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**Diversity-Stability Dynamics of the Amphibian Skin Microbiome and
Susceptibility to a Lethal Viral Pathogen.**

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Conflict of Interest

The authors declare no conflict of interest

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20 **ABSTRACT**

21 Variation among animals in their host-associated microbial communities is increasingly
22 recognised as a key determinant of important life history traits including growth, metabolism
23 and resistance to disease. Quantitative estimates of the factors shaping the stability of host
24 microbiomes over time at the individual level in non-model organisms are scarce. Addressing
25 this gap in our knowledge is important, as variation among individuals in microbiome stability
26 may represent temporal gain or loss of key microbial species and functions linked to host
27 health and/or fitness. Here we use controlled experiments to investigate how both
28 heterogeneity in microbial species richness of the environment and exposure to the emerging
29 pathogen *Ranavirus* influence the structure and temporal dynamics of the skin microbiome
30 in a vertebrate host, the European common frog (*Rana temporaria*). Our evidence suggests
31 that altering the bacterial species richness of the environment drives divergent temporal
32 microbiome dynamics of the amphibian skin. Exposure to ranavirus effects changes in skin
33 microbiome structure irrespective of total microbial diversity, but individuals with higher pre-
34 exposure skin microbiome diversity appeared to exhibit higher survival. Higher diversity skin
35 microbiomes also appear less stable over time compared to lower diversity microbiomes, but
36 stability of the 100 most abundant ('core') community members was similar irrespective of
37 microbiome richness. Our study highlights the importance of extrinsic factors in determining
38 the stability of host microbiomes over time, which may in turn have important consequences
39 for the stability of host-microbe interactions and microbiome-fitness correlations.

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43 INTRODUCTION

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45 Animals are host to diverse communities of microbes, collectively referred to as the
46 microbiome. Variation among individuals in their microbiomes has been linked to variation in
47 host resistance to pathogens (Ford & King 2016; King et al 2016; Villarino et al 2016; Antwis
48 & Harrison 2018; Warne et al 2019), and disruption of the microbiome by external stressors
49 (e.g. antibiotics) can have long term negative effects on host health (Theriot et al 2014; Knutie
50 et al 2017; Warne et al 2019). Though there is growing evidence that perturbation of the
51 microbiome can have deleterious effects on host physiology, an understanding of the drivers
52 of individual microbiome dynamics over time, and resistance to perturbation, remain
53 relatively scarce in non-model organisms (Loudon et al 2014a,2016; Videvall et al 2019).
54 Addressing this shortfall in our knowledge is of fundamental importance to understanding the
55 adaptive value of microbiomes for host health and fitness, as microbiome-health correlations
56 may not be stable over time if microbiome flux represents loss of key microbial species and/or
57 genes critical for optimal host physiology. Variation among individuals in their resistance to
58 microbiome perturbation, and resilience following perturbation, could be a critical
59 determinant of the distribution and stability of traits such as resistance to pathogens in
60 natural populations.

61

62 The amphibian skin microbiome is rapidly becoming established as a model system for
63 understanding the tripartite relationships between host, microbiome and pathogens (e.g.
64 Harris et al 2009; Longo et al 2015; Kueneman et al 2016; Bates et al 2018; Campbell et al
65 2018a,b, 2019, Ross & Hoffman 2019). Production of metabolites by skin-associated bacteria
66 is a crucial component of immune defence against lethal fungal pathogens such as
67 *Batrachochytrium dendrobatidis* (Bd) (e.g. Brucker et al 2008; Becker et al 2009) and *B.*
68 *salimandrivorans* (Muletz-Wolz et al 2017). Anti-fungal metabolite production by bacteria
69 increases dramatically when the bacteria are co-cultured (Loudon et al 2014b), suggesting
70 that microbiome-mediated host protection is likely a function of synergistic interactions
71 among community members. Greater microbiome diversity may therefore offer increased
72 protection from pathogens (e.g. Piovia-Scott et al 2017; Antwis & Harrison 2018, Greenspan
73 et al 2019; but see Becker et al 2019), but the ecological processes structuring and
74 maintaining microbial diversity on amphibian skin remain relatively understudied, especially

75 at the level of the individual (Loudon et al 2014a; 2016; Longo & Zamudio 2017; Hughey et al
76 2019). For example, the diversity-stability hypothesis predicts that more diverse communities
77 should be more resistant to disturbance, and several empirical studies support this hypothesis
78 in plant community assemblages (McCann 2000; Costello et al 2012), but it is unclear whether
79 this theory is also relevant at the scale of host-associated microbial communities (Costello et
80 al 2012, but see Koskella et al 2017). Though several studies have sought to measure the
81 influence of pathogenic infection on host microbiome structure (Jani & Briggs 2014; Longo et
82 al 2015; Longo & Zamudio 2017), investigations of whether the magnitude of microbiome
83 disruption for infected hosts is modulated by initial microbiome state remains relatively
84 scarce (see Jani et al 2017; Jani & Briggs 2018).

85

86 Here, we use experiments to examine how both the diversity of the environmental microbial
87 reservoir and exposure to the lethal pathogen ranavirus influence skin microbial community
88 dynamics in a native UK amphibian species, the European Common frog (*Rana temporaria*).
89 The emerging infectious disease (EID) ranaviriosis represent a significant threat to ectothermic
90 vertebrate health, and infection with ranaviruses is associated with mass mortality,
91 population extirpations and declines in biodiversity at a global scale (Jancovich et al 2005, Fox
92 et al 2006, Bigarre et al 2008, Ariel et al 2009, Une et al 2009, Whittington et al 2010, Jensen
93 et al 2011, Allender et al 2013, Earl et al 2014, Stark et al 2014, Price et al 2014,2019; Brunner
94 et al 2015, George et al 2015, Miaud et al 2016, Rijks et al 2016, Rosa et al 2017). Ranavirus
95 was responsible for multi-species amphibian declines in continental Europe (Price et al 2014),
96 and of the common frog in the United Kingdom (Teacher et al 2010), but also alters the age
97 structure of remnant UK common frog populations (Campbell et al 2018a). The frequency and
98 severity of disease outbreaks are predicted to worsen alongside human-mediated range
99 expansion of ranaviruses (Jancovich et al 2005, Schloegel et al 2009, Price et al 2016, 2019).
100 To manipulate environmental microbiome diversity, we assembled experimental units that
101 either contained a complex natural bacterial reservoir (*complex* habitats, containing a soil
102 substrate and leaf litter) or simplified one (*simple* habitats, containing stony terrestrial
103 substrates and no leaf litter). We performed two sequential experiments. In the first
104 experiment, we group-housed 96 *R. temporaria* metamorphs in blocks of 6 individuals (n= 48
105 individuals per habitat treatment). For the second experiment, we individually housed 48
106 individuals in habitat treatments (n=24 per habitat). Detailed experimental protocols are

107 listed in the Methods section below. Both experiments allowed us to measure the influence
108 of environmental microbiome on host microbiome structure and disruption of the host
109 microbiome by pathogen exposure. Experiment 1 was designed to allow us to measure
110 habitat-dependent mortality following exposure to ranavirus. Conversely, individual housing
111 of frogs in Experiment 2 allowed us to track individual habitat- and pathogen-dependent
112 microbiome trajectories over time, as well as within-individual changes in microbiome
113 stability. Specifically, we sought to test whether i) more diverse environmental bacterial
114 reservoirs elicited more diverse frog skin microbiomes, ii) more diverse skin microbiomes
115 were more stable over time; and iii) whether microbiome diversity predicted differences in
116 resistance to ranavirus, manifesting as lower infection burdens and/or higher survival
117 following exposure.

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122 **METHODS**

123 Ethical Statement

124 All experimental procedures and husbandry methods were approved by the ZSL Ethics
125 Committee before any work was undertaken and was done under licensing by the UK Home
126 Office (PPL 70/7830, P8897246A). Animal health and welfare was monitored daily during both
127 the rearing and experimental periods and all animals were fed *ad libitum* (Tetra Tabimin for
128 tadpoles, small crickets dusted with calcium and the Vetark Nutrobal vitamin supplement for
129 metamorphosed frogs) throughout.

130

131 Experimental Protocols

132 *Animal Rearing: R. temporaria* metamorphs were reared from tadpoles hatched from
133 clutches sourced from UK garden ponds where ranaviriosis had not been reported to the
134 Garden Wildlife Health project (<https://www.gardenwildlifehealth.org/>). Animals that
135 completed metamorphosis were cohoused in large groups (no more than 30 per enclosure)
136 in 460 X 300 X 170mm Exo Terra Faunaria containing cleaned pea gravel, a large, cork bark
137 cover object and sloped to accommodate a small aquatic area. Experimental animals were
138 haphazardly selected from four group enclosures.

139 *Preparation of habitat treatment enclosures:* The general layout of both habitat types
140 was shared in that they both contained a filled, plastic PCR tip box (terrestrial platform) with
141 a cover object, elevated above an aquatic area filled with aged tapwater and autoclaved pea
142 gravel formed into a slope leading from the aquatic area to the platform. The two key
143 differences were that; i) the terrestrial platforms in complex habitats contained garden
144 compost as a substrate, whilst the terrestrial platforms in simple habitats contained standard
145 and autoclaved pea shingle and; ii) leaf litter collected from Regents Park, London, was added
146 to the aquatic area in the complex habitats. Complex habitat enclosures were left uncovered
147 and outdoors for two weeks prior to the start of experiments, while simple habitat enclosures
148 were prepared the day before frogs were transferred into replicates. During the experiment,
149 uneaten cricket corpses were removed from simple habitat enclosures, but left in complex
150 habitat enclosures. Experiment 1 comprised 16 replicate blocks, each housing six recently
151 metamorphosed frogs (8 blocks / 48 frogs per habitat treatment). Experiment 2 comprised 48
152 smaller units each housing an individual frog (24 frogs per habitat treatment). Following

153 rearing in an outdoor facility, animals were moved to a procedure room and housed
154 individually for seven days in Perspex boxes with a cover object and damp paper towel as
155 substrate to acclimatize to experimental conditions prior to any manipulations. Individuals
156 were randomly assigned to experimental replicates and treatments (complex or simple
157 habitats) using a script written in R (R Core Team 2019). We note that our habitat
158 manipulation altered both the bacterial richness in the environment and the structural
159 composition of habitats (e.g. pea shingle vs soil as a terrestrial substrate). Though this could
160 have influenced the results, for example by changing the dynamics of host contact with the
161 environment, these differences may be more representative of natural variation in
162 microbiome-habitat relationships, where we would expect habitat heterogeneity to covary
163 with microbiome structure.

164

165 *Swabbing Protocols:* For both experiments, we rinsed individuals in sterilised aged tap water
166 to remove transient environmental microbes, and then swabbed the skin of the body and
167 limbs of frogs with MW100 DrySwabs (Medical Wire Equipment, UK). In experiment 1, all
168 animals were swabbed on Day 1 immediately preceding transfer to experimental units, then
169 again on day 14, the latter referred to as the ‘pre-exposure’ swab. Following the day 14 swab,
170 we exposed individuals to either ranavirus or the control (see protocol below), and then
171 swabbed all individuals again on Day 17 to measure the effect of ranavirus exposure (‘post-
172 exposure swab’). We swabbed all animals alive at the end of the experiment on Day 30, but
173 do not include these data here as sample size per habitat-treatment group combination was
174 low and unbalanced. For experiment 2, individuals were swabbed more frequently at Day 1,
175 Day 7, Day 14 (pre-exposure) and Day 16 (post-exposure). For experiment 1 we present the
176 pre- and post-exposure swabs as a 2-level time variable, whereas for experiment 2 we present
177 all 4 time points as a time series.

178 Environmental swab samples (two per experimental unit, one terrestrial and one
179 aquatic) were also collected on day 14 preceding pathogen exposure procedures. Terrestrial
180 swabs were taken by running the swab over the terrestrial substrate and inside the cover
181 objects twice. Aquatic swabs were taken by submerging the swab in the aquatic portion of
182 the tank. These swabs allow us to assess how environmental microbiome diversity influences
183 host skin microbiome diversity.

184

185 *Ranavirus Exposure*: Experimental units were randomly assigned to pathogen treatment
186 group (ranavirus or sham) for both experiments using a script written in R. Prior to this,
187 *Ranavirus* (FV3-like isolate RUK13, Cunningham et al 2007) was cultured in EPC cells at 27°C,
188 harvested after the cell layer had completely cleared, subjected to three rounds of freeze-
189 thaw and then cleared of cells and cellular debris by centrifugation at 800g for ten minutes
190 and discarding the cell pellet. Virus titre was estimated using a 50% Tissue culture Infective
191 Dose assay (TCID₅₀) and calculated following the method of Reed and Muench (1938). Sham
192 exposure media was produced by harvesting the supernatant of a pure culture of EPCs after
193 the same 800g, ten minute spin. For exposures, animals were transferred either as co-housed
194 groups (Experiment 1) or individually (Experiment 2) to 90 mm petri dishes containing 19 mL
195 of aged tap water. Depending on treatment, either 1 mL of stock virus culture at 2×10^6
196 TCID₅₀/mL (giving a final exposure concentration of 1×10^5 TCID₅₀/mL) or 1 mL of sham media
197 was added to the petri dish. Animals were exposed in petri dishes for six hours before being
198 returned to their habitat treatment enclosures. We used daily health and welfare checks
199 throughout the experiment to monitor survival rates. We also used daily checks to monitor
200 for signs of disease commonly associated with ranavirosis (see below: Price et al 2016). We
201 ended Experiment 1 on day 30 when all surviving frogs appeared physically healthy and when
202 mortality had subsided, and Experiment 2 on day 16 following the post-exposure swab.

203

204 16S Sequencing and Bioinformatics

205 16S metagenetic library preparation was carried out using a modified version of the protocol
206 detailed in Kozich *et al* (2013) that amplifies the v4 section of the 16S rRNA gene. Sequencing
207 was performed using 250bp paired-end reads on an Illumina Miseq using a v2 chemistry 500
208 cycle cartridge (detailed information in supplementary file 'Detailed Amplicon Sequencing
209 Methods'). Experiment 1 and 2 were processed on separate MiSeq runs, but all comparisons
210 and statistical tests are made among samples *within* runs, so negating batch effects and inter-
211 run variability. We processed raw 16S reads in the DADA2 pipeline (Callahan et al 2017), using
212 standard parameters as per the online tutorial. We used *phyloseq* (McMurdie and Holmes
213 2013) for downstream sequence processing. In both experiments we removed amplicon
214 sequence variants (ASVs) present in the no-template controls (Experiment 1: 54 ASVs of
215 11,640; Expt. 2: 466 of 14,963). To focus on differences in high abundance ASVs and to remove
216 any potential bias introduced by small differences in low-abundance reads, we removed all

217 ASVs from the dataset with fewer than 100 reads (e.g. Longo & Zamudio 2017), leaving
218 5,796,063 reads of 1446 ASVs for Experiment 1, and 7,068,790 reads of 1969 ASVs for
219 Experiment 2 used in downstream analysis. Reads per sample ranged from 6237 – 66993
220 (Experiment 1) to 16406 – 59607 (Experiment 2). We rarefied data to the minimum per-
221 experiment sequencing depth prior to analysis.

222

223 Viral Load Quantification

224 Liver samples were extracted with DNeasy Blood & Tissue kits (Qiagen) following the
225 manufacturer's protocol. We quantified viral loads in all individuals using the qPCR method
226 of Leung et al (2017), which normalises viral DNA quantities relative to host DNA in the
227 sample.

228

229 Statistical Analysis

230 We conducted all statistical analyses in R. Due to differences in experimental design, the sets
231 of analyses employed vary by experiment. For example, we did not track individuals group-
232 housing in Experiment 1 and so do not examine drivers of within-individual changes in
233 microbiome stability, but do so in Experiment 2. Likewise we did not assay survival in
234 Experiment 2, but do present survival analyses for Experiment 2.

235 We fitted mixed effects models in the R package *lme4* (Bates et al 2015) and ranked
236 competing models by AICc using the R package *MuMIn* (Barton 2019). We considered all
237 models within 6 AICc units of the best supported AICc model to have relatively equal support
238 in the data. To remove overly complex models from consideration we also applied the nesting
239 rule (see Richards 2008; Harrison et al 2018) to remove models that were more complex
240 versions of models with better AIC support. Where we refer to the 'top model set', we refer
241 to the delta-6-AICc model set after the nesting rule has been applied. Where appropriate, we
242 refitted models in a Bayesian framework using the *Stan* computational framework ([http://mc-](http://mc-stan.org/)
243 [stan.org/](http://mc-stan.org/)) accessed with the *brms* package (Bürkner 2017,2018). The advantage of the
244 Bayesian framework is that it allows quantification of uncertainty in parameters such as
245 slopes and r^2 values. Where appropriate, we specified mildly informative priors for
246 parameters such as the correlation between random effects and slopes to speed up sampling
247 and optimise convergence. We assessed convergence of chains using the Gelman-Rubin

248 statistic, and inspected plots of posterior draws to verify adequate mixing of chains and
249 sampling. Detailed descriptions of all statistical analyses and code are provided as an R
250 Markdown document.

251 *Diversity Indices:* We calculated two metrics of alpha diversity: i) richness as the
252 exponent of the Shannon diversity index, also referred to as the effective number of species;
253 and ii) evenness, measured as the Shannon index divided by the log of the number of
254 observed sequences in a sample. To derive measures of beta diversity, we performed Non-
255 Metric Multidimensional Scaling (NMDS) ordinations on Bray-Curtis distance among bacterial
256 community ASV abundances distances using the R package *vegan* (Oksanen et al 2015). We
257 also extracted NMDS1 values from these ordinations for analysis in statistical models (see
258 below)

259 *Experiment 1:* We fitted a model containing the three-way interaction among time
260 (pre- vs post-exposure), habitat (Complex vs Simple) and exposure (ranavirus vs control) as
261 predictors of alpha diversity, with separate models for richness and evenness. All models
262 included a random intercept term for block ID (experimental tank) and used a Negative
263 Binomial error structure. We performed PERMANOVA analysis in the R package *vegan* to test
264 for differences among samples in beta diversity, also containing the time:habitat:pathogen
265 three way interaction, and marginalising the effect of block ID.

266 *Experiment 2:* We fitted a model containing day, day² (to permit non-linear effects of
267 time), habitat (Complex vs Simple) and exposure (ranavirus vs control) as well as an
268 interaction between day² and habitat as predictors of alpha diversity. All models included a
269 random intercept for individual, and a random slope for day given individual. More complex
270 models could not be fitted given the data available and produced convergence warnings. We
271 used a Negative Binomial error structure for alpha diversity models to control for
272 overdispersion (see Harrison 2014) and a Gaussian error structure for beta diversity models.
273 We performed PERMANOVA on Bray-Curtis distances among samples using the R package
274 *vegan* to test for differences in beta diversity. We fitted a model containing a 3-way
275 interaction between habitat, day and ranavirus exposure, permuted 999 times to derive *p*
276 values for effects. We also fitted a linear mixed effects model to examine factors predicting
277 NMDS1 variation among individuals, and included habitat, day, day² and pathogen exposure
278 as main effects, as well as habitat:pathogen exposure, habitat:day and habitat:day² as

279 predictors. All models included a random intercept for individuals. We could not include a
280 random slope for day given individual as this produced convergence warnings.

281 *Survival Analysis:* We used the R package *coxme* (Therneau 2015) to examine
282 differences in survival dependent on habitat and pathogen exposure whilst controlling for
283 block ID in Experiment 1. Sample size for this analysis was 85 individuals (42 in Simple Habitats
284 and 43 in Complex habitats) across 8 habitat blocks per habitat type. We censored eight
285 individuals because they died prior to exposure. We ranked survival models by AICc to derive
286 a top model set.

287 *Predicted Functional Analysis:* We used the ASV abundance matrices from Day 7 to
288 predict functional profiles of microbial communities using PIPHILLIN (Iwai et al 2016) and
289 tested for differences in functional profiles dependent on habitat using Constrained
290 Correspondence Analysis (CCA) in the R package *vegan*. We used the May 2017 release of the
291 KEGG database and 97% identity cutoff. We visualised differences in predicted functional
292 repertoire by plotting the axes of a Constrained Correspondence Analysis (CCA) model fitted
293 in *vegan* where we specified the two-level habitat predictor as the constrained variable. We
294 performed predicted functional analysis only on Experiment 2 data as controlling for block
295 effects in DESeq2 is difficult, and individual housing of Experiment 2 obviates the need for this
296 and so should more tightly control the false positive rate.

297 *Stability over Time:* We calculated microbiome stability as the correlation between
298 ASV abundances across two time points, following Lahti et al (2014). That is, microbiome
299 stability over time is estimated as the correlation between the two vectors of microbial
300 community abundances from an individual for two time points, where stronger correlations
301 indicate greater stability. We used Day 7 and 13 in Experiment 2 to quantify baseline stability,
302 and tested variation in stability dependent on habitat using a t-test. We also calculated
303 stability following exposure to a pathogen using the Day 13 and Day 16 ASV abundances and
304 tested for a correlation between pre- and post-infection stability using Spearman's
305 correlation tests. We also calculated change in stability across the two time points by
306 subtracting pre-infection stability from post infection stability.

307 We used ANOVA to test whether change in stability was explained by habitat or pathogen
308 treatment. We repeated the above analyses restricting the dataset to the top 100 most
309 abundant ASVs in each habitat to represent the 'core' microbiome.

310

312 RESULTS

313 Environment and Pathogen Exposure Modify Skin Microbiome Structure (Experiment 1)

314 *Alpha Diversity:* Bacterial richness and evenness of common frog skin was directly
315 influenced by the complexity of the bacterial species reservoir in the environment. Individuals
316 in habitats with higher environmental bacterial species richness possessed greater mean skin
317 bacterial diversity ($r = 0.82$, $p=0.001$; Fig 1A; Fig. S1). There was some evidence that overall
318 effective number of species increased over time, but only weak evidence that this effect was
319 dependent on habitat treatment (Fig. 1B; Table S1). There was no evidence of an effect of the
320 interaction between time, habitat and pathogen exposure, or a main effect of ranavirus
321 exposure on overall species richness of the microbiome (Table S1). The top model
322 investigating drivers of differences in community evenness contained interactions between
323 ranavirus exposure and time, as well as habitat and time (Table S2a). As for species richness,
324 these results indicated that community evenness was lower in Simple Habitats prior to
325 ranavirus exposure (Table S2b). There was also some evidence that evenness increased over
326 time for complex habitats, but differences due to ranavirus exposure were not clear (Fig. S2;
327 Table S2b).

328 *Beta Diversity:* PERMANOVA analysis controlling for block identified the dominant
329 source of variation in microbial communities to be habitat (simple vs complex, $r^2 = 15.9\%$,
330 $p=0.001$; Fig. 1C). Exposure to ranavirus also influenced microbial community structure
331 (infection main effect, $r^2 = 2.8\%$, $p=0.001$), but critically operated via habitat:infection and
332 infection:time interactions. The habitat:infection:time interaction was not significant ($p=0.08$;
333 Table S3).

334

335 Survival Following Exposure to Ranavirus (Experiment 1)

336 Individuals in simple habitats exposed to ranavirus exhibited higher rates of mortality
337 (68.4%) than individuals in complex habitats exposed to ranavirus (52.2%). The best-
338 supported model contained effects of both habitat complexity and disease treatment on
339 survival (Fig. 2A, Table S4). A model containing only disease treatment received marginally
340 less support ($\Delta AICc = 0.22$). Though the model containing the interaction between habitat
341 and treatment was in the $\Delta 6$ AIC model set, it was a more complex version of a simpler model
342 with better AIC support and so was removed under the nesting rule (Richards 2008). Model

343 averaged coefficients [and 95% confidence intervals] from the survival model were: Habitat
344 0.57 [-0.167,1.3] and ranavirus exposure 2.26 [1.2,3.33].

345 There was no difference between habitats in likelihood of exhibiting gross signs of
346 disease (Binomial GLMM, mean probability of exhibiting signs of disease [95% credible
347 intervals]: complex 0.48 [0.11,0.82]; simple 0.5 [0.1,0.85]; $p_{\text{MCMC}} = 0.92$) or in severity of
348 visible signs of disease (Ordinal GLMM, mean probability of being scored category 0 [95%
349 credible intervals]; complex 0.51 [0.12,0.9]; simple 0.46 [0.06,0.93]; $p_{\text{MCMC}} = 0.88$). Individuals
350 that died following exposure to ranavirus had higher viral loads than those that were still alive
351 at the end of the experiment (Fig. 2B). The best supported model examining variation in viral
352 loads contained only the main effect of mortality (r^2 38.7% [95% CI 15.1-56.1%]), as all other
353 models with weaker support were removed under the nesting rule (Table S5). Three
354 individuals in the Control treatments died following exposure and exhibited weak ranavirus
355 infections; these were inconsistent with the higher infection loads observed in other
356 individuals that died after exposure (Fig. 2B).

357

358 Environment Alters Host Microbiome Dynamics Over Time (Experiment 2)

359 *Alpha Diversity:* All individuals had similar bacterial species richness on Day 0 when
360 they entered the experimental habitats, but the dynamics of host microbiome bacterial
361 species difference over time differed markedly depending on habitat treatment (Fig 3A). The
362 best supported model explaining differences in richness contained an interaction between
363 day² and habitat. When marginalising the effects of time (sampling day) and variation among
364 individuals in their change in diversity over time, individuals in complex habitats had greater
365 skin bacterial diversity than those in simple habitats (Fig. S3). The top model explained 18.91%
366 of variation in alpha diversity (95% credible interval 7.38 – 32.17%). There was no evidence
367 that ranavirus exposure altered the dynamics of richness over time (Table S6, S7). When
368 considering only the pre-exposure (Day 13) and post-exposure (Day 16) time points, the
369 modal response was an increase in richness across the two time points. The top model
370 examining factors predicting microbial community evenness contained only effects of day and
371 day², but no habitat main effect or interactions. The null model was also retained in the top
372 model set (Table S8). These data corroborate those from experiment 1 indicating a change in
373 evenness over time. However, it is the environment (habitat) that appears to drive changes
374 predominantly in the dynamics of microbial richness of amphibian skin over time.

375 *Beta Diversity:* PERMANOVA performed on Bray-Curtis distances revealed significant
376 effects of habitat, day and a habitat:day interaction (all $p=0.001$) on bacterial beta diversity
377 (Fig. 3B, Table S9). Collectively these terms explained roughly 15% of the variation in variation
378 among individuals in bacterial community structure. There was no evidence that exposure to
379 ranavirus modified the structure of bacterial communities (all interaction terms containing an
380 effect of ranavirus exposure, $p>0.05$), nor evidence of a ranavirus main effect ($p=0.28$, Table
381 S9). Linear modelling of factors predicting NMDS1 revealed clear evidence of habitat-
382 dependent variation in beta diversity trajectory over time for all individuals (Fig 3C). The only
383 model in the top model set explaining predictors of NMDS1 contained an interaction between
384 habitat type and day² (Fig. 3C, Fig. S4), corroborating the results of the PERMANOVA above.

385 *Functional Traits:* Predicted functional analysis using PIPHILLIN revealed distinct separation in
386 the functional repertoires of the amphibian skin bacterial microbiome based on habitat after
387 7 days (CCA analysis, effect of habitat $F_{1,45} = 3.15$, $p = 0.01$, Fig. S5). Analysis using DESeq2
388 revealed 12 pathways that were significantly more abundant in simple Habitats, and 8
389 pathways more abundant in complex habitats (Table S8).

390 *Viral Load Data:* Viral loads of frogs following exposure to ranavirus in Experiment 2 were
391 weak; mean viral load was 0.0013 viral copies per host cell [range 0.0001 – 0.01]. There was
392 no significant difference in the mean viral load between animals in Complex and Simple
393 habitats (Wilcoxon rank sum test, $W = 88$, $p = 0.19$, Fig. S6). No control animals in either
394 habitat treatment had detectable levels of virus (Fig S5).

395

396 Patterns of Microbiome Stability Varied by Habitat (Experiment 2)

397 When considering all ASVs, complex habitats exhibited decreased stability over time prior to
398 infection when compared to simple habitats ($t=5.8$, $df=43.3$, $p<0.001$; Fig. 4A). However, an
399 individual's microbiome stability appeared consistent over time when comparing stability
400 prior to pathogen exposure and stability following pathogen exposure (Fig 4B; Fig. S7A)

401 When considering only the top 100 ASVs, the difference in stability over time pre-infection
402 was no longer apparent ($t=1.2$, $df=42$, $p=0.21$; Fig. 4C). Forty-seven ASVs from 5 Phyla were
403 common to both sets of 100 most abundant ASVs by habitat (Table S9). The most common
404 Phylum of shared ASVs was *Proteobacteria*, comprising 32 of the 47 ASVs (68%).
405 *Actinobacteria* and *Bacteroidetes* accounted for 13% each of the shared ASV taxonomy. At
406 the genus level, notable shared ASVs were classified as *Citrobacter*, *Acinetobacter*,

407 *Chryseobacterium*, and *Stenotrophomonas*, all of which have been associated with production
408 of metabolites that inhibit other amphibian pathogens like *B. dendrobatidis* (e.g. Antwis &
409 Harrison 2018).

410 Both habitats still exhibit consistent levels of stability either side of exposure to the
411 pathogen (Fig. 4D; Fig. S7B), though the correlation is weaker. There was no evidence that
412 habitat treatment, pathogen exposure or their interaction affected the magnitude of change
413 in stability over time, for either all ASVs or the analysis restricted to the top 100 most
414 abundant ASVs (ANOVA, all $p > 0.27$).

415

416

417 DISCUSSION

418

419 Though our knowledge of the factors shaping the structure of the host-associated microbiota
420 is increasing, studies directed at understanding the predictors of longitudinal variation of the
421 microbiome in non-model organisms are relatively scarce (e.g. Smith et al 2015; Videvall et al
422 2019). Our results from two experiments suggest that the structure and temporal dynamics
423 of the amphibian skin microbiome are influenced by both the environment and exposure to
424 a lethal pathogen. Overall alpha diversity of the microbiome appeared to influence temporal
425 stability, where more 'species-rich' microbial communities were less stable over time
426 compared to less diverse communities. Crucially, this effect disappeared when considering
427 only the top 100 most abundant bacterial taxa, suggesting 'core microbiome' stability may be
428 relatively uniform irrespective of total diversity. Finally, our survival data suggest that higher
429 skin microbiome diversity may correlate with greater survival following exposure to the lethal
430 pathogen ranavirus. Our results have important implications for our understanding of factors
431 driving variation among individuals in the stability of both their microbiomes and the strength
432 of host-microbe interactions over time, and in turn how both traits may be compromised by
433 external stressors such as exposure to pathogens.

434

435 *Environmental Microbial Diversity Influence Temporal Microbiome Dynamics*

436 By manipulating the microbial reservoir in the environment, we elicited differential
437 patterns of microbiome diversity on the skin of common frogs. Microbial communities
438 differing in diversity also exhibited distinctive signatures of change over time. Higher diversity
439 skin microbiomes appeared less stable over time, an effect driven primarily by weak
440 correlations over time in the abundances of rarer bacterial taxa. The 'core microbiome' of the
441 most abundant ASVs in each habitat type appeared stable irrespective of overall diversity.
442 Most strikingly, microbiome stability itself appeared conserved over time: stability between
443 the first two time points correlated strongly with the two time points bracketing exposure to
444 the pathogen. Our data therefore support the idea of *consistent* variation among individuals
445 in microbiome stability over time, where structure itself is a function of the environment that
446 an individual inhabits. These data support previous work indicating that environmental
447 context and complexity is a key determinant of the assembly and stochasticity dynamics of
448 host microbiomes, with important consequences for host resistance to disease (Becker et al

449 2017). It is notable that 47 of the 100 most abundant per-habitat ASVs were common to both
450 habitats, alluding to a constrained core microbiome structure irrespective of habitat microbial
451 diversity and structure.

452 A major outstanding question is what are the consequences of consistent within and
453 among-individual variation in microbiome stability over time? Predicted functional analysis
454 highlighted that differences in overall microbial community diversity also reflected
455 differences in functional repertoire (see also Bletz et al 2016) which could reflect variation in
456 the presence or strength of key interactions between microbes and hosts such as production
457 of antimicrobial metabolites that defend the host from pathogens (e.g. Brucker et al 2008;
458 Becker et al 2009; Antwis & Harrison 2018). Measures of temporal stability or stochasticity of
459 microbiomes, and the processes that drive these traits, are critical for understanding the
460 consistency of microbe-mediated functions over time. Most of the data we have on *within-*
461 *individual* microbiome dynamics and stability come from human or model organism studies
462 (e.g. Fink et al 2013; Kelly et al 2016; Schirmer et al 2018; Gilbert et al 2018), with relatively
463 few on non-model organisms (e.g. Videvall et al 2019; Antwis et al 2019), and fewer still from
464 controlled experiments (Kueneman et al 2016; Grotoli et al 2018). Though several studies
465 have measured seasonal dynamics of microbiome in species such as aphids (Smith et al 2015)
466 and mosquitoes (Novakova et al 2017); they rely on population-based metrics of microbiome
467 structure that may mask substantial among-individual variation in microbiome dynamics.
468 Amphibians and their skin microbiomes provide a model for understanding the processes
469 shaping the forces of colonisation, competition and coexistence of microbial species on a
470 vertebrate host, and quantifying the emergent functional properties of these microbial
471 communities and their consequences for the host. The properties of this system make it well
472 suited to testing the applicability of established ecological theory derived from eukaryotic
473 communities to prokaryotic assemblages associated with animals, plants and soils, including
474 the relationship between biodiversity and ecosystem function (e.g. Greenspan et al 2019; see
475 Koskella et al 2017).

476

477 *Exposure to Ranavirus Disrupts the Host Skin Microbiome*

478 Our data from experiment 1 revealed that exposure to ranavirus elicited subtle but
479 significant changes to the structure of the amphibian skin microbiome after 48 hours. We
480 predicted that more diverse microbial communities should be more resistant to perturbation

481 by the ranavirus, but our data suggest that the skin microbiomes of individuals in both habitat
482 treatments were affected by the pathogen. This supports previous work showing that
483 pathogens like *Bd* can destabilise host microbiomes (e.g. Jani & Briggs 2014; Walke et al 2015;
484 Longo & Zamudio 2017). Though we didn't detect a similar time:habitat:pathogen interaction
485 in Experiment 2, this can be explained by the relatively low infection burdens in this
486 experiment. Results from our experimental work here are well supported by counterpart
487 investigations into the structure of the microbiota of wild common frogs. These studies have
488 illustrated distinct differences in bacterial community structure at sites suffering mass
489 mortality events due to ranavirus compared to sites where no such outbreaks have been
490 detected (Campbell et al 2018b, Campbell et al 2019), even after accounting for differences
491 among populations (Campbell et al 2019). These correlative data from wild frogs could
492 represent bacterial communities in some populations associated with protection of the host
493 from viral infection, or marked shifts in microbiome structure in populations suffering
494 ranaviral infection. Use of pesticides has been associated with increased prevalence of
495 ranaviruses (North et al 2015) and could theoretically be mediated by disruption of the both
496 environmental and host-associated bacterial communities. Considering these data with
497 results from our experiments hints that both processes may be responsible for the patterns
498 observed in nature. Disruption of the host microbiome by pathogens of wild vertebrates is
499 likely to be far more common than the existing literature suggests. The scarcity of studies
500 directed at quantifying microbiome disruption by pathogens means we currently lack the
501 ability to compare the magnitude of the perturbation effect among host species and both
502 host and pathogen taxonomic groups.

503

504 *Links Between Microbiome and Survival Following Ranavirus Exposure*

505 Our controlled infection experiment revealed that individuals with less diverse
506 microbiomes exhibited higher mortality following exposure to ranavirus compared to
507 individuals with higher diversity microbial communities, consistent with our predictions. In
508 our models, greater resistance to pathogenic infection as an emergent property of
509 microbiome diversity would be evidenced by a diversity (habitat) by pathogen exposure
510 interaction term. We note that though there was reasonable support for a model containing
511 this interaction in our top model set, it was not retained under the nesting rule. As such, there
512 exists some model selection uncertainty regarding the effect of microbiome diversity on

513 resistance to ranavirus infection. Several studies have provided evidence consistent with a
514 correlation between overall microbiome diversity and susceptibility to infectious disease and
515 costs associated with host responses to pathogen exposure (e.g. Cariveau et al 2014;
516 Kueneman et al 2016), though these effects are not always consistent (e.g. Becker et al 2019;
517 Ma et al 2019). Disruption of the normal microbiome by administration of antibiotics to
518 laboratory mice can permit successful infection of *Clostridium difficile* (Theriot et al 2014),
519 loss of microbiome diversity in amphibians can increase susceptibility to the fungal pathogen
520 *Batrachochytrium dendrobatidis* (*Bd*) (Kueneman et al 2016), and disruption of the
521 microbiome in early life can increase downstream susceptibility to parasites (Knutie et al
522 2017). Notably, augmentation of low diversity skin microbiomes with key taxa from the more
523 diverse wild-type microbiome can reverse the observed increase in susceptibility to a lethal
524 pathogen like *Bd* (Kueneman et al 2016). Our habitat treatments differed in overall physical
525 structure as well as microbial diversity, as complex habitats contained different terrestrial
526 substrate as well as leaf litter in the water. Traits such as host microbiome richness, temporal
527 dynamics and resistance to disease will be governed by both extrinsic processes (microbial
528 diversity present in the environment capable of colonising the host) and intrinsic factors, such
529 as host immunogenetic variation (e.g. Bolnick et al 2014) and physiological stress (e.g. Lokmer
530 & Wegner 2015). Though variation in host stress due to structural heterogeneity between
531 habitat treatments could have influenced microbiome dynamics, we believe this effect would
532 be minimal in our data as all individuals were reared under captive conditions as tadpoles,
533 and so were acclimated to conditions found in the simple habitat treatments. Nevertheless,
534 future work will standardise the environmental structure of the habitats and manipulate only
535 the microbial reservoir to remove the potential for such differences between treatments.

536 The mechanisms underpinning diversity-disease relationships in amphibians warrant
537 further investigation. Microbiome diversity alone cannot be considered a beneficial trait for
538 hosts; rather diversity itself is an emergent property of ecological processes playing out within
539 the host (Shade 2017) that underpin the true mechanism. More diverse microbiomes could
540 be more likely to contain species producing antiviral compounds such as bacteriocins (see
541 Drider et al 2016), or to prime the host immune system to produce anti-microbial peptides
542 (Woodhams et al 2019) that can inactivate ranavirus virions (Chinchar et al 2004). As
543 expected, ranavirus-exposed individuals that died during the experiment had higher viral
544 loads than those that survived. Higher survival in individuals with more diverse microbiomes

545 could represent microbe-mediated defence preventing infection burdens from reaching
546 lethal thresholds. Indeed, our predicted functional analysis of skin bacterial microbiomes
547 revealed distinct differences dependent on diversity. Though complex skin microbiomes were
548 predicted to differ in relative abundance of pathways linked to human viral infections, the
549 relevance of such differences to amphibian defence against ranavirus remains to be
550 determined. An important priority for future work is to quantify the true functional genetic
551 repertoire of amphibian skin microbiomes to permit identification of potential metabolic
552 pathways linked to disease, and examine how their relative abundance changes in concert
553 with overall microbiome diversity and microbial species composition. Addressing this
554 knowledge gap requires integration of further 'omic tools such as shotgun metagenomics and
555 metabolomics with more common amplicon sequencing metagenetics (Rebollar et al 2016).
556 Finally, Warne et al (2019) recently showed that disruption of the gut microbiome in early life
557 can influence host metabolism and susceptibility to ranavirus in later life. Given that the oral
558 cavity and alimentary canal are major routes of infection for ranaviruses (e.g. Robert et al
559 2011; Salcedo et al 2019), one possibility is that measurements of skin microbiome diversity
560 in amphibians are also reflective of gut microbiome diversity. Future work should quantify
561 this covariation between multi-site microbiome dynamics and seek to understand the
562 functional consequences of increased skin and gut microbiome diversity in hosts vulnerable
563 to ranavirus.

564

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570

571 AUTHOR CONTRIBUTIONS

572 XAH and TWJG designed the experiment. XAH, SJP, WTML, CS and TWJG conducted the
573 experiment. XAH and KPH sequenced the microbial data. SJP and WTML produced ranaviral

574 infection load data. XAH analysed the data. XAH, SJP and TWJG wrote the manuscript, with
575 input from all authors.

576 CONFLICT OF INTEREST

577 The authors declare no conflict of interest.

578

579 DATA ACCESSIBILITY

580 R markdown scripts and data sets permitting full reproduction of all analyses are hosted on
581 FigShare at <https://doi.org/10.6084/m9.figshare.c.4607198>. Sequences have been uploaded
582 to the NCBI Sequence Read Archive under BioProject accession numbers PRJNA559513
583 (Experiment 1) and PRJNA559522 (Experiment 2).

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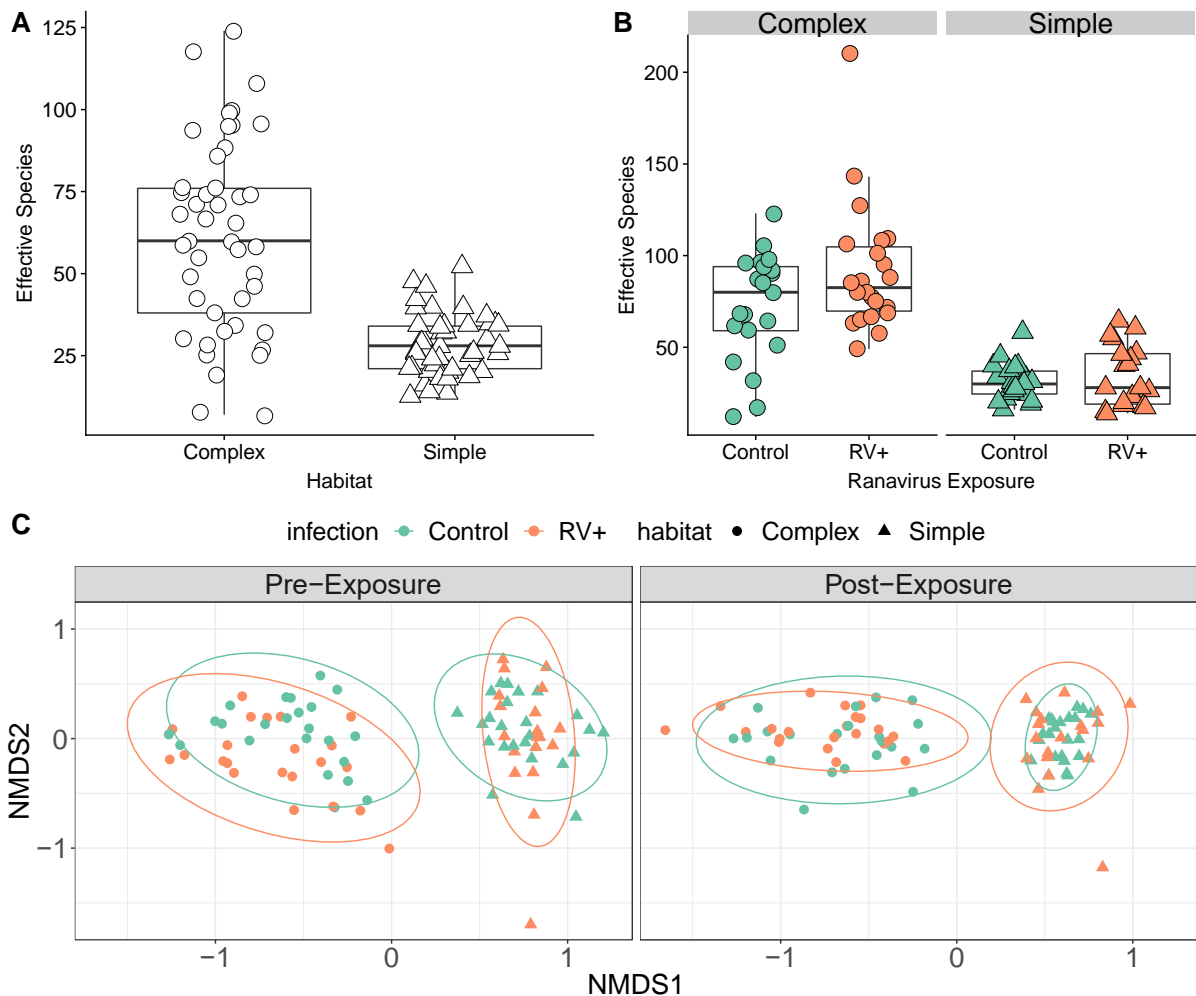
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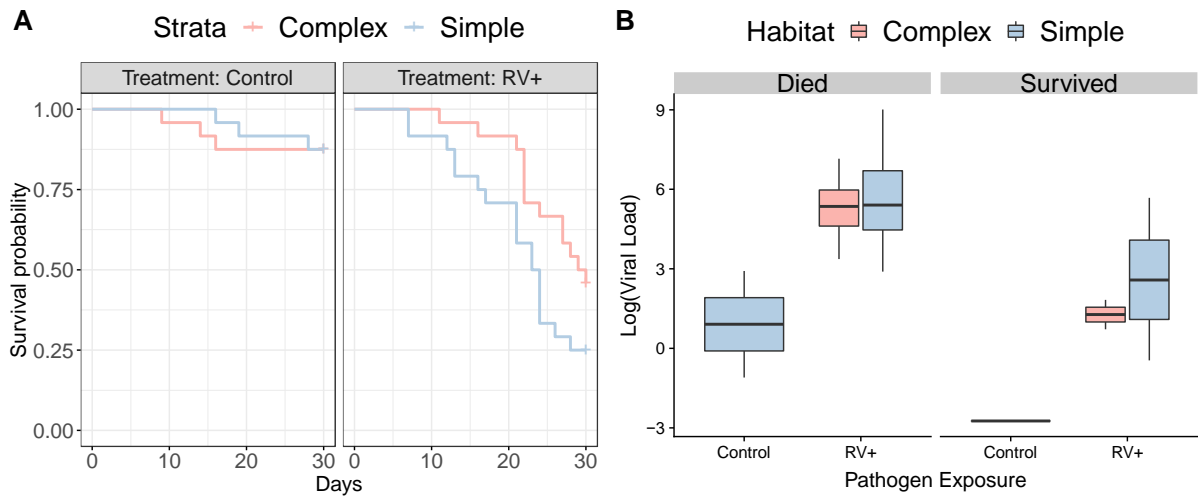
837 **FIGURES**

838

839 **FIGURE 1. ENVIRONMENT MODIFIES HOST SKIN BACTERIAL COMMUNITY STRUCTURE**

840 **(A)** Effect of environmental bacterial richness on host skin microbiome diversity (Experiment
 841 1, Time Point 2 [pre-exposure]). Individuals in habitats with more species rich environmental
 842 bacterial reservoirs also had higher skin bacterial richness (Complex) compared to individuals
 843 in habitats with lower diversity bacterial reservoirs (Simple). **(B)**. There was no effect of
 844 exposure to ranavirus on mean levels of bacterial richness in either habitat type (Experiment
 845 1, Time Point 3 [post-exposure]; See Table 1). **(C)** Skin bacterial community structure (beta
 846 diversity) differed significantly based on habitat and time (pre- vs post-exposure). We
 847 detected a significant three-way interaction between time, habitat and pathogen exposure,
 848 suggesting that ranavirus exposure causes shifts in community structure dependent on
 849 habitat complexity. Note that no individuals were exposed to ranavirus in the *Pre-Exposure*

850 panel, but individual points are coloured by pathogen treatment (ranavirus vs control) in both
851 panels to allow comparison of groups across time.
852



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854 FIGURE 2. SURVIVAL FOLLOWING EXPOSURE TO RANAVIRUS

855 A) Survival data for frogs exposed to Ranavirus or Control, in both Complex (red shading) and

856 Simple (blue shading) habitats. The top model explaining variation in survival contained

857 effects of both habitat and pathogen exposure. The model containing the habitat:pathogen

858 interaction was not retained under the nesting rule. B) ranaviral infection loads following

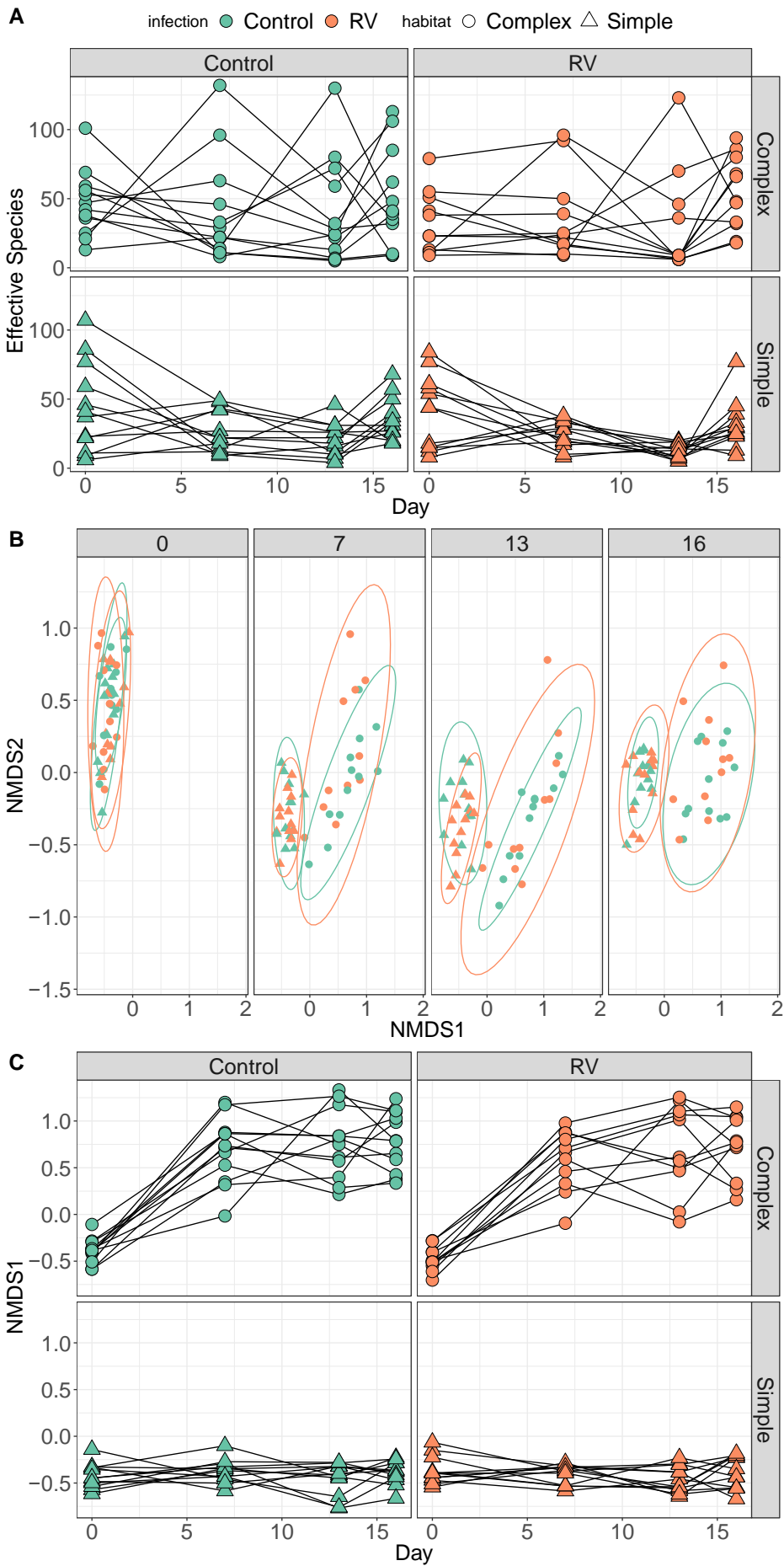
859 exposure to ranavirus, split by whether individuals died following exposure or were still alive

860 on Day 30 at the close of the experiment. There was no difference in ranaviral infection

861 burdens based on habitat treatment, but individuals that died had significantly higher

862 infection loads. Three individuals in the control group exhibited weak infection.

863



865 FIGURE 3. DYNAMICS OF FROG SKIN BACTERIAL COMMUNITIES OVER TIME

866 (A) Trends over time in bacterial alpha diversity (effective number of species) depending on
867 Habitat treatment (rows) and pathogen exposure (columns). Dynamics of alpha diversity over
868 time were significantly different in Complex habitats (see Table 1). When marginalising the
869 effects of time (day), individuals in Complex habitats possessed higher bacterial species
870 richness compared to individuals in Simple habitats (see Fig. S2).

871 **(B)** Trends over time in bacterial community structure (beta diversity).

872 Individuals were exposed to ranavirus or control between days 13 and 16, but points are are
873 coloured by disease treatment at all time points to allow tracking of beta diversity over time
874 for different groups.

875 **(C)** Temporal trends in primary axis of NMDS ordination (beta diversity) depending on Habitat
876 treatment (rows) and pathogen exposure (columns). As with (A), there was strong support in
877 the data for an interaction between day and habitat on beta diversity trajectories over time
878 (see Table 2).

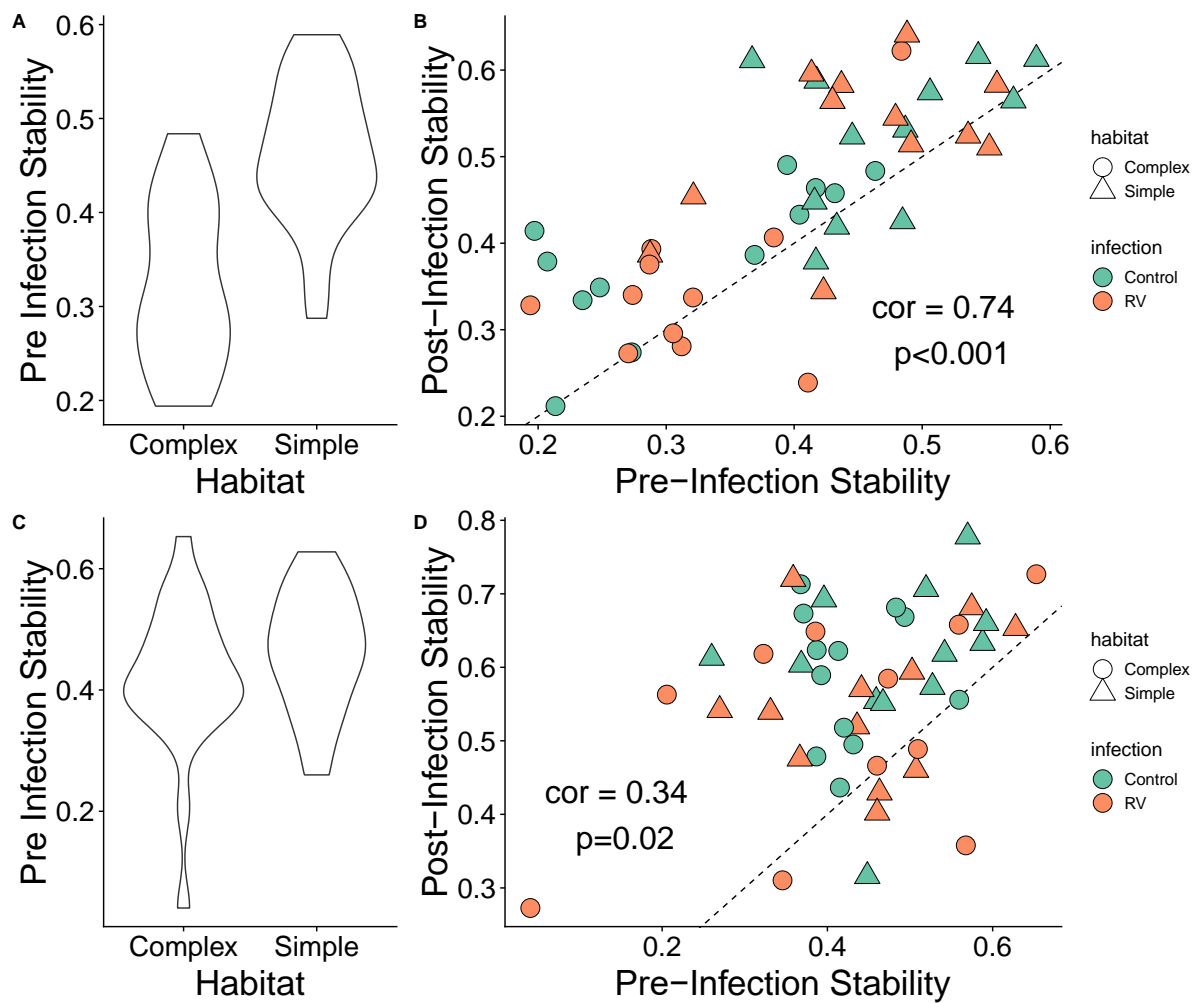
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886 FIGURE 4. MICROBIOME STABILITY OVER TIME

887 **(A)** Microbiome stability, measured as the correlation between ASV abundances across two
 888 times points, prior to pathogen exposure. Frogs in Simple habitats appear to have more stable
 889 microbial communities than those in Complex Habitats. **(B)** Scatterplot of microbiome
 890 stability over two sampling points prior to pathogen exposure (x axis) and two sampling points
 891 either side of pathogen exposure (y axis). Individual microbiome stability appears relatively
 892 consistent over time, irrespective of habitat or pathogen exposure. Dashed line represents
 893 1:1 line of perfect correlation. **(C)** and **(D)** are identical to plots A and B, but use only the top
 894 100 most abundant ASVs for each habitat type, representing a 'core microbiome'. There is no
 895 difference between habitat types in stability for the core microbiome **(C)**, and the correlation
 896 between stability values over time remains, though the relationship is weaker **(D)**.

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