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SYMPOSIUM

Variation in Immune Defense Shapes Disease Outcomes in Laboratory and Wild *Daphnia*

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Synopsis Host susceptibility may be critical for the spread of infectious disease, and understanding its basis is a goal of ecological immunology. Here, we employed a series of mechanistic tests to evaluate four factors commonly assumed to influence host susceptibility: parasite exposure, barriers to infection, immune responses, and body size. We tested these factors in an aquatic host–parasite system (*Daphnia dentifera* and the fungal parasite, *Metschnikowia bicuspidata*) using both laboratory-reared and field-collected hosts. We found support for each factor as a driver of infection. Elevated parasite exposure, which occurs through consumption of infectious fungal spores, increased a host's probability of infection. The host's gut epithelium functioned as a barrier to infection, but in the opposite manner from which we predicted: thinner anterior gut epithelia were more resistant to infectious spores than thick epithelia. This relationship may be mediated by structural attributes associated with epithelial cell height. Fungal spores that breached the host's gut barrier elicited an intensity-dependent hemocyte response that decreased the probability of infection for some *Daphnia*. Although larger body sizes were associated with increased levels of spore ingestion, larger hosts also had lower frequencies of parasite attack, less penetrable gut barriers, and stronger hemocyte responses. After investigating which mechanisms underlie host susceptibility, we asked: do these four factors contribute equally or asymmetrically to the outcome of infection? An information-theoretic approach revealed that host immune defenses (barriers and immune responses) played the strongest roles in mediating infection outcomes. These two immunological traits may be valuable metrics for linking host susceptibility to the spread of infectious disease.

Introduction

Susceptibility of hosts to parasites may hold the key to how disease spreads, but it remains one of the most beguiling aspects of disease ecology. At the heart of host susceptibility is the immune system. All living organisms are threatened by parasites, and many have evolved a suite of immunological defenses to prevent infection. As such, ecological immunology provides a framework to link host susceptibility to parasite dynamics and disease spread (Hawley and Altizer 2011; Martin et al. 2016). However, several challenges confront empirical work at the interface of eco-immunology and disease ecology. Immunological defenses can be challenging to measure and interpret (Sheldon and Verhulst 1996; Graham et al. 2011; Moreno-García et al. 2013). Furthermore, it is often unknown which immune defenses regulate particular host–parasite interactions (Boughton et al. 2011). Finally, immunity is complex, highly integrated, and exceedingly variable (Schulenburg et al. 2009; Pedersen and Babayan 2011). Amidst all of the immunological noise, how can we find the signal for susceptibility?

Susceptibility and its immunological basis may be captured by decomposing host–parasite interactions into functional steps (e.g., Johnson and Hartson 2009; Auld et al. 2010, 2012a; Hall and Ebert 2012; Lafferty et al. 2015). These steps include parasite

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exposure, parasite entry into the host, and parasite survival within the host until the point of transmission. At each step, host strategies attempt to prevent passage of the parasite to the subsequent step (*sensu* Combes 2001). For instance, avoidance behaviors limit exposure (Buck et al. 2018), barriers impede entry (Söderhäll 2010; Davis and Engström 2012), and immune responses inhibit parasite survival. By isolating each step, we can first identify key host traits that govern success or cessation of infection. Then, by examining all steps together, we can determine which host traits most strongly determine susceptibility.

A plankton system shows great promise for determining the extent to which host susceptibility explains patterns of infectious disease. In this system, a virulent fungus, *Metschnikowia bicuspidata*, infects a crustacean host, *Daphnia dentifera*. *Daphnia* possess broad variation in susceptibility, which can contribute to the failure or emergence of natural epidemics as well as epidemic size (Strauss et al. 2018; Stewart Merrill 2019). Furthermore, descriptions of the parasite's within-host life cycle provide direct links from host traits (including immune defenses) to infection outcomes (Stewart Merrill and Cáceres 2018). With these new developments, we decompose the infection process into its functional steps and compare four factors that may govern infection: exposure to parasites, barriers to parasite entry, internal immune responses against parasites, and body size. These commonly-invoked drivers distill complex host–parasite interactions into a linear set of tractable mechanisms (Fig. 1). We test them using laboratory-reared and field-collected *Daphnia* to forge a balance between tight experimental control and broad ecological reality.

In the first part of our study ("Identifying Mechanisms of Infection"), we mechanistically test the four drivers of infection in isolation to understand their biology and to explore the range of host variation present at each infection step. We present each driver of infection as a unique module, such that each driver has its own background, methods, and results. In the second part of our study ("Integrating Infection Steps to Understand Susceptibility"), we unite the four drivers of infection to determine which play the strongest roles in shaping *Daphnia* susceptibility. Finally, we discuss how the biology of each infection driver informs our broader understanding of host susceptibility.

General methods

The study host, *D. dentifera*, is a cladoceran zooplankton found in freshwater lakes across North America. The study parasite, *M. bicuspidata* (formerly, *Monospora bicuspidata*; Metschnikoff 1884), is an ascomycete fungus that commonly causes epidemics in *Daphnia* populations (Cáceres et al. 2006; Cáceres et al. 2014). *Metschnikowia* is transmitted when *Daphnia* ingest fungal spores (hence, exposure is through feeding). The needle-shaped spores must then pierce through the *Daphnia* gut epithelium, which represents a barrier. If penetration succeeds, the fungus enters the body cavity of its host and must survive defense by host hemocytes (immune cells). The fungus then undergoes 8–10 days of morphological development and reproduction before reaching its terminal stages (the conidia and ascus stages, outlined in Supplementary material S1; Metschnikoff 1884; Stewart Merrill and Cáceres 2018). Terminal infections are those from which the host does not recover; the body cavity fills with new spores that kill the host. Host death is required to release spores back to the environment (i.e., to enable transmission). Because *Daphnia* are transparent, the full sequence of events from spore ingestion to terminal infection can be visualized *in vivo*. In this study, we experimentally inoculated *Daphnia* with fungal spores and observed the early steps of this interaction, during which spores are consumed and invade the body cavity. During our observations, we quantified a series of host and parasite metrics (Table 1) to mechanistically test the four drivers of infection. We then tracked hosts until 9 days post-inoculation (when they had either recovered from infection or entered the terminal infection stage) to evaluate which of the four drivers played the strongest role in determining terminal infection outcomes.

In the laboratory, we reared 10 unique multi-locus *Daphnia* genotypes originally collected from lakes in Central Indiana and Michigan. In our rearing protocol, we sought to eliminate maternal effects using standardized laboratory conditions for three generations (Lynch and Walsh 1998). Experimental individuals were collected from standardized mothers as neonates and were inoculated when they were 8-days-old. Field-collected *Daphnia* were sampled from six lakes in Central Indiana between 4 June and 4 December 2017. Experimental inoculations occurred 24 h after collection. Further description of laboratory conditions (containment, temperature, and resources) is provided in the Supplementary material S2.

The dose used for experimental inoculation differed for laboratory-reared versus field-collected *Daphnia*. In the laboratory study (2015), we used 500 spores/mL of *Metschnikowia*. This dose produced a high prevalence of terminal infections, with low

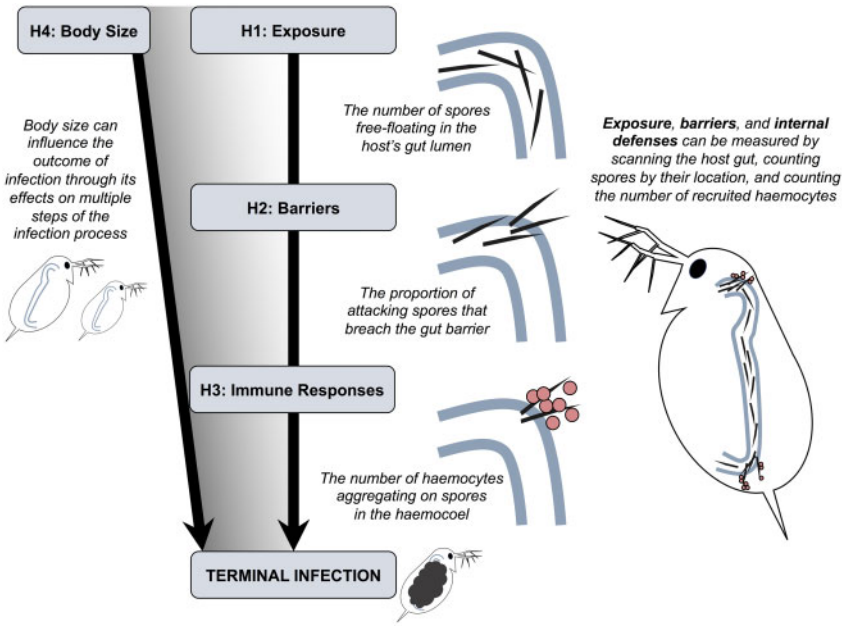

Fig. 1 The four drivers of infection, as well as associated empirical measurements in *D. dentifera*. Hypotheses one to three (H1:H3) focus on three sequential steps of the infection process, any of which may be the strongest driver of terminal infection. Hypothesis 4 (H4) proposes that body size influences the outcome of terminal infection through its potential effects (gray shading) on the full set of drivers. Terminal infection is reached when the host possesses late infection stages from which it cannot recover (described in Supplementary material S1). To measure exposure (H1), barriers (H2), and immune responses (H3), we scanned the full length of the *Daphnia* gut and classified spores based on their location within the host's body (following a set of metrics and calculations [Table 1]). For example, the enlarged gut diagram depicted here has four lumen spores, two barrier spores, and two hemocoel spores, resulting in an exposure value of 8, attack of 4, and infection of 2. For gut thickness (barriers, H2), we measured height of epithelial cells at the anterior (top) and posterior (bottom) bends of the gut, where spores most commonly penetrate (see full body diagram, to right). For our measure of immune response (H3), we counted host hemocytes. Here, the enlarged gut diagram has seven hemocytes aggregating on the spores in its hemocoel, or 3.5 hemocytes per spore.

host recovery rates. In the field study (2017), we used a more field-relevant dose (200 spores/mL) to enable greater host recovery. In both studies, after a 24-h inoculation period in tubes containing spores and 10 mL filtered lake water, live *Daphnia* were examined visually using a Leica DMLB compound microscope paired with a 40× objective (yielding total magnification of 400×). The full length of each host's gut and body cavity was scanned to quantify host and parasite metrics (defined in Table 1 and illustrated in Fig. 1). Field-collected *Daphnia* that had prior terminal infections with *Metschnikowia* were excluded from all analyses.

We tested our predictions using general linear models and ANOVA, with the individual host as the unit of replication. Statistical models were constructed for both laboratory-reared and field-collected *Daphnia* whenever the two datasets contained the required variables. Sample sizes are available (Table 1) and consolidated statistical output is provided (see Supplementary material S3). All models were fit in R version 3.3.3 (R core team 2013). Residuals were evaluated for normality, homoscedasticity, and over-dispersion to ensure compliance with model assumptions.

Identifying mechanisms of infection

H1: exposure drives infection

Background

Parasite exposure may strongly predict infection. For instance, low prevalence of parasites in natural systems often reflects low exposure, caused by limited infectious propagules or upstream hosts (Skirnisson and Galaktionov 2002; Hechinger and Lafferty 2005; Fredensborg et al 2006; Byers et al. 2008). Of course, exposure represents only a first step in the infection process, and subsequent steps may decouple exposure-infection relationships. For instance, while foraging behaviors amplify exposure in *Daphnia* (Hall et al. 2010; Shocket et al. 2018), broad unexplained variation in exposure-infection relationships exists in this and other systems (Thieltges and Reise 2007; Bertram et al. 2013; Sánchez et al. 2013; Izhar and Ben-Ami 2015; Izhar et al. 2015). We tested whether exposure drives infection by measuring

Table 1 Metrics used to quantify steps and mechanisms of the infection process

Metric	Description or equation	Laboratory *N*	Field *N*
Lumen spores	Ingested spores free-floating in the gut lumen	58	2039
Barrier spores	Spores only partially embedded in the gut epithelial barrier	136	2263
Hemocoel spores	Spores in the body cavity that can develop to later stages	136	2266
Hemocytes	Immune cells aggregated on spores in the body cavity	108	2065
Gut thickness	The width of the gut epithelium where spores penetrate	79	–
Body size	Length from center of the eye to base of the tail spine	112	1786
Terminal infection	Terminal infection status at 9 days post-inoculation	–	510
Exposure	Σ (lumen spores, barrier spores, hemocoel spores)	58	2039
Attack	Σ (barrier spores, hemocoel spores)	136	2263
Infection	= hemocoel spores	136	2266
Attack frequency	Attack/exposure	59	2023
Gut penetrability	Infection/attack	136	2262

Note: "Terminal infections" were not measured on laboratory-reared animals and "gut thickness" was not measured on field-collected animals. For each measure, we provide its description or equation, along with sample sizes (*N*) for laboratory-reared and field-collected hosts. Sample sizes varied due to the ability to accurately quantify a particular metric. For instance, we did not count "lumen spores" in individuals where spores could not be reliably distinguished from other material in the gut. In addition, "hemocytes" could not be counted for individuals without penetration of "hemocoel spores."

infection success of *Metschnikowia* spores after they are ingested by *Daphnia* hosts.

Methods

To develop and reproduce, *Metschnikowia* spores must first undergo a three-part journey. Spores must be ingested by hosts, cross the gut's epithelial barrier, and enter the body cavity (hemocoel). Therefore, we characterized *Metschnikowia* spores based on their location (Fig. 1, Table 1). "Lumen spores" represent spores that were free-floating in the gut lumen (hollow) following ingestion. Because the gut is a high flow-through system, lumen spores represent a snapshot of spore ingestion and approximate how many spores a host generally eats. "Barrier spores" represent spores that became partially embedded in the gut epithelium but failed to penetrate into the body cavity, that is, spores that were blocked by the gut barrier. "Hemocoel spores" represent spores that successfully crossed the gut epithelium and entered the host body cavity. The cumulative tally of spores within the host's body (lumen + barrier + hemocoel) represents total "exposure"; similarly, the number of "attacking" spores (*sensu* Lafferty et al. 2015) was the sum of those which attempted to cross the gut epithelium (barrier + hemocoel). Each host's level of "infection" refers directly to the number of spores infecting the body cavity. Extended definitions of spore types are provided in the Supplementary material S1.

If exposure (eating spores) drives infection, then ingested spores should predict successful penetrations into the body cavity. Tracing this path, we tested relationships between (1) lumen spores and spores embedded in gut epithelia (barrier spores), (2) barrier spores and infecting spores (hemocoel spores) and, ultimately, we tested whether (3) lumen spores predicted hemocoel spores.

Results

In laboratory-reared *Daphnia*, the number of lumen spores predicted barrier spores (Fig. 2A; df = 56, estimate[est] = 0.195, $P < 0.001$, $R^2 = 0.399$), but barrier spores did not predict hemocoel spores (Fig. 2B; df = 134, est = −0.028, $P = 0.455$, $R^2 = 0.004$). The lumen to body cavity path was decoupled at the gut barrier; hence, lumen spores did not ultimately predict hemocoel spores (Fig. 2C; df = 56, est = 0.013, $P = 0.510$, $R^2 = 0.008$). In the highly replicated experiment with field-collected *Daphnia*, each relationship was statistically significant. More lumen spores led to more barrier spores (Fig. 2D; df = 2037, est = 0.071, $P < 0.001$, $R^2 = 0.032$), then more barrier spores led to more hemocoel spores (Fig. 2E; df = 2260, est = 0.086, $P < 0.001$, $R^2 = 0.024$); hence, more lumen spores increased hemocoel spores (Fig. 2F; df = 2036, est = 0.036, $P < 0.001$, $R^2 = 0.026$). However, each relationship was generally weak in field-collected *Daphnia* (i.e., R^2 between 2.4 and 3.2%; Fig. 2) and laboratory-reared *Daphnia* also had weak associations ($R^2 < 0.01$) once spores began moving into the body cavity. These results indicate that the *Metschnikowia* path to infection is riddled

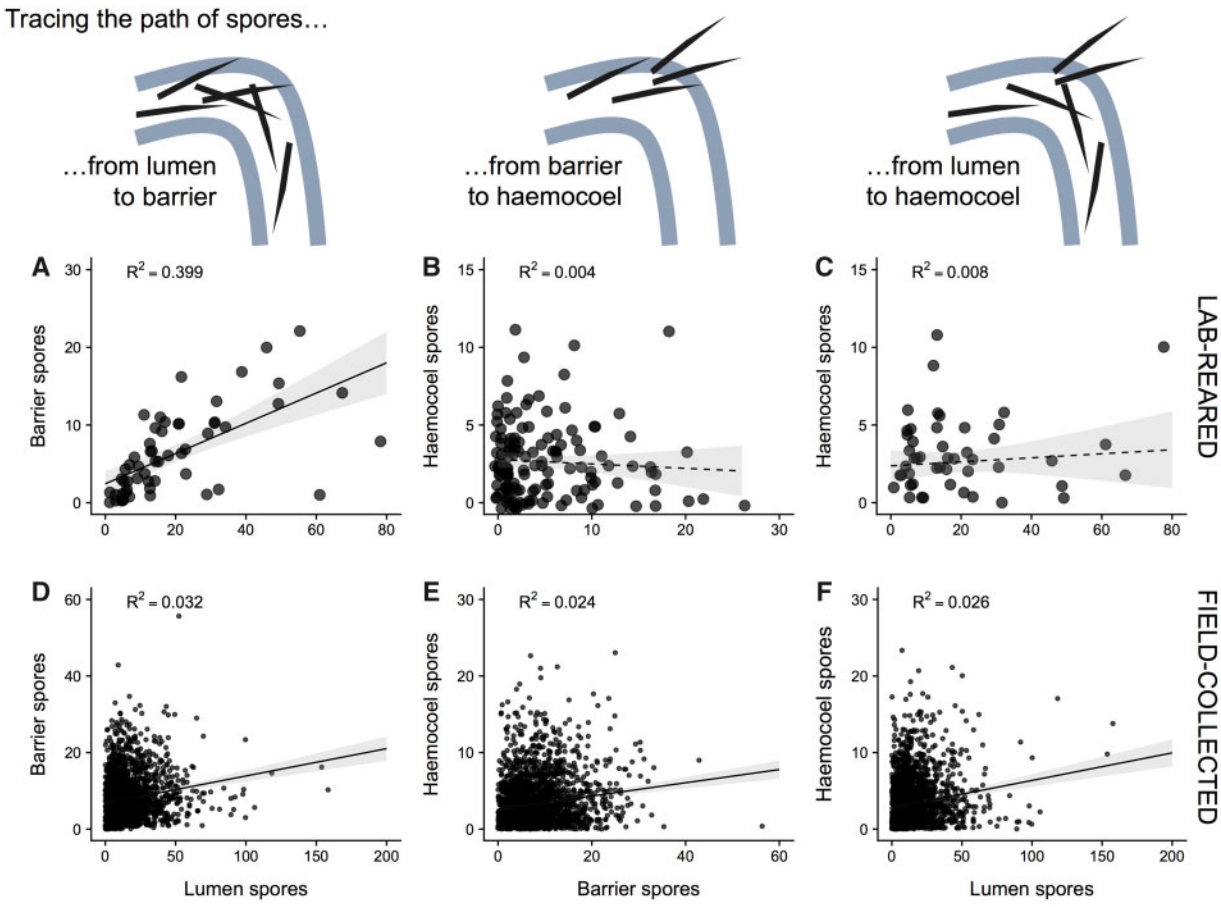

Fig. 2 Testing H1 ("exposure drives infection") by tracing the path of fungal spores after they are ingested. Exposure-infection relationships become decoupled as spores move from the gut lumen, across the gut barrier, and into the host body cavity. Lumen spores are positively associated with barrier spores (left column: **A** and **D**). Weak or non-significant associations occur between barrier spores and successfully penetrated hemocoel spores (central column: **B** and **E**). Ultimately, number of lumen spores explains less than 3% of successfully penetrated hemocoel spores (right column: **C** and **F**). Top row plots (**A**–**C**) are laboratory-reared *Daphnia*, and bottom row (**D**–**F**) are field-collected *Daphnia*; each point represents a single individual. Solid regression lines indicate significant relationships, dashed lines indicate non-significant relationships, and gray shading around the regression lines represents the standard error of the fit regression. Further information on the path spores take and how they are classified is provided in Fig. 1 and Table 1.

with host variation. The weight of evidence for exposure driving infection is low.

H2: gut thickness creates a barrier to infection

Background

To contend with parasite exposure, organisms possess diverse physical and chemical barriers that resist infection (Söderhäll 2010; Davis and Engström 2012). For ingested parasites, such barriers occur within the host's intestinal tract (Garcia-Garcia et al. 2013). For instance, *Wuchereria bancrofti* are killed and melanized during passage across the fly gut epithelium (Michalski et al. 2010), and mosquito-vectored arboviruses may be physically inhibited by the thickness of the mosquito's midgut basal lamina (Grimstad and Walker 1991; Franz et al. 2015). *Daphnia* exhibit strong genetic variation in parasite resistance (Stewart Merrill 2019), and the midgut epithelium likely mediates susceptibility (Auld et al. 2010, 2012b). Because the *Daphnia* midgut epithelium is one cell layer thick, tall epithelial cells (thicker epithelia) may inhibit spores from crossing into the body cavity. To evaluate whether gut thickness creates a barrier to infection, we measured thickness of gut epithelia and how penetrable they were by *Metschnikowia* spores.

Methods

Gut epithelia of live hosts were imaged at high resolution (400×) with Leica Imaging Software. Using ImageJ (Schneider et al. 2012), we measured the height of midgut epithelial cells (basal to apical surface) at both 90-degree bends in the C-shaped gut, where the majority of fungal spores penetrate (Fig. 1; Stewart Merrill and Cáceres 2018). Three epithelial

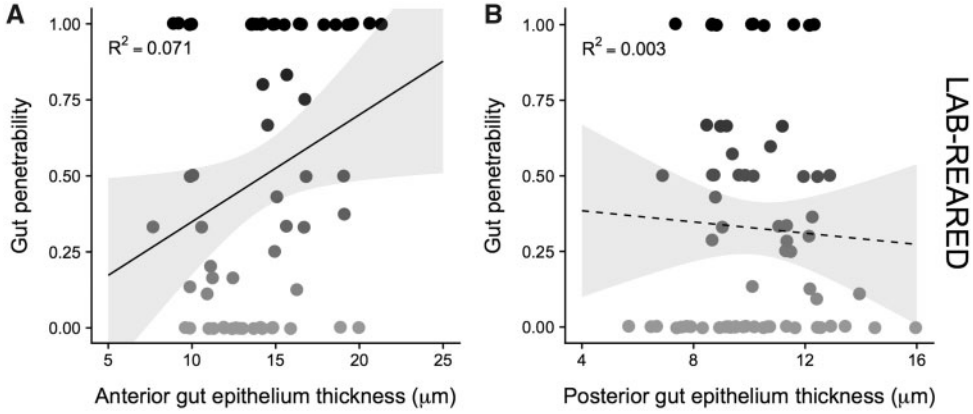

Fig. 3 Testing H2 ("gut thickness creates a barrier to infection"), we assessed whether gut penetrability (i.e., proportion of attacking spores that successfully penetrate the body cavity: Table 1) was explained by the thickness of the gut epithelium. (**A**) Thicker anterior epithelia are associated with higher gut penetrability. (**B**) In the posterior region of the gut, there is no association between gut epithelium thickness and gut penetrability. In both panels, each point represents a unique laboratory-reared individual, and points shade from gray to black as gut penetrability increases (light gray: 0%; black: 100%). The solid line indicates a significant relationship, the dashed line indicates a non-significant relationship, and gray shading around the regression lines represents the standard error of the fit regression.

cells were measured at each bend and, from these values, we calculated the average anterior (top bend) and posterior (bottom bend) epithelium thickness. Cell heights at the three points were strongly correlated, indicating high measurement consistency (average anterior $r = 0.91$; average posterior $r = 0.87$). To measure the penetrability of the gut barrier, we used the spore locations from H1 to relate each host's level of infection to its level of attack. Gut penetrability is the proportion of attacking spores that successfully infected the body cavity (Table 1). Larger values indicate higher gut penetrability, while zero represents impenetrability.

Results

Daphnia gut penetrability varied from entirely penetrable (100%) to entirely impenetrable (0%), indicating that the gut epithelium can act as a barrier to infection, but that *Daphnia* possess substantial variation in the strength of this barrier. Counter to our prediction, anterior gut thickness increased gut penetrability by spores (Fig. 3A; df = 61, est = 0.035, $P = 0.035$, $R^2 = 0.071$). Alternatively, posterior gut thickness was not associated with gut penetrability (Fig. 3B; df = 66, est = −0.009, $P = 0.672$, $R^2 = 0.003$). *Metschnikowia* spores, which average 45 μm in length (Ebert 2005), are at least two times longer than the thickest epithelium we observed (22.8 μm), highlighting that epithelium thickness alone does not create a realistic barrier for the spores. The weight of the evidence for gut thickness explaining the gut barrier is intermediate; in the anterior region of the midgut, gut thickness explained 7% of the variation in gut penetrability (but in the opposite manner from which we predicted).

H3: hemocytes mediate recovery

Background

Parasites that bypass their invertebrate host's barriers face cellular defenses. Host hemocytes are recruited to the site of infection and can kill invading parasites via phagocytosis, melanization, and secretion of humoral effectors (Bayne et al. 2001; Lemaitre and Hoffmann, 2007; Bartholomay et al. 2007; Moreno-García et al. 2013). But linking hemocytes to host recovery presents an interpretation problem (Dittmer et al. 2011; Auld et al. 2012b). Hemocytes kill parasites but are also up-regulated during infection. Hence, interpreting hemocytes as mediators of recovery or symptoms of susceptibility is difficult without knowing the host's intensity of infection. By measuring each host's intensity of infection and tracking their infection fate (whether they recovered from infection or succumbed to terminal infection), we examined if hemocytes merely increase following infection or more directly mediate host recovery.

Methods

To measure hemocytes, we counted the number of hemocytes aggregating on hemocoel spores (Fig. 1). This gave us two values: total hemocyte recruitment (the total count of hemocytes on spores), and the number of hemocytes per spore. We first tested whether hemocytes were up-regulated in response to infection by evaluating the relationship between total hemocyte recruitment and spores infecting the

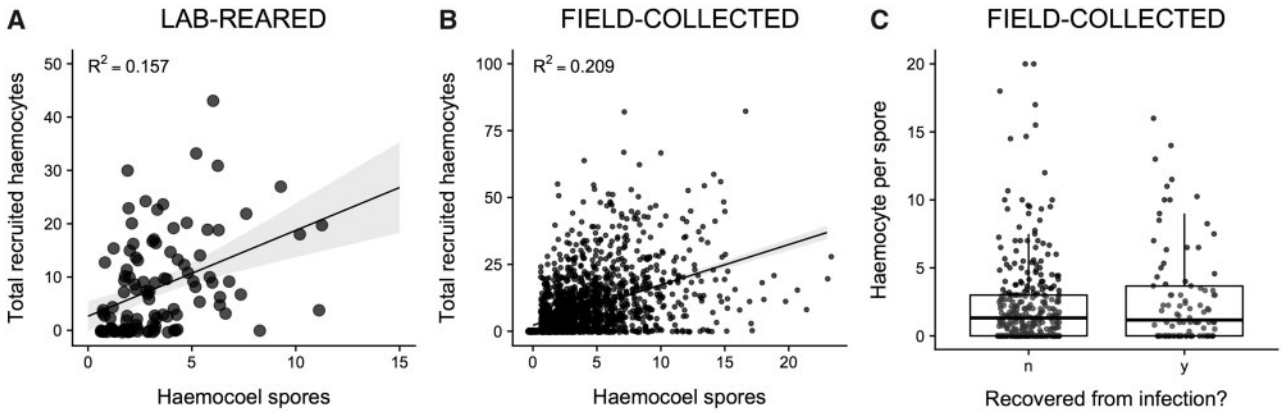

Fig. 4 Testing H3 ("hemocytes mediate recovery"), we examined hemocytes as symptoms of infection and causes of recovery. For both laboratory-reared (**A**) and field-collected (**B**) *Daphnia*, recruited hemocytes increased as a function of spores infecting the body cavity, suggesting that infection intensity may be an important factor for interpreting hemocyte-recovery relationships. (**C**) Field-collected individuals that achieved early infections were tracked until they recovered from infection or succumbed to terminal infection and the number of hemocytes per spore was not associated with recovery. Solid lines indicate significant relationships and shading around the line represents the standard error of the fit regression.

body cavity. Then, we tested whether hemocytes were associated with recovery. Having tracked field-collected *Daphnia* until 9 days post-inoculation, we were able to separate previously infected hosts (those that had spores infecting the body cavity following inoculation) into two categories: hosts that recovered and hosts that succumbed to terminal infection. We compared the number of hemocytes per spore among these two classes.

Results

Total recruited hemocytes increased with the number of infecting spores in both laboratory-reared *Daphnia* (Fig. 4A; df = 106, est = 1.609, $P < 0.001$, $R^2 = 0.157$) and field-collected *Daphnia* (Fig. 4B; df = 1931, est = 1.508, $P < 0.001$, $R^2 = 0.209$). Of 510 inoculated and tracked *Daphnia*, 13% never became infected (their gut barriers resisted infection), 19% recovered from infection, and 68% succumbed to terminal infection. However, recovery from infection was not associated with the number of hemocytes per spore (Fig. 4C; $F_{1,410} = 1.237$, $P = 0.267$). Although hemocytes were upregulated in an apparent attempt at recovery, they had no detectable impact on recovery. Thus, the weight of the evidence for hemocytes mediating recovery is low in our study.

H4: body size influences infection

Background

Body size itself may determine infection outcomes (Hall et al. 2007; Poulin 2013). For instance, as organisms grow, they can accumulate more parasites over time. Greater host size may also increase encounter rates with parasites that are consumed. In addition, large organisms may provide a higher quality resource for feeding and developing parasites. However, size can exert opposing effects on other infection mechanisms. For example, large organisms may have more resources to invest in energy-dependent immune responses (Rantala and Roff 2005; Sparkman and Palacios 2009). Therefore, the role of size in host susceptibility depends on the size-dependence and relative importance of each step of the infection process (Downs et al. 2019). In *Daphnia*, body size increases exposure (foraging rate; Ebert 1995; Hall et al. 2007) as well as the size of the resource base (Hall et al. 2009; Civitello et al. 2015). Here, we evaluate the effects of body size on the full set of steps comprising the *Daphnia*–*Metschnikowia* interaction.

Methods and results

Body length was measured from the center of the eye to the base of the tail spine. We first tested if body size increased spore ingestion (as lumen spores). Body size increased lumen spores in both laboratory-reared (Fig. 5A; df = 54, est = 27.73, $P = 0.041$, $R^2 = 0.075$) and field-collected *Daphnia* (Fig. 5B; df = 1622, est = 9.434, $P < 0.001$, $R^2 = 0.008$). Second, we tested whether body size increased the frequency of parasite attack. Attack frequency is the proportion of spores a host is exposed to that attempt to cross the gut barrier (attack/exposure; Table 1). Attack frequency trended negatively with body size in laboratory-reared *Daphnia* (Fig. 5C; df = 55, est = −0.112, $P = 0.080$, $R^2 = 0.055$) and decreased with body size in field-collected

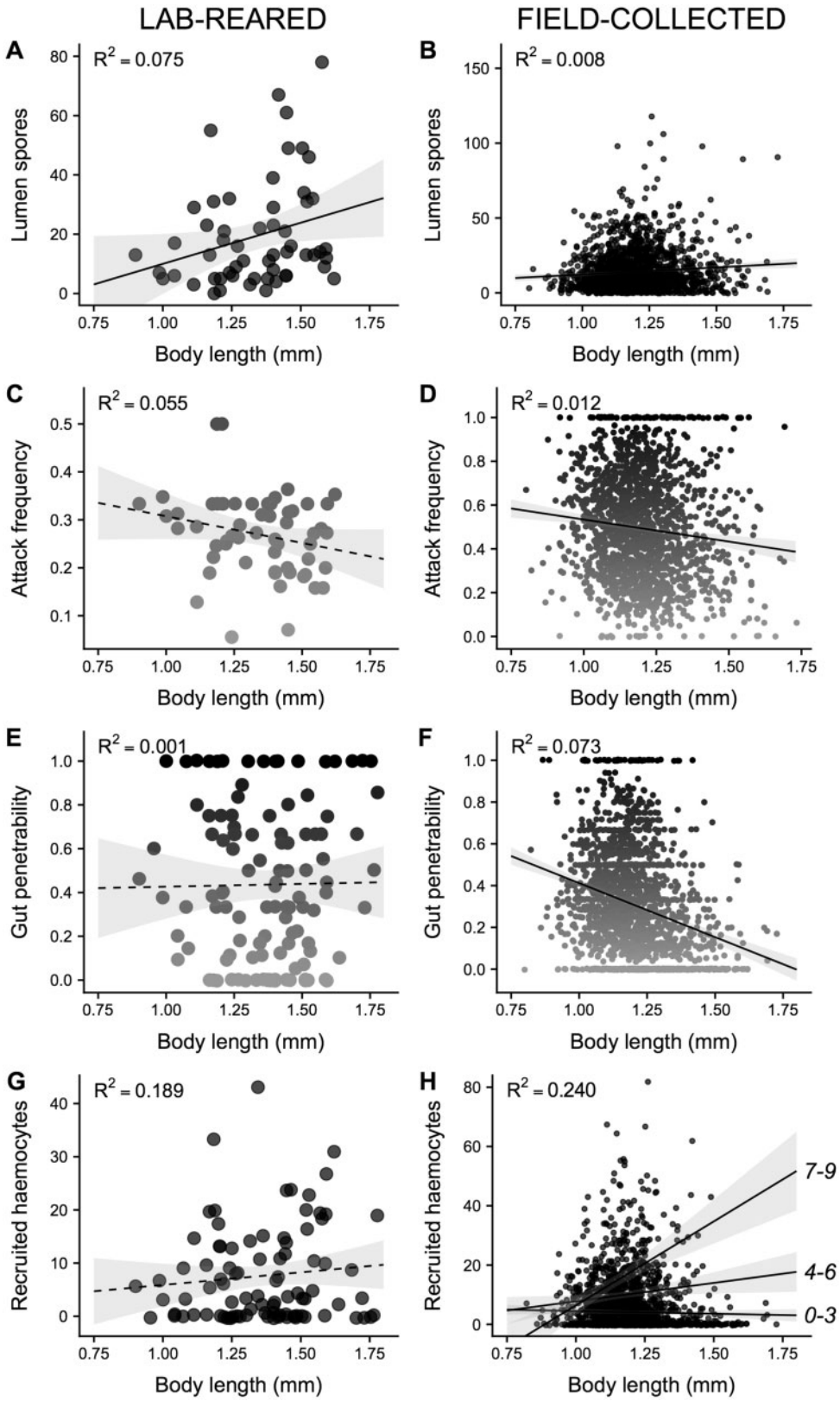

Fig. 5 Body size can have a complex relationship with host susceptibility due to its potentially opposing effects on multiple steps of infection. Testing H4 ("body size influences infection"), we examined the effects of body size on spore consumption, attack frequency, gut penetrability, and the hemocyte response, for laboratory-reared (left column) and field-collected (right column) hosts. Although body size (**A**, **B**) increased spore consumption, both the (**C**, **D**) attack (attack/exposure) and (**E**, **F**) gut penetrability (infection/attack) decreased with host body size. The dose-dependent hemocyte response (**G**, **H**) also increased with body size for field-collected

Daphnia (Fig. 5D; df = 1609, est = −0.201, $P < 0.001$, $R^2 = 0.012$). Larger *Daphnia* may have larger gut epithelial cells, so may also have higher gut penetrability. We tested for correlations among gut thickness and body size, and tested whether body size increased gut penetrability. Body size was only weakly correlated with gut epithelium thickness in laboratory-reared *Daphnia* (anterior gut epithelium: $r = 0.21$, $P = 0.060$; posterior gut epithelium: $r = 0.22$, $P = 0.069$) and did not predict gut penetrability of laboratory-reared *Daphnia* (Fig. 5E; df = 110, est = 0.026, $P = 0.886$, $R^2 = 0.001$). However, we found a strong negative relationship between body size and gut penetrability in field-collected *Daphnia* (Fig. 5F; df = 1728, est = −0.518, $P < 0.001$, $R^2 = 0.073$). Finally, we tested whether body size increased immune responses by evaluating body size, hemocoel spores and their interaction on total recruited hemocytes. Here, the interaction effect between body size and hemocoel spores tells us how size influences the response of hemocytes to a given level of infection. In laboratory-reared *Daphnia*, we did not detect an interaction between body size and hemocoel spores (Fig. 5G; df =86, est = 2.475, $P = 0.251$, $R^2 = 0.189$). In field-collected *Daphnia*, the interaction between body size and hemocoel spores was strong: larger *Daphnia* had greater hemocyte responses for a given level of infection (Fig. 5H; df = 1437, est = 5.975, $P < 0.001$, $R^2 = 0.240$). The weight of the evidence for body size influencing infection was intermediate and mixed. The amount of variation that body size explained ranged from $R^2 = 0.00$ to $R^2 = 0.24$ for multiple infection steps. Knowledge of which steps of infection (exposure, barriers, or immune responses) are the most important for determining terminal infection outcomes will clarify the role of body size in influencing this host–parasite interaction.

Laboratory to field comparisons with standardized regression coefficients

Laboratory environments can introduce artificial biases into our experiments and a common concern is whether interactions observed in a laboratory approximate those that occur in the natural world. We wanted to know: were the infection drivers we uncovered consistent across laboratory and natural *Daphnia* populations? We tested for consistency in relationships among laboratory-reared and field-collected *Daphnia* by comparing standardized model coefficients with a paired *t*-test. Fit to *z*-transformed data, these standardized coefficients scaled all relationships to the same currency. With them, we compared the following *y* by *x* relationships: (1) hemocoel spores by lumen spores, (2) hemocytes by spores, (3) lumen spores by body size, (4) attack frequency by body size, (5) gut penetrability by body size, and (6) hemocytes by body size (Fig. 6). Standardized regression coefficients did not differ among field-collected and laboratory-reared *Daphnia* but fell on or near the 1:1 line (df = 5, $t = -0.033$, $P = 0.975$). The processes and traits that drive the steps of infection were highly consistent from laboratory to field.

Integrating infection steps to understand susceptibility

Isolating the steps of infection provided multiple sound alternative hypotheses. *Daphnia* may face greater risk of infection as their spore ingestion increases and may be particularly susceptible to infection if they have penetrable gut barriers. Susceptibility may be further tuned by the hemocytes produced for a given level of infection, and by the host's body size. Here, we bring these drivers together to determine what factors underlie susceptibility. More specifically, we competed models with AIC (Burnham and Anderson 2002) and determined which hypothetical drivers of infection (1–4) best fit empirical data on terminal infection outcomes.

Having tracked field-collected *Daphnia* until 9 days post-inoculation, we had binary data for their terminal infection status (terminal infection: 1; recovery from infection: 0). We constructed generalized linear models (binomial distribution, logit link) assessing how terminal infection status at Day 9 was affected by predictors measured post-inoculation. We generated seven model sets within which we manipulated the number and type of interaction

Daphnia, where lines indicate the intensity of infection at 0–3, 4–6, and 7–9 hemocoel spores (93% of individuals had infections within the range of 0–9 hemocoel spores). Although the direction of relationships was fairly consistent among the two populations, not all relationships were significant for laboratory-reared *Daphnia* (given less power of tests). Across all panels, points represent unique individuals, and points shade from gray to black as attack frequency and gut penetrability increases (light gray: 0%; black: 100%). Solid lines indicate significant relationships, dashed lines indicate non-significant relationships, and shading around the line represents the standard error of the fit regression.

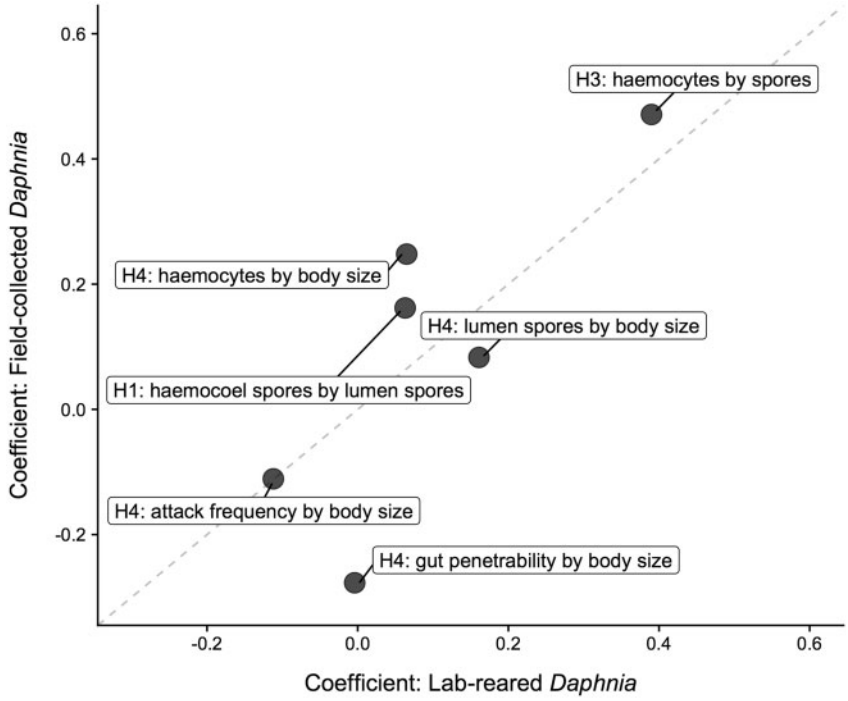

Fig. 6 Comparing hypothetical drivers of infection among laboratory-reared and field-collected *Daphnia*. Each point represents the regression coefficient for a given *y* by *x* analysis from H1, H3, and H4. Coefficients were standardized to the same currency by performing analyses on *z*-transformed data. The gray dashed 1:1 line indicates perfect correspondence among coefficients. In H1 ("exposure drives infection"; Fig. 2), we tested whether ingested lumen spores predicted successfully penetrated hemocoel spores, here indicated by "H1: hemocoel spores by lumen spores." We could not compare the results of H2 ("gut thickness creates a barrier to infection") because we did not have gut thickness measurements for field-collected animals. In H3 ("hemocytes mediate recovery"; Fig. 4), we tested whether total hemocyte recruitment increased with the number of spores infecting the body cavity, here indicated by the label "H3: hemocytes by spores." We could not compare the effects of hemocytes on recovery because we did not have terminal infection status for laboratory-reared animals. In H4 ("body size influences infection"; Fig. 5), we assessed how body size affected multiple steps of the host–parasite interaction: spore ingestion ("H4: lumen spores by body size"), attack frequency ("H4: attack frequency by body size") and gut penetrability ("H4: gut penetrability by body size"). In H4, we also tested whether body size increased immune responses by evaluating the effects of body size, hemocoel spores, and their interaction on total recruited hemocytes—the coefficient for the interaction between body size and hemocoel spores is plotted here as "H4: hemocytes by body size." Relationships are highly consistent among laboratory-reared and field-collected *D. dentifera*, with no difference in standardized regression coefficients among the two populations.

effects (Table 2). Because terminal infections require exposure, all models (except the null) included exposure as a covariate (defined in Table 1), which allowed us to determine which factors best explained variation in the exposure-terminal infection relationship.

In the first model set (1), "exposure," exposure is the sole predictor of terminal infection. This model assumes that *Daphnia* do not vary in their susceptibility; terminal infection only depends on the cumulative number of spores that enter their bodies. The second model set (2), "body size," included exposure and body size, and consisted of two models containing their additive or interactive effects. In the third model set (3), "barriers," spores that enter the host are inhibited by the gut barrier, and the model set consisted of two models containing the additive or interactive effects of exposure and gut penetrability. In the fourth model set (4), "immune responses," the fungus is killed by host hemocytes, and the two models included the additive or interactive effects of exposure and hemocytes per spore. Exposure, body size, and gut penetrability were combined in the fifth model set (5), "pre-body cavity interactions," which consisted of five models containing their additive and interactive effects (i.e., all possible interactions, then subsets of interactions). Here, terminal infection is primarily determined by processes occurring *before* spores enter the body cavity, including the effects of body size. Then, exposure, body size, and hemocytes per spore were paired in the sixth model set (6), "within-host battle," consisting of five models containing their additive and interactive effects. Here, terminal infection is primarily determined by interactions occurring *within* the host's body cavity, including the effects of body size. Finally, in (7), "total defenses," barriers and immune

Table 2 Generalized linear models assessing potential predictors of terminal infection outcomes

Model set	Predictors	K	ΔAIC	w_i
(7) Total defenses	**exposure*guts* hemocytes**	**8**	**0.00**	**0.84**
(7) Total defenses	**exposure + guts* hemocytes**	**5**	**4.41**	**0.09**
Global model	exposure*size*guts* hemocytes	16	4.91	0.07
(7) Total defenses	exposure*hemocytes + guts	5	14.21	0.00
(5) Pre-body cavity	exposure + size*guts	5	17.07	0.00
(3) Barriers	exposure + guts	3	17.38	0.00
(3) Barriers	exposure*guts	4	17.77	0.00
(5) Pre-body cavity	exposure + size + guts	4	19.23	0.00
(7) Total defenses	exposure + guts + hemocytes	4	19.37	0.00
(5) Pre-body cavity	exposure*guts + size	5	19.70	0.00
(7) Total defenses	exposure*guts + hemocytes	5	19.77	0.00
(5) Pre-body cavity	exposure*size*guts	8	20.26	0.00
(5) Pre-body cavity	exposure*size + guts	5	20.70	0.00
(6) Within-host battle	exposure*size* hemocytes	8	32.21	0.00
(6) Within-host battle	exposure + size*hemocytes	5	34.35	0.00
(6) Within-host battle	exposure*hemocytes + size	5	40.12	0.00
(2) Body size	exposure + size	3	42.31	0.00
(4) Immune responses	exposure*hemocytes	4	42.85	0.00
(2) Body size	exposure*size	4	43.86	0.00
(6) Within-host battle	exposure + size + hemocytes	4	44.25	0.00
(1) Exposure	exposure	2	44.94	0.00
(6) Within-host battle	exposure*size + hemocytes	5	45.80	0.00
(4) Immune responses	exposure + hemocytes	3	46.89	0.00
Null model	Intercept-only	1	48.31	0.00

Numbers in parentheticals indicate the model set that each model belongs to (1), Exposure; (2), Body size; (3), Barriers; (4), Immune responses; (5), Pre-body cavity interactions; (6), Within-host battle; (7), Total defenses. Predictors include "exposure" (cumulative number of all spores within the host's body), "guts" (gut penetrability), "hemocytes" (average number of hemocytes per spore), and "size" (body size of the host). Additive effects of predictors are indicated with "+". We use "*" to denote when a model combines both the additive and interactive effects of predictors. Provided for each model are *K* (the number of estimated parameters), ΔAIC (indicating model performance relative the best-ranked model), and relative likelihood (w_i, the probability that the model fits best, given the suite of models considered). Additional output (AIC and estimates) is presented in Supplementary material S4. Models from the "Total defenses" set (7), which incorporated exposure, gut penetrability ("guts"), and average hemocytes per spore ("hemocytes") were the highest ranked with a combined relative likelihood (w_i) of 0.93 (indicated with bold text). The interaction between gut penetrability and the hemocyte response (guts*hemocytes) was consistently represented among the three top-ranked models, suggesting an important interaction effect between host barriers and immune responses.

responses act together to defeat parasites (absent body size), and five models were constructed that included the additive and interactive effects of exposure, gut penetrability, and hemocytes per spore. All models contained an intercept and, within our model competition, we also included an intercept-only null model, and a global model containing all predictors and their interactions. Models were ranked by their AIC values, with the lowest AIC value representing the most likely model given the data. We then compared models based on their performance relative the best-ranked model (ΔAIC) and by their model weights (w_i). Model weights represent the probability that a model fits best, given the suite of models considered (Burnham and Anderson 2002).

The two host immune defenses (barriers and hemocytes) acted in concert to best explain variation in the exposure-terminal infection relationship. The top-ranked model emerged from model set (7), "total defenses," and contained all interactions between exposure, gut penetrability and hemocytes per spore. In this winning model, terminal infection is dictated by how spores are blocked by the gut barrier and met by the hemocyte response. The best-ranked model had an Akaike weight of 0.84, indicating its high explanatory power relative the other models. Because the second most competitive model was another variant of the "total defenses" set, model set (7) contained over 93% of the weight of the evidence (Table 2).

In our model competition, the interaction between gut penetrability and hemocytes per spore (gut pen*hemocytes; Table 2) came out as a consistently important predictor: this interaction was only included in three models, and all three models containing the interaction were also the best ranked. We examined the probabilities predicted from the winning model to explore what this interaction means for *Daphnia* (Fig. 7). While hemocytes decreased terminal infection probability for *Daphnia* with high gut penetrability, they were associated with increased terminal infection probability for *Daphnia* with low gut penetrability (Fig. 7). This interaction effect helps resolve why hemocytes were not associated with recovery in H3 and suggests that hemocytes may mediate recovery for only some *Daphnia*, while signaling susceptibility in others.

Discussion

We found statistical support for four drivers of *Daphnia* infection: (1) exposure, where spore ingestion increased the number of spores that infected the

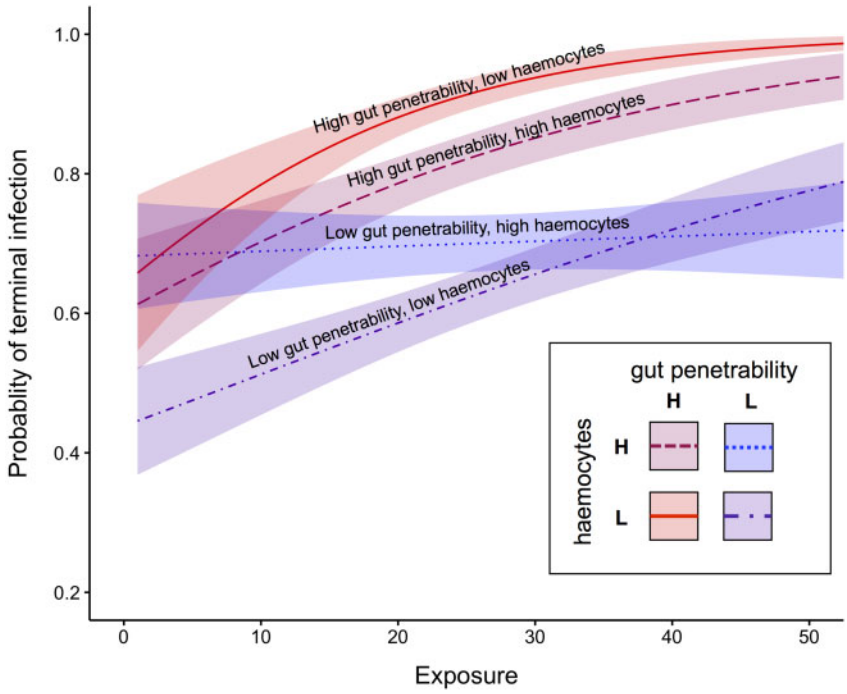

Fig. 7 Predicted terminal infection probabilities from the top ranked "total defenses" model (Table 2) are plotted as a function of exposure (see Table 1). To illustrate the interaction between gut penetrability and hemocytes, we plot lines and standard error shading for four host classes, categorized by whether they fall above (H: high) or below (L: low) the median level of gut penetrability and the median hemocyte response (hemocytes per spore). Low gut penetrability generally decreases the risk of terminal infection: the probability of terminal infection is highest for *Daphnia* with high gut penetrability and lowest for *Daphnia* with low gut penetrability. Intermediate terminal infection risk emerges for *Daphnia* with high gut penetrability and high hemocyte responses: when *Daphnia* barriers are poor, hemocytes aid in recovery. While the three aforementioned classes share similar exposure-terminal infection curves, the fourth class (low gut penetrability, high hemocytes) shows consistently high susceptibility over the range of exposure. These *Daphnia* may be highly susceptible to terminal infection when their barriers fail, such that hemocytes are more a symptom of susceptibility than a cause of recovery.

host; (2) barriers, where attacking spores were blocked by the gut barrier and thinner anterior epithelia conferred greater resistance; (3) immune responses, where spores that infected the host elicited an intensity-dependent increase in recruited hemocytes; and (4) host body size, which influenced multiple steps of infection, increasing spore ingestion, decreasing attack frequency and gut penetrability, and increasing the magnitude of the hemocyte response. However, when considered alone, each driver exhibited substantial host variation and generally low effect sizes. The weight of the evidence in support of exposure (H1) and immune responses (H3) was low, whereas we found intermediate support for barriers (H2) and body size (H4). By integrating these four drivers, we sought to absorb variation in the complete infection process. We found that each driver of infection differed in its contribution to terminal infection outcomes. Model comparison revealed that host immune defenses, that is, the combination of gut barriers and hemocytes, explained the most variation in the exposure-terminal infection relationship. Host body size was present in the third best-ranked model but could not compete with barriers and hemocytes. Our results illustrate the hierarchical nature of host immune defenses and raise questions about potential tradeoffs occurring at each step of infection.

Parasite exposure increases disease risk, and avoidance behaviors are a first line of defense for limiting exposure (Buck et al. 2018; Weinstein et al. 2018a). For example, spiny lobsters detect viral infection in conspecifics and reduce their risk of transmission by limiting physical contact (Behringer et al. 2006). But avoidance behaviors may be costly when parasite encounter is tightly coupled with feeding. In this case, hosts must balance the risk of disease against the need for food (Lozano 1991; Hall et al. 2009, 2010). Whether avoiding parasite consumption is costly or beneficial for a host should depend on the parasite's pathogenicity and density in the environment. For low pathogenicity parasites, heightened risk of infection can be worth the benefit of a meal (Lafferty and Morris 1996; Weinstein et al. 2018b),

and when parasites are dense across the environment, avoidance may be futile. *Metschnikowia* is both highly pathogenic and highly abundant during epidemics (Stewart Merrill 2019) and wild *Daphnia* may be forced to feed amidst unavoidable levels of risk. We found only weak relationships between spore ingestion and infection, suggesting that downstream defenses decouple exposure from infection and relax foraging-infection tradeoffs.

For parasites that must be ingested to infect, the gut epithelium presents a physical barrier to infection (Söderhäll 2010; Garcia-Garcia et al. 2013). In our test of whether thicker guts were less penetrable by *Metschnikowia*, we were surprised by the result: thicker anterior epithelia were more, rather than less, penetrable by the needle-shaped spores. Given this finding, as well as the large difference between average spore length (45 µm) and average gut thickness (15 µm), it may not be cell height per se that is driving penetrability, but rather, structural attributes that are correlated with cell height. We suspect that the penetrability of the gut barrier is related to its cells' ability to absorb nutrients. In addition to being a site of infection, the anterior midgut is important for resource assimilation and requires its permeability. Anterior midgut epithelia are actively involved in resource absorption (Quaglia et al. 1976; Schultz and Kennedy 1976) and have been observed to shrink during periods of starvation (Theilacker and Watanabe 1989; Elendt and Storch 1990). The potential reliance of parasite resistance and resource assimilation on contrasting aspects of gut morphology could generate a foraging-infection tradeoff at the gut barrier. Similar tradeoffs have been detected in *Drosophila*, where strong pathogen resistance by the peritrophic membrane decreases its permeability and nutrient absorption (Kuraishi et al. 2011; Shibata et al. 2015). Given the broad diversity of parasites that infect via the host gut, future work on host resistance may benefit from the dual consideration of the gut's defensive and digestive properties (Miguel-Aliaga et al. 2018).

Internal immunological responses are a final defense against parasites that cross host barriers. Hemocytes are among the most well-studied immune responses of invertebrates (Bayne et al. 2001; Bartholomay et al. 2007; Lemaitre and Hoffmann 2007; Moreno-García et al. 2013), but their role in combatting parasites has been called into question in *Daphnia*. In his classic study of invertebrate immunity, Metschnikoff (1884) described *Daphnia* hemocytes attacking *Metschnikowia* spores, highlighting their role in host defense. More recently, Auld et al. (2010, 2012b) observed the strongest hemocyte responses in the most susceptible *Daphnia*, suggesting that hemocytes were merely a symptom of upstream susceptibility (Graham et al. 2011). Our findings revealed a complicated relationship between hemocyte responses and parasite infection. In particular, our model comparison revealed a strong interaction between gut penetrability and the hemocyte response: among *Daphnia* that had more penetrable guts, higher hemocyte levels were associated with decreased terminal infection risk, whereas hemocytes did not dampen terminal infection risk in *Daphnia* with low gut penetrability. The source of the disparate relationship between hemocytes, terminal infection risk, and gut penetrability is unclear, but may stem from tradeoffs between immune defense types. Vertebrates are thought to differentially invest in innate or acquired defenses (Lochmiller and Deerenberg 2000), and invertebrates may likewise invest either in resistant barriers or effective immune responses. If barriers are weak and parasites can easily enter the body cavity, a *Daphnia* host should rely on a well-operating immune response. Alternatively, *Daphnia* with robust barriers may have high internal susceptibility and hemocytes may be a symptom of internal susceptibility. Greater resolution on this potential tradeoff may be achieved by assessing hemocyte quality (in addition to quantity), as well as other immune responses that act against *Metschnikowia*.

Body size often increases exposure to parasite propagules (Ebert 1995; Hall et al. 2007; Civitello et al. 2015) but may have variable effects on a host's susceptibility (Downs et al. 2019). While our results confirmed that body size increases spore ingestion, we found negative size-susceptibility relationships for all subsequent infection steps. In support of Izhar et al. (2015) and Izhar and Ben-Ami (2015), the cumulative effects of body size generally resulted in decreasing terminal infection risk with increasing body size (Supplementary material S4). Our "total defenses" model, which included exposure, barriers, and the hemocyte response, substantially outperformed all other models in explaining terminal infection outcomes. But body size was a strong contender. Body size was included in the third most competitive model (the global model), suggesting its important role in infection. That the effects of body size were statistically overwhelmed by those of gut penetrability and hemocytes raises the question of how body size affects those two key traits. Hemocyte responses increased with body size, which is in line with both theory (Sheldon and Verhulst 1996; Sadd and Schmid-Hempel 2009) and empirical work on energy-dependence of immune defense (Siva-Jothy and Thompson 2002; Valtonen et al. 2010; Triggs and Knell 2012).

Gut penetrability decreased with body size, which warrants consideration of the physical processes by which spores enter their hosts. We measured gut penetrability functionally, asking: what proportion of spores does the gut epithelium block? Because this measure results from the interaction of two players (gut and spore), it should depend on both the epithelial cell's permissiveness to puncture as well as the force and direction of the spores. Guts are like pipes, and while increasing host body size will increase the length and surface area of the gut (Hall et al. 2007), it will increase its volume at a faster rate. Hence, as body size increases, there are more opportunities for spores to occupy the gut lumen than contact its edges. In large hosts (big pipes), spores may just barely contact the gut epithelium, with the majority of the spore's length residing in the gut lumen. In small hosts (small pipes), the intraluminal space may be tight enough that spores get stuck and pierce through the gut barrier. For ingested parasites more generally, such scaling relationships between body size, feeding rate, and gut morphology may be important determinants of the body size-infection relationship.

Natural ecological conditions can be varied and unpredictable, such that measurements taken under laboratory settings might paint an artificial picture of an organism's capacity to fight infection (Boughton et al. 2011; Pedersen and Babayan 2011). Our laboratory-reared *Daphnia* were standardized to remove maternal effects and were raised in a high-food, low competition, and parasite-free environment (until inoculation, at least). Alternatively, our field-collected *Daphnia* were of unknown genetic/epigenetic background and possessed diverse histories with resources, competitors, and exposure to parasites. In spite of these differences, our results were remarkably consistent among laboratory-reared and field-collected hosts. For instance, the relationship between body size and attack frequency had almost identical standardized coefficients in both populations, lending credence to the idea that spore attack is a purely physical process. Through robust sample sizes, the field-collected *Daphnia* allowed us to detect noisy relationships, and also provided greater insight into one driver of infection: the gut barrier. The strongest deviation in standardized coefficients was in the body size and gut penetrability relationship, which was strong and negative for field-collected *Daphnia* but weak and near zero for laboratory-reared *Daphnia*. Interestingly, individuals from the field also had much lower gut penetrability on average (field mean: 0.30; laboratory mean: 0.44). Given the importance of gut penetrability for both parasite resistance and resource assimilation, we suspect that resources, which are often poorer quality in the field, and parasites, which are abundant and diverse in the field, may be driving these differences.

Host susceptibility is a simple concept in theory but difficult to measure in practice. By decomposing the infection process, we gained new biological insight into four drivers of infection in *Daphnia*. When considered in isolation, these drivers weakly explained infection outcomes, but when united, they became powerful predictors of *Daphnia* susceptibility. In both laboratory-reared and field-collected *Daphnia*, we found dramatic variation in the four host traits that drive infection. Spore ingestion ranged over two orders of magnitude, gut penetrability varied from completely penetrable (100%) to completely impenetrable (0%), and hemocytes varied both in number and ability to prevent infection. It is no stretch to link this variation to resources. Host feeding brings spores into the body, the epithelial cells comprising the gut barrier process resources, and hemocytes are likely resource-dependent. A resource perspective (e.g., Hall et al. 2009, 2010, 2012; Cressler et al. 2014) on the genetic and plastic components of susceptibility will propel research in *Daphnia* disease, as well as other systems where parasites infect through host feeding.

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Supplementary data

Supplementary data available at *ICB* online.

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