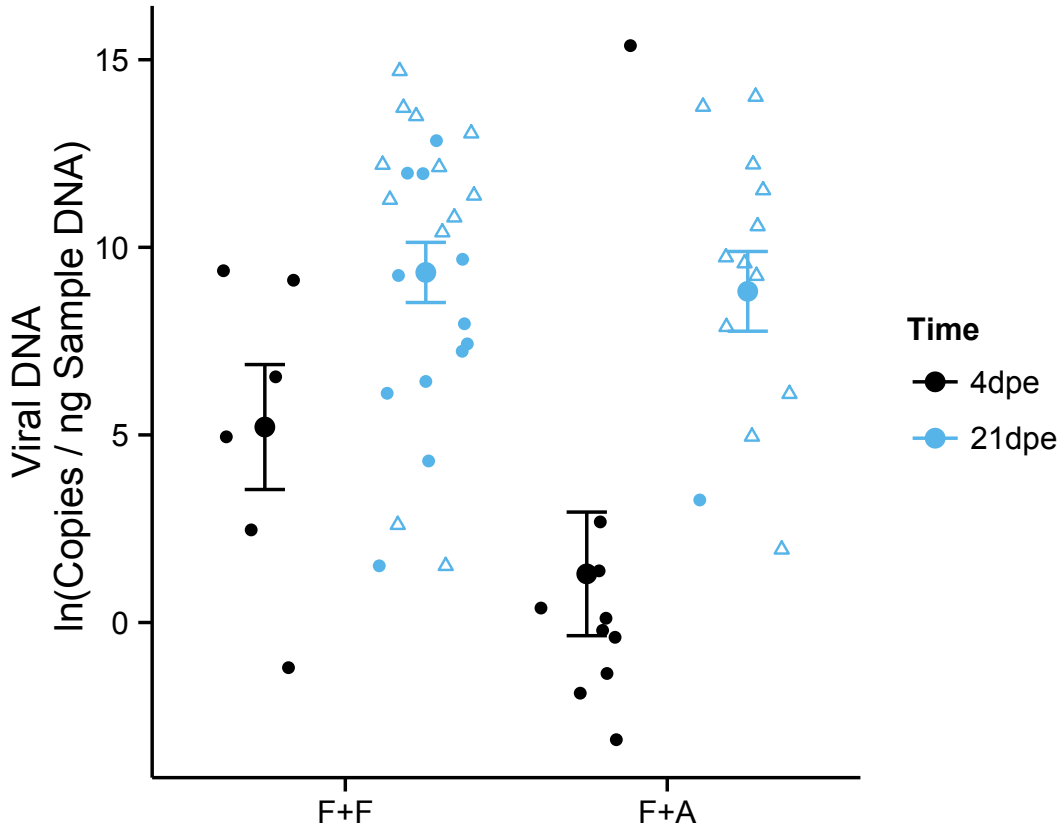
703 **Figure 2**. Proportion of *Pseudacris triseriata* infected individuals between time points and
704 treatments in experiment 2. Time point are distinguished by color, as depicted in the legend.
705 Large, bold circles represent the mean prevalence, and error bars represent one standard error of
706 this mean. Smaller and more opaque circles represent the prevalence of the replicate larval
707 populations. Note that all 3 replicates of the FV3-only treatment at 21dpe had the same
708 prevalence.



709

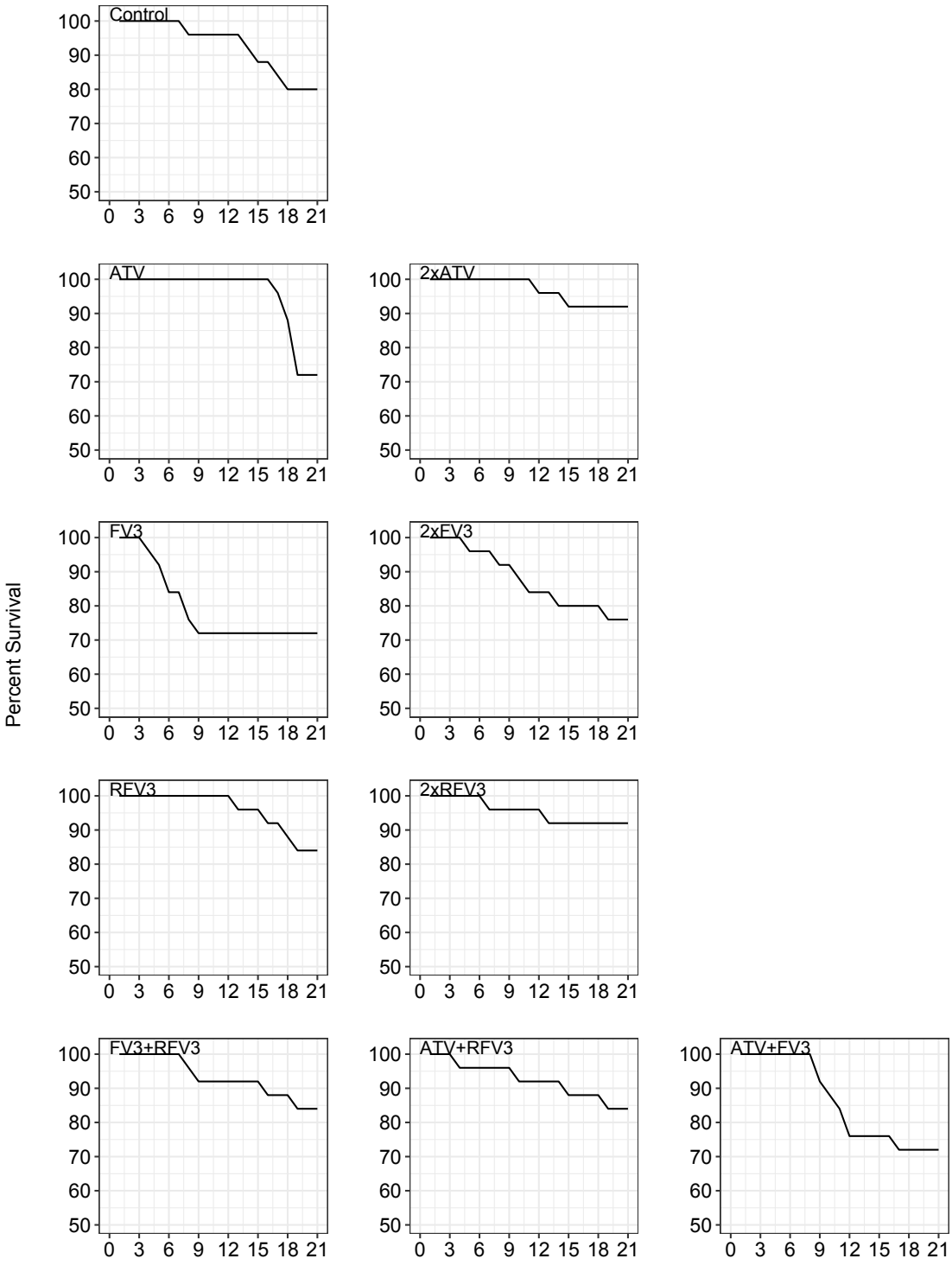
710

711 **Figure 3**. Viral copy number across time points and treatments in experiment 2 with *Pseudacris*
712 *triseriata*. ATV-only treatments are not shown, because only 3 total individuals became infected,
713 all in the 21dep treatments (one individual per replicate). Time points are distinguished by color,
714 as depicted in the legend. Large, closed circles represent the mean viral load, and error bars
715 represent one standard error of the mean. Smaller closed circles represent the viral load of
716 infected individuals that survived until the end of the experiment (or until destructive sampling in
717 the case of 4dpe replicates). Open triangles represent infected individuals that died prior to the
718 end of the experiment. A jitter is added to the data for ease of interpretation. Notice the most
719 heavily infected individual from the FV3+ATV treatment at 4dpe.
720



721

722 **Figure A1**. Survival curves for all treatments in experiment 1 with *Rana aurora*.



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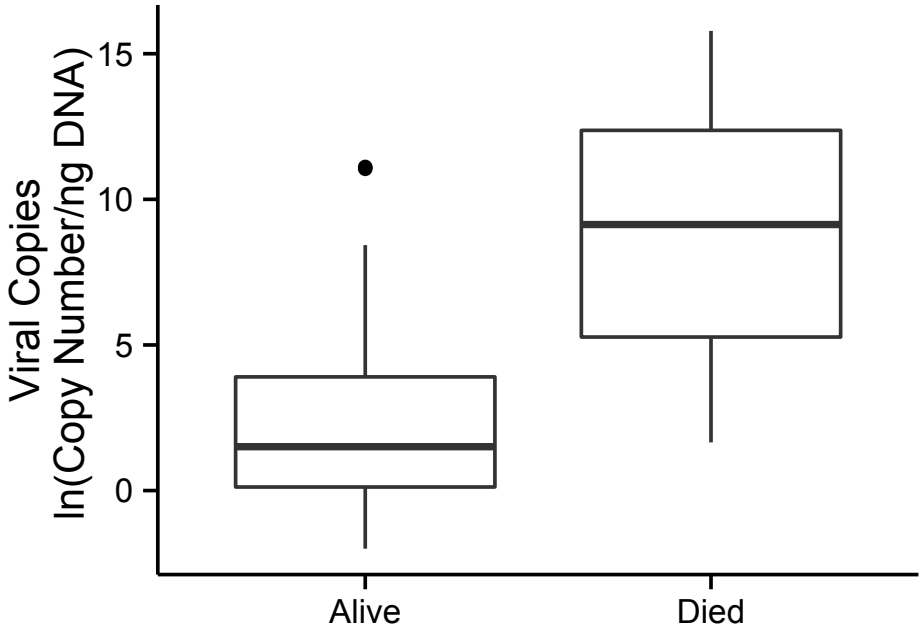
725 **Figure A2**. Boxplots (as in Figure 2) of viral load for infected *Rana aurora* that survived until

726 the end of the experiment (Alive) or that died due to infection (Died).



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