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1 **Disparate effects of antibiotic-induced microbiome change and enhanced fitness**

2 **in *Daphnia magna***

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12 ***Abstract***

13 It is a common view that an organism's microbiota has a profound influence on host fitness;
14 however, supporting evidence is lacking in many organisms. We manipulated the gut
15 microbiome of *Daphnia magna* by chronic exposure to different concentrations of the
16 antibiotic Ciprofloxacin (0.01 – 1 mg L^{-1}), and evaluated whether this affected the animals'
17 fitness and antioxidant capacity. In line with our expectations, antibiotic exposure altered the
18 microbiome in a concentration-dependent manner. However, contrary to these expectations,
19 the reduced diversity of gut bacteria was not associated with any fitness detriment. Moreover,
20 the growth-related parameters correlated negatively with diversity indices; and, in the
21 daphnids exposed to the lowest ciprofloxacin concentrations, the antioxidant capacity,
22 growth, and fecundity were even higher than in control animals. These findings suggest that
23 ciprofloxacin exerts direct stimulatory effects on growth and reproduction in *Daphnia*, while
24 microbiome- mediated effects are of lesser importance. Thus, although microbiome profiling
25 of Daphnia may be a sensitive tool to identify early effects of antibiotic exposure,
26 disentangling direct and microbiome-mediated effects on host fitness is not straightforward.

27

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29 ***Introduction***

30 In multicellular organisms, the microbiome contributes to critical aspects of host development
31 and physiology (1). In ecological, evolutionary and ecotoxicological research, there is
32 growing recognition that environmental stresses imposed upon the microbiome may drive
33 physiological responses, life-history penalties and adaptation capacity of their hosts (2), (3) 4)
34 . Consequently, coping with various environmental insults would involve both the host and its
35 microbiome responses.

36 The gut microbiota participates directly in food digestion and nutrient assimilation, which
37 affects the host's energy acquisition and growth (5). In addition to this, the host immune
38 system is influenced by the gut microbes via a number of different mechanisms, e.g.,
39 competition with pathogens as well as suppression and modification of virulence factors via
40 metabolite production (6). Symbiotic bacteria are also capable of enhancing the host innate
41 immune system by, for example, up-regulation of mucosal immunity, induction of
42 antimicrobial peptides and antibodies (7, 8). Considering the biological effects triggered by
43 the host-microbiome interactions, a disruption of mutualistic bacterial communities may
44 result in increased susceptibility to pathogens and infections, while simultaneously
45 affecting the growth and development of the host via compromised nutrition. In various
46 gnotobiotic animal models, poor survival, growth and fecundity are commonly observed,
47 reflecting a physiological impairment due to some dysbiotic state of microflora (3, 9).

48 If growth penalties are to be expected in animals with perturbed microbiota, then it should be
49 possible to manipulate animal fitness by targeting its resident bacteria with antibacterial
50 substances. In line with this, retarded development has been observed in the copepod *Nitocra*
51 *spinipes* upon antibiotic exposure, and linked to structural changes in its microbiota (10). It
52 was suggested that aberrant digestion was behind these changes as has also been observed in
53 *Daphnia magna* following a short-term antibiotics exposure (9,11). Moreover, an altered

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54 microbiota composition was reported in *Daphnia* following a long-term exposure to the
55 antibiotic oxytetracycline, concurrent with reduced host growth (12). While perturbed
56 microbiota can manifest itself directly as decreased nutrient uptake, another outcome can be
57 effects on host antioxidant production, with concomitant effects on immunity and growth
58 (13). However, short antibiotic exposure may not necessarily result in any significant growth
59 penalties in the long run. The outcome of any chronic exposure to antibiotics would largely
60 depend on the resilience of the bacterial communities, and their capacity to recover and re-
61 establish any functional interaction(s) relevant to the host (16,17,18,19,20).

62 To study the relationships between microbiome composition and host performance, a
63 common set of model species and methods to manipulate their microbiomes is needed. In
64 ecology, evolution and ecotoxicology, *Daphnia* species are used routinely as model
65 organisms because of their well-known physiology, rapid reproduction, and sensitivity to
66 environmental factors (19). The microbiome of the laboratory-reared *Daphnia magna* has
67 been recently presented in several studies using different approaches, from cloning to shotgun
68 sequencing (22, 23). Regardless of the sequencing platform, origin of specimens, and culture
69 conditions, the core microbiome appears relatively stable, mainly comprised of
70 *Betaproteobacteria*, *Gammaproteobacteria* and facultative anaerobic *Bacteroidetes* species.
71 At the genus level, *Limnohabitans* has been reported as one of the most stable and dominant
72 members in *Daphnia* gut, and variations in its abundance have been tied to the animal
73 fecundity (22). Although some studies have addressed the dependence of *Daphnia* on its
74 microbiota (9) and some short-term effects on fitness following exposure to antibiotics have
75 been observed in *Daphnia magna* (25, 13), the relationship between microflora perturbation
76 and host fitness is still unclear, as is the involvement and modulating role of antioxidants in
77 these relationships.

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78 In this study, the relationship between antibiotic-mediated gut microbiome modulation and
79 host fitness were addressed experimentally using a model cladoceran *Daphnia magna*. We
80 monitored changes in the gut microbiome, host longevity, growth, and reproduction, as well
81 as antioxidant levels in the exposed animals following ciprofloxacin exposure. We
82 hypothesized that the diversity and abundance of the gut-associated microflora would
83 decrease with increasing concentration of antibiotics. Furthermore, we expected longer
84 exposure time and higher antibiotic concentrations to have negative effects on somatic
85 growth, reproductive output, and antioxidant capacity. These reductions we expected would
86 be due to reduced bacterial diversity in particular, and to some extent, an altered community
87 composition. These hypotheses were tested by combining (1) long-term (21 d) exposure
88 experiments with life-table analysis, (2) microbiome profiling using the next generation
89 sequencing of 16S rRNA gene and taxonomic assignment, and (3) measurements of daphnid
90 total antioxidant capacity, growth, and fecundity.

91

92 ***Material and methods***

93 ***Test species and culture conditions***

94 The cladoceran *Daphnia magna*, originating from a single clone (Environmental pollution test
95 strain *Clone 5*, Federal Environment Agency, Berlin, Germany), was used in this experiment.
96 The animals were cultured in groups of 20 individuals in 3-L beakers with M7 medium
97 (OECD standard 202 and 211), and fed a mixture of the green algae *Pseudokirchneriella*
98 *subcapitata* and *Scenedesmus subspicatus* three times a week; the algae were grown
99 axenically.

100

101 ***Ciprofloxacin stock solutions***

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102 Ciprofloxacin hydrochloride (CAS: 86393-32-0; Sigma) the antibiotic utilized in this study
103 and is a broad spectrum fluoroquinolone, active against both Gram-positive, G+, and Gram-
104 negative, G-, bacteria. Its mode of action is the inhibition of the gyrase and / or
105 topoisomerase enzyme of microbes which determines the supercoiling state of DNA, and
106 critical to bacterial replication, repair, transcription and recombination (24). Selection of this
107 drug was due to its rapid absorption, long half-life in the test system, and the absence of
108 acute toxicity in *D. magna* within the range of the concentrations tested (25). A singular
109 stock solution of ciprofloxacin (1 mg/ml) was prepared in M7 medium and stored at -20°C
110 during the course of the experiment.

111

112 ***Experimental design***

113 We employed three drug concentrations (0.01, 0.1 and 1 mg/L) and a control treatment (M7
114 medium). For each treatment, 25 neonates (< 24 h) of *D. magna* were placed individually in
115 40 mL of M7 medium, with or without ciprofloxacin; the medium was changed every
116 second day. The test design followed a standard procedure for the reproduction test with
117 *Daphnia* (OECD standard 211). The animals were fed daily with a suspension of green
118 algae *Pseudokirchneriella subcapitata* (0.2 mg C d^{-1}; axenic culture) and incubated at 22°C
119 with 16^L: 8^D photoperiod. Under these conditions, the animals matured and started to
120 reproduce 8-9 d after the start of the experiment. All jars were inspected daily and mortality
121 recorded. Upon release of neonates, counts were made, offspring discarded, and brood size
122 recorded for each female and within each brood. In conjunction with brood release, four
123 randomly selected individuals from each treatment were placed in antibiotic-free medium. In
124 this manner, we collected females after their 1st, 2nd, 3rd, and 4th clutch, with the last
125 individuals sacrificed on day 21, when the experiment was terminated. When sampling, the
126 images of females were acquired by scanning live animals on a glass surface in a drop of

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127 water (CanoScan 8800F 13.0), and their body length (BL, mm) was measured using ImageJ
128 software (26). For each individual, the gut was dissected using a sterile needle and a pair of
129 forceps, washed with nuclease-free water, transferred individually to Eppendorf tubes and
130 stored at -80°C until DNA extraction. The degutted body was transferred to a fresh
131 Eppendorf tube, stored at -80°C and tested for antioxidant levels based on Oxygen Radical
132 Absorbance Capacity (ORAC) and protein content.

133

134 ***DNA Extraction***

135 DNA was extracted from the gut samples using 10% Chelex (24) and purified with
136 AMPure^{R} XP beads (Beckman Coulter, Brea, CA, USA) following the manufacturer's
137 instructions. Initial DNA concentrations following purification were evaluated using Quant-
138 iT PicoGreen dsDNA Assay kit (ThermoFisher, USA) following the instructions from
139 L(27)). Absorbance was measured at 530 nm, using a Tecan Ultra 384 SpectroFluorometer
140 (PerkinElmer, USA).

141

142 ***16S rRNA gene amplification and sequencing library preparation***

143 Bacterial diversity of the samples was analyzed by sequencing of amplicons generated from
144 the V3-V4 region of the 16S rRNA gene using the MiSeq Illumina platform. Two-stage PCR
145 amplification was performed using forward primer 341F: (CCTACGGGNGGCWGCAG) and
146 reverse primer 805R: (GGACTACHVGGGTWTCTAAT). The first PCR was carried out in
147 25-µl PCR reactions and comprised 0.02 U μl^{-1} Phusion polymerase (ThermoFisher, USA),
148 0.2 mM dNTP, 1 mM $MgCl_2$, 1 × Phusion reaction buffer, 0.5 µM of each primer as well as 5
149 ng of DNA template). The amplification protocol consisted of an initial denaturation at 98 °C
150 for 30 seconds followed by 35 cycles of 10 sec at 98 °C, 30 sec at 55 °C and 72 °C, and, a

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151 final extension step (72 °C for 10 min). PCR products were purified using Agencourt AMPure
152 XP beads (Beckman Coulter, Brea, CA, USA). Following this, amplicon PCR was performed
153 on 5 µl of equimolar amounts of PCR product using Nextera XT primers (Index 1 (N7XX)
154 and Index 2(S5xx)), targeting the same region of the 16S rRNA genes (8 cycles of 30 sec at
155 95 °C, 30 sec at 55 °C and 35 sec at 72 °C). The products were purified with Amplicons
156 AMPure XP Beads (Beckman Coulter) according to the manufacturer's protocol and
157 concentrations estimated using Quant-iT PicoGreen dsDNA Assay kit (ThermoFisher, USA).
158 Individually barcoded samples were mixed in equimolar amounts, and DNA sequencing
159 adaptor indexes ligated using the TruSeq DNA PCR-free LT Library Preparation Kit
160 (Illumina). Quality control was performed on an Agilent 2100 BioAnalyser using high
161 sensitivity DNA chip. PhiX DNA (10%) was added to the denatured pools, and sequencing
162 was performed on an Illumina MiSeq using the MiSeq V3 reagent kit (600-cycles) on the
163 Illumina MiSeq platform. De-multiplexing and removal of indexes and primers were done
164 with the Illumina software v. 2.6.2.1 on the instrument according to the standard Illumina
165 protocol.

166

167 ***Processing of sequencing data***

168 Following initial upstream de-multiplexing and index removal, sequences were analysed
169 using the *DADA2* v. 1.6 module (28) as implemented in the R statistical software v. 3.4.2
170 (29). The pipeline consisted of quality-filtering, trimming of bad quality (< Q30) stretches,
171 error estimation and de-replication of reads, merging of forward and reverse reads and finally,
172 removal of chimeric sequences. All remaining sequences were assigned taxonomy on the
173 genus level using the Silva Ribosomal RNA database version v.128. Subsequent statistical
174 analyses and visualization were done with the *Phyloseq* R-module v.1.22.3 (30) unless
175 otherwise stated.

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176

177 ***Analysis of Oxygen Radical Absorbance Capacity and protein content***

178 As a proxy for antioxidant capacity, we assayed oxygen radical absorbance capacity (ORAC)
179 according to (31) with minor modifications and normalized values to protein content. This
180 biomarker represents the water-soluble fraction of antioxidants and has been applied in
181 daphnids (32). Samples for ORAC and protein measurements were homogenized in 100 µL of
182 PPB buffer (75 mM, pH 7.4). Fluorescein was applied as a fluorescent probe (106 nM) and 2,
183 2- azobis (2-amidinopropane) dihydrochloride (AAPH) (152.66 mM) as a source of peroxyl
184 radicals. Trolox (218 µM, Sigma–Aldrich) was used as the standard. The assay was conducted
185 in 96-well microplates while 20 µL of homogenate sample was added to each well and mixed
186 with 30 µL of AAPH and 150 µL of fluorescein. Fluorescence was measured at 485nm/520nm.
187 Protein content of the supernatant was determined by the bicinchoninic acid method using a
188 Pierce BCA Protein Assay kit 23227 (Thermo Scientific) according to the microplate procedure
189 with some modifications. In each well, 25 µl of blank, standard or samples was added to 200
190 µl of working solution. Absorbance was measured at 540 nm using a FluoStar Optima plate
191 reader (BMG Lab Technologies, Germany). Antioxidant capacity was expressed as mg trolox
192 eq. mg protein^{-1}.

193

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194 ***Data analysis and statistics***

195 *Life- history traits*

196 Survival probability was calculated using Kaplan-Meier analysis, which estimates
197 the probability of an event (i.e., death) occurring in a given period (33). The logrank test was
198 used to evaluate differences in the survivorship between the treatments using package *survival*
199 in R (34).

200

201 The empirical von Bertalanffy growth model was applied to determine growth parameters
202 using length-at-age data fitted to the equation:

203 $$BL = BL_{max} \times (1 - exp^{(-K \times t)})$$

204 where BL is the total length at time t (days); BL_{max} is the length reached at an infinite time,
205 defined as the maximum potential length attained under the prevailing conditions; and K is the
206 growth rate. Statistical differences in BL_{max} and K between each treatment and control were
207 determined by non-overlapping 95% confidence intervals.

208 To analyze the effects of exposure time and ciprofloxacin concentration on the daphnid
209 fecundity, we used generalized linear models (GLM) with Poisson distribution and identity
210 link function. Residuals were checked visually, and nonsignificant interaction terms were
211 dropped from the analysis. A post hoc Tukey HSD test was used to compare the brood size
212 among the treatments for each clutch.

213 The daphnid population growth rate (PGR) was estimated according to Euler-Lotka's
214 equation using (R Core Team, 2018) (Appendix S10):

215 $$\sum_{x=\alpha}^{\beta} l(x)\ m(x) e^{-rx} = 1$$

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216 where $l(x)$ is the fraction of individuals surviving to age x and $m(x)$ is the birth rate per capita
217 for the mothers of age x. Bootstrapping (999 permutations) was used to estimate 95%
218 confidence limits of the PGR values in each treatment, and statistical differences in r between
219 each treatment and control were determined by non-overlapping 95% confidence intervals.

220

221 *Analysis of microbiota communities*

222 Diversity indices analysis

223 To assess the alpha diversity of the bacterial communities, we calculated commonly used
224 indices of diversity and evenness (ACE, Chao1 and Fisher´s alpha). Effects of time and
225 concentration on the diversity indices were tested by GLM with normal error structure and
226 log-link. Quantile plots were used to evaluate the distribution of the residuals and deviance
227 was used to access goodness of the model. Interaction (*time* × *concentration*) was first
228 included in every model but omitted if found not significant. The Principal coordinates
229 analysis (PCoA) with Bray-Curtis dissimilarity index was used to visualize differences in
230 community composition among the treatments (35). Differences in the community structure
231 at the family level were tested by permutational multivariate analysis of variance
232 (Permanova) using variance stabilized Bray-Curtis dissimilarity. Multivariate homogeneity
233 of treatment dispersion was assessed using the beta-disperser in the *vegan* package (36).

234

235 Connecting the microbiome to host fitness

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236 The R-package *edgeR* (37) was used to identify differentially abundant bacterial taxa (false
237 discovery rate-corrected *P*-values, $\alpha = 0.05$, FDR=1%) that were associated with high or low
238 growth rate (somatic and reproductive) of the daphnids. As a measure for somatic and
239 reproductive growth, we used BL and fecundity, respectively. For each trait, we created two
240 classes, *high* (above the group mean, coded as 1) and *low* (below the group mean, coded as 0)
241 using zeta scores for individual BL and fecundity measurements. Zeta scores (zero mean, unit
242 variance) were calculated based on clutch-specific mean values (all treatments included) and
243 corresponding standard deviations to account for the changes in BL and fecundity with the
244 daphnid age.

245

246 **Results**

247 *Survival and individual growth*

248 The survival rate was moderate to high (84% to 92%), not differing significantly among the
249 treatments (log rank test, $p > 0.8$), although the antibiotic-exposed animals had slightly higher
250 survival compared to the controls (Figure S1**)**. According to the individual growth curve
251 analysis, the animals exposed to the lowest ciprofloxacin concentration (0.01 mgL^{-1}) had a
252 significantly greater maximal body length (BL_{max}) compared to the control animals, whereas
253 the K values were similar across the treatments (Figure 1).

254 **Figure 1**. **This is the Figure 1 title.** Individual growth curve analysis

255

256 **This is the Figure 1 legend.**

257 Estimated BL_{max} and K values and corresponding 95%-confidence limits for *Daphnia*
258 *magna* grown in 0.01, 0.1 and 1 mg / L ciprofloxacin and the control.

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259

260 *Reproduction*

261 The average brood size was significantly higher in all ciprofloxacin treatments compared to
262 the control (GLM, $t_{263, 267} = 12.97, p < 0.001$; Figure 2), with the increase varying from 36% in
263 the 0.01 mg/L treatment ($t_{263, 267} = 4.347$; $p < 0.001$) to 42% in the 0.1 mg/L treatment ($t_{263, 267}$
264 $= 4.05$; $p < 0.001$). Also, there was a significant negative effect of time ($t_{263, 267} = -2.74$; $p <$
265 0.05), which was mainly related to the low fecundity in the last brood (Tukey HSD, $z_{(4-1)}$:-
266 3.084, $p_{(4-1)} < 0.01$; $z_{(4-2)}$: -5.97, $p_{(4-2)} < 0.01$; $z_{(4-3)}$: -3.34, $p_{(4-3)} < 0.005$). The total number of
267 offspring produced during the experiment per individual female was 27-36% higher in the
268 daphnids exposed to ciprofloxacin compared to controls.

269

270 **Figure 2**. **This is the Figure 2 title.** Reproduction of *Daphnia magna* (brood size and time of
271 reproduction) during a 21-d exposure to ciprofloxacin (0.01, 0.1, and 1 mg / L) and a control.
272 **This is the Figure 2 legend.** Breadth of the box indicates an extended period for clutch
273 release within a treatment, i.e., non-synchronous reproduction. Note that the last clutch was
274 estimated from both the number of offspring released and the number of embryos in the brood
275 chamber at the termination of the experiment.

276

277 *Population growth rate*

278 The population growth rate (PGR) varied from 0.26 to 0.30 among the treatments and was
279 higher in the exposed daphnids relative to the control by 17%, 19% and 15 % in the animals
280 exposed to 0.01, 0.1 and 1 mgL^{-1}, respectively. The differences from the control were
281 significant for all treatments (Table S1).

282

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283 *Characterization of the gut microbiota in Daphnia*

284 A total of 1314 high-quality sequences were obtained after trimming and assembly. The core
285 gut microbiome of our test animals was dominated by Proteobacteria, which contributed on
286 average 74% (ranging from 25% to 95% in individual specimens). When all treatments were
287 considered, Actinobacteria (15%), Bacteroidetes (7%), Firmicutes (1%) and
288 Verrucomicrobia (1%) were also common. In the non-exposed animals, the contributions
289 were different, with Proteobacteria, Bacteroidetes and Verrucomicrobia being the most
290 common (Figure S2e). Together, these five phyla formed the core microbiome of the gut and
291 comprised on average 99% of the OTUs assigned to phylum level (Table S5a).

292

293 The major classes of bacteria found in all treatments, in order of prevalence, were
294 Betaproteobacteria (35% of total OTUs), Gammaproteobacteria (29%), Actinobacteria
295 (14%), Alphaproteobacteria (9%), Cytophagia (5%), and Verrucomicrobia (1%). In the non-
296 exposed animals, Cytophagia was the third most abundant group, contributing 8 to 36%
297 throughout the experiment, whereas Actinobacteria contributed less than 2% on average.
298 Bacilli, Sphingobacteria and Bacteroidia were found together in about 3% of total reads
299 assigned at class level (Table S5b).

300

301 We found members of 62 orders in all treatments (Table S5c). Predominant orders included
302 Burkholderiales (34%), Oceanospirillales (15%), Alteromonadales (10%), Rhizobiales (7%),
303 Micrococcales (5%), and Cytophagales (5%), which was the second most represented order
304 (16%) in the non-exposed animals. The core gut microbiome were formed by these orders
305 along with Propionibacteriales, Corynebacteriales, Pseudomonadales and Methylophilales
306 representing almost 89% of the OTUs assigned at the order level.

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307

308 Members of 101 families comprising 252 genera were identified as unique reads and assigned
309 at the family and genus level. Across the treatments, Comamonadaceae (33%),
310 Halomonadaceae (15%), Shewanellaceae (10%), and Cytophagaceae (5%) were the most
311 common (Table S5e). In the non-exposed animals, Comamonadaceae (65%) and
312 Cytophagaceae (17%) were the most common. When all treatments were considered, the most
313 abundant genera were *Limnohabitans, Shewanella, Halomonas, Bosea*, and *Leadbetterella*.
314 These genera contributed on average 71% (ranging from 57% to 81%) to the gut microbiota.
315 In the non-exposed animals, however, *Bosea* was not contributing to the core microbiome
316 (Figure S2a).

317

318 *The effects of ciprofloxacin on the gut microbiota*

319 Chao1, ACE and Fisher´s alpha indices were negatively co-related to ciprofloxacin
320 concentration (Figure 3a and Table S3 and S4). According to the PCoA, populations
321 exposed to 0.1 and 1 mgL^{-1} clustered closely to each other, with higher loadings on the
322 second axis, which separated them from the control (Figure 4). The homogeneous dispersion
323 (Betadisper, $p > 0.05$, Table S3a) met the assumption for further pairwise comparison
324 between the treatments, and a permutation test detected significant differences between the
325 communities exposed to ciprofloxacin and those in control (Permanova, pairwise
326 comparison $p < 0.05$, Table S4). Differential abundance analysis showed the most
327 Ciprofloxacin sensitive bacteria to be *Leadbetterella* (Bacteroidetes), and *Hydrogenophaga*
328 and *Methylotenera*, both Betaproteobacteria. On the opposite end of the scale (most
329 refractory) were *Pseudorhodoferax*, *Shewanella*, and *Halomonas* (Beta- and Gamma-

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330 Proteobacteria), as their abundance in the exposed animals had increased significantly
331 following antibiotic exposure (Figure 5a, Table S6).

332

333 **Figure 3**. **This is the Figure 3 title.** Alpha diversity indices (Chao1, ACE, and Fisher) obtained
334 for gut microbiota

335 **This is the Figure 3 legend.** Communities grouped by (a) ciprofloxacin concentration and (b)
336 clutch number during the 21-day exposure. Clutch "0" indicates initial diversity of individuals.
337 Points indicate specific values for individual daphnids.

338

339 **Figure 4**. **This is the Figure 4 title**. Principal coordinate ordination (PCoA) of the 16S rRNA
340 gene libraries based on the Bray-Curtis dissimilarity.

341 **This is the Figure 4 legend**

342 Colors indicate treatments, i.e., concentration of ciprofloxacin (Control: 0, 0.01, 0.1, and 1 mg
343 / L). The ellipsoids represent a 95% confidence interval of normal distribution surrounding each
344 group. Point labels indicate day of sampling. Plot shows the clear clustering of bacterial
345 communities in the treatments exposed to the two highest concentrations of ciprofloxacin (0.1
346 and 1 mg / L), as well as between communities in the controls and the lowest exposure
347 concentration (0.01 mg / L).

348

349 **Figure 5**. **This is the Figure 5 title.** Differential abundance analysis of gut bacteria
350 **This is the Figure 5 legend.** Bacterial genera significantly associated with (a) exposure to
351 ciprofloxacin; (b) high somatic growth and fecundity of the host observed during the

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352 experiment. The fold change (log2FC) and the associated statistics were derived by the edgeR
353 test.)
354
355

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356

357 *Changes of the gut microbiota with time*

358 Although diversity (Fisher´s alpha) increased with time of exposure, concentration had a more
359 profound than time on this index (Figure 3b; Table S2). Chronic exposure to ciprofloxacin,
360 resulted in a significantly lower diversity in the exposed animals (Figure 3a, Table S2). All
361 diversity indices showed a similar trend over time, with a high diversity during the first two
362 weeks (the first clutch), a decrease observed at the time of the second clutch, following by an
363 increasing trend. However, the time effect was not significant (Table S2).

364

365 *Linkages between the gut microbiome and life-history traits*

366 The diversity indices correlated negatively with fecundity, while only Fisher's alpha had a
367 positive correlation with body size. The differential abundance analysis indicated that genera
368 *Bosea* and *Hydrogenophaga* were more abundant in the daphnids with high and low somatic
369 growth, respectively (Table S7; Figure 5b). Moreover, *Bosea* and *Galbitalea* were
370 significantly more abundant in the daphnids with higher fecundity, whereas abundances of
371 *Leadbetterella* and *Hydrogenophaga* in these individuals were significantly lower (Table S7,
372 Figure 5b). Thus, *Bosea* and *Hydrogenophaga* were consistently associated with high and low
373 growth phenotypes, respectively.

374

375 *Biomarker ORAC/Protein responses to antibiotic exposure*

376 The total antioxidant capacity (ORAC, g Trolox eq. g protein^{-1}) was significantly higher in the
377 animals exposed to lower concentrations of ciprofloxacin (0.01 and 0.1 mgL^{-1}) (Figure 6,
378 Table S8). Moreover, there was a significant positive relationship between the individual
379 ORAC and body length (GLM; Wald stat. = 5.83, $p < 0.02$; Table S9, Supporting

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380 Information) across the concentrations tested. The correlations between the ORAC values and
381 diversity indices were negative and marginally significant (Table S10, Supporting
382 information).
383
384 **Figure 6**. **This is the Figure 6 title.** . *Daphnia magna*: response of the total antioxidant
385 capacity (ORAC, g Trolox eq. g protein^{-1}) to the ciprofloxacin concentration.
386 **This is the Figure 6 legend.** The individuals sampled after their fourth clutch were excluded,
387 because some of them contained eggs in the brood chamber. The non-matching letters
388 indicate significant differences between the groups (Tukey's multiple comparisons test; $p < 0.05$
389). See Table S9 for details on the statistical comparisons.
390
391
392 **Discussion**
393 The intestinal microbiome plays an essential role in regulating many aspects of host
394 physiology, and its disruption through antibiotic exposure has been implicated in microbiota-
395 mediated consequences on host fitness. We examined effects of chronic antibiotics exposure
396 on *Daphnia magna* gut microbiota in concert with fitness-related responses of the host. As
397 hypothesized, the exposure to ciprofloxacin resulted in profound changes in the microbiome
398 and a reduced microbial diversity at all concentrations tested (0.01 to 1 mgL^{-1}). Surprisingly,
399 no negative effects on daphnid antioxidant levels, fitness and mortality were observed.
400 Moreover, the negative changes in the microbiome coincided with increased antioxidant
401 capacity, individual growth and host reproduction and, as a result, significantly higher
402 population growth in the animals exposed to ciprofloxacin. Thus, the hypothesized positive
403 correlation between microbiome diversity and host performance was not observed. Our

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404 findings indicate that reliance on shifts in taxonomic composition of bacterial community
405 generates an incomplete picture of the functional effect of antibiotic intervention in a non-
406 target eukaryote. A full mechanistic understanding will require further study of the specific
407 functional relationships between the host and its core microbiome, and the integration of
408 metabolomic and phenotypic data. Moreover, in case of antibiotic-mediated intervention, we
409 need to disentangle direct effects of the exposure on host physiology. This is already evident
410 in human microbiome study where drug effects on mitochondrial activity are known to
411 confound (38,39).
412
413 *Core microbiome of* Daphnia magna
414 Proteobacteria, Actinobacteria and Bacteroidetes comprise a core microbiome of the *Daphnia*
415 *magna* intestine. Most taxa (or their close relatives) identified in this study as a part of core
416 microbiome have previously been reported in *Daphnia* (21,40,41). The Comamonadaceae
417 family of Burkholderiales have been shown to be the most abundant family in *Daphnia* gut
418 microbiota (41,42), and were most prevalent in our test animals. Other taxa found in high
419 abundance were the Gammaproteobacteria orders Oceanospirillales and Alteromonadales, and
420 the families *Nocardioidaceae*, *Microbacteriaceae*, and *Moraxellaceae* (21,12). On the genus
421 level, more differences between earlier reported daphnid associated taxa and our dataset were
422 evident. In addition to *Limnohabitans*, other identified microbial taxa were *Pseudorhodoferax*
423 and *Hydrogenophaga* (Burkholderiales) but not the previously reported *Bordetella*,
424 *Cupriavidus* (43), *Ideonella* and *Leptothrix* spp. (41). Also, *Enhydrobacter* was the dominant
425 genus of Moraxellaceae in our study (Table S5e), while *Acinetobacter* spp. was reported in
426 other studies (12,20). *Methylibium* was only found in the animals that were exposed to 0.01
427 mg / L of Ciprofloxacin and not in the non-exposed individuals, suggesting that this genus is
428 relatively rare if normally present. Together, our results present a relatively stable bacterial

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429 composition in the *Daphnia* gut from a higher taxonomic level, suggestive of functional or
430 other redundancy in the preferred association of daphnids with their microbiota components.
431
432 *Effects of Ciprofloxacin on the Daphnia gut microbiome*
433 Drug exposure significantly altered the microbiome, with a decrease or even the
434 disappearance of many taxa by the end of the experiment at lowest exposure concentration
435 and within a first week at higher concentrations (Figure 3b, Table S5). Although diversity
436 decreased with both ciprofloxacin concentration and exposure time, only the concentration
437 effect was significant (Table S2, Figure 3). G+ bacteria, mostly *Actinobacteria* and
438 *Firmicutes*, were better able to withstand ciprofloxacin effects as their relative abundance
439 increased with drug concentration (Figure. S4a), while the G- bacteria had divergent
440 responses (Figure. S4b). For example, *Hydrogenophaga* and *Pseudorhodoferax*, both
441 belonging to the G- genus *Burkholderiales*, had clearly opposite responses, decreasing and
442 increasing, respectively, with increasing concentration. This is in line with earlier studies that
443 demonstrated higher susceptibility to Ciprofloxacin among G- bacteria, as compared with co-
444 occurring G+ species (44). This is evident for the typically low minimum inhibitory
445 concentrations, MICs, estimated for Alphaproteobacteria, such as Escherichia/Shigella,
446 (commonly in the low μM range) as compared with that for many Firmicutes, which are
447 usually in the mM range.
448 At higher concentrations of Ciprofloxacin, several genera representative of the core
449 microbiome declined to non-detectable levels; the *Limnohabitans* genus was replaced by
450 *Halomonas* and *Shewanella*, whose relative abundances increased with drug concentration
451 (Table S5e). *Shewanella* is a known acid producer (45) and may alter the pH balance in the
452 gut microenvironment when at higher densities. This would suppress the growth of
453 *Limnohabitans* who grow preferentially under neutral and alkaline conditions (46). Such

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454 community-level effects probably play a significant role in the dynamics of specific bacterial
455 taxa as a result of the exposure to antibiotics.
456

457 *Effects of Ciprofloxacin on Daphnia life history traits and antioxidant levels*

458 Previous studies on aposymbiotic daphnids showed that disruption in gut microbiota, either
459 by drugs or diet, had adverse effects on nutrition (40) (11), immunity (8), growth (12),
460 fecundity (22), and longevity (47). The effects that we observed however, were most
461 prominent at low antibiotic concentrations. Despite the ciprofloxacin-induced shifts in the
462 microbiome composition, ORAC levels, growth and reproduction in the daphnids were
463 similar or even significantly higher than in controls. The discrepancy between the microbiome
464 and the organism-level responses may result from the variable susceptibility of various
465 microbes to the broad-spectrum Ciprofloxacin and additional variability related to induction
466 of the SOS response pathways in different taxa.

467 The mismatch between microbiome change and host response suggests that other drivers,
468 such as a direct effect of Ciprofloxacin on the host, were involved, leading to the observed
469 effects on growth and reproduction. In line with this, a biphasic dose-response to
470 ciprofloxacin observed in human fibroblast cells, manifesting as increased cell proliferation
471 and viability when compared to non-exposed controls (48). In *Daphnia magna*, the
472 reproduction response to ciprofloxacin was also biphasic, with stimulatory effects at
473 concentrations below 5 mg/L (49). This is in line with the positive response induced by the
474 test concentrations utilized in our study (0.01-1 mg/L). In mice, ciprofloxacin has also been
475 shown to improve survival by enhancing immune efficiency via stimulating cytokine
476 production (50). In addition, several *in vitro* and *in vivo* studies using animal and tissue
477 models have revealed that fluoroquinolones like ciprofloxacin, induce oxidative stress via
478 reactive oxygen species (ROS) production, in a dose- and time-dependent manner (49,51).

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479 Measurable ROS production was observed following an exposure to ciprofloxacin at
480 concentrations as low as 0.025 mM (53), which is within the concentration range used in our
481 study. At low levels of such pro-oxidative exposure, the additional production and/or activity
482 of the endogenous antioxidant enzymes and low-molecular weight antioxidants to remove the
483 continuously generated free radicals would increase (54). In the daphnids exposed to the
484 lowest Ciprofloxacin concentration, a significant increase in ORAC levels (Figure S3)
485 suggests that exposure had direct stimulatory effects on the antioxidant production.
486 Moreover, we observed a positive correlation between the ORAC levels and animal body size
487 across the treatments indicating a possible primary mechanism behind the observed effects
488 being a hormetic shifting of redox environment by pro-oxidative ciprofloxacin, antioxidant
489 response and the resulting beneficial effects on growth. Such effects are in agreement with a
490 concept of physiological conditional hormesis (55) and suggest a possible mechanism for the
491 direct response of *Daphnia magna* to Ciprofloxacin exposure at environmentally relevant
492 concentrations. An important caveat is that hormesis, also shown to occur in several
493 microbes' response to quinolones and fluoroquinolones (the so-called paradoxical effect) (56)
494 might be universal and thus ciprofloxacin may be suboptimal for the uncomplicated study on
495 microbiome involvement following dose-response relationships.

496

497 *Microbiome-fitness relationships*

498 Although elevated growth and reproduction were associated with some bacterial taxa, there
499 was no clear signal for involvement of the gut microbiome in the high-growth phenotype.
500 This is suggestive of a form of redundancy in host-microbiome function, i.e., microbes can be
501 exchanged with little or no penalty. Moreover, as mechanisms governing most observed
502 associations are not well understood, definitive conclusion of direct effects by specific
503 microbes is intuitively discouraged. In particular, several taxa (*Bosea* and *Shewanella*)

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504 significantly associated with fitness-related variables have been shown to be highly resistant
505 to ciprofloxacin (57,58). Thus selection although acting directly on the polymicrobial
506 community, it does so differentially and although the effect may be due to absolute numbers
507 of microbes, the cumulative physiological and metabolic state may matter more. In line with
508 this, the relative abundance of those genera that were associated with higher fecundity and
509 growth barely comprise 5% of the organism's core microbiome (Table S5), suggesting that
510 sheer abundance was unlikely to be the primary factor driving host fitness.

511 An important caveat is that hormesis, also shown to occur in several microbes' response to
512 quinolones and fluoroquinolones (the so-called paradoxical effect) (56) might be universal
513 and thus ciprofloxacin may be suboptimal for the uncomplicated study on microbiome
514 involvement following dose-response relationships.

515 It is a common view that strains capable of supplying essential elements for reproduction and
516 growth would benefit the host. For example, the key components of *Daphnia* gut microbiota,
517 *Limnohabitans*, *Aeromonas* and methanotrophic bacteria (47), have been linked to acquisition
518 of essential amino acids (58,38), polyunsaturated fatty acids (PUFA) and sterols (60) that
519 positively affect *Daphnia* growth and reproduction (9,61). Surprisingly, none of these taxa
520 were associated with elevated growth and fecundity in our study. This also speaks for
521 functional redundancy although additional studies would be required to show this. At the
522 genus level, only *Bosea* and *Galbitalea* had significantly positively association with *Daphnia*
523 growth and fecundity, whereas *Leadbetterella* and *Hydrogenophaga* correlated negatively.
524 *Leadbetterella* and *Hydrogenophaga* were previously found to be associated to 8 Daphnia
525 genotypes (62). More interestingly however, the Bradyrhizobiaceae (*Bosea*) and
526 Microbacteriaceae (*Galbitalea*) are bio-degraders capable of producing hydrolytic enzymes
527 such as chitinase, cellulase, glucanase, protease, etc. (57,63). As these are positively
528 correlated with fecundity and host fitness, it suggests that increased network density and

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529 number of degradation pathways may contribute by providing essential nutrients from more
530 available substrates (64). Regardless of the mechanisms underlying their increased
531 abundance, resistance, or at the very least, refractoriness to Ciprofloxacin cannot be ignored.
532 Such effects would be evident in perturbed outcome of inter- and intra-species competition
533 and illustrates one of the difficulties facing future studies into host-microbiome interactions.

534

535 ***Acknowledgements***

536 The computations were performed on resources provided by SNIC through Uppsala
537 Multidisciplinary Center for Advanced Computational Science (UPPMAX) under project
538 2018/8-68. Sequencing and analysis of microbiome results were made possible by grant #
539 20160933 from the Stockholm County Council (SLL) to KU.

540

541 **Conflict of Interest**

542 The authors declare no conflict of interest.

543

544

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719

720 **Supporting information**

721 **Figure S1**. Survival of *Daphnia magna* exposed to ciprofloxacin (0.01, 0.1 and 1 mg/L) and in
722 the control (M7 only) during the 21-d exposure.

723

724 **Figure S2**. Relative abundance of bacterial taxa in the microbiome of *Daphnia magna* from
725 the non-exposed treatments: (a) genera, (b) families, (c) orders, (d) classes, and (e) phyla. The
726 data are grouped by the exposure week, 1 to 4 (Y-axis). Animals collected at the termination
727 of the experiment are included in the week 4.

728

729 **Figure S3**. Change in the total antioxidant capacity (ORAC, g Trolox eq. / g protein) in
730 individual daphnids during the course of the experiment. The data are shown for each
731 treatment (ciprofloxacin exposure, 0.01, 0.1 and 1 mg / mL) and the control. The regression
732 line and the 95%-confidence interval are shown to indicate the overall direction of change
733 over time; no trends are significant ($p > 0.05$).

734

735 **Table S1**. Population growth rate (*r*) of *Daphnia magna* in the control and ciprofloxacin
736 exposure (0.01 – 1 mg/L) and the corresponding 95-% confidence interval estimated by
737 bootstrapping. Asterisk indicates significant difference from the control; when the confidence
738 intervals were not overlapping, the difference was considered significant.

739

740 **Table S2**.

741 Diversity indices were calculated using individual data rarefied to equal sequencing depth at
742 treatment level. Effects of concentration and time on the diversity indices (Fisher´s alpha,

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743 Chao1 and ACE) were evaluated using GLM. Interactions were included first in each model
744 but omitted when found not significant.
745
746 **Table S3**. Multivariate homogeneity of groups' dispersions (betadisper) of samples analyzed
747 according to treatment (Ciprofloxacin concentration).
748
749 **Table S4**. PERMANOVA output with Bray-Curtis dissimilarity testing differences between
750 treatments at family level.
751
752 **Table S5**. Relative contributions of the ten most common bacterial taxa to gut microbiota of
753 *Daphnia magna* exposed to ciprofloxacin (0.01. 0.1. and 1 mg/L) and in control (0 mg/L) as
754 well as the average relative abundance for all treatments.
755
756 **5Table S6**. Differential abundance of individual genera estimated by edgeR-function and
757 testing taxa-specific responses to ciprofloxacin exposure. The positive log2FC values indicate
758 increased relative abundance in the exposed daphnids compared to the controls. Significance
759 presented at false discovery rate of 5%. (FDR<0.05). See also Figure 7a.
760
761 **Table S7**. Differential abundance analysis of individual genera estimated by the edgeR-
762 function and testing associations between the microbiome and host fitness. The genera
763 positively associated with high growth or fecundity of *D. magna* have positive log2FC values.
764 All values reported are significant at false discovery rate of 1%. (FDR<0.01). See also **Error!**
765 **Reference source not found.**b.
766

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767 **Table S8**. Effect of ciprofloxacin concentration (mg mL^{-1}) on antioxidant capacity in
768 *Daphnia magna*: (A) ANOVA results testing overall effect, and (B) Pair-wise comparisons
769 using Tukey's multiple comparisons test; $p < 0.01$: **, $p < 0.05$: *; and $p > 0.05$: ns. The
770 individuals sampled at the termination of the experiment were excluded, because some
771 daphnids contained eggs in the brood chamber. As the reference group, we used the daphnids
772 exposed to the highest concentration. See also Figure S3.
773
774 **Table S9**. Generalized linear model output linking antioxidant capacity to daphnid body
775 length across the concentrations tested. Normal error structure and log-link function were
776 applied. The animals collected at the termination of the experiment were excluded, because
777 they had eggs in the brood chamber, which may affect the ORAC values.
778
779 **Table S10**. Spearman rank correlation between the ORAC values in the daphnids and
780 diversity indices of their gut microbiome.
781
782 **Appendix S11.**
783 R script used to calculate population growth rate of daphnids applying Euler-Lotka equation:
784

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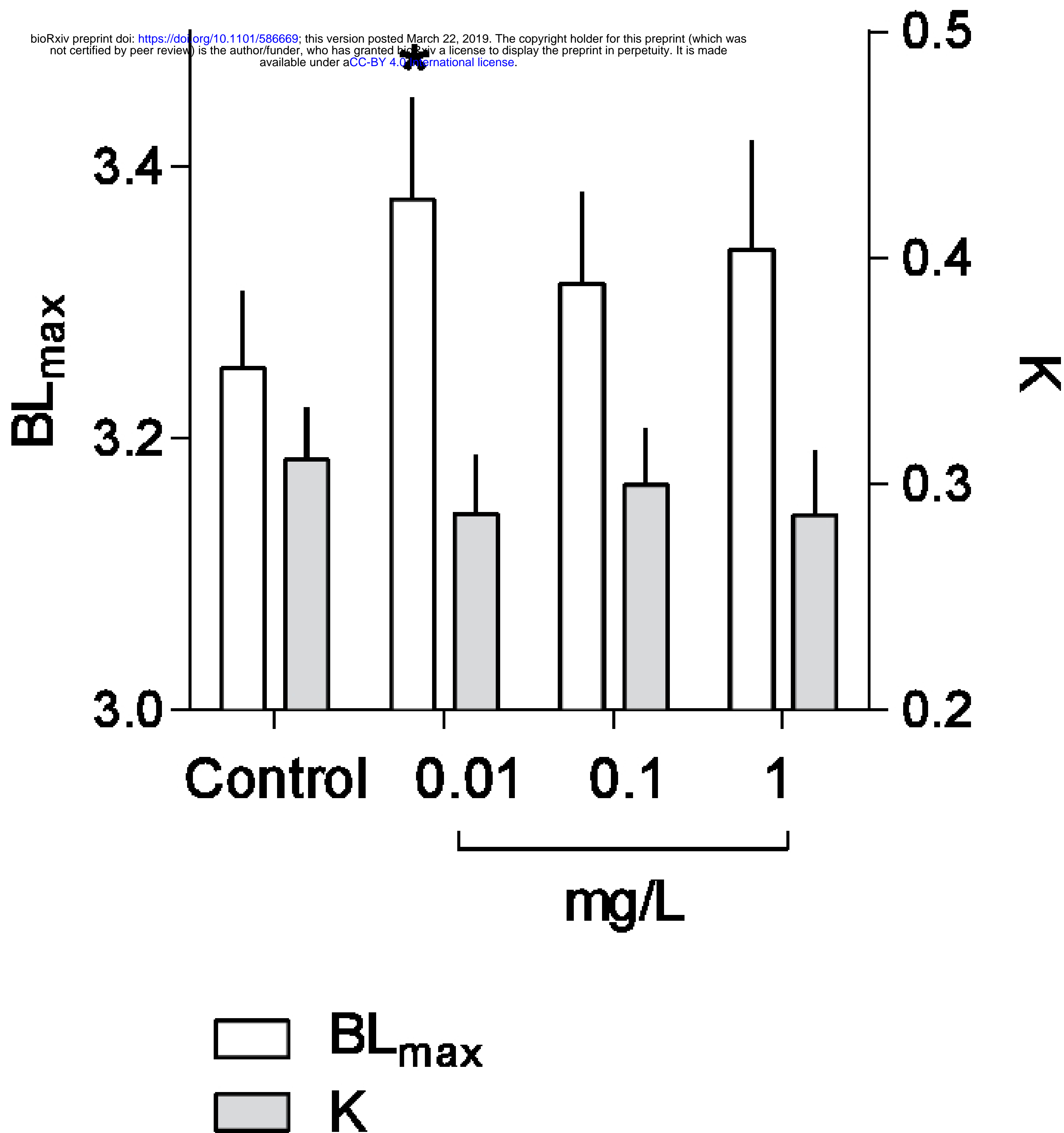


Figure 1

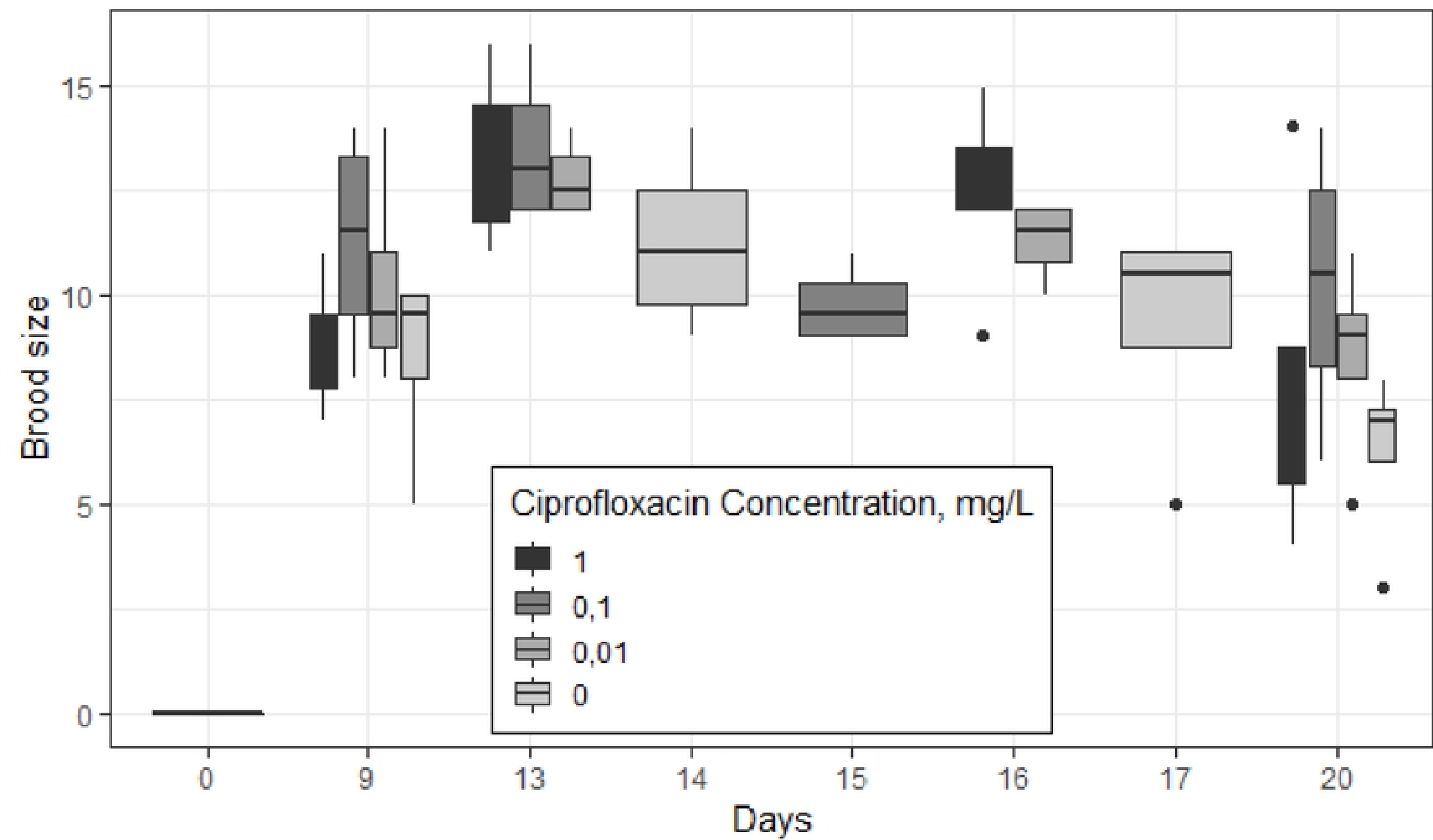


Figure 2

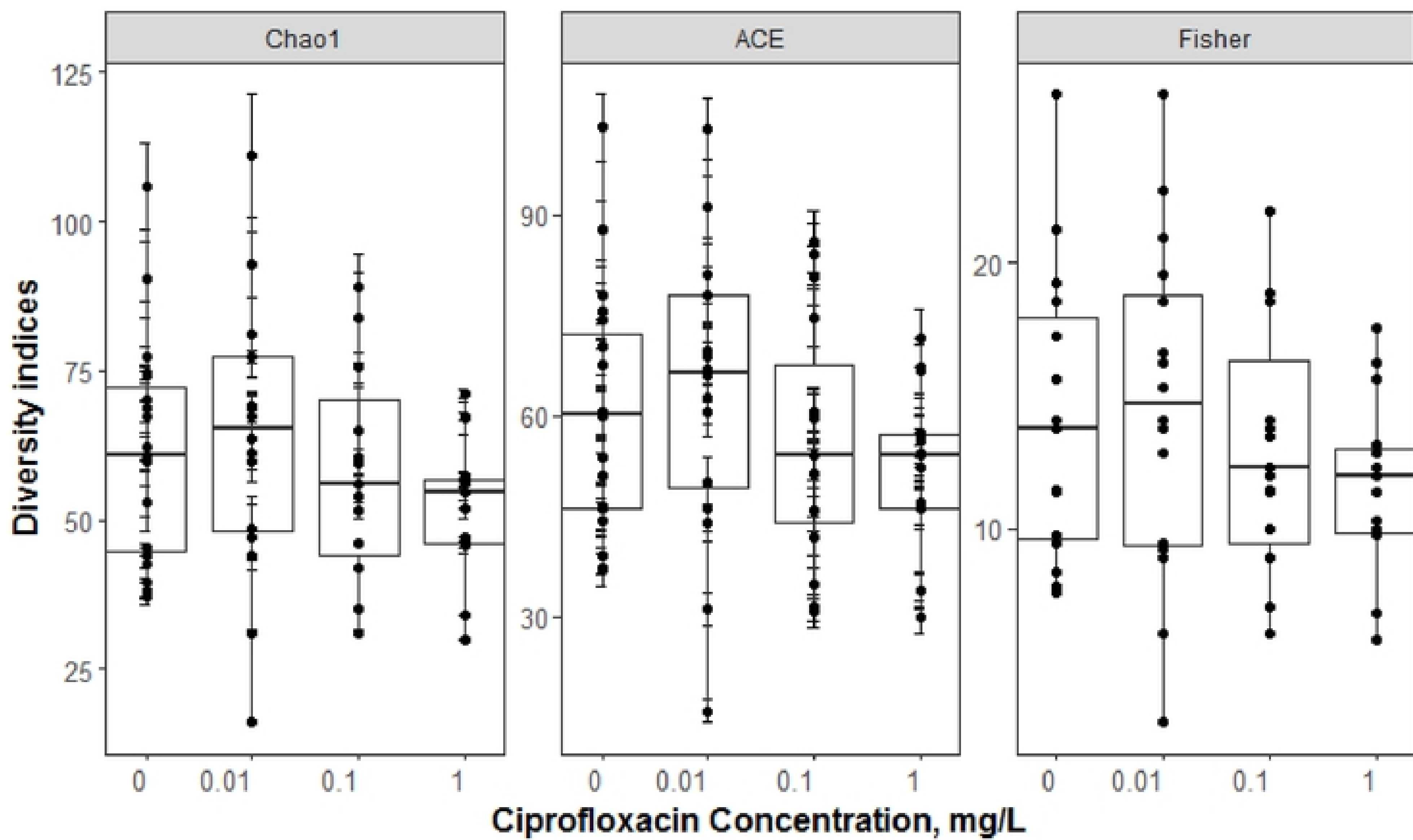


Figure 3a

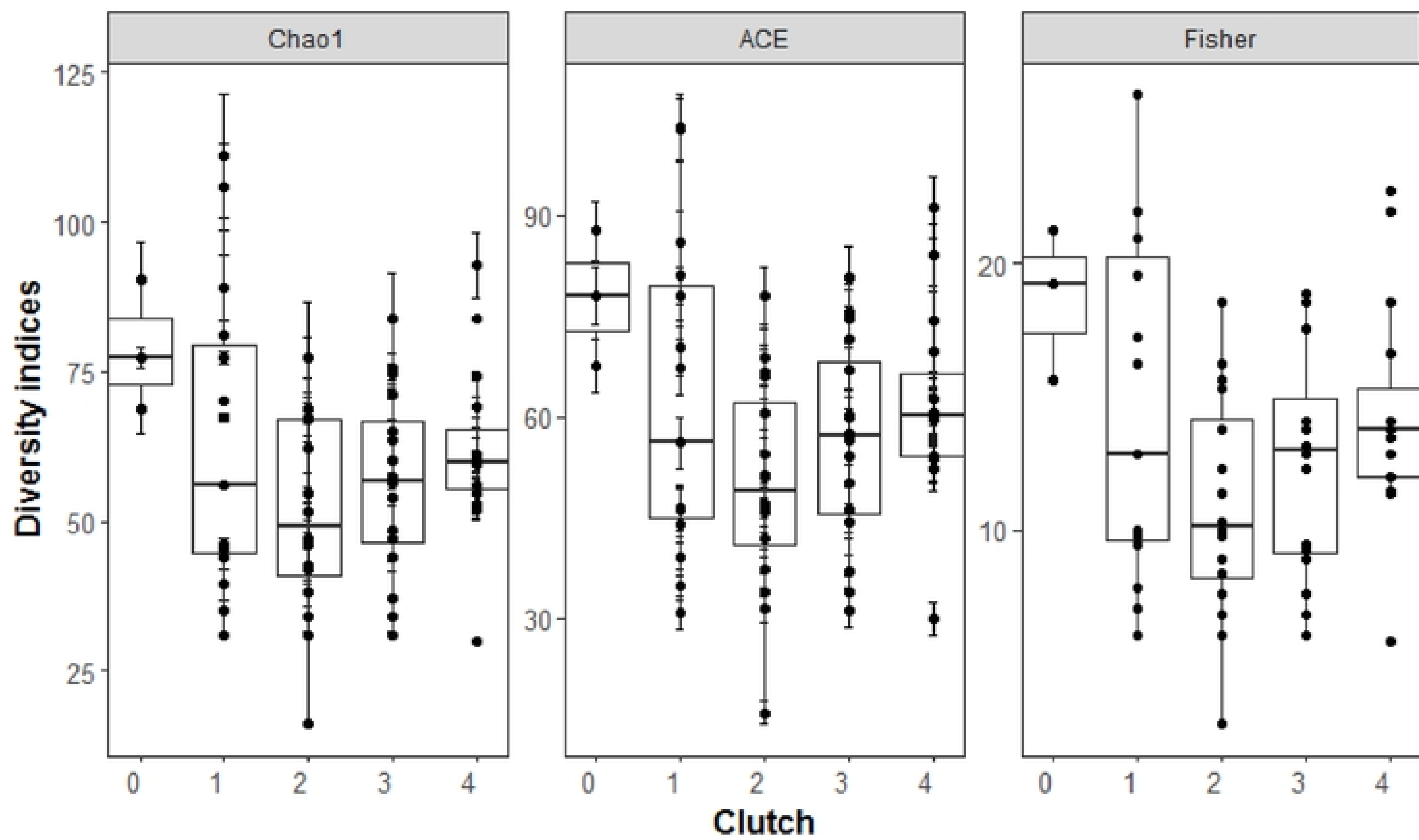


Figure 3b

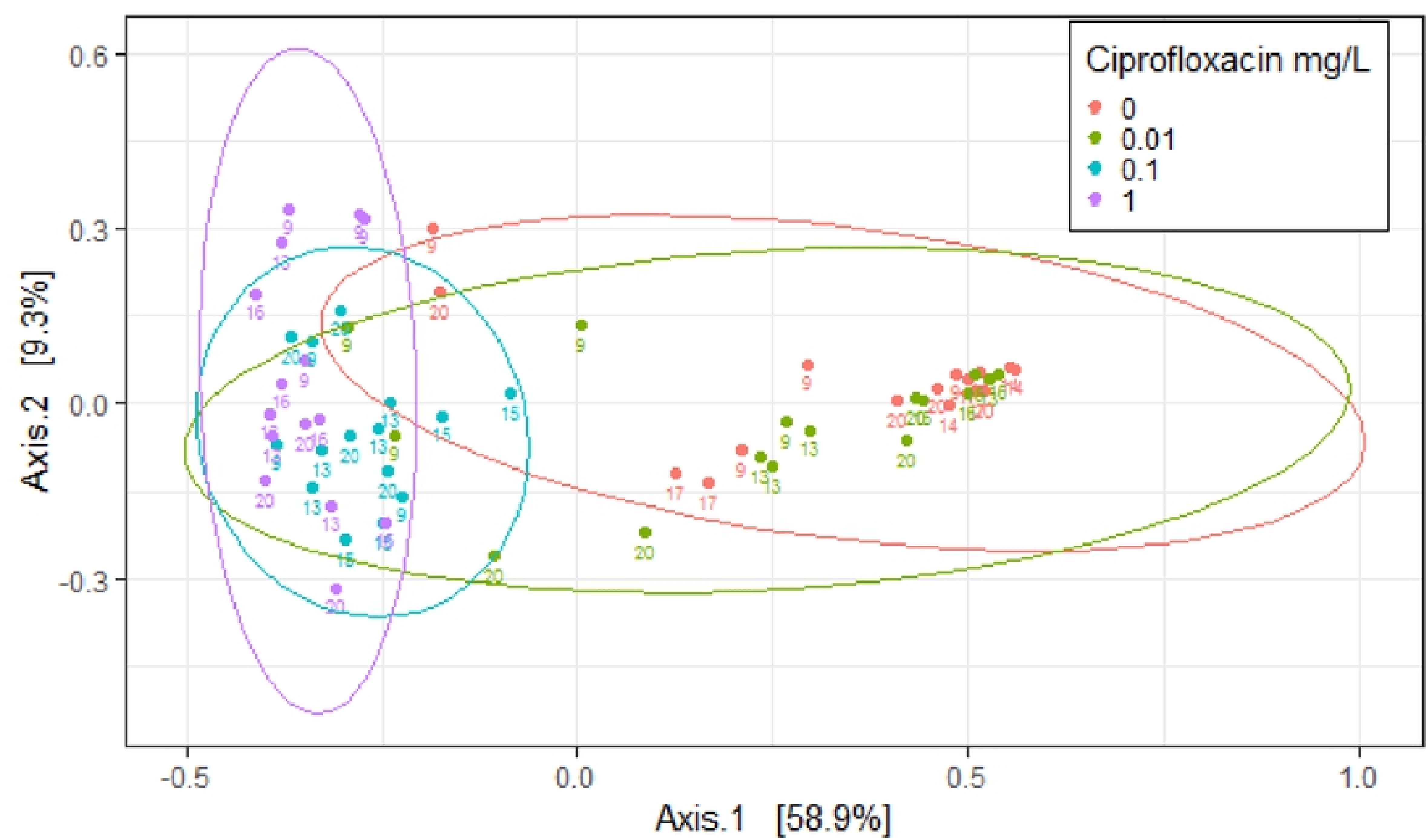
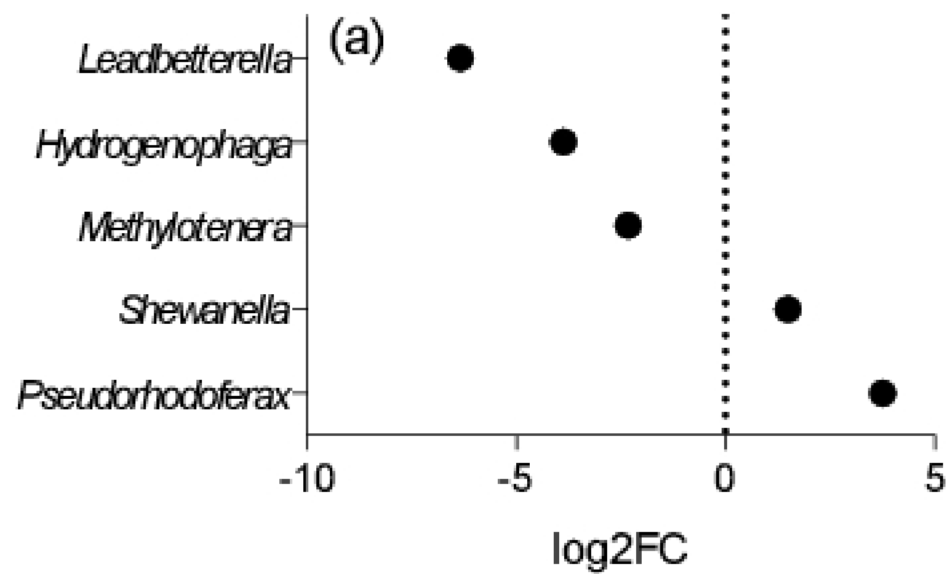


Figure 4



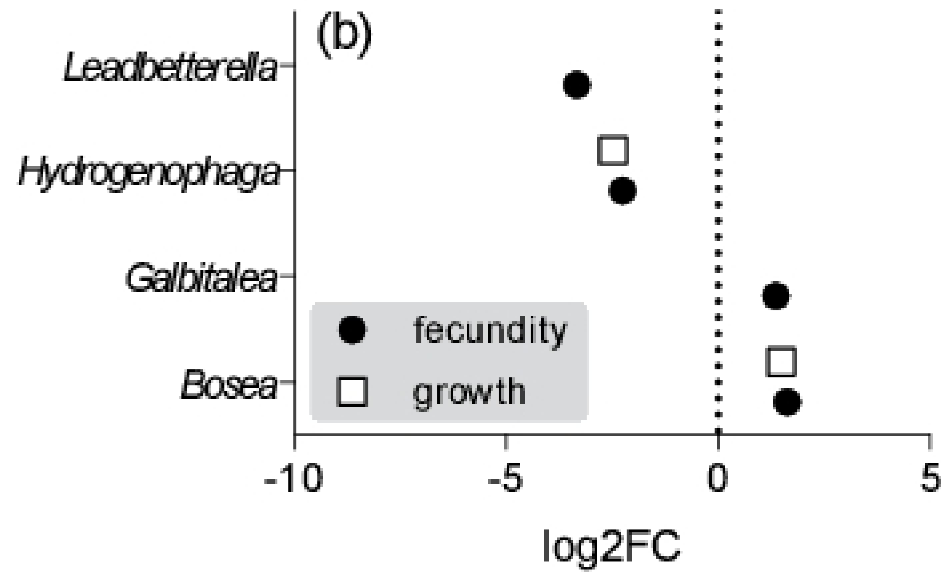


Figure 5

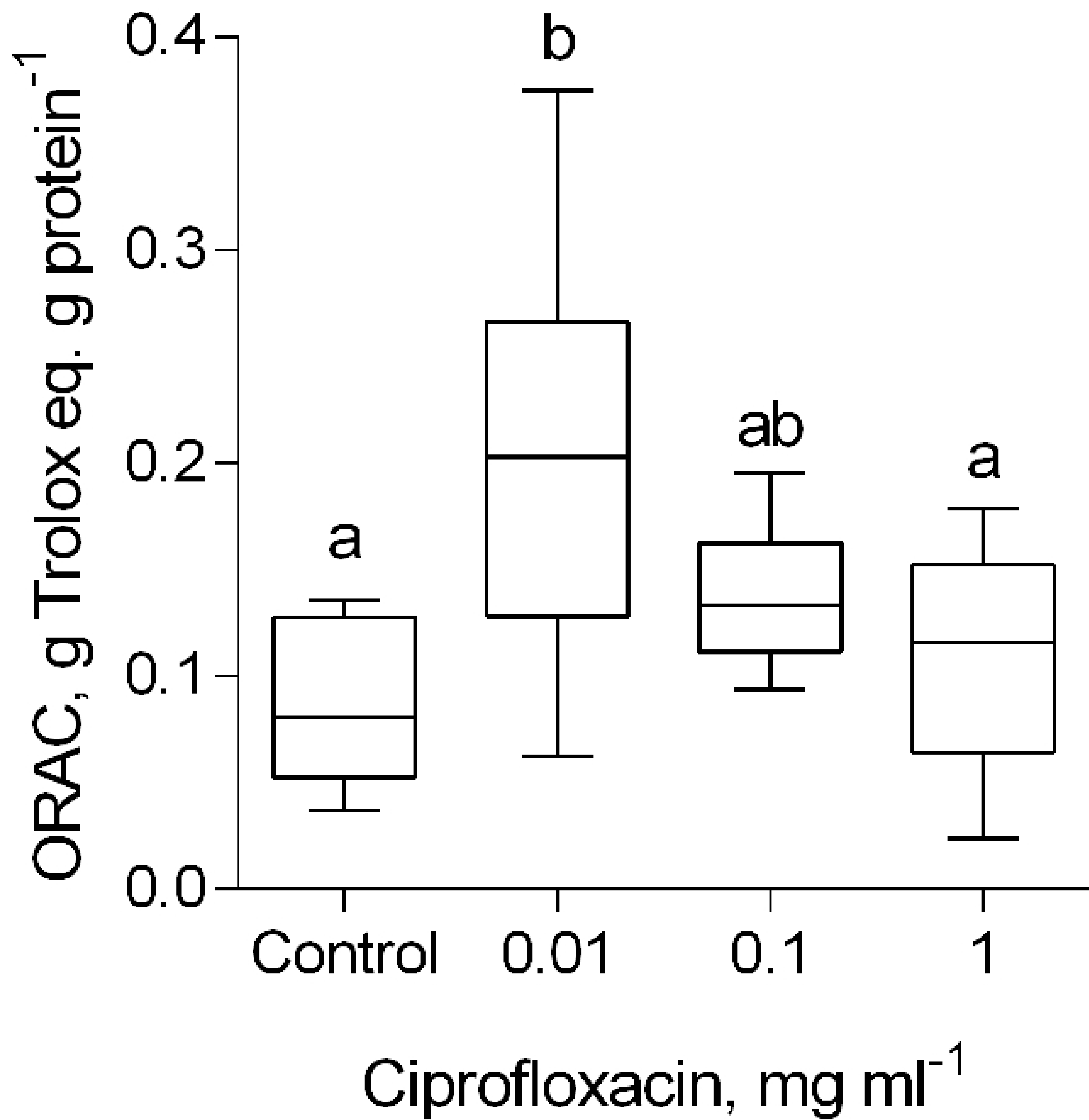


Figure 6