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Examining Behavioral Defenses of the Fungus-growing Ant Trachymyrmex septentrionalis Against Trichoderma-derived Metabolites

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Examining Behavioral Defenses of the Fungus-growing Ant *Trachymyrmex septentrionalis* Against *Trichoderma*-derived Metabolites

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Abstract

Fungus-growing ants defend their fungal mutualist against pathogens through a number of chemical and behavioral mechanisms. Work by Dr. Jonathan Klassen's lab has found that *Trachymyrmex septentrionalis* ants maintain their fungal symbionts' health through physical removal ("weeding") upon infection with pathogenic fungi in the genus *Trichoderma*. Ants also exhibited weeding behavior when the fungus was inoculated with peptaibols, a class of secondary metabolites produced by *Trichoderma*, suggesting that these molecules act as chemical cues that induce ant weeding. However, the mechanisms by which weeding behaviors are communicated between the ants and their fungal symbiont are unknown. Behavioral experiments demonstrated that *T. septentrionalis* ants exhibit weeding behaviors in response to *Trichoderma* metabolite cues and to cultivar death and that peptaibol-induced weeding has a characteristic temporal pattern that is no longer present after cold treatment. This work provides several directions for further behavioral and molecular analysis to determine if weeding behaviors in response to different stimuli are mediated through similar or distinct mechanisms.

Introduction

Fungus-growing or attine ants are a group of about 200 species that maintain a symbiotic relationship with a coevolved "cultivar" fungus as their primary source of nutrition [1]. Attine ants construct underground "fungus garden" structures in their nests where they protect their cultivar and supply it with foraged substrates such as plant material. In exchange, the fungus produces specialized hyphal growths that the ants use as their main food source [1–2]. This relationship is an obligate mutualism, meaning that the organisms involved benefit from each other in a way that makes them dependent on each other for survival. Therefore, pathogens that target the cultivar may also leave the ants vulnerable and vice versa. The ants protect their cultivar through several chemical and behavioral

measures that can be described as an "extended" defense response against threats to the mutualism, such as grooming cultivar hyphae with their mouth parts to remove pathogenic spores [4]. Furthermore, many fungus-growing ants participate in another defensive symbiosis with antibiotic-producing *Pseudonocardia* bacteria [5–6]. However, it is unknown how such extended responses are communicated and mediated between the ant host, fungal mutualist, and bacterial symbionts.

Work by Dr. Jonathan Klassen's lab and others has established *T. septentrionalis* as an especially suitable model system for studying the attine ant-fungus mutualism. As the northernmost attine ant, *T. septentrionalis* occurs widely in the eastern US and overlaps minimally with related species [7], minimizing possible variability from cultivar switching. Furthermore, its small colony size allows for the collection and maintenance of colonies in a lab setting to carry out experiments. Recent work by the Klassen lab found using ITS2 community amplicon sequencing that *Trichoderma* spp. were the most abundant non-cultivar fungus in wild *T. septentrionalis* fungus gardens collected across the Eastern US [8]. *Trichoderma* is a genus of soil fungi that exhibit broad-spectrum antifungal activity through the production of antibiotics and cell wall-degrading enzymes [9–10]. In vitro experiments with *T. septentrionalis* fungus gardens treated with *Trichoderma* isolates showed that *Trichoderma* acts as an opportunistic pathogen of the cultivar and overwhelms the fungus garden in the absence of the ants. However, ants prevented *Trichoderma* from killing the cultivar fungus, notably by deconstructing ("weeding") infected parts of the fungus garden [8]. Metabolomics analysis of fungus garden waste generated during these experiments found that peptaibols, a class of secondary metabolites produced by *Trichoderma* [11–12], were abundant during infection. Similar analysis with field samples detected peptaibols in several wild fungus gardens, suggesting that *Trichoderma* infects *T. septentrionalis* fungus gardens in the wild and that peptaibols serve as a key marker of infection [8]. Furthermore, ants displayed elevated weeding behavior when the fungus garden was treated with peptaibol-rich fractions of *Trichoderma* extract and with purified peptaibols [8], suggesting that peptaibols are a chemical signal of *Trichoderma* infection that is sensed by the ant-fungus mutualists through an unknown mechanism.

Therefore, the principal goal of this project was to clarify how peptaibol-induced defenses are sensed and mediated between these mutualistic partners. The primary hypothesis that I tested was that *T. septentrionalis* mutualists sense fungal infection through one of the following mechanisms: 1) the ants detect infection of the cultivar directly or 2) the cultivar produces a chemical signal when infected that induces a response in the ants and/or their bacterial symbionts.

Results

Freezing experiments: To experimentally test the hypothesis that either the ants or fungus sense *Trichoderma* infection, I assessed ant behavior when crude *Trichoderma* extract was applied to dead fungus gardens. Killing methods were first evaluated by creating subcolonies consisting of 8 ants and fungus gardens that had been heated or frozen at different time intervals. At 72 hours post-treatment, all treatment groups except for the -80 °C/15 minutes and all -20 °C treatments showed highly elevated waste production compared to the negative control (Figure 1). Visual analysis of frozen fungus gardens under a microscope also showed substantial hyphal loss for the -80 °C treatments but little for the -20 °C treatments (Figure 2). These findings suggested that the cultivar fungus had some degree of cold tolerance or did not undergo the same physical or chemical death cues as the other temperature treatment groups where weeding was induced, raising questions about how peptaibol-induced weeding would be influenced by this "mild" treatment compared to the "harsh" (i.e., the -80 °C and heating) treatments.

To evaluate the effect of different freezing treatments on weeding behavior, ant subcolonies were formed from cold-treated fungus gardens at -20 °C and -80 °C and exposed to crude *Trichoderma* extract. Experiments were replicated across five different colonies and trials of each colony were carried out at multiple points in the ants' active season (Table C). In the room temperature group, cumulative weeding after 24 hours was greater in *Trichoderma* extract-treated fungus gardens compared to the negative controls treated with DMSO or left untreated (Figure 3). A similar pattern was observed for the -20 °C treatment groups but not the -80 °C treatment groups, where overall weeding rates were instead highly variable. While the average for all trials in the -80 °C/*Trichoderma* extract treatment group was higher than that of the corresponding no treatment/DMSO groups, individual trials did not show a consistent

trend between the extract-treated and no treatment/solvent-treated groups, suggesting that *Trichoderma* extract treatment did not yield a clear increase in weeding behavior at -80 °C.

Because weeded masses were measured at both 6 and 24 hours, I also compared weeding rates between the 0–6-hour and 6–24-hour intervals (Figure 4). Notably, the room temperature fungus + *Trichoderma* extract treatment exhibited highly elevated weeding rates at the 6-hour interval that declined sharply over the next 18 hours, suggesting that *Trichoderma* metabolites induce a rapid behavioral response when dripped onto the fungus garden. This pattern was distinct from that of the -20 °C fungus + *Trichoderma* extract treatment, where overall weeding rates were elevated compared to the -20 °C untreated and DMSO groups but did not show the same strong weeding response within the first 6 hours. Finally, weeding rates for all -80 °C treatments showed a high degree of variation between individual trials but did not suggest that there were significant differences between each treatment group.

Fungus garden incorporation: *T. septentrionalis* colonies reared in lab conditions incorporate sterilized corn meal into their fungus garden, with significant incorporation occurring shortly after colonies are collected. To evaluate the effect of peptaibols on incorporation behavior, *T. septentrionalis* subcolonies were presented with *Trichoderma* extract-treated corn meal (with corresponding untreated and DMSO-treated subcolonies as controls) and assessed qualitatively using still and time-lapse images. After 12 to 13 days post-treatment, a low baseline level of incorporation was observed among the replicates and there were no discernable patterns when comparing between extract-treated corn meal and solvent-treated or untreated corn meal subcolonies (Figure 5). However, one group of replicates from each colony (Replicate 1 for JKH000448 and Replicate 2 for JKH000449) exhibited higher weeding rates than the other replicate from the colony, as evidenced by noticeable shrinkage of the fungus garden between the beginning and end of the experiment. The behavior of these replicates may have been influenced by several factors. First, the higher-weeding replicates had been placed a row closer to a light source than the lower-weeding replicates, which may have induced stress or simulated different nesting or seasonal conditions. Additional possible factors include variation in initial fungus garden mass, unaccounted-for task division between workers, or variation in colony behavior due to the small number

of replicates, although it is unclear what factors are most responsible for the observed differences and how these factors may interact. Nevertheless, the presence of a consistent behavior pattern across the higher-weeding replicates suggests that it may be valuable to investigate potential causes beyond colony variation.

Bead experiments: Studies have used chemical-coated beads to investigate how ant cuticular hydrocarbon cues influence behaviors such as foraging decisions and colony-mate recognition [13–14]. Given this, another major objective of this project was to evaluate the utility of *Trichoderma* extract-coated beads in studying weeding behavior. To create the treated beads, 1 mm-sized silica beads were left untreated or were treated with crude *Trichoderma* extract or 0.5% DMSO. Behavioral experiments were first conducted to assess how ants would react to bead treatment of the fungus garden. Qualitative assessment of subcolonies after approximately 24 hours showed that the ants readily removed the beads from the fungus garden with no clear trend of bead removal or elevated weeding behavior between the no treatment/DMSO and extract-treated groups (Figure 6).

Experiments with only ants and beads were also carried out to isolate the ants' role in detecting *Trichoderma* metabolites without the fungus garden. Qualitative assessment of subcolonies for up to 5 days showed that the ants manipulated the beads at highly variable rates and that there were no clear trends of manipulation between the bead treatment groups (Figure 7). Some replicates extensively deconstructed the pile of beads (e.g., by the second day: JKH000460 NT 1 and DMSO 1; by the fifth day: JKH000442 DMSO 1 and JKH000462 DMSO 1, crude 1, and crude 3), while others did not manipulate the beads for the duration of the experiment. The observation of substantial variation between replicates within the same colony and treatment group may be explained by task division among workers or the random nature of the ants' decisions to move the beads. One notable confounding variable was the timing of the experiments, which were conducted near the end of the season when many colonies began to weed at elevated rates. Although colonies used for the experiments were selected because they were not displaying this end-of-season behavior, each of the mother colonies began to weed at elevated rates after subcolony creation. To confirm whether peptaibols still induced weeding in each colony, it would

have been necessary to accompany the beads-only experiments with liquid treatment controls (i.e., subcolonies treated with 200 μL of *Trichoderma* extract and 0.5% DMSO).

In summary, the ants interacted with both the *Trichoderma* extract-treated beads and the untreated or solvent-treated beads in some replicates. However, without the liquid treatment controls, it cannot be concluded that the ants do not detect peptaibols in the absence of the fungus garden because it would first be necessary to establish that peptaibol-induced weeding still occurs. Some alternative explanations for the observed results could include task division and random decision-making as described previously; furthermore, it is plausible that seasonal variation distorted the results so that the ants manipulated the beads more readily or less readily than they would have earlier in the season.

Discussion

Overall, my data revealed several trends of interest that may serve as a starting point to better understand weeding behaviors in the ant-fungus mutualism. Ants weeded their fungus gardens at higher rates when the room-temperature fungus was treated with liquid *Trichoderma* extract compared to the no treatment/DMSO groups, replicating a pattern found in previous work by the Klassen lab [8], and exhibited characteristically high weeding rates within the first 6 hours after treatment. Freezing the fungus at -20 °C yielded a similar overall relationship of a low baseline weeding rate for the no treatment/DMSO groups and elevated weeding for the *Trichoderma* extract treatment group, although the latter did not exhibit the same strong initial weeding response as the corresponding room temperature treatment. Finally, ants weeded at variable rates with the harsher -80 °C treatment with a high average for all treatment groups.

Given that ants weeded in response to peptaibol treatment and to freezing at -80 °C, these results raise questions about the extent to which peptaibol-induced weeding is distinct from a more general response to cultivar death or stress. The amphipathic structure of peptaibols suggests that they have membrane-disrupting functions, which is further supported by studies showing that they exhibit inhibitory activity against a variety of fungi and other organisms [12, 15–16]. It is conceivable that weeding responses may be induced both by signals produced by the fungus in response to peptaibols or by ants

detecting physical signs of cultivar death caused by peptaibols. To answer this question, it may be valuable to assess whether peptaibol-induced weeding is localized only to treated areas of the garden or if it is systemic, which may suggest the presence of signaling in response to treatment. This could potentially be done using alternative treatment methods: *Trichoderma* extract was dripped directly onto the fungus garden in this project, but subcolonies could also be created with fungus gardens that had been submerged in extract or contained treated and untreated portions in the same enclosure. Another approach may be to conduct the experiments over a longer timeframe than the 24 hours used in these experiments, as longer-term experiments may reveal if weeding remains localized to the treated area or if the fungus garden is completely disassembled, which would suggest a broader response**.** Behavioral data and molecular analysis of samples generated from such experiments may be useful in understanding if weeding behavior is induced by signaling, physiological changes in the fungus, or both, and could potentially identify genes or signaling molecules associated with weeding behaviors.

Weeding in the -20 °C/*Trichoderma* extract treatment group showed a different temporal pattern compared to the room-temperature/extract-treated group, suggesting that peptaibol-induced weeding responses are somehow affected by the freezing treatment. This difference raises questions about how peptaibol-induced weeding behavior may be affected by gene expression in the cultivar. It is plausible that genes for cold tolerance in the cultivar were more highly expressed than those responsible for peptaibol signaling during the freezing treatment, preventing a strong initial weeding response from the 0–6 hour interval. Molecular analysis of samples generated from this work or similar experiments done in the future may reveal transcriptional differences that could provide additional evidence for this hypothesis. This analysis may help to uncover the signaling mechanisms inducing the weeding response, although additional experimentation is required to obtain a more comprehensive set of data and samples to reinforce the initial observations from this project. Furthermore, an experimental approach to this question may be to freeze a fungus garden at -20 °C, then wait for a certain time interval before *Trichoderma* extract treatment to assess whether the weeding pattern of the room temp./extract treatment

is restored (i.e., a strong weeding response at the 0–6-hour interval), which could corroborate any potential findings about transcriptional differences.

The incorporation and bead experiments revealed several behavioral patterns that may warrant continued investigation, but further work is required to establish experimental methods more firmly for future study. For the incorporation experiments, the low baseline incorporation rates among the subcolonies made it difficult to assess the effects of substrate treatment on incorporation. It may be necessary to conduct these experiments at an earlier time after collection or use larger subcolonies or whole colonies to assess whether stress from subcolony formation prevents incorporation. Additionally, it would be valuable to quantify the results by measuring the masses of weeded material and incorporated substrate throughout the course of the experiment.

For the bead treatments, additional work may be required to establish a *Trichoderma* extract bead treatment that is comparable to using liquid extract. Beads were treated with 200 µL of 10 mg/mL *Trichoderma* extract so that the amount of extract used was equivalent to the liquid treatment in the freezing experiments, but this approach introduced challenges with drying and potential degradation of the metabolites that may have impacted the results. It would be valuable to use different solvents, drying methods, bead materials, or quantities of extract to assess if these factors are relevant to the ants' response to *Trichoderma* metabolites. Furthermore, quantitative measurements of the manipulated bead mass were not presented because of challenges obtaining consistent measurements for the beads. Alternative quantification methods could include counting the number of manipulated beads between the intervals or coloring the beads to facilitate automated analysis.

Methods

Evaluating killing methods: Pieces of fungus garden weighing 0.5 grams from colony JKH000451 were weighed out and placed into a 50 mL centrifuge tube. Three tubes were used for each treatment group (-80 °C, -20 °C, 50 °C, and 100 °C) and one was used for the negative control (room temperature), for a total of 13 tubes/pieces of fungus garden. For the -80 °C and -20 °C treatment groups, the tubes were placed in the corresponding freezer, and each tube was removed at 15 minutes, 1 hour, and

3 hours. For the 50 °C and 100 °C treatment groups, the tubes were placed closed in a dry, pre-warmed heat block, and tubes removed at the same intervals. Temperature treatments were staggered so that the tubes could be left to return to room temperature for approximately 1 to 2 hours. The fungus gardens were then placed into plaster-lined boxes that had been saturated with deionized water. Pictures were taken immediately after treatment and at 24, 48, and 72 hours after treatment. Time-lapse images were also taken every 5 seconds during the experiment duration using a GoPro Hero5. After 72 hours, fungus garden waste was weighed and recorded. For the no treatment control, weeded mass was instead measured at 96 hours. Comparison with time-lapse images showed that the fungus garden's appearance did not differ significantly between 72 and 96 hours, a conclusion that is also supported by the low overall weeding rate.

To create the microscope images, three small pieces (~1 mm in diameter) and one larger piece (~8 mm in diameter) of fungus garden from colony JKH000460 were removed and placed on a microscope slide. To simulate different freezing conditions, slides were placed in the -80 °C freezer for 1 hour or 6 hours and the -20 °C freezer for 1 hour or 6 hours. Still images were taken at 40x total magnification under a compound light microscope immediately before freezing, immediately after freezing, and 6 hours and 24 hours after freezing.

Weeding experiments: For each treatment group, a quarter-sized amount of fungus garden was removed from the mother colony (refer to Table C for colonies used and experimental setups) and placed in a clear plastic box. One fungus garden for each treatment group was placed in the -20 °C or -80 °C freezer for 1 hour or left at room temperature for 1 hour. Fungus gardens were then removed from the freezer, left to thaw for approximately 30 minutes, then placed in a plastic box lined with plaster of Paris and saturated with deionized water. The fungus garden from each treatment group was then left untreated or was treated with 200 µL of 0.5% DMSO in ultrapure water or 200 µL of 10 mg/mL *Trichoderma* str. MB101 crude extract in 0.5% DMSO dripped onto the fungus garden by pipet. Still images of each fungus garden were taken immediately after treatment. After the fungus was left to sit for another 30 minutes, subcolonies were created by adding 8 ants from the mother colony to each box, and another still

image of each subcolony was taken immediately afterwards. Timelapse photos were also taken every 60 seconds for 24 hours with a GoPro Hero5. At 6 hours after subcolony creation, fungus garden waste was removed, weighed, and stored in 1.5 mL microcentrifuge tubes in the -20 °C freezer. Still images were taken before and after removal of weeded material. At 24 hours, the additional waste generated since the 6-hour interval was removed, weighed, sampled in the corresponding 1.5 mL microcentrifuge tubes from the 6-hour interval, and stored dry in the -80 °C freezer.

In experiments conducted during the last week of August, colonies exhibited a much higher baseline level of weeding in the room temp./no treatment negative controls (37.08–82.82% this week vs. 0.39–4.48% before). Therefore, all experiments conducted after August 16th were excluded from figures and analysis. This observation is likely due to seasonal variation in colony activity, although further testing is necessary to better understand seasonal behavior and how it impacts peptaibol-induced weeding.

Incorporation experiments: Approximately two weeks after colonies were collected and acclimated to lab conditions, colonies JKH000448 and JKH000449 were selected for use in the incorporation experiments because the ants were actively incorporating corn meal into the fungus garden. Quarter-sized pieces of fungus garden were removed from the mother colony and placed into clear plastic enclosures. Each enclosure consisted of a plaster-lined box saturated with deionized water to maintain 100% relative humidity connected to a side box containing a weigh boat filled with enough corn meal to barely cover the bottom of the weigh boat (~0.5 grams of corn meal pre-treatment). The corn meal was either left untreated or treated by pipetting 200 µL of 0.5% DMSO in ultrapure water or 200 µL of 10 mg/mL *Trichoderma* str. MB101 crude extract in 0.5% DMSO onto the corn meal. Eight ants were then added to each box to create subcolonies. Two replicates were carried out for each treatment, making for a total of 6 subcolonies per colony. For colony JKH000449, still images were taken immediately after subcolony creation as well as 13 days post-subcolony formation. Time-lapse images were also taken every 5 seconds for a period of 13 days. For colony JKH000448, images were taken up to 12 days post-treatment instead of 13. Comparisons with time-lapse images showed that subcolonies did not change significantly between 12 and 13 days.

Approximately two weeks after colonies were collected again in August, another round of incorporation experiments was started, accompanied by additional controls where the fungus was treated with 200 µL of 10 mg/mL crude *Trichoderma* extract or 0.5% DMSO and the cornmeal was left untreated (refer to Table C). However, these experiments were not completed because of problems collecting data and abnormally high ant mortality.

Bead experiments: To create the silica bead treatments, 0.5 g of 1 mm diameter silica disruption beads were placed onto a plastic weigh boat. The beads were either left untreated or treated with 100 µL of 0.5% DMSO in ultrapure water or 100 µL of 10 mg/mL *Trichoderma* str. MB101 crude extract in 0.5% DMSO. The beads were then air-dried overnight at room temperature to evaporate the solvent. For the fungus/bead experiments with colonies JKH000448 and JKH000460, one quarter-size piece of fungus garden was taken from the colony for each bead treatment group (refer to the first two entries of Table D for experimental setup). For colony JKH000448, one replicate was carried out for all treatments. For colony JKH000460, two replicates were carried out for each bead treatment group and one replicate was carried out for the liquid treatment controls. The beads were scattered over the fungus garden, and any beads that landed on the plaster were repositioned on top of the garden using tweezers. Still images were taken immediately after subcolony creation and approximately 24 hours post-treatment. Timelapse photos were taken every 10 seconds during the same interval. Weeded masses and removed/unremoved bead masses were also recorded after around 24 hours but were excluded from further analysis because of challenges separating the beads from the fungus garden trash and clearly determining which beads were manipulated. For example, it was difficult to distinguish whether beads adjacent to or under the fungus garden were manipulated by the ants or if the beads shifted position after application.

For the beads-only experiments, beads were instead treated with 200 µL of 0.5% DMSO in ultrapure water or 200 µL of 10 mg/mL *Trichoderma* str. MB101 crude extract in 0.5% DMSO. After drying, beads were then placed into a pile at the center of a plaster-lined box that had been saturated with deionized water. Subcolonies were created by taking 8 ants from each colony that were in the main box but not on the fungus garden. For the NT/DMSO/crude group 1 treatments of colony JKH000442, the

ants were instead taken from the trash box of the mother colony's enclosure. For all colonies in the experiment except colony JKH000462, 2 replicates were carried out for each treatment (refer to Table D for colonies used). For colony JKH000462, 3 replicates were performed. Still images were taken immediately after subcolony creation and at subsequent intervals of approximately every 24 hours for 5 days. Timelapse photos were also taken every 10 seconds during the same interval.

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Figures

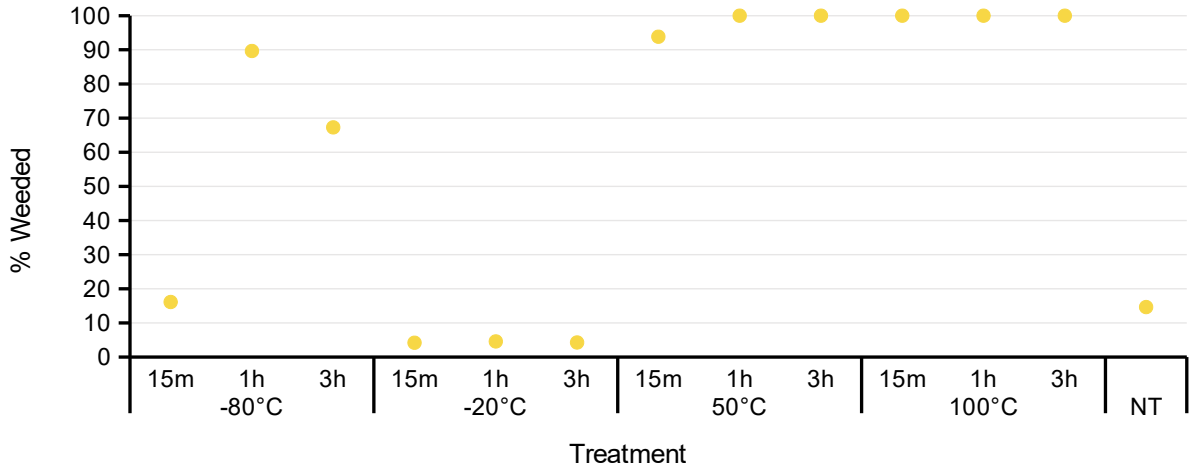

Figure 1: Ants weed in response to cultivar death. Fungus gardens were treated at the described temperatures and duration to observe weeding behavior after approximately 72 hours. For the NT (no treatment) negative control, weeded mass was instead weighed at 96 hours. Weeded masses are expressed in percent form to account for differences in initial mass. All trials were conducted with ants and fungus garden from colony JKH000451.

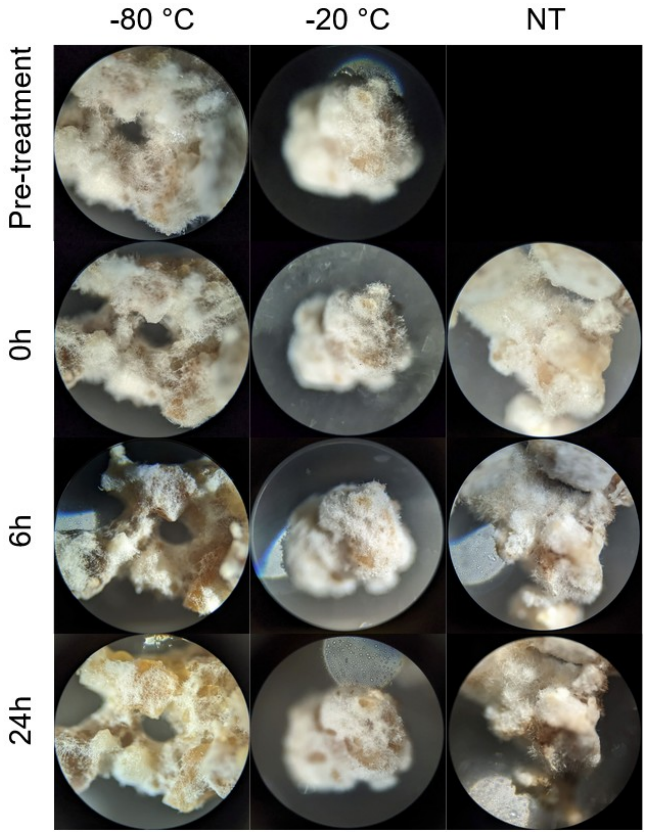

Figure 2: Fungus garden hyphae are inhibited by treatment at -80°C. Small pieces of fungus garden were placed on microscope slides and frozen at -20°C or -80°C for 1 hour (a 6 hour treatment was also performed but is not shown). Still images of the fungus garden were taken before treatment and 0, 6, and 24 hours after treatment. All images were taken under a compound microscope at 40x total magnification.

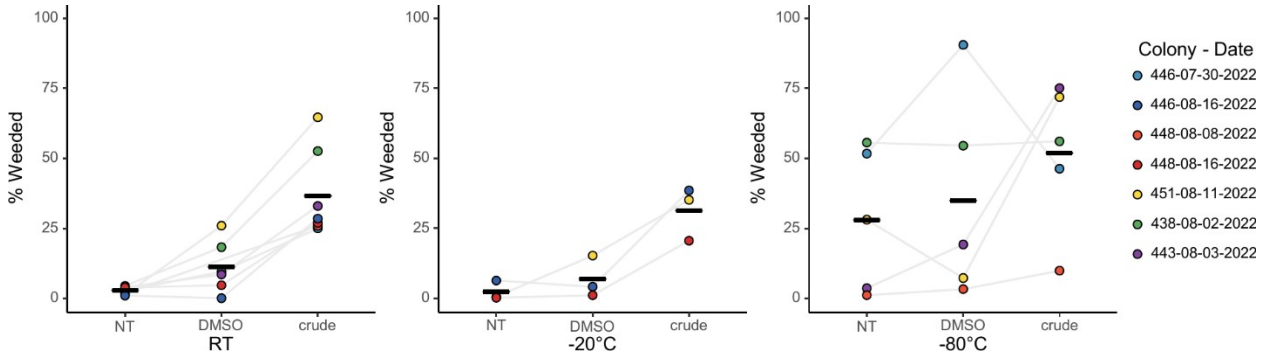

Figure 3: Ants weed in response to *Trichoderma* extract treatment of room temperature and -20°C fungus gardens and at variable rates in response to -80°C cold treatment. Room temperature (RT) or cold-treated (-20°C or -80°C) fungus gardens were left untreated or treated with crude *Trichoderma* extract or 0.5% DMSO. Fungus garden waste was measured at 6 and 24 hours post-treatment, and the mass at both intervals was added to obtain the cumulative amount weeded after 24 hours. Weeded masses are expressed as a percent of the total fungus garden mass to account for variations in fungus garden mass.

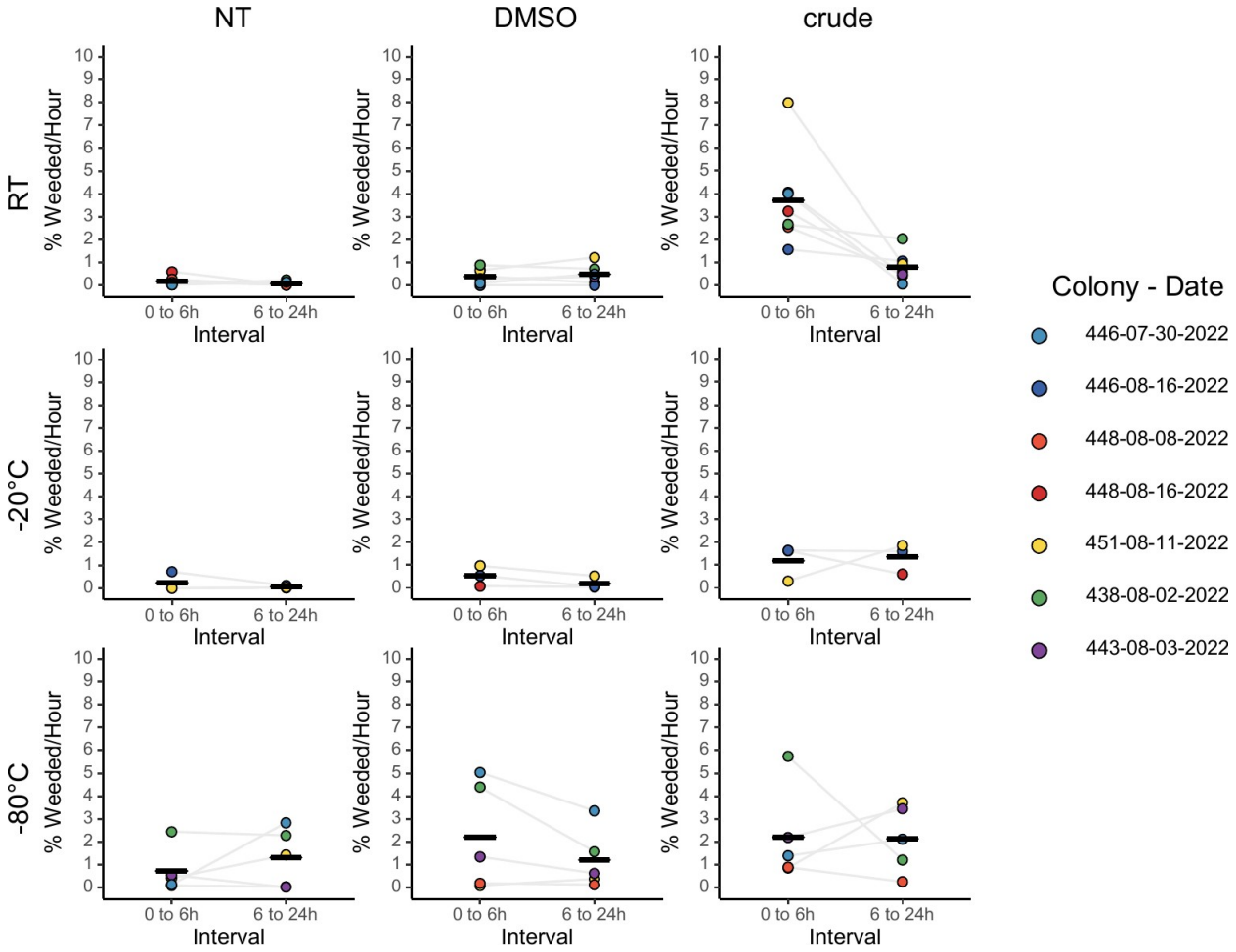

Figure 4: Ants weed room temperature/*Trichoderma* extract-treated fungus gardens at an elevated rate from 0 to 6 hours and at a lower rate from 6 to 24 hours. Weeded mass was removed and weighed after approximately 6 and 24 hours after each treatment, and weeding rates were calculated for intervals from 0–6 hours post-treatment and 6–24 hours. Weeding rates are expressed in percent/hour to normalize for variations in fungus garden mass and account for the different lengths of each interval.

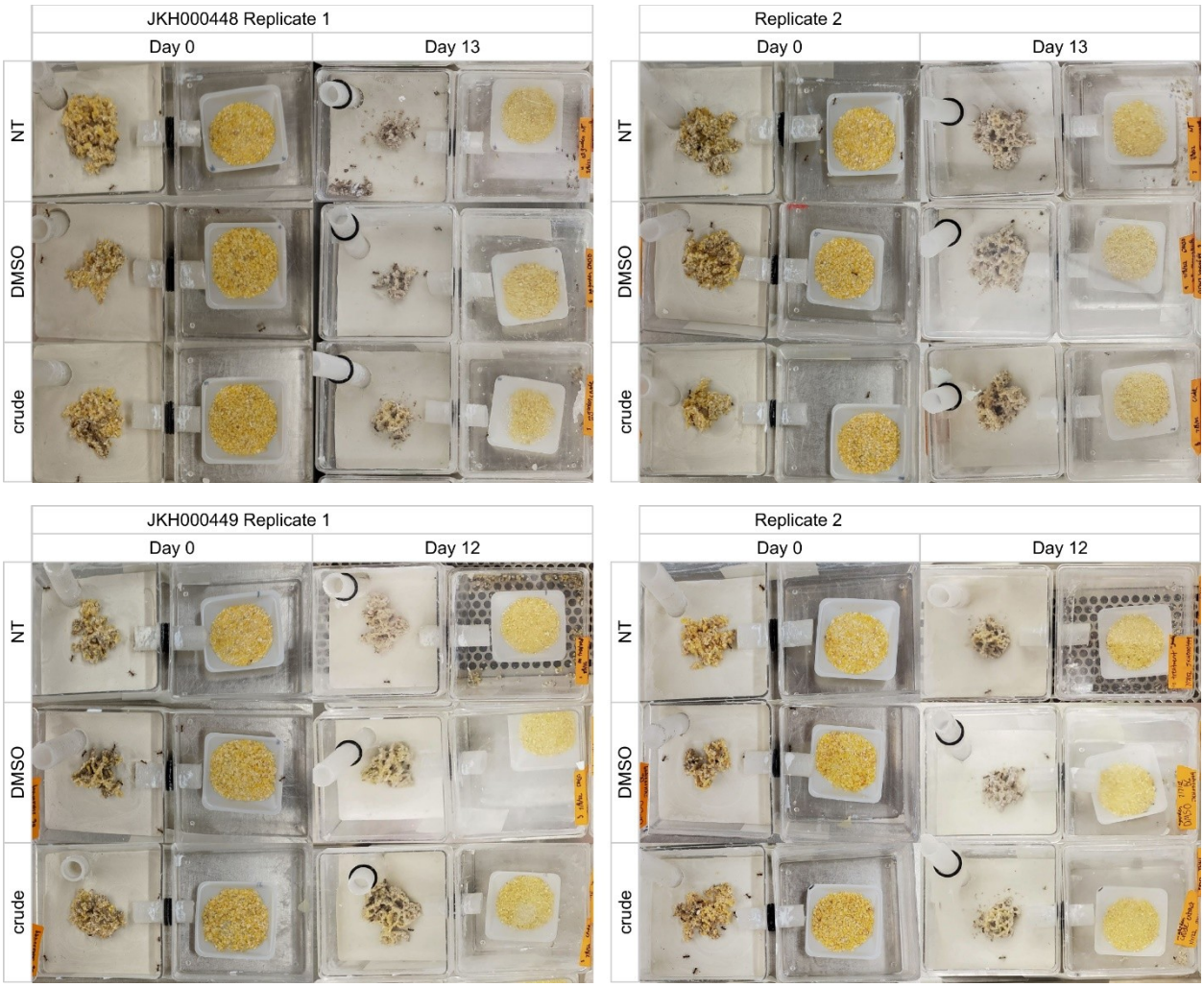

Figure 5: Ants incorporate treated corn meal into the fungus garden at variable rates. Subcolonies were presented with treated or untreated corn meal to assess the effect of *Trichoderma* extract on substrate incorporation. Pre- and post-treatment images were taken to complement time-lapse images used for video analysis. For experiments with colony JKH000448, high-resolution pictures were taken 13 days post-treatment instead of 12. Comparison with time-lapse images showed that subcolony appearance did not change significantly between Day 12 and Day 13.

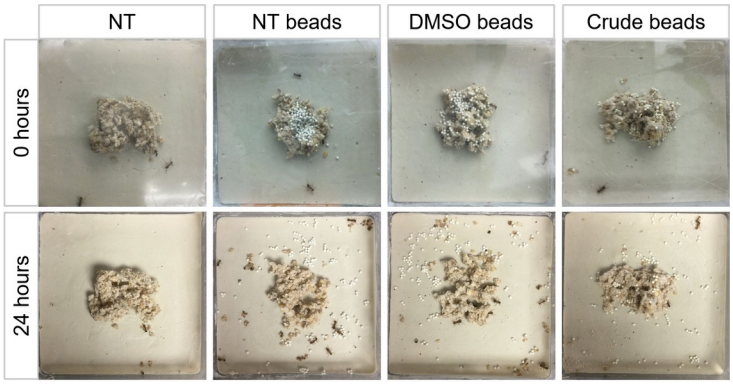

Figure 6: Ants remove treated and untreated silica beads from the fungus garden. Subcolonies were treated with 0.5 g of untreated silica beads or beads coated with *Trichoderma* extract/0.5% DMSO solvent. The images above for each subcolony were taken immediately after treatment (above) and 24 hours after treatment (below). Subcolonies pictured in the figure were

formed from colony JKH000448. Another fungus/bead treatment experiment was performed using subcolonies from colony JKH000460 but is not pictured because the subcolonies exhibited highly elevated weeding behavior in the negative controls.

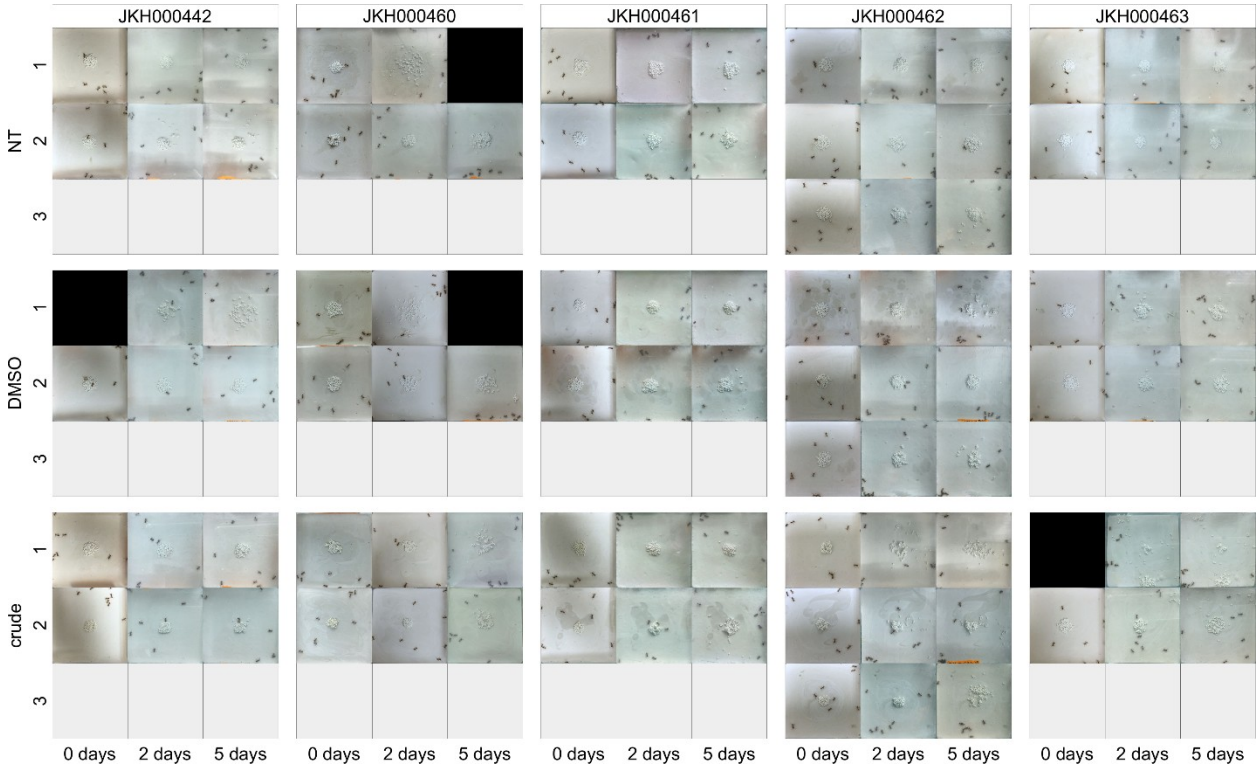

Figure 7: Ants manipulate beads at variable rates. Subcolonies were formed using 8 ants and 0.5 g of DMSO/*Trichoderma* extract-treated or untreated beads. Images of each subcolony were taken every 24 hours for at least 6 days after subcolony formation, along with timelapse image data. White squares indicate that a third replicate was not carried out for that colony, and black squares indicate that there was no image data for a particular time point.

Tables

A. Field collection metadata for all *T. septentrionalis* colonies used in the project.

Dataset	Colony ID	State	Park	Location	Collection date
Freezing	JKH000438	New Jersey	Wharton State Forest	Hawkins Bridge	2022-06-21
Beads	JKH000442	New Jersey	Wharton State Forest	Hawkins Bridge	2022-06-21
Freezing	JKH000443	New Jersey	Wharton State Forest	Hawkins Bridge	2022-06-21
Freezing	JKH000446	New Jersey	Wharton State Forest	Batona Campsite	2022-06-22
Freezing, incorp-oration	JKH000448	New Jersey	Wharton State Forest	Batona Campsite	2022-06-22
Incorp-oration	JKH000449	New Jersey	Wharton State Forest	Batona Campsite	2022-06-22
Freezing	JKH000451	New Jersey	Wharton State Forest	Batona Campsite	2022-06-22
Beads	JKH000460	New Jersey	Wharton State Forest	Quaker Bridge	2022-06-24
Beads	JKH000461	New Jersey	Wharton State Forest	Quaker Bridge	2022-06-24
Beads	JKH000462	New Jersey	Wharton State Forest	Quaker Bridge	2022-06-24
Beads	JKH000463	New Jersey	Wharton State Forest	Quaker Bridge	2022-06-24
Incorp-oration	JKH000481	New Jersey	Wharton State Forest	Washington Turnpike	2022-07-29
Incorp-oration	JKH000482	New Jersey	Wharton State Forest	Washington Turnpike	2022-07-29

B. Incorporation assays.

Date	Treatments	Controls (+/–)	Short Colony ID	Included in analysis?
7/8-7/20	Crude *Trichoderma* extract-treated corn meal	Untreated/DMSO-treated corn meal (-)	448	Yes
7/9-7/20	Crude *Trichoderma* extract-treated corn meal	Untreated/DMSO-treated corn meal (-)	449	Yes
8/16-8/26	0.5 g crude *Trichoderma* extract-treated corn meal +	0.5 g untreated corn meal + untreated fungus garden (-)	481	No— experiment was stopped early due to data

	untreated fungus garden	0.5 g DMSO-treated corn meal + untreated fungus garden (-) 0.5 g untreated corn meal + 200 µL DMSO-treated fungus garden (-) 0.5 g untreated corn meal + 200 µL crude *Trichoderma* extract-treated fungus garden (+)		collection issues and abnormally high ant mortality
8/25-8/26	0.5 g crude *Trichoderma* extract-treated corn meal + untreated fungus garden	0.5 g untreated corn meal + untreated fungus garden (-) 0.5 g DMSO-treated corn meal + untreated fungus garden (-) 0.5 g untreated corn meal + 200 µL DMSO-treated fungus garden (-) 0.5 g untreated corn meal + 200 µL crude *Trichoderma* extract-treated fungus garden (+)	482	No— experiment was stopped early due to data collection issues and abnormally high ant mortality

C. Weeding assays.

Date	Treatments	Controls (+/–)	Short Colony ID	Included in analysis?
7/18-7/23	Killing methods trial: -80 °C 15 min/1 hr/3 hr -20°C 15 min/1 hr/3 hr 50 °C 15 min/1 hr/3 hr 100 °C 15 min/1 hr/3 hr	NT (no treatment) (-)	451	No - experimental design trial
7/22-7/23	-80 °C 1 hr + NT/DMSO/crude	Room temp.+ NT/0.5% DMSO (-) Room temp. + crude (+)	451	No - experimental design trial
7/28-7/29	-80 °C 1 hr + NT/DMSO/crude 50 °C 1 hr + NT/DMSO/crude	Room temp.+ NT/0.5% DMSO (-) Room temp. + crude (+)	448	No - experimental design trial
7/29-7/30	-80 °C 1 hr + NT/DMSO/crude	Room temp.+ NT/0.5% DMSO (-) Room temp. + crude (+)	446	Yes
8/2-8/3	-80 °C 1 hr + NT/DMSO/crude	Room temp.+ NT/0.5% DMSO (-) Room temp. + crude (+)	438	Yes

8/3-8/4	-80 °C 1 hr + NT/DMSO/crude	Room temp.+ NT/0.5% DMSO (-) Room temp. + crude (+)	443	Yes
8/8-8/10	-80 °C 1 hr + NT/DMSO/crude	Room temp.+ NT/0.5% DMSO (-) Room temp. + crude (+)	448	Yes
8/11-8/12	-80 °C 1 hr + NT/DMSO/crude -20 °C 1 hr + NT/DMSO/crude	Room temp.+ NT/0.5% DMSO (-) Room temp. + crude (+)	451	Yes
8/16-8/17	-20 °C 1 hr + NT/DMSO/crude	Room temp.+ NT/0.5% DMSO (-) Room temp. + crude (+)	446	Yes
8/16-8/17	-80 °C 1 hr + NT/DMSO/crude -20 °C 1 hr + NT/DMSO/crude	Room temp.+ NT/0.5% DMSO (-) Room temp. + crude (+)	448	Yes
8/21-8/22	-80 °C 1 hr + NT/DMSO/crude -20 °C 1 hr + NT/DMSO/crude 50 °C 1 hr + NT/DMSO/crude	Room temp.+ NT/0.5% DMSO (-) Room temp. + crude (+)	438	No – observed highly elevated baseline weeding due to end-of-season behavior
8/22-8/23	-80 °C 1 hr + NT/DMSO/crude -20 °C 1 hr + NT/DMSO/crude	Room temp.+ NT/0.5% DMSO (-) Room temp. + crude (+)	446	No – negative/positive controls were consistent with earlier trials but experiment was excluded due to date
8/23-8/24	-80 °C 1 hr + NT/DMSO/crude -20 °C 1 hr + NT/DMSO/crude	Room temp.+ NT/0.5% DMSO (-) Room temp. + crude (+)	451	No - observed highly elevated baseline weeding due to end-of-season behavior
8/24-8/25	-80 °C 1 hr + NT/DMSO/crude -20 °C 1 hr + NT/DMSO/crude	Room temp.+ NT/0.5% DMSO (-) Room temp. + crude (+)	443	No - observed highly elevated baseline weeding due to end-of-season behavior
8/24-8/25	-80 °C 1 hr + NT/DMSO/crude -20 °C 1 hr + NT/DMSO/crude	Room temp.+ NT/0.5% DMSO (-) Room temp. + crude (+)	451	No - observed highly elevated baseline weeding due to end-of-season behavior

D. Bead assays.

Date	Treatments	Controls (+/–)	Short Colony ID	Included in analysis?
8/11-8/12	NT/DMSO/crude beads on fungus	Fungus + NT (-)	448	Yes (Note: beads were treated with 100 µL of solvent instead of 200 µL)
8/28-9/6	NT beads-only DMSO beads-only Crude extract beads-only Fungus + NT beads Fungus+ DMSO beads Fungus+ crude beads	Fungus + NT or 200 µL 0.5% DMSO (-) Fungus + 200 µL of 10 mg/mL crude extract (+)	460	Yes – however, beads/ fungus garden trials were excluded from analysis due to end-of-season weeding behavior
8/29-9/7	NT beads-only DMSO beads-only Crude extract beads-only	n/a	463	Yes
9/1-9/7	NT beads-only DMSO beads-only Crude extract beads-only	n/a	442	Yes
9/1-9/7	NT beads-only DMSO beads-only Crude extract beads-only	n/a	461	Yes
9/1-9/7	NT beads-only DMSO beads-only Crude extract beads-only	n/a	462	Yes