

RELATIVE EFFECTS OF TEMPERATURE, LIGHT, AND HUMIDITY ON CLINGING BEHAVIOR OF METACERCARIAE-INFECTED ANTS

C. F. Botnevik, J. Malagocka, A. B. Jensen, and B. L. Fredensborg

Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark.
Correspondence should be sent to: blf@plen.ku.dk

ABSTRACT: The lancet fluke, *Dicrocoelium dendriticum*, is perhaps the best-known example of parasite manipulation of host behavior, which is manifested by a radically changed behavior that leaves infected ants attached to vegetation at times when transmission to an herbivore host is optimal. Despite the publicity surrounding this parasite, curiously little is known about factors inducing and maintaining behavioral changes in its ant intermediate host. This study examined the importance of 3 environmental factors on the clinging behavior of red wood ants, *Formica polyctena*, infected with *D. dendriticum*. This behavior, hypothesized to involve cramping of the mandibular muscles in a state of tetany, was observed in naturally infected *F. polyctena* under controlled temperature, light, and humidity conditions. We found that low temperature significantly stimulated and maintained tetany in infected ants while light, humidity, ant size, and infection intensity had no influence on this behavior. Under none of the experimental conditions did uninfected ants attach to vegetation, demonstrating that tetany was induced by *D. dendriticum*. Temperature likely has a direct impact on the initiation of clinging behavior, but it may also serve as a simple but reliable indicator of the encounter rate between infected ants and ruminant definitive hosts. In addition, temperature-sensitive behavior manipulation may protect infected ants from exposure to temperatures in the upper thermal range of the host.

Parasite manipulation of host behavior with the aim of increasing transmission of infective stages to a next host is a widespread phenomenon described from most parasite phyla (Moore, 2002). Unfortunately, the underlying mechanisms of host behavior manipulation are unknown in all but a few host–parasite models (Adamo, 2013; van Houte et al., 2013). In many cases, the behavioral changes of infected hosts are relatively subtle and may be manifested as a general increase in activity or of behaviors also performed in uninfected individuals. However, some parasites induce behaviors uniquely associated with infection (Thomas et al., 2002; Hoover et al., 2011), which occur specifically when the parasite is infective to a next host (Bethel and Holmes, 1974; Hughes et al., 2011) and/or which location in the host demonstrates a link between infection and behavioral modification of the host (Romig et al., 1980; Helluy, 1983; Vyas et al., 2007). Those parasites may be particularly amenable to the investigation of proximate causes triggering host behavior manipulation, and they may in turn help to unravel the mechanistic basis for inducing and regulating those behaviors.

The trematode *Dicrocoelium dendriticum* represents a classic textbook example of the ability of parasites to radically change host behavior to complete their life cycle. In addition, it is of financial importance because the adult worm is the causative agent of dicrocoeliasis, which is a chronic inflammation of the bile ducts in wild and domestic ruminants (Otranto and Traversa, 2003). The unusual life cycle of this species remained enigmatic until about 60 yr ago when ants were discovered as the required second intermediate hosts (Krull and Mapes, 1952). Adults live in the bile ducts of wild or domestic ruminants, and eggs are voided with host feces to the external environment.

The first intermediate host is a terrestrial snail which accidentally ingests the eggs of *D. dendriticum*. Sporocysts located in the digestive gland release mature cercariae, which migrate to the respiratory chamber, where they provoke an excess production of slime, and the cercariae are shed into the environment in host-produced slime balls. Ants of the genus *Formica* act as the second intermediate hosts and they become infected by ingestion

of slime balls containing cercariae. After ingestion, cercariae penetrate the gut wall and enter the hemocoel of the ant. After a short resting period, the cercariae travel toward the brain, and 1 (or more rarely 2) cercaria (the “brainworm”) lodges itself against the subesophageal ganglion (Schneider and Hohorst, 1971; Romig et al., 1980). The brainworm somehow induces migration of the remaining cercariae back to the gaster where they develop into metacercariae (Schneider and Hohorst, 1971).

Infected ants climb vegetation and cling by use of the mandibles to a leaf or a stem. Previous literature and the currently-accepted hypothesis states that clinging behavior is caused by a temporary cramping of mandibular muscles in a state of tetany (Paraschivescu and Raicev, 1980). However, the role of the mandibular muscles in clinging behavior remains to be verified. It is believed that the brainworm is responsible for the behavior manipulation, but it is unknown what stimuli induce the altered behavior and what underlying mechanisms are involved (mechanical and/or neuromodulatory). Thus, despite the iconic nature of host behavior manipulation by *D. dendriticum*, the clinging behavior in ants has never undergone experimental scrutiny, and most of the natural history of this host–parasite interaction is based on anecdotal evidence.

Observations from field studies report infected ants in tetany primarily in the morning and evening hours (Badie et al., 1973; Spindler et al., 1986; Schuster, 1991; Manga-González et al., 2001; Manga-González and González-Lanza, 2005) at times when the probability of trophic transmission to grazing herbivores presumably is highest (Hester et al., 1996). The state of tetany is reversible, and infected ants will detach from the vegetation and climb down to presumably resume normal ant behavior. In all previous studies temperature was suspected to determine when ants enter tetany because it typically takes place in the cool hours of the day, but it is unknown whether tetany is induced by a single cue or by a suite of interacting environmental stimuli. Thus, other environmental variables such as light and relative humidity covary with temperature. The contribution of each of those variables cannot easily be determined in field studies, and to our knowledge no studies have experimentally tried to tease apart the effects of different environmental stimuli on the initiation or duration of tetany in infected ants.

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In this study we used naturally infected ants to test the effects of temperature, light, and relative humidity on tetany in ants under controlled experimental conditions. We also tested if tetany was unique to infected ants and if ant size or the abundance of metacercariae located in the gaster affected this behavior. Thus, this study is the first to systematically investigate the impact of multiple environmental factors on the initiation and maintenance of a key behavioral trait presumably associated with parasite-induced trophic transmission in *D. dendriticum*.

MATERIALS AND METHODS

Field collections

Formica polyctena worker ants were collected 6 October 2013 and again 16 October 2014 from the Bidstrup Forests situated south of Roskilde Fjord, Zealand, Denmark (55.58°N, 11.87°E). *Formica polyctena* habitat was characterized as mixed stands of mature hardwood and coniferous trees, and ant hills were typically located adjacent to stands of Norway spruce (*Picea abies*) on the edge of clearings dominated by grasses and annual herbs. Seven sites consisting of single ant nests or super-colonies with interconnecting trails were identified as sources of infections and therefore included in the collections of infected and uninfected ants. Worker ants found in tetany (2013: $n = 96$, 2014: $n = 77$) were collected from the vegetation within a perimeter of 1 m from the ant nests where most infected ants occurred (Badie and Rondelaud, 1988). The leaf/grass blade, which the infected ant was attached to, was gently cut off and each transferred to a 15-ml test tube.

The control group (2013: $n = 93$, 2014: $n > 100$) was collected from the ant nests at the same time by placing a handkerchief on top of the ant nest for 5 min for the ants to latch on to. Control ants were transported back to the lab in a plastic container (17 × 11 × 6 cm). Infected ants and ants in the control group were kept at 5 °C for 12–48 hr prior to testing.

Sampling in both years occurred between 1400 and 1700 hr. This time period may not be optimal in terms of achieving the greatest number of infected ants, which are more common in the morning and evening hours (Spindler et al., 1986). However, it was selected to avoid collecting ants infected by the entomopathogenic fungus *Pandora formicae*, which induces the same behavior as *D. dendriticum* (Malagocka, 2016). Ants infected with *P. formicae* are almost exclusively found in tetany in the evening after which the fungus rapidly kills the ant, produces conidiophores, and spreads its conidia spores during the night and early morning hours (Malagocka, 2016).

Light and temperature study

Experiments controlling for light and temperature were performed in temperature- and light-controlled incubators over 3 days in the beginning of October 2013. The selection of temperatures used in the experiments was based on a pilot study on 22 infected ants where the occurrence of tetany was observed in an incubator at temperatures ranging from 10–26 °C. Seventy-two infected and 72 uninfected specimens were haphazardly selected, and each ant was placed in a 15-ml conical bottom test tube containing a fresh blade of grass (*Dactylis glomerata*). The test tubes were individually marked, sealed with a plastic cap, and placed in racks in an upright position after which the ants were acclimatized to room temperature (21 °C) for at least 1 hr. The infected and uninfected ants were evenly divided into 3 groups of 24 individuals and transferred to an incubator held at a constant temperature of 12, 20, or 25 °C. Within each temperature group, 8 infected and 8 uninfected ants were separated into a light and a dark treatment group while 8 infected and 8 uninfected ants were exposed to 6 hr of light followed by 6 hr of dark within each of the 3 temperatures. Racks with ants assigned to the dark treatment group were covered with black plastic but kept in the same incubators as the light treatment group. The ants were inspected every 3 hr for 12 hr, and the presence-absence of tetany (attachment to the grass) was noted for each ant. The relative humidity (RH) in the test tubes was not controlled but, as the test tubes were sealed, the RH was assumed to quickly reach equilibrium when held at constant temperature and remain stable for the duration of the study.

After the experiment all ants were measured from the tip of the mandibles to the end of the gaster and dissected to confirm the infection

status and to count the number of metacercariae in the gaster. The length and width of 5 metacercariae were measured under a compound microscope at ×100. The brainworm(s) could not be identified by dissection, and ants harboring metacercariae in the gaster were assumed to also harbor at least 1 unencysted metacercariae at the subesophageal ganglion as described in previous studies (Schneider and Hohorst, 1971; Romig et al., 1980). In addition, the presence and identification of *D. dendriticum* in the head and gaster was confirmed by DNA extraction and sequencing (see below).

Relative humidity and temperature study

In October 2014, the effect of humidity on the state of tetany was tested with 2 different RH levels, a low (~30% RH) and a high (~80–90% RH). Humidity settings were selected based on the natural humidity preferences for a close relative, *Formica rufa* (North, 1991). A saturated salt solution, which yielded the lowest relative humidity while remaining stable at the 3 different temperatures (Winston and Bates, 1960), was chosen as the low humidity treatment. To keep a high RH, a container with 100 ml tap water was placed inside the incubator. A low RH was kept by a saturated salt solution of magnesium chloride (54.3 g/100 ml water) in a container inside the incubator. Light condition was set to dark and the same 3 temperatures used in the light and temperature experiments were applied. Gauze was used to cover the test tubes instead of a cap to expose the ants to the same relative humidity as that of the incubator. Similarly to the temperature and light study, the experiment was performed within 48 hr of collection. Fifty-six infected ants and 60 uninfected ants were used in the experiments with 10 individuals per group. Unfortunately, due to mortality among the infected ants, the high temperature-high humidity treatment had to be completed with a sample size of 5 infected individuals. The experiment was ended after 6 hr. After the experiment all ants were dissected to confirm infection status and to count the number of metacercariae in the gaster as described above.

Presence and identification of *D. dendriticum*

The brainworm is difficult to detect and identify by dissection. We therefore verified the presence and species identification of the brainworm as well as metacercariae located in the gaster. We did this by separating the head from the rest of the body in 3 ants collected in tetany and, from 1 ant, metacercariae were extracted by opening the gaster. Total genomic DNA was extracted from these 7 subsamples using the 2×CTAB method of Doyle and Doyle (1987).

A fragment of the ITS II was amplified using *D. dendriticum*-specific primers (Dd ITS-2_F: 5' ACACACCTAGTTATCAGACAGG 3') and (Dd ITS-2_R: 5' CACCACACGAGATGTTCTACAG3') (Martinez-Ibeas et al., 2011). Amplification was carried out in a 20-μl reaction volume containing 12.5 μl 2×Taq PCR Master Mix Kit (Qiagen, Hilden, Germany), 1 μl primer Dd ITS-2_F at a concentration of 10 μM, 1 μl primer Dd ITS-2_R at a concentration of 10 μM, 5.5 μl MilliQ water, and 5 μl of DNA extraction. The amplification was done on Biometra® T1 thermocycler (Biometra, Göttingen, Germany) with initial denaturation at 92 °C for 2 min followed by 35 denaturation cycles (95 °C, 30 sec), annealing (63 °C, 30 sec) and extension (72 °C, 1 min), with a final extension phase at 72 °C for 10 min. PCR products were electrophoretically separated on 2% agarose gels in TAE buffer and visualized with EZvision One® (Solon, Ohio). PCR products were cleaned using a QIAquick® PCR purification kit (Qiagen) and sent to Beckman Coulter Genomics (Essex, U.K.) for sequencing with both forward and reverse primers. Sequences were assembled with Sequencher® version 5.3 and the sequences were subjected to a BLASTN search in NCBI (Morgulis et al., 2008).

Statistics

In both experiments a stepwise multiple logistic regression analysis was used to test the effect of environmental stimuli (temperature, light, and humidity) and intensity of infection on tetany in infected ants at any of the observation time points during the study. In the first experiment the predictor variables were temperature (12, 20, and 25 °C), light (light and dark), temperature × light, the intensity of infection, and ant body length. Because light showed no significant effect on tetany, the treatment involving 6 hr of light followed by 6 hr of dark was included in the analysis for each temperature treatment. In the second experiment the predictor variables were temperature (12, 20, and 25 °C), relative humidity (30 and 80%), temperature × relative humidity, and intensity of infection. In the

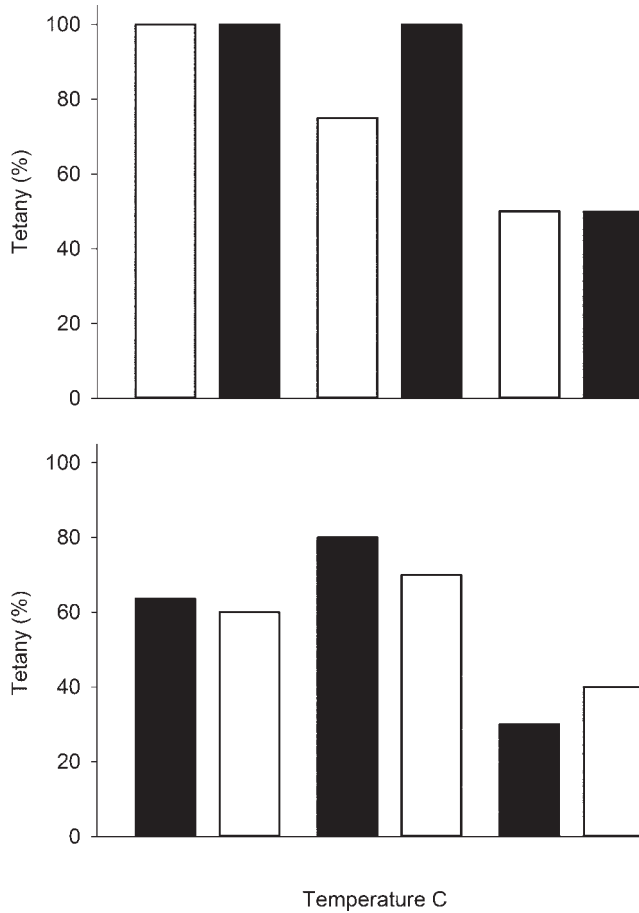


FIGURE 1. Effects of temperature and light (A) and temperature and relative humidity (B) on the percentage of infected red wood ants, *Formica polyctena*, observed in tetany on at least 1 observation (3, 6, 9, or 12 hr post-incubation) at 1 of 3 temperatures (12, 20, or 25 C) and 1 of 2 light conditions (light [white bars] or dark [black bars]) (A), and at 1 of 2 relative humidity conditions (low [30–40%; white bars] or high [80–90%; black bars]) (B). In (A), each treatment combination consisted of 8 individuals. In (B), each treatment combination consisted of 10 individuals, except the high relative humidity–high temperature treatment, where only 5 individuals were used.

analyses, the predictor variables were entered in a hierarchical manner starting with variables independently explaining the greatest amount of variation in the probability of entering tetany. Only variables contributing a significant contribution were retained in the model. Planned contrasts among the three levels of temperature were conducted for both experiments.

Because tetany is reversible, it was also of interest to know if temperature had an effect on the duration of attachment among ants displaying this behavior. Therefore, in the light and temperature experiment a chi-square (χ^2) test (3×4 contingency table) was used to compare the frequency of ants observed in tetany at 1, 2, 3, or all 4 observation points (3, 6, 9, and 12 hr) for the 3 temperatures. Statistical analyses were conducted using the JMP® 11.0 software package (SAS Institute Inc., Cary, North Carolina) and used $\alpha = 0.05$ as the significance level.

RESULTS

Our results from both studies showed that none of the uninfected ants ($n = 129$) displayed tetany regardless of the experimental conditions. Therefore, only infected ants were

TABLE I. Stepwise multiple logistic regression results for test of predictor variables explaining the state of tetany among infected *Formica polyctena* workers in experiment 1. The dependent variable was the state of tetany (0 or 1) and the predictor variables were temperature (12, 20, or 25 C), light (light or dark), the interaction between temperature and light, the number of metacercariae in the gaster, and ant body length. Note: $R^2 = 0.291$ (Nagelkerke); Model $\chi^2 (1) = 15.49$, $P = 0.0004$.

Predictor	β	SE (β)	Wald's χ^2	df	P^*
Constant	−1.58	0.41	14.91	1	0.0001
Temperature			11.25	2	0.0036
12 C vs. 25 C	3.14	1.10	8.12	1	0.0040
20 C vs. 25 C	1.61	0.68	5.55	1	0.0180
12 C vs. 20 C	−1.53	1.16	1.73	1	0.1880
Predictors not included					
Light	—	—	—	—	—
Light \times temperature	—	—	—	—	—
Number of metacercariae	—	—	—	—	—
Ant body length	—	—	—	—	—

* P -values in bold indicate variables with a statistically significant contribution to the model and significant differences in the planned contrasts among the 3 levels of temperature.

considered for the analyses of the effect of temperature, light, and relative humidity on the probability that ants would enter tetany at least at 1 observation time point during the experiments.

Temperature, light, and relative humidity

Temperature had a significant effect on tetany in infected ants in both experiments with ants being more likely to display tetany at 12 or 20 C compared to 25 C (Fig. 1A; Tables I, II). Light did not significantly affect the probability of tetany, and it did not interact significantly with temperature (Table I).

The intensity of infection varied from 1 to 216 metacercariae in the light and temperature experiment (mean = 52.2 ± 5.4 , $n = 72$) and varied from 1 to 170 metacercariae in the temperature and relative humidity experiment (mean = 39.6 ± 4.9 , $n = 56$). The

TABLE II. Stepwise multiple logistic regression results for test of predictor variables explaining the state of tetany among infected *Formica polyctena* workers in experiment 2. The dependent variable was the state of tetany (0 or 1) and the predictor variables were temperature (12, 20 or 25 C), relative humidity (30% or 80%), the interaction between temperature and relative humidity, and the number of metacercariae in the gaster. Note: $R^2 = 0.170$ (Nagelkerke); Model $\chi^2 (1) = 7.57$, $P = 0.023$.

Predictor	β	SE (β)	Wald's χ^2	df	P
Constant	−0.79	0.54	2.14	1	0.144
Temperature			6.89	2	0.032
12 C vs. 25 C	1.41	0.72	3.88	1	0.049
20 C vs. 25 C	1.89	0.75	6.39	1	0.011
12 C vs. 20 C	0.48	0.70	0.47	1	0.492
Predictors not included					
Humidity	—	—	—	—	—
Humidity \times temperature	—	—	—	—	—
Number of metacercariae	—	—	—	—	—

* P -values in bold indicate variables with a statistically significant contribution to the model and significant differences in the planned contrasts among the 3 levels of temperature.

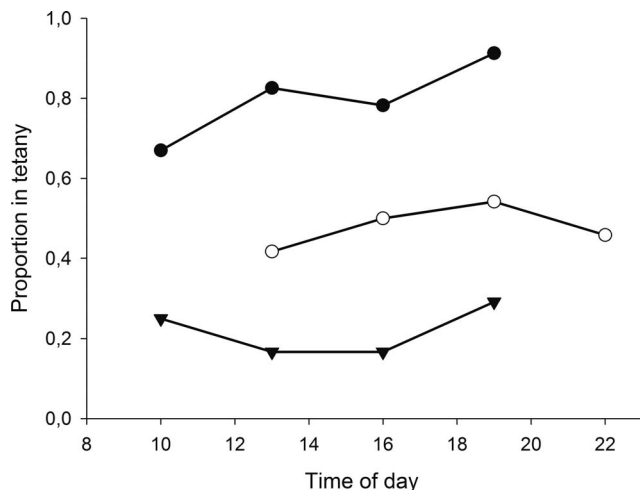


FIGURE 2. Proportion of infected *Formica polyctena* in tetany in relation to time of day during a 12-hr study at the 3 temperatures of 12°C (closed circles), 20°C (open circles), and 25°C (closed triangles). $n = 24$ individuals per temperature.

mean intensity of infection did not differ among the 3 temperature groups (light and temperature experiment: Kruskal Wallis test, $H = 0.960$, $df = 2$, $P = 0.619$, $n = 72$; relative humidity and temperature experiment: Kruskal Wallis test, $H = 1.474$, $df = 2$, $P = 0.479$, $n = 56$). The intensity of infection did not significantly affect the probability of an infected ant displaying tetany in any of the 2 experiments (Tables I, II).

Mean ant size, which was recorded in the light and temperature experiment, was not related to the intensity of infection (linear regression: $F = 0.731$, $df = 1$, $P = 0.396$, $R^2 = 0.012$) nor did it significantly affect tetany of infected ants (Table I).

The relative humidity did not affect tetany in infected ants, and no interaction was detected between relative humidity and temperature (Fig. 1B; Table II). In addition to affecting the probability of tetany, temperature also influenced the duration of this behavior, as indicated by the number of ants observed in tetany on 1, 2, 3, or 4 observation times ($\chi^2 = 21.297$, $df = 6$, $P = 0.002$). Thus, 68.2% of ants in tetany at 12°C were observed in the manipulated state on more than half of the observation points compared to only 16.7% of ants in tetany at 25°C.

The light and temperature experiment conducted for 12 hr did not indicate any periodicity in tetany, and the percentage of ants in tetany remained almost constant after the first 6 hr (Fig. 2).

Presence and identification of *D. dendriticum*

A PCR band was produced from heads, gaster, and clean metacercariae. The 94 bp ITS II fragment sequenced from 3 ant heads, 3 ant bodies, and metacercariae were identical, except for 1 ambiguous A/C position, and were 100–99% similar to all published *D. dendriticum* sequences. The closest relatives, *Dicrocoelium chinensis* and *Dicrocoelium orientalis*, differed with 9–10 bp.

DISCUSSION

Our study demonstrated that tetany is a behavior uniquely observed in infected ants, confirming previous anecdotal evidence

that *D. dendriticum* manipulates ants to attach to vegetation. This behavior was strongly affected by temperature while light and humidity did not have a significant effect, neither alone nor in combination, with changes in temperature. Infected ants were significantly less likely to be observed in tetany at 25°C compared to 12 and 20°C, while there was no significant difference between the latter 2 temperatures. Thus, our data are in accordance with previous field studies reporting ants in tetany primarily at temperatures at or below 20°C (Badie et al., 1973; Spindler et al., 1986). The intensity of infection did not influence whether infected ants were found in tetany, and our study thus confirms previous assumptions and studies on another trematode–arthropod relationship, that only the brainworm manipulates the behavior of the host (Thomas et al., 2000).

In addition, our study showed that time spent in tetany was strongly influenced by temperature. Ants entering tetany at 12°C remained attached for the majority of the study while ants in tetany at 25°C were much more likely to detach from the vegetation. It was therefore evident that the manipulation of host behavior by *D. dendriticum* relies on temperature as the most important environmental cue, although it remains to be tested why temperature is important. This could be due to multiple alternative explanations which are not necessarily mutually exclusive. First, temperature may be directly linked to the mechanism of behavioral manipulation. The brainworm in *D. dendriticum* is located adjacent to the subesophageal ganglion at the origin of the mandibular nerves (Romig et al., 1980). Interestingly, the location of the brainworm is also the region in the subesophageal ganglion which contains the highest concentration of cell bodies of the mandible closer muscle motor neurons (Paul and Gronenberg, 2002). Unlike metacercariae located in the gaster, the brainworm is unencysted or only surrounded by a thin cyst layer of 1–2 μm compared to a cyst wall thickness of 11–12 μm in metacercariae found in the gaster (Romig et al., 1980). It therefore seems plausible that the incomplete development of the cyst wall in the brainworm is essential to manipulate mandible muscle function. The role of temperature in this process is unclear, but it is possible that low temperature decreases the rate of stimulation required to achieve unfused tetany of muscles.

It is also possible that the effect of temperature is primarily indirect by acting as a simple but reliable indicator (time giver) of the optimal window of transmission from the ant second intermediate host to an herbivore definitive host. In south and central Europe, a strong diurnal periodicity in the occurrence of infected ants in tetany has been observed, with the maximum number of ants in tetany observed in the early morning and evening hours while relatively few ants occur in tetany during the afternoon (Badie et al., 1973; Paraschivescu, 1983; Spindler et al., 1986; Manga-González et al., 2001; Manga-González and González-Lanza, 2005). If the diurnal pattern is an adaptation to maximize transmission, then temperature should also be a reliable indicator of the activity of the hosts. Field studies showed that daily temperature cycles strongly influenced activity of *F. polyctena* with a peak activity on the trails observed at a ground temperature of 20–25°C (De Bruin and Kruk-De Bruin, 1972; Mabelis, 1979). Light itself was not a controlling factor for ant density on the trails, and ants were observed foraging at all hours (Rosengren, 1977; Mabelis, 1979; North, 1993). However, combined light:dark and temperature cycles produced the strongest periodicity in ant activity, presumably with changes in

temperature as the time giver for initiating activity (North, 1993). Those findings correspond with field studies demonstrating a bimodal pattern of activity peaking in the morning followed by a second, less pronounced peak around dusk (Rosengren, 1977; Mabelis, 1979; North, 1993).

Studies on diurnal activity patterns of potential herbivore definitive hosts indicate that grazing is generally most pronounced around dusk and dawn (Georgii, 1981; Cederlund, 1989; Carranza et al., 1991; Hester et al., 1996; Kamler et al., 2007), coinciding with the cooler times of the day. However, grazing activity does not cease during the day, and roe deer (*Capreolus capreolus*) in particular, presumably the most important definitive host for *D. dendriticum* at our sampling site, may have additional bouts of grazing throughout the day (Cederlund, 1989).

Finally, temperature-sensitive behavioral manipulation may serve to avoid exposing the infected ant to potentially lethal temperatures during the hottest time of the day. This is supported by field observations where more ants in tetany are observed throughout the day during overcast conditions when the temperature is lower than usual (Manga-González et al., 2001). Ants used for our study were collected in the afternoon, so tetany is clearly not restricted to mornings and evenings at our collection site. In the laboratory, time of day (1000 to 2000 hr) did not seem to affect the percentage of ants in tetany, which remained stable after 3–6 hr, suggesting that the effect of temperature overrides the internal clock of the ants. However, the study was not designed to investigate the circadian rhythm of ant behavior in relation to infection, and keeping the ants at 5 °C in dark conditions up to 48 hr could have disrupted the circadian clock of the ants. Further studies allowing ants to maintain their diurnal activity pattern are therefore clearly needed.

Interestingly, a very similar behavior occurs in ants infected by some entomopathogenic fungi (Hughes et al., 2011; Malagočka, 2016). In the Bidstrup Forest, *F. polycytena* is infected by *P. formicae*, which induces tetany immediately preceding the death of the host and the subsequent release of infectious conidial spores. It remains unknown if the mechanisms involved in tetany in the 2 parasites are similar. However, in *P. formicae* the attachment to vegetation and transmission of spores is restricted to the night and early morning hours, indicating that the circadian rhythm is the driving force in ant behavioral manipulation by parasitic fungi (Malagočka, 2016).

In conclusion, our study demonstrated a significant effect of temperature on tetany in *F. polycytena*, while neither light nor relative humidity influenced this behavior. We suggest that temperature acts both as an important time giver in the circadian rhythm of ant behavior and as the main stimulus for attachment of infected ants to vegetation. Together, those 2 effects allow *D. dendriticum* to maintain an otherwise unlikely transmission event to ruminants.

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