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ARTICLE

Disease Ecology

Influence of melatonin on the successful infection of *Daphnia dentifera* by *Metschnikowia bicuspidata*

Ashley G. Hughes[1] | **Jeannette E. Cullum**[2,3] | **Molly J. Fredericks**[4] | **Patrick J. Wilson**[3,4] | **Anke Schwarzenberger**[5] | **Carla E. Cáceres**[1,2,3,4]

[1]School of Integrative Biology, University of Illinois Urbana Champaign, Urbana, Illinois, USA

[2]Program in Ecology, Evolution, and Conservation Biology, University of Illinois Urbana Champaign, Urbana, Illinois, USA

[3]Carl R. Woese Institute for Genomic Biology, University of Illinois Urbana Champaign, Urbana, Illinois, USA

[4]Department of Evolution, Ecology, and Behavior, University of Illinois Urbana Champaign, Urbana, Illinois, USA

[5]Universität Konstanz, Limnological Institute, Constance, Germany

Correspondence
Carla E. Cáceres
Email: cecacere@illinois.edu

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Abstract

The levels of the hormone melatonin fluctuate daily, with higher concentrations often found at night. These fluctuations likely influence multiple aspects of physiology, including the immune response. We demonstrated that the addition of exogenous melatonin increased the proportion of the freshwater zooplankton *Daphnia dentifera* that became infected by the fungal pathogen *Metschnikowia bicuspidata*, during the day but not at night. To determine the stage of this host–pathogen interaction at which melatonin may increase susceptibility, we conducted a series of laboratory experiments in which we raised *Daphnia* in the presence and absence of exogenous melatonin. To complete its life cycle, *Metschnikowia* must encounter a foraging host, overcome the host's barrier resistance (gut wall), and evade the host's immune response (internal clearance). We quantified encounter rate by measuring the gut passage time and the number of spores that entered the gut. We also measured the number of spores that successfully entered the body cavity (barrier resistance) and the hemocyte response to spores entering the body cavity (one metric of internal clearance). Finally, we quantified the effect of exogenous melatonin on triggering molting. The addition of exogenous melatonin lengthened gut passage time and decreased the number of spores present in the gut. We found no effect of melatonin on the percentage of gut spores successfully entering the host's body cavity, nor on the hemocyte response. Melatonin is known to influence the timing of molting and hosts that molted during exposure were more likely to become infected, likely due to a decrease in barrier resistance. In a fully factorial experiment, there was a high death rate, low infection rate, and therefore no discernible effect of melatonin on molting, nor molting or melatonin on infection. Our results provide insight into the stages of infection where melatonin does and does not have significant effects.

KEYWORDS
disease, immunity, lake, molecular, parasite, plankton

INTRODUCTION

Daily synchronized dynamics occur from the level of gene expression to ecosystem processes in aquatic and terrestrial systems. In many cases, these diel dynamics result from an underlying circadian clock that through differential gene expression influences an individual's physiology and behavior (Panda, 2016; Rund et al., 2016; Stanton et al., 2022). These cycles in turn influence how individuals interact with conspecifics, competitors, resources, predators, and pathogens (Pfenning-Butterworth et al., 2022; Resco de Dios & Gessler, 2018; Schwarzenberger et al., 2021). Despite these coupled cycles from cells to ecosystems, it is often difficult to make meaningful connections due to limited studies incorporating both molecular and ecological approaches. This lack of integration limits our ability to make predictions about the outcome of community dynamics.

At the molecular level, the common neurotransmitter melatonin plays a key role in mediating several physiological processes and is a promising candidate to begin linking changes at the cellular level to community dynamics such as host–pathogen interactions (Carrillo-Vico et al., 2005; Poeggeler, 1993; Schwarzenberger et al., 2021). Melatonin is an ancient indole amine that is present in species throughout the tree of life with an original function believed to be cellular protection via free radical scavenging (Hardeland & Poeggeler, 2003; Schippers & Nichols, 2014; Tan et al., 2006; Tosches et al., 2014). Since its initial discovery in 1958, the various roles of melatonin in plants, animals, and microbes continue to be explored (Biggio et al., 2021; Farooqi et al., 2022; Markowska et al., 2009; Poeggeler, 1993). In crustaceans, melatonin has been shown to influence a number of traits, including the timing of molting (Sainath & Reddy, 2010), response to predators (Bentkowski et al., 2010; Schwarzenberger et al., 2014), and the expression of genes related to immune function (She et al., 2019). These melatonin-induced trait changes could affect population and community dynamics by altering the outcome of predator–prey interactions as well as host–pathogen interactions.

In particular, melatonin may influence environmentally transmitted diseases through a variety of mechanisms, including altering the encounter rate of host and pathogen by changing host behavior, altering barrier resistance by changing molting or other forms of cuticle penetration, or influencing the rate of infection clearance by changing immune response (Decaestecker et al., 2002; Fels et al., 2004; Johnson et al., 2017). Many pathogens of aquatic crustaceans rely on the host's swimming and feeding behavior for encounter (Ebert, 2005). Therefore, any changes in these behaviors may change host–pathogen encounter rates. The period directly following molting is thought to be a time of increased susceptibility to pathogens in arthropods due to the ease with which the pathogens can penetrate the softened cuticle (Corteel et al., 2009; Moret & Moreau, 2012). Molting also alters the immune response, which in crustaceans includes cellular processes and humoral processes (e.g., hemocytes, antimicrobial peptides, melanization, Toll and Toll-like pathways, redox signaling).

In *Daphnia pulex*, Schwarzenberger and Wacker (2015) identified diel gene expression rhythms of the core clock genes *tim*, *per*, *clk*, *cyc*, and *cry2* and of arylalkylamine N-transferase (AANAT)—the rate-limiting enzyme in melatonin synthesis. They also found that the peak of AANAT gene expression was followed by a peak of melatonin production. Although the authors did not prove that the increase in gene expression of AANATs actually caused the observed increase in AANAT enzyme synthesis and thus melatonin synthesis, the pattern of increased gene expression followed by a peak in melatonin production was conspicuous. Rund et al. (2016) identified both clock genes and immune genes that were rhythmically expressed in *D. pulex*, and Cai et al. (2020) found a circadian rhythm in two putative immune responses, the accumulation of reactive oxygen species (ROS) (more in darkness), and the antioxidant enzyme gene (more activation in the light) in the same species. Although there is undoubtedly a link between melatonin, the expression of core clock genes, and immunity, additional research is needed to understand the extent of the connections.

We used the crustacean host *Daphnia dentifera* and the fungal pathogen *Metschnikowia bicuspidata* to address questions regarding the role of melatonin in disease dynamics. *D. dentifera* is a freshwater zooplankton located in many lakes in North America, and *M. bicuspidata* is a fungal pathogen that commonly causes epidemics in many species of *Daphnia* (Cáceres et al., 2014). *Daphnia* ingest needle-shaped *Metschnikowia* spores while filter-feeding in the water column (encounter). Only some of those ingested spores successfully penetrate the gut epithelium (barrier resistance) and begin growing inside the host (Stewart Merrill & Cáceres, 2018). Penetrating spores are attacked by hemocytes, a process that may or may not clear the infection. If the host immune response fails to clear the infection, *Metschnikowia* will develop in the host's body cavity and eventually kill the host (Rogalski et al., 2021; Stewart Merrill et al., 2019, 2021).

We conducted a series of laboratory assays to explore the role of melatonin in the process of *M. bicuspidata* infecting *D. dentifera*. Some studies (e.g., Maestroni, 2024; Vielma et al., 2014) have found beneficial effects of melatonin for resistance to infection, whereas others (Crespo et al., 2020) have determined that innate immune activity

was hindered by exogenous melatonin addition. In short, melatonin's effects on innate immunity might be more complex than previously thought. We started by asking whether melatonin would increase susceptibility to infection. If so, infection rates should be higher at night (when melatonin is known to be highest, Schwarzenberger & Wacker, 2015) and in the presence of exogenous melatonin.

We tested three specific hypotheses regarding the effect of melatonin on particular stages of susceptibility (encounter, barrier resistance, internal clearance): (1) melatonin alters feeding behavior to increase the encounter rate between host and pathogen, (2) melatonin alters the ability of the pathogen to gain entrance into the host (barrier resistance), and (3) melatonin alters the ability of the immune system to clear early-stage infections. We predicted that in the presence of exogenous melatonin, gut passage times would be longer, giving spores more time to puncture the gut wall. Hence, we expected more spores would successfully penetrate the gut wall with melatonin (lower barrier resistance, high gut penetrability). Finally, we predicted that there would be fewer hemocytes per spore (cellular immune response) in the presence of melatonin. Although it is known that putative immune genes follow a circadian rhythm (Rund et al., 2016), recent research suggests that likely candidates, such as the phenoloxidase cascade, do not seem to influence disease in some freshwater zooplankton, indicating the need for additional work to understand the *Daphnia* immunity and the phenomenon of infection rates often being higher at night (Pfenning-Butterworth et al., 2022; Terrill Sondag et al., 2023). Our work seeks to address the role of melatonin on immune response in *D. dentifera*.

MATERIALS AND METHODS

To quantify how melatonin may influence encounter rate, barrier resistance, and clearance rate, and thus overall susceptibility, we conducted a series of laboratory experiments with nine unique genotypes of *D. dentifera* collected from seven lakes in Indiana, Illinois, and Michigan, USA (Table 1). Clonal lines were raised under low-density conditions for more than three generations to standardize maternal effects (Lynch, 1984). Experimental individuals were obtained from mothers at fourth clutch or later and were less than 24 h old at the time of collection. In all experiments, *Daphnia* were fed on Monday, Wednesday, and Friday with 2 mg C/L of *Ankistrodesmus falcatus* and were incubated at 20°C in an environmental chamber with a 12:12 photoperiod (on:off). The specific genotypes used in each experiment depended on their availability at the time of the experiment. We included multiple genotypes in each experiment given the known genetic variation in traits (Rogalski et al., 2021; Stewart Merrill et al., 2019, 2021). However, we were not interested in the responses of any particular genotype, and therefore do not include "genotype" as a factor in any of the statistical models. At times, this leads to considerable variance, which is illustrated in the figures. Including "genotype" in the models does not alter the results for melatonin, and was only significant in the case of gut penetrability.

Experiment 1: Exogenous melatonin and the day/night cycle

In the first experiment, we examined the role of exogenous melatonin on susceptibility by exposing a total of 297 *D. dentifera* to *M. bicuspidata* in the presence or absence of a 2×10^{-6} M melatonin solution. The dosage was selected based on the work conducted by Schwarzenberger et al. (2014). Neonates (<24 h old) were raised individually in 50-mL centrifuge tubes containing 45 mL of filtered lake water. On the evening of Day 6, half the individuals were exposed to *Metschnikowia* for the 12 nighttime hours in the incubator by transferring them to individual 15-mL centrifuge tubes with 200 spores/mL of *Metschnikowia* added to either 10 mL of filtered lake

TABLE 1 List of genotypes used in each experiment.

Experiment	W2	A45	ST	Clear 1	IL 14	IL 16	Mid 37	BD	DW 22
1. Melatonin day/night	X	X							
2. Gut passage time			X	X		X	X		
3. Spore penetration and hemocyte response			X	X		X	X		
4. Melatonin and molting	X		X	X	X	X	X	X	X

Note: Genotypes W2, A45, and ST were isolated from two lakes in southwest Michigan, USA. Clear 1 was isolated from Clear Lake in Kickapoo State Recreation Area, Vermilion County, IL, USA. The remaining clones were isolated from Midland Lake (Mid 37), Inland Lake (IL 14, IL 16), Beaver Dam Lake (BD), and Downing Lake (DW) in Central Indiana, USA. Multiple genotypes were used in each experiment because *Daphnia* are known to maintain genetic variation in most traits. The particular genotypes used in each experiment were determined by their availability at the time of each experiment.

water or 10 mL of the melatonin solution. The following morning, the individuals that had been exposed during the night were transferred back to their 50-mL tubes, and the remaining individuals were exposed to *Metschnikowia* for 12 daytime hours, using the same protocol. Following the 12-h daytime exposure, those individuals were moved back to their 50-mL tubes. All hosts continued to be fed daily with water changes occurring every Monday, Wednesday, and Friday. On Days 10, 12, and 14 post exposure, all individuals were examined for late-stage infection.

We performed a binomial (individuals are either infected or not) ANOVA with a logit link in R 4.3.3 (R Core Team, 2024) to assess the effects of time of infection (day vs. night), presence or absence of exogenous melatonin, and their interaction on infection.

The experiments conducted by Schwarzenberger et al. (2014) used different host species (*Daphnia magna* and *D. pulex*). Hence, to determine the melatonin dose that produced the highest infection rate in *D. dentifera*, we then conducted an experiment in which we compared five doses of melatonin and measured infection rates. In this experiment, we compared the melatonin dose used in Experiment 1 (2×10^{-6} M), to three additional doses (1×10^{-6} M, 4×10^{-6} M, 2×10^{-5} M), and a control (no melatonin). The infection rates of the five treatments were significantly different from each other (p 0.0066), and the 4×10^{-6} M dose had the highest rates of infection, so 4×10^{-6} M was the dose used in all remaining experiments.

Experiment 2: Gut passage time

We next investigated gut passage time as a possible explanation for increased susceptibility in the presence of melatonin. If the presence of exogenous melatonin slows gut passage time, ingested spores would have longer time to penetrate the gut wall, thereby overcoming barrier resistance. Sixty *Daphnia* were placed individually in 50-mL centrifuge tubes filled with 45 mL of filtered lake water. When they were one week old, during the day, half were exposed to exogenous melatonin (4×10^{-6} M) and half remained in filtered lake water. This treatment was crossed with a *Metschnikowia* treatment where half were inoculated with 100 spores/mL, resulting in four treatments: with exogenous melatonin and spore exposure, with exogenous melatonin without spore exposure, no melatonin with spore exposure, and no melatonin without spore exposure (control). The gut passage time of 48 *Daphnia* was examined 12–24 h after exposure. Each individual sat for 30 s in a Carmine red dye solution. The *Daphnia* ingest the dye, which can then be tracked through the gut. Followed by two brief (15 s) rinses in filtered lake water, the *Daphnia* were moved to a small Petri dish with *A. falcatus* for 2 min. The animals were then examined under a microscope, and gut passage time was determined as the amount of time between introduction to the test food and when the first red particle emerged from the anus. We used the aov function in R 4.3.3 (R Core Team, 2024) with melatonin, *Metschnikowia*, and their interaction as factors for analysis.

Experiment 3: Gut penetrability

We then asked whether melatonin could increase the penetrability of the single-cell-thick *Daphnia* gut. If longer gut passage time increases the time available to penetrate, it also decreases the number of spores that pass through the gut. During the day, 80 *Daphnia* (20 per genotype) were evenly separated into two treatment groups: with exogenous melatonin and control (filtered lake water). All *Daphnia* were exposed to 100 spores/mL during the day when they were 7 days old. The next day, each individual was examined under a Leica DMLB compound microscope with 400× magnification. We recorded (1) the number of spores in the gut, (2) those that had entered the epithelium but had not penetrated the basal cell layer, and (3) those that had penetrated the basal cell layer and entered the body cavity (Stewart Merrill et al., 2019). We used these data to establish a metric of barrier resistance, "gut penetrability," which is defined as the number of spores that successfully entered the body cavity (3 above) divided by the number of spores that fired into the gut (the sum of 2 and 3 above). For those spores that had entered the body cavity, we also recorded the number of hemocytes attached to each spore. Each metric was analyzed with a t test in R 4.3.3 (R Core Team, 2024) with the presence or absence of melatonin as the factor.

Experiment 4: Molting, melatonin, and infection

The fourth experiment simultaneously examined the effect of melatonin on molting (which can reduce barrier resistance), melatonin on infection, and molting on infection by exposing 416 *D. dentifera* to the presence or absence of melatonin crossed with the presence and absence of *Metschnikowia*. To begin, neonates (<24 h old) were collected over a 2-day period. Genotypes were grown separately by rearing 10 neonates in 200 mL of filtered lake water for the first 3 days. On Day 5, the density

was reduced to 5 *Daphnia*/200 mL. Water changes were made every other day, and all beakers were fed every other day with *A. falcatus* at 2 mg C/L. On Day 10, *Daphnia* were transferred individually to 10 mL of water in six-well tissue culture plates. Those in the infection treatment received 200 spores/mL of *M. bicuspidata* during the day. *Daphnia* in the melatonin treatments received a 4×10^{-6} M melatonin solution during the day. After 24 h, the water was changed, and molts were recorded by identifying those that had molted and given birth. Each individual was then placed in a 50-mL tube filled with 45 mL of water and labeled as "molted" or "unmolted." Water changes were made every other day until the 10th day after exposure, at which point infection status was determined for both the molted and unmolted categories. We performed two binomial ANOVAs (individuals either molted or did not and were either infected or not) with a logit link in R 4.3.3 (R Core Team, 2024). The first model asked whether the presence of melatonin or *Metschnikowia* influenced molting. The second model asked whether melatonin, molting, or their interaction influenced infection. Because this experiment resulted in only 19 infected individuals, we also explored the effect of molting alone by exposing 245 *D. dentifera* from three additional clones to *Metschnikowia* and recorded which individuals molted during exposure. The effect of molting on infection was determined with a binomial (animals were infected or not) t test with a logit link, with molting as the factor in R 4.3.3 (R Core Team, 2024).

RESULTS

Experiment 1: Exogenous melatonin and the day/night cycle

In the first experiment that investigated the role of exogenous melatonin under day versus night conditions, the percentage of hosts infected increased from 34% infection during the day to over 57% at night (χ^2_1 26.2, $p < 0.0001$; Figure 1). The addition of exogenous melatonin increased overall infection from 41% to 50% (χ^2_1 13, p 0.0003) with a highly significant time × melatonin interaction (χ^2_1 11.3, p 0.0008). The addition of melatonin nearly doubled susceptibility during the day but had no effect at night.

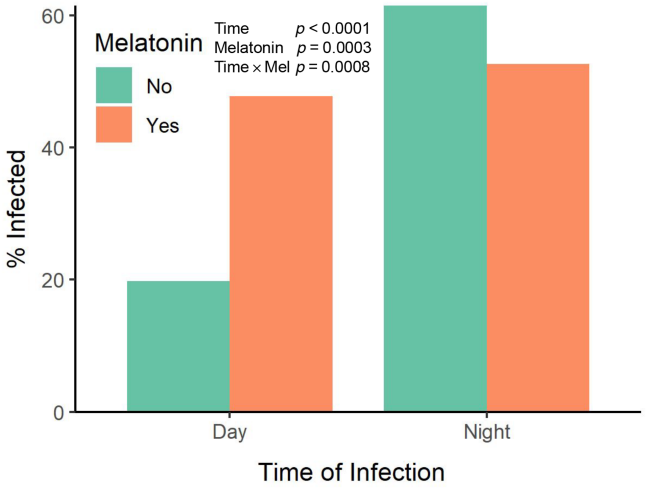

FIGURE 1 The addition of exogenous melatonin increased overall infection with a highly significant time–melatonin interaction. The addition of melatonin nearly doubled susceptibility during the day but had no effect at night. Because every individual is either infected or not, there are no error bars to display.

Experiments 2 and 3: Gut passage time and gut penetrability

We assessed potential changes in exposure rate by measuring how long spores remain in the gut, how that gut passage time influenced the number of spores ingested, and the success of those ingested spores at penetrating the gut wall. Gut passage time varied from 3 to 44.5 min, with most values falling between 8 and 20 min (Figure 2). Melatonin increased gut passage time ($F_{1,46}$ 20.57, p 0.00004) from 8.8 ± 0.9 (mean ± SE) min to 20.2 ± 2.59 min. Adding *Metschnikowia* resulted in gut passage times trending longer than in the absence of spores, but there was no significant effect ($F_{1,46}$ 2.54, p 0.117). There was also no significant interaction ($F_{1,46}$ 0.08, p 0.775).

As predicted, the longer gut passage times in the presence of melatonin reduced the number of spores found in the gut from 11 ± 1 to 7.35 ± 0.9 (t_{50} 2.1, p 0.039). However, despite there being fewer spores in the gut, the addition of melatonin did not have a significant effect on the number of spores that successfully entered the host's body (t_{50} 0.166, p 0.869; Figure 3A,B). The average gut penetrability for individuals treated with melatonin was 0.36 ± 0.06 while that without melatonin was 0.38 ± 0.05. Average hemocytes per spore (Figure 3C), our metric of the internal clearance due to an immune response, did not differ between treatments (t_{32} 0.689, p 0.496). The average number of hemocytes per spore was 4.32 ± 1.21 with melatonin and 5.29 ± 0.72 without melatonin.

Experiment 4: Molting, melatonin, and infection

We found no effect of either the addition of spores (χ^2_1 1.31, p 0.252) or melatonin (χ^2_1 1.20,

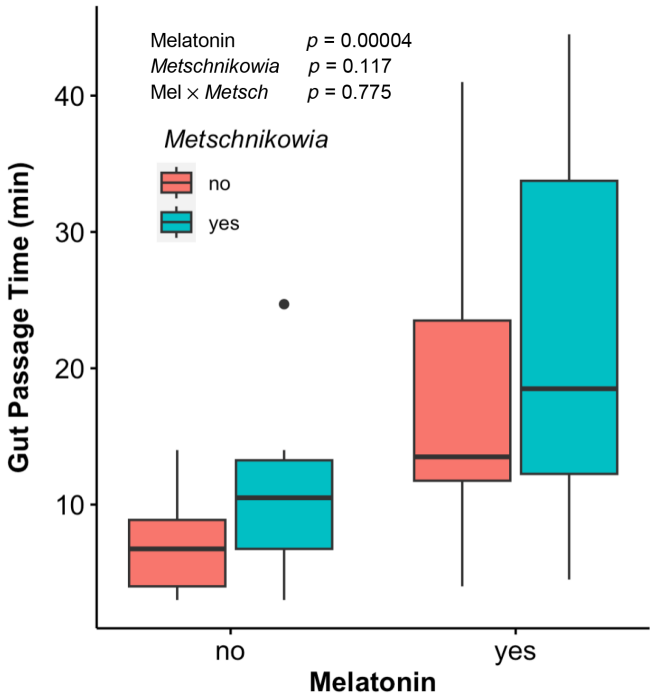

FIGURE 2 The addition of exogenous melatonin increased the gut passage time both in the presence and absence of *Metschnikowia*, as shown in a box-and-whiskers plot. The midline in each box is the 50th percentile, and the top and bottom of each box are the 75th and 25th percentiles. Whiskers depict the smallest and largest values within 1.5 times the interquartile range. The individual outlier is represented by a datapoint. The considerable among-host variation in the *Daphnia* exposed to melatonin resulted in no significant effect of the addition of *Metschnikowia* on gut passage time.

p 0.273) on *Daphnia* that molted during the 24-hour exposure (Figure 4A). There was also no significant interaction (χ^2_1 0.278, p 0.599). Infection rate was also not influenced by molting (χ^2_1 0.404, p 0.517), melatonin (χ^2_1 0.718, p 0.397), or their interaction (χ^2_1 0.713, p 0.399; Figure 4B). For unknown reasons, mortality was high in this experiment, with 56% of the exposed individuals in the treatment with exogenous melatonin and 51% of individuals in the treatment without exogenous melatonin dying before a terminal infection could be determined (as compared with the treatments without spores added, where 46% of the *Daphnia* died in the double control and 44% died in the treatment without spores but with melatonin). Of the 94 exposed *Daphnia* that survived, only 19 of 94 *Daphnia* developed terminal infections. A final experiment with a larger sample size of infected individuals (150 of 245) indicated that molting increased susceptibility significantly (χ^2_1 16.6, $p < 0.0001$; Figure 4C).

DISCUSSION

Our results from the first experiment show that the addition of exogenous melatonin during the day increases overall infection, but there was no significant effect when adding exogenous melatonin at night (when endogenous melatonin is already high). In a series of experiments designed to uncover the mechanisms driving this pattern, we found that the addition of melatonin resulted in a reduced encounter rate between host and pathogen (fewer spores ingested, potentially benefiting the host), but a longer gut passage time, which we predicted could provide the spores with more time to penetrate the gut wall, which would benefit the pathogen. Contrary to our prediction, the addition of melatonin did not influence the number of spores successfully penetrating the gut wall and entering the host's body. Our measure of immune response, the number of hemocytes per spore, also did not differ in the presence or absence of melatonin. In the experiment that simultaneously exposed hosts to melatonin and *Metschnikowia*, neither the addition of spores nor the addition of exogenous melatonin significantly influenced the molting rate of hosts, which could influence barrier resistance. The addition of exogenous melatonin and *Metschnikowia* resulted in a mortality rate that precluded interpretation of the infection rate in the presence of both factors. However, in an experiment that excluded melatonin, hosts that molted during exposure were more likely to become infected than those that did not.

Although our study reveals some clear patterns between melatonin and disease, the full mechanistic explanation remains unresolved. In particular, as we are currently only able to successfully culture *Metschnikowia* in live hosts, we had no way to disentangle the effects of the exogenous melatonin on the *Daphnia* versus the *Metschnikowia*. Two recent studies suggest that melatonin may act directly on fungal pathogens to influence disease. Li et al. (2022) found that melatonin inhibited fungal growth and infection of 13 fungal pathogens of plants. Similarly, Kong et al. (2021) found that 5-methoxyindole, a homolog of melatonin, inhibited formation, growth, and conidia germination of the fungal pathogen *Fusarium graminearum*. They demonstrated a significant downregulation in *F. graminearum* genes involved in scavenging ROS. We know little about the physiology of *M. bicuspidata* inside the *Daphnia* host, in particular, what triggers the osmotic cannon required to launch the spore through the gut wall. It is possible that melatonin may simultaneously influence both host and parasite physiology in our experiments. Our attempts to culture the parasite outside of the host have failed, limiting our ability to assess the direct effects of melatonin on the parasite.

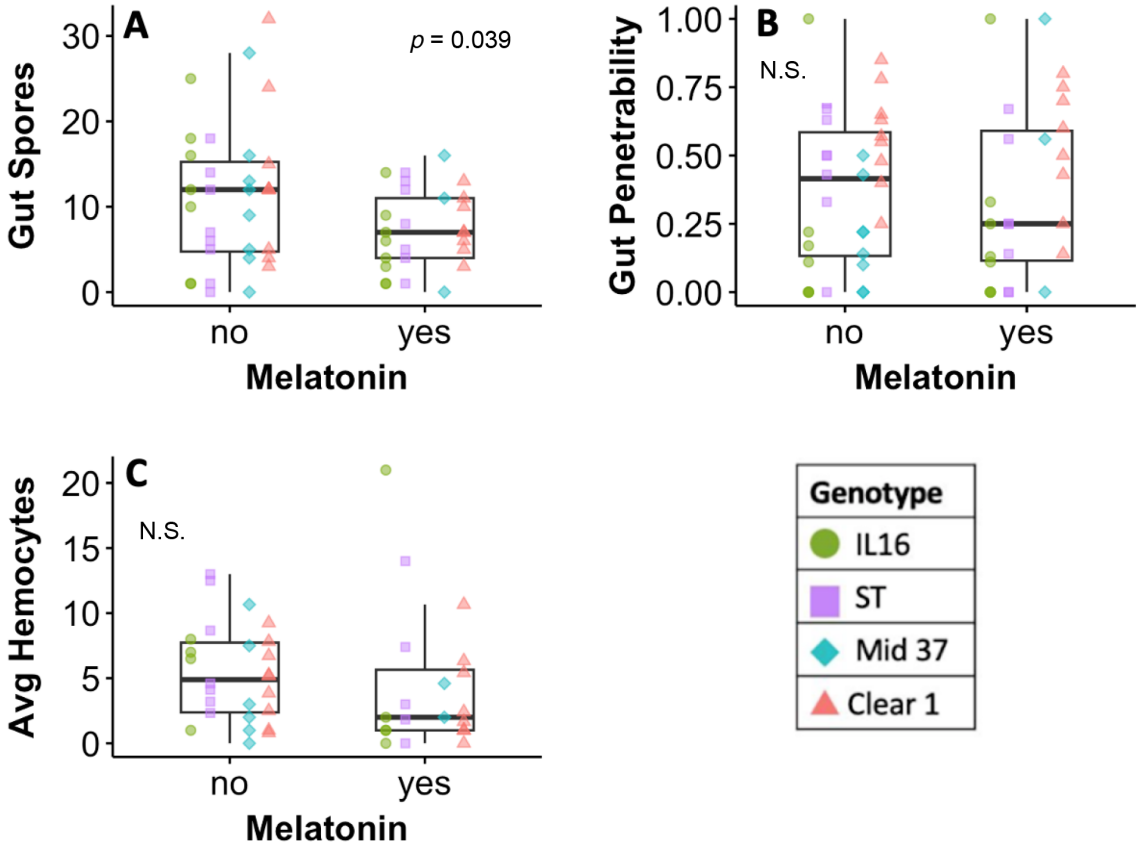

FIGURE 3 (A) Longer gut passage times resulted in significantly fewer spores being found in the gut in the presence of exogenous melatonin. (B) Being exposed to exogenous melatonin did not influence the proportion of spores that successfully entered the host body cavity nor (C) the average number of hemocytes attached to each of those penetrating spores. Genotype, which was not included in the final model, is shown in the figure by the various colored symbols to illustrate the degree of variation. The midline in each box is the 50th percentile, and the top and bottom of each box are the 75th and 25th percentiles. Whiskers depict the smallest and largest values within 1.5 times the interquartile range.

We predicted that the addition of melatonin could influence disease dynamics via behavioral or physiological responses given several prior studies addressing the role of melatonin on the physiology and behavior of *Daphnia*. Bentkowski et al. (2010) showed that in the presence of fish kairomones, exogenous melatonin changed the depth distribution of *D. magna*. Swimming and feeding behavior, which can be linked to depth distribution, have been hypothesized to influence the contact rate between host and pathogen. For example, Decaestecker et al. (2002) demonstrated that infection risk in *Daphnia* increases when they change their swimming behavior to avoid predation by fish. Moreover, Pfenning-Butterworth et al. (2021, 2022) demonstrated that there is a circadian rhythm to feeding behavior, with feeding rates being higher at night. They link this pattern of increased feeding to increased encounter between host and pathogen at night. Other physiological effects were found by Kaas et al. (2009), who demonstrated that the addition of exogenous melatonin significantly decreased heart rate in *D. magna*. Schwarzenberger and Wacker (2015) determined that *Daphnia* synthesizes melatonin at night with circadian rhythms and that the addition of melatonin decreases *Daphnia's* response to stress (Schwarzenberger et al., 2014). We add to the understanding of these physiological changes by demonstrating that melatonin slows gut passage time. Lengthened gut passage time suggests that in the presence of melatonin, *Daphnia* exhibit a slower consumption rate for both food and spores. Although we do not know whether melatonin influences swimming speeds, that is also possible given Bentkowski et al.'s (2010) reported change in depth distribution.

Although encounter rate was influenced by melatonin, the addition of exogenous melatonin had no effect on barrier resistance. This result differed from our hypothesis that there would be more penetrating spores because of increased infection rates found in preliminary experiments. From there, we predicted that more penetrating spores would result in an increase in infection unless there was an immune response to the spores via

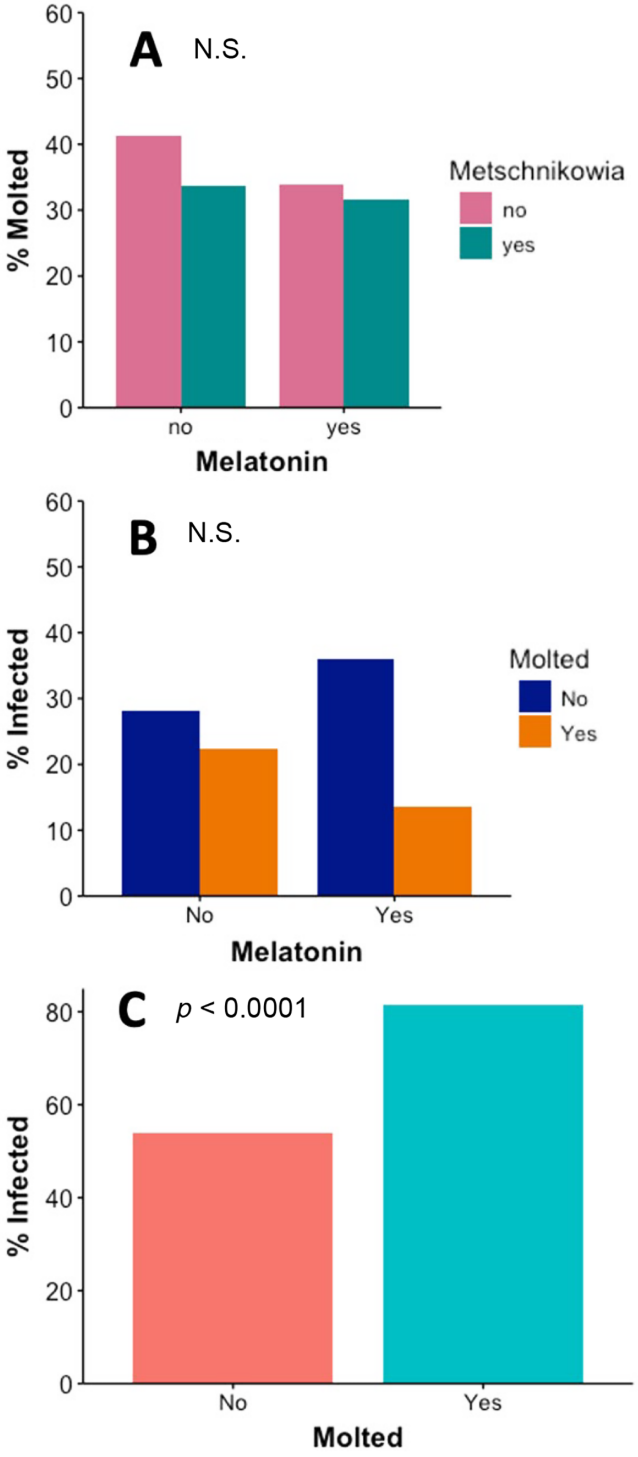

FIGURE 4 (A) Neither the addition of exogenous melatonin nor the addition of *Metschnikowia* significantly changed the percentage of individuals that molted during a 24-h exposure. (B) In the fully factorial experiment, there was no effect of molting, melatonin, or their interaction on infection. (C) In a larger study, individuals that molted during exposure were more susceptible to infection.

hemocytes. This is not the observed outcome, which highlights that there are more factors that influence susceptibility to infection. We found evidence in one experiment that infection was increased by molting, which likely influences barrier resistance. Although it may not seem surprising that molting increases susceptibility, to our knowledge, this is the first time data have been reported for this system. Although we found no effect of melatonin on molting, that could have been because the exposure duration was too short. Sainath and Reddy (2010) exposed crabs (*Oziotelphusa senex senex*) to exogenous melatonin over a month-long experiment and found accelerated molting.

This study sheds light on the relationship between melatonin and infection dynamics, as regulated through encounter, barrier resistance, and the immune response in *D. dentifera*. Although the addition of exogenous melatonin during the day increased overall infection and lengthened gut passage time, it also resulted in decreased spores in the gut and had no effect on gut penetrability. *Daphnia*'s immune response, characterized by the number of hemocytes responding to penetrating spores, was also seemingly unaffected by the addition of exogenous melatonin. Additionally, the presence of melatonin had no effect on molting or infection rates in this experiment. What then, could be responsible for the results seen in the first experiment? Crustaceans lack an adaptive immune system and consequently rely on their innate immune system for protection against pathogens (Huang et al., 2020). Part of this innate immune system consists of cellular responses in which pattern-recognition receptors within hemocytes detect and rid the body of pathogens (Huang et al., 2020). An additional defense in many arthropods is the release of ROS to kill the pathogen (Paiva & Bozza, 2014). Could melatonin's free-radical scavenging benefit pathogens? These ROSs, some of which are also free radicals, damage lipids, proteins, and DNA.

Additional work to determine the mechanisms underlying our observed patterns is clearly needed. Ongoing work in metabolomics on both infected and healthy individuals is addressing these additional potential immune system mechanisms. Moreover, we suspect melatonin produced by the host might also have an effect on *Metschnikowia*, such as altering its ability to complete its lifecycle within the host. We are also investigating the role of artificial light at night in disease spread, given the potential direct connection with the circadian clock and melatonin production. Finally, it has not been investigated whether melatonin also influences immune gene expression in *Daphnia*, and whether this would influence the interaction between *Daphnia* and a fungal parasite such as *Metschnikowia*.

AUTHOR CONTRIBUTIONS

All authors designed the study. Ashley G. Hughes, Molly J. Fredericks, and Carla E. Cáceres collected the data. Ashley G. Hughes, Jeannette E. Cullum, Patrick

J. Wilson, and Carla E. Cáceres conducted the analyses. All authors contributed to the writing of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data (Hughes et al., 2024) are available from Dryad: https://doi.org/10.5061/dryad.1zcrjdg1b.

ORCID

Jeannette E. Cullum https://orcid.org/0009-0009-4752-1843

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