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 of zooplankton (*Daphnia* sp.) that has significant impacts on zooplankton population dynamics. Despite its ecological importance, little is known about *Spirobacillus'* virulence and transmission biology. These knowledge gaps stymie our understanding of *Daphnia*-*Spirobacillus* interactions and the ecology of the newly described family *Silvanigrellaceae,* of which *Spirobacillus* is the longest studied member. Here, we present our work to fill these gaps, experimentally. We found that *Spirobacillus* is among the most virulent of *Daphnia* pathogens, killing its host within a week of exposure and reducing host fecundity. Our experiments further suggest that *Spirobacillus* requires host death to transmit horizontally i.e., it is an "obligate killer", since it did not transmit horizontally among hosts unless the host died or was destroyed. In a series of further experiments characterizing the dynamics of horizontal transmission, we found that *Spirobacillus'* per capita rate of transmission is low, does not change with host density or pathogen inoculum size, and decreases rapidly with time spent in the environment. Lastly, we complemented our experimental work with a field survey of *Spirobacillus'* prevalence within ephippia, seed-like cases containing *Daphnia* offspring. We found *Spirobacillus* DNA in almost 40% of ephippia, suggesting that *Spirobacillus* is vertically transmitted and/or can invade ephippia and use them to endure environmental stress, as their hosts do. Our work fills critical gaps in our understanding of this ecologically important pathogen's biology.

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 large 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, to what extent it reduces host survival and reproduction (i.e., how virulent it is) 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 sacks. 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* populations in Michigan, where *Spirobacillus'* dynamics have been most intensively studied, *Spirobacillus* causes large epidemics, depleting the numbers of hosts and increasing their vulnerability to predation (4, 14, 15). *Spirobacillus'* relatives in the *Silvanigrellaceae* may also be host-associated, and perhaps even pathogenic, as evidenced by their presence on frog skin and in the tissues of a sick person (16, 17). By better understanding the biology of *Spirobacillus* we thus stand to gain a deeper understanding

of the eco-epidemiological dynamics of zooplankton and the biology of a newly described group of bacteria, the *Silvanigrellaceae*.

A first step toward this goal is to fill critical gaps in our understanding of *Spirobacillus*' virulence and transmission biology, which will together drive its evolutionary and epidemiological dynamics (18). These gaps exist because *Spirobacillus*' has been difficult to establish in the laboratory; nevertheless, field studies, limited laboratory investigations and genomic analyses have yielded some insights and hypotheses. For example, the observation that *Spirobacillus*-infected, asexually reproducing *Daphnia* rarely bear offspring and always die from infection (11, 19), gave rise to the hypothesis that *Spirobacillus* is an "obligate-killer" i.e., a pathogen that requires host death to transmit horizontally. This hypothesis remains untested, however. Similarly, very little is known about the dynamics of *Spirobacillus'* horizontal transmission or the ecological factors that impact it. An experimental investigation demonstrated that *Spirobacillus* transmits via water (19), presumably by motile free-living stages and/or spore-like stages that have been observed via microscopy and for which there is genomic evidence (10, 20). The infectivity of free-living stages, and the rate at which their infectivity decays, are critical drivers of the epidemiological dynamics and virulence of environmentally transmitted pathogens (21–23) and can be impacted by a range of abiotic and biotic factors (22, 24, 25). Thus, understanding the ecology of horizontal transmission is essential if we are to explain *Spirobacillus'* epidemic dynamics. Lastly, to better understand how *Spirobacillus* persists between the seasons when epidemics occur, further investigation is required to establish if *Spirobacillus* is capable of vertical, as well as horizontal, transmission, as prior observations suggest (11).

In 2016, we overcame a major obstacle to filling these knowledge gaps by establishing *Spirobacillus* in *in vivo* culture (19) and thereafter performed a series of experiments to answer fundamental questions about *Spirobacillus'* infection biology, which we present here. Our

experiments demonstrate that *Spirobacillus* is a highly virulent, obligate killer that dramatically reduces host fecundity. Consistent with previous laboratory observations, but surprising in the light of the large epidemics that have been observed in the field, we show that the transmissibility of *Spirobacillus* is consistently very low, does not vary with host density or parasite inoculum size (i.e., the amount of bacterium to which hosts were exposed, hereafter "dose") and declines rapidly after host death. Lastly, consistent with the hypotheses that *Spirobacillus* is capable of vertical transmission or invades ephippia while in a free-living state in the water column, we show that a high frequency of field-collected ephippia harbor *Spirobacillus*. Our work advances our understanding of the basic biology of this little studied pathogen and leads to multiple, testable hypotheses as to how *Spirobacillus* can persist in *Daphnia* populations, despite its apparently low horizontal transmission rate.

Materials & Methods

Individual-scale experiments

To investigate the virulence and transmission dynamics of *Spirobacillus*, we conducted several "individual-scale" experiments, wherein individual *Daphnia* (hereafter "recipients') were exposed to either infected hosts (hereafter "donors") or their homogenized remains (Table 1). Briefly, in experiment 1 we investigated whether *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. In experiments 2 and 3, we respectively investigated whether pathogen dose

Exp.	Question(s)	**Independent variable** (treatments)	**Pathogen dose** (# host's worth material)	**Duration exposed**	**Duration monitored** *(block)*
1	Is *Spirobacillus* an obligate killer? What is the impact of infection on host fitness?	Status of donor (alive, dead, crushed, unexposed)	1	6hr	21d
2	Is *Spirobacillus'* transmission rate dose-dependent?	# donors per beaker (2, 3, unexposed)	0, 2, 3	continuous	11d *(a)* 14d (*b)*
3	Does *Spirobacillus'* transmission rate change with host density?	Density of hosts per beaker (1, 2)	1	continuous	10d *(a, b)* 12d *(c, d)*
4	How does *Spirobacillus'* infectiousness change following host destruction?	Time since donor death (0, 1, 2, 4, 6d)	0.5	24hr	14d

Table 1: Focal questions addressed by individual-scale experiments. Exp, experiment; d, days; hr, hours. "continuous" exposure means that animals remained in infectious slurry for the experiment's duration.

and host density, two variables that commonly alter infectious disease transmission (26, 27), impact the per capita rate of *Spirobacillus* transmission. Lastly, since *Spirobacillus* is a water-borne pathogen whose transmission dynamics are likely to depend on its survival in the environment, in experiment 4 we investigated how the infectiousness of *Spirobacillus* varies with increasing time spent incubating in the environment, in the absence of hosts. 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. Each of these individual-scale experiments shared a similar format and schedule, as follows.

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 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 (19).

Experimental exposure & monitoring

To expose recipients to *Spirobacillus,* we either added donors or their homogenized remains to the water in which recipients were housed (Table 1). Specifically, on the first day of the experiment, denoted day 0, we placed recipients in 50mL beakers filled with 25mL filtered lake water. In experiment 1, we exposed recipients to live donors, the remains of hosts that had died the previous day, or a “slurry” made of homogenized hosts. To make the slurry, we homogenized donor hosts in MilliQ water using a motorized pestle and brought the solution up to a known volume. We then mixed the “donor-slurry” thoroughly and added a fraction of the slurry to each beaker so that each beaker received ~ one donor host’s worth of putatively infectious material. In addition, we included an “unexposed” treatment that was treated the same way except that we did not add live donors, dead infected hosts or crushed hosts to the beakers.

In experiments 2-4, we exposed recipients via donor slurry only. We introduced a volume of slurry equivalent to between 0.5 to 3 donor host’s worth of infectious material (depending on the experiment, cf. Table 1). We varied parasite inoculum in this way because methods to quantify the amount of *Spirobacillus* in a sample via e.g., microscopy or CFU have not been developed. In experiment 2, we also included an “unexposed” treatment that did not receive any donor slurry.

In experiment 4, we sought to understand how environmental conditions – independent of hosts – alter the infectiousness of *Spirobacillus* over time. Accordingly, we added donor-slurry to each beaker before the recipient was added. We then placed the water-slurry solution in an incubator at 22°C, until a predetermined interval (0, 1, 2, 4 or 6 days) had elapsed, at which point we added a recipient.

In experiments 2 & 3, we exposed recipients for the duration of the experiment. In experiments 1 & 4, we exposed recipients for 6 and 24 hours respectively, after which time they were removed, rinsed in filtered lake water and placed in clean filtered lake water. We chose an exposure period of 6-hours in Experiment 1 based on preliminary data on mean survival time of symptomatic, *Spirobacillus*-infected hosts (Fig. S1). These data suggested that greater than 50% of alive donors would remain alive during the exposure period. Nevertheless, 48 of 79 donors in the alive treatment died during the exposure period; none of the recipients became infected, however.

We monitored recipients daily for the above-described symptoms of infection (19). Although our data (cf. Results) suggests that hosts that appear asymptomatic after a week or more are not infected, we cannot exclude the possibility that “uninfected” hosts became infected but subsequently cleared the infection. As such, our experiments are best interpreted as evidence of the effects of experimental treatment on the probability that symptomatic *Spirobacillus*-infection is observed.

In experiments 1, 2 and 4, we allowed infection to proceed until host death; we hence use these experiments to quantify the impact of infection on survival. In experiment 3, recipients were preserved for subsequent analyses (not presented here) as soon as they became symptomatic. To quantify the impact of infection on fecundity, we removed and counted the number of offspring produced by each host in experiment 1, three times a week, and in block b of experiment 2 (daily). In all other experiments, we removed offspring from beakers daily but did not quantify their number.

In experiment 1, we preserved recipients that died from an unknown cause or were alive on the last day of the experiment in 1.5mL tubes filled with 180µL TE buffer and froze them at -

20°C. To assess whether asymptomatic infection occurred, we assessed a subset of the stored, asymptomatic hosts for infection by PCR, as previously described (19).

Husbandry

All experiments were conducted under a 16-8 hour light-dark cycle at 22°C. Animals were fed 250μl of a 1 million cells/ml solution of *Ankistrodesmus falcatus* daily (yielding 10,000 cells/mL food), with the exception of experiment 4 wherein animals were fed 500μl (20,000 cells/mL) per day.

<u>Mesocosm-scale experiments</u>

We supplemented our individual-level experiments with investigations of the dynamics of *Spirobacillus* infection in mesocosms, akin to those used to maintain infection in the laboratory. All experiments were maintained under a 16-8 hour light-dark cycle at 22°C and fed 20ml of algal solution (1 million cells/mL *A. falcatus*), three times a week.

To complement experiment 4, and to assess the extent to which mesocosms contain infectious material as time elapses, we performed an experiment of the following design. We added 80 recipients, denoted the `primary cohort', to 1L beakers containing 800ml filtered lake water. We then added 4 donors to this mesocosm. After a predefined interval (1, 3, or 5 days), we removed the primary cohort to a secondary beaker containing filtered lake water and added a cohort of 80 `secondary' recipients to the original mesocosm. We monitored whether any infected animals appeared in the primary and secondary cohorts for 15 days following their initial exposure to the water in the original beaker. Accordingly, we used the 'primary' cohort to indicate whether the focal mesocosm was *ever* infectious, and the secondary cohort to indicate whether any

infectious material remained in the beaker after the time interval had lapsed. We ran 3 replicates of each of the 1 and 3-day treatments and 4 of the 5-day treatment.

To quantify the dynamics of infections in *in vivo* cultures, we monitored the prevalence of infection (as indicated by symptomatic animals) and total host density in 1L mesocosms. On day 0, we filled each 1L mesocosm with 800ml filtered lake water. We added 75 recipients to each mesocosm. To estimate the density of animals and prevalence of infection in mesocosms, we took 20 x 20ml samples on each sampling day using a handmade scoop. We demonstrated in preliminary experiments that the estimate of infection prevalence stabilizes at or before 20 samples were taken. We temporarily retained each sample, counted the number of symptomatic and asymptomatic animals and then stirred the mesocosm before taking the next sample. After all samples were taken, we replaced the water and animals back into mesocosm.

Quantification of *Spirobacillus* in field-collected ephippia

To assess whether *Spirobacillus* DNA was present inside ephippia, we collected ephippia from the field and quantified *Spirobacillus* via ddPCR.

In May and November 2019, we collected sediment from Pickerel Lake (Washtenaw County, Michigan, USA), wherein *Spirobacillus* infected animals have been observed over several years (28). To separate the ephippia from the sediment, we placed small portions of the sediment (~4 grams) on a 243µm plankton net filter and rinsed it with deionized water (diH_2O) to remove small particles and liquid reside. We transferred the material retained on the filter to a watch glass containing diH_2O, inspected it under a dissecting microscope and isolated ephippia of *Daphnia dentifera* following Rogalski et al. (29). We then surface-sterilized the ephippia by placing them in 70% ethanol for 2 mins and finally placed each ephippium in a separate 1.5 ml

centrifuge tube filled with PBS buffer. We homogenized the ephippium using a sterile motor-powered pestle and stored the tubes at -20℃.

Next, we extracted whole genomic DNA from each ephippium using the DNeasy Blood and Tissue kit (Qiagen®), initiated by adding Proteinase K into the thawed solution and then following the manufacturer's protocol. Extracted DNA was stored at -20℃ prior to analysis by digital PCR, as follows. Each reaction contained 20µl of eluted DNA, 10µl of mastermix (QIAcuity™ Probe PCR Kit), 0.72 µl of forward primer Spiro70F (5'-AACTGTAGCTAAGACCGCGT-3') and 0.72 µl of reverse primer Spiro210R (5'-CCAGCTATCCATCTTCGCCT-3'), 0.36 µl of dual-quenched probe SpiroP (5'-ACCGTAAGGCCAGCAGCCATAAGATGA-3') labeled with fluorescent Dye ROX at the 5' end and Iowa Black® RQ dark quencher at the 3' end, 0.5 µl of restriction enzyme EcoRI and 13.5 µl of RNase-free water. We used a high volume of DNA because we expected that the ephippia would contain a low concentration of bacterial DNA. The PCR was performed in a 24-well Nanoplate 26K (Qiagen®), using a QIAcuity One Digital PCR System (Qiagen®). PCR conditions were 2 min at 95℃, followed by 40 cycles of 15 sec at 95℃ followed by 1 min at 55℃. We first performed post-PCR imaging using an exposure time of 300ms and, when it was necessary to increase the separation of signal from noise, reimaged the plates using either 200ms or 100ms. The results were analyzed with QIAcuity Software Suite 2.0.20. Finally, we converted the output – number of gene copies in the reaction mix – to the total copy number/ephippium. Note that, since we do not know the number of copies of the 16SrRNA copies in the *Spirobacillus* genome, copy number should not be interpreted as an absolute estimate of the number of cells contained within each ephippium.

We designed the aforementioned primers and probe using NCBI Primer-BLAST (30) to target the *Spirobacillus* 16S rRNA gene. We verified the assay's specificity by using it to assay DNA of three other members of the family *Silvanigrellaceae, Silvanigrella aquatica*, *Silvanigrella paludirubra* and *Fluviispira multicolorata.* The assay did not amplify DNA from these species.

Statistical analysis

We performed statistical analysis using R (version 4.2.1). To test for an association between treatment and the probability of infection, we ran Chi-square tests or (when expected values<5) Fisher's Exact Tests, following (31). To verify the result of these analyses and to obtain estimates of per capita infection rates (and confidence intervals around said estimates) for presentation in figures, we ran binomial general linearized models (GLMS) and (where necessary) controlled for block. In all case, the two approaches yielded the same results *vis-a-vis* the significance of treatment. We removed asymptomatic animals that died on or before day 8 – the last day on which we ever observed symptoms – from the analysis of transmission rates, reasoning that these animals may not have had sufficient time to display symptoms.

To quantify the impact of infection on fecundity, we used Poisson GLMs. We corrected for overdispersion, which was found in all cases following (32) i.e., using a quasi-Poisson model. Animals whose fecundity was not recorded throughout their lifetime were removed from this analysis.

To analyze the effect of treatment on time to death and symptoms, we employed survival analyses using the *surv* package (33). These analyses allow us to include all the individual-level time series data, including data relating to animals that died before day 9 or which were not tracked throughout the entirety of a time-period. We fit Kaplan-Meier survival curves to time-series data *vis-à-vis* host death and the appearance of symptoms and used them to derive

estimates of the median (+/- 95% CI) time-to -death and -symptoms, respectively. To ask whether *Spirobacillus* had a significant impact on lifespan, we used log rank tests to compare the survival curves. In addition, in Figure 1b we plot the results of (i) a “competing risks” analysis, which we used to quantify the probability that an animal either becomes symptomatic or dies without showing symptoms (dashed line), and (ii) a “multi-state” analysis, which we used to quantify the probability that an animal died following the appearance of symptoms (black line) (34).

Results

Virulence

Symptomatic *Spirobacillus* infection (“infection”, hereafter) had a 100% fatality rate in our individual-level experiments (Fig. 1a). Symptoms developed rapidly – on average, by day 6 – and were followed by death within a day, on average (Fig. 1b; Fig. S1, restricted mean survival time of a symptomatic animal 9.6 hours). As a result, *Spirobacillus* infection had a significant impact on host lifespan (χ^2 = 290, df = 1, p<0.001). In experiment 1, the median survival time of infected animals fell from over 21 days (i.e., longer than the duration of the experiment) in infected, but exposed, animals to to 7 days (95% CI 7-8) in uninfected animals.

Spirobacillus infection significantly reduced the fecundity of infected animals, as compared to uninfected treatment-mates (Fig. 2a, *infection status* δAIC= 187, df=1). Over the course of experiment 1, for example, infected animals had ten times fewer offspring than uninfected animals in the same treatment. The effect of *Spirobacillus* on host fecundity does not seem to stem only from its effect on host lifespan. During the first week of experiments 1 and 2**,** recipients that later became symptomatic had fewer offspring than those that did not become

infected (Fig. 2b). Thus, the negative effects of *Spirobacillus* infection on host fitness are apparent even before symptoms are detectable.

Transmission among asexually reproducing *Daphnia*

Spirobacillus required host death or destruction to transmit (Fig. 3). In experiment 1, symptomatic infection was only observed in animals exposed to material from donors that had been destroyed by homogenization or had died naturally (Fig. 3). To assess whether asymptomatic infections may have arisen, 46 asymptomatic animals (including 20 that were exposed to alive donors) were assessed for infection via PCR. None were positive (data not shown). These results suggest that *Spirobacillus* is an obligate killer and that asymptomatic infection does not occur.

Across all individual-scale experiments, the mean per capita probability of infection did not exceed 50% in any treatment (Figs. 3-5). The per capita probability of infection was not impacted by pathogen dose (Fig. 4a, $p= 0.7$) or the density of animals in the beaker (Figure 4b, $p = 0.2$).

The infectiousness of *Spirobacillus* declined with time spent in water in the absence of hosts (Fig. 5*, time since host destruction* $\chi^2 = 31$, df =1, $p<0.001$), in experiment 4 were we made observations at the individual scale. *Spirobacillus* that was not incubated in the environment for any period generated infected hosts at a rate of 20**%**. Our data suggests that, after just 3 days of incubation, the probability that a host became infected fell to <1% (Fig. 5). We observed a similar decline in infectivity in mesocosm-scale experiments (where, unlike in experiment 4, we cannot disentangle the effect of hosts and the environment on *Spirobacillus'* infectivity). None of 3 mesocosms that generated infections in the “primary cohort” were infectious 5 days after inoculation and only 1 (of 3) generated any infections 3 days after inoculation.

We did not observe classical epidemic dynamics in mesocosms (Fig. 6). With the exception of 1 mesocosm, the prevalence of symptomatic hosts cycled up and down at a level below or equivalent to the initial prevalence. In no mesocosm did the prevalence of symptomatic hosts ever grow for a period longer than the incubation period of 7 days (which, given that *Spirobacillus* is an obligate killer, is equivalent to the time-to-death of symptomatic hosts as estimated above). Furthermore, the density of symptomatic hosts did not lag behind that of asymptomatic hosts, as is expected (21); indeed, the dynamics of these two cohorts bear scant relationship to one another.

<u>Presence within ephippia</u>

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

Discussion

Despite *Spirobacillus cienkowksii*'s long history of study and widespread distribution, fundamental aspects of its biology have remained uncharacterized. Our experiments reveal that *Spirobacillus* is among the most virulent of *Daphnia* pathogens yet described (33–35), killing its host almost twice as rapidly as *Metschnikowia bicuspidata* (35), the pathogen of *Daphnia dentifera* with the next most rapid measured impact on host death. In addition to killing hosts rapidly, *Spirobacillus* reduces host fitness by reducing host fecundity. *Spirobacillus'* impact on host reproduction is equivalent to that of another common parasite of *Daphnia*, the castrator *Pasteuria ramosa*, but *Spirobacillus'* effect likely comes about via a different mechanism. *Pasteuria* sterilizes hosts weeks before it kills them, presumably via chemical manipulation of the host (36). By contrast, *Spirobacillus* primarily impacts offspring production by reducing host lifespan, though it also reduces fecundity prior to their early death. Given that we have

observed field- and laboratory- infected *Daphnia* birth on the day they die (data not shown), we think it unlikely that *Spirobacillus* directly interferes with host reproductive functions but rather reduces host reproduction by inducing e.g., nutritional stress.

We demonstrated that *Spirobacillus'* effects on mortality are intimately connected with its capacity to transmit horizontally. Specifically, in experiment 1, we showed that *Spirobacillus* requires the death of its host to transmit horizontally consistent with the hypothesis that it is an obligate killer. Given that mechanical destruction of the host permitted infection, host death may not need to be induced by the pathogen, however. Rather, destruction of hosts by "sloppy" predators may facilitate transmission (37, 38). Though our experiments were not explicitly designed to explore the timescale of transmission following host death, the "alive" treatment of experiment 1 reveals something about it. Several of the "alive" donors died within the 6-hour exposure period, yet none of the recipients became infected**.** This implies that either there is a lag between host death and the production of viable transmission stages (unlikely given that crushing hosts yielded infections) or, more likely, that it takes some time for motile pathogens to escape and find a new host.

In these laboratory conditions, the per capita rate at which susceptible hosts become infected (or the force of *Spirobacillus* infection (FOI)) was low and was not influenced by two ecological factors that are expected to change FOI. FOI is a product of a transmission constant, the density of infected hosts or pathogen in the environment, and susceptible host density (27). Yet *Spirobacillus*' FOI did not increase with pathogen dose in experiment 2, nor between experiment 3 (where recipients were exposed to 1 host worth of parasite) and experiment 2 (where recipients were exposed to 2-3 host's worth of parasite). This behavior contrasts markedly with that of *Pasteuria ramosa*, for example. In *D. magna* housed in similar experimental conditions, only a small fraction (~0.001%) of the *Pasteuria* spores contained in a fully infected host is

sufficient to infect ~50% of new hosts, and a dose of ~10% of the spores in a fully infected host all but guarantees infection ((39–41)(42). Our data are not sufficient to conclude that there is no relationship between pathogen dose and *Spirobacillus'* FOI, however. Dose-response relationships are rarely linear, and, instead, tend to be decelerating (42, 43). One hypothesis for our observations, therefore, is that we were working in the decelerating region of the dose response curve in experiments 2 and 3 and that had we explored a larger range of smaller doses, we would have observed a relationship between inoculum size and FOI. Similarly, there may well be a relationship between host density and FOI, that went unobserved because of experiment 3's limited sample size and/or range of treatments. Indeed, that new infections arose in our mesocosms – which contained a higher density (~2.5x) of susceptible hosts but were initiated with a much smaller inoculum than our individual-scale experiments – implies that density-dependence may play a role in *Spirobacillus'* transmission dynamics. Ironically, we were unable to explore a wide range of density and dose treatments or boost the power of our experiments via greater replication because the poor transmission of *Spirobacillus* in *in vivo* cultures limited the availability of donors.

Spirobacillus' limited environmental persistence, as demonstrated in experiment 4, combined with its low force of infection, likely explain why we did not observe epidemics in *in vivo* cultures. The extent to which environmentally transmitted pathogens can persist in the environment is a critical determinant of their fitness and epidemiological dynamics (22, 25, 44–46). For obligate killers like *Spirobacillus*, whose survival in the environment (at least in our experiments) does not exceed the timescale of host population dynamics, the ability to invade a host population increases with transmission rate and virulence, and declines as the decay rate of infectious stages increases (47). So, although *Spirobacillus* is highly virulent, its low FOI and rapid rate of decay in the environment may provide a "break" on its ability to cause epidemics in the laboratory. Instead, we observe peaks and troughs in the prevalence of infection, with a

periodicity of ~6 days (at least at the beginning of infections, when small differences among individuals have not accumulated to dampen the cycles). We hypothesize that these dynamics are a result of the strong, relatively invariant periodicity of the time to symptoms (6-7 days) and time to death (7-8 days), combined with *Spirobacillus'* limited environmental persistence.

Why, then, does *Spirobacillus* generate epidemics in the field (and not in the lab)? We have two hypotheses. Our first assumes that *Spirobacillus* is an obligate parasite and attributes our findings to a discrepancy between the conditions in the lab and those under which transmission occurs in nature. For example, in a study of *Spirobacillus* in the water-column, Thomas et al. (48) found the highest density of *Spirobacillus* at the surface-water interface, a region characterized by low temperatures, oxygen and light (49, 50). If this region is the location of transmission in nature, our experiments poorly represent them. Alternatively, it is possible that our recipient hosts were less susceptible than the hosts that become infected in nature e.g., because of their age or genotype or nutritional status. Given that we have observed infection across host ages in the field and have generated infections at equivalent rates in other hosts clones (unpublished data), we think that differences in the abiotic conditions of the lab vs. the field are more likely to explain our inability to recover epidemic dynamics in the lab, than differences in their biotic conditions.

Our second, more heterodox, hypothesis is that *Spirobacillus* is a facultative pathogen of *Daphnia,* whose epidemic dynamics are not entirely a function of replication within asexually reproducing *Daphnia*. If our experiments do reflect *Spirobacillus* behavior in nature, it is unlikely that it could make a living as an obligate pathogen because of its poor horizontal transmission rate. Instead, it is possible that *Spirobacillus* primarily replicates on particles (including ephippia) in the water column and/or sediment and employs the same traits that allow for particle-finding, -attachment and -exploitation to parasitize hosts, when the opportunity arises. In accordance

with this hypothesis, *Spirobacillus* and the other *Silvanigrellaceae* exhibit a suite of traits, including pleiomorphism, small size and the genomic capacity to make flagella and perform chemotaxis (20), that are characteristic of copiotrophic aquatic microbes capable of facultative predation or parasitism, e.g., the *Vibrios* and *Bdellovibrios* (51, 52). Under this model, either (i) *Spirobacillus* outbreaks in *Daphnia* result from "blooms" in the particle-associated population that spillover into the *Daphnia* population, or (ii) there is an ecological cue that stimulates a switch from a free-living or particle associated lifestyle to a *Daphnia*-associated lifestyle. Comparative genomic analyses of the *Spirobacillus* genome with that of predatory and/or parasitic species, and investigations of the distribution and abundance of *Spirobacillus* in different particulate and non-particulate fractions of the water column, could shed further light on this hypothesis.

Our discovery that *Spirobacillus* is harbored within ephippia is consistent with the idea that *Spirobacillus* may not entirely rely on asexually reproducing hosts for its fitness and could help us to explain a range of observations made in the past and in this study. We found *Spirobacillus* DNA in almost 40% of the ephippia assayed. This observation, combined with a previous observation that ephippia inside infected *Daphnia* contain bacteria (11), suggests that *Spirobacillus* may be vertically transmitted from sexually reproducing females to ephippia. (We note that vertical transmission would include bacteria that are contained within the ephippium but not the embryo itself.) That said, these data do not exclude the possibility that ephippia become colonized by *Spirobacillus* after they had been released from *Daphnia* and, given that *Spirobacillus'* prevalence rarely exceeds 10% even during epidemics, its comparatively high prevalence in ephippia (39%) is difficult to explain with respect to vertical transmission alone. Irrespective of the mechanism by which *Spirobacillus* enters the ephippia, the ability to exploit ephippia could explain *Spirobacillus'* inter-epidemic persistence and hypervirulence. Rodrigues *et al*. observed that *Spirobacillus*-infected hosts hatch directly from ephippia (11). This

observation, coupled within our findings, suggests that *Spirobacillus* might use the hardy ephippia to persist through the winter, before emerging from them and seeding the next year's epidemics. Given the high energetic costs of sporulation (53), invading a hardy structure rather than making one could have significant advantages. The capacity to survive within ephippia might also allow *Spirobacillus* to offset or avoid paying the costs of virulence, if it is an obligate pathogen, per the "curse of the pharaoh" hypothesis (23).

Our work reveals much about the life history of a bacterium that has a long and significant history in bacteriology and sets a foundation for the use of *Spirobacillus* as a model of host-parasite interactions at the individual host and evolutionary timescale. Carotenoid production in *Spirobacillus* was first described in 1959 at the dawn of carotenoid chemistry (12, 54), and bacterial carotenoids have since received significant attention in biotechnology and medicine, wherein carotenoids are considered virulence factors (55). Here, we found that *Spirobacillus* infection is virulent, reliably symptomatic and proceeds from initiation to death within a week. These features, coupled with our prior description of natural variation in the color of infected hosts (and thence, presumably, carotenoid production) (19), could make *Spirobacillus* a tractable, *in vivo* model with which to study how bacterial secondary metabolites alter infection dynamics and virulence. Future work to identify conditions under which infection can be guaranteed will further boost its tractability in this context. Indeed, future work aimed at elucidating the mode(s) of *Spirobacillus* transmission could help us to make new insights about the evolutionary dynamics of virulence, as well as carotenoid's role in it. The dynamics of virulence evolution in pathogens capable of both horizontal and vertical (i.e., "mixed-mode") transmission and/or environmental replication are poorly understood as compared to pathogens with strictly horizontal and/or direct transmission (56); virulence evolution in obligate killers is 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.

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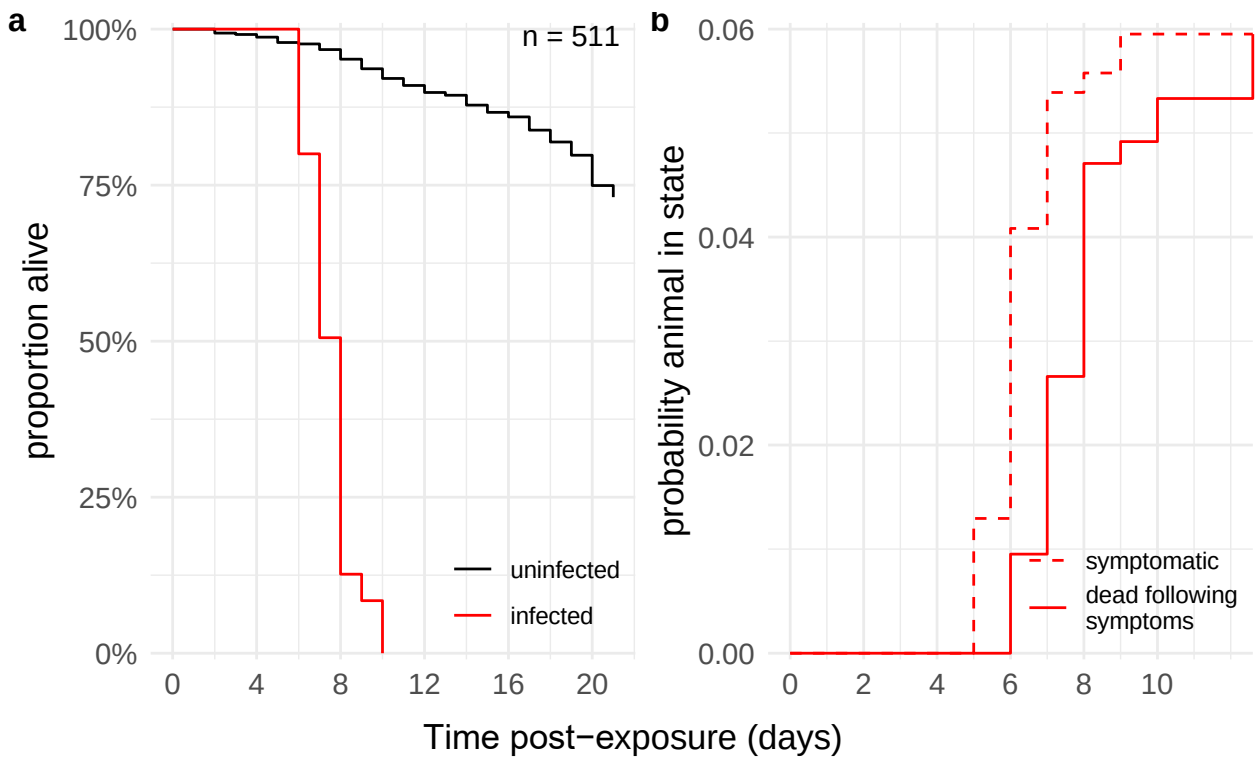

Fig 1. *Spirobacillus* kills its host shortly after symptoms appear, dramatically reducing host lifespan. a) Mean probability of survival (+/- 95% confidence interval, shaded area) following exposure to *Spirobacillus* of infected (red) or uninfected (black) hosts in experiments 1-4. Estimates derived 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, 2 and 4. The estimated probabilities take such small values because most of the animals exposed in these experiments did not become infected (cf. Fig. 3, 4a, 5)

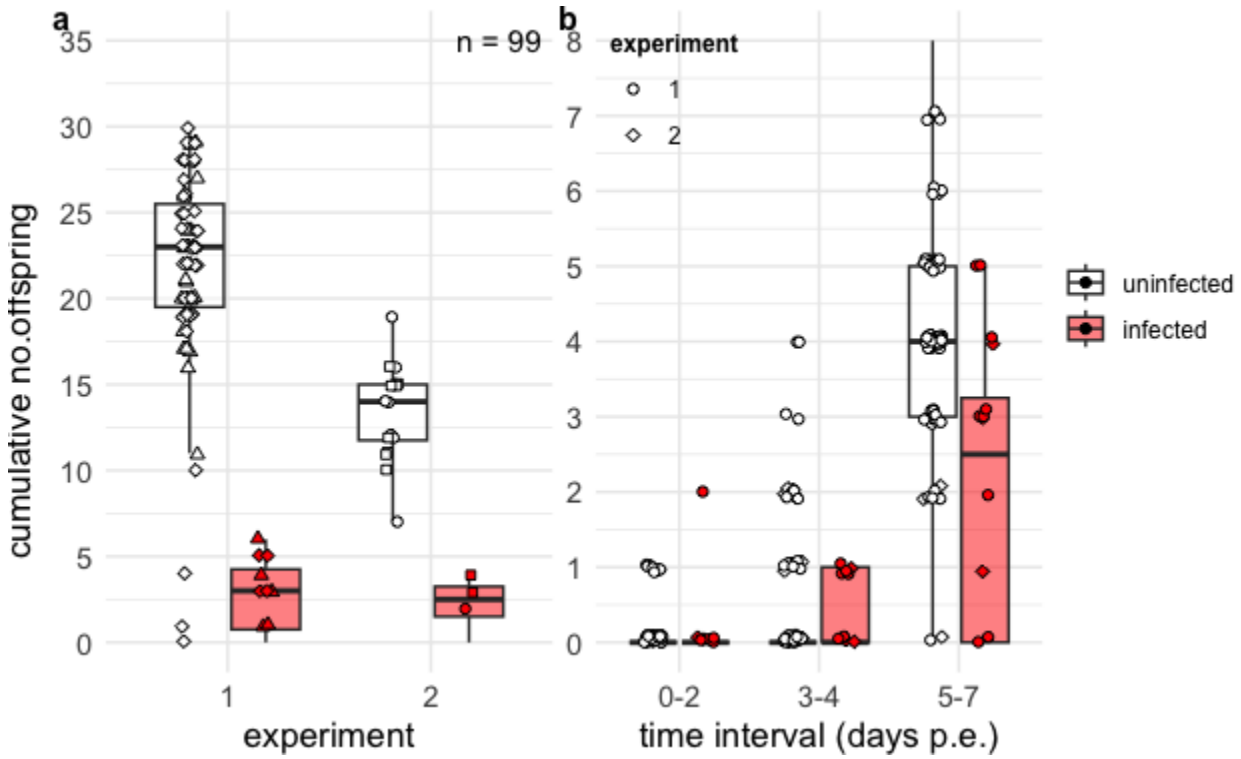

Fig 2. *Spirobacillus* infection reduces the fecundity of infected *Daphnia*, even during the short period that they are alive. a) Cumulative no. of offspring produced per host over the course of experiments 1 and 2. b) Total offspring produced in the first week post exposure (p.e.), by hosts that went on to be infected (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.

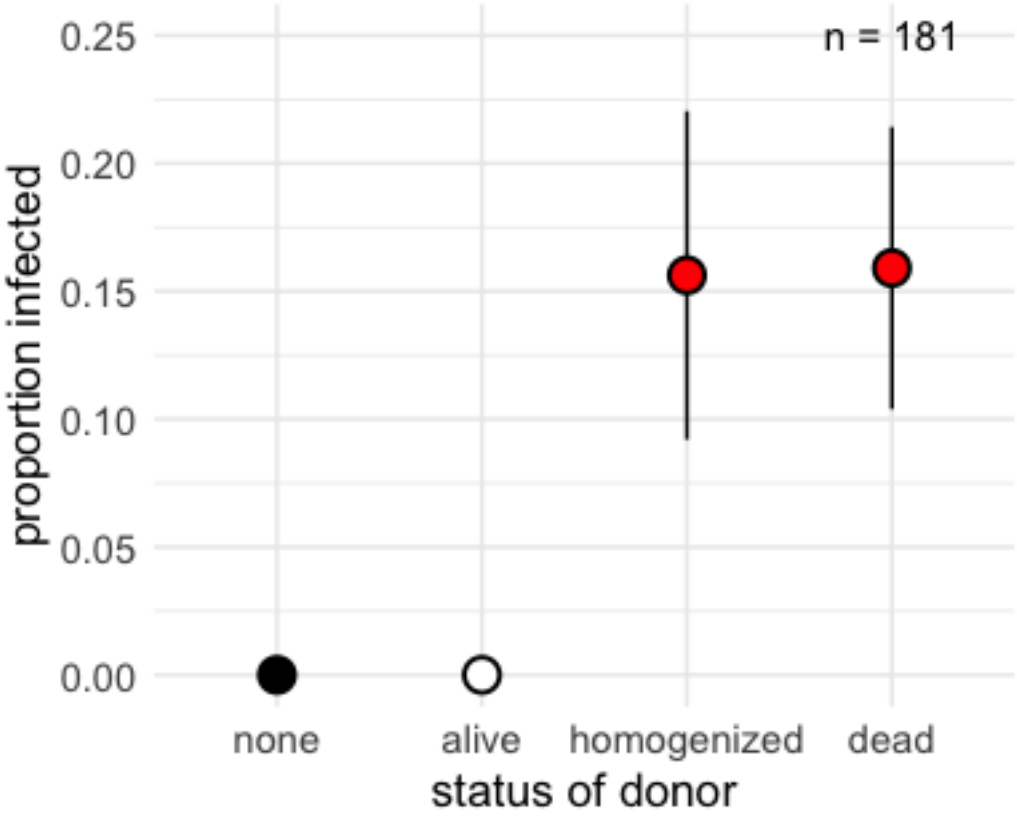

Fig. 3 *Spirobacillus* requires the demise of its host to transmit horizontally. Mean infection rates (+/- standard error) in each treatment of experiment 3, as estimated from a generalized linear model.

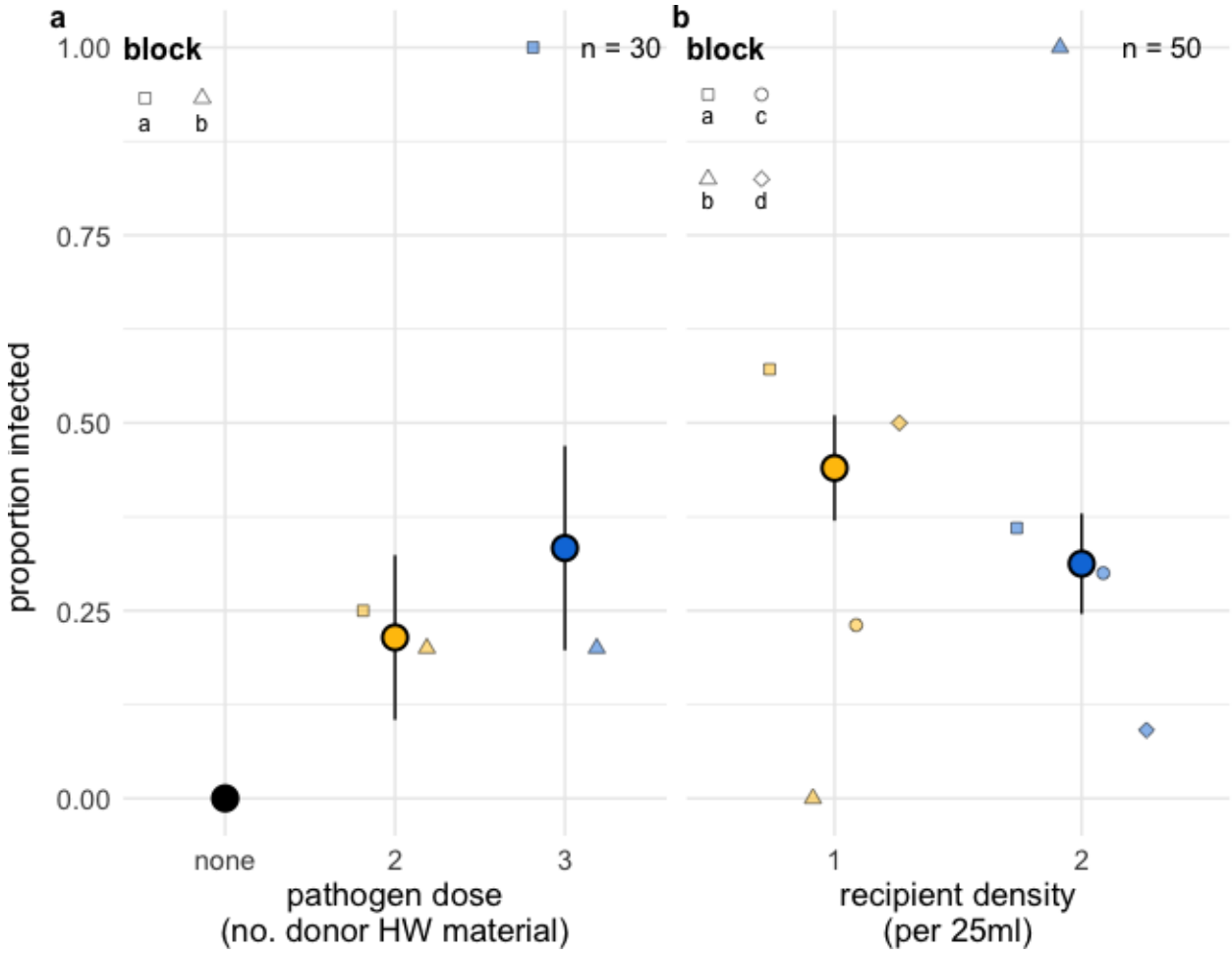

Fig. 4 The per capita transmission rate of *Spirobacillus* is not altered by inoculum size or recipient host density. Mean infection rates (+/- standard error) in a) experiment 2 and b) experiment 3, as calculated from a generalized linear model specifying treatment as the sole predictor variable. Smaller 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.

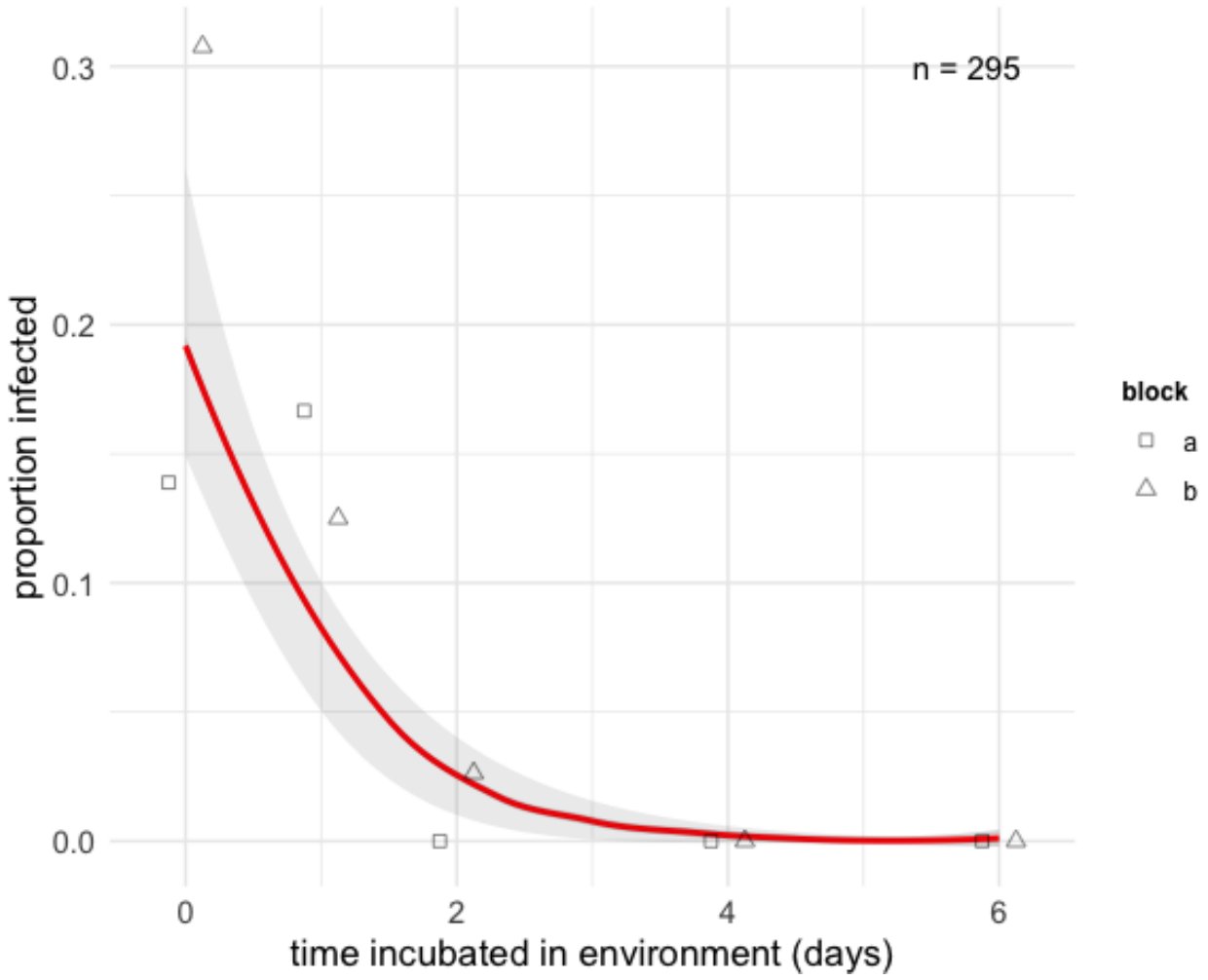

Fig. 5 The infectivity of *Spirobacillus* declines rapidly with time. Mean proportion of animals infected (red line), +/- standard error (grey shading). Quantities estimated from a generalized linear model with time since donor death as the sole predictor variable. Points indicate the proportion infected 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.

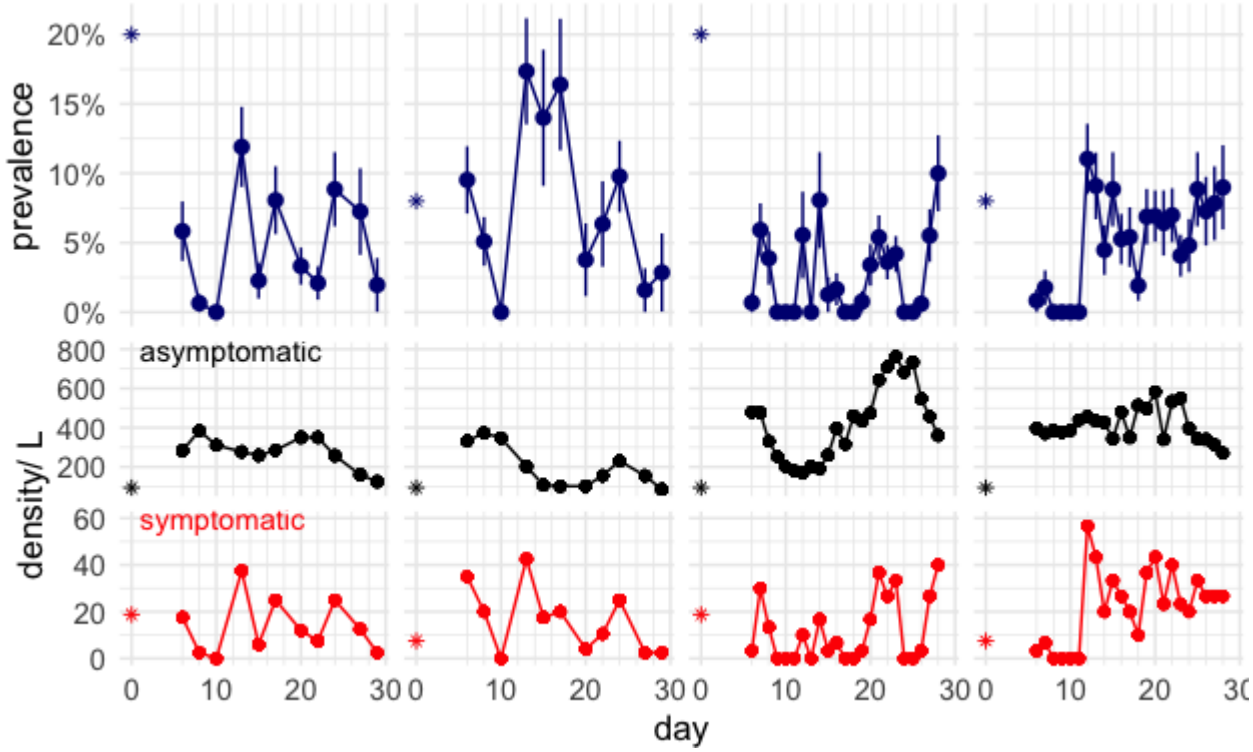

Fig. 6 Dynamics of *Spirobacillus* infection in laboratory mesocosms. The prevalence of symptomatic hosts (blue), and the density of asymptomatic host (black) and symptomatic hosts (red), in 800ml mesocosms over 28 days. Asterisks indicate the initial value of parameters in day 0, when the mesocosm were established. Error bars around prevalence estimated from a binomial distribution.

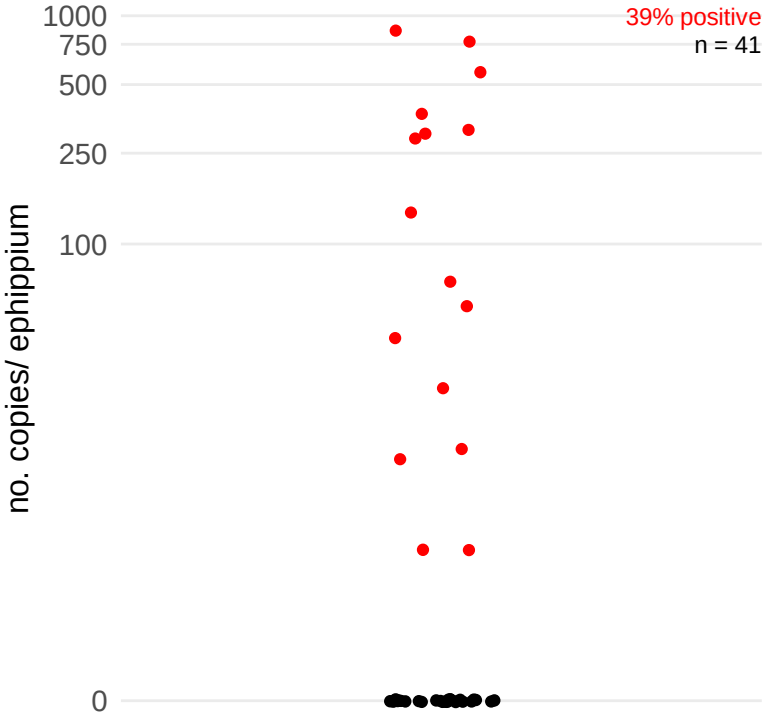

Fig. 7. *Spirobacillus* is present in field-collected *Daphnia dentifera* ephippia (resting eggs). Quantity of *Spirobacillus* rRNA gene copies within individual ephippia collected from a Michigan lake where *Spirobacillus* epidemics have previously been observed.