

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

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 (Appendix S1: 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



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 (Appendix S1: 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.

than 130 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 (unpublished data). 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-photosynthetic 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 producing them. Indeed *Myxococcus* colonies produce few carotenoids and remain yellow if they are maintained in the dark, even if they are in stationary phase (Burchard and Dworkin 1966). In preliminary experiments where *Daphnia* were infected with *Spirobacillus* in the presence and absence of light (Appendix S1), light-exposed hosts had a more intense coloration than those exposed in the dark (Fig. 2). This suggests that *Spirobacillus* may, like *Myxococcus*, restrict the production of carotenoids in the dark. Under this hypothesis, we expect *Daphnia* living in lakes that are rich in dissolved organic compounds, which readily absorb carotenogenesis-inducing blue light (Wetzel 2001), or that dwell in the dark depths of lakes (such as

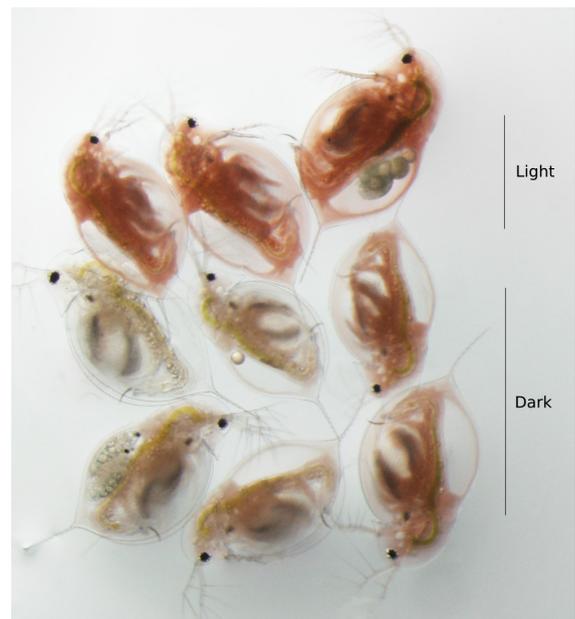


FIG. 2. The color of infected *Daphnia* changes with the light conditions in which they were infected. The most intensely colored *Spirobacillus*-infected hosts taken from (top) three infected microcosms maintained under a 16 h:8 h light-dark cycle and (bottom) six infected microcosms maintained in the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

D. pulicaria) to appear more yellow than red in the terminal phase of infection.

A second factor that could contribute to variation in terminal coloration is predation. Both fish and salamanders preferentially feed on red-pigmented copepods in ponds and shallow lakes (Byron 1982) and bluegill are two to three times more likely to eat red *Spirobacillus*-infected *Daphnia* than healthy *Daphnia* (Duffy et al. 2005). If *Spirobacillus* cannot survive the digestive system of such predators, predation could significantly reduce its transmission (as per Packer et al. 2003) and hence exert strong selective pressure against pigment production. On the other hand, it is possible that the red pigment renders infected hosts partially concealed, at least in certain light environments. Water readily absorbs red light, so it does not penetrate even a few meters below the surface (Wetzel 2001). As a result, objects that appear red in white light lose their color underwater (Cronin et al. 2014). Red, infected *Daphnia* might thus be more camouflaged relative to those infected with light-colored bacteria, at least on a dark background. So predation could either select for or against the “blushing” phenotype. The effect of infection-induced coloration on a predator’s capacity to see *Daphnia* will depend on the extent to which it causes *Daphnia* to contrast with their surrounding environment (e.g., Johnson et al. 2006), as perceived by the eyes of the predator. Tools and approaches from “visual ecology” (Cronin et al. 2014) will thus be essential for understanding the direction and extent to which predation exerts selection on pigment production in *Spirobacillus*.

The color of *Spirobacillus*-infected hosts may thus be shaped by a variety of ecological forces, both inside and outside of the host. These forces may differentially favor pigment production by the bacteria and interact to drive both the color variation that we have described and, if pigment production impacts parasite fitness as we hypothesize, epidemiological dynamics. Color is a trait with a storied history of study in evolutionary, but not disease, ecology. Variation in host coloration in this system could represent an excellent opportunity to study how selection pressures at different levels of biological organization impact parasite ecology and evolution.

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Supporting Information. A colorful killer: *Daphnia* infected with the bacterium *Spirobacillus cienkowskii* exhibit unexpected color variation. Nina Wale, McKenna L. Turrill, and Meghan A. Duffy. *Ecology*. 2018.

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 those used in 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.

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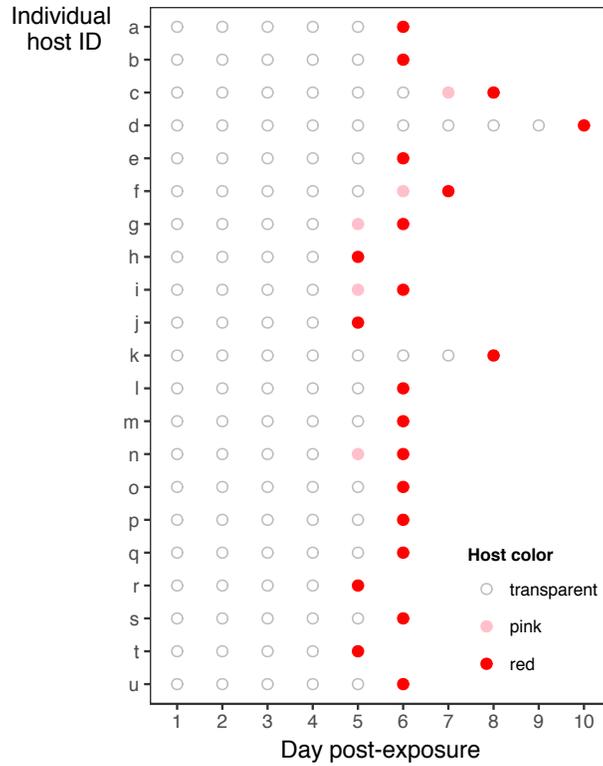


Fig. S1: The red color associated with *Spirobacillus* infection appears at the end of infection, 5-6 days after exposure. Each row represents an individual *Daphnia* exposed to *Spirobacillus* in an individual beaker (see supplementary text for details). The appearance of each host to the naked eye 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.