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1 **Captivity and exposure to the emerging fungal**
2 **pathogen *Batrachochytrium salamandrivorans* are linked to perturbation**
3 **and dysbiosis of the amphibian skin microbiome**
4

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26 **Abstract**

27 1. The emerging fungal pathogen, *Batrachochytrium*
28 *salamandrivorans* (*Bsal*) is responsible for the catastrophic decline of
29 European salamanders and poses a threat to amphibians globally.
30 2. The amphibian skin microbiome is strongly associated with disease
31 outcome for several host-pathogen systems, yet its role
32 in *Bsal* infection remains unresolved. In addition, many *in-*
33 *vivo Bsal* studies to date have relied on specimens that have been kept
34 in captivity for long periods without considering the influence of
35 environment on the microbiome and how this may impact the host
36 response to pathogen exposure.
37 3. We characterised the impact of captivity and exposure to *Bsal* on the
38 skin bacterial and fungal communities of two co-occurring European
39 newt species, the smooth newt (*Lissotriton vulgaris*) and the great-
40 crested newt (*Triturus cristatus*).
41 4. *Bsal* infection and subsequent mortality in both newt species was
42 associated with perturbation of the skin microbiome and possible
43 dysbiosis. In addition, reduced microbial diversity and changes in
44 microbiome structure accompanied the transition of newts from the wild
45 to captivity, suggesting a possible decline in microbe-associated
46 protection and increased risk of infection by opportunistic pathogens.
47 5. Our findings advance current understanding of the role of host-
48 associated microbiota in *Bsal* infection and highlight important
49 considerations for *ex-situ* amphibian conservation programmes.

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50 **Introduction**

51 Microbial communities associated with amphibian skin are increasingly
52 recognised for their ecological complexity and importance in pathogen
53 defence. In particular, studies have demonstrated that skin-associated
54 bacteria are linked to disease outcome in amphibians infected by the chytrid
55 fungus *Batrachochytrium dendrobatidis* (*Bd*) (Lauer *et al.* 2007; Harris *et al.*
56 2009; Jani & Briggs 2014; Kueneman *et al.* 2016; Bates *et al.* 2018).
57 Specifically, host-associated microbes may offer protection through
58 production of pathogen-inhibiting compounds (Brucker *et al.* 2008; Woodhams
59 *et al.* 2017), preventing pathogen colonisation (Buffie & Pamer 2013) or by
60 outcompeting harmful microbial invaders (Kamada *et al.* 2012). In addition to
61 the host benefits conferred by the microbiome, some microbes can promote
62 pathogen growth (Stacy *et al.* 2016), while perturbations of host-associated
63 microbial communities can negatively impact host health in a process called
64 dysbiosis (Croswell *et al.* 2009). In recent years, the recognition of the
65 microbiomes' role in disease has led to a search for pathogen-inhibiting
66 probiotics and microbial manipulations that could mitigate host infection and
67 subsequently be utilised as a tool in wildlife conservation (Bletz *et al.* 2013;
68 Kueneman *et al.* 2016).

69

70 In 2013 a novel pathogenic chytrid fungus, *Batrachochytrium*
71 *salamandrivorans* (*Bsal*), was discovered that has caused mass mortalities of
72 caudates in Europe (Martel *et al.* 2013) and threatens amphibians worldwide
73 (Yap *et al.* 2015). While *Bd* and *Bsal* are closely related phylogenetically and
74 occupy similar niches as the only known species within the Chytridiomycota

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75 capable of infecting vertebrates (Berger *et al.* 1998; Martel *et al.* 2013), they
76 show marked differences in their biology. *Bd* infects over 500 amphibian
77 species (Fisher, Garner & Walker 2009) that span all amphibian orders,
78 whereas *Bsal* has a narrower host range limited mostly to caudates (Martel *et*
79 *al.* 2014). *Bd* and *Bsal* also differ in their pathogenesis with *Bd* causing
80 hyperkeratosis and hyperplasia of the amphibian epidermis, compared to
81 lesions and focal necrosis in *Bsal* (Martel *et al.* 2013). Prior studies have
82 shown variability in *Bsal* susceptibility between caudate species (Martel *et al.*
83 2014), however little is known of the determinants of disease outcome. Given
84 its importance in *Bd* infection, the amphibian skin microbiome is a candidate
85 driver of within- and between-species variability in response to *Bsal* exposure.
86 While a great deal is known regarding the impact of *Bd* on the host
87 microbiome, no *in-vivo* studies have investigated the microbiome response to
88 *Bsal*. Importantly, it is not possible to predict the impact of *Bsal* on amphibian
89 microbiota based on prior *Bd* studies, or to presume a microbiome response
90 similar to that of *Bd*. This is due to a range of factors including intrinsic
91 biological differences between *Bd* and *Bsal* (Farrer *et al.* 2017), and the
92 variable responses that single bacterial strains can have with different
93 pathogen isolates. For example, it is well established that the same bacterial
94 strain can be either inhibitory or growth-promoting depending on what
95 genotype of *Bd* it is in co-culture with (Antwis *et al.* 2015; Antwis & Harrison
96 2018). In addition, more recent studies have shown that certain bacteria
97 isolated from amphibian skin inhibit *Bsal*, but not always *Bd*, *in-vitro* (Muletz-
98 Wolz *et al.* 2017) and that *Bd* and *Bsal* metabolites can modulate growth of
99 different bacteria (Woodhams *et al.* 2017). Taken together, these findings

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100 show that differences exist in the way *Bsal* and *Bd* interact with the
101 microbiome of amphibians, reinforcing the importance of investigating the *in-*
102 *vivo* host response to *Bsal* exposure.
103
104 A key component of wildlife disease mitigation for highly threatened species is
105 the establishment of assurance populations or disease treatment in captivity
106 (Mendelson *et al.* 2006; Gascon 2007). However, despite recognition of the
107 importance of the microbiome in host health and pathogen defence, few
108 studies have investigated the potential impact of captivity on the amphibian
109 skin microbiome (Becker *et al.* 2014; Loudon *et al.* 2014; Bataille *et al.* 2016;
110 Kueneman *et al.* 2016; Sabino-Pinto *et al.* 2016) and none have investigated
111 this with respect to *Bsal* mitigation. Prior studies have yielded mixed results
112 with reductions in bacterial alpha diversity and depletion of chytrid-inhibiting
113 bacteria in captive compared to wild individuals for some host species
114 (Loudon *et al.* 2014; Bataille *et al.* 2016; Kueneman *et al.* 2016; Sabino-Pinto
115 *et al.* 2016) while increased alpha diversity was seen in other species (Becker
116 *et al.* 2014). In addition, studies investigating the impact of captivity on the
117 amphibian skin microbiome have neglected to examine microbial kingdoms
118 other than bacteria, despite recent advances demonstrating that fungi may be
119 equally important to host health and disease resistance (Kearns *et al.* 2017).
120 Further, cross-kingdom responses to captivity may not be uniform making it
121 essential that a more holistic outlook of the skin microbiome is taken.
122 Understanding the effect of captivity on the host microbiome is especially
123 important with regard to *Bsal* exposure since field based interventions are
124 unlikely to be successful owing to disease transmission occurring at low

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125 population density (Schmidt *et al.* 2017) and with the only currently effective
126 treatments being captivity based (Blooi *et al.* 2015a; Blooi *et al.* 2015b).
127 Gaining insights into how both *Bsal* exposure and the transition from the wild
128 to captivity affect the caudate skin microbiome is therefore an important
129 advancement in our understanding of infection as well as being valuable in
130 informing future captivity-based conservation interventions.
131
132 In this study, we combine field and laboratory studies to investigate how the
133 amphibian skin microbiome changes with the transition from the wild to
134 captivity followed by subsequent exposure to *Bsal*. We focus on two UK
135 caudate species, the smooth newt (*Lissotriton vulgaris*) and the great crested
136 newt (*Triturus cristatus*). While *L. vulgaris* is ubiquitous in the UK, *T. cristatus*
137 is rarer, more localised and declining in many parts of its natural range
138 (Edgar, Griffiths & Foster 2005). *T. cristatus* is also listed as a protected
139 species in Annexes II and IV of the European Commission Habitats Directive
140 and under the UK Wildlife and Countryside Act 1981. Consequently, the long-
141 term population viability of *T. cristatus* is particularly vulnerable to local
142 disease outbreaks. The susceptibility of captive-raised *T. cristatus* to *Bsal* has
143 been tested in a prior study (Martel *et al.* 2014) which showed mortality in all
144 infected animals. Meanwhile, no study to date has investigated the lethality of
145 *Bsal* in *L. vulgaris*. Testing the effect of *Bsal* on endemic UK species and the
146 possible risk it poses to wild populations is vital given the recent emergence of
147 *Bsal* in private collections (Cunningham *et al.* 2015) and the continued spread
148 of pathogenic chytrids through the global trade (O'Hanlon *et al.* 2018)
149 suggesting a wild outbreak is possible. Understanding the effects of *Bsal*

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150 exposure and the influence of captivity on the amphibian skin microbiome
151 could therefore not only improve the capacity for developing adequate
152 national response protocols to disease outbreaks, but also inform effective
153 captivity based measures that seek to maximise natural pathogen protection.
154

155 **Methods**

156 ***Field sampling and captivity experiment***

157 A total of 15 adult *Triturus cristatus* and 15 adult *Lissotriton vulgaris* were
158 collected from a reserve in Cambridgeshire, UK. Individual newts were
159 generally found under rocks at night and represented less than 0.1% of the
160 total estimated site population. Using a single sterile MW100 rayon tipped dry
161 swab (MWE Medical Wire, Corsham, UK), the skin microbiome was sampled
162 by swabbing the ventral and dorsal surfaces 10 times, and the fore- and
163 hindlimbs five times. Swabs were stored at -80°C until processed. Animals
164 were transferred to individual 1.6L plastic boxes containing moss collected
165 from the field site and transported to the Central Biomedical Services (CBS)
166 Unit at Imperial College London. In captivity animals were housed individually
167 under semi-natural conditions in plastic boxes containing a damp paper towel
168 substrate and a cover object. Enclosures were cleaned with Rely+On Virkon
169 (Antect International Ltd., Suffolk, UK) and animals were fed mealworms
170 (*Tenebrio molitor*) or crickets (*Acheta domesticus*) *ad libitum* twice weekly.
171 The animal room was kept on a 12 hour light/dark cycle and was maintained
172 at 16°C. At two weeks post capture animals were swabbed again to measure
173 the effects of captivity on the skin bacterial and fungal community.
174

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175 ***Bsal exposure experiment***

176 In order to compare species response to *Bsal*, experiments were designed to
177 be as similar as possible to those described in a previous study (Martel *et al.*
178 2014). *Batrachochytrium salamandrivorans* (isolated from a *Salamandra*
179 *salamandra* outbreak in the Netherlands, isolate AMFP13/1) was grown in
180 25cm^3 Nunc tissue culture flasks (Thermo Fisher Scientific, Massachusetts,
181 USA) containing mTGhL liquid media (8g tryptone, 2g gelatin hydrosylate, 4g
182 lactose, 950ml distilled water) and incubated at 15°C. Ten individuals from
183 each caudate species were randomly assigned to a treatment group and
184 exposed to 500uL of mTGhL media containing 50 x 10^4 *Bsal* zoospores. The
185 remaining five individuals from each species were assigned to a control group
186 and exposed to 500uL of mTGhL liquid media. The inoculum was pipetted
187 directly onto the dorsum of the animal. During exposure, *T. cristatus* were
188 placed individually in 0.7L plastic boxes and *L. vulgaris* were placed in sterile
189 petri dishes for 22 hours.

190

191 Animals were weighed and swabbed prior to *Bsal* infection on day 0 of the
192 experiment and then every 7 days post infection for a period of 58 days. On
193 day 58 of the experiment surviving animals were euthanized by an overdose
194 of tricaine methanesulfonate (MS222) and subsequent destruction of the brain
195 following UK Home Office animal procedure guidelines. In agreement with
196 ethical protocols, any animals exhibiting pre-defined endpoint criteria (lack of
197 righting reflex within five seconds of being inverted, persistent skin lesions
198 covering over 20% of the body or that became septic, greater than 20% loss
199 in body weight) were euthanized prior to day 58.

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200 ***Sample processing, DNA extraction and quantification of Bsal infection***

201 Genomic DNA was extracted from swabs using a bead beating protocol
202 (Boyle *et al.* 2004) and diluted 1/10 before undergoing subsequent PCR
203 based analyses. Quantification of *Bsal* infection load was done using qPCR
204 amplification following a modified published method (Boyle *et al.* 2004) that
205 included a *Bsal* specific probe (STerCVIC), forward primer (STerFC) and
206 reverse primer (STerT). Each sample was run in duplicate and with *Bsal*
207 standards of 100, 10 and 1 genomic equivalents (GE). A distilled water
208 negative control was also included. Samples were considered positive if both
209 wells gave a GE of greater than 0.1.
210

211 ***Bacterial microbiome sample processing***

212 DNA extracted from swabs was used to amplify the V4 region of the 16S
213 rRNA gene using custom barcoded primers and PCR conditions adapted from
214 a prior study (Kozich *et al.* 2013). PCR conditions consisted of a denaturing
215 step of 95°C for 15 min, followed by 28 cycles of 95°C for 20s, 50°C for 60s,
216 72°C for 60s and a final extension step of 72°C for 10 min. Each PCR
217 including a negative water control was performed in triplicate. Amplicons were
218 visualized on a 2% agarose gel and pooled yielding a final per sample volume
219 of 24µl. Pooled amplicon DNA was purified using an Ampure XP PCR
220 purification kit (Beckman Coulter, California, USA). Following purification, 1ul
221 of each combined sample was pooled into a preliminary library and the
222 concentration was determined using Qubit fluorometric quantification (Life
223 Technologies, California, USA). Amplicon quality and incidence of primer
224 dimer was assessed using an Agilent 2200 TapeStation system (Agilent

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225 Technologies, California, USA). A titration run of 300 sequencing cycles was
226 performed on a MiSeq instrument (Illumina, California, USA) to quantify the
227 number of reads yielded per sample from the preliminary library. An equimolar
228 concentration of each sample was then pooled into a final composite library
229 based on the index representation from the titration run and subsequently
230 sequenced on a 500 cycle MiSeq run with a 250 bp paired-end strategy.
231
232 ***Fungal mycobiome sample processing***
233 DNA extracted from swabs was used to amplify the ITS2 region of the fungal
234 internal transcribed spacer (ITS) using custom barcoded primers (Kozich *et al.*
235 2013) and the following PCR conditions: denaturing step of 95°C for 2 min,
236 followed by 35 cycles of 95°C for 20s, 50°C for 20s, 72°C for 5 min and a final
237 extension step of 72°C for 5 min. Each PCR plate included a negative swab
238 control and negative water control, and was performed in duplicate.
239 Amplicons were visualized on a 1.5% agarose gel and pooled yielding a final
240 per sample volume of 50µl. Pooled amplicon DNA was purified using AMPure
241 XP bead clean-up (Beckman Coulter, California, USA). Qubit fluorometric
242 quantification (Life Technologies, California, USA) was used to determine the
243 concentration of each purified sample, which were equimolar pooled to create
244 the final library sample. This pooled sample was run on an Agilent 2200
245 TapeStation system (Agilent Technologies, California, USA) to assess
246 amplicon distribution and presence of primer dimer. The sample underwent
247 300bp paired-end sequencing using v3 chemistry on an Illumina MiSeq
248 platform.
249

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250 ***Bacterial microbiome analysis***

251 Sequences were processed using MOTHUR (Schloss *et al.* 2009) following a
252 previously described method (Kozich *et al.* 2013). Paired-end reads were split
253 by sample and assembled into contigs. Sequences were quality filtered by
254 removing ambiguous base calls, removing homopolymer regions longer than
255 8 bp, and trimming reads longer than 275 bp. Duplicate sequences were
256 merged and aligned with 16S reference sequences from the SILVA small-
257 subunit rRNA sequence database (Pruesse *et al.* 2007). A pre-clustering step
258 grouped sequences differing by a maximum of 2 bp. Chimeric sequences
259 were removed using UCHIME (Edgar *et al.* 2011) as implemented in
260 MOTHUR. 16S rRNA gene sequences were clustered into groups according
261 to their taxonomy at the level of order and assigned operational taxonomic
262 units (OTUs) at a 3% dissimilarity level. Sequences were taxonomically
263 classified with an 80% bootstrap confidence threshold using a naïve Bayesian
264 classifier with a training set (version 9) made available through the Ribosomal
265 Database Project (http://rdp.cme.msu.edu) (Wang *et al.* 2007). Sequences
266 derived from chloroplasts, mitochondria, archaea, eukaryotes or unknown
267 reads were eliminated. The number of sequences per sample ranged from
268 17804 to 63367. To mitigate the effects of uneven sampling (Schloss, Gevers
269 & Westcott 2011) all samples were rarefied to 17804 sequences
270 corresponding to the size of the lowest read sample. OTUs making up less
271 than 0.01% of the total reads were removed (Bokulich *et al.* 2013).
272 Downstream analysis of OTUs was carried out using the package Phyloseq
273 (McMurdie & Holmes 2013) in R version 3.4.1 (R Development Core Team
274 2017).

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275 ***Fungal mycobiome analysis***

276 Analysis of fungal communities was performed for the wild versus captive
277 experiment only. Following sequencing, forward and reverse reads were
278 assigned to samples according to dual index combinations and were paired
279 using Paired-End reAd mergeR (PEAR) (Zhang *et al.* 2014). Paired-end reads
280 were trimmed by per-base quality score using MOTHUR (Schloss *et al.* 2009)
281 and reads shorter than 50bp or containing ambiguous base calls were
282 removed. UCHIME (Edgar *et al.* 2011) was used to identify and remove
283 chimeric sequences, and remaining sequences were clustered into
284 Operational Taxonomic Units (OTUs) based on 97% similarity using Cd-hit (Li
285 & Godzik 2006). The most abundant sequence in each OTU was used for
286 BLASTn searches against the User-friendly Nordic ITS Ectomycorrhiza
287 (UNITE) database (Koljalg *et al.* 2005). Unidentified sequences or those
288 belonging to kingdoms other than "fungi" were removed, as were fungal
289 sequences with BLASTn search result e-values $>e^{-20}$ or identity <85%. The
290 number of sequences per sample ranged from 3252 to 39493. Downstream
291 analysis of OTUs was carried out using the package Phyloseq (McMurdie &
292 Holmes 2013) in R version 3.4.1.
293

294 ***Statistical analysis***

295 To determine the effect of captivity and *Bsa*l exposure on the microbiome, we
296 calculated both alpha and beta diversity metrics using the phyloseq package
297 (McMurdie & Holmes 2013) in R version 3.4.1 (R Development Core Team
298 2017). Shannon diversity was calculated for all samples and a mixed linear
299 model (package lme4 (Bates *et al.* 2015)) was used to investigate changes in

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300 diversity in captive versus wild animals whilst taking into account repeated
301 sampling. For the exposure experiment, separate mixed linear models were
302 used for each newt species to investigate the effect of day of sampling,
303 experimental group, mass, *Bsal* infection intensity and survival on Shannon
304 diversity. P-values were calculated using the Kenward-Roger approximation
305 of degrees of freedom in the afex package (Singmann *et al.* 2017)**.** A Bray-
306 Curtis distance matrix was used to calculate beta diversity for both the
307 captivity study and *Bsal* challenge experiment. Beta diversity for both the
308 captivity study and *Bsal* challenge experiment was visualised using Detrended
309 Correspondence Analysis (DCA) plots. For the captivity study, the effects of
310 captivity, host species and their interaction on skin microbial community
311 structure was assessed using permutational multivariate analysis of variance
312 (PERMANOVA) (Anderson 2001) using the adonis function in the vegan
313 package (Oksanen *et al.* 2016). For the *Bsal* challenge experiment differences
314 in beta diversity based on treatment, *Bsal* infection status and survival were
315 investigated using PERMANOVA. Differentially abundant bacterial OTUs in
316 both wild and captive individuals of each species and for each outcome group
317 at day 28 of the infection experiment were determined using indicator analysis
318 (Dufrene & Legendre 1997) using the labdsv package (Roberts 2016). An
319 indicator score of ≥ 0.7 and *q*-value <0.05 was used as a cut-off (Becker *et al.*
320 2015; Longo & Zamudio 2017). Differences in survival among animals in the
321 *Bsal* exposure experiment was investigated using a cox proportional-hazard
322 regression model in the survival package (Therneau 2015) with mass at the
323 beginning of the experiment, species, and GE at time of death or at the end of
324 the experiment included as covariates.

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325 **Results**

326 Our analysis found rapid reductions in bacterial and fungal Shannon diversity
327 associated with the transition from the wild to captivity for both host species
328 ($p<0.0001$**,** Fig. 1a, b). Bacterial and fungal beta diversity differed in wild
329 versus captive conditions (PERMANOVA, bacteria wild-captive: Pseudo-
330 $F_{(1,56)}=66.84$, $R^2=0.505$, $p=0.001$, fungal wild-captive: Pseudo-$F_{(1,28)}=8.48$,
331 $R^2=0.217$, $p=0.001$) and a host species effect was present for bacterial
332 communities (PERMANOVA, host species: Pseudo-$F_{(1,56)}= 5.46$, $R^2=0.041$,
333 $p=0.007$, host species*wild-captive: Pseudo-$F_{(1,56)}=4.02$, $R^2=0.030$, $p=0.016$,
334 Fig. 1c-d). Interestingly, changes in alpha and beta diversity associated with
335 captivity were mirrored for bacteria and fungi demonstrating a common
336 response across microbial kingdoms. Captivity was associated with
337 compositional changes in the core microbiome of both species with reductions
338 in taxa such as *Cladosporium* and *Pseudomonas* (Fig. 1.e,f). Indicator
339 analysis of wild versus captive specimens identified 178 and 167 differentially
340 abundant bacterial OTUs in *T. cristatus* and *L. vulgaris* respectively (Fig. 2a,b;
341 SI Table 1,2). Significant differences in fungal OTU abundance associated
342 with captivity were also evident with 45 indicator OTUs for *T. cristatus* and 18
343 indicator OTUs for *L. vulgaris* (SI Table 3,4). Interestingly both host species
344 demonstrated similar indicator OTU profiles of major bacterial groups (Fig.
345 2a,b) with changes in abundance of potentially important taxa such the
346 Actinomycetales that are a major source of antimicrobial compounds (Berdy
347 2005) and *Lysobacter* (SI Table 1,2) that was previously identified as
348 inhibitory against the closely related *Batrachochytrium dendrobatidis* (Brucker
349 *et al.* 2008). In addition to the reduction in putatively protective microbes, in

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350 captive *T. cristatus* there was an increase in pathogenic fungi such as
351 *Basidiobolus ranarum* (SI Table 3) which has previously been shown to cause
352 disease in amphibians (Taylor *et al.* 1999).
353
354 *Bsal* exposure resulted in infection in 40% of *L. vulgaris* and 60% of *T.*
355 *cristatus*. Prevalence and infection intensity fluctuated throughout the
356 experiment for both species (SI Table 5, Fig. 3) with *T. cristatus* exhibiting
357 consistently higher infection intensity and prevalence than *L. vulgaris*.
358 Lesions were evident in 50% of *T. cristatus* and 75% of *L. vulgaris* that tested
359 *Bsal* positive (SI Table 5, SI Fig. 1). Of the animals that tested positive for
360 *Bsal*, 50% of *T. cristatus* died, while 25% of *L. vulgaris* died over the 58 days
361 of the experiment. Of the four *L. vulgaris* that became infected with *Bsal*, three
362 animals cleared infection, while for *T. cristatus* only one of six *Bsal* positive
363 animals cleared infection. Survival analysis showed that infection intensity but
364 not species or mass were significantly associated with mortality (hazard-
365 ratio=1.07, p=0.022, SI Table 6).
366
367 Bacterial Shannon diversity differed based on day of sampling and mass for
368 *T. cristatus* (day: $F_{(2,29.2)}$=15.75, p=0.0001, mass: $F_{(1,13.7)}$=4.68, p=0.05), but
369 did not significantly alter over the course of the experiment in *L. vulgaris* (day:
370 p=0.16, mass: p=0.96). Beta diversity differed only on day 28 based on
371 treatment for *T. cristatus* (PERMANOVA Pseudo-$F_{(1,11)}$=2.34, R^2=0.108,
372 p=0.045) and for both host species based on disease status (PERMANOVA
373 *T. cristatus* Pseudo-$F_{(1,11)}$=4.48, R^2=0.199, p=0.002, *L. vulgaris* Pseudo-
374 $F_{(1,11)}$=4.95, R^2=0.247, p=0.001, Fig. 4a, b) and survival (PERMANOVA *T.*

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375 *cristatus* Pseudo-$F_{(1,11)}$=4.59, R^2=0.204, *p*=0.006, *L. vulgaris* Pseudo-
376 $F_{(1,11)}$=2.44, R^2=0.122, *p*=0.048). In *L. vulgaris*, on day 28 two individuals
377 demonstrated microbiome perturbation, but cleared infection and returned to a
378 microbiome state that did not differ significantly from control animals by day
379 56 (Fig. 4a). Indicator analysis of microbiome samples based on control, *Bsal*
380 negative and *Bsal* positive animals from day 28 identified ten and six
381 differentially abundant bacterial operational taxonomic units (OTUs) in *L.*
382 *vulgaris* and *T. cristatus* respectively (Fig. 4c,d**,** SI Table 7). Both *L. vulgaris*
383 and *T. cristatus* exhibited different indicator taxa profiles with the exception of
384 *Stenotrophomonas* (Fig. 4e,f), which was strongly associated with *Bsal*
385 infection in both species.
386
387 **Discussion**

388 We demonstrate that both captivity and exposure to *Bsal* impact the
389 amphibian skin microbiome. The decrease in microbial Shannon diversity and
390 changes in beta diversity associated with captivity supports findings of
391 previous studies (Becker *et al.* 2014; Kueneman *et al.* 2016) and is likely
392 correlated with a reduction in environmental microbes and changes to the
393 host environment (Harrison *et al.* 2017). Importantly, both fungi and bacteria
394 exhibited similar changes in alpha and beta diversity suggesting that
395 commonalities exist in response to selection pressures across both microbial
396 kingdoms. The perturbation in microbial diversity that occurs due to the
397 transition from the wild to captivity may also change the ecological dynamics
398 and functional capacity of the skin by altering host-microbe interactions in
399 such a way that biases host- or microbe-mediated control of the microbiome

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400 (Foster *et al.* 2017). Specifically, the divergence in microbiome structure of
401 different host species in captivity may be indicative of stronger host-mediated
402 control, with host species differing in their selection of microbes in a common
403 captive environment. This in turn may lead to decline of some potentially
404 pathogenic taxa as signified by the reduction in OTUs belonging to the
405 Chlamydiales (Fig. 2a,b) which have previously been associated with
406 amphibian epizootics (Reed *et al.* 2000). Conversely, the reduced microbial
407 diversity associated with captivity in both host species may also be a
408 consequence of greater microbe-mediated control with diminished ecological
409 resistance of the host skin microbiome rendering it more susceptible to
410 potentially harmful invaders that may subsequently dominate the microbial
411 community (Piovia-Scott *et al.* 2017). This hypothesis is supported by a
412 reduction of putatively beneficial bacterial groups in captivity such as the
413 Actinomycetales (Fig. 2a,b) which are well known for producing antimicrobial
414 compounds (Berdy 2005). In addition, taxa belonging to the genus *Lysobacter*
415 that have previously been associated with inhibition of other pathogenic
416 chytrids (Brucker *et al.* 2008; Bates *et al.* 2018) showed a reduction in
417 abundance in captivity. Captivity was also associated with an increase in
418 abundance of the amphibian fungal pathogen *Basidiobolus ranarum* for both
419 host species (though only significant in *T. cristatus*) further suggesting captive
420 conditions may favour host-pathogen interactions as protective aspects of
421 microbial diversity are lost. While the overall reduction in microbial diversity
422 associated with captivity may impact host resistance to disease, the additional
423 perturbation of microbial community structure may lead to dysbiotic effects
424 (Zaneveld, McMinds & Thurber 2017). These findings suggest that captivity

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425 induced changes in the microbiome may reduce host capability to evade
426 pathogens and potentially predispose such individuals towards adverse health
427 outcomes. Importantly, this is the first study of its kind to measure the effect of
428 captivity on fungal communities, which despite being overlooked relative to
429 bacteria, have been shown in some cases to confer higher rates of chytrid
430 inhibition (Kearns *et al.* 2017). In light of the major perturbation of fungal
431 communities, it is imperative that future studies utilise a more holistic view of
432 the microbiome to include kingdoms other than bacteria. Overall, our results
433 highlight that host microbial ecology should carefully be considered when
434 transferring animals from the wild to captivity to reduce possible microbiome
435 disturbance, prevent proliferation of opportunistic pathogens and preserve
436 microbes that are beneficial to host health. For *ex-situ* conservation
437 programmes that aim to rescue or re-establish wild amphibian populations
438 using captive-bred stock, the implications are complex and suggest that
439 maintenance or ‘rewilding’ of the skin microbiome would be an essential
440 aspect before reintroduction.
441
442 Exposure to *Bsal* resulted in mortality in both *L. vulgaris* and *T. cristatus*.
443 While mortality was higher in *T. cristatus*, this was not statistically significant
444 when compared to *L. vulgaris*. The intensity of infection was found to be
445 higher in *T. cristatus,* which had a maximum GE of 338 compared to a
446 maximum GE of 195 for *L. vulgaris*. These data, while preliminary, suggest
447 that *T. cristatus* may be more susceptible to *Bsal* infection than *L. vulgaris*.
448 Further, the greater zoospore burdens that *T. cristatus* harbour, coupled with
449 their larger size and rates of zoospore shedding, suggest that they will be

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450 more important drivers of outbreak dynamics than *L. vulgaris*. Interestingly,
451 our findings counter those of a previous study that found 100% mortality in *T.*
452 *cristatus* and a species effect on survival (Martel *et al.* 2014). The increased
453 survival of *T. cristatus* here compared to the prior study (Martel *et al.* 2014)
454 may be explained by a range of factors. In particular, the studies used
455 animals collected from different countries (the UK versus the Netherlands)
456 and there will likely be differences in host genetics and immunity. In addition,
457 the initial microbiome of the animals will almost certainly differ which may
458 have an impact on the subsequent host microbial response to infection.
459 These findings highlight the importance of investigating a geographically
460 diverse range of hosts from the same species when conducting species level
461 risks assessments of emerging pathogens. While this additional confirmation
462 of *Bsal* induced death in *T. cristatus* warrants serious concern, the higher
463 survivorship shown here may suggest a more optimistic outlook in the event
464 of a wild *Bsal* outbreak for this species than previously predicted.
465
466 Bacterial community structure of individuals in the experiment differed
467 significantly for both species based on infection status and survival. These
468 findings are consistent with results of prior studies on *Bd* (Jani & Briggs 2014),
469 suggesting that infection by *Bsal* and *Bd* are both associated with microbiome
470 disruption. Two *L. vulgaris* individuals tested positive for *Bsal* and
471 demonstrated significant microbiome perturbation that may be indicative of
472 dysbiosis on day 28 of the experiment, but subsequently cleared infection by
473 day 56. In both cases *Bsal* clearance was associated with a change in
474 microbiome profile that was similar to that of control animals. Determining

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475 what underpins this recovery in *L. vulgaris*, whether driven by host factors
476 such as secretion of antimicrobial peptides or *Bsal* inhibition by microbes, will
477 be vital in determining the key parameters underpinning infection outcome.
478 Indicator analysis revealed that the bacterial taxa associated with disease
479 state were different based on host species with the exception of one OTU
480 classified as *Stenotrophomonas.* Interestingly, a prior study demonstrated
481 *Stenotrophomonas* as inhibitory to *Bsal* and several *Bd* genotypes (Muletz-
482 Wolz *et al.* 2017). Meanwhile, another study identified an isolate of
483 *Stenotrophomonas* as inhibitory to *Bd in-vitro*, however when applied as a
484 probiotic on an amphibian host exposed to *Bd in-vivo*, mortality was higher
485 than animals that were exposed to only *Bd* (Becker et al. 2015). Ultimately,
486 determining whether *Stenotrophomonas* is associated with microbe mediated
487 defence or synergistic growth with *Bsal* will require further experiments that
488 take into account microbial function. While both newt species shared few taxa
489 that were associated with disease outcome, the common perturbation in beta
490 diversity associated with death in both *L. vulgaris* and *T. cristatus* may
491 suggest that *Bsal* survival is not linked to specific taxa, but rather perturbation
492 of a stable host microbiome resulting in an overall negative impact on health
493 (Zaneveld, McMinds & Thurber 2017). Further support to this hypothesis is
494 given by the return to a stable microbiome state in animals that cleared
495 infection.
496
497 Our results build on the work of prior studies by demonstrating significant and
498 potentially negative effects of captivity on the amphibian skin microbiome. In
499 addition, we provide vital insight into the disease process of *Bsal* by

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500 demonstrating a close link between infection outcome and microbiome
501 structure. Overall, our findings demonstrate that it is vital for host microbial
502 ecology to be considered in future *Bsal* studies and captive based
503 conservation programmes.
504

505 **Data accessibility**

506 Sequence data have been deposited on the BioProject database under
507 accession code PRJNA430498. All other data are available upon request from
508 the authors.
509

510 **References**

511 Anderson, M.J. (2001) A new method for non-parametric multivariate analysis
512 of variance. *Austral Ecology,* 26**,** 32-46.
513 Antwis, R.E. & Harrison, X.A. (2018) Probiotic consortia are not uniformly
514 effective against different amphibian chytrid pathogen isolates.
515 *Molecular Ecology,* 27**,** 577-589.
516 Antwis, R.E., Preziosi, R.F., Harrison, X.A. & Garner, T.W. (2015) Amphibian
517 symbiotic bacteria do not show a universal ability to inhibit growth of
518 the global panzootic lineage of *Batrachochytrium dendrobatidis*.
519 *Applied and Environmental Microbiology,* 81**,** 3706-3711.
520 Bataille, A., Lee-Cruz, L., Tripathi, B., Kim, H. & Waldman, B. (2016)
521 Microbiome variation across amphibian skin regions: Implications for
522 chytridiomycosis mitigation efforts. *Microbial Ecology,* 71**,** 221-232.
523 Bates, D., Machler, M., Bolker, B.M. & Walker, S.C. (2015) Fitting linear
524 mixed-effects models using lme4. *Journal of Statistical Software,* 67**,** 1-
525 48.
526 Bates, K.A., Clare, F.C., O'Hanlon, S., Bosch, J., Brookes, L., Hopkins, K., . . .
527 Harrison, X.A. (2018) Amphibian chytridiomycosis outbreak dynamics
528 are linked with host skin bacterial community structure. *Nature*
529 *Communications,* 9**,** 693.
530 Becker, M.H., Richards-Zawacki, C.L., Gratwicke, B. & Belden, L.K. (2014)
531 The effect of captivity on the cutaneous bacterial community of the
532 critically endangered panamanian golden frog (*Atelopus zeteki*).
533 *Biological Conservation,* 176**,** 199-206.
534 Becker, M.H., Walke, J.B., Cikanek, S., Savage, A.E., Mattheus, N., Santiago,
535 C.N., . . . Gratwicke, B. (2015) Composition of symbiotic bacteria
536 predicts survival in panamanian golden frogs infected with a lethal
537 fungus. *Proceedings of the Royal Society B: Biological Sciences,* 282**,**
538 20142881.

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539 Berdy, J. (2005) Bioactive microbial metabolites - a personal view. *Journal of*
540 *Antibiotics,* 58**,** 1-26.
541 Berger, L., Speare, R., Daszak, P., Green, D.E., Cunningham, A.A., Goggin,
542 C.L., . . . Parkes, H. (1998) Chytridiomycosis causes amphibian
543 mortality associated with population declines in the rain forests of
544 australia and central america. *Proceedings of the National Academy of*
545 *Sciences of the United States of America,* 95**,** 9031-9036.
546 Bletz, M.C., Loudon, A.H., Becker, M.H., Bell, S.C., Woodhams, D.C.,
547 Minbiole, K.P.C. & Harris, R.N. (2013) Mitigating amphibian
548 chytridiomycosis with bioaugmentation: Characteristics of effective
549 probiotics and strategies for their selection and use. *Ecology Letters,*
550 16**,** 807-820.
551 Blooi, M., Martel, A., Haesebrouck, F., Vercammen, F., Bonte, D. & Pasmans,
552 F. (2015a) Treatment of urodelans based on temperature dependent
553 infection dynamics of *Batrachochytrium salamandrivorans*. *Scientific*
554 *Reports,* 5**,** 8037.
555 Blooi, M., Pasmans, F., Rouffaer, L., Haesebrouck, F., Vercammen, F. &
556 Martel, A. (2015b) Successful treatment of *Batrachochytrium*
557 *salamandrivorans* infections in salamanders requires synergy between
558 voriconazole, polymyxin E and temperature. *Scientific Reports,* 5**,**
559 11788.
560 Bokulich, N.A., Subramanian, S., Faith, J.J., Gevers, D., Gordon, J.I., Knight,
561 R., . . . Caporaso, J.G. (2013) Quality-filtering vastly improves diversity
562 estimates from illumina amplicon sequencing. *Nature Methods,* 10**,** 57-
563 U11.
564 Boyle, D.G., Boyle, D.B., Olsen, V., Morgan, J.A.T. & Hyatt, A.D. (2004) Rapid
565 quantitative detection of chytridiomycosis (*Batrachochytrium*
566 *dendrobatidis*) in amphibian samples using real-time taqman pcr assay.
567 *Diseases of Aquatic Organisms,* 60**,** 141-148.
568 Brucker, R.M., Baylor, C.M., Walters, R.L., Lauer, A., Harris, R.N. & Minbiole,
569 K.P. (2008) The identification of 2,4-diacetylphloroglucinol as an
570 antifungal metabolite produced by cutaneous bacteria of the
571 salamander *Plethodon cinereus*. *Journal of Chemical Ecology,* 34**,** 39-
572 43.
573 Buffie, C.G. & Pamer, E.G. (2013) Microbiota-mediated colonization
574 resistance against intestinal pathogens. *Nature Reviews. Immunology,*
575 13**,** 790-801.
576 Croswell, A., Amir, E., Teggatz, P., Barman, M. & Salzman, N.H. (2009)
577 Prolonged impact of antibiotics on intestinal microbial ecology and
578 susceptibility to enteric *Salmonella* infection. *Infection and Immunity,*
579 77**,** 2741-2753.
580 Cunningham, A.A., Beckmann, K., Perkins, M., Fitzpatrick, L., Cromie, R.,
581 Redbond, J., . . . Fisher, M.C. (2015) Emerging disease in UK
582 amphibians. *The Veterinary Record,* 176**,** 468.
583 Dufrene, M. & Legendre, P. (1997) Species assemblages and indicator
584 species: The need for a flexible asymmetrical approach. *Ecological*
585 *Monographs,* 67**,** 345-366.
586 Edgar, P.W., Griffiths, R.A. & Foster, J.P. (2005) Evaluation of translocation
587 as a tool for mitigating development threats to great crested newts

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588 (*Triturus cristatus*) in England, 1990–2001. *Biological Conservation,*
589 122**,** 45-52.
590 Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C. & Knight, R. (2011)
591 Uchime improves sensitivity and speed of chimera detection.
592 *Bioinformatics,* 27**,** 2194-2200.
593 Farrer, R.A., Martel, A., Verbrugghe, E., Abouelleil, A., Ducatelle, R.,
594 Longcore, J.E., . . . Cuomo, C.A. (2017) Genomic innovations linked to
595 infection strategies across emerging pathogenic chytrid fungi. *Nature*
596 *Communications,* 8**,** 14742.
597 Fisher, M.C., Garner, T.W. & Walker, S.F. (2009) Global emergence of
598 *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in
599 space, time, and host. *Annual Review of Microbiology,* 63**,** 291-310.
600 Foster, K.R., Schluter, J., Coyte, K.Z. & Rakoff-Nahoum, S. (2017) The
601 evolution of the host microbiome as an ecosystem on a leash. *Nature,*
602 548**,** 43-51.
603 Gascon, C., Collins, J.P., Moore, R.D., Church, D.R., McKay, J.E. &
604 Mendelson, J.R. III (eds) (2007) *Amphibian conservation action plan :*
605 *Proceedings IUCN/SSC amphibian conservation summit 2005*. World
606 Conservation Union (IUCN), Gland, Switzerland.
607 Harris, R.N., Brucker, R.M., Walke, J.B., Becker, M.H., Schwantes, C.R.,
608 Flaherty, D.C., . . . Minbiole, K.P. (2009) Skin microbes on frogs
609 prevent morbidity and mortality caused by a lethal skin fungus. *The*
610 *ISME journal,* 3**,** 818-824.
611 Harrison, X.A., Price, S.J., Hopkins, K., Leung, W.T.M., Sergeant, C. &
612 Garner, T. (2017) Host microbiome richness predicts resistance to
613 disturbance by pathogenic infection in a vertebrate host. *bioRxiv*.
614 Jani, A.J. & Briggs, C.J. (2014) The pathogen *Batrachochytrium dendrobatidis*
615 disturbs the frog skin microbiome during a natural epidemic and
616 experimental infection. *Proceedings of the National Academy of*
617 *Sciences of the United States of America,* 111**,** E5049-5058.
618 Kamada, N., Kim, Y.G., Sham, H.P., Vallance, B.A., Puente, J.L., Martens,
619 E.C. & Nunez, G. (2012) Regulated virulence controls the ability of a
620 pathogen to compete with the gut microbiota. *Science,* 336**,** 1325-
621 1329.
622 Kearns, P.J., Fischer, S., Fernandez-Beaskoetxea, S., Gabor, C.R., Bosch, J.,
623 Bowen, J.L., . . . Woodhams, D.C. (2017) Fight fungi with fungi:
624 Antifungal properties of the amphibian mycobiome. *Frontiers in*
625 *Microbiology,* 8**,** 2494.
626 Koljalg, U., Larsson, K.H., Abarenkov, K., Nilsson, R.H., Alexander, I.J.,
627 Eberhardt, U., . . . Ursing, B.M. (2005) Unite: A database providing
628 web-based methods for the molecular identification of ectomycorrhizal
629 fungi. *New Phytologist,* 166**,** 1063-1068.
630 Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K. & Schloss, P.D.
631 (2013) Development of a dual-index sequencing strategy and curation
632 pipeline for analyzing amplicon sequence data on the miseq illumina
633 sequencing platform. *Applied and Environmental Microbiology,* 79**,**
634 5112-5120.
635 Kueneman, J.G., Woodhams, D.C., Harris, R., Archer, H.M., Knight, R. &
636 McKenzie, V.J. (2016) Probiotic treatment restores protection against

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637 lethal fungal infection lost during amphibian captivity. *Proceedings of*
638 *the Royal Society B: Biological Sciences,* 283**,** 20161553.
639 Lauer, A., Simon, M.A., Banning, J.L., Andre, E., Duncan, K. & Harris, R.N.
640 (2007) Common cutaneous bacteria from the eastern red-backed
641 salamander can inhibit pathogenic fungi. *Copeia,* 2007**,** 630-640.
642 Li, W.Z. & Godzik, A. (2006) Cd-hit: A fast program for clustering and
643 comparing large sets of protein or nucleotide sequences.
644 *Bioinformatics,* 22**,** 1658-1659.
645 Longo, A.V. & Zamudio, K.R. (2017) Temperature variation, bacterial diversity
646 and fungal infection dynamics in the amphibian skin. *Molecular*
647 *Ecology,* 26**,** 4787-4797.
648 Loudon, A.H., Woodhams, D.C., Parfrey, L.W., Archer, H., Knight, R.,
649 McKenzie, V. & Harris, R.N. (2014) Microbial community dynamics and
650 effect of environmental microbial reservoirs on red-backed
651 salamanders (*Plethodon cinereus*). *The ISME journal,* 8**,** 830-840.
652 Martel, A., Blooi, M., Adriaensen, C., Van Rooij, P., Beukema, W., Fisher,
653 M.C., . . . Pasmans, F. (2014) Recent introduction of a chytrid fungus
654 endangers western palearctic salamanders. *Science,* 346**,** 630-631.
655 Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher,
656 M.C., . . . Pasmans, F. (2013) *Batrachochytrium salamandrivorans* sp
657 nov causes lethal chytridiomycosis in amphibians. *Proceedings of the*
658 *National Academy of Sciences of the United States of America,* 110**,**
659 15325-15329.
660 McMurdie, P.J. & Holmes, S. (2013) Phyloseq: An r package for reproducible
661 interactive analysis and graphics of microbiome census data. *PLoS*
662 *One,* 8**,** e61217.
663 Mendelson, J.R., Lips, K.R., Gagliardo, R.W., Rabb, G.B., Collins, J.P.,
664 Diffendorfer, J.E., . . . Brodie, E.D. (2006) Biodiversity - confronting
665 amphibian declines and extinctions. *Science,* 313**,** 48-48.
666 Muletz-Wolz, C.R., Almario, J.G., Barnett, S.E., DiRenzo, G.V., Martel, A.,
667 Pasmans, F., . . . Lips, K.R. (2017) Inhibition of fungal pathogens
668 across genotypes and temperatures by amphibian skin bacteria.
669 *Frontiers in Microbiology,* 8**,** 1551.
670 O'Hanlon, S.J., Rieux, A., Farrer, R.A., Rosa, G.M., Waldman, B., Bataille, A.,
671 . . . Fisher, M.C. (2018) Recent asian origin of chytrid fungi causing
672 global amphibian declines. *Science,* 360**,** 621-627.
673 Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara,
674 R.B., . . . Wagner, H. (2016) Vegan: Community ecology package. *R*
675 *package version 2.3-4*.
676 Piovia-Scott, J., Rejmanek, D., Woodhams, D.C., Worth, S.J., Kenny, H.,
677 McKenzie, V., . . . Foley, J.E. (2017) Greater species richness of
678 bacterial skin symbionts better suppresses the amphibian fungal
679 pathogen *Batrachochytrium dendrobatidis*. *Microbial Ecology,* 74**,** 217-
680 226.
681 Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J. &
682 Glockner, F.O. (2007) Silva: A comprehensive online resource for
683 quality checked and aligned ribosomal rna sequence data compatible
684 with arb. *Nucleic Acids Research,* 35**,** 7188-7196.

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685 R Development Core Team (2017) *R: A language and environment for*
686 *statistical computing.* R Foundation for Statistical Computing, Vienna,
687 Austria.
688 Reed, K.D., Ruth, G.R., Meyer, J.A. & Shukla, S.K. (2000) *Chlamydia*
689 *pneumoniae* infection in a breeding colony of african clawed frogs
690 (*Xenopus tropicalis*). *Emerging Infectious Diseases,* 6**,** 196-199.
691 Roberts, D.W. (2016) Labdsv: Ordination and multivariate analysis for
692 ecology. *R package version 1.8.0*
693 Sabino-Pinto, J., Bletz, M.C., Islam, M.M., Shimizu, N., Bhuju, S., Geffers, R.,
694 . . . Vences, M. (2016) Composition of the cutaneous bacterial
695 community in japanese amphibians: Effects of captivity, host species,
696 and body region. *Microbial Ecology,* 72**,** 460-469.
697 Schloss, P.D., Gevers, D. & Westcott, S.L. (2011) Reducing the effects of pcr
698 amplification and sequencing artifacts on 16s rrna-based studies. *PLoS*
699 *One,* 6**,** e27310.
700 Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister,
701 E.B., . . . Weber, C.F. (2009) Introducing mothur: Open-source,
702 platform-independent, community-supported software for describing
703 and comparing microbial communities. *Applied and Environmental*
704 *Microbiology,* 75**,** 7537-7541.
705 Schmidt, B.R., Bozzuto, C., Lotters, S. & Steinfartz, S. (2017) Dynamics of
706 host populations affected by the emerging fungal pathogen
707 *Batrachochytrium salamandrivorans*. *Royal Society Open Science,* 4**,**
708 160801.
709 Singmann, H., Bolker, B., Westfall, J. & Aust, F. (2017) Afex: Analysis of
710 factorial experiments. *R package version 0.18.0*.
711 Stacy, A., Fleming, D., Lamont, R.J., Rumbaugh, K.P. & Whiteley, M. (2016)
712 A commensal bacterium promotes virulence of an opportunistic
713 pathogen via cross-respiration. *MBio,* 7**,** e00782-00716.
714 Taylor, S.K., Williams, E.S., Thorne, E.T., Mills, K.W., Withers, D.I. & Pier,
715 A.C. (1999) Causes of mortality of the wyoming toad. *Journal of*
716 *Wildlife Diseases,* 35**,** 49-57.
717 Therneau, T.M. (2015) A package for survival analysis in s. *R package*
718 *version 2.38*.
719 Wang, Q., Garrity, G.M., Tiedje, J.M. & Cole, J.R. (2007) Naive bayesian
720 classifier for rapid assignment of rrna sequences into the new bacterial
721 taxonomy. *Applied and Environmental Microbiology,* 73**,** 5261-5267.
722 Woodhams, D.C., LaBumbard, B.C., Barnhart, K.L., Becker, M.H., Bletz,
723 M.C., Escobar, L.A., . . . Minbiole, K.P.C. (2017) Prodigiosin, violacein,
724 and volatile organic compounds produced by widespread cutaneous
725 bacteria of amphibians can inhibit two *Batrachochytrium* fungal
726 pathogens. *Microbial Ecology,* 75**,** 1049-1062.
727 Yap, T.A., Koo, M.S., Ambrose, R.F., Wake, D.B. & Vredenburg, V.T. (2015)
728 Averting a north american biodiversity crisis. *Science,* 349**,** 481-482.
729 Zaneveld, J.R., McMinds, R. & Thurber, R.V. (2017) Stress and stability:
730 Applying the Anna Karenina principle to animal microbiomes. *Nature*
731 *Microbiology,* 2**,** 17121.
732 Zhang, J.J., Kobert, K., Flouri, T. & Stamatakis, A. (2014) Pear: A fast and
733 accurate illumina paired-end read merger. *Bioinformatics,* 30**,** 614-620.
734

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735 **Acknowledgements**

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743

744 **Author contributions**

745

746 K.A.B. and V.L.M. conducted field surveys and animal experiments. K.A.B.,
747 V.L.M., K.H., X.A.H., and J.M.G.S. performed data processing and analysis.
748 K.A.B., M.C.F., S.P., X.A.H., V.L.M. and J.M.G.S. wrote the manuscript.

749

750 **Competing interests**

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752 The authors declare no competing financial interests.

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754 **Ethic statement**

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756 Animal work was conducted under UK Home Office Project Licence PPL
757 70/8402 held by Matthew Fisher and was reviewed by the Imperial College
758 London Animal Welfare Ethical Review Board for approval. The experiment
759 was carried out in accordance with The Animals (Scientific Procedures) Act of

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760 1986 Directive 2010/63/EU and followed all of the Codes of Practice which
761 reinforce this law, including all elements of housing, care and euthanasia.
762 Animals were collected in the wild under Natural England Licence 2015-
763 15771-SCI-SCI and following ethical review by the board of Froglife.
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Figures

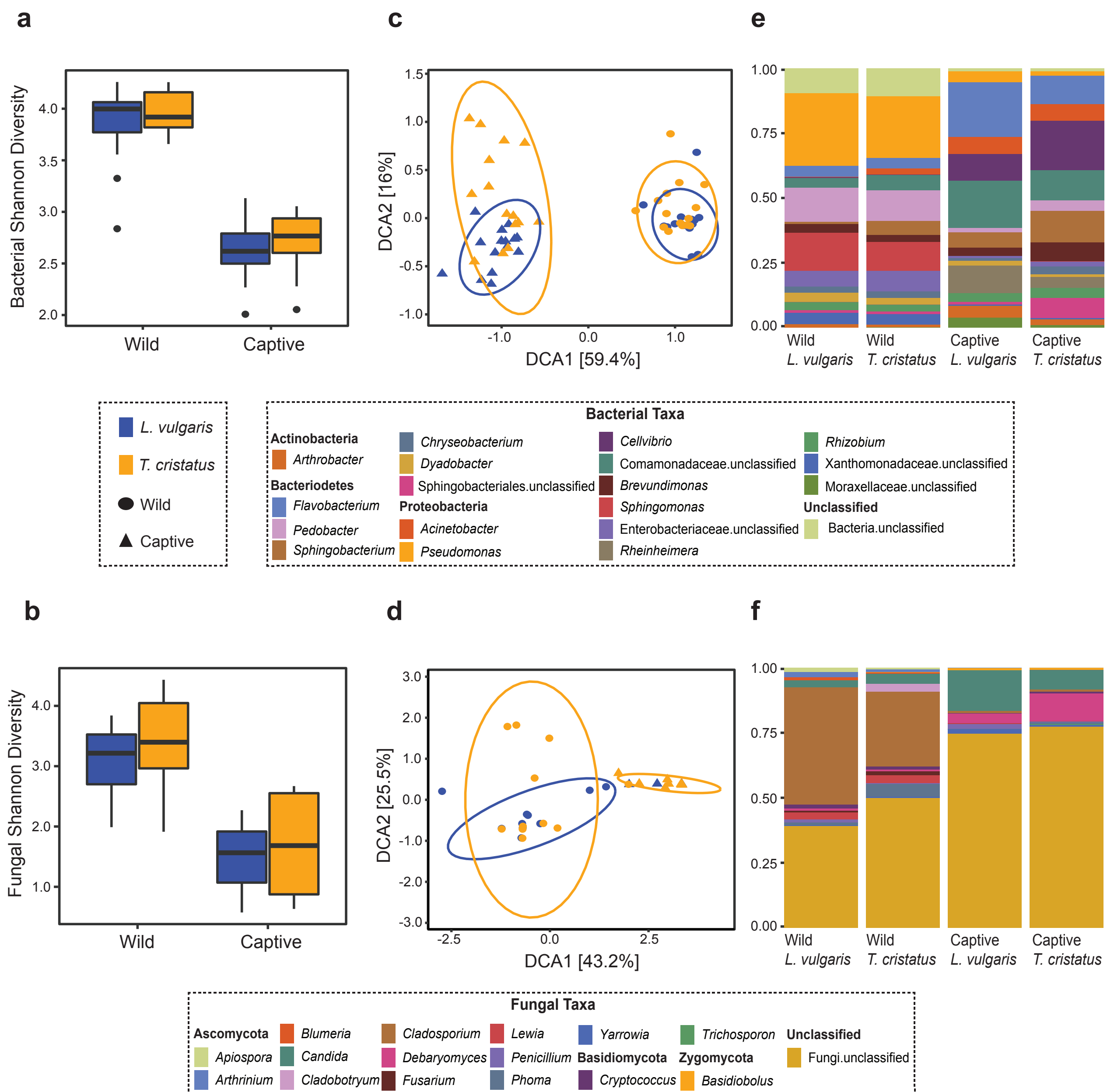

Figure 1. Boxplots displaying Shannon diversity in wild and captive *Lissotriton vulgaris* and *Triturus cristatus* for **(a)** bacteria **(b)** fungi. Detrended correspondence analysis (DCA) plots of beta diversity in wild and captive *L. vulgaris* and *T. cristatus* for **(c)** bacteria **(d)** fungi. Ellipses indicate 95% confidence intervals. Where ellipses are absent, insufficient samples were present. Stacked bar plots of **(d)** bacterial taxa with relative abundance > 1% and **(e)** fungal taxa with relative abundance > 0.5%.

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Figure 2. Heatmap of normalised relative abundance of bacterial indicator OTUs for wild and captive animals labelled by order for **(a)** *T. cristatus* **(b)** *L. vulgaris*.

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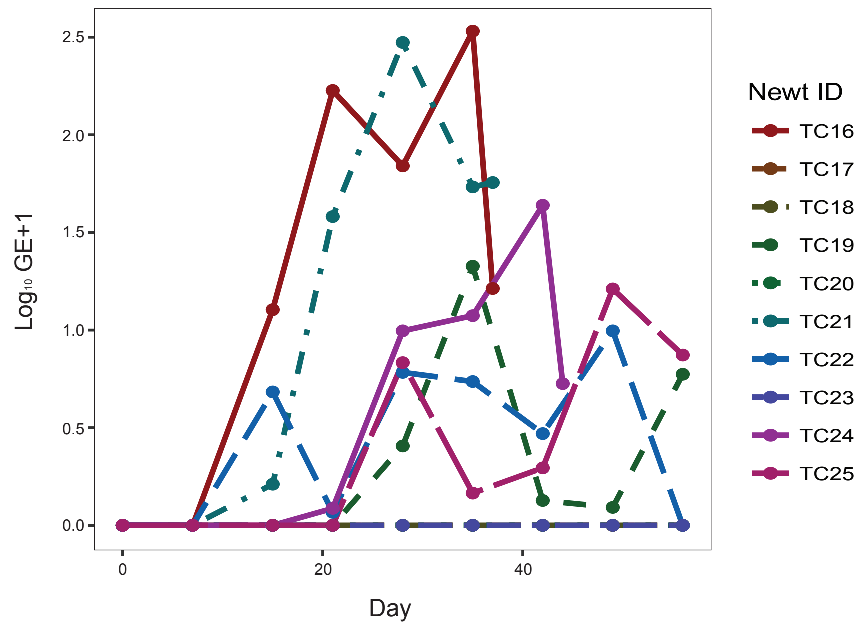

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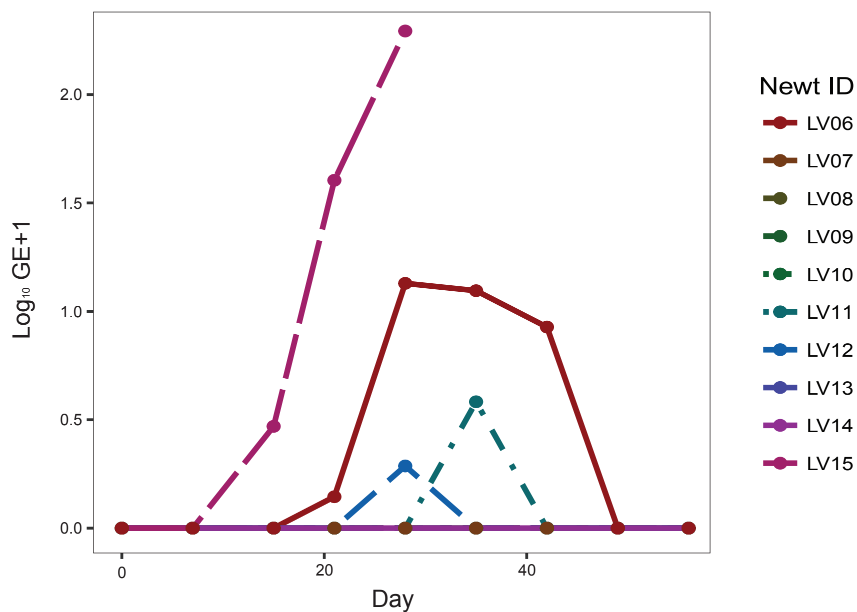

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799 **Figure 3.** Plot of infection intensity for *Bsal* exposed animals for **(a)** *T.*

800 *cristatus* **(b)** *L. vulgaris*.

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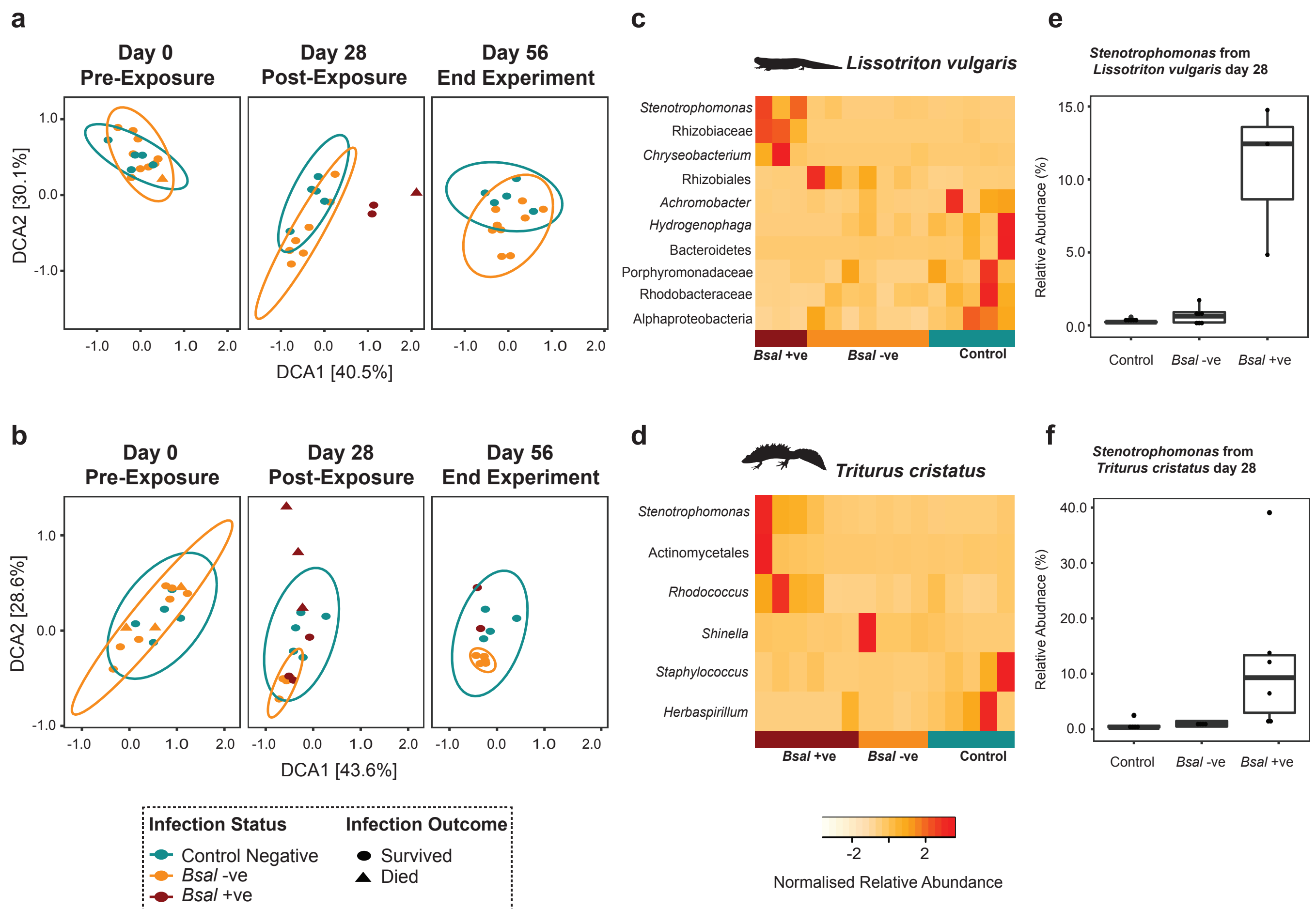

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805 **Figure 4.** Detrended correspondence analysis (DCA) plots displaying

806 temporal variation in bacterial beta diversity in **(a)** *L. vulgaris* **(b)** *T. cristatus*.

807 Heatmap of normalised relative abundance of top bacterial indicator taxa

808 associated with different disease outcomes in **(c)** *L. vulgaris* **(d)** *T. cristatus*.

809 Boxplot of OTU016 *Stenotrophomonas* identified as an indicator OTU for *Bsal*

810 infection in both **(e)** *L. vulgaris* and **(f)** *T. cristatus*.