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

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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 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 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 *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 prove 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 biological levels of biological organization impact parasite ecology and evolution.

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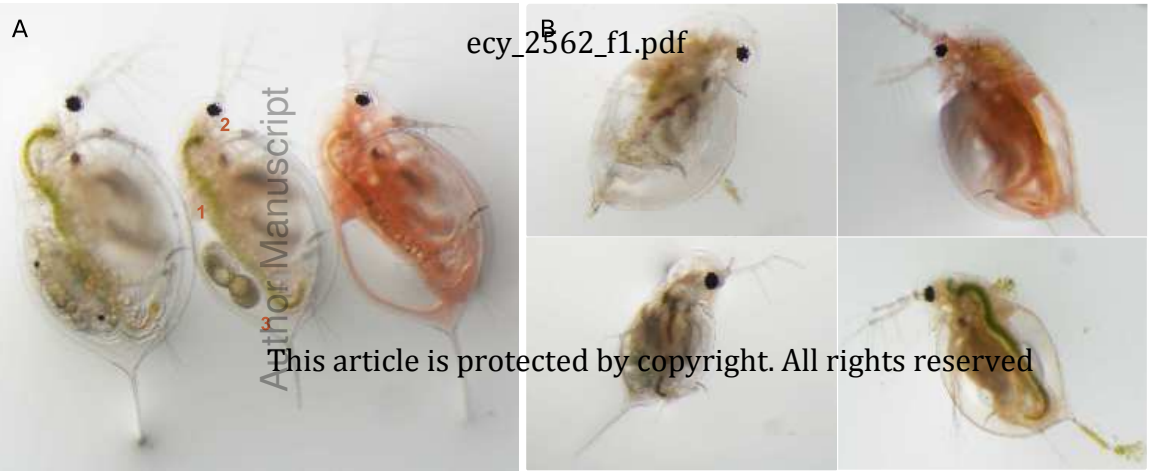
Figure Legends

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.

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).



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