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Within-host complexity of a plankton-parasite interaction

Parasite life cycles can be complex, involving multiple hosts and life history stages. For instance, the protozoan parasite *Toxoplasma gondii* moves from the environment to a small mammal host, and then via trophic transmission to a feline host, all the while undergoing four developmental transformations. Such among-host complexity has informed research at the community level by linking hosts through their parasites and by indicating the strength of host interactions (Lafferty et al. 2008). Moreover, it has solidified a community-level perspective on parasite regulation, since the completion of parasite life cycles often depends on the availability and interaction of host species.

Parasite life cycles are also complex at the within-host level. Parasites can exhibit morphological diversity across within-host developmental stages and, through the infection process, can move among diverse host tissues. Addressing complexity at the within-host level can enrich our understanding of both hosts and parasites. For example, through observing trematode morphological variation in snails, Hechinger et al. (2011) discovered social organization among flatworms, bringing sociality to a new phylum and raising questions about its evolutionary origins and ecological consequences. Additionally, recent work on trypanosomes has moved beyond the vector bite and into the vector midgut, where the majority of fly-trypanosome interactions play out. This shift to within-host interactions has revealed that the fly's midgut prevents the majority of infections from succeeding (Sloan and Ligoxygakis 2017), elevating the role of host defenses in regulating parasites. By peeling back the host exterior and focusing on interactions within, new questions arise, assumptions can be overturned, and we can attain a deeper understanding of how within-host processes scale to the community level.

The complicating factor of within-host life cycles is that they are concealed in a host, which can limit opportunities for observing complete parasite natural histories. Many plankton species are transparent, providing an opportunity to observe host–parasite interactions in vivo, and we used this characteristic to describe the within-host interactions of the fungus, *Metschnikowia bicuspidata,* and its host, *Daphnia dentifera*. *Metschnikowia bicuspidata* (Metschnikov) Kamenski is an ascomycete fungus that parasitizes freshwater zooplankton, including *Daphnia*. Designated an "obligate killer," *Metschnikowia* exhibits a parasitoid life history strategy (sensu Lafferty and Kuris 2002) requiring the death of its host for transmission (Ebert 2005). Portions of the infection process (spore, conidia, and ascus stages; see Appendix S1 for glossary of terms) were described, as was the host haemocyte response, in the late 19th century (Metschnikoff 1884). But since then, the within-host life cycle has been largely neglected, despite this parasite's prominence in studies of freshwater disease (Cáceres et al. 2014). This oversight has led to a pervasive assumption that *Daphnia* cannot and do not recover from infection (Hall et al. 2007, Duffy et al. 2009), which has directed research away from within-host processes and toward broad-scale ecological factors that mediate exposure. Herein, we describe the complete within-host life cycle of *Metschnikowia*, which we resolved by observing *Daphnia* over a 10-d period following inoculation. Our observations produced a sophisticated picture of within-host events (Fig. 1) and yielded novel findings and questions regarding host immunity and cryptic infections. First, we detail the progression of parasite development that occurs across five morphological stages. We then discuss how the resolution of this interaction overturns the assumption of no recovery and informs our understanding of the within-host controls of parasites.

Metschnikowia infects the host passively via ingestion when the *Daphnia* is filter feeding (Ebert 2005). Twenty-four hours after exposure to *Metschnikowia*, we observed spores in the lumen of the host gut (mean number of spores, 46.2; range, 10–114; Fig. 2A) moving posteriorly with intestinal contents (e.g., ingested algae). Most of the ingested spores were passed with waste. However, some "attacking" spores punctured the gut epithelium (mean number of spores, 8.8; range, 2–26; Fig. 2B) and a subset of attacking spores fully crossed the epithelium and entered the host body cavity (mean number of spores, 2.3; range, 0–10; Fig. 2C). All exposed hosts had at least two spores attacking their gut membranes, and approximately 75% of exposed hosts had spores that fully crossed the gut barrier to infect the body cavity. Spores that did not cross the gut barrier gradually left the lumen, potentially from digestive action or the shedding of the gut lining during molt. The *Daphnia* gut is C-shaped, and most attacking spores (85%) were located at the anterior and posterior bends, where the gut approximates two right angles. This pattern suggests that spore penetration is a physical process; the spores are needle-shaped and those that are unable to make the turn at the bends are shunted into the epithelium. Host feeding and digestion traits may contribute to the number of spores that puncture by providing peristaltic force. Structural host defenses, like a robust gut epithelium, may prevent spores from infecting.

Spores that infected the body cavity began producing hyphae 2–4 d after exposure (Fig. 2D). The web-like hyphae extended from the spores and adhered to nearby surfaces. During the spore and hyphae stages, host haemocytes (immune cells) proliferated and moved rapidly through the body cavity. Haemocytes attached to spores upon contact, resulting in large congregations of haemocytes on spores and/or hyphae (Fig. 2E). As observed by Metschnikoff (1884), congregating haemocytes regularly coalesced to engulf the parasite.

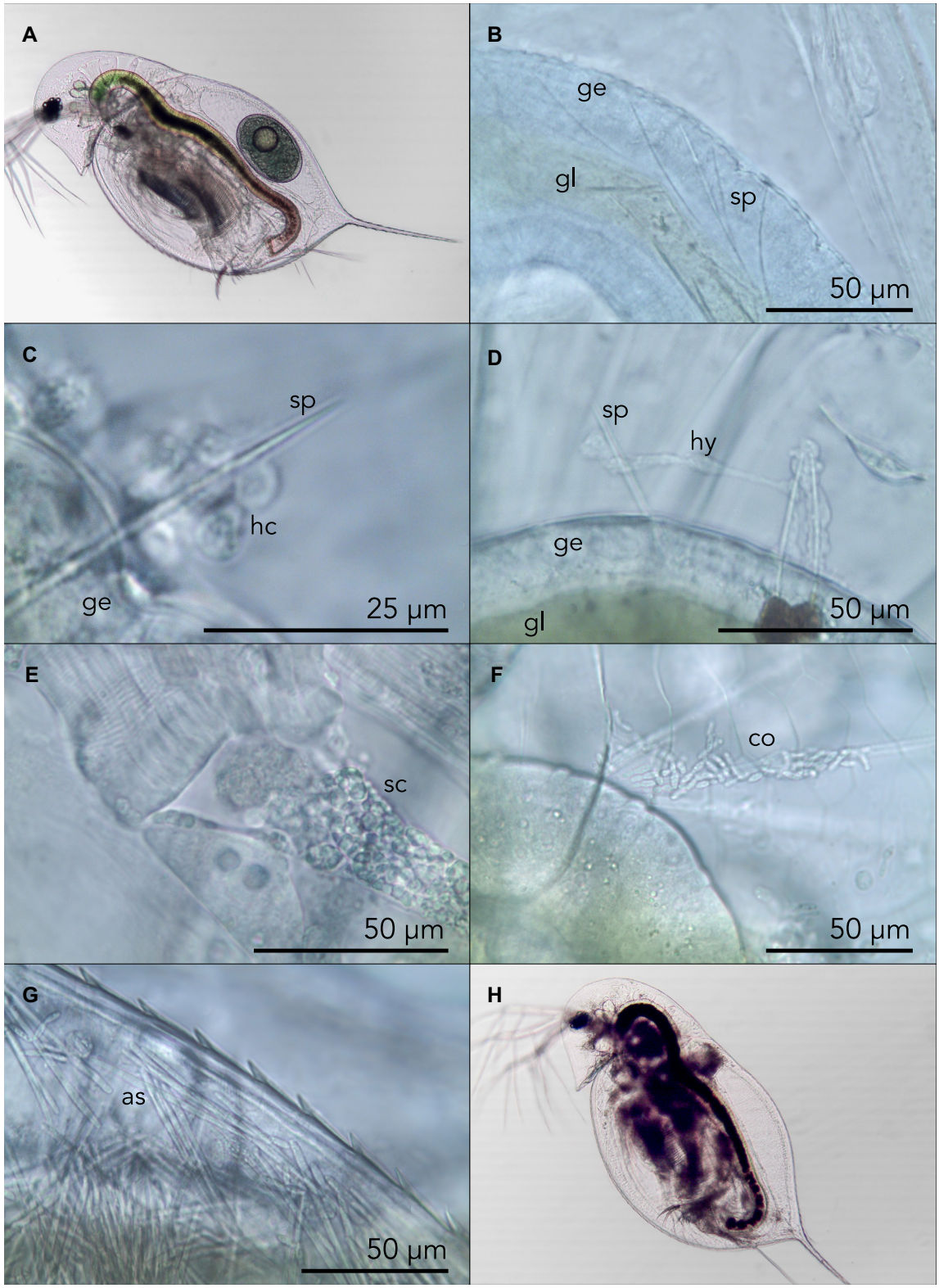

FIG. 1. The progression of infection of *Metschnikowia bicuspidata* in its host *Daphnia dentifera*. (A) Uninfected hosts consume ascospores, (B) some of which attack the gut membrane. (C) Spores that cross the gut barrier are met by the host haemocyte response. (D) Surviving spores produce hyphae that develop into (E) sporocysts. (F) Sporocysts rupture and release conidia that replicate and spread throughout the body. (G and H) Fully infected hosts are filled with asci. Labels: ge, gut epithelium; gl, gut lumen; sp, spore; hc, haemocyte; hy, hyphae; sc, sporocyst; co, conidia; as, ascus.

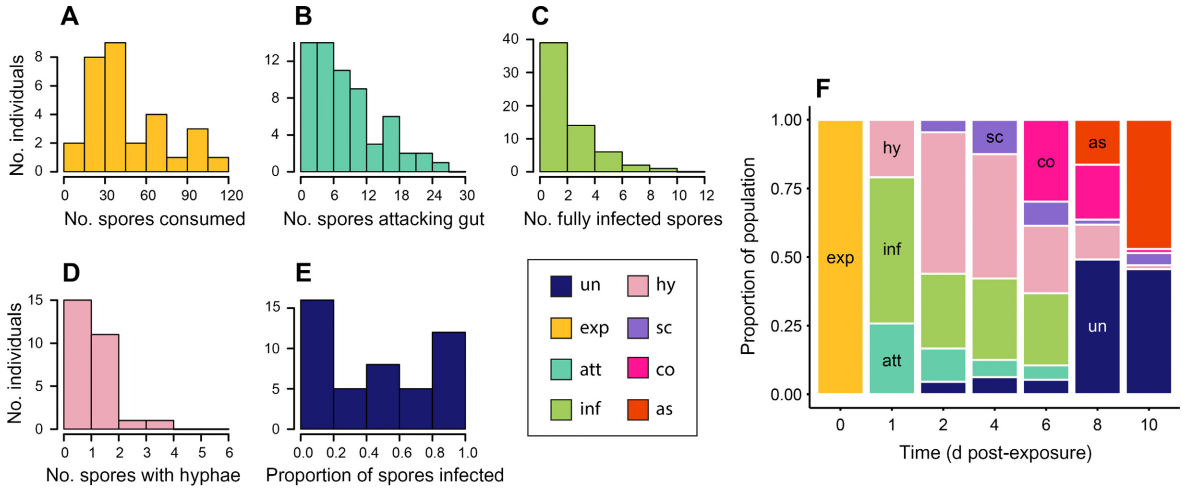

FIG. 2. *Metschnikowia* spores can be decomposed into multiple steps of infection, each with a declining probability of success. (A) Hosts are exposed to spores while feeding and (B) a subset of consumed spores attack the gut. (C) Some attacking spores enter and infect the body cavity, but (D) few produce hyphae. (E) Hosts vary in the proportion of spores they defend with haemocytes. (F) Hosts transition among infection stages through time and infection clearance is evidenced by the increased proportion of uninfected individuals toward the end of the 10-d period. Labels: un, uninfected (dark blue); exp, exposed (yellow); att, attacked (teal); inf, infected (green); hy, hyphae (pale pink); sc, sporocyst (purple); co, conidia (dark pink); and as, ascus (red).

Hyphae that survived the haemocyte response developed sporocysts 4–5 d after exposure. Sporocysts are small, sack-like structures that contain the next stage (conidia). Sporocysts appear to grow through time as conidia develop within them, and sporocysts were often found aggregated near the mother spore. Sporocysts were also observed overtaking large muscular tracts of the host, and in some cases, floated freely through the host body cavity. Because many sporocysts are produced from a single spore and its hyphae, this stage may represent the point at which the infection becomes pathogenic (sensu Lafferty and Kuris 2002).

Conidia, which are small and oblong-elliptical in shape, emerged from sporocysts 6–8 d after exposure and rapidly increased in abundance via budding. The conidia are another pathogenic phase of infection; a small group of localized conidia can proliferate into thousands, dispersing and filling the host body in as few as 24 h. Early in this stage, small clusters of conidia can be found almost anywhere within the host. We have observed conidia along the gut, concentrated in the post-abdominal claw, in the antennae, and even hugging the eye's ommatidia.

The conidia developed into the final stage, the ascus, around 9–10 d after exposure. The ascus is an elongated cylindrical structure that houses the needle-shaped ascospore that is infective to susceptible hosts. Asci, which number in the thousands to tens of thousands, are released into the water column when the host dies. Morphological descriptions of the ascus were provided by Miller and Phaff (1998).

Fully infected hosts appear opaque due to the dense clustering of asci throughout the body. Because asci are conspicuous at low magnification, the ascus stage has traditionally designated hosts as infected (Cáceres et al. 2006). However, with low magnification (40×) an advanced conidia infection can present as an uninfected host, and spores, hyphae, and sporocysts are not detectable. Only one-fifth of this parasite's life history stages are observable using conventional methods, leading to a high false-negative rate and a vast underestimate of infection prevalence and parasite abundance (see also Fontes et al. 2017, Stewart et al. 2018). Indeed, in one natural population, we observed ascus prevalence at 4% while the prevalence of all five stages combined was 64% (T. E. Stewart Merrill, *unpublished data*). Disease ecology is predicated on understanding the roles of ecological processes in mediating parasite exposure, and our results highlight how traditional measures of prevalence can fail as metrics for exposure. For parasites whose late stages are used to detect infection, prevalence is the final manifestation after both exposure to a host and success within that host have played out. While our field observation of 4% ascus prevalence could suggest low exposure, this value may also be an outcome of high exposure and low host susceptibility. By integrating life cycles into our observations, exposure and susceptibility can be disentangled, allowing for the identification of environmental vs. host controls of transmission. Further, the inclusion of early infections provides better estimates of parasite abundance, highlighting their potential importance for regulating host populations.

Our observations provided us with new information regarding *Daphnia* immune defenses, and in particular, overturned the paradigm that *Daphnia* cannot recover from infection (Hall et al. 2007, Duffy et al. 2009). At exposure, most *Daphnia* experience early infections with spores or hyphae; however, only a subset of early infections ultimately produce late infections (Fig. 2F). By tracking cohorts and individuals through time, we have found that *Daphnia* can clear spores, hyphae, and sporocysts. Although rare, we have observed one *Daphnia* recovering from an ascus infection. Clearance of early infections is common under laboratory conditions, but may be sensitive to environmental stressors.

Future work should explore its incidence in natural populations to determine its epidemiological importance.

The immune defense responsible for clearing infections appears to be haemocytes, which we observed attacking spores, hyphae, and in some cases, sporocysts. *Daphnia* are highly variable in the number of spores they defend (Fig. 2E) and in the magnitude of their haemocyte response. Identifying the immunological controls of parasites is an emerging goal in ecology (Hawley and Altizer 2011) that can be realized with simple observation in this system, and we encourage using the haemocyte response to investigate questions at the intersection of eco- immunology and disease ecology. By capitalizing on the ability to observe haemocytes in real time, future work can examine within- and among-population variation in immunity, sensitivity of immune responses to ecological factors, and associations between immunological activity, parasite fitness, and transmission.

Our observations indicate that the path from exposure to infection is anything but simple: there is variation at every step and both host and parasite are involved in a dynamic interaction that ultimately determines their fitness. Within-host complexity is a general black box in disease ecology that, if opened, has broad potential to shift and complement our current understanding. Our description of the *Metschnikowia* life cycle allows the quantification of parasite exposure, attack rate, and infection success. The five resolved life history stages allow one to gauge disease progression and disentangle exposure and susceptibility. The host immune response can be estimated by counting haemocytes and quantifying infection clearance. Ultimately, the resolution of this interaction provides a new and powerful system for examining the contribution of within-host interactions to parasite ecology.

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