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Article

Exploring chemical cue perception in gordiid worms: A behavioral assessment

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SUMMARY

Nematomorph parasites, or horsehair worms, are probably best known for their ability to force their final insect hosts to enter water, where the worms emerge to complete their life cycle. To date, not much is known about these fascinating organisms and huge knowledge gaps remain about large parts of the nematomorph life history. With this project, we wanted to address questions regarding nematomorph behavior outside the final host and investigate whether adult behavior is influenced by chemical cues in the environment. We hypothesized that freshwater nematomorphs use chemical cues for both mate finding and predator avoidance. To test this, we exposed nematomorphs to a) water exposed to worms of the opposite sex (mate finding), b) water that contained ground nematomorphs to simulate the presence of injured or dead animals (predator avoidance), and c) plain freshwater (negative control). Although we did not detect any significant effects of the tested cues, our findings highlight both the challenges of studying nematomorphs and the potential of chemical ecology as a promising avenue for future research. By establishing a foundation for examining nematomorph responses to environmental stimuli, this work underscores the need for continued efforts to uncover the sensory and behavioral mechanisms that shape the ecology of these unique parasites.

INTRODUCTION

Parasites play crucial roles in ecological, economic, and evolutionary contexts (1–3). Despite their significance, many parasite taxa remain understudied, including the nematomorphs. Nematomorphs, commonly known as horsehair worms, represent one of the few animal phyla composed exclusively of parasites, and they share a close evolutionary relationship with nematodes (4, 5). To date, approximately 350 freshwater (Gordiida) and five marine (Nectonematida) species have been described (6). However, only recently have scientists begun using genetic markers, such as the mitochondrial cytochrome oxidase subunit 1 gene (*CO1*), to aid in nematomorph species identification (7, 8). Because their external features offer limited distinguishing traits, numerous additional species are probably still unrecognized and await formal description.

Gordiida exhibit a unique life cycle, beginning their early development as dormant cysts inside aquatic insect larvae (**Figure 1**). As these first insect hosts mature and transition to terrestrial environments, so too do the developing nematomorphs (4). Although gordiids mature in terrestrial hosts, they must return to water to reproduce (4). Currently it is unclear how this transition between environments happens and might be different for different gordiid species.

To complete this transition from a terrestrial to an aquatic environment, gordiids exert control over their hosts' behavior, altering their responses to environmental cues. Infected hosts first display altered phototaxis (i.e., the movement of an organism in response to light) that brings them closer to water (9). This is followed by a drastic shift that causes them to enter the water and drown, enabling adult gordiids to emerge from the hosts' abdomen (4). Once free, adult gordiids display their characteristic behavior: within hours of encountering one another, males and females form tangled masses known as "Gordian knots" (10). After mating, females deposit egg strings on submerged surfaces such as rocks and twigs. Within weeks, larvae develop and infect various invertebrate hosts (10, 11).

Despite their unique life history, or maybe because of it, gordiids remain poorly studied. Research has primarily focused on systematics, aspects of their life cycle, or anatomical descriptions (4, 10, 12, 13, 15, 16). However, fundamental aspects of their ecology and physiology, particularly their interactions with the environment and their sensory capabilities, remain largely unexplored. Although

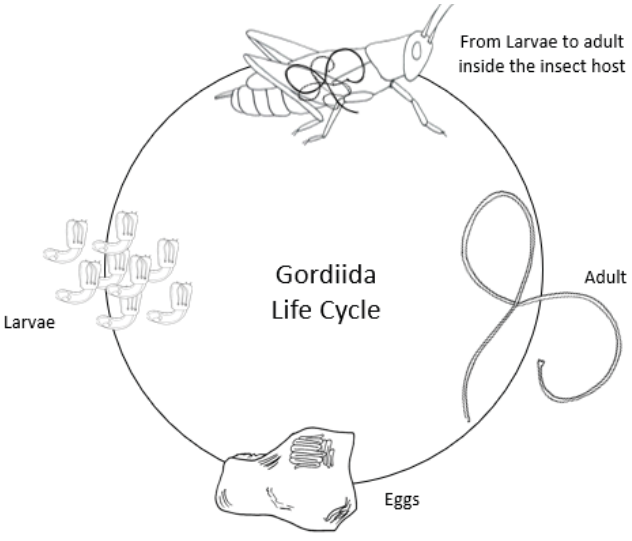


Figure 1: Diagram of a typical gordiid life cycle. Adult worms are free-swimming. Females lay many eggs that hatch into larvae, which are parasitic and infect their host. Inside the insect hosts, larvae grow into adults, which emerge from the insects once mature.

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some gordiid species possess sensory receptors, existing ultrastructural studies provide minimal insight into their function (17, 18). This raises key questions. First, how do adult gordiids interact with their surroundings? And second, can adult gordiids detect and respond to environmental cues?

An intriguing question in gordiid ecology centers around how adults locate mating partners after emerging from their hosts. In dioecious species (i.e., species where male and female reproductive organs are in separate individuals), individuals must find the opposite sex to reproduce, yet the mechanisms underlying mate attraction in gordiids remain unknown. It is unclear whether gordiids rely on chemical cues, such as pheromones, or if encounters occur purely by chance. Studies indicate that both males and females are capable swimmers, but whether directed movement plays a role in mate location is unresolved (4, 10). Understanding how gordiids navigate their environment could thus provide valuable insights into their sensory capabilities and reproductive strategies.

In addition to their reproductive ecology, little is known about gordiid predators as well. Most available data come from stomach content analysis of aquatic animals (19, 20). During a recent study, researchers conducted feeding trials with *Gordius robustus* and found that most of the tested fish species ignored nematomorphs as prey (19). However, in a different study green sunfish (*Lepomis cyanellus*) actively consumed both individual worms and Gordian knots (20). Whether nematomorphs have additional natural predators or if predation occurs indirectly through consumption of infected hosts remains unclear. Predation risk often drives the evolution of chemical communication in aquatic systems. Many organisms detect and respond to chemical alarm signals, which can be released directly by predators or indirectly by injured conspecifics (i.e., other animals of the same species) or other prey species (21, 22). Understanding whether gordiids use such chemical cues could provide insight into their ability to detect and respond to threats in their environment.

To address these knowledge gaps, we tested the effects of chemicals released by conspecifics on mate-finding behavior, and predator-avoidance behavior. We hypothesized that *Gordius aquaticus*, a gordiid species, uses chemical cues for both mate finding and predator avoidance. Specifically, we predicted that *G. aquaticus* would exhibit a change in movement in response to chemical cues associated with the presence of a mate as well as the presence of chemicals related to predator-prey interactions.

RESULTS

To test whether *G. aquaticus* uses chemical environmental cues for mate finding and predator avoidance, we exposed adult *G. aquaticus* to different treatment media and recorded their behavioral response. Specifically, we recorded how often the head region of the test animals crossed the lines on a grid at the bottom of our testing arena. These line crossings will be referred to as "movement" for the reminder of the manuscript (for example, one movement equals one line crossing). As a negative control, we exposed a subset of individuals to water from their original sampling location (Burschenplatz Water, BPW). To test for mate-finding behavior, we exposed *G. aquaticus* to Conditioned Medium (CM), which had contained individuals of the opposite sex for at least 48 hours. Finally, to assess predator-avoidance responses, we exposed *G. aquaticus* to water containing ground *G. aquaticus* tissue (GT), thereby simulating the presence of injured or dead conspecifics. For each trial, we recorded the movement of the focal animal for 10 minutes in untreated mineral water to establish baseline behavior, followed by an additional 10 minutes after the introduction of the respective test medium (BPW, CM, GT). We added the test media by forcefully dispensing the contents of two disposable 3-mL plastic pipettes on the opposite side of the dish relative to the focal animal. A total of three individuals were tested in BPW, three in CM, and seven in GT. We removed one outlier from the GT treatment, as it showed movement levels 5–10 times higher than its conspecifics.

In the BPW group, two individuals showed an overall increase in movements, while one showed a decrease in movements after treatment (**Figure 2**). In contrast, in the CM group, two individuals showed an overall decrease in movements, while one showed a very slight increase in movements (**Figure 2**). Lastly, in the GT group, half of the animals showed an increase in movements and half of the animals showed a decrease in movements (**Figure 2**). However, there was no statistically significant difference between pre- and post-treatment for any of the groups: BPW ($t(2) = -0.13$, p-value = 0.91), CM ($t(2) = 1.28$, p-value = 0.33), GT ($v = 12.5$, p-value = 0.75) . Although our observations are not statistically significant and show no clear pattern, they highlight the potential of chemical ecology as a promising avenue for future research.

DISCUSSION

In this project, we aimed to provide first insights into the role of chemical cues in the mate-finding and predator-avoidance behaviors of *G. aquaticus*. During sampling, we collected a higher number of males than females, which led to us only using males as focal animals in the experimental trials. While this decision was necessary due to specimen availability, it may have influenced the observed behavioral patterns. Previous research consistently shows that natural populations of adult gordiids do not exhibit equal numbers of males and females, though the pattern of bias is not the same across species. For instance, *G. robustus* populations have been reported to exhibit female-skewed ratios, whereas *Gordius difficilis* populations have been reported to have more males (23, 24). Additionally, male gordiids often accumulate towards the end of the mating season, possibly due to differences in lifespan or developmental timing (10). Since we do not know the exact timing of *G. aquaticus* emerging at the sampling location, we might have sampled late in the season and therefore mainly caught males.

Male gordiids frequently engage in intense competition for mates, which could alter their responsiveness to environmental cues compared to females (14). This makes it surprising that we did not find any change in behavior after adding CM. However, this might also be explained by our sampling time. Laboratory observations on *Paragordius varius*, another species of horsehair worms, suggest that males initiate mating with females even before completely

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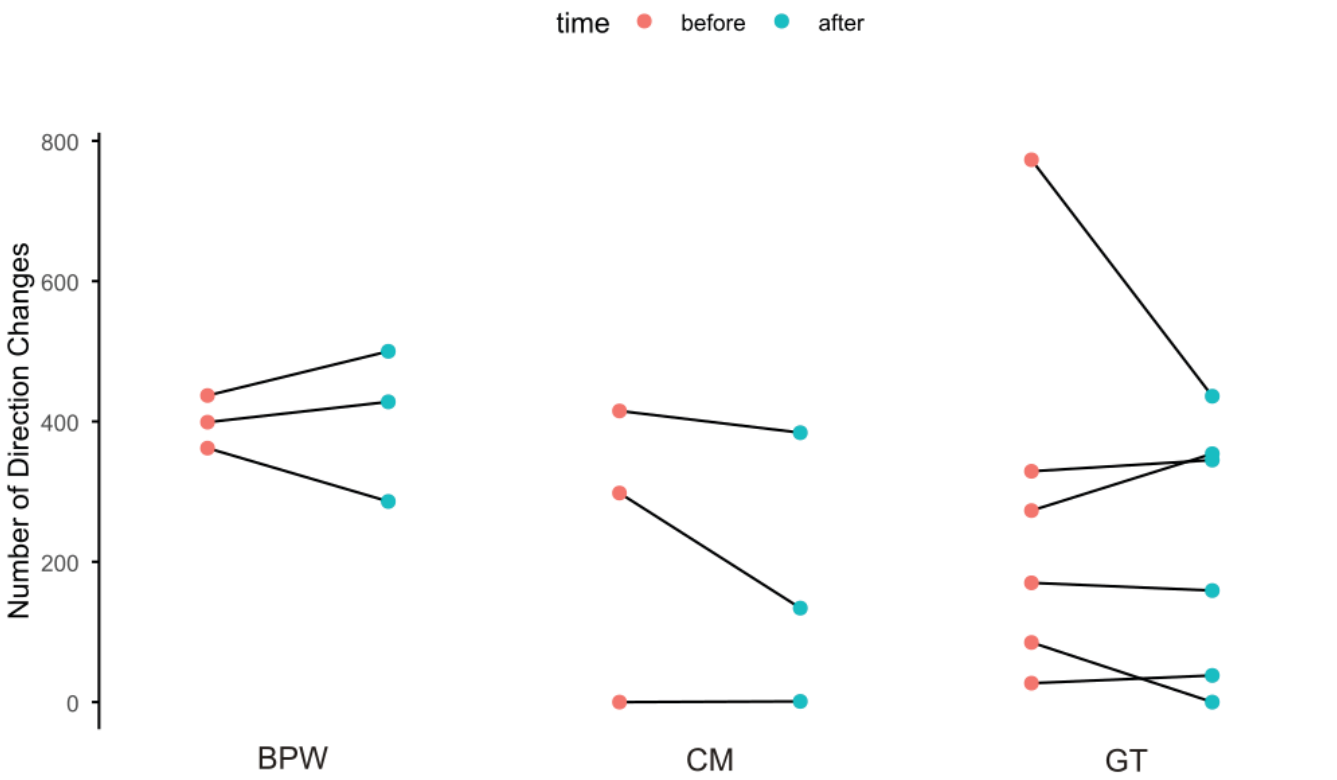


Figure 2: Number of movements (Number of movements) of focal animals before and after treatment with different media. The figure shows the number of movements of the focal animal before (orange) and after (orange) the addition of Burschenplatz Water (BPW), Conditioned Medium (CM), or Ground Tissue (GT). No significant differences in the number of movements after the addition of BPW ($t(2) = -0.13$, $p = 0.91$), CM ($t(2) = 1.28$, $p = 0.33$), or GT ($v = 12.5$, $p = 0.75$) were recorded. A total of three adult male Gordius aquaticus were tested in BPW, three in CM, and seven in GT. We removed one outlier from the GT treatment, as it showed movement levels 5–10 times higher than its conspecifics.

exiting the host (25). When *G. difficilis* worms were monitored through daily field collections, almost every female examined (over 90%) contained sperm, implying that mating happens rapidly following emergence from the host (10). Consequently, our sampling window may have coincided with a post-mating period, reducing responsiveness to chemical cues in our experiments. However, since the timing of the *G. aquaticus* life cycle at our sampling location has not yet been recorded, we were not aware of being late in the season and had initially planned to sample as soon as we saw the first worms emerge. A possible solution would have been to test for the presence of sperm in the few females we collected, but due to time, financial and technical constrains, this was not possible. We still found it important to test mate-finding behavior.

Supporting the hypothesis that we collected our samples late in the *G. aquaticus* life cycle is the fact that we observed a rapid decline in the health of collected individuals in the laboratory. Although the hosting lab has been successful in the past in keeping wild-caught, adult gordiids alive in the lab, even for weeks, many of our collected *G. aquaticus* died just days after being collected, with females showing a higher mortality rate. This rapid mortality is consistent with previous reports that *Gordius* tissues in the free-living phase often exhibit poor fixation results, likely due to the onset of degeneration processes (26). This degeneration may have compromised the worms' behavior and overall responsiveness to experimental conditions. For the same reason, we might not have found any response to the GT medium because there might not have been enough energy left to initiate predator-avoidance behavior.

Prey species commonly rely on a blend of sensory information to evaluate predation risk, drawing on signals that come directly from predators as well as indirect cues from the environment or other organisms. Evidence suggests that the presence of both cue types usually elicits a more pronounced antipredator behavior than either type alone (27). In our study, we used a single cue – the presence of ground tissue from conspecifics – to test an antipredator response. While this method has been effective in various aquatic animal groups, it may not be sufficient to induce a behavioral response in *G. aquaticus* (28–30). This could indicate that *G. aquaticus* requires direct predator cues to trigger antipredator behavior or that chemical alarm cues alone are insufficient for eliciting a response in this species.

The persistence of chemical cues in water varies widely, with reported half-lifes ranging from 0.2 to 126 hours across different species (31, 32). To avoid potential degradation over time, we froze our GT samples as aliquots and thawed them on the day of the experimental trial. Studies suggest that freezing can preserve alarm cues in some species. For instance, alarm cues from the freshwater snail *Physella acuta* degrade within 41 hours at room temperature, but freezing

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prolongs their activity (33). However, the effectiveness of frozen cues is not universally applicable across taxa, and it remains uncertain whether freezing maintains the integrity of *G. aquaticus* chemical cues. If the active compounds in the alarm cues were volatile, peptide-based, or otherwise sensitive to freezing, they may have degraded, leading to the absence of a response.

Another possible explanation for the lack of antipredator response in *G. aquaticus* is that adult gordiids prioritize reproduction over predator avoidance. Once gordiids reach adulthood, they stop nutrient uptake, in some species the mouth opening is fully closed at that stage, and most internal space is taken up by gonads (34). Given that mating occurs quickly after, or potentially while exiting the host, adult gordiids may exhibit reduced sensitivity to predation risk, as avoiding predators offers no evolutionary advantage once reproductive success is achieved (10, 25). This should especially be relevant for males, as they can mate immediately upon finding a female without the additional energy investment required for egg laying. As a result, antipredator behaviors may be largely irrelevant for *G. aquaticus* males at this life stage, potentially explaining the absence of a response to alarm cues in our study.

How organisms react to chemical cues often depends on the concentration of those cues in their surroundings (29, 31). Cue sensitivity varies greatly across taxa, and due to resource constraints, we did not assess whether the concentration used in our study was appropriate for *G. aquaticus* males. Future experiments should explore dose-dependent responses to determine the threshold at which these worms detect and react to chemical cues. Additionally, stress from handling, changes in environmental conditions, or captivity effects could have further affected the behavior, potentially masking any antipredator or mate-finding responses.

It is also entirely possible that mating behavior in *G. aquaticus* is not controlled by chemical cues. Although previous research has demonstrated that some gordiid species possess sensory receptors, their functional roles remain unclear (17, 18). Specifically, it is not yet known whether these receptors detect chemical signals, mechanical stimuli such as pressure, or factors such as temperature or light. Gaining a clearer understanding of the function of these sensory structures should therefore be a key priority for future research.

Lastly, another limitation of our study is the low number of tested individuals. With such a small sample size, subtle behavioral changes may have gone undetected, and our results should be interpreted with caution. Increasing the number of replicates should be a priority for future studies to improve statistical reliability and allow for more robust conclusions about *G. aquaticus'* responses to chemical cues.

Despite these challenges, our findings contribute to a growing body of knowledge on the sensory ecology of gordiids. Further investigations should focus on optimizing experimental protocols, such as adjusting collection timing and the concentration of chemicals tested. Additionally, studies should explore other potential environmental cues, such as visual or tactile stimuli, that could be involved in mate finding and predator avoidance. Future research should also incorporate live animals of the opposite sex in experimental trials to assess whether their presence influences behavioral responses. Investigating these questions further will allow future studies to better characterize how gordiids perceive their surroundings and what drives their reactions to different environmental cues.

MATERIALS AND METHODS

Animal Collection and Husbandry

Free-living *G. aquaticus* from puddles at Burschenplatz (Jena, Germany) were collected on June 27th and July 4th, 2024, when the stream had nearly dried out, leaving only small, isolated puddles. A total of 16 males (only 12 were used in the experiments due to errors in determining the sex of 4 animals) and 2 females were collected and transported to the Institute of Zoology and Evolutionary Research at the University of Jena. The animals were transported for less than an hour in a simple plastic container filled with water from the sampling site and kept at approximately room temperature. At the University of Jena, the worms were housed individually in aerated mineral water (Volvic Water) at a room temperature of 17°C for two days before the behavioral experiments. This acclimation period allowed for recovery from the stress of capture while preventing prolonged captivity, which could lead to mortality. There was no need to feed the animals during that time, because in their adult aquatic stage of life they do not consume any kind of food and even reduce their digestive system to make room for reproductive organs. The sex of each worm was determined microscopically by examining the posterior end: males have a bilobed structure while females are unbranched.

Behavioral Experiment

To assess worm activity, behavioral trials were conducted using a round dish (diameter = 24 cm) to eliminate corner effects. At the beginning of each trial, the focal animal was placed in 200 mL of fresh mineral water, which was replaced before each trial. Mineral water was used specifically since the host laboratory had success housing nematomorphs in this water in the past and uses it for their experiments with other aquatic invertebrate organisms. The focal animals were recorded from directly above using an iPhone at 47 cm (**Figure 3**). As a negative control, a subset of individuals were exposed to water from their original sampling location (Burschenplatz Water, BPW), which was collected on the day of gordiid collection. To test for mate-finding behavior, worms were exposed to Conditioned Medium (CM), which was mineral water that had contained individuals of the opposite sex for at least 48 hours. Specifically, a single worm was kept in about 50 mL of mineral water, which was used without dilution for the experimental trial. Finally, to assess predator-avoidance response, worms were exposed to mineral water containing ground *G. aquaticus* tissue (GT), simulating the presence of injured or dead conspecifics. To make the GT medium, two of the collected gordiids were ground into small parts of tissue with a small plastic pestle. The ground-up worms were equally divided into six 1.5-mL microreaction tubes along with enough mineral water to fill the tubes. These GT samples were frozen at -80°C and thawed on the day of the trial. For each trial, the movement of the focal animal were recorded for 10 minutes in untreated mineral water to establish

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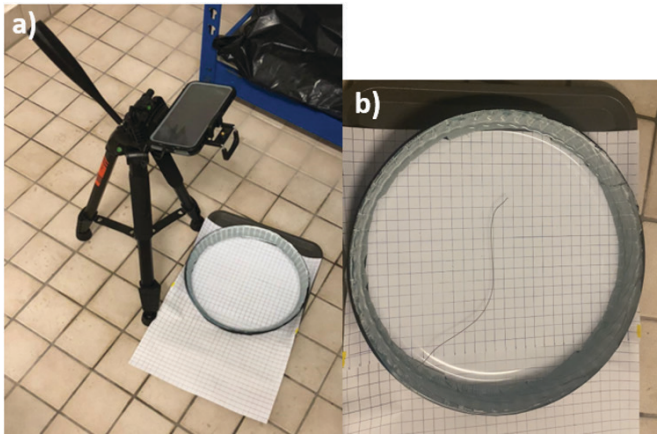


Figure 3: Experimental setup to record movement of the focal animal. a) The camera (IPhone) was placed at a distance of 47 cm above the focal animal. We recorded the movement of the focal animal for 10 minutes in untreated mineral water to establish baseline behavior, followed by an additional 10 minutes after the introduction of the respective test medium. b) Example image of what the camera recorded for each of the treatments, with the parasite currently in the middle of the dish.

baseline behavior, followed by an additional 10 minutes after the introduction of the respective test medium. The treatment was added by forcefully dispensing two disposable 3-mL plastic pipettes on the opposite side of the dish relative to the focal animal.

Data Analysis

For the data analysis, a free event logging software for video/audio coding called BORIS (Behavioral Observation Research Interactive Software) (35) was used. In BORIS a new project with the recorded footage was created. To analyze the data, the specific events "line crossing upwards", "line crossing downwards", "line crossing leftwards" and "line crossing rightwards" were defined. With this it was possible to precisely monitor and capture the nematomorph's movements. A line crossing or movement was defined as follows: When the front, or head region, of the focal animals crossed a line of the grid at the bottom of the testing arena. No other part of the body was considered, as we wanted to test for directed movement. Due to time and resource constraints, each video was only analyzed by one person (three people in total).

All statistical analyses and graph creation was done in R studio (RStudio Team, 2020; version 2024.12.0) (36). The graph was then edited in Inkscape (Inkscape Project, 2020) (37). Normal distribution of the data was assessed via Shapiro-Wilks-Test and visual inspection. The data for BPW and CM were normally distributed, allowing for the use of a paired t-test to compare the number of movements before and after treatment. In contrast, the data for GT did not meet the assumption of normality; therefore, the Wilcoxon signed-rank test was used to evaluate differences in movement.

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