Running Title Color variation in parasitized *Daphnia*

Title A colorful killer: *Daphnia* infected with the bacterium *Spirobacillus cienkowskii* exhibit unexpected color variation.

Authors Nina Wale[1*], McKenna L. Turrill[1] & Meghan A. Duffy[1]

[1]University of Michigan, Department of Ecology & Evolutionary Biology, Biological Sciences Building, 1105 North University Avenue, Ann Arbor, Michigan, 48109
*****Corresponding Author. nwale@umich.edu

Word Count 1505 (exclusive of in-text refs)

When Elie Metchnikoff peered into a pond in the autumn of 1885, he saw something unusual. Among the many small, clear zooplankton that lived there a few *'distinguished themselves by their scarlet red color'* (Metchnikoff 1889). These animals were *Daphnia* infected with a lethal bacterium that Metchnikoff described and named *Spirobacillus cienkowskii*. Despite its wide distribution across the Northern Hemisphere and among many species of daphniid (Rodrigues et al. 2008), this bacterium has since been the subject of limited study. In this note, we (re)describe how the characteristic scarlet symptoms of *Spirobacillus* infection develop (Fig. 1A) and show that there is hitherto unrecognized variation in the color of infected hosts (Fig. 1B). In addition to the scarlet red color that caught Metchnikoff's eye, animals in the terminal stage of *Spirobacillus* infection may appear milky white, custard yellow, or even muddy brown.

When we first observed *Spirobacillus*-infected *Daphnia dentifera*, while surveying natural populations of *Daphnia* and their parasites in Michigan, USA, we were as struck by their color as Metchnikoff – so much so that we called the bacterium "scarlet". However, we soon began to wonder whether this nickname was entirely appropriate. As well as their color, *Daphnia* infected with *Spirobacillus* are characterized by the 'glittery' appearance of their hemolymph and we often observed animals whose hemolymph had this glittery appearance but were light gray or beige rather than red**.** We suspected that these animals might also be infected with *Spirobacillus*, a suspicion that only strengthened when we had Metchnikoff's original work translated. In field-collected animals, Metchnikoff saw **'***the natural yellow color of the* Daphnia*…became grayish*

yellow, then slightly pink only to become…scarlet red'. Perhaps the beige animals that we had observed were simply in the early throes of infection?

In 2016, we established an *in vivo* laboratory culture of *Spirobacillus,* which allowed us to experimentally infect hosts and closely investigate the progression of the symptoms of infection. Healthy *Daphnia dentifera* were placed alone in a beaker of water along with the crushed remains of an infected red individual. After five or six days, the *Daphnia* turned red and, without exception, died within a day (Fig. S1). During one such experiment, we noticed that an exposed individual appeared 'dense' to the naked eye. Under a stereomicroscope, we saw a light beige, glittery material in the hemolymph of the *Daphnia*, which was distributed in a similar way as the red material within a *Daphnia* exhibiting typical symptoms. Over the next day, this animal's hemolymph turned from beige to pink to red, causing the animal to appear red to the naked eye. So more than a hundred and thirty years after he made them, Metchnikoff's observations of field-collected animals were replicated in the laboratory: the hemolymph of *Daphnia* at the early stage of *Spirobacillus* infection has a glittery, pale beige appearance (Fig.1A, middle); only at the very end of infection does the characteristic scarlet symptom of infection appear (Fig.1A, right) as the host's death knell.

But an animal that isn't red may yet find itself dead. Motivated by a desire to validate our experimental observations in the field, we collected animals with beige hemolymph from several lakes and observed them, with the hope of watching their red color develop. In multiple cases, it did not. Though the hemolymph of all animals became more saturated

with color as it filled with bacteria, in some animals the color the hemolymph became was white, yellow or brown rather than red (Fig. 1B). Even as these *Daphnia* entered the terminal phase of infection, they remained uncolored to the naked eye. Using a species-specific polymerase chain reaction assay, we confirmed that the animals that died with white, yellow or a brown hemolymph were infected with *Spirobacillus*. So, the signature symptom of *Spirobacillus* infection is in fact an unreliable one. The 'terminal coloration' of infected animals, the color that they exhibit at or just before death, can vary markedly (Fig. 1B).

Why might a bacterial infection cause its host to change color? Let's first address the classical symptoms of *Spirobacillus* infection – the host's red appearance at the end of infection. We hypothesize that *Spirobacillus* produces orange-red pigments to protect itself from damaging reactive oxygen species (ROS) that it encounters inside the host. Previous work showed that the red color of *Spirobacillus*–infected cladocera is caused by a carotenoid produced by the bacteria (Green 1959), as opposed to a host product, and we have several lines of preliminary evidence consistent with this conclusion. Bacteria produce a wide variety of secondary metabolites such as carotenoids during 'stationary phase', when the size of the bacterial population stagnates, resources become scarce and oxidative stress caused by ROS increases (Navarro Llorens et al. 2010). To quench ROS, some bacteria produce carotenoids, which are powerful antioxidants (Takano 2016). For example, colonies of *Myxococcus*, a member of the same class of proteobacteria as *Spirobacillus*, turn from white to orange at the onset of stationary phase (Burchard and Dworkin 1966). The accumulation of color as *Spirobacillus* fills the host's hemolymph

may similarly reflect the induction of carotenogenesis as the bacterial population reaches carrying capacity. An additional, but not mutually exclusive, hypothesis is that *Spirobacillus* produces carotenoids to protect itself from the oxidative activity of the *Daphnia* immune system (Auld 2014), facilitating a larger and more virulent infection, as in two bacterial pathogens of vertebrates (Liu et al. 2004, 2005). Under this hypothesis, we might expect *Spirobacillus* cells to produce carotenoids throughout the infection; the intensification of the color of infected animals with time would thus result from increasing cell density. Quantifying the per bacteria production of pigment, or the expression of genes associated with its production, during the course of infection could help to discriminate between these hypotheses.

If carotenoids are potentially beneficial in the context of the within-host environment, why do we see variation in terminal coloration? Our first hypothesis is that *Spirobacillus* differentially produces carotenoids depending on the intensity and/or wavelength of light to which it is exposed while living inside its transparent host. As such, variation in lake light conditions could drive variation in the terminal coloration of *Spirobacillus*-infected *Daphnia*. The plastic induction of carotenogenesis is common among free-living, non-phototrophic bacteria and, intriguingly, these bacteria often produce carotenoids in response to blue light (Takano 2016), which dominates in clear water (Wetzel 2001). In this photic context, the ROS-quenching capacity of carotenoids proves beneficial, since ROS are generated upon the absorption of light by photosensitizing molecules within the bacteria (Elias-Arnanz et al. 2011). However, in the absence of light (and the ROS that it induces), the benefits of carotenoids may not outweigh the heavy energetic costs of

122 producing them. Indeed *Myxococcus* colonies produce few carotenoids and remain
123 yellow if they are maintained in the dark, even if they are in stationary phase (Burchard
124 and Dworkin 1966). In preliminary experiments where *Daphnia* were infected with
125 *Spirobacillus* in the presence and absence of light (Supplementary Text), light-exposed
126 hosts had a more intense coloration than those exposed in the dark (Fig. 2). This suggests
127 that *Spirobacillus* may, like *Myxococcus,* restrict the production of carotenoids in the
128 dark. Under this hypothesis, we expect *Daphnia* living in lakes that are rich in dissolved
129 organic compounds, which readily absorb carotenogenesis-inducing blue light (Wetzel
130 2001), or that dwell in the dark depths of lakes (such as *D. pulicaria*) to appear more
131 yellow than red in the terminal phase of infection.
132
133 A second factor that could contribute to variation in terminal coloration is predation.
134 Both fish and salamanders preferentially feed on red-pigmented copepods in ponds and
135 shallow lakes (Byron 1982) and bluegill are two to three times more likely to eat red
136 *Spirobacillus*-infected *Daphnia* than healthy *Daphnia* (Duffy et al. 2005). If
137 *Spirobacillus* cannot survive the digestive system of such predators, predation could
138 significantly reduce its transmission (as per (Packer et al. 2003) and hence exert strong
139 selective pressure against pigment production. On the other hand, it is possible that the
140 red pigment renders infected hosts partially concealed, at least in certain light
141 environments. Water readily absorbs red light, so it does not penetrate even a few meters
142 below the surface (Wetzel 2001). As a result, objects that appear red in white light lose
143 their color underwater (Cronin et al. 2014). Red, infected *Daphnia* might thus be more
144 camouflaged relative to those infected with light-colored bacteria, at least on a dark

145 background. So predation could either select for or against the 'blushing' phenotype. The
146 effect of infection-induced coloration on a predator's capacity to see *Daphnia* will
147 depend on the extent to which it causes *Daphnia* to contrast with their surrounding
148 environment (e.g. (Johnson et al. 2006)), *as perceived by the eyes of the predator*. Tools
149 and approaches from 'visual ecology'(Cronin et al. 2014) will thus prove essential for
150 understanding the direction and extent to which predation exerts selection on pigment
151 production in *Spirobacillus*.
152
153 The color of *Spirobacillus*-infected hosts may thus be shaped by a variety of ecological
154 forces, both inside and outside of the host. These forces may differentially favor pigment
155 production by the bacteria and interact to drive both the color variation that we have
156 described and, if pigment production impacts parasite fitness as we hypothesize,
157 epidemiological dynamics. Color is a trait with a storied history of study in evolutionary,
158 but not disease, ecology. Variation in host coloration in this system could represent an
159 excellent opportunity to study how selection pressures at different biological levels of
160 biological organization impact parasite ecology and evolution.
161
162 Acknowledgments
163 We would like to thank Alexander Strauss for help establishing the *in vivo* culture,
164 Charlotte Blumrosen for translating Metchnikoff's original text and Stephen B. Heard
165 and David Kehoe for comments on this manuscript. This work was supported by NSF
166 DEB-1305836 and DEB-1655856.
167

168 Bibliography

169 Auld, S. K. J. R. 2014. Immunology and Immunity. Pages 219–234 *in* N. N. Smirnov,
170 editor. The Physiology of Cladocera. Elsevier Inc.
171 Burchard, R. P., and M. Dworkin. 1966. Light-induced lysis and carotenogenesis in
172 *Myxococcus xanthus*. Journal of Bacteriology 91:535–545.
173 Byron, E. R. 1982. The adaptive significance of calanoid copepod pigmentation: a
174 comparative and experimental analysis. Ecology 63:1871–1886.
175 Cronin, T. W., S. Johnsen, N. J. Marshall, and E. J. Warrant. 2014. Visual Ecology.
176 Princeton University Press.
177 Duffy, M. A., S. R. Hall, and A. J. Tessier. 2005. Selective predators and their parasitized
178 prey: are epidemics in zooplankton under top-down control? Limnology and
179 Oceanography 50:412–420.
180 Elias-Arnanz, M., S. Padmanabhan, and F. J. Murillo. 2011. Light-dependent gene
181 regulation in nonphototrophic bacteria. Current Opinion in Microbiology 14:128–
182 135.
183 Green, J. 1959. Carotenoid pigment in *Spirobacillus cienkowskii Metchnikoff*, a pathogen
184 of Cladocera. Nature 183:56–57.
185 Johnson, P. T. J., D. E. Stanton, E. R. Preu, K. J. Forshay, and S. R. Carpenter. 2006.
186 Dining on disease: how interactions between infection and environment affect
187 predation risk. Ecology 87:1973–1980.
188 Liu, G. Y., A. Essex, J. T. Buchanan, V. Datta, H. M. Hoffman, J. F. Bastian, J. Fierer,
189 and V. Nizet. 2005. *Staphylococcus aureus* golden pigment impairs neutrophil killing
190 and promotes virulence through its antioxidant activity. The Journal of Experimental

Medicine 202:209–215.

Liu, G. Y., K. S. Doran, T. Lawrence, N. Turkson, M. Puliti, L. Tissi, and V. Nizet. 2004. Sword and shield: Linked group B streptococcal β-hemolysin/cytolysin and carotenoid pigment function to subvert host phagocyte defense. Proceedings of the National Academy of Sciences 101:14491–14496.

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

Navarro Llorens, J. M., A. Tormo, and E. Martínez-García. 2010. Stationary phase in gram-negative bacteria. FEMS Microbiology Reviews 34:476–495.

Packer, C., R. D. Holt, P. J. Hudson, K. D. Lafferty, and A. P. Dobson. 2003. Keeping the herds healthy and alert: implications of predator control for infectious disease. Ecology Letters 6:797–802.

Rodrigues, J. L. M., M. A. Duffy, A. J. Tessier, D. Ebert, L. Mouton, and T. M. Schmidt. 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.

Takano, H. 2016. The regulatory mechanism underlying light-inducible production of carotenoids in nonphototrophic bacteria. Bioscience, Biotechnology, and Biochemistry 80:1265–1274.

Wetzel, R. G. 2001. Light in Inland Waters. Pages 49–69 *in* Limnology. Third Edition.

Figures

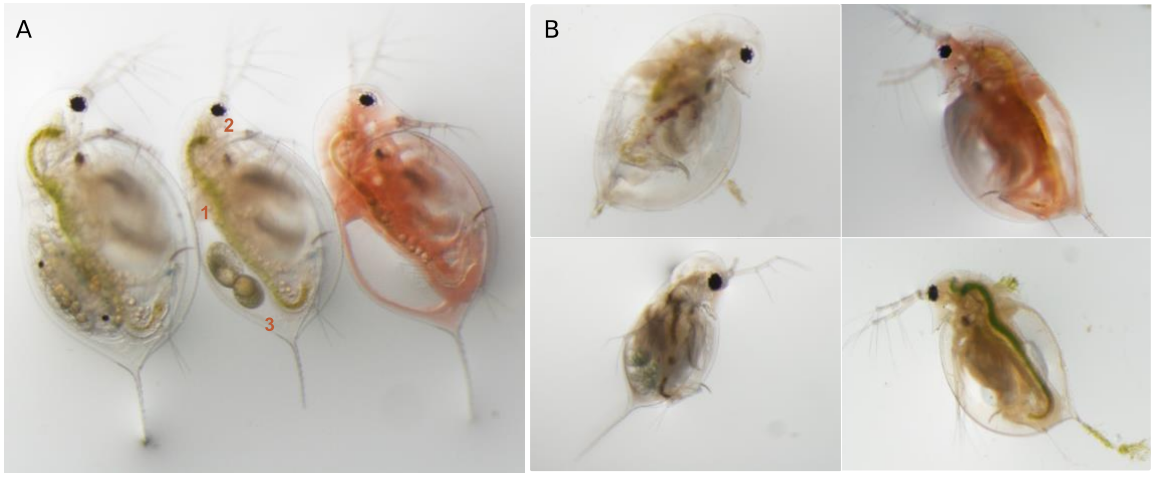

Fig. 1 Color variation in *Daphnia dentifera* infected with *Spirobacillus cienkowskii.* A) The color of infected animals varies as the infection progresses. From left to right, an uninfected *Daphnia dentifera*, an experimentally infected animal with the beige coloration indicative of the early stage of infection and an experimentally infected animal with the scarlet coloration indicative of the late, terminal stage of infection; the latter is the hallmark symptom of *Spirobacillus* infection. In the early stage of infection, colored material first appears around the heart (1), eye (2) and in the hemolymph around the brood chamber (3). A day after this photograph was taken, the middle animal had the appearance of the animal on the right. Note that animals infected with *Spirobacillus* have a similar appearance to those with an abundance of hemoglobin in their hemolymph but can be distinguished from the latter by their opacity, when visualized using darkfield microscopy, and the `glittery' appearance of their hemolymph (Fig. S2). B) Variation in the terminal coloration of field-collected *Daphnia dentifera*. Pictures were taken either not long before or after the animals' death.

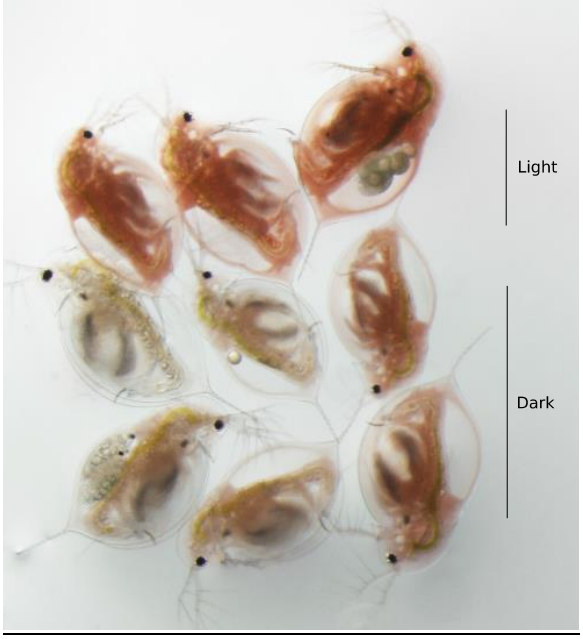

Fig. 2 The color of infected *Daphnia* changes with the light conditions in which they were infected. The most intensely colored *Spirobacillus*-infected hosts taken from (top) 3 infected microcosms maintained under a 16-8 hour light-dark cycle and (bottom) 6 infected microcosms maintained in the dark (see Appendix S1 for details).

Appendix S1

Below we provide more details of the methods used to study *Spirobacillus cienkowskii* in the laboratory. Two additional figures are also included: the first shows how the colorful symptoms of *Spirobacillus* infection develop (Fig. S1), the second contrasts the appearance of *Spirobacillus*-infected and hemoglobin-producing *Daphnia* to help the reader distinguish between these host types (Fig. S2).

Materials & Methods

Spirobacillus cienkowskii has been maintained in *in vivo* culture in the Duffy Lab at the University of Michigan since February 2016, having originally been established by Alex Strauss (Indiana University). Cultures are set up in 1000ml beakers filled with 800ml filtered lake water (FLW) collected from North Lake (Washtenaw County, Michigan, USA) and initiated with 6 infected and 75 uninfected *D. dentifera* of the L6D9 clone, originally collected from Dogwood Lake (Greene-Sullivan State Forest, Indiana, USA). An alternative protocol, using 250ml beakers containing 200ml FLW, 20 uninfected animals and 5 infected animals is also used. New cultures are made every 10-14 days, using infected animals from previously established cultures and uninfected L6D9 individuals; the latter are collected from uninfected stock cultures that are maintained separately.

Infection assays

We present representative methods and data (Fig. S1) for individual-level infection experiments. For these individual exposure assays, 5 day old *D. dentifera* of the L6D9 clone were placed individually in 50ml beakers filled with 25ml FLW. Each beaker was fed 0.25ml of a 1,000,000 cells per ml solution of *Ankistrodesmus falcatus* daily, and maintained at 22°C in an incubator on a 16-8 hour light-dark cycle. Infected animals were collected from *in vivo* cultures into a 1.5ml tube and crushed using a motorized pestle; the contents were carefully mixed and evenly distributed among the beakers. Daily, individuals were checked for symptoms of infection by holding each beaker over white paper to better facilitate the detection of colored hosts. In this experiment, red animals were preserved before they died for further analysis. However, we have never seen a *Spirobacillus*-exposed animal recover after turning red. Indeed, in a recent experiment, red animals had an hourly mortality rate of 5%.

Assessment of infection status by PCR

DNA was extracted from animals using the DNeasy Blood & Tissue kit (Qiagen) according to manufacturer's instructions. The presence of *Spirobacillus* infection was assessed using a species-specific PCR assay. Each 50µl reaction contained GoGreenTaq Mastermix (Promega) and primers (0058F, 462R; (Rodrigues et al. 2008, Thomas et al. 2011)) at a final concentration of 1x and 400nM**,** respectively, and 10µl of extracted DNA. Cycling conditions were the same as (Rodrigues et al. 2008), with the exception that 40 rather than 30 cycles of denaturation/annealing/extension were used. Gel electrophoresis was used to confirm that a fragment of the appropriate length had been amplified and hence that *Spirobacillus* DNA was present.

The impact of light on the color of infected Daphnia

12 250ml beakers were filled with 200ml FLW and 20 4-5 day old uninfected hosts of the L6D9 clone. 45 infected hosts were collected, crushed and distributed evenly among 9 of the beakers; 3 beakers were left unexposed in order to assess the impact of darkness on the color of uninfected hosts. 3 'exposed' cultures were placed in a clear plastic box with a lid; the remaining 6 'exposed' and further 3 'unexposed' in a similar box completely covered in light-blocking vinyl (BlackOut Cling Vinyl, Delta Photography Supplies, USA). Both totes were then placed in an incubator on a 16-8 hour light-dark cycle. Each beaker was fed 4ml of 1,000,000 cells per ml solution of *Ankistrodesmus falcatus* daily; animals in the 'dark' treatment were fed in a dark room devoid of light except for a red headlight worn by the experimenter (NW). 6 days after they were established**,** the cultures were inspected and the brightest colored animal from each of the replicate cultures selected and photographed (as shown in Fig. 2). Light had no apparent impact on the color of the unexposed hosts. This experiment does not preclude the possibility that *Spirobacillus* infection, and the characteristic red symptoms associated with it, takes longer to develop in the dark etc. and further investigations of the impact of light on carotenoid production are needed.

References

Rodrigues, J. L. M., M. A. Duffy, A. J. Tessier, D. Ebert, L. Mouton, and T. M. Schmidt. 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.

Thomas, S. H., C. Bertram, K. van Rensburg, C. E. Cáceres, and M. A. Duffy. 2011. Spatiotemporal dynamics of free-living stages of a bacterial parasite of zooplankton. Aquatic Microbial Ecology 63:265–272.

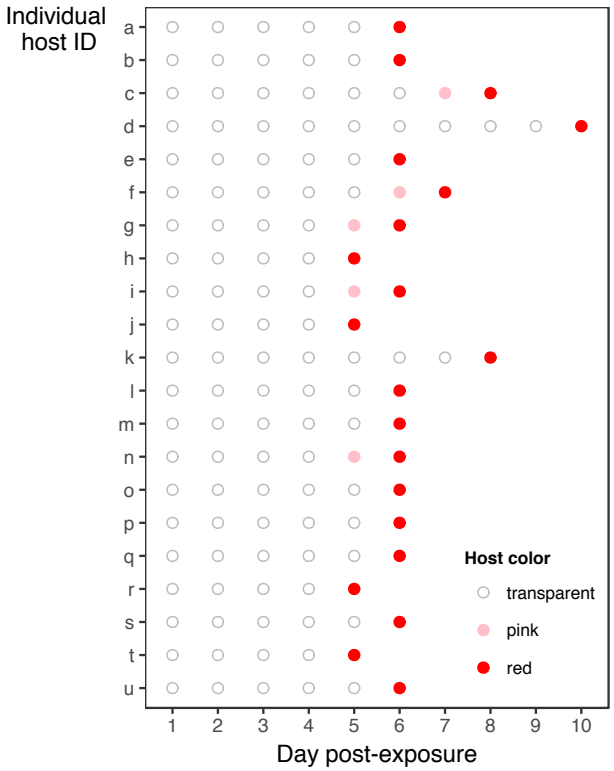

Fig. S1: The red color associated with *Spirobacillus* infection appears at the end of infection, 5-6 days after exposure. Each row represents an individual *Daphnia* exposed to *Spirobacillus* in an individual beaker (see supplementary text for details). The appearance of each host to the naked eye was recorded daily and is indicated by the circles' fill color. In this experiment, red animals were preserved before they died for further analysis but we have never observed a *Spirobacillus*-infected animal recover from infection after turning red.

Fig. S2: *Spirobacillus*-infected animals can be distinguished from hemoglobin-rich animals using dark field microscopy. The animal on the left of each photograph is infected with *Spirobacillus*, while the animal on the right is uninfected but producing hemoglobin in abundance. When viewed with bright field microscopy (left photograph), it can be challenging to distinguish *Spirobacillus* from hemoglobin. However, when viewed with dark field microscopy (right photograph), the *Spirobacillus*-infected *Daphnia* is more opaque than its hemoglobin-producing counterpart. In addition, when viewed live the hemolymph of *Spirobacillus*-infected hosts is characterized by a glittery appearance that hemoglobin-rich hemolymph lacks. Note that, due to the limited availability of hemoglobin-producing *D. dentifera* at the time that these photographs were taken, this figure contrasts a *Spirobacillus*-infected *D. dentifera* and a hemoglobin-producing *D. pulicaria*. The increased opacity of *Spirobacillus*-infected hosts is consistent across species.