

Research Article**Trematode parasites have minimal effect on the behavior of invasive green crabs**Hyejoo Ro¹, Amy E. Fowler², Chelsea L. Wood¹ and April M.H. Blakeslee^{3,*}¹University of Washington, School of Aquatic and Fishery Sciences, 1122 NE Boat St, Seattle, WA 98195-5020, USA²George Mason University, Department of Environmental Science and Policy, Potomac Science Center, 650 Mason Ferry Ave, Woodbridge, VA 22191, USA³East Carolina University, Department of Biology, 101 E 10th St, Life Sci Bldg, Greenville, NC 27858, USA

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OPEN ACCESS**Abstract**

Trophically-transmitted endoparasites can manipulate the behavior of intermediate hosts to increase transmission to definitive hosts. Less clear, however, is whether these relationships exist when parasites and hosts have limited coevolutionary history, e.g., a native parasite infecting an invasive host. We investigated infection by the northeastern North American trematode *Microphallus similis* in non-native green crabs (*Carcinus maenas*) to assess whether infection by *M. similis* influenced feeding behaviors in *C. maenas* and if this changed with time post-infection. We manipulated infection by randomly assigning crabs to parasite exposure and control groups. We then measured individual crab behavior at five time points (pre-infection, 72 h, 1 week, 2 week, and 3 week post-infection) with an established ethogram that recorded multiple behavioral types. We also conducted righting response trials at each time point and additionally at 4 and 5 weeks post-exposure. Compared to controls, infected crabs showed little difference in recorded behaviors, and burden of infection (i.e., number of trematode cysts) was not correlated to any behavioral metric. This lack of behavioral impact occurred at all stages of infection. Active parasite penetration/establishment early in the infection process did not provoke greater behavioral response than later stages, when the encysted parasite becomes relatively dormant. Although *M. similis* is capable of infecting non-native *C. maenas*, our results suggest that it does not manipulate host behavior, possibly because of limited coevolutionary history with *C. maenas* in the region, or because host manipulation is unnecessary for successful completion of its life cycle. In nature, this may have implications for the crab's invasion success in the western Atlantic, where it has escaped much of its native parasite diversity.

Key words: *Carcinus maenas*, coevolutionary history, enemy release, manipulation, microphallid, *Microphallus similis*, non-native

Introduction

Parasitic species exhibit diverse adaptations for manipulating the behavior of their hosts (Lafferty and Shaw 2013; Poulin 2013; Weinersmith and Earley 2016; Hughes and Libersat 2019). Host manipulation tends to evolve in host–parasite systems where modifications of host appearance or behavior can enhance the parasite's fitness, particularly when such manipulations increase the likelihood of trophic transmission from a prey

host to a predator host (Poulin and Maure 2015), also referred to as parasite-increased trophic transmission (PITT) (Lafferty 1999). A well-known example of PITT occurs in the California killifish (*Fundulus parvipinnis*), which is infected by a trematode parasite (*Euhaplorchis californiensis*) that encysts in its brain. Infected killifish hosts display conspicuous behaviors that make them more vulnerable to predation by birds, which are the definitive hosts of the parasite (Lafferty and Morris 1996). Another example involves nematode parasites (*Myrmeconema neotropicum*), which induce changes in both appearance and behavior in the tropical canopy ant (*Cephalotes atratus*); in this case, uninfected *C. atratus* are black, while infected *C. atratus* turn bright red and walk in an elevated position, which increases their conspicuousness to predatory birds (Yanoviak et al. 2008). Further, by changing host geotactic responses, clinging behaviors, and attraction to predator cues, acanthocephalans can manipulate the behavior of amphipod intermediate hosts, such that hosts are more likely to be eaten by aquatic fish and birds; in addition, the acanthocephalan is also morphologically conspicuous in infected individuals (Bethel and Holmes 1973; Bauer et al. 2005; Perrot-Minnot et al. 2007). In these examples, hosts are manipulated to display bolder or even suicidal behaviors, allowing the parasite to advance its life cycle in the next host (Lafferty and Morris 1996; Sato et al. 2012). However, not all behavioral changes observed in parasitized hosts are due directly to host manipulation (Poulin 2000, 2010, 2013) but could be the result of the infection process and/or the resultant host immune response (Kekäläinen et al. 2014). No matter the mechanism, parasite-influenced behavioral changes are important to identify because they can influence trophic dynamics and alter ecological communities (Reisinger and Lodge 2016; Morton 2018; Friesen et al. 2020).

Digenean trematodes are a class of parasites with complex life cycles that often include multiple intermediate hosts, enhancing selective pressures for the evolution of manipulative behaviors (Lafferty 1999; Choisy et al. 2003). Many examples of behavioral manipulation have been documented among the digeneans (e.g., Arundell et al. 2019; Goodman and Johnson 2011; Filion et al. 2017; Friesen et al. 2019); however, one family of digenean trematodes infecting crustacean intermediate hosts—the Microphallidae—have mixed evidence of behavioral manipulation (Poulin and Maure 2015). For example, amphipods infected by microphallids have shown altered swimming behaviors (i.e., more commonly found swimming at the surface and nearshore) and positive phototactic and negative geotactic behavior (Helluy and Thomas 2003; Guler et al. 2015). In another example, crayfish (*Orconectes rusticus*) infected with *Microphallus* spp. metacercarial cysts performed fewer feeding behaviors (Sargent et al. 2014), but were bolder and exhibited more foraging behaviors than their uninfected conspecifics when in the presence of predatory fish (Reisinger and Lodge 2016). Further, Hansen and Poulin (2005) found infection of *Microphallus* spp. in

isopod intermediate hosts to enhance swimming activity and reduce evasive response to a simulated predator relative to uninfected isopods; moreover, these behaviors were intensity-dependent. Similarly, grass shrimp (*Palaemonetes pugio*) infected by *Microphallus turgidus* metacercariae displayed reduced levels of swimming stamina and were more likely to be consumed by predators (Kunz and Pung 2004). In contrast, there are also documented examples where no behavioral effect is observed. For example, red damselflies (*Xanthocnemis zealandica*) infected with putative *Microphallus* spp. metacercariae exhibited no observable differences in behavior related to activity or boldness (Filion et al. 2017). Thus, in some intermediate hosts, microphallid infection induces a clear and measurable behavioral response, while in other cases, there may be limited or no detectable response.

In the North Atlantic, a prevalent microphallid species, *Microphallus similis*, uses the European green crab (*Carcinus maenas*) as a second intermediate host in both its native range in the northeast Atlantic and its non-native range in the northwest Atlantic (Torchin et al. 2001; Blakeslee et al. 2009; Bojko et al. 2018). Genetic evidence (Blakeslee et al. 2020a; Barnard et al. *in revision*) shows that *M. similis* is broadly distributed in multiple populations throughout the North Atlantic; this evidence, along with its usage of multiple native first- and second- intermediate hosts on both coasts (Stunkard 1957; James 1968), suggests a cosmopolitan distribution in the North Atlantic. Moreover, Blakeslee et al. (2020a) found evidence of host-switching by native lineages of microphallid trematode species, including *M. similis*, between native crabs, *Cancer irroratus*, and the non-native *C. maenas* in Newfoundland. Furthermore, genetic evidence from Blakeslee et al. (2020a) and Barnard et al. (*in revision*) show that *M. similis* identified using characteristic cercarial and sporocyst morphology in first-intermediate snail hosts, *Littorina obtusata* and *L. saxatilis*, is genetically the same as *M. similis* metacercarial cysts detected in *C. irroratus* and *C. maenas* from several eastern North American locations. Thus, herein, we refer to the *M. similis* from *Littorina* spp. and *C. maenas* hosts as native to eastern North America.

Microphallus similis begins its life cycle by infecting periwinkle snails (*Littorina* spp.) as first-intermediate hosts, asexually reproducing in the snails' gonads, and consequently castrating the snail. Free-swimming larvae, called cercariae, are produced and intermittently emerge from the snail into the water column. These cercariae find and encyst as metacercariae in their second-intermediate host (identified host species include *C. maenas*, *Cancer pagurus*, and *Cancer irroratus*; Stunkard 1957; Blakeslee et al. 2020a). The *M. similis* life cycle is completed when the crab is consumed by a definitive shorebird host, such as *Larus argentatus* (herring gull) and *Sterna hirundo* (common tern) (Stunkard 1957). The metacercariae excyst in the bird's gastro-intestinal tract and sexually reproduce; eggs

containing miracidia are deposited into the marine environment through the bird's feces. Grazing snails accidentally ingest the eggs, and the cycle continues (see life cycle diagram in Blakeslee et al. 2015).

Previous work in this system suggests that infection by *M. similis* may have minimal long-term impacts on *C. maenas* behavior and physiology. Specifically, Blakeslee et al. (2015) identified few detectable effects in *C. maenas* dependent on infection status or infection intensity four weeks post-infection, including energy storage, gonad condition, calorimetry, lipid content, foraging behaviors, and shelter-use behaviors (Blakeslee et al. 2015). However, early in the infection process, there were some apparent differences. For example, crab righting response significantly differed between infected and uninfected crabs 72 hours post-infection; moreover, an immune response was also detected 72 hours post-infection but had dissipated by four weeks (Blakeslee et al. 2015). In addition, cyst intensity was positively associated with the handling time of a prey item (blue mussel; Blakeslee et al. 2015), suggesting that when in greater numbers, these parasites may affect specific crab activities. Indeed, during the course of infection, the most damage to host tissue and organs occurs when the parasite enters the host and migrates to the hepatopancreas (Meissner and Bick 1999). Therefore, potential indirect behavioral effects as a result of host immune response may be more likely during earlier stages of infection (Blakeslee et al. 2015).

In our study, we sought to determine when behavioral effects become apparent post-infection, following cercarial penetration and during cyst maturation within *C. maenas*. This is because time-since-infection, cyst development, and cyst intensity could all be key factors for understanding how and when a parasite could modify its host's behavior (Poulin et al. 1994). In nature, this could manifest as the host being more vulnerable to predation by definitive hosts at different stages of the infection process (i.e., at early stages where there is active cercarial penetration versus later encysted stages where the parasite is primarily dormant). For example, the acanthocephalan *Pomphorhynchus laevis* induces behavioral changes in its amphipod (*Gammarus pulex*) intermediate host during the parasite's cystacanth stage, which is the most infective stage for its definitive hosts (often fish or birds) (Franceschi et al. 2008).

To determine the potential impacts of *M. similis* on *C. maenas* behavior through time, we conducted a series of experimental trials prior to induced infection and at four time points post-infection. We examined a novel stimulus response and general behavioral assays (i.e., shelter use, foraging patterns, and righting response) across one pre-infection and four post-infection time points (72 hours, 1 week, 2 weeks, 3 weeks) to determine whether there were changes in behavior related to activity and conspicuity over the course of infection, especially before the signal dissipates at four weeks as reported by Blakeslee et al (2015). Past work has also suggested

that the trematode is fully encapsulated and patent 2–3 weeks post-infection (Stunkard 1957; Blakeslee *unpublished*), and thus, the influence of trematode infection on host activity and conspicuity may change with metacercarial cyst development. Moreover, we examined whether infection burden (i.e., number of cysts) may affect host response, as has been observed in past studies of microphallid infection in intermediate hosts (e.g., Hansen and Poulin 2005; Blakeslee et al. 2015). We hypothesized that higher burdens would be associated with greater changes in behaviors as compared to control crabs. Altogether, our study advances understanding of parasite-induced behavioral responses on hosts and how these may develop through time post-infection.

Materials and methods

To obtain *Carcinus maenas* with few preexisting infections of *Microphallus similis*, we sampled crabs ($n = 44$) in June 2018 from Adams Point (43.0898°N; 70.8662°W; Durham, New Hampshire, USA), a site with naturally low abundances of *M. similis* in *C. maenas* and upstream *Littorina* spp. snail hosts (Blakeslee and Byers 2008; Blakeslee et al. 2009, 2015). The crabs were transported to Shoals Marine Laboratory (SML) on Appledore Island (Maine) (42.9878°N; 70.6144°W), where they were assorted as evenly as possible by sex and size using a stratified random sampling design to place them into control and exposed groups ($n = 22$ crabs per group). To keep track of individuals, we recorded carapace width (CW) and sex; we then painted unique identifying numbers on each crabs' carapace. Our control group consisted of 14 females (CW = 55.74 mm $\pm$ 5.52) and 8 males (CW = 56.07 mm $\pm$ 5.24), and the exposed (experimentally infected) group consisted of 16 females (CW = 55.73 mm $\pm$ 4.85) and 6 males (CW = 56.07 mm $\pm$ 4.44).

To induce microphallid trematode infection in *C. maenas*, we required first-intermediate snail hosts with moderate levels of infection by *M. similis*. We collected snails (*Littorina obtusata* and *L. saxatilis*, $n \geq 600$) from sites where we have previously recorded moderate prevalence of *M. similis* infection in snails, including York (Maine), and sites on Appledore Island (Byers et al. 2008, 2016; Blakeslee et al. 2015). The control and exposed treatment groups were placed in separate ~ 50 L clear containers filled with ~ 30 L of aerated seawater. Both containers had metal mesh strainers suspended at the top of the water column, but only the container with the exposed treatment had strainers containing snails. Two batches of snails ($n = 300$ snails in each batch) were alternated every 12 hours to allow one batch to desiccate while the other was used for inducing infection. Desiccation and re-submergence in water mimics a tidal cycle, which stimulates the release (shedding) of the asexual, infective stage of *M. similis* from upstream snail hosts (Combes et al. 1994; Blakeslee et al. 2015; Blakeslee et al. 2020b). Evidence of cercarial emergence was checked every

12 hours (0800 and 2000) by pipetting five 10 mL aliquots of water from each corner and the middle of each container. The water samples were then examined for cercariae under a stereomicroscope (40x power). After the evening cercarial check, 50% water changes were performed. *Carcinus maenas* were exposed to these treatments for 72 hour to induce a moderate level of cyst intensity in exposed crabs (Blakeslee et al. 2015). Crabs were not fed during the exposure period.

After the 72-hour infection period, *C. maenas* were maintained at SML in individual plastic aquaria (9.13"L × 6.00"W × 6.12"H) with slits to allow water flow. Individual housing was to prevent cannibalism and easily identify individuals. Each aquarium was submerged in a large, flow-through sea table for the duration of behavioral experiments (3–5 weeks). *Carcinus maenas* were maintained by feeding them locally collected green algae (*Ulva* spp.) *ad libitum*. This provided sufficient sustenance but also deprived the crabs of animal protein, such that crabs would be more likely to elicit detectable behaviors when offered *Mytilus edulis* (blue mussel) tissue during the novel stimulus trials (see below).

Behavioral Assays

To determine whether and when behaviors were altered as a response to infection and cyst abundance, we conducted behavioral assays at five time points: pre-infection, and 72 h (3 days), 168 h (1 week), 336 h (2 weeks), and 504 h (3 weeks) post-infection. A behavioral arena (50 L container wrapped with black plastic and filled with ~ 30 L of natural seawater) was set up with a common shelter that crabs would use in the rocky intertidal zone: a rock standardized for size (~ 6"L × 4"W × 2"H) with 40 ± 5 g of seaweed (*Fucus vesiculosus*) attached to it with rubber bands. The shelter and water were replaced between every trial. The rocks were washed and dried in sunlight for 24 hours before re-using in subsequent trials, while the *Fucus* fronds were unique for each trial. Between trials, the position of the shelters in the arena was alternated randomly from one end to the other. Each crab was allowed 30 minutes to acclimate to the behavioral arena prior to starting observations. The trials lasted for 15 minutes, and *C. maenas* behavior was noted every 30 seconds ($n = 30$ observation points/crab) using an established ethogram (Blakeslee et al. 2015). Recorded behaviors included walking, standing still, and attempting to climb up the sides of the chamber. We also noted where the crab was located with regard to the shelter (next to, on top, or under). To ensure consistency in assessing behaviors, the same individual watched all trials in person. To minimize the impact of human presence, *C. maenas* behavior was observed from behind a screen with eye slits in a dark room where windows were covered with fabric; the only light came from a series of red lights strung overhead (Nguyen et al. 2017).

We also employed a novel stimulus response as a measure of boldness. Prior work has shown that bolder individuals approach a novel stimulus sooner than shyer individuals (Gherardi et al. 2012). The novel stimulus was a standardized mass ($4.25 \text{ g} \pm 0.25$) of mussel tissue, which was dropped ~ 10 inches from the shelter in the behavioral arena after the 15-minute observation period. We then timed how long it took for the crab to reach the novel stimulus. If the crab did not reach the mussel within 15 minutes, the trial was terminated.

We tested righting response to determine whether individual *C. maenas* would differ in their ability to right themselves over the course of infection and as a function of cyst abundance. In this behavioral assay, we added two additional time points beyond those tested above: 672 h (4 weeks) and 840 h (5 weeks) post-infection. Though Blakeslee et al. (2015) found no difference in righting response time between infected and uninfected crabs at 4 weeks post-infection, that study did not examine the influence of infection burden through time on righting response as we have done here. To perform these righting response trials, we placed an individual crab on its dorsal side in the middle of a ~ 50 L container without water. We then timed how long it took the crab to right itself (i.e., flip back over). Each crab was tested three times to obtain an average righting response time. If a crab had not righted itself within five minutes, the trial was terminated.

Crab dissections

After all behavior trials were completed, all crabs ($n = 44$) were dissected to confirm and quantify infection burden using protocols outlined in Blakeslee et al. (2015). Briefly, all crabs were anesthetized by freezing for at least 1 hour at 0°C ; then the upper carapace was separated from the lower carapace with a sterilized razor blade. A series of 10 tissue samples (“snips”) were taken from different regions of the crab (1 from the thoracic ganglion, 1 from the gonad, and 8 from the hepatopancreas), placed onto glass slides, and squashed with a $22\text{-mm} \times 22\text{-mm}$ glass cover slip. More tissue was taken from the hepatopancreas because it is where *M. similis* cysts tend to concentrate (Torchin et al. 2001, 2002, Blakeslee et al. 2009, 2015). Slides were examined under a compound microscope at 40x for *M. similis* cysts, which were tallied using a handheld counter. Experimental (new) cysts were differentiated from pre-existing cysts based on the presence and thickness of the parasite’s cyst wall, as recently formed cysts have thin walls and grow in thickness over time (Stunkard 1957; Blakeslee et al. 2015). Moreover, pre-existing cysts are morphologically quite different in appearance to melanized cysts, which are products of the host immune response (Bryan-Walker et al. 2007; see images in Blakeslee et al. 2020b-supplemental Fig. 2).

Statistical analyses

The recorded behaviors from the behavior trials were grouped by activity level (walking, climbing on shelter/side of arena, or standing still) and position

relative to the shelter (within/touching shelter or out in the arena). The four categories of grouped behaviors were the following: (1) near the shelter and active (ShelterActive), (2) near the shelter but inactive (ShelterInactive), (3) not near the shelter but active (NoShelterActive), and (4) not near the shelter and inactive (NoShelterInactive). Behaviors classified as “Active” included walking and climbing on the shelter or along the side of the arena, while the “Inactive” behaviors were simply the absence of any movement (standing still). These groupings were created to decrease the number of behaviors and increase power to detect any effects in the models.

We tested for infection-mediated behavior change by using two variants of the response variable. First, we used raw counts of the grouped behaviors to explore whether there were any common effects of infection amongst all *C. maenas*. In addition, because individual crabs differ from one another in their behavior profiles (Griffen et al. 2012), we also calculated the change from the initial behavior of individuals over time. To examine how time-since-infection impacted behavior and activity patterns, each time point post-infection was compared to the pre-infection values for each category for each individual crab.

To assess which factor(s) (i.e., infection status, time, crab sex, crab size, and cyst abundance) influenced change over time in behavior, we ran a series of linear mixed-effect models on each of the four behavioral categories (ShelterActive, ShelterInactive, NoShelterActive, NoShelterInactive). Two models were run for each category: the summed behavioral points for each time point, irrespective of the pre-infection values, and the calculated difference across crab individuals as compared to the pre-infection behaviors. The fixed effects were treatment (i.e., control or infected), time (i.e., 72 h, 168 h, 336 h, and 504 h post-infection), crab sex (i.e., male versus female), crab size (i.e., carapace width in mm), and infection burden (i.e., total number of metacercarial cysts). We included a random effect of individual crab ID to account for repeated measures within each crab. In the first iterations, models included an interaction effect between treatment and time, but the interaction was not significant and was therefore removed to conserve degrees of freedom. The linear mixed effects models took the form:

$$\text{Change of behavior from initial (or Summed Total)} \sim \text{Treatment} + \text{Time} + \text{Sex} + \text{Size} + \text{Infection Burden} + (1 \mid \text{Individual Crab ID}),$$

where the response variable was the summed total of each behavior or the change in behavior compared to the pre-treatment (initial) behaviors. All models were fit using maximum likelihood and Gaussian error structure with the lme4 package in R (R Core Team, 2019).

Similarly, we ran series of linear mixed-effects models that tested whether infection status and time-since-infection impacted *C. maenas* novel stimulus response and righting response. The two models took the form described above.

Results

Experimentally-infected crabs had a higher *M. similis* burden (range: 0 to 3820 cysts; mean $\pm$ SE = 328 ± 109) as compared to control crabs (range: 0 to 11 cysts; mean $\pm$ SE = 4 ± 3) (t test; $t = -3.319$, $df = 21.001$, $p < 0.005$). Most cysts were new, as a result of induced infection in our experiment, but 17 of the 44 crabs had at least one pre-existing cyst (maximum number of pre-existing cysts in a single crab was 11). However, because these pre-existing metacercarial burdens represented a small proportion of the overall cyst intensity, infected crabs with pre-existing cysts were included in our models. At the end of the experiment, we dissected a subset of the snails used to induce infection, and of these ($n = 280$), 2% were infected with *M. similis*. While we also observed other trematode species present in some *Littorina* snails, including *Cryptocotyle lingua*, *Renicola roscovita*, and *Cercaria parvicaudata*, they do not utilize *C. maenas* as an intermediate host (Blakeslee and Byers 2008). Based on prior work in the system, we had *a priori* expected higher infection prevalence in *Littorina* spp. snails, closer to 10% (Blakeslee et al. 2015); even so, our water samples routinely demonstrated *M. similis* cercariae in the infection chambers, and post-experimental dissections of crabs also revealed newly-acquired *M. similis* cysts in the *C. maenas* exposure group.

Behavioral assays demonstrated no significant impact of *M. similis* infection (Figures 1–3) on *C. maenas* behavior at any time point. In addition, there were no trends uncovered in the raw sums of the grouped behaviors (Figure 1A–D); although there was an increase in “ShelterActive” behaviors over time, but this included both the control and exposed treatments (Figure 1C). Moreover, we found no evidence of *M. similis* influence on behavioral changes post-exposure when compared to initial grouped behaviors (Figure 1E–H). There was also no evidence of an impact on righting response through time, either in terms of raw sums (Figure 2A) or change from initial (Figure 2B). Likewise, with the novel stimulus response, we found no trends in reaction time between the control and infected crabs of the raw sums (Figure 3A) or change from initial (Figure 3B). To check for collinearity between infection burden and treatment, we evaluated the variance inflation factor (VIF) for all models. All variables in all models yielded VIFs < 5 , so we retained all fixed effects in the models (Supplementary material Table S1).

For the mixed effects models with the raw sums data only, there was support for an increasing trend over time; however, since this applied to both the control and exposed treatments, this effect was not due to parasite infection (Table S2, LMM; coefficient = 0.010, SE = 0.002, $df = 177.7$, $t = 2.246$, $p \leq 0.01$). Meanwhile, there were no significant predictors for the change from initial grouped behavior values (Table S3). As for the righting response mixed models, we also found no significant effects for the raw sums

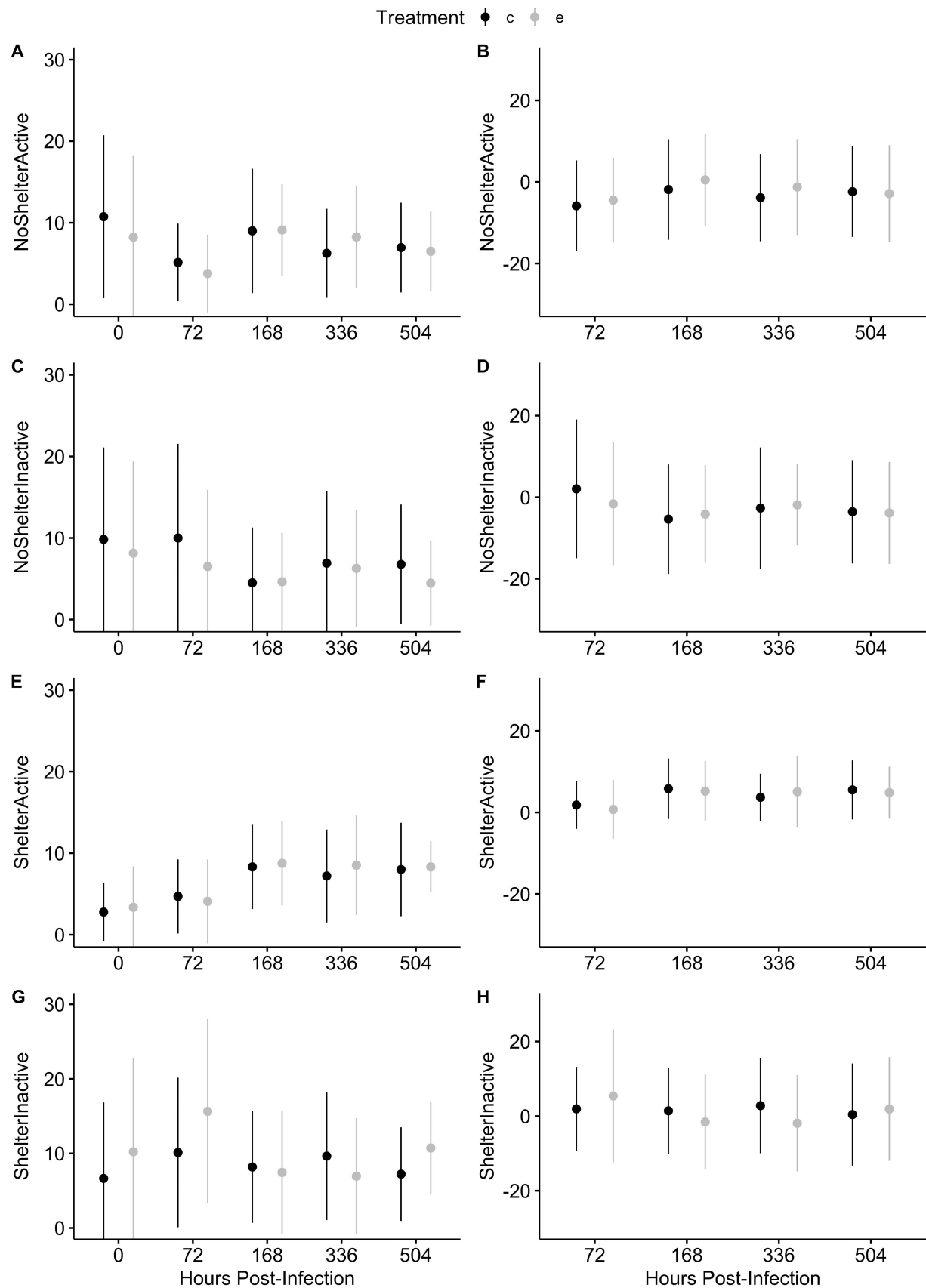


Figure 1. Grouped behavior response over time with raw sums (A, C, E, G) and changes from initial behavior (B, D, F, H). In all panels, the central point is the mean, and the lines are standard deviation. The black lines are the control treatment individuals, and the gray lines are the exposed treatment individuals. Panel “A” and “B” are behaviors classified as NoShelterActive, Panels “C” and “D” are behaviors classified as NoShelterInactive, Panels “E” and “F” are behaviors classified as ShelterActive, and Panels “G” and “H” are behaviors classified as ShelterInactive.

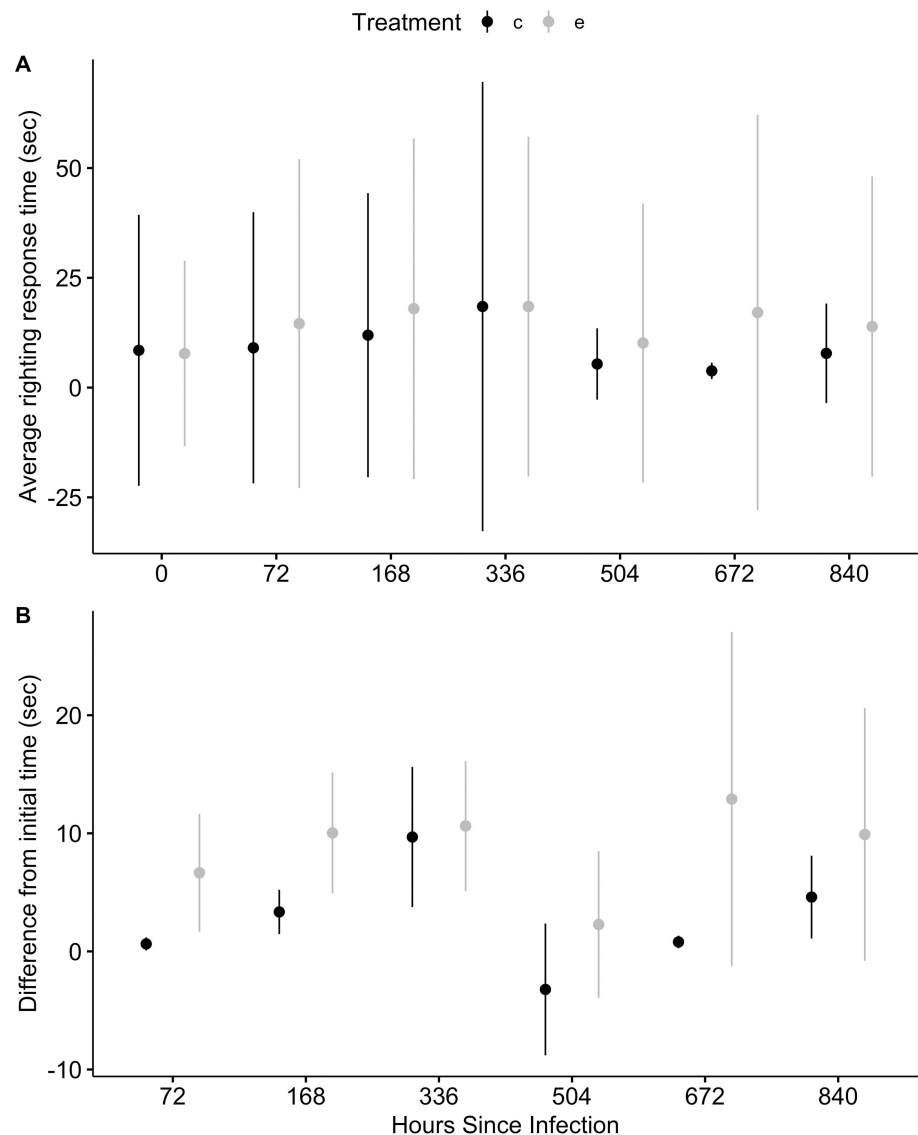


Figure 2. Righting response times of A) raw sums, and B) change from initial through time. The central point is the mean with lines as standard deviation. The black lines are the control treatment and the gray lines are the exposed treatment.

(Table S4) or change from initial (Table S5). For the models testing the novel stimulus response, there were no significant effects of our predictor variables (e.g., sex, size, time, treatment, cyst abundance) for the raw sums data (Table S6), or the change from initial data (Table S7).

Discussion

To enhance the likelihood of life cycle completion, parasites may manipulate their host's phenotypes (i.e., representing an "extended phenotype", Dawkins 1982) to increase transmission success and overall fitness (Lafferty 1999; Poulin 2010; Thomas et al. 2010; Hughes and Libersat 2019). These modifications can be morphological and/or behavioral and often occur in intermediate hosts (Lafferty 1999). While behavioral manipulations could make an intermediate host a more conspicuous prey item to a competent final host—thus increasing transmission likelihood—these manipulations

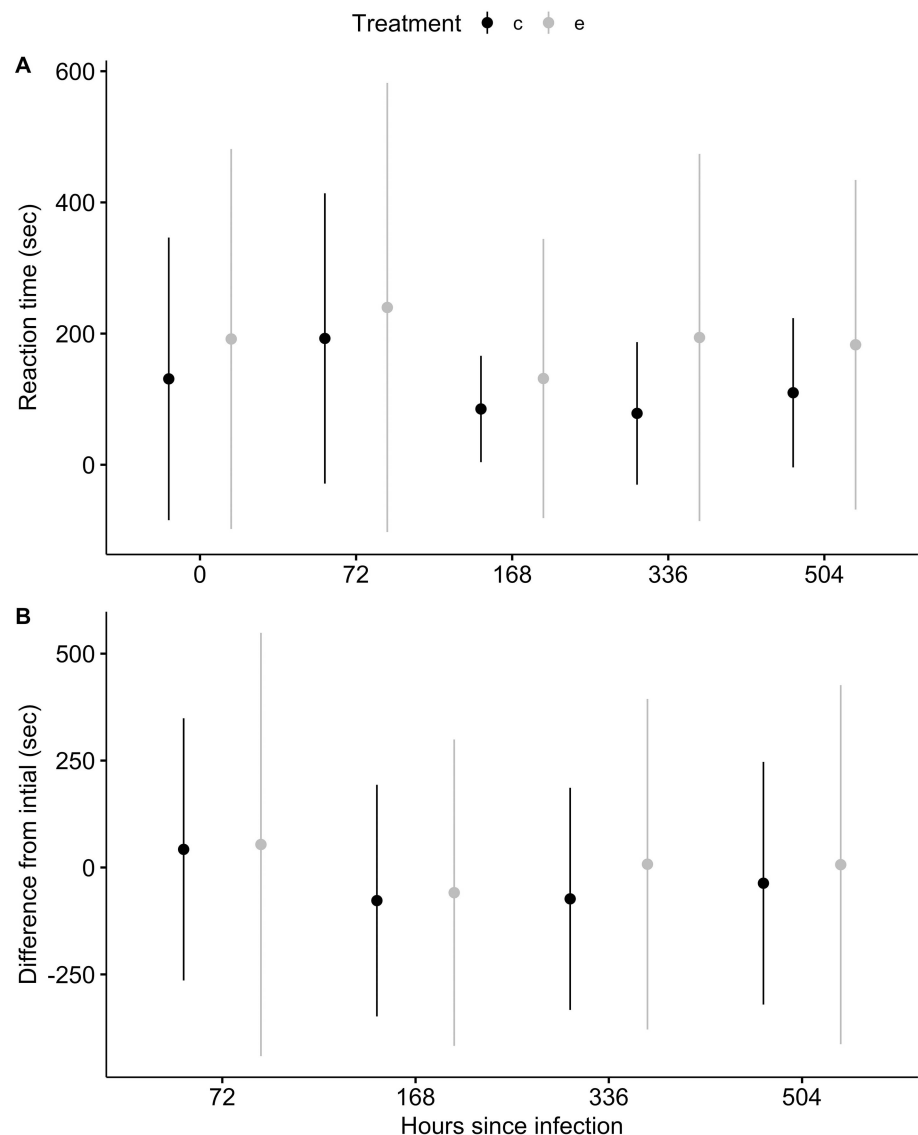


Figure 3. Novel stimulus reaction times of A) raw sums and B) change from initial through time. The central point is the mean with lines as standard deviation. Black lines are the control treatment and gray lines are the exposed treatment.

are energetically costly and thus may not always represent the most cost-effective way for a parasite to successfully complete its life cycle (Maure et al. 2013; Thomas et al. 2005). Instead, simply being parasitized (without induced behavioral manipulation by the parasite) may be enough for transmission success, by draining the host's energy and influencing its ability to escape predators (i.e., the *energy drain hypothesis*), leading to higher predation rates on parasitized individuals (Hafer and Milinski 2016). Moreover, the potential behavioral impacts a parasite may have on its second-intermediate host can also depend on the parasite's developmental stage in the host, as well as the developmental stage of the host itself (Thomas et al. 1995; Schotthoefer et al. 2003; Gabagambi et al. 2019). For our study, specifically, the latter is less relevant given that we only used adult crabs. Related to the former, it can take weeks for a digenean, like *M. similis*, to be fully patent within the second-intermediate host (Stunkard

1957; Blakeslee et al. 2015), and if the parasite is under-developed at the time of consumption by the final host, it may not be viable (Bethel and Holmes 1973; Dianne et al. 2011). To avoid such premature transmission, the parasite may elicit a behavioral response in the host to suppress predation and decrease the chance of an early transmission to a final host during a potentially non-infective stage (i.e., the *predation suppression hypothesis*; Parker et al. 2009; Dianne et al. 2011). Indeed, modelling performed by Parker et al. (2009) suggests that predation suppression behaviors in hosts could evolve more quickly than manipulative behaviors. Moreover, the evolution of manipulative behaviors could be unnecessary if transmission success is high without it; for example, in our system, if *M. similis* is successful at broadly infecting common prey items (i.e., crabs) for avian final hosts, it may not require manipulative behaviors for successful and widespread transmission. In addition, because the host, *C. maenas*, is an introduced species, *M. similis* has only been in contact with the crab in eastern North America for < 200 years (Blakeslee et al. 2020b); thus, it is possible that the time needed to evolve manipulative behaviors in this host–parasite system has been insufficient. Below, we further discuss our results in the context of these potential hypotheses for a lack of conspicuous behaviors in infected individuals, as well as their implications for community interactions in the region.

*Lack of conspicuous host behavior with M. similis infection:
possible explanations*

Our study joins a number of others documenting a lack of host behavioral manipulation due to infection by a *Microphallus* spp. trematode. One possible explanation for a lack of perceived behavioral response by *C. maenas* could be the site of encystment by the trematode species. Some microphallidae encyst in the nervous systems of their hosts and produce or regulate neurotransmitters that could impact host behavior (Øverli et al. 2001). *Microphallus similis*, however, is primarily found in the hepatopancreas (i.e., liver and pancreas, where energy storage occurs) of *C. maenas*, and rarely in the gonad tissues or thoracic ganglia (i.e., nerve center) (Blakeslee et al. 2015). Similar to our study, an investigation by Sparks and Hibbits (1981) found no behavioral differences between Dungeness crabs (*Cancer magister*) with and without microphallid metacercarial cysts, although these cysts were found in the nerve center of these crabs. In other trematode systems, behavioral manipulation can be difficult to detect or may be irrelevant to predators. For example, freshwater snails (*Potamopyrgus antipodarum*) infected by *Microphallus* spp. were more likely to be consumed by waterfowl in field experiments, but there were no differences in snail behavior to explain this trend (Levri and Fisher 2000). Similarly, isopods (*Auvidotea annectens*) infected by trematodes (*Maritrema poulini*) had

higher levels of activity, but the rate of predation remained unchanged between infected and uninfected individuals (Friesen et al. 2019).

It is also possible that we may have been recording behaviors during a window of time when manipulative, conspicuous behaviors may have been less likely to be detected. This is because the formation and full development of metacercarial cysts for some microphallid species can take up to 42 days (Galaktionov et al. 1996, 1997). While a trematode is transitioning from the cercarial to metacercarial stage in its intermediate host and is not fully competent to infect its definitive host, it may elicit behavioral responses in its host to avoid predators and decrease the chance of early transmission (i.e., *predator suppression*; Parker et al. 2009; Médoc and Beisel 2011; Dianne et al. 2011). However, once a cyst is fully formed, behavioral manipulations that increase predation risk through conspicuity could become more apparent (Gabagambi et al. 2019). In our study, all trematode cysts were fully formed at 5 weeks when crabs were dissected (albeit with a thin cyst wall); thus this hypothesis is less likely for our system. To our knowledge, experimental evidence demonstrating a shift from predator suppression to manipulative behaviors depending on cyst competency has not been documented in our system. However, such a strategy has been identified in an acanthocephalan parasite, *P. laevis*, infecting the amphipod, *G. pulex*. Dianne et al. (2011) found predator suppression behaviors (more hiding) in the amphipod and less predation during the uninfected stage of the parasite's life cycle but enhanced vulnerability to predation during the parasite's infective stage. Moreover, in multi-parasite systems, parasites have been shown to sabotage a co-infecting parasite's manipulative behaviors by suppressing that behavior in favor of the parasite's own strategy (Hafer and Milinski 2016). Thus, strategies related to enhanced versus decreased host conspicuity could change depending on the parasite's development stage or co-infection with other parasites.

Another reason that a parasite may not induce manipulative behaviors which enhance conspicuity is that the parasite may instead have an energetic effect on a host that increases a host's lethargy and lessens its ability to escape a predator (i.e., *energy drain*; Lafferty and Shaw 2013; Hafer and Milinski 2016). Digenean trematodes use a diversity of sites within the host's body, including the central nervous system where manipulation is more likely to evolve, but also in the body cavity, muscles, internal organs and skin (Lafferty and Shaw 2013). Indeed, associated energy drain behaviors may more readily evolve in parasite life stages that utilize host energy storage tissues, like *M. similis* cysts in *C. maenas*. In our study, however, we did not see any direct evidence of behavioral differences in infected and uninfected hosts throughout the study period that might suggest energy drain. Moreover, righting response trials did not suggest greater lethargy in infected versus uninfected hosts. In both our study and in Blakeslee et al. (2015), there were also no apparent differences

in shelter usage between infected versus uninfected crabs – thus we did not detect any differences that could be attributed to energy drain on crab hiding behaviors. Blakeslee et al. (2015) did find evidence of longer righting response times 72 hours post exposure in infected crabs, which may provide some support for energy drain or some other impact of the parasite on the host's physiology shortly after infection. We did not see the same response in our study; however, cyst intensity was lower in our study compared to Blakeslee et al. (2015), which may partially explain these different outcomes. Finally, in prior work, Blakeslee et al. (2015) found no evidence of lessened investment in a crab's storage tissues for infected versus uninfected crabs, nor with increasing cyst intensity. Taken together, our data, at this point, appear to argue against an energy drain effect of *M. similis* on *C. maenas*.

We also found no evidence of *M. similis* influence on crab personality in terms of boldness, an aspect of animal personality that may be affected by parasites (Barber and Bingemanse 2010). Previous work has shown that parasitized individuals can show greater boldness (e.g., Giles 1983; Gering et al. 2021), less boldness (e.g., Coats et al. 2010), or no difference in boldness (e.g., Hagmayer et al. 2020) compared to non-parasitized conspecifics. Parasitism could thus influence boldness in one direction or the other depending on the parasite's objective (i.e., *PITT* versus *energy drain* versus *predation suppression*). In our study, we used a novel stimulus response as a measure for boldness (Sih et al. 2015) and found no significant effect of cyst burden on novel response time at either the group or individual levels, suggesting that *M. similis*-induced behavioral modifications are not reflected by our metric of boldness in *C. maenas*. However, our boldness assay did not directly test for foraging behavior of hosts (e.g., prey handling times), which were positively correlated with infection intensity in Blakeslee et al. (2015). A longer foraging time could possibly enhance predation risk, but this would need to be tested in the field to determine if higher infection burden of *M. similis* cysts might translate to higher predation rates in natural conditions.

Lastly, we did not detect any influence of infection burden (abundance of metacercarial cysts) on *C. maenas* behavior. In past work, the magnitude of host behavioral change has correlated linearly with the number of cysts in some studies (Carreon et al. 2011; Bolliat et al. 2020), while in others, behavioral impacts were nonlinearly dependent on infection intensity (e.g., Kekäläinen et al. 2014) and only occurred after a threshold in the number of cysts was reached (Rees 1955). For *C. maenas*, evidence of reproductive fitness loss as a result of trematode infection has only been observed at high infection intensities (> 1,000 metacercariae per crab) (Zetlmeisl et al. 2011). In North America, natural *M. similis* cyst intensities can reach upwards of 6,000 cysts in *C. maenas* (Blakeslee et al. 2015). In our study, the highest cyst intensity per infected crab was 3,820 cysts, with an average of 521 cysts

(SD = 806). Blakeslee et al. (2015), using the same infection protocols, estimated infection intensities as high as 40,000 cysts for crabs exposed to infected snails for 120 hours, while those exposed for 72 hours (as in our study here) had on average 4,383 cysts (range = 129–18,908 cysts). Thus, the lower infection burdens we achieved in our study may have affected our ability to detect a behavioral effect. Even so, Blakeslee et al. (2015) found few behavioral or physiological differences with increasing cyst intensity. Behaviors in our experiments were extremely variable within and between crabs across time points, adding noise to comparisons of infected versus uninfected crabs. This high variation is common in behavioral assays (e.g., Brown and Robinson 2016; Hartmann et al. 2016; Kain and McCoy 2016), but also suggests that a more narrow assay may be better poised to detect differences, if they exist. Behavioral manipulations may be subtler than we had anticipated and therefore difficult to quantify with the assays we used here (Filion et al. 2017). Further experimentation using different, more targeted behavioral assays would be valuable.

Influence of host–parasite coevolution

Coevolutionary history could also play a role in behavioral influences of parasites on their hosts. In our study system, the host–parasite relationship is complicated, in that *C. maenas* is parasitized by *M. similis* in both its native range (western Europe) and non-native range (eastern North America) (Blakeslee et al. 2015, 2020a, b). Thus *M. similis* has a cosmopolitan distribution in the North Atlantic (Blakeslee et al. 2020a; Barnard et al. *in revision*). However, the barcoding markers used in these studies cannot resolve whether there has been any recent gene flow of *M. similis* associated with *C. maenas* introductions over the past few centuries, or whether these connections represent associations with hosts native to both coasts (e.g., *Littorina* spp. snails and *Larus* spp. gulls; Blakeslee et al. 2020a). Herein, we characterize *M. similis* as native in North America given its association with native North American hosts; however, more genetic work is needed to determine whether there has been additional gene flow from Europe. Further, recent work shows that invasive *C. maenas* in North America are more susceptible to *M. similis* than are *C. maenas* crabs sourced from their native range in Europe, demonstrating a strong association of coevolutionary history with infection susceptibility (Blakeslee et al. 2020b). This may play a role in perceived behavioral effects of *M. similis* on *C. maenas* in eastern North America if the short-term history between the host and its parasite is insufficient for the evolution of manipulative behaviors. It would be informative to examine behavioral effects of *M. similis* on *C. maenas* in the crab's native range compared to the invasive range to determine if there are differences between the regions associated with coevolutionary history.

Moreover, *C. maenas*' greater susceptibility to North American *M. similis* (Blakeslee et al. 2020b) could be playing a role in the crab's infection

prevalence in the region. Indeed, in some populations, like Appledore Island (ME) where we carried out our experiment, infection prevalence in *C. maenas* can be 100% (Blakeslee et al. 2009) with cyst intensity as high as 1,500 cysts per crab (Blakeslee et al. 2015). High infection prevalence and intensity would enhance the encounter rates of *M. similis* with its final hosts, such as *Larus* spp. gulls (Stunkard 1957). *Carcinus maenas* are common prey items of these gulls (Dumas and Whitman 1993), and there is clear evidence that *M. similis* readily transmits from *C. maenas* to *Larus* spp. gulls in the region (Stunkard 1957). This could lessen any selective pressure on *M. similis* to evolve potentially costly manipulative effects in crab hosts if encounter rates and susceptibility of *C. maenas* are enhanced—resulting in high infection prevalence in the intermediate crab hosts and the consumption by final gull hosts of a large proportion of infected prey. Indeed, this could be the most adaptive solution for this digenean in this region. Once again, it would be valuable to analyze *C. maenas* infection by *M. similis* in native European populations and compare to invasive populations in North America to identify any differences between the regions in terms of infection prevalence associated with host–parasite coevolutionary history.

Conclusions

Overall, our results suggest that infected *C. maenas* show no behavioral effects when compared to uninfected crabs, and we found no effect of cyst intensity in any of our behavioral assays. Here, we discussed possible mechanisms for a lack of detectable manipulative behaviors including energy drain, predator suppression, and host–parasite coevolutionary history. A repeated study, with greater replication and a narrower behavioral assay, may yet uncover behavioral changes not detected in this study. Furthermore, investigations throughout the crab's native range in Europe could provide insight into whether *C. maenas* is associated with parasite manipulative behaviors in the native range. This could also help us target future behavioral assays in the invasive range. Finally, our results could have implications for the crab's invasion success in eastern North America and other non-native regions, since *C. maenas* can harbor over 70 symbionts which can all have varied effects on the crab host in terms of their pathologies and possible behavioral influences (Bojko et al. 2018; Bojko et al. 2021). For example, a recent study by Frizzera et al. (2021) found higher microparasite infection in introduced Argentinian populations of *Carcinus* spp. than co-occurring native crabs, but no infection of green crabs by macroparasites in the same region. Indeed, with the possibility for co-infections of macro- and micro- parasites with varying transmission strategies, intensities, and pathologies, host behavioral response could be quite variable and potentially difficult to

unravel. More studies across the native and invasive ranges of *C. maenas* would provide greater insight into host–parasite interactions and potential influences throughout its global distribution.

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Author's contribution

HJR, AEF, AMHB: research conceptualization; HJR, AEF, AMHB: sample design and methodology; HJR: investigation and data collection; HJR, AEF, CLW, AMHB: data analysis and interpretation; AMHB: funding provision; HJR, AEF, CLW, AMHB: original draft; HJR, AEF, CLW, AMHB: review and editing.

Ethics and permits

This project did not involve the use of vertebrate animals or human subjects. Collections of invertebrates proceeded under the Shoals Marine Lab general collecting permit.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Variance inflation factors for all models used for analysis. VIF stands for variance inflation factor.

Table S2. Estimated regression coefficients, standard error, and p-values for each grouped behavior model run with the raw summed values.

Table S3. Estimated regression parameters, standard error, and p-values for each grouped behavior model run with calculated change from initial behaviors.

Table S4. Estimated regression parameters, standard error, and p-values for raw sums of righting response model.

Table S5. Estimated regression parameters, standard error, and p-values for righting response model run on change from initial values.

Table S6. Estimated regression parameters, standard error, and p-values for novel stimulus model run on raw sums.

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