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RESEARCH ARTICLE

Host and parasite functional morphology jointly explain parasite specificity

Syuan-Jyun Sun[1,2] | Siobhan K. Calhoun[1] | Meghan A. Duffy[1]

[1]Department of Ecology & Evolutionary Biology, University of Michigan, Ann Arbor, Michigan 48109, USA

[2]International Degree Program in Climate Change and Sustainable Development, National Taiwan University, Taipei, 10617, Taiwan

Correspondence
Syuan-Jyun Sun
Email: sjs243@ntu.edu.tw

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Abstract

1. Host–parasite coevolution is a major diversifying force. However, while the genetic determinants of host–parasite coevolution have received substantial attention, it remains unresolved how morphological adaptations contribute to host–parasite coevolutionary dynamics.
2. We used a well-studied and ecologically important host–parasite system to explore morphological adaptation in host–parasite interactions. In this daphniid-fungal parasite system, infection occurs when fungal spores puncture the host gut and enter the body cavity. Prior work found genetic differences in the parasite associated with spore size.
3. We studied how host gut traits, parasite spore size and host immune responses influenced the infection process. We collected parasite spores from two host species, the larger *Daphnia dentifera* and the smaller *Ceriodaphnia dubia*, and exposed both host species to spores sourced from each host.
4. The ability of a spore to embed in the host gut and to penetrate into the body cavity was influenced by the host species that was exposed to the parasite ('exposure host species') and the species from which the spores were sourced ('source host species'). Spores sourced from *D. dentifera* were better able to attack both hosts, but were especially good at attacking *D. dentifera*. These differences likely resulted from morphological differences, with a striking correspondence between the diameter of host guts and the size of the parasite spores.
5. Immune responses were influenced by both exposure and source host, with *D. dentifera*-sourced spores triggering a larger immune response in *D. dentifera* than in *C. dubia*. In addition, in *C. dubia* exposure hosts, *D. dentifera*-sourced spores triggered a greater immune response than did *C. dubia*-sourced spores.
6. Only 13.5% of hosts that had at least one parasite spore penetrate ended up with terminal infections; all but one of these infections occurred in *D. dentifera* hosts exposed to *D. dentifera*-sourced spores.
7. Overall, infection was influenced by morphological traits of both hosts and parasites, with the outcome at each step of the infection process—and the likelihood of terminal infection—being determined by both the exposure host and the source host.

KEYWORDS
Ceriodaphnia, coevolution, *Daphnia*, host–parasite interactions, *Metschnikowia*, morphological adaptations

1 | INTRODUCTION

Coevolutionary interactions between hosts and their parasites are a major force driving genetic and phenotypic diversity in both parties (Betts et al., 2018; Gandon, 2002; Schulte et al., 2013). Antagonistic interactions pose strong selective forces favouring adaptive changes in traits such as parasite virulence and host resistance, resulting in a coevolutionary arms race of reciprocal adaptation (Woolhouse et al., 2002). While there is ample evidence demonstrating the genetic processes (e.g. recombination and mutation) underlying host–parasite coevolution within and among populations (Brockhurst et al., 2004), it is unclear whether similar processes are manifested in morphological characteristics, especially for parasites that require specialized morphological structures to establish a successful infection. It is likely that phenotypic and genetic aspects of coevolution are interdependent, and investigating the morphological aspects of host–parasite coevolution can complement molecular studies, providing an integrated understanding of local adaptation. Existing evidence regarding morphological aspects of host–parasite coevolution largely comes from macroparasites (Abuzeid et al., 2020; Fain, 1994; Nagler & Haug, 2016; Walter & Proctor, 2013), such as mites and nematodes. These parasites commence external or internal attachment to the hosts using their specialized anchoring structures (e.g. mouthparts), thereby ensuring effective host exploitation (Baillie et al., 2019). In addition, some nematophagous fungi use similar strategies, attaching to the host nematodes using adhesive traps and spores (Li et al., 2015). Thus, while there is evidence that morphological characteristics are under selection in host–parasite interactions, studies of morphological adaptations have primarily focused on parasite attachment to hosts; to date, the morphological adaptation underlying infection processes per se remains unresolved.

Freshwater zooplanktonic hosts in the family Daphniidae and their fungal parasite *Metschnikowia bicuspidata* present an ecologically important and experimentally tractable system in which we can explore morphological adaptation in host–parasite interactions. *M. bicuspidata* is widely distributed geographically but has surprisingly little genetic variation (Duffy & Sivars-Becker, 2007; Searle et al., 2015; Shaw et al., 2021; Wolinska et al., 2009); however, recent work found genetic variation that was associated with spore size and the ability to infect different host species, suggesting potentially adaptive morphological differentiation between *M. bicuspidata* clades (Shaw et al., 2021).

Successful infection by *M. bicuspidata* is likely to be a mechanical process and occurs when hosts incidentally ingest parasite spores along with their similarly sized phytoplankton food. Infections are most common at the anterior and posterior bends in the gut (Stewart Merrill et al., 2019), where spores puncture the gut epithelium and, in successful infections, penetrate into the body cavity (Ebert, 2005). Upon host death, spores are released into the environment and can be ingested by new hosts of the same or different species to complete their life cycle (Ebert, 2005). Interestingly, in lakes with two host species (*Daphnia dentifera* and *Ceriodaphnia dubia*), *M. bicuspidata* often causes an outbreak in one host but not the other (Shaw et al., 2021). These hosts differ substantially in body size, with *D. dentifera* being significantly larger than *C. dubia* (Thorp & Covich, 2010). Notably, *M. bicuspidata* spores have been found to be smaller in *C. dubia* than those in *D. dentifera* (Shaw et al., 2021). Similar differences in spore size associated with host size were also found in *M. bicuspidata* spores infecting two different *Daphnia* species in England (Stirnadel & Ebert, 1997). These differences between species in host and parasite traits suggest a potential mechanism underlying variation in the ability to infect different hosts, as has been found experimentally (Auld et al., 2017; Shaw et al., 2021).

Here, we studied how host gut traits and spore size influenced the ability of *M. bicuspidata* to infect two of its zooplanktonic hosts, *D. dentifera* and *C. dubia*. To do this, we compared spores sourced from *D. dentifera* with those sourced from *C. dubia*. Prior work has shown that spore size is determined by the parasite genotype rather than the host species in which it is reared (Shaw et al., 2021). First, we predicted that large (*D. dentifera* sourced) spores would be more likely to pierce the host gut than small (*C. dubia* sourced) spores. Second, successful infection requires that spores not only embed in the gut but also penetrate through the epithelium into the body cavity. Thus, we predicted that larger hosts might differ in resistance to attacking spores because of their thicker gut epithelia, although the unimodal relationship between gut thickness and resistance (Sun et al., 2023) made it hard to predict whether larger guts would be more or less resistant. We predicted that larger spores would be more likely to pierce through the gut. Finally, we were also interested in how these processes related to terminal infections—that is, infections that yield mature transmission spores that can go on to infect a future host. Terminal infections require not only that a parasite attack the host and penetrate into the body cavity, but that it also manages to avoid being stymied by the immune response: parasites can enter the body cavity but then fail to result in a full-blown infection, presumably due to attack by the host immune system (Stewart Merrill et al., 2019). Thus, we also explored variation in the immune responses of the two host species, and whether those differed depending on the source of the spores to which a host was exposed.

2 | MATERIALS AND METHODS

2.1 | Study system

D. dentifera and *C. dubia* commonly coexist in the same lakes in North America, and both species host the virulent fungal parasite, *M. bicuspidata*, although lakes tend to have an outbreak in one host but

not the other (Shaw et al., 2021). In this experiment, we used the 'Standard' genotype of *D. dentifera*, which was collected from a lake in Barry County in Michigan, US; this clone has been the subject of extensive study, as has the 'Standard' genotype of *D. dentifera*-associated *M. bicuspidata*, which also originated from a lake in Barry County. Because we did not have any *Ceriodaphnia* or *Ceriodaphnia*-associated *M. bicuspidata* in culture, we isolated *C. dubia* ('Gosling 9' genotype) and its associated *M. bicuspidata* from Gosling Lake (Livingston County, Michigan, USA); this lake is known to have outbreaks of *M. bicuspidata* in *C. dubia* that occasionally spill over into *D. dentifera* (Shaw et al., 2021). Stocks of 'Standard' *D. dentifera* and 'Gosling 9' *C. dubia* were maintained as small populations of five individuals per 150 mL beaker filled with 100 mL filtered lake water (40 beakers in total per species). All animals were fed three times a week with a phytoplankton food (*Ankistrodesmus falcatus*, 20,000 cells/mL). The *D. dentifera*-sourced and *C. dubia*-sourced *M. bicuspidata* were maintained following protocols as described in detail elsewhere (Sun et al., 2023). In brief, sources of stock *D. dentifera* or *C. dubia* were exposed to *D. dentifera*-sourced or *C. dubia*-sourced spores respectively. The *D. dentifera*-sourced spores were the 'Standard' isolate, and the *C. dubia*-sourced spores were a new isolate ('Gos21') generated for this study by grinding up a single infected individual and then rearing spores in the Gosling 9 genotype. All exposed animals were checked daily for survival, and upon death, infected animals were placed in a 1.5 mL tube filled with 100 μL filtered lake water and stored in a refrigerator before use.

2.2 | Experimental design

To test for the effects of host and parasite traits on infection processes in our host–parasite system, we conducted a cross-infection experiment by infecting *D. dentifera* and *C. dubia* with *M. bicuspidata* spores from either the 'Standard' (hereafter: *D. dentifera* sourced) or 'Gos21' (hereafter: *C. dubia* sourced) isolates. We collected neonates (aged 24 h) of both *D. dentifera* and *C. dubia* from laboratory colonies of the 'Standard' *D. dentifera* genotype and the 'Gosling 9' *C. dubia* genotype. All individuals were maintained in 50 mL beakers filled with 50 mL filtered lake water (16:8 light:dark, 20°C), and fed three times a week with a phytoplankton food (*Ankistrodesmus falcatus*, 20,000 cells/mL).

The infection process was conducted in a standardized approach by transferring a single 5-day-old juvenile to a 10 mL beaker filled with 5 mL lake water. A dose of spore solution (250 spores/mL) of either *D. dentifera*- or *C. dubia*-sourced spores was added to a beaker; each beaker was fed 20,000 cells/mL *A. falcatus* at the same time. After a 24 h inoculation period, we examined the infection and spore penetration process under an Olympus BX53F compound microscope (200–400× magnification). Because successful infection requires spore penetration into the body cavity through the gut epithelium, we screened the anterior and posterior ends of the gut. We classified the spores into two categories (based on Stewart Merrill et al., 2019): embedded spores (i.e. partially embedded in the gut epithelium) or penetrated spores (i.e. successfully penetrated into the body cavity). This categorization allowed us to examine whether the gut is a physical barrier to spore penetration, by the proportion of penetrated spores. To determine immune responses associated with the infection process, we counted the number of haemocytes attached to each penetrated spore. For host traits, we determined gut epithelium thickness and gut diameter from images taken at high resolution (400×) as we examined spore penetration. Three epithelial cells in the anterior midgut (at the 90-degree bend in the gut) were haphazardly selected, and the average gut epithelium thickness and the average gut diameter measured using cellSens Standard Software (Olympus, version 1.18). For parasite traits, we determined the spore length by sampling *D. dentifera*-sourced *M.* bicuspidata grown in *D. dentifera* and *C. dubia*-sourced spores grown in *C. dubia*. From each spore source species, a 10 μL aliquot of a homogenous spore solution was added to a Neubauer haemocytometer. We then haphazardly selected the first 20 different individual spores in view at high magnification (200–400×) with an Olympus BX53F compound microscope and measured the length of each individual spore using the cellSens Standard Software (Olympus, version 1.18).

After the examination, all individuals were transferred to 50 mL beakers filled with 50 mL spore-free filtered lake water and fed three times a week as previously maintained. Eleven days postparasite exposure, we terminated the experiment and checked for terminal infection outcomes. The experiment was conducted in two time blocks.

2.3 | Statistical analysis overview

All statistical analyses were conducted in R version 4.1.2. Generalized linear mixed models (GLMMs) were conducted with the glmer function in the LME4 package (Bates et al., 2015). Once significant interactions were detected from GLMMs, Tukey *post-hoc* comparisons were conducted to assess differences between individual treatments in the EMMEANS package (Lenth, 2021). Nonsignificant interaction terms were removed from the final models. All of the final models were significant compared to a null model (Table S1). Analysis of variance (type III sums-of-squares) was conducted in the CAR package (Fox et al., 2021). In all GLMMs, experimental block was included as a random factor since the experiments were conducted in two blocks.

2.3.1 | Parasite attack and physical barrier

We analysed the number of attacking spores with a Poisson distribution by including exposure host species (*D. dentifera* or *C. dubia*), sources of spores (*D. dentifera* or *C. dubia*) and their interaction as fixed effects. For exposed hosts that had at least one attacking *M. bicuspidata* spore, we also analysed the proportion of penetrating spores (the number of penetrating spores given the number of attacking spores) and the total number of penetrating spores. We analysed the proportion of spores penetrating animals by including the number of penetrated spores as a

dependent variable and the natural log of number of attacking spores [ln (*x*+1)] as an offset term. Host species, sources of spores and their interaction were included as fixed effects. We analysed the number of penetrating spores with a Poisson distribution by including exposure host species (*D. dentifera* or *C. dubia*), source of spores (*D. dentifera* or *C. dubia*) and their interaction as fixed effects. In addition, we analysed interspecific differences in gut epithelial thickness and gut diameter with a Gaussian distribution by including host species (*D. dentifera* or *C. dubia*) as a fixed effect.

2.3.2 | Host immune response

We analysed the total number of haemocytes with a Poisson distribution and haemocytes per spore with a Gaussian distribution by including exposure host species (*D. dentifera* or *C. dubia*), sources of spores (*D. dentifera* or *C. dubia*) and their interaction as fixed effects. We compared the total number of haemocytes and haemocytes per spore between treatments using nonparametric Wilcoxon rank sum tests with the wilcox.test function, since there was no proper error structure for fitting parametric GLMMs.

2.3.3 | Terminal infection

We analysed the probability of terminal infection (terminal infection: 1; no terminal infection: 0) by fitting a logistic regression model applying Firth's correction to the likelihood in the LOGISTF package (Heinze et al., 2022). Firth's regression is well suited to addressing the problem of separation (when one variant is associated with only one type of outcome), which causes problems for maximum likelihood estimation, and is also well suited to analysing rare events (Heinze & Schemper, 2002). We included exposure host species (*D. dentifera* or *C. dubia*), sources of spores (*D. dentifera* or *C. dubia*) and their interaction as fixed effects.

3 | RESULTS

3.1 | Parasite attack and physical barrier

The ability to attack the host gut and successfully penetrate into the body cavity was influenced by exposure host species, depending on the source of the spores (attack: source host×exposed host: $\chi^2=16.06$, $p<.001$, Figure 1a; penetration: source host×exposed host: $\chi^2=3.51$, $p=0.061$, Figure 1b). Spores sourced from *D. dentifera* were better able to attack both hosts, but especially good at attacking *D. dentifera* hosts ($z=-4.22$, $p<0.001$; Figure 1a). Spores sourced from *C. dubia* were less able to attack both hosts (as compared to *D. dentifera*-sourced spores), but did better at attacking *C. dubia* hosts (as compared to *D. dentifera*; $z=2.21$, $p=0.027$; Figure 1a). Of the *C. dubia* hosts that did successfully attack *D. dentifera*, almost none successfully penetrated into the body cavity. One likely explanation for this is a difference in the size of the host gut and the parasite spores (Figure 2). *D. dentifera*-sourced spores (56.20±0.43 μm; mean±SD) were almost twice as long as *C. dubia*-sourced spores (32.94±0.51 μm; mean±SD), with their length showing a striking similarity to the diameter of *D. dentifera* guts (57.34±0.66 μm; mean±SD). These results suggest the smaller size of the *C. dubia*-sourced spores made it difficult for them to lodge into the gut wall. Overall, the number of successfully penetrating spores was determined by an interaction between the exposure host species and the source of the spores (source host×exposed host: $\chi^2=8.15$, $p=0.004$; Figure 1c). *D. dentifera*-sourced spores consistently outperformed *C. dubia*-sourced spores, to a greater extent when infecting *D. dentifera* ($z=-6.23$, $p<0.001$) than *C. dubia* ($z=-4.74$, $p<0.001$). Furthermore, *D. dentifera*-sourced spores were more effective at establishing in *D. dentifera* than *C. dubia* ($z=-4.11$, $p<0.001$), whereas *C. dubia*-sourced spores were marginally more effective at infecting *C. dubia* than *D. dentifera* ($z=1.79$, $p=0.073$).

3.2 | Host immune response

Immune responses among hosts in which at least one spore successfully penetrated were influenced by both exposure and source host (Figure 3), but there was no significant interaction between the two ($\chi^2<0.5$, $p>0.5$ for both total haemocytes and haemocytes per spore). *D. dentifera*-sourced spores triggered a higher total number of haemocytes ($W=219$, $p=0.014$; Figure 3a) and a marginally higher number of haemocytes per penetrated spore ($W=159.5$, $p=0.066$; Figure 3b) in *D. dentifera* than in *C. dubia* (Figure 3). In *C. dubia*, *D. dentifera*-sourced spores triggered a higher total number of haemocytes (comparing blue bars in Figure 3a; $W=108.5$, $p<0.001$) and haemocytes per penetrated spore (Figure 3b; $W=13$, $p<0.001$).

3.3 | Terminal infection

D. dentifera-sourced spores were more likely to penetrate host guts, particularly when in *D. dentifera* exposure hosts (Figure 1c). At the same time, *D. dentifera*-sourced parasites encountered a more robust immune response once inside the host (Figure 3). Putting these together to consider terminal infections, the former (high penetration) favours high fitness of *D. dentifera*-sourced parasites in *D. dentifera* hosts, but the latter (strong immune response) should reduce the fitness of *D. dentifera*-sourced parasites. The former appears to outweigh the latter, because almost all terminal infections occurred in *D. dentifera* exposed to *D. dentifera*-sourced parasites. Only one of the terminal infections occurred when *D. dentifera* was exposed to *C. dubia*-sourced parasites, and no *C. dubia* developed terminal infections (Figure 4). Overall, the likelihood of a terminal infection was determined independently by both exposure host ($\chi^2=8.60$, $p=0.003$) and the source of spores ($\chi^2=6.09$, $p=0.014$). Focusing on *D. dentifera*-sourced parasites in *D. dentifera*, neither total haemocyte number ($\chi^2=0.26$, $p=0.611$) nor haemocytes per spore ($\chi^2=0.92$, $p=0.337$)

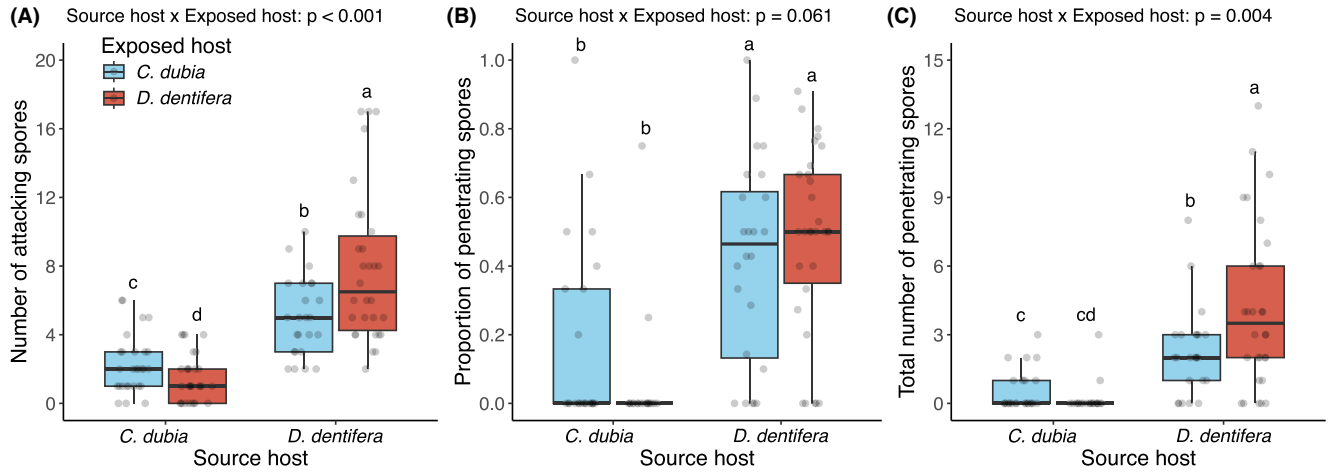

FIGURE 1 Exposure host species and spore source host species jointly determined the infection process in the initial infection stages. (a) The number of attacking spores, (b) proportion of penetrating spores and (c) number of penetrating spores were all influenced by the source and exposed host. Attacking spores include both embedded spores (i.e. spores partially embedded in the gut epithelium) and penetrated spores (i.e. spores that successfully penetrated into the body cavity); the proportion of penetrating spores is the number of penetrating spores divided by the number of attacking spores. There was a significant source host × exposed host interaction for number of attacking spores and total number of penetrating spores, and a marginally significant interaction term for the proportion of penetrating spores (see main text for more information). Letters on each panel indicate statistically significant differences of pairwise comparisons; the 'cd' on panel c indicates that treatment is marginally different than the one labelled 'c' (see main text). The box plots show median values, the 25th and 75th percentiles, and interquartile ranges, with the raw data overlain (grey points).

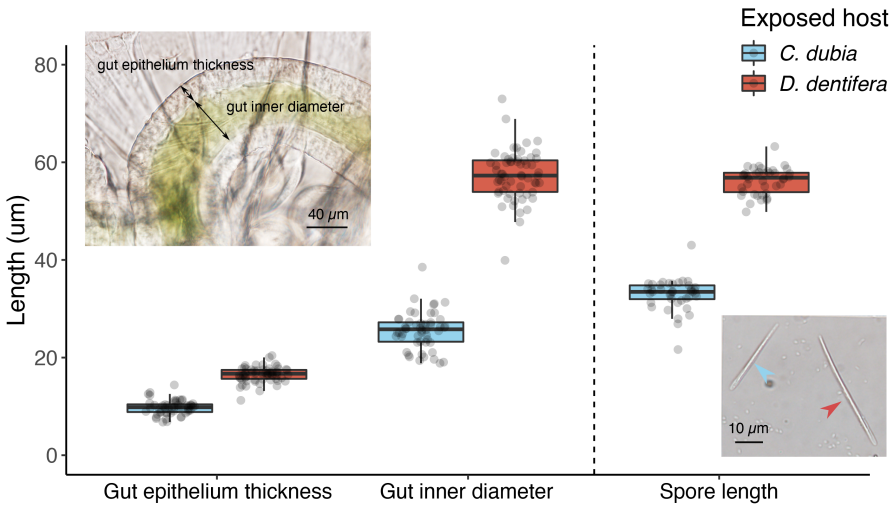

FIGURE 2 *Daphnia dentifera* (red) are substantially larger than *Ceriodaphnia dubia* (blue) in key traits associated with the infection process in the gut (i.e. gut epithelium thickness and inner diameter); spore length of *D. dentifera*- (red) and *C. dubia*-sourced (blue) *Metschnikowia bicuspidata* also differ, paralleling differences in host gut traits. The box plots show median values, the 25th and 75th percentiles, and interquartile ranges, with the raw data overlain (grey points).

explained variation in the probability of infection. Notably, most parasites that successfully entered the host body cavity did not result in terminal infections—only 7 of the 52 hosts that had at least one parasite spore penetrate the body cavity (across all source host × exposed host combinations) ended up with terminal infections.

4 | DISCUSSION

Understanding key functional traits associated with host–parasite interactions is important since it provides insight into parasite specificity and host defence. In this study, we found compelling evidence that the infection process of the parasite *Metschnikowia bicuspidata* in their hosts *Daphnia dentifera* and *Ceriodaphnia dubia* was determined by interspecific differences in morphological traits of both the hosts and the parasites.

Conventionally, studies of host–parasite coevolutionary dynamics have particularly focused on host-genotype-by-parasite-genotype specificity, in which hosts experience selection for alleles that confer resistance and parasites experience selection to overcome host defences (Barribeau et al., 2014; Carius et al., 2001). The focus on genotype-by-genotype specificity has led to other factors, including quantitative traits influencing morphology and physiology, being largely neglected. The system of *M. bicuspidata* and their daphniid hosts provides a valuable opportunity for exploring these traits, since it has long been hypothesized that *M. bicuspidata* infection is a

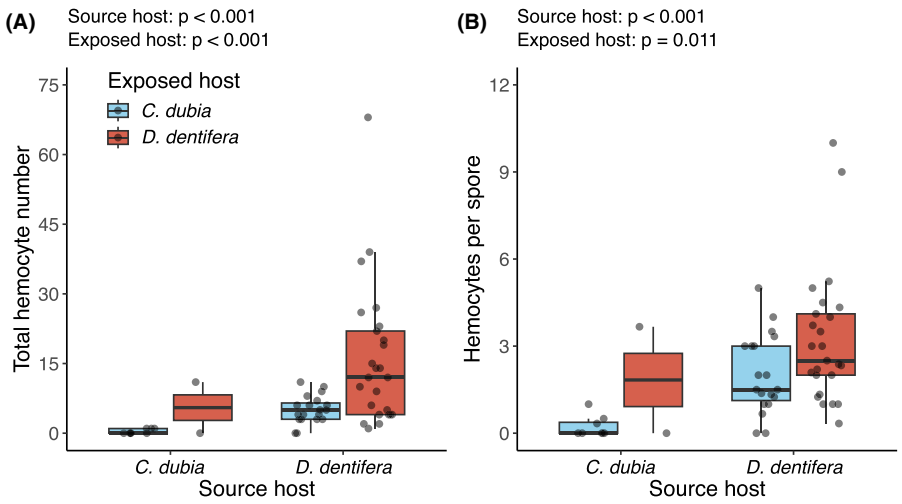

FIGURE 3 Immune responses depended on both the host species and the source of the parasite spores. Immune responses were characterized as (a) total haemocyte number and (b) haemocytes per spore in hosts in which at least one spore penetrated into the body cavity. The box plots show median values, the 25th and 75th percentiles, and interquartile ranges, with the raw data overlain (grey points). For both total haemocyte number and haemocytes per spore, the source host × exposed host interaction was not significant; therefore, results on the figure are for a simplified model that does not include the interaction term.

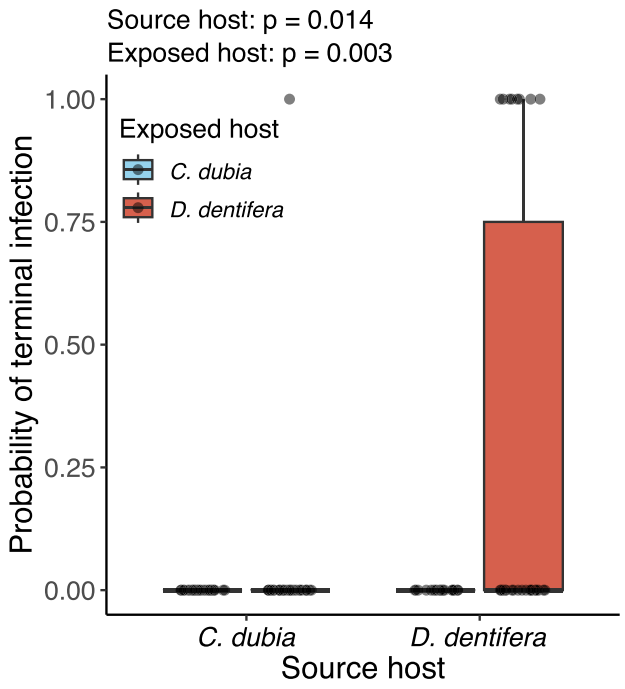

FIGURE 4 Probability of terminal infection of exposed host in response to differently sourced parasite spores. The box plots show median values, the 25th and 75th percentiles, and interquartile ranges, with the raw data overlain (grey points).

mechanical process, requiring successful penetration of the spores into the host's body cavity (Metchnikoff, 1884; Stewart Merrill et al., 2019). Although our previous work showed that two main *M. bicuspidata* clades were found to be associated with *D. dentifera* and *C. dubia*, and that outbreaks tended to happen in one host species or the other, each of these *M. bicuspidata* clades can also be found in both host species (Shaw et al., 2021). Our reciprocal transplant infection experiments explored differences in host species and parasite isolates in two mechanical processes: (1) embedding in the gut epithelium and (2) penetration into the body cavity, both of which are linked to key traits of the hosts and the parasites.

We found that the spore length of both *D. dentifera*- and *C. dubia*-sourced *M. bicuspidata* spores near-perfectly matched the gut inner diameter of *D. dentifera* and *C. dubia* respectively (Figure 2). Consistent with this and with a mechanical process of infection, we found that *D dentifera*-sourced spores were much more likely to pierce the gut wall than *C. dubia*-sourced spores. This suggests specificity in this system at the level of host species × parasite clade: *D. dentifera*-associated *M. bicuspidata* is better able to attack *D. dentifera*, and *C. dubia*-associated *M. bicuspidata* is better able to attack *C. dubia*.

If the outcome of infection is a largely mechanical process, the difference in gut diameter and spore size should mean that more spores would attack *C. dubia* than *D. dentifera*, since its smaller gut diameter should make it easier for spores to attack the gut. However, we found the opposite: *D. dentifera*-sourced spores had more spores attacking the gut in *D. dentifera* than in *C. dubia*. This is likely due to a higher feeding rate in larger sized *D. dentifera*, which likely increased the number of spores ingested. Filtration rates in cladocerans vary as a power function of body length (Porter et al., 1983), so the substantial size difference between *D. dentifera* and *C. dubia* likely translated to very different filtration (and, therefore, spore ingestion) rates. Meanwhile, shorter *C. dubia*-sourced spores may easily follow the curve of the gut lumen without colliding with the epithelium, particularly in larger *D. dentifera* hosts. This is likely why *C. dubia*-sourced spores had more spores attacking the gut in *C. dubia* than in *D. dentifera*.

Upon attacking, a greater proportion of *D. dentifera*-sourced spores successfully penetrated into the body cavity compared to *C. dubia*-sourced spores, with this difference being more pronounced

when infecting *D. dentifera*. This suggests that thicker gut epithelial cells, especially in *D. dentifera*, may prevent spore penetration when spores are not long enough. In studies of *D. dentifera*-associated *M. bicuspidata* infecting *D. dentifera*, gut penetrability initially increases as gut epithelial cells shift from being thin to moderately thick (Stewart Merrill et al., 2019; Sun et al., 2023), but then decreases as cells shift from moderately to very thick (Sun et al., 2023). This combination of results—lower penetrability of the much smaller *C. dubia* guts (this study) and a unimodal relationship for *D. dentifera* guts (Sun et al., 2023)—warrants further exploration to understand the overall relationship between gut epithelium thickness and resistance to penetration by attacking spores. In our study, both host and parasite traits influenced the likelihood of spores attacking and penetrating hosts.

The immune responses of hosts generally reflected the differences in spore penetrability. *D. dentifera*-sourced spores triggered a higher haemocyte response than *C. dubia*-sourced spores did, and, in *C. dubia*, *D. dentifera*-sourced spores induced a higher number of total haemocytes. These trends still hold in terms of haemocyte number per penetrating spore (Figure 3b). Along with previous work (Auld et al., 2012; Stewart Merrill et al., 2019), we also found that the magnitude of haemocyte recruitment did not explain the probability of recovery from parasite infection. Therefore, our data did not support the hypothesis that haemocyte recruitment helps hosts avoid terminal infection. Yet, of the hosts that had at least one spore enter the body cavity, most did not develop a terminal infection, which is also consistent with earlier studies (Stewart Merrill et al., 2021; Sun et al., 2023). Further investigation of the factors that lead to these failed infections—including whether they result from an immune response of the host—is important for understanding the dynamics in this host–parasite system, including reciprocal coevolutionary change. We were unable to precisely evaluate the immune responses of *D. dentifera* when exposed to *C. dubia*-sourced spores because only two of 30 individuals contained successfully penetrated spores. While it might be worth increasing sample size and/or exposing with higher densities of spores to obtain sufficient numbers of *D. dentifera* infected by *C. dubia*-sourced spores, our results present robust evidence that *D. dentifera* are comparatively resistant to infection by smaller *C. dubia*-sourced spores in the first step of the mechanical process of infection. Our findings were also in line with a previous study showing that *D. dentifera*-sourced spores are more capable of infecting both *D. dentifera* and *C. dubia*, whereas smaller *C. dubia*-sourced spores are largely restricted to *C. dubia* (Shaw et al., 2021).

Overall, the results of our study suggest that the outcome of infection in this system depends on both the host species and parasite genotype, providing evidence for host–parasite specificity at the level of host species × parasite clade, which is consistent with the results of an earlier study that considered these higher taxonomic levels (Shaw et al., 2021). In contrast, studies that have sought to find host–parasite genotype specificity in this system within a host species (*D. dentifera*) and parasite clade (*D. dentifera*-associated *M. bicuspidata*) have failed to do so (Duffy & Sivars-Becker, 2007; Searle et al., 2015). This highlights the potential for variation in whether there is host–parasite specificity depending on the taxonomic level that is considered. These results also highlight the value of considering morphological adaptation by hosts and parasites, as well as other key traits associated with infection processes, in order to explain diversification processes and specificity in host–parasite systems. In this case, longer spores were more effective at piercing through the gut epithelium, and thicker gut epithelia were a physical barrier that prevented smaller spores from penetrating. Overall, this study demonstrates that key morphological traits of both hosts and parasites influence the infection process and shape host–parasite specificity.

AUTHOR CONTRIBUTIONS

Syuan-Jyun Sun and Meghan A. Duffy conceived the study. Syuan-Jyun Sun, Siobhan K. Calhoun and Meghan A. Duffy designed the experiments. Siobhan K. Calhoun and Syuan-Jyun Sun isolated and maintained cultures used in this study. Syuan-Jyun Sun collected the data. Syuan-Jyun Sun performed the data analysis. Syuan-Jyun Sun wrote the initial draft of the manuscript, and all authors contributed critically to the draft and gave final approval for publication.

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CONFLICT OF INTEREST STATEMENT

The authors declare there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data used for this study are available on Zenodo https://zenodo.org/record/7754587#.ZBnM_3bP02w.

ORCID

Syuan-Jyun Sun https://orcid.org/0000-0002-7859-9346
Meghan A. Duffy https://orcid.org/0000-0002-8142-0802

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1: Statistical significance of the final models compared to null models.

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