Virulence and transmission biology of the widespread, ecologically important pathogen of zooplankton, *Spirobacillus cienkowskii*

Authors

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Abstract

Spirobacillus cienkowskii (*Spirobacillus,* hereafter) is a widely distributed bacterial pathogen that has significant impacts on the population dynamics of zooplankton (*Daphnia spp.*), particularly in months when *Daphnia* are asexually reproducing. Yet little is known about *Spirobacillus'* virulence, transmission mode and dynamics. As a result, we cannot explain the dynamics of *Spirobacillus* epidemics in nature or use *Spirobacillus* as a model pathogen, despite *Daphnia's* tractability as a model-host. Here, we work to fill these knowledge gaps experimentally. We found that *Spirobacillus* is among the most virulent of *Daphnia* pathogens, killing its host within a week and reducing host fecundity. We further found that *Spirobacillus* did not transmit horizontally among hosts unless the host died or was destroyed (i.e., it is an "obligate killer"). In experiments aimed at quantifying the dynamics of horizontal transmission among asexually reproducing *Daphnia*, we demonstrated that *Spirobacillus* transmits poorly in the laboratory. In mesocosms, *Spirobacillus* failed to generate epidemics; in experiments wherein individual *Daphnia* were exposed, *Spirobacillus'* transmission success was low. In the (limited) set of conditions we considered, *Spirobacillus'* transmission success did not change with host density or pathogen dose and declined following environmental incubation. Lastly, we conducted a field survey of *Spirobacillus'* prevalence within egg-cases (ephippia) made by sexually reproducing *Daphnia*. We found *Spirobacillus* DNA in ~40% of ephippia, suggesting that, in addition to transmitting horizontally among asexually reproducing *Daphnia*, *Spirobacillus* may transmit vertically from sexually reproducing *Daphnia*. Our work fills critical gaps in the biology of *Spirobacillus* and illuminates new hypotheses vis-à-vis its life-history.

Importance

Spirobacillus cienkowskii is a bacterial pathogen of zooplankton, first described in the 19th Century and recently placed in a new family of bacteria, the *Silvanigrellaceae*. *Spirobacillus* causes epidemics in lake zooplankton populations and increases the probability that zooplankton will be eaten by predators. However, little is known about how *Spirobacillus* transmits among hosts, its impact on host survival and reproduction (i.e., how virulent it is) in laboratory conditions and what role virulence plays in *Spirobacillus'* life cycle. Here, we experimentally quantified *Spirobacillus*' virulence and showed that *Spirobacillus* must kill its host to transmit horizontally. We also found evidence that *Spirobacillus* may transmit vertically via *Daphnia*'s seed-like egg cases. Our work will help scientists to (i) understand *Spirobacillus* epidemics, (ii) use *Spirobacillus* as a model pathogen for the study of host-parasite interactions and (iii) better understand the unusual group of bacteria to which *Spirobacillus* belongs.

Introduction

Interactions among pathogens and *Daphnia* can have significant impacts on *Daphnia* population dynamics, evolution and trophic interactions, and serve as important models of host-pathogen interactions (1–8). One of the oldest *Daphnia* pathogens known to science is *Spirobacillus cienkowskii*, a gram-negative bacterium that belongs to the newly characterized family *Silvanigrellaceae* (9). First found in *Daphnia magna* in Ukraine in the 1880s (10), *Spirobacillus cienkowskii* (hereafter *Spirobacillus*) has since been found in a wide range of Cladocera at locations across the globe (11–13). In *Daphnia*, *Spirobacillus* causes large epidemics and increases their vulnerability to predation (4, 14, 15). *Spirobacillus* also produces pigments, which cause *Daphnia* to become a red color that predators can readily detect (6). Accordingly, there has been interest in using the *Spirobacillus-Daphnia* system as a laboratory model of predator-parasite-host interactions and the evolution of pigment-production in pathogens (16).

At present, however, we have a limited capacity to explain *Spirobacillus'* dynamics in nature or to harness it as a laboratory model because we do not understand fundamental aspects of *Spirobacillus*' biology. There are three classes of knowledge gaps, all of which stem from the difficulty of growing *Spirobacillus* in the laboratory. First, although field observations suggest that *Spirobacillus* infection is lethal and reduces fecundity (11, 14, 16), we do not yet have quantitative estimates of the impact of *Spirobacillus* infections on host lifespan or lifetime reproduction (i.e., how virulent *Spirobacillus* is). Second, there are a suite of open questions vis-à-vis the mode and dynamics of *Spirobacillus'* transmission among asexually reproducing *Daphnia*; *Daphnia* reproduction is primarily asexual during the periods when *Spirobacillus* epidemics occur. For example, while there is circumstantial evidence that *Spirobacillus* is an "obligate-killer" (i.e., a pathogen that requires host death to transmit) this hypothesis has not been rigorously tested (11, 16). Furthermore, we have no quantitative measures of key epidemiological parameters such as *Spirobacillus'* force of infection (that is, the per capita rate at which susceptible hosts become infected), the degree to which its transmission depends on pathogen-dose or host density, or *Spirobacillus'* ability to maintain its capacity to infect a host (that is, its infectivity) while in the environment. Third – and related to the previous point – we do not yet know how *Spirobacillus* persists between epidemics in nature. Genomic analyses imply that this bacterium has the capacity to make spores (similar to other *Daphnia* pathogens such as *Pasteuria ramosa* (17)), but prior observations suggest that it may be capable of being incorporated into the hardy "resting eggs" (ephippia) that *Daphnia* use to traverse seasons (11).

Here, we present our work to fill some of these knowledge gaps, following our successful establishment of *Spirobacillus* in the laboratory. In a series of experiments, we show that *Spirobacillus* is a highly virulent obligate killer that dramatically reduces both host lifespan and fecundity. Next, we quantitatively describe the epidemic dynamics of *Spirobacillus* in laboratory mesocosms, showing that – consistent with previous observations – it does not generate

classical epidemic dynamics (at least in the conditions we used). Further experiments designed to elucidate quantitative aspects of horizontal transmission provide possible explanation(s) for this observation. Specifically, they demonstrate that a single infected host generates a secondary infection ~30% of the time and, furthermore, the infectivity of *Spirobacillus* (as measured by the per capita probability of infection) declines rapidly with time spent in the environment. Lastly, we show that a high frequency of field-collected ephippia harbor *Spirobacillus*. Our work advances our understanding of the transmission mode and virulence of this little studied pathogen and provides a preliminary explanation for why it has been difficult to establish in the laboratory. We suggest multiple, testable hypotheses as to how these difficulties can be remedied and for why *Spirobacillus* can nevertheless persist in *Daphnia* populations, despite its apparently low horizontal transmission rate.

Materials & Methods

Overview

To quantify the virulence and transmission dynamics of *Spirobacillus*, we conducted several experiments wherein uninfected *Daphnia dentifera* (hereafter "recipients') were exposed to either infected *D. dentifera* (hereafter "donors") or their homogenized remains (Table 1). Experiments took the form of either "mesocosm-scale" experiments, wherein populations of recipients were exposed to *Spirobacillus,* or "individual-scale" experiments, wherein individual recipients were exposed to either infected hosts or their homogenized remains. Briefly, in experiment 1, we tested the hypothesis that *Spirobacillus* is an obligate killer by exposing hosts to either live infected animals, the homogenate of an infected animal or water in which infected animals had died. Next, we performed a series of experiments to investigate the epidemiological dynamics of *Spirobacillus* in the laboratory setting. Specifically, in experiment 2 we described the population dynamics of *Spirobacillus* in replicate populations of *Daphnia dentifera* in the laboratory. In experiment 3 and 4, we investigated whether pathogen dose and host density, two

variables that commonly impact infectious disease transmission (18, 19), impact the per capita probability of *Spirobacillus* infection (its "transmission success"). Lastly, since *Spirobacillus* is a water-borne pathogen whose transmission dynamics are likely to depend strongly on its capacity to maintain its infectivity (i.e., ability to successfully find and infect a new host) while free-living (20–25), we performed experiments 5a and 5b to investigate how much the transmission success of *Spirobacillus* varies with time spent incubating in the environment. For each experiment, we report the proportion of individuals infected, as an estimate of the "per capita probability of infection". We refrain from using the term "force of infection" (i.e., the per capita probability that a host becomes infected per unit time) because our experiments varied in length (Table 1).

In the following section, we first describe methods that were in common among all experiments and then provide additional detail for each experiment in turn.

Hosts & parasites

"Recipient" hosts were 5-6 day old *Daphnia dentifera* of the L6D9 clone, which was originally collected from Dogwood Lake (Greene-Sullivan State Forest, Indiana, USA). Infected, 'donor' hosts were harvested from *in vivo* cultures that we maintained in the Duffy Lab at the University of Michigan from February 2016 until the spring of 2020, when a laboratory shutdown associated with the COVID-19 pandemic caused their loss. These cultures originated from infected animals also isolated from Dogwood Lake. We identified donor hosts by their opacity and red coloration, which signifies the terminal stage of infection (16). Note that, since the availability of donors was limited by the number of donors in our *in vivo* cultures on the day of experimental setup, the sample size of each individual experiment was limited and some experiments were necessarily performed in several blocks.

157 *Experimental exposure & monitoring*

158 To expose recipients to *Spirobacillus,* we either added donors (experiment 1, 2) or their
159 homogenized remains (experiment 1, 3-5) to the water in which recipients were housed (Table
160 1). To make a donor homogenate or "slurry", we homogenized donor hosts in MilliQ water using
161 a motorized pestle, brought the solution up to a known volume and mixed the slurry thoroughly.
162 We inoculated vessels with a fraction of the slurry, so that each vessel received an amount of
163 infectious material equivalent to a predefined number of hosts (precise numbers depend on the
164 experiment, see Table 1). Note that we use the units "donor hosts worth of material", because
165 methods to quantify the amount of *Spirobacillus* in a sample via, for example, microscopy have
166 not been developed.

167

168 We monitored recipients daily for the above-described symptoms of infection (16). Although our
169 data (cf. Results) suggests that hosts that appear asymptomatic after a week or more are not
170 infected, we cannot exclude the possibility that "uninfected" hosts became infected but
171 subsequently cleared the infection. As such, our experiments are best interpreted as evidence
172 of the effects of experimental treatment on the probability that symptomatic *Spirobacillus*
173 infection is observed.

174

175 In experiments 1, 3 and 5b, we allowed infection to proceed until host death; we hence use
176 these experiments to quantify the impact of symptomatic infection on survival. To quantify the
177 impact of infection on fecundity, we removed and counted the number of offspring produced by
178 each host in experiment 1, three times a week, and in block b of experiment 3 (daily). In all other
179 experiments, we removed offspring from beakers daily but did not quantify their number.

180

181 *Husbandry*

182 On the first day of the experiment, denoted day 0, we placed recipients in a vessel: either (i) for

183 individual-scale experiments, a 50mL beaker filled with 25mL filtered lake water, or (ii) for
184 mesocosm-scale experiments, a 1L beaker filled with 800ml filtered lake water.
185 All experiments were conducted under a 16-8 hour light-dark cycle at 22°C. In individual-
186 scale experiments, animals were fed 250ml of a 1 million cells/ml solution of *Ankistrodesmus*
187 *falcatus* daily (yielding 10,000 cells/mL food), with the exception of experiment 4 wherein
188 animals were fed 500ml (20,000 cells/mL) per day. In mesocosm-scale experiments, animals
189 were fed 20ml of algal solution (1 million cells/mL *A. falcatus*) three times a week.
190
191 Experiment 1: elucidating whether *Spirobacillus* is an obligate killer
192 To elucidate whether *Spirobacillus* is an obligate killer, like many other *Daphnia* pathogens (26),
193 we exposed recipients to either live donors, the remains of hosts that had died the previous day
194 or a "slurry" made of homogenized donors. In addition, we included an unexposed treatment.
195 We exposed recipients for 6 hours, after which time they were removed, rinsed in filtered lake
196 water and placed in clean water. We chose an exposure period of 6-hours based on preliminary
197 data on mean survival time of symptomatic, *Spirobacillus*-infected hosts (Fig. S1). These data
198 suggested that greater than 50% of alive donors would remain alive during the exposure period.
199 Nevertheless, 48 of 79 donors in the alive treatment died during the exposure period; none of
200 the recipients became infected, however.
201 To assess whether asymptomatic hosts were infected, we assayed a subset of
202 asymptomatic recipients for infection via PCR. Briefly, we stored asymptomatic recipient
203 animals that died before the end of the experiment or were alive on the last day of the
204 experiment in 1.5mL tubes filled with 180μL TE buffer and froze them at -20°C. We assessed
205 46/151 (~30%) of these animals for infection by PCR, as previously described (16). 20 of these
206 animals were exposed to alive donors that died during the exposure period. Note that – since
207 these animals were assayed days after they were putatively exposed and standard PCR has
208 limited sensitivity – this assay does not definitely exclude the possibility that hosts became

209 infected and then cleared the infection.

210

211 Experiment 2: quantifying epidemic dynamics in mesocosms

212 To quantify the dynamics of infections in laboratory populations of *Daphnia*, in experiment 2 we
213 monitored the prevalence of infection (as indicated by symptomatic animals) and total host
214 density in 1L mesocosms. On day 0, we added 75 recipients to each of four mesocosms and
215 either 6 or 15 donors. To estimate the density of animals and prevalence of infection in
216 mesocosms, we took 20 x 20ml samples on each sampling day using a handmade scoop. We
217 demonstrated in preliminary experiments that the estimate of infection prevalence stabilizes at
218 or before 20 samples were taken. We temporarily retained each sample, counted the number of
219 symptomatic and asymptomatic animals, and then stirred the mesocosm before taking the next
220 sample. After all samples were taken, we replaced the water and animals back into mesocosm.
221 We repeated this procedure from d6 to d29.

222

223 Experiments 3 & 4: elucidating the dose- and density- dependence of *Spirobacillus'*
224 transmission

225 The transmission success of a pathogen is a function of two probabilities: the probability that a
226 host becomes infected upon contact with a pathogen and (ii) the probability the host encounters
227 an infected host or, in the case of environmentally transmitted pathogens, an infectious, free-
228 living parasite (27, 28). Pathogen dose commonly impacts the former probability while the latter
229 can vary with host density (although not in the case of pathogens with so-called "frequency-
230 dependent" transmission) (18). In experiments 3 and 4, respectively, we investigated the dose-
231 and density- dependence of *Spirobacillus* transmission success using the "individual-scale"
232 experimental design outlined above and varying the parameters in Table 1.

233

234 Experiment 5: quantifying the impact of environmental incubation on *Spirobacillus* transmission

235 The epidemic dynamics observed in experiment 2 suggested that *Spirobacillus* may not remain
236 capable of initiating a symptomatic infection (i.e., infectious) long after the death of an infected
237 individual. We sought to quantify the effect of time spent in the environment (environmental
238 incubation) on *Spirobacillus'* transmission success, as measured by the probability that either a
239 population of host(s) or individual host became infected. To do this we performed a mesocosm-
240 and individual- scale experiment (experiments 5a and 5b, respectively).
241 For the mesocosm-scale experiment, we first established a mesocosm containing 80
242 recipients, denoted the `primary cohort', to which we added 4 donors. After a predefined interval
243 (1, 3, or 5 days), we removed the primary cohort to a secondary vessel and added a cohort of
244 80 `secondary' recipients to the original mesocosm. We monitored whether any infected animals
245 appeared in the primary and secondary cohorts for 15 days following their initial exposure to the
246 water in the original beaker. Accordingly, we used the 'primary' cohort to indicate whether the
247 focal mesocosm was *ever* infectious, and the secondary cohort to indicate whether any
248 infectious material remained in the mesocosm after the time interval had lapsed. We ran 3
249 replicates of each of the 1 and 3-day treatments and 4 of the 5-day treatment.
250 Experiment 5a was limited in size and confounded the effect of hosts (i.e., the primary
251 cohort) and environmental incubation *per se* on *Spirobacillus'* transmission success. Therefore,
252 we conducted a second individual-scale experiment (5b). Here, we added donor-slurry to each
253 and placed the water-slurry solution in an incubator at 22°C until a predetermined interval (0, 1,
254 2, 4 or 6 days) had elapsed, at which point we added a recipient. Recipients were exposed for
255 24 hours, after which time they were removed, rinsed in filtered lake water and placed in clean
256 water for monitoring.
257
258 <u>Quantification of *Spirobacillus* in field-collected ephippia</u>
259 To assess whether *Spirobacillus* DNA was present inside ephippia, we collected ephippia from
260 the field and quantified *Spirobacillus* via digital PCR (dPCR), using methods similar to those in

261 Davenport et al. (29).

262

263 In May and November 2019, we collected sediment from Pickerel Lake (Washtenaw County,
264 Michigan, USA), wherein *Spirobacillus* infected animals have been observed over several years
265 (30). To separate the ephippia from the sediment, we placed small portions of the sediment (~4
266 grams) on a 243μm plankton net filter and rinsed it with deionized water (diH_2O) to remove
267 small particles and liquid residue. We transferred the material retained on the filter to a watch
268 glass containing diH_2O, inspected it under a dissecting microscope and isolated ephippia of
269 *Daphnia dentifera* following Rogalski et al. (31). We then surface-sterilized the ephippia by
270 placing them in 70% ethanol for 2 mins and finally placed each ephippium in a separate 1.5 ml
271 centrifuge tube filled with PBS buffer. We homogenized the ephippium using a sterile motor-
272 powered pestle and stored the tubes at -20℃.

273

274 Next, we extracted whole genomic DNA from each ephippium using the DNeasy Blood and
275 Tissue kit (Qiagen®), initiated by adding Proteinase K into the thawed solution and then
276 following the manufacturer's protocol. Extracted DNA was stored at -20℃ prior to analysis by
277 digital PCR, as follows. Each reaction contained 20μl of eluted DNA, 10μl of mastermix
278 (QIAcuity™ Probe PCR Kit), 0.72 μl of forward primer Spiro70F (5'-
279 AACTGTAGCTAAGACCGCGT-3') and 0.72 μl of reverse primer Spiro210R (5'-
280 CCAGCTATCCATCTTCGCCT-3'), 0.36 μl of dual-quenched probe SpiroP (5'-
281 ACCGTAAGGCCAGCAGCCATAAGATGA-3') labeled with fluorescent Dye ROX at the 5' end
282 and Iowa Black® RQ dark quencher at the 3' end, 0.5 μl of restriction enzyme EcoRI and 13.5 μl
283 of RNase-free water. We used a high volume of DNA because we expected that the ephippia
284 would contain a low concentration of bacterial DNA. The PCR was performed in a 24-well
285 Nanoplate 26K (Qiagen®), using a QIAcuity One Digital PCR System (Qiagen®). PCR

286 conditions were 2 min at 95℃, followed by 40 cycles of 15 sec at 95℃ followed by 1 min at 55℃.
287 We first performed post-PCR imaging using an exposure time of 300ms and, when it was
288 necessary to increase the separation of signal from noise, reimaged the plates using either
289 200ms or 100ms. The results were analyzed with QIAcuity Software Suite 2.0.20. Finally, we
290 converted the output – number of gene copies in the reaction mix – to the total copy
291 number/ephippium. Note that, since we do not know the number of copies of the 16S rRNA
292 copies in the *Spirobacillus* genome, copy number should not be interpreted as an absolute
293 estimate of the number of cells contained within each ephippium.
294
295 We designed the aforementioned primers and probe using NCBI Primer-BLAST (32) to target
296 the *Spirobacillus* 16S rRNA gene. We verified the assay's specificity by using it to assay DNA of
297 three other members of the family *Silvanigrellaceae, Silvanigrella aquatica*, *Silvanigrella*
298 *paludirubra* and *Fluviispira multicolorata.* The assay did not amplify DNA from these species.
299
300 Statistical analysis
301 We performed statistical analysis using R (version 4.2.1). To test for an association between
302 treatment and the per capita probability of infection, we ran Chi-square tests or (when expected
303 values were less than 5) Fisher's Exact Tests, following (33). To verify the result of these
304 analyses and to obtain estimates of per capita infection rates (and confidence intervals around
305 said estimates) for presentation in figures, we ran binomial general linearized models (GLMs)
306 and (where necessary) controlled for block. In all cases, the two approaches yielded the same
307 results *vis-a-vis* the significance of treatment. We removed asymptomatic animals that died on
308 or before day 8 – the last day on which we ever observed symptoms – from the analysis of
309 transmission rates, reasoning that these animals may not have had sufficient time to display
310 symptoms.
311

312 To quantify the impact of infection on fecundity, we used Poisson GLMs. We corrected for
313 overdispersion, which was found in all cases using a quasi-Poisson model, following (34) .
314 Animals whose fecundity was not recorded throughout their lifetime (due to experimental error,
315 such as inadvertently knocking over a beaker) were removed from this analysis.
316
317 To analyze the effect of treatment on time to death and symptoms, we employed survival
318 analyses using the *surv* package (35). These analyses allow us to include all the individual-level
319 time series data, including data relating to animals that died before day 9 or which were not
320 tracked throughout the entirety of a time-period. We fit Kaplan-Meier survival curves to time-
321 series data *vis-à-vis* host death and the appearance of symptoms and used them to derive
322 estimates of the median (+/- 95% CI) time-to -death and -symptoms, respectively. To ask
323 whether *Spirobacillus* had a significant impact on lifespan, we used log rank tests to compare
324 the survival curves. In addition, in Figure 1b we plot the results of (i) a "competing risks"
325 analysis, which we used to quantify the probability that an animal either becomes symptomatic
326 or dies without showing symptoms (dashed line), and (ii) a "multi-state" analysis, which we used
327 to quantify the probability that an animal died following the appearance of symptoms (black line)
328 (36).
329
330 **Results**

331 Virulence

332 Symptomatic *Spirobacillus* infection ("infection", hereafter) had a 100% fatality rate in our
333 individual-level experiments (Fig. 1a). Symptoms developed rapidly – on average, by day 6 –
334 and were followed by death within a day, on average (Fig. 1b; Fig. S1, restricted mean survival
335 time of a symptomatic animal 9.6 hours). As a result, *Spirobacillus* infection had a significant
336 impact on host lifespan (χ^2 = 290, df = 1, p<0.001). In experiment 1, the median survival time of

337 uninfected, but exposed, animals was greater than the length of the experiment (that is, over 21
338 days), while infected animals lived for just 7 days (95% CI 7-8) on average.

339 *Spirobacillus* infection significantly reduced the fecundity of infected animals, as compared to
340 uninfected treatment-mates (Fig. 2a, *infection status* ∆AIC= 187, df=1). Over the course of
341 experiment 1, for example, infected animals had ten times fewer offspring than uninfected
342 animals in the same treatment. The effect of *Spirobacillus* on host fecundity does not seem to
343 stem only from its effect on host lifespan. During the first week of experiments 1 and 2**,**
344 recipients that later became symptomatic had fewer offspring than those that did not become
345 infected (Fig. 2b). Thus, the negative effects of *Spirobacillus* infection on host fitness are
346 apparent even before symptoms are detectable and are not solely attributable to a reduction in
347 lifespan.

348 <u>Mode of horizontal transmission among asexually reproducing *Daphnia*</u>

349 *Spirobacillus* required host death or destruction to transmit (Fig. 3). In experiment 1,
350 symptomatic infection was only observed in animals exposed to material from donors that had
351 been destroyed by homogenization or had died naturally (Fig. 3). To assess whether or not
352 asymptomatic hosts were infected, we assayed 46/151 (30%) of the asymptomatic animals from
353 experiment 1 (including 20 that were exposed to alive donors) for infection via PCR. None were
354 positive (data not shown). These results suggest that *Spirobacillus* is an obligate killer and that
355 asymptomatic hosts are unlikely to be infected. These data do not, however, preclude the
356 possibility that asymptomatic hosts became infected but cleared the infection before they were
357 assayed.

358 <u>Dynamics of horizontal transmission among asexually reproducing *Daphnia*</u>

359 *Spirobacillus* did not exhibit classical epidemic dynamics in mesocosms (Fig. 4). In all but 1

360 mesocosm, the prevalence of symptomatic hosts cycled up and down at a level below or
361 equivalent to the initial prevalence. In no mesocosm did the prevalence of symptomatic hosts
362 ever grow for a period longer than the incubation period of 7 days (which, given that
363 *Spirobacillus* is an obligate killer, is equivalent to the time-to-death of symptomatic hosts as
364 estimated above). Furthermore, the density of symptomatic hosts did not lag behind that of
365 asymptomatic hosts, as is expected (20); indeed, the dynamics of these two cohorts bear scant
366 relationship to one another.

367 Across all individual-scale experiments, the per capita probability of infection did not exceed
368 50% in any treatment (Figs. 3, 5, 6). Furthermore, the per capita probability of infection was not
369 impacted by pathogen dose (Fig. 5a, p= 0.7) or the density of animals in the beaker (Figure 5b,
370 p = 0.2) in the conditions we tested.

371 *Spirobacillus'* infectivity – i.e., its capacity to generate (symptomatic) infections in exposed hosts
372 – declined with time spent in the environment (water). In experiment 5a, we added a secondary
373 cohort of recipients to mesocosms either 1, 3 or 5d after they were initially inoculated with
374 donors and a primary cohort of recipients. The primary cohort was used to assess whether the
375 donors were infectious and was removed prior to the addition of the secondary cohort. The
376 proportion of mesocosms that generated infections in the secondary cohort declined with the
377 time post-inoculation (Fig. 6a). The same pattern holds when we consider only those
378 mesocosms that were confirmed to be infectious (i.e., that generated infections in the primary
379 cohort; Fig. 6a, open circles). In experiment 5b, we exposed individual recipients to water that
380 had been inoculated with *Spirobacillus* and incubated for various time intervals (0-6d). We found
381 a similar-decline in transmission success with time spent in the environment (Fig. 6b, time
382 incubated in the environment χ^2 = 31, df =1, p<0.001). Indeed, our data implies that after just 3
383 days of incubation *Spirobacillus'* has almost no chance (<1%) of successfully infecting a host
384 (Fig. 6b). These results suggest that, in these laboratory conditions, *Spirobacillus* exhibits

385 limited capacity to maintain its infectivity in water.

386 Presence within ephippia

387 We found *Spirobacillus* DNA in 39% of the 41 *Daphnia dentifera* ephippium assayed**.** Positive
388 ephippia contained 83 copies of the 16s rRNA gene, on average (range 4 to 860) (Figure 7).
389

390 **Discussion**

391 Despite *Spirobacillus cienkowksii*'s long history of study and widespread distribution,
392 fundamental aspects of its biology have remained uncharacterized. As a result, *Spirobacillus'*
393 potential as a model pathogen has gone unfulfilled and we are unable to explain its dynamics
394 and persistence in nature. Here, we showed that *Spirobacillus* is a highly virulent pathogen of
395 *Daphnia* that – at least in standard laboratory conditions – transmits poorly among clonally
396 reproducing *Daphnia* and exhibits limited environmental persistence. Our results help explain
397 why it has been so difficult to establish *Spirobacillus* in the laboratory (despite repeated
398 attempts) and indicate several new avenues of investigation vis-à-vis its life history and that of
399 the newly described family of bacteria to which it belongs.
400

401 Our data suggest that *Spirobacillus* is among the most virulent of *Daphnia* pathogens yet
402 described, killing its host almost twice as rapidly as *Metschnikowia bicuspidata* (37), the
403 pathogen of *Daphnia dentifera* with the next most rapid measured impact on host death. In
404 addition to killing hosts rapidly, *Spirobacillus* reduces host fitness by reducing host fecundity.
405 Given that we have observed field- and laboratory- infected *Daphnia* produce offspring on the
406 day they die (data not shown), we suggest that *Spirobacillus'* impact on fecundity stems from
407 physiological stress rather than a direct reproduction-interference mechanism. An alternative
408 hypothesis is *Spirobacillus* selectively infects hosts that are less fit. Given that we controlled
409 genotype and age and husbandry conditions, we view this possibility as less likely.

410

411 Our work confirms the longstanding hypothesis that – like many other *Daphnia* pathogens –
412 *Spirobacillus* is an obligate killer; that is, *Spirobacillus* must kill its host to transmit horizontally
413 among asexually reproducing hosts, which dominate during the period of epidemics (11, 16).
414 Nevertheless, our data do not imply that this pathogen must directly kill its host to transmit,
415 since mechanical destruction of symptomatic hosts also permitted infection. This observation
416 has two implications. First, it implies that, in nature, destruction of hosts by "sloppy" predators
417 may facilitate transmission, as in the case of other *Daphnia* pathogens (38, 39). Second, in the
418 context of using *Spirobacillus* as an infection model, it enables the experimenter to control the
419 timing and dose of infection that experimental animals receive via a "host slurry" (as in most of
420 our individual-scale experiments).

421

422 Although our experiments were not explicitly designed to measure the timescale of transmission
423 following host death, the "alive" treatment of experiment 1 yields some insight into this process.
424 Several of the "alive" donors died within the 6-hour exposure period, yet none of the recipients
425 became infected**.** Two hypotheses could explain this. First, there is a lag between host death
426 and the production of transmissible *Spirobacillus* – this hypothesis is unlikely given that living
427 hosts that were crushed yielded infections. Alternatively, it may take >6 hours for *Spirobacillus*
428 to escape the carcass of its host and find a new one. Testing either of these hypotheses is
429 presently impossible because we do not know the morphology of *Spirobacillus* during
430 transmission (although we and others have observed motile stages within dead or dying
431 *Daphnia* and there is genomic evidence to suggest that *Spirobacillus* possesses the genomic
432 machinery to make flagella and spores (10, 40)). Further microscopic study of *Spirobacillus* will
433 thus be invaluable to further developing this pathogen as a model system.

434

435 While *Spirobacillus* shares the same transmission mode as many other *Daphnia* pathogens, it

436 does not share their capacity to efficaciously generate epidemics in laboratory mesocosms (Fig.
437 4), as experiment 2 starkly shows. For obligate killers like *Spirobacillus*, whose survival in the
438 environment (at least in our experiments) does not exceed the timescale of host population
439 dynamics, the ability to generate an epidemic within a host population increases with its
440 transmission rate and virulence and declines with the rate at which free-living stages lose their
441 infectivity (41). The results of experiments 3, 4 and 5 thus provide a putative explanation for our
442 failure to observe epidemic dynamics: although *Spirobacillus* is highly virulent, its low
443 transmission rate and rapid rate of decay in the environment may reduce its ability to cause
444 epidemics. Instead of a classical epidemic curve, we observed peaks and troughs in the
445 prevalence of infection, with a periodicity of ~6 days (at least at the beginning of infections,
446 when small differences among individuals have not accumulated to dampen the cycles). We
447 hypothesize that these dynamics are a result of the strong, relatively invariant periodicity of the
448 time to symptoms (6-7 days) and time to death (7-8 days), combined with *Spirobacillus'* limited
449 ability to maintain its infectivity while in the environment (as found in experiment 5a,b).
450
451 In individual-scale experiments, we found *Spirobacillus'* transmission success did not vary with
452 pathogen dose or susceptible host density. However, we do not feel able to make definitive
453 conclusions about *Spirobacillus'* dose- or density-dependence due the limited scope and scale
454 of these experiments. The per capita probability of infection did not change with pathogen dose
455 in experiment 3, nor between experiment 1 (where recipients were exposed to 1 host worth of
456 parasite) and experiment 3 (where recipients were exposed to 2-3 host's worth of parasite). This
457 behavior contrasts markedly with that of *Pasteuria ramosa*, for example. In *D. magna* housed in
458 similar experimental conditions, only a small fraction (~0.001%) of the *Pasteuria* spores
459 contained in a fully infected host is sufficient to infect ~50% of new hosts, and a dose of ~10%
460 of the spores in a fully infected host all but guarantees infection (42–44). One hypothesis for our
461 observations is that the dose-response curve has a decelerating shape (as is common, (45, 46))

462 and we were working in the decelerating region of the dose response curve. Under this
463 hypothesis, we would expect to observe a relationship between pathogen dose and per capita
464 probability of infection were we to repeat the experiment with a large range of (lower) doses.
465 Similarly, we cannot conclude much about whether *Spirobacillus'* transmission dynamics lie on
466 the spectrum between frequency and density dependence because of experiment 4's limited
467 sample size and range of treatments. Indeed, that new infections arose in our mesocosms trials
468 (experiment 2) – which contained a higher density (~2.5x) of susceptible hosts but were initiated
469 with a much smaller inoculum than our individual-scale experiments – implies that density-
470 dependence may play a role in *Spirobacillus'* transmission dynamics.
471
472 Our results raise the question "why does *Spirobacillus* generate epidemics in the field (and not
473 in the lab)?". We have two hypotheses. Our first assumes that *Spirobacillus* is an obligate
474 parasite and attributes our findings to a discrepancy between the conditions in the lab and those
475 under which transmission occurs in nature. For example, in a study of *Spirobacillus* in the water-
476 column, Thomas et al. (47) found the highest density of *Spirobacillus* at the surface-water
477 interface, a region characterized by low temperatures, oxygen and light (48, 49). If this region is
478 the location of transmission in nature, our experiments poorly represent them. Alternatively, it is
479 possible that our recipient hosts were less susceptible than the hosts that become infected in
480 nature, perhaps because of their age, genotype or nutritional status. Given that we have
481 observed infection across host ages in the field and have generated infections at equivalent
482 rates in other hosts clones (unpublished data), we think that differences in the abiotic conditions
483 of the lab vs. the field are the most likely to explain our inability to recover epidemic dynamics in
484 the lab.
485
486 Our second, more heterodox, hypothesis is that *Spirobacillus* is a facultative pathogen of
487 *Daphnia,* whose epidemic dynamics are not entirely a function of replication within asexually

488 reproducing *Daphnia*. Instead, it is possible that *Spirobacillus* primarily replicates on particles
489 (including ephippia) in the water column and/or sediment and employs the same traits that allow
490 for particle-finding, -attachment and -exploitation to parasitize hosts, when the opportunity
491 arises. In accordance with this hypothesis, *Spirobacillus* and the other *Silvanigrellaceae* exhibit
492 a suite of traits, including pleiomorphism, small size and the genomic capacity to make flagella
493 and perform chemotaxis (40), that are characteristic of copiotrophic aquatic microbes capable of
494 facultative predation or parasitism, e.g., the *Vibrios* and *Bdellovibrios* (50, 51). Under this model,
495 either (i) *Spirobacillus* outbreaks in *Daphnia* result from "blooms" in the particle-associated
496 population that spillover into the *Daphnia* population, or (ii) there is an ecological cue that
497 stimulates a switch from a free-living or particle associated lifestyle to a *Daphnia*-associated
498 lifestyle. Comparative genomic analyses of the *Spirobacillus* genome with that of predatory
499 and/or parasitic species and investigations of the distribution and abundance of *Spirobacillus* in
500 different particulate and non-particulate fractions of the water column could shed further light on
501 this hypothesis.
502
503 Our discovery that *Spirobacillus* is harbored within ephippia is consistent with the idea that
504 *Spirobacillus* may not entirely rely on asexually reproducing hosts for its fitness and could help
505 us to explain a range of observations made in the past and in this study. We found *Spirobacillus*
506 DNA in almost 40% of the ephippia assayed. This observation, combined with a previous
507 observation that ephippia inside infected *Daphnia* contain bacteria (11), suggests that
508 *Spirobacillus* may be vertically transmitted from sexually reproducing females to ephippia. (We
509 note that vertical transmission would include bacteria that are contained within the ephippium
510 but not the embryo itself.) That said, these data do not exclude the possibility that ephippia
511 become colonized by *Spirobacillus* after they had been released from *Daphnia* and, given that
512 *Spirobacillus'* prevalence rarely exceeds 10% even during epidemics, its comparatively high
513 prevalence in ephippia (39%) is difficult to explain with respect to vertical transmission alone.

514 Irrespective of the mechanism by which *Spirobacillus* enters the ephippia, the ability to exploit
515 ephippia could explain *Spirobacillus'* inter-epidemic persistence and hypervirulence. Rodrigues
516 *et al*. observed that *Spirobacillus*-infected hosts hatch directly from ephippia (11). This
517 observation, coupled within our findings, suggests that *Spirobacillus* might use the hardy
518 ephippia to persist through the winter, before emerging from them and seeding the next year's
519 epidemics. Given the high energetic costs of sporulation (52), invading a hardy structure rather
520 than making one could have significant advantages. The capacity to survive within ephippia
521 might also allow *Spirobacillus* to offset or avoid paying the costs of virulence, if it is an obligate
522 pathogen, per the "curse of the pharaoh" hypothesis (22).
523
524 Our work reveals much about the life history of a bacterium that has a long and significant
525 history in bacteriology and sets a foundation for the use of *Spirobacillus* as a model of host-
526 parasite interactions at the individual host and evolutionary timescale. Here, we found that
527 *Spirobacillus* infection is virulent, proceeds from initiation to death within a week and causes its
528 host to express readily detectable symptoms. These features, coupled with our prior description
529 of natural variation in the color of infected hosts (and thence, presumably, carotenoid
530 production) (16), could make *Spirobacillus* a tractable, *in vivo* model with which to study how
531 bacterial secondary metabolites alter infection dynamics and virulence. That being said, clearly,
532 the limited transmissibility of *Spirobacillus* in the laboratory reduces its tractability as a model.
533 Identifying the conditions that optimize transmission is thus a priority if it is to be used in this
534 context. Indeed, future work aimed at elucidating the mode(s) of *Spirobacillus* transmission
535 could help us to make new insights about the evolutionary dynamics of virulence. The dynamics
536 of virulence evolution in pathogens capable of both horizontal and vertical (i.e., "mixed-mode")
537 transmission and/or environmental replication are poorly understood as compared to pathogens
538 with strictly horizontal and/or direct transmission (53); virulence evolution in obligate killers is
539 also relatively poorly studied. Our work suggests that mixed-mode transmission and/or

environmental replication is possible in *Spirobacillus*. Better understanding these aspects of *Spirobacillus'* biology could help us to build and test ideas about how obligate killers evolve when their fitness does not rely solely on horizontal transmission. In conclusion, by establishing *Spirobacillus* in the laboratory for the first time, we established critical facets of *Spirobacillus'* infection biology that can be harnessed by biomedical biologists and ecologists alike.

Acknowledgements

We thank Libby Davenport (U. Michigan) for designing the dPCR assay and Martin Hahn (U. Innsbruck) for the samples of *Silvanigrellaceae* that we used to validate said assay. We are grateful to the National Science Foundation (DEB-1655856) and the Moore Foundation for funding.

Bibliography

1. Duncan AB, Little TJ. 2007. Parasite-driven genetic change in a natural population of *Daphnia*. Evolution 61:796–803.

2. Duffy MA, Ochs JH, Penczykowski RM, Civitello DJ, Klausmeier CA, Hall SR. 2012. Ecological context influences epidemic size and parasite-driven evolution. Science 335:1636–1638.

3. Duffy MA, Brassil CE, Hall SR, Tessier AJ, Cáceres CE, Conner JK. 2008. Parasite-mediated disruptive selection in a natural Daphnia population. BMC evolutionary biology 8:80.

4. Duffy MA, Hall SR, Tessier AJ, Huebner M. 2005. Selective predators and their parasitized prey: Are epidemics in zooplankton under top-down control? Limnol Oceanogr 50:412–420.

5. Johnson PTJ, Stanton DE, Preu ER, Forshay KJ, Carpenter SR. 2006. Dining on disease: how interactions between infection and environment affect predation risk. Ecology 87:1973–1980.

6. Wale N, Fuller RC, Johnsen S, Turrill ML, Duffy MeghanA. 2021. The visual ecology of selective predation: Are unhealthy hosts less stealthy hosts? Ecol Evol https://doi.org/10.1002/ece3.8464.

7. Ebert D. 2008. Host–parasite coevolution: Insights from the *Daphnia*–parasite model system. Curr Opin Microbiol 11:290–301.

8. Ebert D. 2022. Daphnia as a versatile model system in ecology and evolution. Evodevo 13:16.

9. Hahn MW, Pitt A. 2017. Silvanigrella, p. 1–9. *In* Bergey's Manual of Systematics of Archaea and Bacteria.

10. Metchnikoff E. 1889. Contributions a l'etude du pleomorphisme des bacteriens. Annales de l'Institut Pasteur 3:61–68.

11. Rodrigues JLM, Duffy MA, Tessier AJ, Ebert D, Mouton L, Schmidt TM. 2008. Phylogenetic characterization and prevalence of Spirobacillus cienkowskii, a red-pigmented, spiral-shaped bacterial pathogen of freshwater Daphnia species. Applied and Environmental Microbiology 74:1575–1582.

12. Green J. 1959. Carotenoid pigment in Spirobacillus cienkowskii Metchnikoff, a pathogen of Cladocera. Nature 183:56–57.

13. Mioduchowska M, Czyż MJ, Gołdyn B, Kilikowska A, Namiotko T, Pinceel T, Łaciak M, Sell J. 2018. Detection of bacterial endosymbionts in freshwater crustaceans: the applicability of non-degenerate primers to amplify the bacterial 16S rRNA gene. Peerj 6:e6039.

14. Duffy MA, Hall SR. 2008. Selective Predation and Rapid Evolution Can Jointly Dampen Effects of Virulent Parasites on Daphnia Populations. Am Nat 171:499–510.

15. Hall SR, Duffy MA, Cáceres CE. 2005. Selective predation and productivity jointly drive complex behavior in host-parasite systems. The American Naturalist 165:70–81.

16. Wale N, Turrill ML, Duffy MA. 2019. A colorful killer: Daphnia infected with the bacterium Spirobacillus cienkowskii exhibit unexpected color variation. Ecology 100.

17. Ebert D, Rainey P, Embley TM, Scholz D. 1996. Development, life cycle, ultrastructure and phylogenetic position of *Pasteuria ramosa* Metchnikoff 1888: Rediscovery of an obligate endoparasite of *Daphnia magna* Straus. Philosophical Transactions Royal Soc Lond Ser B Biological Sci 351:1689–1701.

18. McCallum H, Fenton A, Hudson PJ, Lee B, Levick B, Norman R, Perkins SE, Viney M, Wilson AJ, Lello J. 2017. Breaking beta: deconstructing the parasite transmission function. Philosophical Transactions Royal Soc B Biological Sci 372:20160084.

19. Bjørnstad ON. 2023. Epidemics, Models and Data Using R. R https://doi.org/10.1007/978-3-031-12056-5.

20. Cortez MH, Weitz JS. 2013. Distinguishing between Indirect and Direct Modes of Transmission Using Epidemiological Time Series. Am Nat 181:E43–E52.

601 21. Turner WC, Kamath PL, Heerden H van, Huang Y-H, Barandongo ZR, Bruce SA, Kausrud
602 K. 2021. The roles of environmental variation and parasite survival in virulence–transmission
603 relationships. Roy Soc Open Sci 8:210088.

604 22. Gandon S. 1998. The curse of the pharoah hypothesis. Proc Royal Soc Lond Ser B Biological
605 Sci 265:1545–1552.

606 23. Li S, Eisenberg JNS, Spicknall IH, Koopman JS. 2009. Dynamics and Control of Infections
607 Transmitted From Person to Person Through the Environment. Am J Epidemiol 170:257–265.

608 24. Ogbunugafor CB, Miller-Dickson MD, Meszaros VA, Gomez LM, Murillo AL, Scarpino
609 SV. 2020. Variation in microparasite free-living survival and indirect transmission can modulate
610 the intensity of emerging outbreaks. Sci Rep-uk 10:20786.

611 25. Rumschlag SL, Roth SA, McMahon TA, Rohr JR, Civitello DJ. 2022. Variability in
612 environmental persistence but not per capita transmission rates of the amphibian chytrid fungus
613 leads to differences in host infection prevalence. J Anim Ecol 91:170–181.

614 26. Ebert D. 2005. Ecology, Epidemiology, and Evolution of Parasitism in Daphnia. National
615 Center for Biotechnology Information, NIH, Bethesda, MD.
616 https://www.ncbi.nlm.nih.gov/books/NBK2036/.

617 27. Keeling MJ, Rohani P. 2011. Modeling Infectious Diseases in Humans and Animals.
618 Princeton University Press.

619 28. BEGON M, BENNETT M, BOWERS RG, FRENCH NP, HAZEL SM, TURNER J. 2002. A
620 clarification of transmission terms in host-microparasite models: numbers, densities and areas.
621 Epidemiology and Infect 129:147–153.

622 29. Davenport ES, Dziuba MK, Jacobson LE, Calhoun SK, Monell KJ, Duffy MA. 2024. How
623 does parasite environmental transmission stage concentration change before, during, and after
624 disease outbreaks? Ecology 105:e4235.

625 30. Gowler CD, Rogalski MA, Shaw CL, Hunsberger KK, Duffy MA. 2021. Density, parasitism,
626 and sexual reproduction are strongly correlated in lake Daphnia populations. Ecol Evol
627 11:10446–10456.

628 31. Rogalski MA, Leavitt PR, Skelly DK. 2017. Daphniid zooplankton assemblage shifts in
629 response to eutrophication and metal contamination during the Anthropocene. Proc Royal Soc B
630 Biological Sci 284:20170865.

631 32. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. 2012. Primer-BLAST:
632 A tool to design target-specific primers for polymerase chain reaction. Bmc Bioinformatics
633 13:134.

634 33. Field A, Miles J, Field Z. 2012. Discovering Statistics Using R. SAGE Publications Ltd.

34. Zuur AF, Ieno EN, Walker NJ, Saveliev AA, Smith GM. 2009. Mixed Effects Modelsand Extensions in Ecology with R.

35. Therneau TM. 2023. A Package for Survival Analysis in R. https://CRAN.R-project.org/package=survival.

36. Therneau T, Crowson C, Atkinson E. Multi-state models and competing risks. https://cran.r-project.org/web/packages/survival/vignettes/compete.pdf. Retrieved 2022.

37. Clay PA, Dhir K, Rudolf VHW, Duffy MA. 2019. Within-Host Priority Effects Systematically Alter Pathogen Coexistence. Am Nat 193:187–199.

38. Duffy MA, Cáceres CE, Hall SR. 2019. Healthy herds or predator spreaders? Insights from the plankton into how predators suppress and spread disease, p. 458–479. *In* Wilson, K, Fenton, A, Tompkins, & D (eds.), Wildlife Disease Ecology: Linking Theory to Data and Application. Cambridge University Press.

39. Cáceres CE, Knight CJ, Hall SR. 2009. Predator–spreaders: Predation can enhance parasite success in a planktonic host–parasite system. Ecology 90:2850–2858.

40. Bresciani L, Lemos LN, Wale N, Lin JY, Strauss AT, Duffy MA, Rodrigues JLM. 2018. Draft genome sequence of "Candidatus Spirobacillus cienkowskii," a pathogen of freshwater Daphnia species, reconstructed from hemolymph metagenomic reads. Microbiology Resource Announcements 7.

41. Tate AT. 2016. The Interaction of Immune Priming with Different Modes of Disease Transmission. Front Microbiol 7:1102.

42. Ben-Ami F, Regoes RR, Ebert D. 2008. A quantitative test of the relationship between parasite dose and infection probability across different hostparasite combinations. Proc Royal Soc B Biological Sci 275:853–859.

43. Ben-Ami F, Ebert D, Regoes RR. 2010. Pathogen Dose Infectivity Curves as a Method to Analyze the Distribution of Host Susceptibility: A Quantitative Assessment of Maternal Effects after Food Stress and Pathogen Exposure. Am Nat 175:106–115.

44. Regoes RR, HOTTINGER JW, SYGNARSKI L, EBERT D. 2003. The infection rate of Daphnia magna by Pasteuria ramosa conforms with the mass-action principle. Epidemiology Amp Infect 131:957–966.

45. Lunn TJ, Restif O, Peel AJ, Munster VJ, Wit E de, Sokolow S, Doremalen N van, Hudson P, McCallum H. 2019. Dose–response and transmission: the nexus between reservoir hosts, environment and recipient hosts. Philosophical Transactions Royal Soc B Biological Sci 374:20190016.

46. Clay PA, Cortez MH, Duffy MA. 2021. Dose relationships can exacerbate, mute, or reverse the impact of heterospecific host density on infection prevalence. Ecology 102:e03422.

47. Thomas S, Bertram C, Rensburg K van, Cáceres C, Duffy M. 2011. Spatiotemporal dynamics of free-living stages of a bacterial parasite of zooplankton. Aquat Microb Ecol 63:265–272.

48. Wetzel RG. 2001. Light in Inland Waters, p. 49–69. *In* Third. Elsevier.

49. Morris RL, Schmidt TM. 2013. Shallow breathing: bacterial life at low O2. Nat Rev Microbiol 11:205–212.

50. Sockett RE. 2009. Predatory Lifestyle of Bdellovibrio bacteriovorus. Microbiology+ 63:523–539.

51. Lutz C, Erken M, Noorian P, Sun S, McDougald D. 2013. Environmental reservoirs and mechanisms of persistence of Vibrio cholerae. Front Microbiol 4:375.

52. O'Hara MB, Hageman JH. 1990. Energy and calcium ion dependence of proteolysis during sporulation of Bacillus subtilis cells. J Bacteriol 172:4161–4170.

53. Pandey A, Mideo N, Platt TG. 2022. Virulence Evolution of Pathogens That Can Grow in Reservoir Environments. Am Nat 199:141–158.

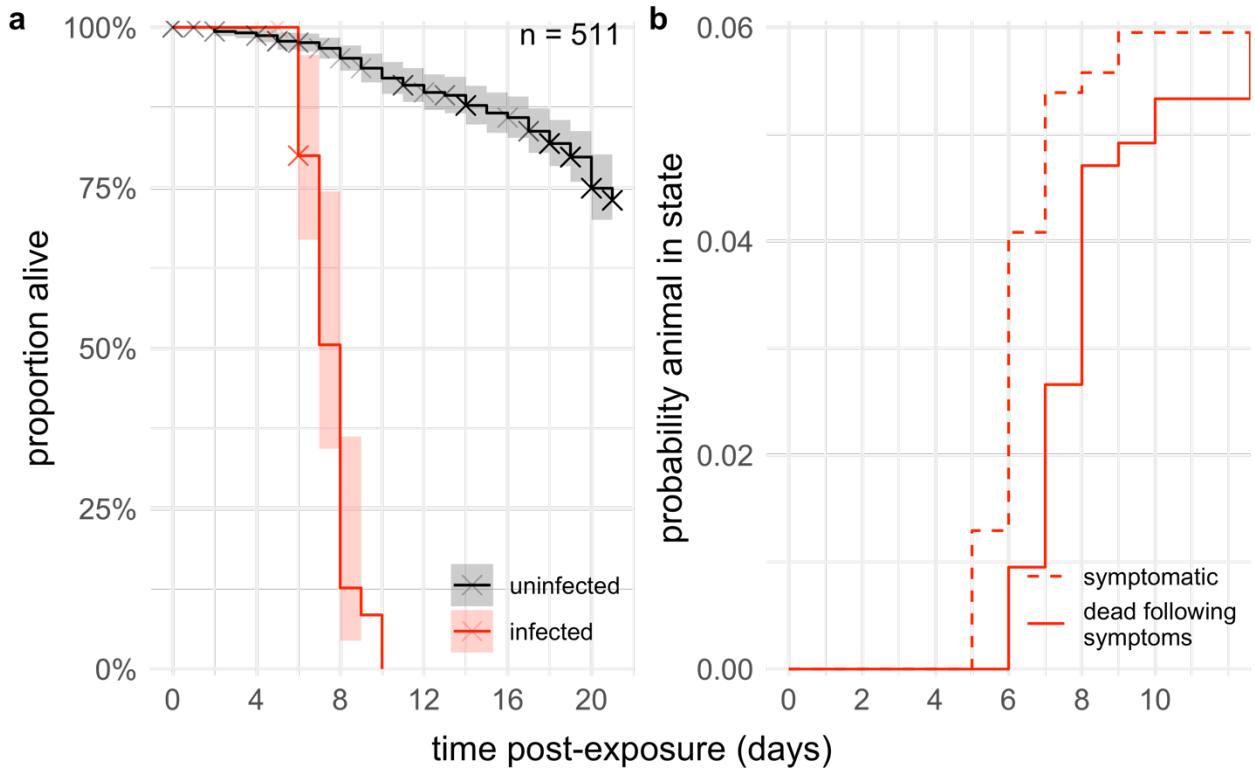

Fig 1. *Spirobacillus* kills its host shortly after symptoms appear, dramatically reducing host lifespan. a) Mean probability of survival (+/- 95% confidence interval, shaded area) following exposure to *Spirobacillus* of infected (red) or uninfected (black) hosts in experiments 1-4. Estimates are derived from a Kaplan-Meier survival curve. Crosses indicate when animals were removed from the experiments for a reason other than death (e.g., the experiment ended or, in experiment 3, when infected animals were preserved before death). b) Estimated probability that a host was symptomatic (dashed line) and dead after displaying symptoms (solid line) following exposure to *Spirobacillus*, across experiments 1, 3 and 5. The estimated probabilities take such small values (note the y-axis scale) because most of the animals exposed in these experiments did not become infected (cf. Fig. 3, 5a, 6).

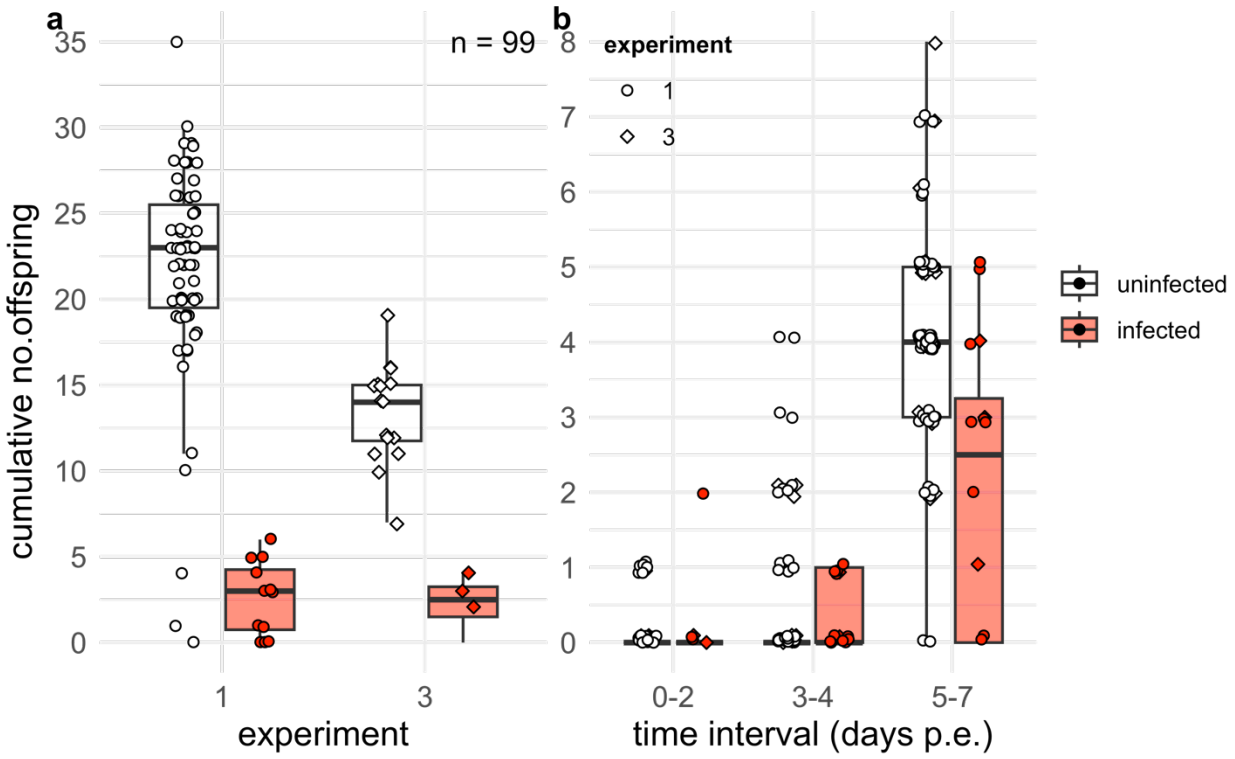

Fig 2. *Spirobacillus* infection reduces the fecundity of infected *Daphnia*, even during the short period that they are alive. a) Cumulative no. of offspring produced per host over the course of experiments 1 and 3. b) Total offspring produced in the first week post exposure (p.e.), by hosts that went on to develop symptomatic infection (red) or remained uninfected (white). Note that animals were exposed while juveniles, hence the limited reproduction between days 0-2. In both plots, each individual point represents data from an individual host.

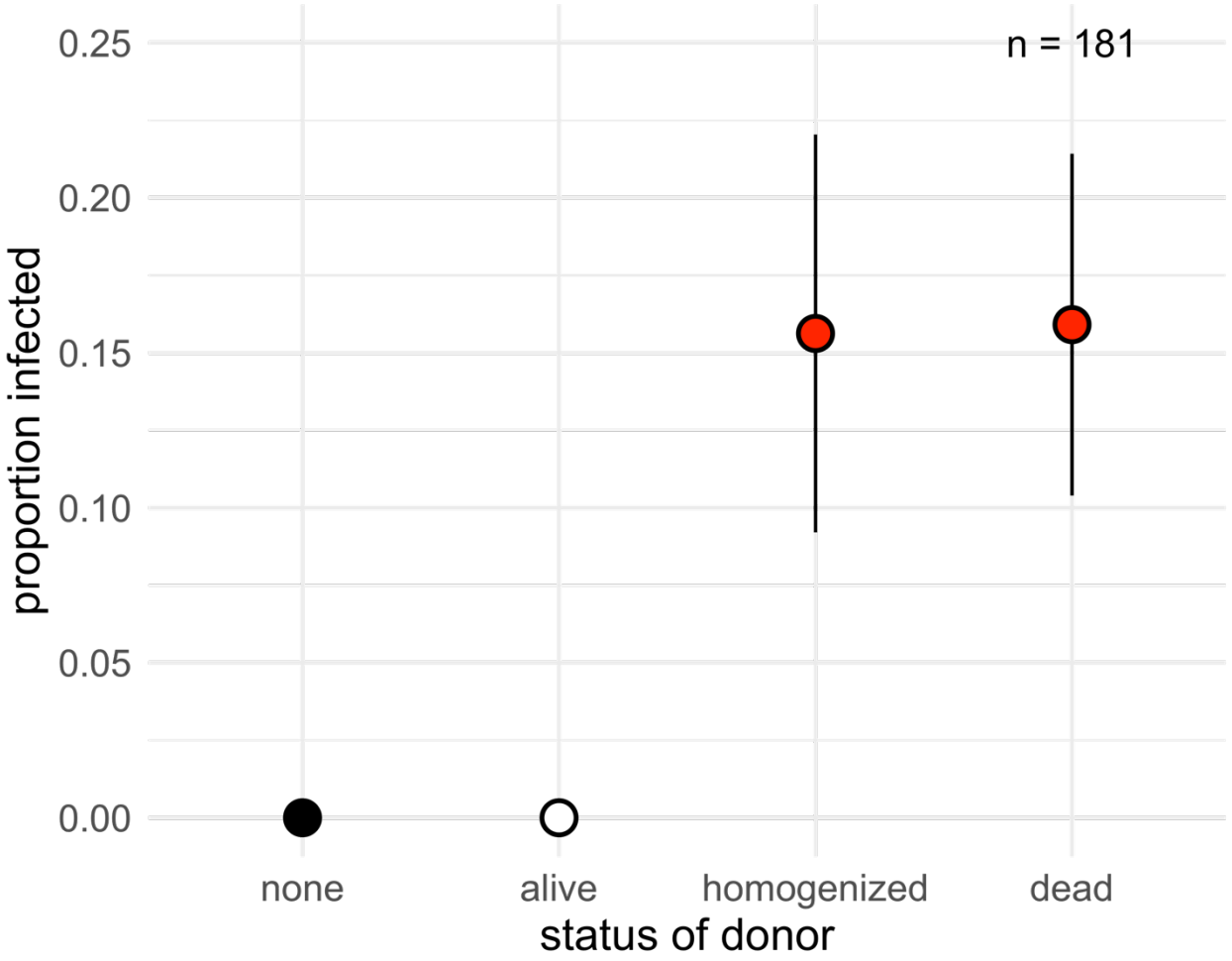

Fig. 3 *Spirobacillus* requires the demise of its host to transmit horizontally. Mean proportion of hosts infected (+/- standard error) in each treatment of experiment 1, as estimated from a generalized linear model.

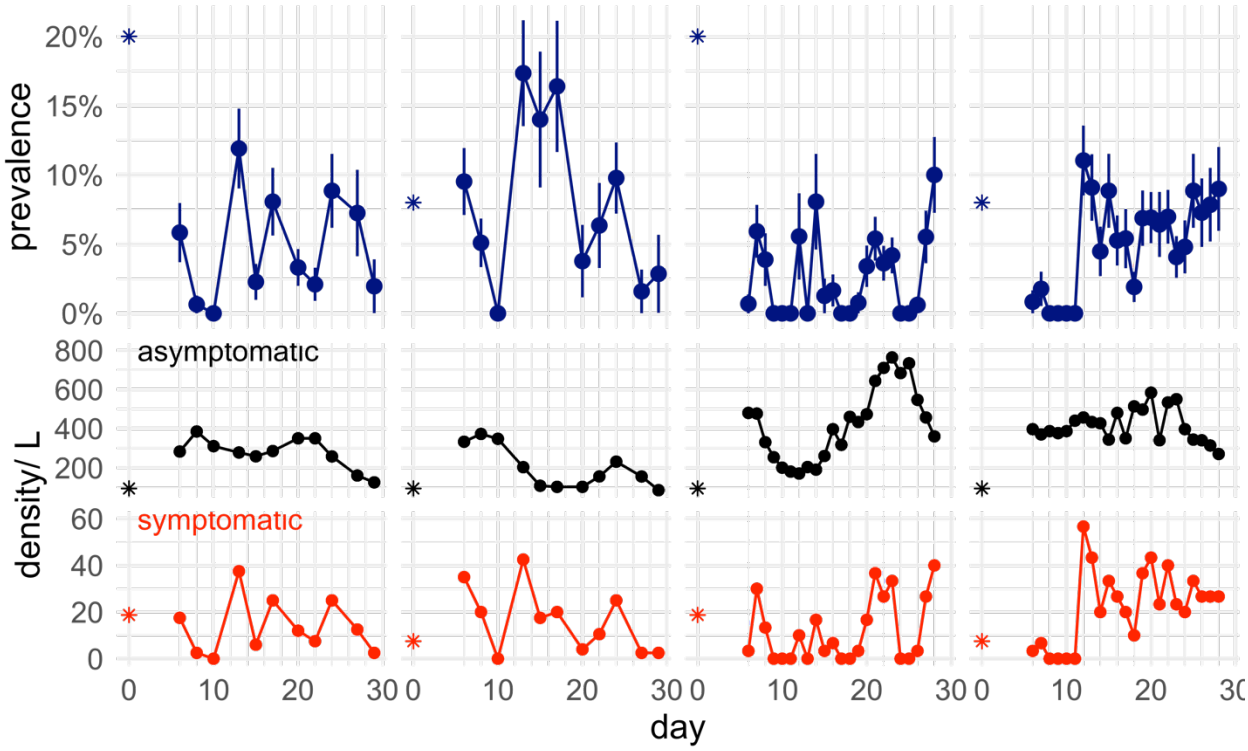

Fig. 4 Dynamics of *Spirobacillus* infection in laboratory mesocosms. The prevalence of symptomatic hosts (blue), and the density of asymptomatic host (black) and symptomatic hosts (red), in 800ml mesocosms over 28 days. Asterisks indicate the initial value of parameters in day 0, when the mesocosm were established. Error bars around prevalence estimated from a binomial distribution.

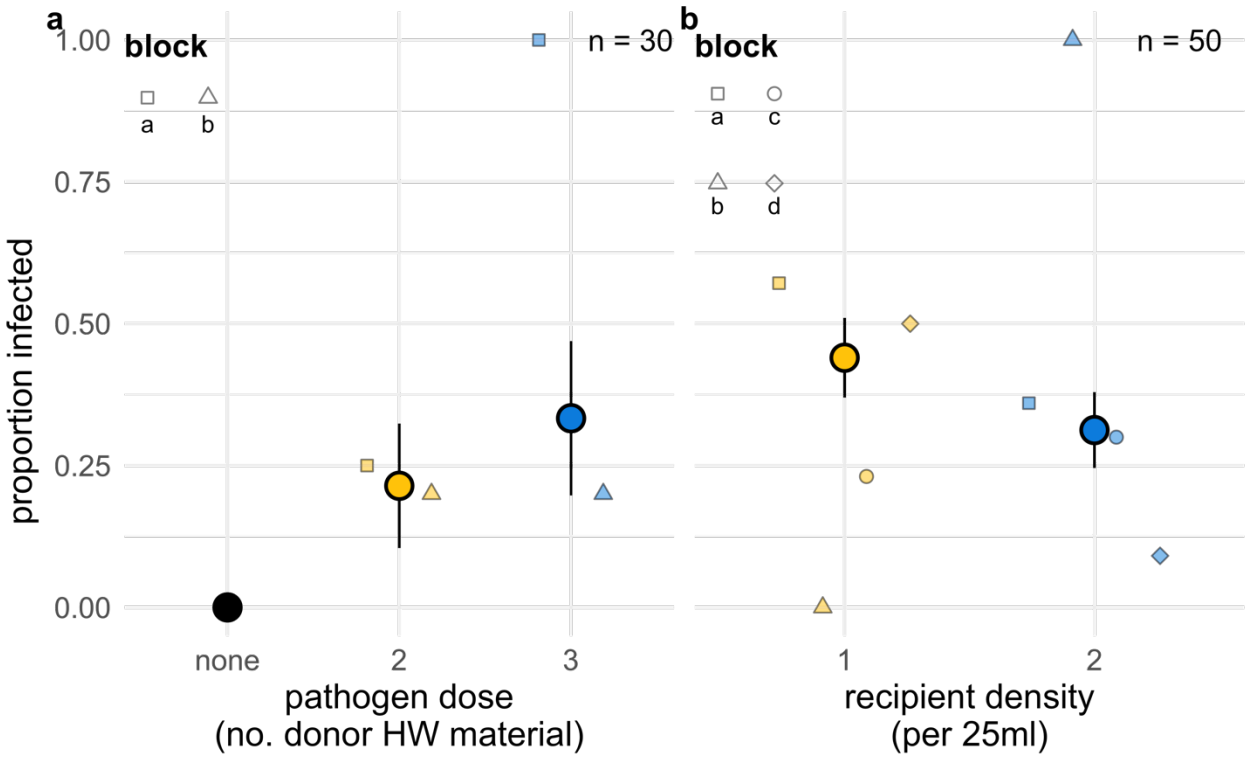

Fig. 5 The transmission success of *Spirobacillus* is not altered by pathogen dose or recipient host density. Mean proportion of individual hosts infected (+/- standard error) in a) experiment 3 and b) experiment 4, as calculated from a generalized linear model specifying treatment as the sole predictor variable. Smaller shaded points indicate the proportion infected in different experimental blocks. Inclusion of block in the statistical model does not change the significance of treatment in either case. The 100% infection rates in blocks a and d of a) and b), respectively are likely an artefact of small sample size (n = 2 in each case). The pathogen dose in panel a is given in terms of the number of donor hosts' worth of material.

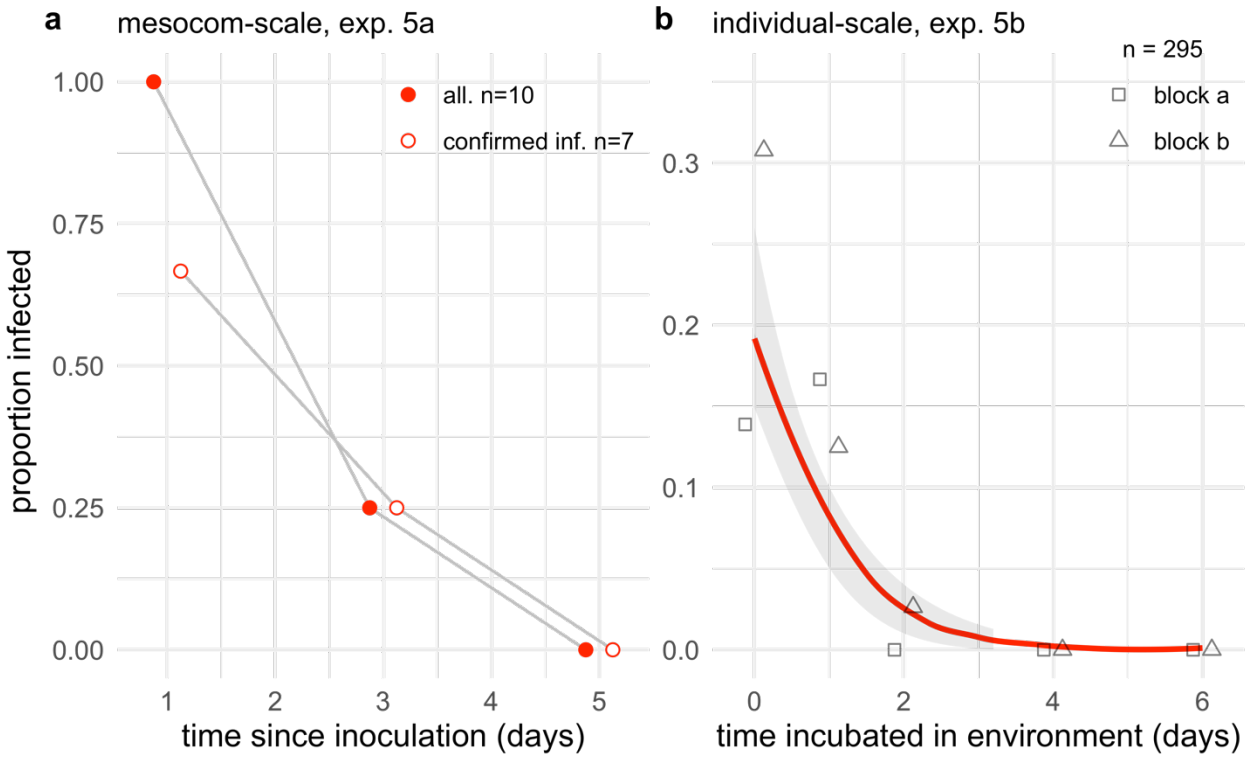

Fig. 6 The infectivity of *Spirobacillus* declines rapidly with time spent in the environment. a) Proportion of mesocosms that generated >=1 symptomatic infections in a secondary cohort of hosts that was added at various intervals post-inoculation. Only a proportion of mesocosms were confirmed to harbor infectious material on d0 (i.e., generated infections in the primary cohort added on day 0). Data from this "confirmed infectious" subset of mesocosms is shown in open circles. The grey lines are plotted to promote readability and do not represent statistical output. b) Mean proportion of individual recipients infected (red line), +/- standard error (grey shading) after exposure to water that had been incubated at different time intervals post-inoculation. Quantities estimated from a generalized linear model with time since donor death as the sole predictor variable. Points indicate the proportion infected in the indicated experimental block. Neither the statistical significance of time since donor death nor the trend indicated changes with the inclusion of block in the model.

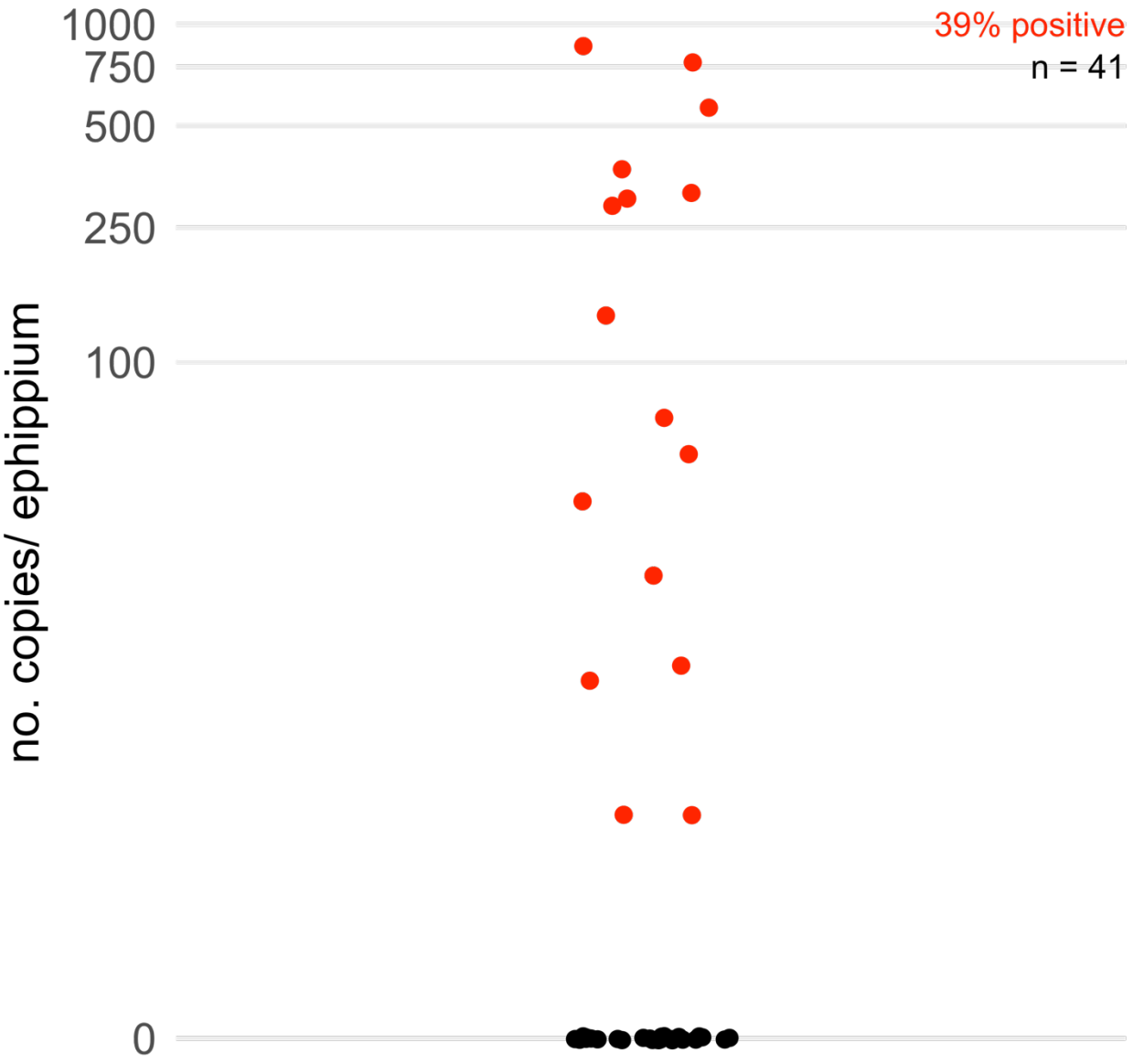

Fig. 7. *Spirobacillus* is present in field-collected *Daphnia dentifera* ephippia (resting eggs). The y-axis gives the absolute quantity of *Spirobacillus* rRNA gene copies (as determined by digital PCR) within individual ephippia collected from a Michigan lake where *Spirobacillus* epidemics have previously been observed.

Exp.	Question(s)	Scale	**Independent variable** (treatments)	**Pathogen dose/ recipient** (# donor host's worth material)	**Duration exposed**	**Duration monitored** *(block)*
1	Is *Spirobacillus* an obligate killer? What is the impact of infection on host fitness?	individual	Status of donor (alive, dead, crushed, unexposed)	0, 1	6hr	21d
2	How do *Spirobacillus* epidemics play out in laboratory populations?	mesocosm	# donors/ vessel (6, 15)	0.08, 0.2	continuous	29d
3	(How) does *Spirobacillus'* transmission success change with pathogen-dose?	individual	# donors/ vessel (2, 3, unexposed)	0, 2, 3	continuous	11d *(a)* 14d (*b)*
4	(How) does *Spirobacillus'* transmission success change with host density?	individual	Density of hosts/ vessel (1, 2)	1	continuous	10d *(a, b)* 12d *(c, d)*
5a	How does *Spirobacillus'* transmission success change with time spent in the environment (environmental incubation)?	mesocosm	Time elapsed after donor addition (1, 3, 5d)	0.05	1, 3, 5d	15d
5b		individual	Time incubated in the environment (water) prior to host exposure (0, 1, 2, 4, 6d)	0.5	24hr	14d

Table 1: Focal question of each experiment. Exp, experiment; d, days; hr, hours. "continuous" exposure means that animals remained in infectious slurry or water to which infected animals were added for the experiment's duration. In all individual-scale experiments, we use the per capita probability that a *Daphnia* became infected as the metric of transmission success.