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1 **Multiple generations of antibiotic exposure and isolation influence host fitness and the**  
2 **microbiome in a model zooplankton species**

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4

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14

15

## 16 **Abstract**

### 17 **Background**

18 Chronic antibiotic exposure impacts host health through changes to the microbiome, increasing  
19 disease risk and reducing the functional repertoire of community members. The detrimental  
20 effects of antibiotic perturbation on microbiome structure and function after one host  
21 generation of exposure have been well-studied. However, much less is understood about the  
22 multigenerational effects of antibiotic exposure and how the microbiome may recover across  
23 host generations.

### 24 **Results**

25 In this study, we examined microbiome composition and host fitness across five generations of  
26 exposure to a suite of three antibiotics in the model zooplankton host *Daphnia magna*. By  
27 utilizing a split-brood design where half of the offspring from antibiotic-exposed parents were  
28 allowed to recover and half were maintained in antibiotics, we aimed to examine recovery and  
29 resilience of the microbiome. Unexpectedly, we discovered that experimental isolation of single  
30 host individuals across generations also exerted a strong effect on microbiome composition,  
31 with composition becoming less diverse over generations regardless of treatment.

32 Simultaneously, *Daphnia magna* body size and cumulative reproduction increased across  
33 generations while survival decreased. Though antibiotics did cause substantial changes to  
34 microbiome composition, the microbiome generally became similar to the no antibiotic control  
35 treatment within one generation of recovery no matter how many prior generations were  
36 spent in antibiotics.

### 37 **Conclusions**

38 Contrary to results found in vertebrate systems, *Daphnia magna* microbiome composition  
39 recovers quickly after antibiotic exposure. However, our results suggest that the isolation of  
40 individual hosts leads to the stochastic extinction of rare taxa in the microbiome, indicating that  
41 these taxa are likely maintained via transmission in host populations rather than intrinsic  
42 mechanisms. This may explain the intriguing result that microbiome diversity loss increased  
43 host fitness.

44

45 Keywords: Antibiotics, Recovery, Invertebrate microbiome, *Daphnia*-microbiota interactions,  
46 Transmission

47

## 48 **Background**

49 Antibiotic exposure can impact host health by changing microbiome abundance and  
50 composition[1–4]. Acute exposure causes rapid[5,6] and sometimes permanent [7] shifts in  
51 microbiome composition, both of which have been associated with increased pathogen  
52 susceptibility[8,9], dramatic changes in host life history [10], and prevalent disease states like  
53 obesity and selection for antibiotic-resistant bacteria during infection[11–13]. In addition to  
54 altering composition, antibiotic exposure reduces the absolute abundance of taxa in the  
55 microbiota, which coincides with increased host mortality or changes in immunity[14,15].  
56 Metabolic functions of the microbiota are lost or altered[16,17]; for example, the microbiota of  
57 mice exposed to antibiotics shifted to produce more carbohydrates and bile acids[18]. In turn,  
58 changes to the microbiota caused by antibiotic exposure may affect the fitness of antibiotic-  
59 exposed hosts' offspring, primarily through affecting transmission of functionally important

60 taxa in the microbiota. While antibiotic effects on microbiome composition and host fitness  
61 after a single generation of exposure are well-documented, far less is understood about the  
62 multigenerational impacts of antibiotic exposure. Because chronic antibiotic exposure across  
63 multiple host generations is becoming increasingly common due to antibiotic use in agriculture  
64 and subsequent environmental contamination[19,20], it is essential to investigate the potential  
65 impacts of antibiotics across generations of exposure.

66

67 Members of the microbiota are acquired either directly from parents[21] (i.e., vertical  
68 transmission) or from environmental microbes primarily shed by conspecifics[22] (i.e.,  
69 horizontal transmission). For environmentally transmitted microbes, acquisition by naïve hosts  
70 is dependent on the abundance of microbes in the local environment, which is affected both by  
71 the number of hosts and the rate of microbial shedding from these hosts [23,24]. Because  
72 microbial abundance is reduced in antibiotic-exposed hosts, shedding rate can be reduced[25],  
73 potentially lowering naïve hosts' probability of encountering functionally important taxa.  
74 Moreover, shed microbes may encounter environmental antibiotics, which can further reduce  
75 transmission rates. Finally, microbes may struggle to successfully colonize naïve hosts that have  
76 been exposed to antibiotics[26]. Together, these findings suggest that antibiotic exposure may  
77 disrupt the conservation of functionally important taxa in host species [8], potentially having  
78 long-lasting effects on the evolutionary trajectory of the microbiota. However, understanding  
79 these long-term impacts can be difficult when studying complex microbiota comprised of  
80 hundreds or thousands of taxa[27] due to the complexity of community responses to antibiotics  
81 and other stressors.

82  
83 We investigated the impacts of antibiotics on host fitness and microbiome composition across  
84 multiple generations in the freshwater zooplankton *Daphnia magna*. *Daphnia magna* has  
85 served as a bioindicator species for aquatic ecosystems[28] and is commonly used as a model  
86 system for ecotoxicology research[29]. Recently, *D. magna* has emerged as a system for  
87 microbiome research due to its fast, clonal reproduction and its relatively simple  
88 microbiota[30]. Multiple studies have found that taxa in the *D. magna* microbiota influence  
89 host fitness, primarily through supporting reproduction and growth[30–32], tolerance to  
90 toxins[33], and nutrient provisioning[34]. Environmental factors, including bacterioplankton  
91 composition[35], influence the *Daphnia magna* microbiota[30,36–41]. As antibiotics are  
92 considered major environmental contaminants[20], aquatic organisms are chronically exposed  
93 to antibiotics. Across multiple generations of exposure, it may become impossible to recover  
94 lost microbiome functions and taxa, potentially leading to permanent loss of fitness.

95  
96 Here we used a split-brood experimental design where offspring of antibiotic-treated *D. magna*  
97 were either maintained with antibiotics or moved to antibiotic-free conditions and allowed to  
98 recover. This design allowed us to ask several key questions. We asked what the effects were of  
99 multiple generations of antibiotic exposure on host fitness and the microbiota, hypothesizing  
100 that *Daphnia magna* continuously exposed to antibiotics for multiple generations would  
101 experience continuous reductions in all measured life history metrics (growth, survival, and  
102 reproduction) and that the microbiota of these hosts would become significantly less diverse  
103 across generations of exposure. We also asked whether the offspring of antibiotic-treated hosts

104 could recover, both in terms of microbiome diversity and host life-history metrics, and how that  
105 recovery was affected by the number of previous generations spent in antibiotics. Here, we  
106 hypothesized that progressive loss of horizontally transmitted taxa would make recovery more  
107 difficult with more generations of previous exposure.

108  
109 Our findings show that *Daphnia magna* are able to recover a microbiome composition similar  
110 to the controls within one generation of recovery regardless of the number of generations  
111 spent in antibiotics, contrary to our hypothesis that detrimental fitness effects would  
112 compound across generations of exposure. Because of our experimental design, we were also  
113 able to investigate the effects of isolation on fitness and microbiome composition: juvenile  
114 *Daphnia magna* were only exposed to microbes shed into the environment by their parents for  
115 a short time period, and then were raised individually in isolation. This corresponded with a  
116 continuous loss of microbiome diversity across generations and increases in *Daphnia magna*  
117 body size and cumulative reproduction, indicating a surprising potential link between the loss of  
118 rare taxa in the microbiome and increased host fitness.

119

## 120 **Methods**

### 121 *Daphnia magna* culturing

122 We maintained cultures of *Daphnia magna* (genotype 8A, isolated at Kaimes Farm, Leitholm,  
123 Scottish Borders[42]) for >5 years in 400mL glass jars at a concentration of 10-30 individuals per  
124 jar, filled with COMBO medium[43] and supplemented with 0.25 mg C/mL/day of

125 *Chlamydomonas reinhardtii* (CPCC 243) as a nutrient source. We maintained these cultures in a

126 16h:8h light: dark cycle at 19°C. Prior to the experiment, we transferred 48 juvenile *Daphnia*  
127 *magna* to individual 35 mL glass vials, where they matured. From those individuals, we pooled  
128 offspring from the second brood and randomly assigned 48 to the initial experimental  
129 treatments.

130

### 131 *Experimental design*

132 We raised experimental *Daphnia magna* individually in 35 mL glass vials for five generations. In  
133 the initial generation (Generation 1), *D. magna* were exposed to one of two treatments: an  
134 antibiotic cocktail of 500 ug/L aztreonam, 400 ug/L erythromycin, and 250 ug/L  
135 sulfamethoxazole shown previously to suppress the *Daphnia* microbiome[32] in 25 mL of  
136 COMBO medium, or a no-antibiotic control treatment of 25 mL COMBO medium. We replaced  
137 the medium for each treatment every two days and all individuals were fed with 0.25 mg  
138 C/mL/day of *Chlamydomonas reinhardtii*. We measured survival and reproduction within  
139 treatments every day, and every four days we measured body size (measured from the top of  
140 the eyespot to the beginning of the apical tail spine). Individuals were monitored for 21 days or  
141 until death, then final body size was measured and animals were pooled for DNA extraction and  
142 microbiome characterization.

143

144 To initiate each new generation of treatments, we pooled second brood offspring of individuals  
145 within a treatment and randomly chose 24 individuals to move into the next generation of  
146 treatment, following a split-brood design (Fig. 1). We placed 24 offspring from control  
147 individuals in the next generation of the control treatment, and 48 offspring from individuals in



148 the antibiotic treatment were randomly assigned to either remain in the antibiotic treatment or  
149 to be allowed to recover in the control treatment (hereafter, all combinations of antibiotic  
150 exposure followed by control are referred to as recovery treatments). For each subsequent  
151 generation, we applied the same transfer method for offspring in the antibiotic and control  
152 treatments, and offspring from the recovery treatment continued to be placed in the no-  
153 antibiotic control (**Figure 1**).

154

#### 155 *DNA extraction and sequencing*

156 We pooled sets of five individuals for DNA extraction and microbiome sequencing (n=4 per  
157 treatment per generation). We amplified the 16S rRNA V4 hypervariable region from DNA  
158 extracted using the Qiagen DNEasy Blood & Tissue Kit with the 515f/806r primer pair[44], with  
159 PCR steps as follows: 95°C for 3 min; 35 cycles of 95°C for 45 sec, 58°C for 30 sec, 72°C for 45  
160 sec; and 72°C for 5 minutes. We generated and normalized sample libraries with the  
161 SequelPrep Normalization Plate Kit and quality controlled with the Agilent High Sensitivity DNA  
162 Kit and the Agilent TapeStation, and the KAPA Library Quantification Kit. We pooled all quality-  
163 checked samples and spiked with PhiX, then sequenced the samples using a MiSeq Reagent Kit  
164 v2 (300-cycles) on the Illumina MiSeq at the Nebraska Food for Health Center (Lincoln, NE,  
165 USA).

166

#### 167 *Data processing and statistical analysis*

168 For both the analyses of both microbiome composition and life history traits, we compared  
169 antibiotic-treated and control composition in the same generations (for example, A1 to C1),

170 recovery treatments to the control in the same generational time point (for example, R1->1 to  
171 C2, R1->2 to C3, and R2->1 to C3, all columns in **Figure 1**), antibiotic-treated and control  
172 composition in the final generation as compared to the first generation (C5 to C1 and A5 to A1),  
173 and recovery treatments in the first generation out of antibiotics to each other (for example,  
174 R1->1 to R2->1 or R2->1 to R4->1).

175  
176 For the life history analyses, we measured *Daphnia magna* fitness using three key host life  
177 history metrics: growth, reproduction, and survival. Growth was quantified for each individual  
178 as the length (mm) of the carapace after 21 days. Reproduction was quantified as the  
179 cumulative number of offspring produced by each individual over 21 days. The analysis of final  
180 size used only data from *D. magna* that survived through the end of each generation, whereas  
181 the analysis of cumulative reproduction includes individuals that did not survive until the  
182 experimental end point. For the growth analysis, we fit a normal linear model with the  
183 interaction of generation and treatment sequence as explanatory variables. To analyze the  
184 cumulative reproduction data, we used a hurdle model fit with the `pscl` package (v1.5.5[45])  
185 with negative binomial random component based on the model's ability to properly account for  
186 excess zeros and overdispersion. The negative binomial hurdle model included the same  
187 explanatory variables as the model for the growth analysis, but the hurdle component of the  
188 model involved only the main effects of treatment sequence and generation because the model  
189 including interactions could not be estimated due to complete separation of the data. For both  
190 growth and reproduction analyses we computed linear contrasts to test for differences in the  
191 response variable between pertinent combinations of treatment sequence and generation

192 number. Comparisons were made using Wald tests adjusted for family-wise error to control for  
193 related tests using the emmeans package (v1.5.4[46]). We used a Cox proportional hazards  
194 model from the survival package (v3.2-10[47]) to examine the effects of both generation and  
195 treatment on survival.

196

197 For microbiome analysis, we used dada2 (v3.11[48]) and phyloseq (v1.32[49]) to identify  
198 amplicon sequence variants (ASVs) and visualize microbiome diversity and composition. To  
199 identify taxa, we used the GTDB taxonomy database formatted for dada2[50]. Differences  
200 among microbial communities across treatments were identified using PERMANOVAs. We  
201 calculated Pearson correlation coefficients to identify the relationship between microbial taxa  
202 of interest and host life history metrics (body size, cumulative reproduction). DESeq2  
203 (v1.30.1[51]) was used to identify differentially abundant ASVs across treatment subsets.

204

## 205 **Results**

206 **[Figure 1 goes here]**

207 Figure 1: A schematic of experimental design, with 24 *Daphnia magna* per treatment. In generation 1, *D. magna*  
208 were placed into antibiotics or no antibiotics (control). In subsequent generations, control *D. magna* offspring  
209 were placed in control, and antibiotic-treated *D. magna* offspring were split between a no antibiotic recovery  
210 treatment and a continued antibiotic treatment. Throughout the paper, we will use the notation R# to refer to the  
211 entire treatment sequence (e.g., R1 denotes the sequence A1, R1->1, R1->2, R1->3, R1->4).

212

213 We examined differences in community composition between samples and across treatments  
214 using Bray-Curtis dissimilarity. As seen in **Figure 2a**, community composition significantly

215 diverged across both generations and across treatments (PERMANOVA, generation pseudo- $F_{4,70}$   
216 = 7.192,  $R^2 = 0.214$ ,  $P = 0.001$ , treatment pseudo- $F_{15,70} = 3.64$ ,  $R^2 = 0.406$ ,  $P = 0.001$ ,  
217 **Supplementary Table S1A**). The *Daphnia magna* microbiota in first-generation control  
218 individuals primarily consisted of Bacteroidia, Gammaproteobacteria, and Alphaproteobacteria  
219 (**Figure 2a**, treatment C1). Within these bacterial classes, the most abundant ASVs belonged to  
220 the genera *Limnohabitans* (6%, ASV5), *Hydromonas* (13%, ASV1), a Chitinophagaceae with  
221 unidentifiable genus-level taxonomic identity (10%, ASV9), *Daejeonella* (7%, ASV7), UBA4466 (a  
222 Crocinitomicaceae genus, 7%, ASV8), and *Flavobacterium* (7%, ASV3). Antibiotic treatment  
223 immediately shifted microbiota composition, substantially increasing the relative abundance of  
224 Bacteroidia and decreasing Gammaproteobacteria (**Figure 2a**, treatment A1).

225

226 **[Figure 2 goes here]**

227 Figure 2: Microbiome composition and differences across treatments. (a) Microbiome community composition at  
228 the class taxonomic rank across all treatments, with samples pooled by treatment. Classes with less than 5%  
229 abundance in a treatment are denoted as “≤5% Abundant/Unidentified”. The sum of numbers in the “R” treatment  
230 labels denote the experimental generation (for example, R1→4 is the fifth generation of animals in antibiotics for 1  
231 generation and removed from antibiotics for 4 subsequent generations). (b) Differentially abundant ASVs in the  
232 fifth generation of the control treatment as compared to the first generation of control ( $\alpha < 0.01$ ). ASVs are named  
233 by their genus or at higher taxonomic ranks if unidentifiable at the genus rank and colored according to bacterial  
234 class. (c) Differentially abundant ASVs in the fifth generation of the antibiotic treatment as compared to the first  
235 generation of antibiotics ( $\alpha < 0.01$ ), named and colored as in (b).

236

237 The microbiota also shifted across generations, although the nature of the shifts depended on  
238 treatment. In the control treatment, Gammaproteobacteria became increasingly dominant

239 through generations until a single *Hydromonas* ASV (ASV1) comprised over 60% of microbiome  
240 composition, and Gammaproteobacteria in total over 65%; correspondingly, alpha diversity  
241 decreased significantly across generations (**Figure 3b**,  $F_{4,7} = 9.555$ ,  $p < 0.0001$ , **Supplementary**  
242 **Table S1C, Supplementary Figure 4**), though the number of unique ASVs identified did not  
243 (**Figure 3c**,  $F_{4,7} = 4.208$ ,  $p = 0.005$ , **Supplementary Table S1D, Supplementary Figure 5**). In  
244 antibiotics, microbiota composition varied considerably across generations but  
245 Chitinophagaceae remained dominant no matter what generation. Microbiota composition also  
246 recovered after generations spent in antibiotics (**Figure 2a**, all treatments beginning with R),  
247 with Gammaproteobacteria returning to higher abundance within a generation or two, and  
248 other members that appeared in the control treatment reappearing, maintaining, or increasing  
249 in abundance as generations of recovery progressed. Detrended correspondence analysis  
250 indicates that samples in the control treatment cluster more closely with each other than those  
251 in the antibiotic or recovery treatments, and that recovery communities are more similar to  
252 control communities than antibiotic communities (**Figure 3a**).

253

254 **[Figure 3 goes here]**

255 Figure 3: Microbiome diversity across generations and treatments. (a) Detrended correspondence analysis of all  
256 samples, with point color corresponding to treatment type and point shape corresponding to generations spent in  
257 a treatment (CTRL = control, REC = recovery, AB = antibiotic). (b) Inverse Simpson Index in each sample across  
258 generations, with point color corresponding to treatment type. (c) The number of unique ASVs identified in each  
259 sample across generations, with point color corresponding to treatment type.

260

261 Because we observed significant differences in composition across generations, we broke these  
262 down further by just examining the impacts of generation on beta diversity in just control and  
263 just antibiotic samples, finding that there were strong effects of generation on both (control  
264 pseudo- $F_{4,17} = 3.457$ ,  $R^2 = 0.515$ ,  $P = 0.001$ ; antibiotic pseudo- $F_{4,13} = 3.119$ ,  $R^2 = 0.581$ ,  $P = 0.001$ ;  
265 **Supplementary Figures 6 & 7; Supplementary Table S1A**). To find what taxa might be driving  
266 these differences, we analyzed the communities with DESeq2, finding taxa with  $q < 0.01$  in both  
267 the control and antibiotic treatments (**Figure 2b & 2c**). In the fifth generation, individuals in the  
268 control treatment had 20 ASVs that were differentially abundant compared to the first  
269 generation; individuals in the fifth generation of antibiotic treatment had 32 ASVs that were  
270 differentially abundant compared to the first generation. The majority of differentially  
271 abundant ASVs in both analyzed treatments belonged to Alphaproteobacteria and Bacteroidia,  
272 with Chitinophagaceae ASVs in the control experiencing the largest changes in relative  
273 abundance ( $2^{-12}$  reduction in ASV16,  $2^{12}$  increase in ASV4; complete results in **Supplementary**  
274 **Tables S1E & S1F**). More rare taxa were increased in abundance in the antibiotic treatment,  
275 with several Gammaproteobacteria taxa experiencing between  $2^8$  and  $2^{15}$  increases in relative  
276 abundance.

277  
278 We also conducted pairwise PERMANOVAs to understand whether the microbiomes of *Daphnia*  
279 *magna* in each treatment type were distinct from each other (**Supplementary Table S1B**).  
280 Recovery from antibiotics caused beta diversity to be more similar to the control (**Figure 3A**).  
281 Beta diversity changed in the fifth generation of control as compared to the first ( $P = 0.033$ ) but  
282 not in the fifth generation of antibiotics as compared to the first ( $P = 0.33$ ). Comparisons of

283 control beta diversity to all other treatments at each generational time point (columns in **Figure**  
284 **1**) did not show any clear trend in differences (**Supplementary Table S1B**). For the first  
285 generation out of antibiotics in each recovery sequence, only one recovery treatment was  
286 significantly different from the control (R3->1 to C4,  $p = 0.033$ ) but most were not (R1->1 to C2,  
287 R2->1 to C3, R4->1 to C5,  $p > 0.05$ ). Comparisons across recovery treatments after their first  
288 generation removed from antibiotics showed that the beta diversity of most treatments were  
289 significantly different from each other (R1->1 to R2->1, R1->1 to R3->1, R1->1 to R4->1, R3->1 to  
290 R4->1,  $P < 0.05$ ), though two were similar (R2->1 to R3->1  $P = 0.05$ , R2->1 to R4->1  $P = 0.08$ ).  
291 Treatment with antibiotics changed composition in the 3<sup>rd</sup> and 4<sup>rd</sup> generations ( $P = 0.026$ ,  
292 0.029, respectively), but not in the 1<sup>st</sup>, 2<sup>nd</sup>, and 5<sup>th</sup> ( $P = 0.067$ , 0.1, and 0.067, respectively).

293

294 **[Figure 4 goes here]**

295 Figure 4: *Daphnia magna* life history and ASV correlations. (a) *Daphnia magna* body size at the experimental  
296 endpoint (mm at 21 days) across generations. Color denotes treatment type (CTRL = control, REC = recovery, AB =  
297 antibiotic). (b) Cumulative reproduction of *Daphnia magna* in the 21-day experiment across generations. Color  
298 denotes treatment type. (c) ASVs with significant Pearson correlations to *Daphnia magna* body size in the CTRL  
299 treatment. The 5 ASVs with the highest mean abundance across all CTRL samples and  $p < 0.05$  are shown here with  
300 confidence intervals around the linear model fit. Point shape denotes generation, while line and confidence  
301 interval color correspond to ASV taxonomic identity at the class rank. (d) ASVs with significant Pearson correlations  
302 to *Daphnia magna* cumulative reproduction in the CTRL treatment, with the same methods as in (d) applied.

303

304 Some *Daphnia magna* life history metrics changed across generations and treatments. We  
305 found that *D. magna* body size significantly increased over the four generations spent in

306 isolation (**Figure 4A**). Mean body length increased by 0.20 mm [95% CI: 0.09, 0.30;  $p < .001$ ] in  
307 the control sequence and 0.32 mm [95% CI: 0.21, 0.43;  $p < .001$ ] in the antibiotic sequence from  
308 generation 1 to generation 5. Comparisons between the control treatment and all other  
309 treatments within each generational time point (e.g., columns in **Figure 1**) yielded no  
310 discernable pattern of significance (**Supplementary Table S1G, Supplementary Figure 1**). For  
311 instance, in generation 3 the mean body size of individuals in C3 was larger than the mean body  
312 size of individuals in either R1->2 (0.18 [0.05, 0.31 mm];  $p < 0.001$ ) or R2->1 (0.18 [0.04, 0.31];  $p$   
313 = 0.002), but in generation 5 the mean body size of individuals in C5 was smaller than the mean  
314 body size of individuals in R1->4 and no different than R2->3 (C5 to R1->4: -0.15 [-0.29, -0.02];  
315  $p = 0.013$ ; C5 - R2->3: -0.03 [-0.16, 0.10];  $p > 0.999$ ). Finally, we tested the differences in mean  
316 body size between all of the recovery treatment sequences at the first generation the antibiotic  
317 exposure was ceased to see with recovery in growth was impacted by the number of successive  
318 generations of exposure. Again, some contrasts were statistically significant (R1->1 to R3->1: -  
319 0.15 [-0.2734423, -0.0399243] mm;  $p = 0.003$ ; R1->1 to R4->1: -0.17 [-0.29, -0.06];  $p < 0.001$ ),  
320 but an overall pattern was not observable (**Supplementary Table S1G**).

321  
322 Expected cumulative reproduction was larger in generation 5 than in generation 1 in both the  
323 antibiotic (**Figure 4b**, +10.03 offspring [95% CI: 7.10, 12.96];  $p < .001$ ) and control treatments  
324 (+2.74 offspring [95% CI: 0.30, 5.20];  $p = 0.024$ ). Comparisons between the control treatment  
325 and other treatments within the same generational time point showed no clear pattern of  
326 significance (**Supplementary Table S1H, Supplementary Figure 2**). For instance, in generation 3  
327 the mean reproduction by individuals in control sequences in was larger than the mean



328 reproduction of R1->2 and R2->1 (C3 – R1->2: 6.25 [2.82, 9.67];  $p < 0.001$ ; C3 - R2->1: 5.51  
329 [1.98, 9.04];  $p < 0.001$ ), but in generation 5 the mean reproduction of individuals in the control  
330 sequences was smaller than the mean reproduction of individuals in R1->4 and in R2->3 (C5 to  
331 R1->4: -7.63 [-11.85, -3.40];  $p < 0.001$ ; C5 to R2->3: -6.10 [-10.10, -2.11];  $p < 0.001$ ). Finally, the  
332 mean reproduction of individuals after their first generation of recovery showed no overall  
333 pattern across the recovery treatments, with some significant differences (R1->1 to R2->1: 3.93  
334 [0.97, 6.9];  $p = 0.004$ ; R1->1 to R3->1: 4.83 [1.95, 7.72];  $p < 0.001$ ; R3->1 - R4->1: -3.45 [-6.28,  
335 -0.63];  $p = 0.009$ ), but other differences were not significant (**Supplementary Table S1H**).

336  
337 Though both growth and cumulative reproduction increased over time, survival did not.  
338 Individuals in later generations had significantly lower survival, with those in generations 3-5  
339 more likely to die than those in the initial generation (all  $p < 0.05$ , generation 3 hazard ratio  
340 (HR) = 11.3, generation 4 HR = 12.9, generation 5 HR = 10.2, **Supplementary Figure 3**,  
341 **Supplementary Table S1I**) and those in antibiotics less likely to survive than those in recovery  
342 or control ( $p = 0.004$ , HR = 2.8, **Supplementary Figure 3**, **Supplementary Table S1I**).

343  
344 To help connect the microbiome and life history results, we examined the relationship between  
345 each ASV's relative abundance and host life-history metrics through Pearson correlation of  
346 average final size for the individuals pooled for each sequencing sample, and the same for  
347 average cumulative reproduction. ASVs correlated with *Daphnia magna* body size primarily  
348 belonged to Alphaproteobacteria and Gammaproteobacteria (**Figure 4c**, **Supplementary Table**  
349 **S1J**), as did the ASVs correlated with cumulative reproduction (**Figure 4d**, **Supplementary Table**

350 **S1K**). Of these, most were found at relatively low abundances with the exceptions of  
351 *Limnohabitans* ASV 5 and *UBA4466* ASV 8, which were positively associated with *Daphnia*  
352 *magna* reproduction ( $R = 0.5$ ,  $p = 0.035$ ) and negatively associated with reproduction ( $R = -0.67$ ,  
353  $p = 0.002$ ), respectively. ASVs from the Alphaproteobacteria were generally correlated with  
354 reduced cumulative reproduction and reduced final size as relative abundance increased (e.g.,  
355 *Sphingopyxis* ASV20, *Sphingorhabdus\_B* ASV30, *Lewinella\_A* ASV 35, and *UBA4466* ASV 8),  
356 while cumulative reproduction increased with increased relative abundance of  
357 Gammaproteobacteria (e.g., *Limnohabitans* ASV 5, Moraxellaceae ASV 22).

358

## 359 **Discussion**

360 Investigating how the microbiome shifts across generations of hosts, both through normal  
361 processes of microbiome transmission and in response to external stressors, is important for  
362 increasing our understanding of microbe-host interactions across time. In this study, we  
363 examined life history outcomes and microbiome composition across five generations of  
364 antibiotic treatment and recovery in the zooplankton *Daphnia magna*. We found significant  
365 impacts of antibiotic treatment on life history, impacting survival and composition across  
366 multiple generations of continuous antibiotic exposure. We also found an effect of generational  
367 isolation on host fitness and microbiome composition. Offspring of individually raised *Daphnia*  
368 *magna* were only exposed to parental microbes for 24 hours before transfer to their individual  
369 vials, and microbiome diversity decreased across generations of this imposed isolation. At the  
370 same time, *Daphnia magna* body size increased, as did cumulative reproduction, and survival  
371 decreased.

372

373 The pairing of increased host fitness (with the exception of survival) and reduced microbiome  
374 diversity presents an intriguing case of microbial loss positively impacting hosts. This pattern  
375 has been observed in *Daphnia magna* before, where it was attributed to a direct positive effect  
376 of antibiotics on the host[37]. However, the same pattern emerging here even in the control  
377 treatment indicates that reduced diversity might directly and positively impact host fitness. In  
378 the control treatment, the most abundant members of the microbiome became more  
379 abundant, comprising nearly 90% of relative abundance by the fifth generation. Across other  
380 treatments, microbiome diversity also decreases, though perturbation with antibiotics makes  
381 that effect less apparent until later generations. While it is possible that external, unmeasurable  
382 factors were changing in this experiment (e.g., food quality or season-dependent life history  
383 changes), observing the same pattern of reduced diversity increasing fitness as in another  
384 *Daphnia magna* study and across multiple treatments indicates that this is unlikely.

385

386 Our results suggest several non-exclusive possibilities. Rare taxa may always be pathogenic and  
387 be maintained through horizontal transmission among hosts. Over generations, these  
388 detrimental rare taxa could be lost stochastically as transmission events would happen rarely in  
389 the restricted transmission setup. Alternatively, this pathogenicity may be context dependent,  
390 with the taxa providing benefits for the host in specific environmental conditions, but harming  
391 the host in other conditions[52]. In this case, host mechanisms may force these taxa from the  
392 microbiome if they are detrimental in the laboratory conditions maintained during our  
393 experiment (isolated individuals in an environment with abundant resources and constant

394 temperature). Alternatively, rare taxa could have no direct impact on host fitness but may be  
395 occupying niche space that would otherwise be occupied by beneficial taxa. Here, host  
396 mechanisms may lead to the deterministic loss of these niche-occupying rare taxa[53]. It is also  
397 possible that these taxa are lost stochastically as they are sustained at high enough population  
398 densities to ensure consistent transmission to naïve hosts across generations. It is likely that  
399 many of these factors are at play in the *Daphnia* microbiome: for example, a low abundance  
400 Chitinophagaceae genome in the *Daphnia* microbiota encodes for the degradation of chitin (a  
401 key component of *Daphnia magna*'s carapace) while other low abundance genomes from  
402 *Polaromonas* and Burkholderiaceae encode for amino acid biosynthesis and export, important  
403 dietary components for *Daphnia*[34]. Our differential abundance analysis also indicates that  
404 several Chitinophagaceae ASVs are significantly less abundant in the final generations of both  
405 the antibiotic and control treatments.

406  
407 Though correlation of a taxon's relative abundance with host traits does not directly imply  
408 causation nor the underlying mechanisms of potential associations, several of the taxa  
409 highlighted in this study have been previously associated with host fitness. In particular, species  
410 in the highly abundant genus *Limnohabitans* have been beneficially linked to host reproduction  
411 across studies[30,31,54]. Potential mechanisms underlying *Daphnia*-associated *Limnohabitans*  
412 benefits to the host include tolerance to toxins, breakdown of complex carbohydrates from  
413 algal food sources, and provision of essential amino acids to the host[34,55,56]. Moraxellaceae,  
414 identified in other work as a family significantly affected by changes in environmental  
415 temperature[38], is positively associated with host fitness in this study. A UBA4466 ASV (ASV 8,

416 in the Crocinitomicaceae family), is negatively associated with both *Daphnia magna* body size  
417 and cumulative reproduction, warranting further investigation into whether this species has  
418 specific negative effects on host fitness or if interactions with other taxa are at play.

419

420 We also found that the *Daphnia magna* microbiome is surprisingly resilient to  
421 multigenerational antibiotic exposure, often recovering quickly in individuals that were  
422 removed from antibiotic exposure. As can be seen in the treatments where *Daphnia* were  
423 exposed to antibiotics for 3 and 4 generations and then allowed to recover, microbiome  
424 composition in the final generation of recovery after was similar to that of the final generation  
425 of control. This effect was diminished in the recovery treatments where *Daphnia* were exposed  
426 for only 1 or 2 generations, presumably due to more generations in isolation with a microbiome  
427 freed from antibiotic suppression, yet these communities were still more similar to the control  
428 communities than the antibiotic-treated communities.

429

430 The *Daphnia magna* microbiome's resilience to antibiotic exposure is intriguing, as the  
431 microbiome of individuals allowed to recover for just one generation is able to return to a  
432 similar composition as the control individuals. This quick recovery may partially be attributed to  
433 the relative simplicity of its microbiome and potential microbe-microbe interactions  
434 maintaining microbiome stability. Antibiotics can permanently alter microbe-microbe  
435 interactions in host-associated microbiomes, shifting the dynamics of microbiome structure and  
436 assembly[57]. We do not observe a significant, consistent change in structure across recovering  
437 *Daphnia* in this study, suggesting that interactions among microbiome members are preserved.

438 The simplicity of *Daphnia magna*'s microbiome may enforce strict microbe-microbe  
439 interactions, as functional relationships in this system are limited to the present microbes while  
440 in more complex microbiomes functional redundancy allows for multiple species to share  
441 interactions, changing microbiome structure depending on which interactions are  
442 occurring[58,59]. Resistance to antibiotics may also play a role, as antibiotic resistance has been  
443 identified in the *Daphnia* microbiome[60,61]. While this may explain why specific community  
444 members increased in relative abundance after antibiotic exposure, the return to more control-  
445 like microbiome composition in recovery strongly supports the strengths of microbe-microbe  
446 interactions in assembly and stability of the *Daphnia* microbiome. Although microbiome  
447 composition appears to mostly be resilient in *Daphnia* allowed to recover, hosts in the  
448 antibiotic treatment and those in antibiotics for more generations prior to recovery were still  
449 more likely to die than those in the control treatment, indicating that there still are some  
450 impacts of antibiotics to the microbiome that are not necessarily observable through examining  
451 relative abundances.

452

453 The environmental pool of microbes available to *Daphnia magna* clearly shapes its  
454 microbiome[33,35,40], but the impact of isolation on microbial diversity shown in this study  
455 underscored how important host shedding of microbes into the environment (e.g., horizontal  
456 transmission) is for maintaining microbial diversity. Isolation reduces microbial diversity,  
457 indicating that environmental transmission of microbes from populations of adult *Daphnia*  
458 *magna* to juveniles allows rare taxa to persist while isolation reduces the chances of rare taxa  
459 to colonize subsequent generations. However, it remains unclear *why* these rare taxa are

460 maintained in host populations. Further genomic and transcriptomic work is needed to identify  
461 the roles these microbes play the *Daphnia* ecosystem, and isolating juveniles to only receive  
462 microbes from their parents provides an interesting method for testing how loss of rare taxa in  
463 the microbiome impacts host fitness across a range of environmental stressors.

464

## 465 **Conclusions**

466 Antibiotics impact host fitness by perturbing the microbiome, but our understanding of how  
467 these perturbations affect host fitness across multiple generations of exposure and recovery is  
468 limited. We utilized a novel split-brood design in *Daphnia magna* to understand the  
469 multigenerational effects of antibiotic exposure on host fitness. We found that the *Daphnia*  
470 *magna* microbiome is able to recover quickly after release from antibiotics, with offspring of  
471 exposed parents able to return to a microbiome composition similar to that of individuals in no  
472 antibiotics. Due to our experimental design, we also find an intriguing link between reduced  
473 microbiome diversity and increased host fitness across generations. Our results suggest that  
474 rare taxa in the microbiome may not play a beneficial role, but instead may be detrimental for  
475 the host in some environmental contexts. Moreover, our results demonstrate that *Daphnia*  
476 *magna* can play an important role as a model organism in exploring the links between  
477 microbiome resilience, function, and diversity.

478

## 479 **Declarations**

### 480 **Ethical Approval and Consent to Participate**

481 Does not apply.

482 **Consent for Publication**

483 Does not apply.

484 **Availability of Supporting Data**

485 16S rRNA read data is available under BioProject PRJNA703930. All code and other data are  
486 available on Github

487 ([https://github.com/reillyowencooper/daphnia\\_multigenerational\\_antibiotics](https://github.com/reillyowencooper/daphnia_multigenerational_antibiotics)), including an  
488 .rds file containing the processed 16S data in a phyloseq object and R Markdown files  
489 documenting all analyses.

490 **Competing Interests**

491 The authors declare that there are no competing interests.

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496 **Author Contributions**

497 ST, JR, ROC, and CEC designed the study. Animal husbandry and data collection was performed  
498 by ST and JR. Data analysis was conducted by ROC and DTN. ROC wrote the first manuscript  
499 draft and ST, JR, DTN, and CEC contributed to manuscript revisions. All authors approved the  
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505

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668

## 669 **Supplementary Figures**

670

671 Supplementary Figure 1: *Daphnia magna* final size across all treatments colored by treatment  
672 type (CTRL = control, REC = recovery, AB = antibiotic).

673

674 Supplementary Figure 2: *Daphnia magna* cumulative reproduction across all treatments colored

675 by treatment type (CTRL = control, REC = recovery, AB = antibiotic).

676

677 Supplementary Figure 3: Proportion of *Daphnia magna* surviving across the 21-day duration of  
678 the experiment, separated by generation.

679

680 Supplementary Figure 4: Alpha diversity of the *Daphnia magna* microbiome across treatments,  
681 measured by the Inverse Simpson Index and colored by treatment type (CTRL = control, REC =  
682 recovery, AB = antibiotic).

683

684 Supplementary Figure 5: Number of unique ASVs found in the *Daphnia magna* microbiome  
685 across treatments, colored by treatment type (CTRL = control, REC = recovery, AB = antibiotic).

686

687 Supplementary Figure 6: Detrended correspondence analysis using only control samples, with  
688 points colored by generations spent in the control.

689

690 Supplementary Figure 7: Detrended correspondence analysis using only antibiotic samples, with  
691 points colored by generations spent in the control.

692











