Mitigating the impact of tawny crazy ant populations on endangered karst invertebrates: quantifying harm and designing environmentally safe control methods



Tawny crazy ants (*Nylanderia fulva*) preying on an *Oxidus gracilius* millipede in Whirlpool Cave, Travis County, Texas.

Traditional Section 6 Grant Final Report

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Abstract -Tawny crazy ants (*Nylanderia fulva*), a South American, invasive ant species, threaten karst invertebrates in Central Texas. There is an urgent need to quantify the impacts TCAs have on karst invertebrates. There is also urgent need for strategies that control TCA populations around karst ecosystems without impacting karst invertebrates. We pursued two parallel strategies for controlling TCA around caves: (1) developing a low toxicity, boric acid-based, poison bait strategy designed to target TCAs without contaminating caves, and (2) conducting research necessary to evaluate a newly discovered microsporidian parasite (MP) of TCAs as a tool for sustainable TCA management.

We found that TCA cave usage was episodic and driven by outside climatic conditions. When surface conditions were stressful, either cold or desiccating (hot and dry), TCAs invaded the cave in moderate to high numbers. When surface conditions were favorable, TCAs were entirely absent from the cave. In general, in invaded habitats, TCA will be present in caves at high densities during hot, dry times during the summers and present a low-density during winter. The summer-time, high-density incursions will likely have the greatest impacts on cave arthropod faunas.

Our ability to assess TCAs impacts on cave fauna was limited by available invaded caves to a single invaded cave. The TCA population at this site was present at only moderate densities, so its episodic incursions into the cave were of lower density than the other invaded cave examined. Despite this, one of the seven karst invertebrates sufficiently abundant to evaluate suffered a significant decline associated with TCA presence. We expect that this low-level impact is not a general property of TCA invasions of caves, but rather a reflection of the relatively low-density of this particular TCA population. We expect impacts to be greatest on species similar to the species that showed significant declines at the impacted cave: troglophilic and troglobitic cave floor-dwelling species. This category includes endangered species like *Texella reyesi*.

Ultimately our attempt to control a TCA population using boric-acid laced bait stations was unsuccessful. By the Fall TCA population peak, there was no significant difference in the population abundance of ants far from the bait stations as compared to close to the bait stations and cave. The second goal of the boric acid bait control study, to not introduce pesticide into the cave, was also not met. Despite the buffer zone without bait stations around the cave, there was evidence that the pesticide penetrated the cave ecosystem. Based on our results, we do not think that alterations in the design of the boric acid-laced bait station approach would achieve the desired result of reducing TCA populations without introducing unacceptable amounts of pesticide into the cave. We cannot recommend this approach to controlling TCA populations.

Our tests to date indicate that *Myrmecomorba nylanderiae* is a highly specific pathogen of *Nylanderia fulva* (TCA), meeting a critical criterion for use as a biological control agent. The factors that govern the course of *M. nylanderiae* infection in wild populations remain mysterious. No consistent association with season and disease prevalence was observed. In the two populations with patchy infection prevalence at the beginning of the study one remained spatially patchy and consistently localized throughout while infection rapidly spread to near universal prevalence in the other. Despite uncertainties about what drives *M. nylanderiae* infection to high prevalence in some TCA populations and what prevents it from reaching high prevalence in others, an emerging pattern is that highly prevalent infections by *M. nylanderiae* are devastating for TCA populations. Our examination of transmission biology in support of developing an inoculation regime revealed that infected workers transmit *M. nylanderiae* reliably to uninfected larvae. No other pathway of intra-colony transmission was observed with frequency. Our attempts at inoculating field populations of TCA were successful with both infected brood and infected workers succeeding in transmitting the infection to the local nests at their site of introduction. Examinations of relative inoculation efficacy and relative spread rates are in process. Thus using *M. nylanderiae* as a tool for biological control seems a feasible and worthwhile prospect. We recommend continued investment in the research needed to realize this goal as well as continued support for research quantifying impacts on karst invertebrates.

Introduction

Tawny crazy ants (*Nylanderia fulva*), a South American, invasive ant species, threaten karst invertebrates in Central Texas. First discovered in Texas in 2002, this species has now spread to 28 counties. Tawny crazy ants (TCA) are poorly understood, and their long-term impacts on karst ecosystems unknown. However, a recent study in the Gulf Coast prairie region demonstrated this species severely impacts arthropod abundance and diversity (LeBrun et al. 2013). Since TCAs prefer humid environments (Meyers 2008), Central Texas caves will be susceptible to invasion as they contain preferred habitat. Central Texas caves support one of the most important cave faunas in the world (Elliott and Reddell 1989), making this threat particularly alarming.

The Austin area is home to six endangered karst invertebrate species (ES) and 25 karst species of concern (SOC) covered by the Balcones Canyonlands Conservation Plan (BCCP) (Service 1996). Two TCA populations in Travis County are within four km of nineteen BCCP caves. By July 2013 TCAs invaded one cave containing BCCP-listed species of concern (Whirlpool Cave) and were foraging 30 m inside the cave. Preliminary in-cave faunal surveys indicate significant displacement of cave fauna within infested areas of this cave (T. Bayless, M. Sanders pers. obs. 2013). By November 2014, a TCA population had spread into one endangered species cave (No Rent), and very likely a second (McNeil Bat Cave). Because TCA populations spread outward 200 m per year (Meyers 2008), and this species is frequently transported by humans (McDonald 2012), TCAs will likely threaten a large fraction of Central Texas BCCP caves in the near future. This project addresses the need stated in the Travis/Williamson, and Bexar County Karst Invertebrates Recovery Plans of "implementing adaptive management to control existing and new threats" by working to design an effective TCA control method for use around karst ecosystems containing endangered karst invertebrates and SOC (USFWS 1994, 2011a).

There is an urgent need to quantify the impacts TCAs have on karst invertebrates. Negative impacts are likely to include both direct impacts upon populations of federally protected karst invertebrates as well as alterations to cave ecosystems that will negatively impact protected species. For example, cave crickets (*Ceuthophilus spp*.), critical nutrient suppliers to caves (Lavoie et al. 2007), are likely to be strongly impacted. Because TCAs nest opportunistically (McDonald 2012), making nests difficult to locate and often inaccessible, the standard U.S. Fish and Wildlife Service (USFWS 2011b) recommended boiling water treatment for red-imported fire ant mounds is not feasible for TCAs. Thus, there is urgent need for strategies that control TCA populations around karst ecosystems without impacting karst invertebrates.

We pursued two parallel strategies for controlling TCAs around caves: (1) developing a low toxicity, boric acid-based, poison bait strategy designed to target TCAs without contaminating caves, and (2) conducting research necessary to evaluate a newly discovered microsporidian parasite (MP) of TCAs as a tool for sustainable TCA management. Achieving effective control with boric acid, while not contaminating caves, requires an enhanced understanding of various aspects of TCA foraging biology that provides guidance on bait station deployment, bait preferences, and boric acid concentrations. Also necessary are experiments assessing whether boric acid is inadvertently introduced into the karst ecosystem. Before employing this MP as a management tool, we required an understanding of the host specificity of this MP, its impacts on TCA populations, and the spatial and temporal dynamics of its infections.

These approaches offer a potential synergy as the MP will likely both lower TCA population densities as well as reduce the rate at which populations of this ant spread into uninvaded habitat, a function of density. If born-out, these effects will facilitate reducing TCA densities around cave entrances below the threshold of impact, as well as allow for proactive treatment of TCA populations spreading into sensitive cave areas.

Objective

(1) Quantify TCA impacts on karst invertebrates in ES/SOC caves; (2) develop a boric acid, poison-bait approach for controlling TCA populations while avoiding contaminating sensitive karst ecosystems; (3) assess whether boric acid indirectly enters the karst system; and (4) investigate potential for using a newly discovered parasite of TCA to reduce populations around karst features.

Research Topics Addressed:

Impacts upon karst systems

1) An assessment of TCA use of cave environments determining depth that TCAs penetrate caves, densities they reach at different depths, and environmental conditions associated with cave occupancy.

2) An assessment of impact of TCA infestations upon karst invertebrate assemblages quantifying changes in karst invertebrate assemblage at different cave depths in parallel with TCA densities.

Control Strategy 1: Boric acid, liquid bait treatment protocol for TCAs in sensitive karst areas.

3) Formulating toxic bait: Determining optimal boric acid concentration that: (a) does not stimulate aversion, and (b) produces delayed toxicity. Determining macro nutrient composition of bait by (c) determining the optimal form of liquid protein and (d) evaluating how different macronutrients (sugars vs. proteins) are shared among the castes of TCA colonies.

4) Quantifying the spatial scale of resource transfer among TCA nests to design spatial dispersion of bait stations.

5) Creating an inexpensive bait station designed to specifically target TCAs.

6) An attempt to control a TCA population in the area around a cave entrance. We will also determine the efficacy of the control attempt by measuring pre and post-treatment TCA abundances using pitfall trap transects, as well as quantifying the duration of TCA population control.

7) An assessment of non-target impacts of boric acid bait.

Control Strategy 2: An evaluation of the biological control potential of a microsporidian pathogen of TCA.

8) An assessment of whether the microsporidian parasite of TCAs also infects native ant species.

9) An understanding of the temporal and spatial dynamics of the parasite's prevalence in TCA populations allowing for the design of efficient inoculation regimes.

10) An assessment of whether high prevalence of this parasite reduces local abundances of TCAs.

11) An evaluation of methods for inoculating uninfected TCA populations with the parasite.

Location

We conducted our study at known TCA populations in Travis and Bastrop Counties of central Texas, and in Brazoria, Harris, and Galveston Counties of southeastern Texas (Fig. 1 and 2). Callouts in Figures 1 and 2 describe the experimental protocols used at each site. Of the two cave impact sites in our study, we conducted TCA chemical and biological control method testing only at the Convict Hill site due to the presence of a federally endangered karst invertebrate, the Bone Cave harvestman (*Texella reyesi*) within caves at the McNeil Site. We performed TCA laboratory assays at University of Texas' Brackenridge Field Lab in Austin, Texas.

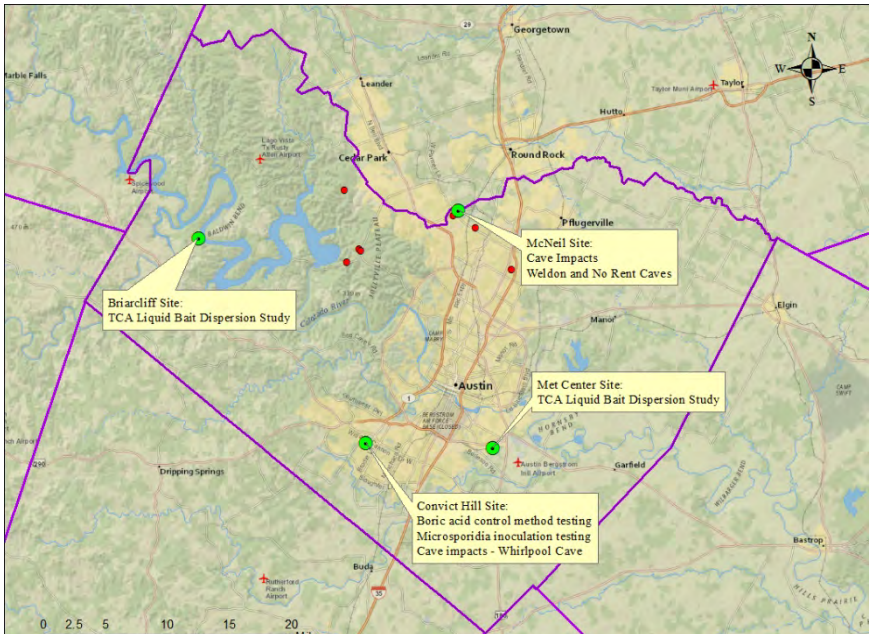


Figure 1: Map of boric acid control method testing, microsporidia inoculation testing, and cave impacts field sites in Travis County, Texas. Green dots indicate field sites where experimental protocols were conducted. Red dots indicate test caves in which Travis County and the City of Austin conducted ongoing faunal surveys that furnished reference data for our cave impacts study.

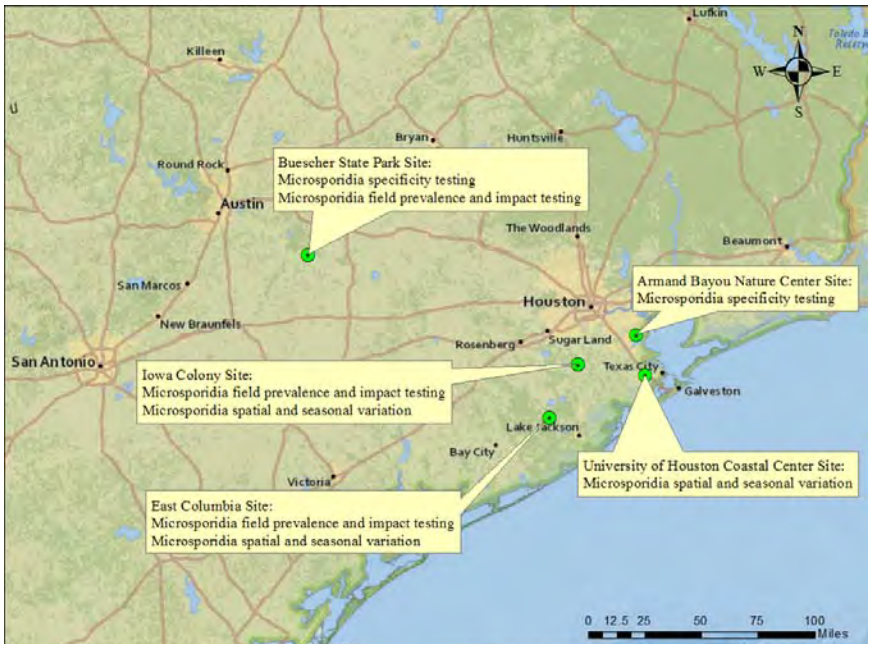


Figure 2. Map of microsporidia specificity testing, microsporidia field prevalence and impact testing, and microsporidia spatial and seasonal variation field sites. Green dots represent infected TCA populations where experimental protocols were conducted. Sites used only for the collection of ant nests for laboratory assays are not included on this map.

Methods

Impacts upon karst systems

1) Assessment of TCA use of cave environments determining depth that TCAs penetrate caves, abundances they reach at different depths, and environmental conditions associated with cave occupancy.

TCA bait based abundance measure

We quantified TCA occurrence and cave use by setting up bait stations outside of the cave entrance and at set distances inside the cave. For larger

caves (Whirlpool and Weldon), we designated bait stations every 10 m beginning above ground near the entrance (-10 m) and from just inside the entrance ("cave drop zone";0 m) to the far end of the survey area (Whirlpool: 0 m to 80 m; Weldon: 0 m to 30 m). Because of No Rent Cave's relatively small size, we designated its bait station array every 5 m to the back of the cave (0 m to 10 m). To assure TCA attraction to bait stations, we provided both protein bait (Bar S hot dog slice) and sugar bait (cotton ball soaked in 30% sucrose solution), deployed in 15 ml falcon tubes. Each bait station consisted of two sets of paired bait tubes, placed >2 m apart from each other. We deployed baits at the same location during each survey using a meter tape pulled from the cave's drop zone to the end of the survey area and/or back of the cave. We left bait stations open for exactly one hour, then collected and uniquely labeled each bait tube, first removing all non-target species collected. We counted and recorded all TCAs collected in each tube, and averaged totals for a final TCA count per bait station.

TCA trail-based density measure

If TCA numbers in a zone exceeded our ability to accurately count them (>20 ants), we quantified TCA density within cave survey zones by measuring all foraging trails observed during our faunal surveys with a flexible tape measure and recording total trail length. We then chose one to four representative TCA trails on the floor of the zone and collected a 1 m sample of each trail with a handheld vacuum coated on the inside with fluon, being careful not to collect non-target species. We collected vacuumed TCA samples in uniquely labeled 50 ml falcon tubes. We later counted and recorded all collected TCAs in each sample, and averaged counts to get an estimate of the total number of ants per survey zone for each quarterly survey. We then divided the total number of ants in each survey zone by the zone's surface area.

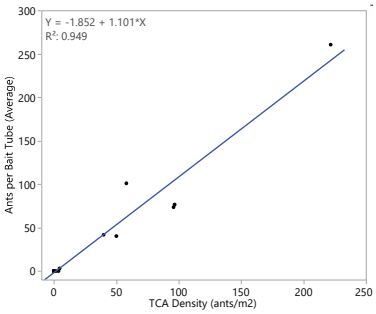


Figure 3: An example of the tight relationship between the bait tube-based measure of abundance and the trail length-based measure of density.

We calculated surface area (m^2) of each survey zone by laying down a 1.7 m^2 canvas tarp across a portion of the cave floor, smoothing and tucking it around the substrate to mimic the cave zone's uneven surface. We then marked the tarp's edges before picking up the tarp, and then reset it along the outside of our marked edges, methodically mapping our work as we made our way across the cave zone floor. Smaller, flatter areas were segmented into geometrical shapes and distinctly measured, mapped, and separately calculated. We repeated this process until the entire cave had been mapped and measured. All individually calculated segments were summed to obtain the total cave zone surface area. These two independent measures of TCA cave usage were very tightly related (Fig. 3).

We analyzed the role of outside abiotic conditions in driving TCA usage of the cave environments by examining how average temperature, average relative humidity, and average vapor pressure deficit related to cave usage by TCAs. We extracted daily measures of the above values from the website Weather Underground for the weather station nearest to the Convict Hill site with reliable historical data, and then calculated a 14 day running average for these measures for the period immediately prior to the cave survey (Weather Underground 2017). Vapor pressure deficit is the most biologically meaningful climate measure for predicting ant activity as it utilizes surface temperature and

relative humidity to create a measure of the desiccating potential of the air, a proxy for ant desiccation risk (Lighton and Feener 1989).

2) An assessment of impact of TCA infestations upon karst invertebrate assemblages quantifying changes in karst invertebrate assemblage at different cave depths in parallel with TCA abundances.

Biological Monitoring

To assess TCA impacts to karst invertebrate assemblages within the cave ecosystem, we performed cave fauna surveys quarterly on Whirlpool, No Rent, and Weldon Caves in spring (May) and fall (November) or summer (August) and winter (mid-January-mid February). We followed methodology and techniques supported by USFWS (2014) that provide results which can be compared between caves throughout the region for better study and analysis. This included designating permanent survey zones in each cave in which all living organisms encountered are identified and enumerated. Survey zones were distinct units of the cave such as a small room or an easily discernible section, so that the size and location of the survey area remained constant during the study for trend comparison. We designated three survey zones in Whirlpool Cave: two zones with TCA infestation and one zone deeper inside the cave unaffected by TCAs. Due to its size, we designated only one survey zone in No Rent Cave which included all humanly accessible areas of the cave's single room. Relative humidity, temperature, nutrient input, and dampness condition were also recorded both outside the cave and at each zone.

We also performed quarterly cave cricket (*Ceuthophilus spp*.) emergence counts as they exited the project caves' entrances to quantify relative abundance of these critical nutrient suppliers to caves (Lavoie et al. 2007). We conducted cave cricket emergence counts for two hours starting at sunset as crickets exited the caves. We documented current weather conditions, surface temperature, and relative humidity for each survey, and placed emerging crickets in one of three age classes: nymph (up to 5 mm), sub-adult/juvenile (5-12 mm) and adult (>12 mm). This methodology has been demonstrated to meet criteria necessary to assess cave cricket relative abundance at caves as well as estimate changes over time in the number of cave crickets that emerge from cave entrances, which is necessary for determining the state of cave ecosystems (Weckerly 2012).

Impact Data Analysis

Our ability to assess the degree to which TCAs impact cave arthropod faunas was severely limited by the number of invaded caves available to be surveyed. Only two caves were invaded during the period of this study: Whirlpool and No Rent Caves. Despite our expectations, Weldon Cave was never invaded. As Whirlpool was used as the site of the boric acid bait control study, only No Rent Cave provided an unambiguous cave for examining impacts of TCAs on karst arthropods. TCAs were only present in No Rent cave for four quarterly TCA abundance surveys. This was insufficient data to attempt to relate TCA abundance to cave arthropod abundances. However we were able to combine data collected during the period of this grant (eight surveys) with data collected prior to the beginning of this grant (seven surveys) to provide a total of four surveys prior to the arrival of TCA at the cave, five surveys during the time TCA were present in the cave area, and six surveys after they disappeared from the area of the cave (see Results).

In order to limit the influence of seasonal and annual variation in arthropod abundance on our results, we utilized surveys from a set of test caves to quantify variation due to seasonal or annual conditions (Fig. 1). These caves were spatially proximate to No Rent Cave and surveyed at the same time intervals. We examined evidence for TCA impact for all arthropod species that occurred in > 90% of the No Rent surveys, with one exception made for *Texella reyesi* (60% occurrence) due to its designation as a federally endangered karst invertebrate. For all caves and all arthropod species examined, we created a normalized abundance index by dividing the observed taxon abundance for a given interval by the maximum abundance of that taxon observed across all surveys in that cave during the survey interval. We then used linear regression to test whether abundance variation in the test caves (average of all normalized test cave abundance scores) explained a significant amount of the variation in normalized abundance in No Rent Cave. If it did, we used the residuals from this relationship, remaining variation not explained by seasonal or annual influence, to test for impact. If the test cave data bore no relationship to the abundance of a taxon in No Rent, we examined impacts using the normalized abundance data. To limit the number of tests, we only ran statistical tests on taxon response patterns that exhibited the response expected for TCA impact: higher abundances prior to TCA arrival than while they were present. However, because the number of surveys limits the power of any analysis, we combined surveys prior to TCA arrival with those after their departure, and contrasted abundance during time periods when TCA were present to abundance when TCA were absent.

Control Strategy 1: Boric acid, liquid bait treatment protocol for TCAs in sensitive karst areas.

3) Formulating toxic bait: Determining optimal boric acid concentration that exhibits (a) delayed toxicity and (b) does not stimulate aversion. (c) Determining macro nutrient composition of bait by evaluating how different macronutrients (sugars vs. proteins) are shared among the castes of TCA colonies.

(a) Boric acid concentration: behavioral acceptance

Colony fragments containing 100 workers, one queen and 0.0625 cc of mixed age brood were housed in 26 x 15 x 17 cm nest boxes with ventilation screening containing 12 ml test tubes half-filled with water and plugged with cotton to provide a humid nest site. Colony fragments were given 48 hours to adjust to their nest box and provided 20% sucrose and crickets during this period. After this period, ants were starved for the 24 hours prior to the assay. Four 30% sucrose solutions were prepared containing 0%, 1%, 2%, 3%, or 4% boric acid by weight.

For the assay, a 1.5 cm x 4.5 cm parafilm rectangle was placed at the far end of the nest box from the nesting tube. A 100 ul droplet of each of the four boric acid solutions was pipetted in a row along the parafilm strip. Order of droplet presentation was determined randomly prior to droplets being introduced. Over 30 minutes, the response of each ant that approached a droplet closely enough for its antennae to touch the droplet. Ants were scored as “accepting” the droplet if they opened their mandibles and drank from the surface of the droplet for at least three seconds. Ants were scored as “rejecting” a droplet if they left without drinking or drank for less than three seconds. Typically, ants that drank for at least three seconds continued to drink until they had filled their crop. If a single ant accepted or refused a droplet multiple times without first leaving the parafilm rectangle, that event was

scored only once. For each fragment, choice assays were conducted once a day on three consecutive days.

(b) Boric acid concentration: time till 50% mortality

The optimal concentration of a pesticide in a bait formulation intended to control ants is generally agreed to that which induces mortality in between 24 to 48 hours and does not induce behavioral aversion in the target ant. Faster acting toxic baits kill workers but are not passed along to queens and developing brood back in the nest.

Colony fragments containing 60 workers were housed in 15 x 15 x 9 cm opaque nest boxes with ventilation screening containing 12 ml test tubes half-filled with water and plugged with cotton to provide humid nest sites. Nest boxes were connected with 10 cm of Tygon™ tubing to an 11.5 x 8 x 5 cm translucent foraging box. All boxes were held at 28°C, and under a 12 hour day length cycle.

To assemble colony components, ants were anesthetized with CO_2. Twenty-four hours after replicates were assembled; any dead workers were removed, counted and replaced with an equal number of live workers. Ants were starved for 24 hours before being provided with cotton plugged test tubes containing 30% sucrose with the desired boric acid concentrations.

Dead ants were removed and counted twice a day: in the morning and evening. The time required for 50% of the starting workers to die (LT_{50}) was used as the response variable.

(c) Macro nutrient composition: relative consumption of macronutrients by castes.

As delivering toxicant to the developing brood and queen, as well as the foraging worker caste is critical to controlling ant populations, we evaluated whether protein and sugars are delivered preferentially to brood or workers in *Nylanderia fulva* colonies and also determined if larvae of different sizes differ in the types of nutrients they consume. To evaluate this, we conducted a study using a tracer dye to track relative macronutrient consumption by different castes.

First, to evaluate whether ants exhibit any behavioral aversion to the consumption of dye we conducted a behavioral acceptance assay. Methods followed the previous assay examining acceptance of boric acid with the exception that the sucrose solutions presented in the choice tests were dyed with 0, 0.5, 1, or 2 mM Fast Green FCF dye.

Colony fragments collected from distinct sites were split each fragment into three nest boxes to serve as a single replicate for each of the three nutrient regime treatments: dyed sucrose (DS), dyed collagen (DC), and control (C). Nest boxes were connected to foraging arenas in an identical manner as those in the boric acid mortality test. Each nest box had 200 workers, 25 second or third instar larvae, and 25 fourth instar larvae. Within the foraging arena, each box was provided with access to 30% sucrose and 10% bovine collagen in two, 5 ml cotton plugged test tubes. For the DS and DC treatments Fast Green FCF dye was added to the appropriate nutrient solution at a 2 mM concentration. Ants were then allowed to feed for 48 hours, collected, sorted to caste, and stored in 100% ethanol. A Li-Cor, Odyssey CLx© near-infrared fluorescence imaging system was used to quantify the amount of dye each ant or larvae ingested. Twenty-five workers, 15 small larvae, and 15 large larvae from each replicate were arranged on the glass plate of the scanner. All replicates from a colony fragment were included in a single scan to ensure that any scan specific variability was shared evenly amongst treatments. The ants were then scanned at 700 nm and 800 nm with a 0.5 mm focus offset. Images were analyzed using

the software Image J™. The 700 nm scan allows for quantification of the fluorescence from the ingested dye while undyed ant tissues autofluoresce at 800 nm. The number of autoflourescing pixels in the 800 nm wavelength allows for a measure of individual body size. The amount of dye an individual ingested was quantified by the brightness of that individual in the 700 nm wavelength: the sum of the pixel intensities (Fig 4). The flourescence intensity and body size of a total of 1323 individual ants (workers + larvae) were measured. The intensities of all individuals of a particular caste within a replicate were averaged and these average values were then analyzed.

For analysis individuals were assigned to one of three caste categories: small larvae, large larvae, and workers. Small larvae were those of median or smaller body size, while large larvae were of greater than median body size. Small larvae were principally made up of first and second instar larvae while large larvae were largely third and fourth instar larvae.

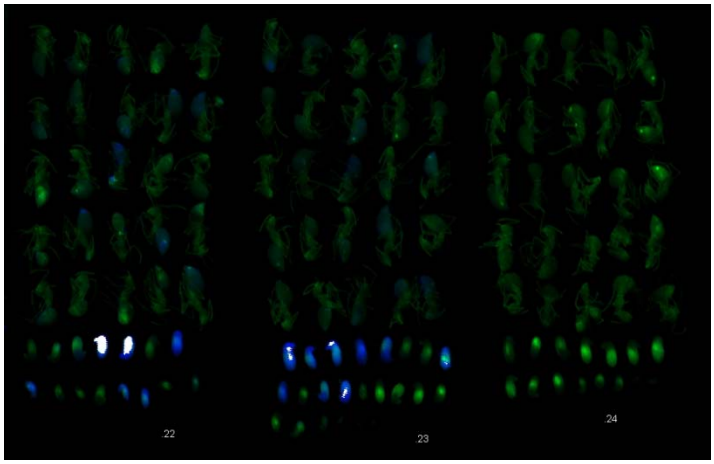


Figure 4: Licor image of dyed ants. Blue light is flourescence from ingested dye in the 700 nm wavelength. Green light is autofluorescence in the 800 nm wavelength. White denotes pixels for which the intensity of 700 nm dye derived flourescence saturated the sensor. The ants on the left (22) are those that fed on dyed sucrose; the ants in the middle (23) fed on dyed collagen, and the ants on the right (24) are the control.

(4) Quantifying the spatial scale of resource transfer among TCA nests to design spatial dispersion of bait stations.

Ants encountering a concentrated sugar solution store it in their crops and pass it to colony mates (workers, larvae, or queens) that have not fed directly from the source via trophallaxis (mouth to mouth reguritation). These ants then pass what they receive onto other colony mates. This process means that poison bait will spread spatially through the ant population from the bait station. To assess the buffer zone needed to prevent this spread from directly contaminating a cave, we performed a study quantifying the spatial spread of dyed food resources through populations of TCA at two sites that differed in TCA abundance: Met Center – low abundance site, and Briarcliff – high abundance site (Fig. 1).

Dye stations consisting of 1 L bottles with 0.5 cm^2 access holes for the ants on the top lip and filled with 500 ml of dyed sugar water solution were set up within areas of high TCA abundances. The dyed solution in the station consisted of 0.024 M Erioglaucine disodium salt (food coloring pigment: FD&C blue No. 1), 0.03% methylparaben (a preservative), and 30% sucrose. Additional dyed sugar solution was added to the station as needed. Ants were allowed to harvest the dyed sugar resource for 168 hours. Dye stations were protected from mammals and birds with poultry mesh.

At 12, 24, 48, 72, and 168 hours post dye placement, four transects of hot dog baits were placed running out from the dye station one every 5 m for 100 m. After one hour, 20 TCA workers were collected off of all baits recruited to by TCA. In the lab, these 20 workers were squashed onto filter paper. The spots left by the squashed ants were scored on a qualitative scale of 0 to 5 with 0 being no detectable blue color and 5 being a fully-saturated, dark-blue spot.

To assess TCA population abundance, immediately after the removal of the dye stations, pitfall traps were installed and run for 24 hours. Pitfall trap consisted of 15 cm long, 3 cm diameter, 50 ml centrifuge tubes. Traps were installed 24 hours before being opened and run to allow ant activity associated with digging to dissipate.

(5) Boric acid bait stations: design, deployment, and measuring usage.

5) (a) Bait station design.

The outer sleeves for bait stations used to deploy boric acid laced baits consisted of 25 cm lengths of 4 inch schedule 40 PVC pipe with end caps. Eight cm from the top a ring of six, 3 cm diameter holes were drilled in the pipe and covered with window screening glued to the interior wall. Five millimeter holes were drilled in the back of the pipe to provide attachment points for wire to affix the station to trees or stakes in the ground. This design ensured that only insects small enough to pass through window screening could potentially access the bait. Finally bait stations were painted brown to blend in with their surroundings and an informational label with contact details attached for inquisitive members of the public (Fig. 5).



Figure 5: Boric acid bait dispensing station.

Inside the PVC sleeve we placed a 500 ml Nalgene™ bottle and a 50 ml Falcon tube. Ten centimeter dowel rods were inserted into both containers to provide additional surface area for ant trails. The bottle was filled with 500 ml of 30% sucrose with boric acid solution, and the Falcon tube was filled with 45 ml of 10% collagen protein powder with boric acid. Boric acid concentration varied across the control interval.

5) (b) Bait station deployment.

Bait stations were maintained and utilization activity measured from March 21 to October 17, 2016. Initially, 16 bait stations were set in a 100 m diameter ring around the entrance to Whirlpool Cave creating a 50 m buffer zone between the bait stations and the cave entrance. Based upon the data gathered in the spatial scale of resource transfer within TCA populations study, this initial buffer zone distance was chosen as sufficient to minimize movement of ants contaminated with boric acid into the cave. Bait stations were charged with sucrose and collagen solutions containing 1.5% boric acid. Solutions and solution containers were changed every two weeks. Based on early bait station utilization data indicating a lack of control of TCA populations, on April 28 the number of bait stations was increased to 24 stations. The bait stations were also moved closer to the cave entrance and set up in two concentric rings: the outer 40 m from the cave entrance and the inner 30 m. Bait station utilization data indicated that TCA abundances continued to increase; so on May 31, boric acid solution concentration was increased to 2%.

(5) (c) Measuring bait utilization

To measure bait utilization, weekly ant activity counts were made. The number of ants exiting the station over a one minute interval on the most active trail was counted. The amount of bait removed from the stations was recorded every two weeks when containers and solutions were changed.

(6) Determining the efficacy of the control attempt.

To assess the efficacy of our boric acid bait station array in reducing TCA populations, we conducted pre, during and post bait station deployment pitfall trapping. All pitfall trap stations were established in the fall of 2015, five months prior to bait station deployment. Pitfall trap stations consisted of 15 cm long, 3 cm diameter PVC sleeves set flush with the ground surface. Into these sleeves, 50 ml centrifuge tubes (3 cm diameter opening) were inserted. During non-trapping intervals these tubes were left capped and served as plugs. During trapping intervals, tubes were replaced with open tubes containing 30 ml of soapy water. Traps were left open for a 24 hour interval. Forty-nine pitfall trapping stations were installed in four areas: six about 10 m from the cave entrance, nine in the region of the original ring of bait stations about 50 m from the cave entrance, 24 in three transects radiating out from the bait station ring for 175 m, and finally nine approximately 400 m from the cave in an area from which we have data on TCA density from previous years.

The entire array of 49 pitfall traps was run three times: the fall of 2015 prior to the experiment, the spring of 2016 immediately prior to bait station deployment, and in the fall of 2016 immediately after bait stations were shut down. In addition, a subset of 27 traps was run monthly throughout the bait station deployment interval. These traps were also run throughout the winter, spring, and summer of 2017 to accumulate a record of TCA abundance fluctuations by which to judge the success of other ongoing and future control efforts. During winter, a period of very low TCA activity, this subset was reduced to 14 traps.

(7) An assessment of non-target impacts of boric acid bait.

To determine to what degree our control attempt indirectly impacts cave fauna, we analyzed boron concentration in tissues of three groups of non-threatened invertebrate species common in caves: cave crickets (*Ceuthophilus* spp.), cave-dwelling spiders (Pholcidae, Lycosidae and *Cryptachaea porteri*), and hothouse millipedes (*Oxidus gracilis*). We also evaluated levels of borate exposure that cause mortality in cave crickets.

Control Strategy 2: An evaluation of the biological control potential of a microsporidian pathogen of TCA.

Diagnosing and quantifying M. nylanderiae infection

Ants were tested for *M. nylanderiae* infection using a combination of a diagnostic PCR assay (for PCR methods see Plowes et al. 2015), and visualization of spores under a phase contrast microscope using air-dried, trichrome blue-stained, smears of homogenized worker tissue (Didier et al. 1995). To remove superficial spore contamination, all individuals were vortexed with two rinses of a 0.2% Triton™ X-100 solution prior to DNA extraction or spore counting preparation. To prevent *N. fulva* exocrine gland products from interfering with DNA extraction (Valles et al. 2012), workers were crushed in

distilled water and the supernatant discarded. DNA was then either extracted from individuals or extracted from a batch of 10 individuals homogenized together per colony fragment. Queens were always tested individually. Positive and negative controls for both DNA extraction and PCR were included in each group.

To confirm infections and assess the intensity of infections, counts of Type 1 DK and Type 2 DK spores (Sokolova and Fuxa 2008) were made on tissue homogenized from 20 workers from fragments that tested positive in the PCR assay. Octospores were uncommon. Their presence was noted but they were not included in spore counts. Infected workers had very large numbers of spores in their tissues, so only a small portion of a dilute sample of tissue homogenate was counted. Workers were bead beaten in 625 ul distilled water for 20 seconds. A 0.3 ul drop of the homogenate was pipetted onto a slide, fixed with ethanol, and stained (Didier et al. 1995). At 1000x magnification under oil immersion, spore counts were made in five non-overlapping field-of-views, covering the middle 2/3 of the fixed tissue stain. Individual worker spore load is not a useful measure of infection in social insects where colonies are the units of reproduction.

8) An assessment of whether the microsporidian parasite of TCA also infects native ant species.

We took two approaches to assess whether *M. nylanderiae* infects native ants: assessing infections in exposed field populations, and attempting to artificially infect colony fragments of a related native ant in the lab. To assess whether ant species other than *N. fulva* were infected with *M. nylanderiae* in the field, we identified two populations that harbored highly prevalent *M. nylanderiae* infections and had a population edge that intersected a diverse ant community. Along that edge we laid out bait transects with baits spaced 15 m apart and, where possible, collected 30 ants of as many species as possible recruiting to the baits. Fifteen meters spacing is sufficient (with the possible exception of polygyne fire ants) to ensure that collected ants come from independent colonies. We also searched opportunistically for nests of co-occurring ants from which to sample. We collected up to 15 samples from independent colonies per species per site. We collected and tested all co-occurring ant species we encountered, not just native species. In addition we collected 10 to 14 samples of *N. fulva* that spanned the edge where co-occurring ants were collected. Collections were first screened with diagnostic PCR. For any samples that tested positive for *M. nylanderiae* DNA, we spore counted homogenized worker tissue.

As an additional assay, we attempted to artificially infect fragments of a congeneric native species to TCA, *Nylanderia terricola*, with *M. nylanderiae* by housing them in environments contaminated with *M. nylanderiae* spores and feeding them diets containing *M. nylanderiae* spores. We utilized two diet treatments: infected TCA brood or infected workers. Feeding on or contacting infected TCA brood or dead workers are the most likely means by which co-occurring species would contract the disease.

Colony fragments (100 workers and 30 larvae) were housed in 15 x 15 x 9 cm opaque nest boxes with ventilation screening containing 12 ml test tubes half-filled with water and plugged with cotton to provide humid nest sites. Boxes also contained an additional cotton-plugged test tube with a 20% sugar solution available *ad libitum* and replaced once per week. All boxes were held at 28°C, and under a 12 hour day length cycle. Boxes were contaminated with dead, infected ants at the beginning of the transmission test by adding 0.1 g midden (dead workers) from an infected colony, and 20 freshly killed

infected workers. Two times per week fragments were either fed 15 pupae or late instar larvae from infected TCA colonies, or a slurry of homogenized, infected TCA worker tissue. Worker-tissue slurry was prepared by homogenizing 200 TCA workers from an infected colony fragment with one adult cricket (*Acheta domesticus*) using a mortar and pestle. The infected brood-fed treatment included 15 colony fragments of *N. terricola* and five of *N. fulva*, while the slurry-fed treatment included seven colony fragments of *N. terricola* and eight of *N. fulva*. Three times per week all pupae or callow workers (recently eclosed workers) were removed from the box and tested for *M. nylanderiae* DNA using diagnostic PCR.

9) An understanding of the temporal and spatial dynamics of the parasite's prevalence in TCA populations allowing for the design of efficient inoculation regimes.

To achieve a better understanding of the spatial and temporal dynamics of *M. nylanderiae* infection, three populations that harbored *M. nylanderiae* were chosen (Fig. 1). At a field site within each population, 15 stations were established separated by a minimum of 200 m. Once in the Spring, Summer, and Fall, each station was sampled by collecting 40 *N. fulva* workers. Spore counts were made of 20 workers per station to assess infection intensity.

10) An assessment of whether high prevalence of this parasite reduces local abundances of TCA.

To assess whether there was evidence that *M. nylanderiae* impact the population densities of TCA under field conditions, we established widely spaced stations (200 m separation) at six field sites with TCA populations: three that harbored *M. nylanderiae* and three that were uninfected. Annually as close as possible to the fall population peak (around September 21), we ran pitfall traps at these stations and sampled workers from baits for *M. nylanderiae* testing. Pitfall trapping methods followed those described in the assessment of efficacy of the boric acid bait treatment. Samples collected for *M. nylanderiae* testing were spore counted following the 20 worker batch homogenization protocol.

Data analysis comprised relating the difference between the fall TCA density peaks in a given year and the preceding year to the intensity of *M. nylanderiae* infection in the fall of the second year. In this analysis, the contrast between the average TCA densities across all stations at a site between the two years comprises a single replicate. Thus in the timeframe of the grant there are few data points available for analysis. To increase the power of this analysis, we went back to the contents of pitfall traps that had been collected from these same sites in years prior to 2015. From the traps that were run close to the fall TCA density peak, we removed 20 TCA workers and spore counted their tissues. Data was only taken for sites where traps were run far from the edge of the TCA population (at least 200 m) and could be expected to be representative measures of the average, equilibrium abundance of TCA for that year. This provided a total 16 contrasts from successive falls with some contrasts dating from TCA collected far back as 2011. To accommodate the wide range of TCA abundances encountered, density changes are presented in units of the pooled standard deviation for the population across the two successive years.

11) An evaluation of methods for inoculating uninfected TCA populations with the parasite.

11a) Assessing how M. nylanderiae *is transmitted within colonies of N. fulva*

In order to design a strategy for inoculating colonies, we must understand what castes and developmental stages within TCA colonies are susceptible to infection and which are capable of transmitting infection. To do this we performed a series of transmission experiments.

Colony fragments were housed in 15 x 15 x 9 cm opaque nest boxes with ventilation screening containing 12 ml test tubes half-filled with water and plugged with cotton to provide humid nest sites. Nest boxes were connected with 10 cm of Tygon™ tubing to an 11.5 x 8 x 5 cm translucent foraging box. Unless otherwise specified, foraging boxes containing a cotton-plugged test tube with a 20% sugar solution available *ad libitum* and replaced once per week, and colony fragments were fed a dead cricket three times per week. All boxes were held at 28°C, and under a 12 hour day length cycle.

Transmission replicates were constructed using independently collected fragments of uninfected ants or brood combined with infected ants or brood from a common source as called for by the particular test. In order to address potential infection due to contamination, control replicates were assembled in an identical manner to transmission replicates. As in treatments, uninfected material introduced into control fragments was from a common source and this source was always independent from the ants used to establish the nest box. To assess transmission, the ant caste being exposed to infection transmission and their matched controls were periodically tested by PCR for *M. nylanderiae* DNA.

Role of queen in transmission

An absence of queen to larvae transmission in a pilot study led us to question the frequency of infection of queens in infected colony fragments in nature. To assess this, we opportunistically performed diagnostic PCR assays on queens collected from colony fragments in which the workers tested positive for microsporidian infection. We tested 42 queens and associated workers collected from 25 nests over one year. In addition, from a single infected colony fragment we performed individual spore counts on 20 queens and 20 workers collected from a single infected nest.

Worker-to-larva transmission

Worker-to-larva transmission was tested by combining 300 infected workers (0.25 g) with uninfected brood (30 eggs and 10 early-instar larvae) and an uninfected queen. Larval development requires 12 days at experimental temperatures (28° C) (Arcila et al. 2002). Pupae produced during the first two weeks were not raised exclusively by infected workers and were removed without testing. After that, pupae were collected weekly, and were tested in batches for the presence of *M. nylanderiae* DNA. Queens were tested at the end of the experiment.

Larva-to-worker transmission

Larva-to-worker transmission was tested by combining brood (60 eggs and 30 mixed-instar larvae) from infected colony fragments with 100 uninfected workers. All developing pupae were removed weekly to prevent them from joining the worker pool. Workers were first tested for infection by diagnostic PCR after 21 days to allow for infection to develop and testing continued weekly until 35 days elapsed. This 35 day interval was chosen because it is longer than the egg to pupa interval of 27 days at these temperatures (Arcila et al. 2002); thus ensuring workers would have reared all inoculation brood to pupal stage. As revealed by molecular testing of removed pupae, consistent infection among the larvae in the brood pile persisted for 21 days with sporadic recovery of infected pupae throughout the remainder of the treatment interval.

Worker-to-worker transmission

Worker-to-worker transmission was evaluated by marking a set of infected ants with paint: 0.1 g of DayGlo™ pink fluorescent paint powder mixed with 5 ml of acetone. This mixture was sprayed using an atomizing spray bottle onto the infected workers. The marked workers were allowed 48 hours to groom and for any mortality due to the marking process to occur. Using a dissecting scope, 50 clearly marked ants from the infected fragment were removed from the pool of surviving marked workers and mixed with 150 unmarked-uninfected ants. No brood or queens were included. After two weeks, ants were anesthetized with CO_2, and all marked workers removed. Workers were first tested for infection by diagnostic PCR 21 days later and testing continued weekly until 35 days elapsed.

Despite the grooming and mortality interval, some infected workers may lose their markings during the two weeks transmission interval as a result of self- or allogrooming. These workers might then be inadvertently included in the test for transmission. To account for this, a manipulation control was performed. Ants were marked and allowed to groom as above, then 200 clearly marked, infected ants were removed and held separately from unmarked, uninfected ants for two weeks. This provided the same opportunity for the removal of paint markings by self- or allogrooming as the mixture of marked and unmarked ants in the treatment. Then, as in treatments, 50 infected ants (marked two weeks prior) were mixed with 150 unmarked, uninfected ants without examining whether workers were still visibly marked. After one hour, all ants with visible marking were removed from the pooled workers. Unmarked ants were then assayed for *M. nylanderiae* DNA. Any infected workers that succeeded in removing their markings during the two week interval will be included in the workers assayed for infection at a similar rate as in the experimental treatment. Thus, in this assay, some infection in the controls may occur and only a much higher level of infection in the treatments would provide evidence for transmission.

Environmental acquisition of infection

The ease by which infection may be acquired from the environment was also tested. For experimental replicates, 300 infected ants (0.25 g) were housed in nest boxes and foraging arenas for two weeks to contaminate the housing materials with spores. After that time infected ants were removed and uninfected ants, brood and queens were housed in the contaminated containers. In addition, one cc of dead workers from an infected colony fragment was introduced into the foraging arena. Controls were treated identically with the exception of being housed in uncontaminated nest boxes and foraging arenas that contained dead, uninfected workers in the foraging arenas. Workers, brood and queens were tested with diagnostic PCR after 21 days and testing continued weekly until 35 days elapsed. Cotton swab samples taken from the floors of treated nest boxes and foraging arenas at the end of the experiment tested positive for *M. nylanderiae* spores by both spore counting and diagnostic PCR confirming that this was an effective method of contaminating the housing apparatus.

11b) Evaluating methods for inoculating uninfected N. fulva *nests with* M. nylanderiae

We conducted a pilot lab test in which infected workers were combined with a larger number of uninfected workers plus queens. Fragments were housed as above. Over a four month period, spore load in the fragments declined as the original infected workers died and then increased above starting spore loads demonstrating that in this highly artificial setup transmission of infection is feasible.

To assess whether this approach could be scaled to inoculate field populations, we conducted an experiment in which infected brood or infected workers were introduced at replicated stations within the Convict Hill field site. We chose to test infected brood and infected workers based upon the results of the transmission experiment. Inoculation fragments were of three types. Infected brood inocula consisted of 0.25 cc of brood from an infected fragment with 50 uninfected workers. Infected worker inocula consisted of 250 workers from an infected colony fragment. Uninfected worker inocula, performed as a sham manipulation control, consisted of 250 workers from an uninfected colony fragment. Inocula were placed into 50 m falcon tubes. Fifteen stations were identified at the site that were separated by a minimum of 70 m. At each station a hot dog bait was placed at each of three locations separated by 20 m. These were the sites of inoculation. Once TCA had recruited to the baits, a falcon tube containing ants of one of the three treatments was opened and placed under a cardboard shade with the opening facing the recruitment trail. Ants in the inocula tubes were observed to rapidly leave the tube and join the trail of recruiting ants. Recruitment trails were followed back to the nest entrance from which they emerged or the point in the leaf-litter beyond which the trail could not be found (typically 0.5-1 m from the bait). This point was marked with ground flagging and was the site from which all future collections of ants to assess infection status were taken. At three week intervals, baits were placed at these sites plus an additional two sites 10 m from the infected brood and infected worker inoculation sites. Forty workers were collected per station and tested for infection status with diagnostic PCR. Positive samples were spore counted to assess infection intensities.

Data Analysis

Data analysis was performed using JMP statistical software. When response data violated distribution specific assumptions such as normality, distribution independent (non-parametric) statistical analyses were employed.

Results

Impacts upon karst systems

1) Assessment of TCA use of cave environments determining depth that TCAs penetrate caves, abundances they reach at different depths, and environmental conditions associated with cave occupancy.

Across nine seasons of monitoring, TCAs never penetrated deeper than 40 m into Whirlpool Cave. Abundances of TCA declined precipitously beyond 20 m from the cave entrance (Fig. 6). TCAs maximum penetrated distance at No Rent Cave was 10 m, which corresponds to the maximum distance of the cave.

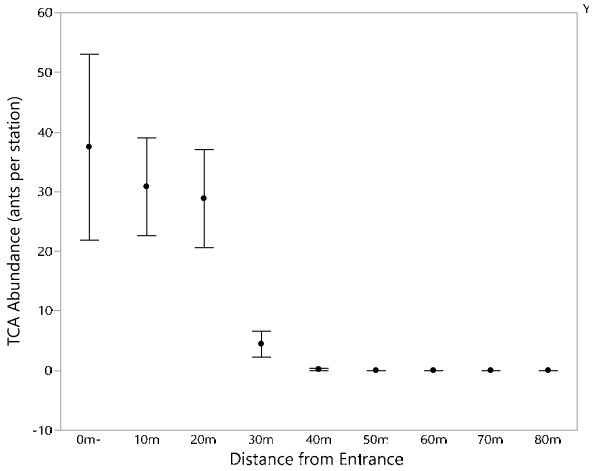


Figure 6: Abundance of TCA inside Whirlpool Cave at various distances from the cave entrance. Data present the average across 9 quarterly surveys of the number of TCA per baited tube.

Bait station deployment at Weldon Cave showed no TCA use on the surface or within the cave, supported by no TCA detections during corresponding cave faunal surveys.

At No Rent Cave, we detected TCAs inside the cave beginning in Fall 2014 while performing cave faunal surveys prior to the grant. Bait deployments began in Summer 2015 and confirmed TCAs within the cave through Winter 2016, but, for unknown reasons, TCAs disappeared from the cave and the areas around its entrance were absent for the remainder of the study (Spring 2016-Summer 2017) (Fig 7A).

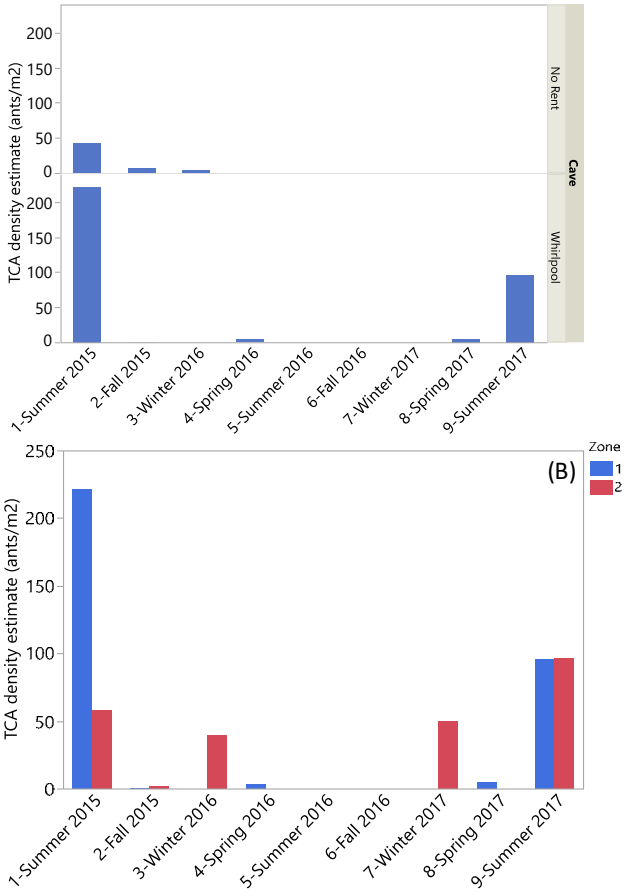


Figure 7: Density estimates of TCA inside (A) No Rent and (B) Whirlpool Caves. (A) top panel shows density at No Rent Cave while bottom shows density for the same time periods at the same distance invervals as Whirlpool Cave. Density estimates were made using the trail length measurement and vacuum subsample technique.

TCA presence inside impacted caves was episodic across the nine seasons of monitoring, and when present densities were highly variable. When TCAs were present at No Rent Cave, densities were low, and ranged from 3.35 – 6.33 ants/m^2 (*N*=2). Whirlpool Cave's TCA densities varied from low to high when present at a comparable depth to No Rent Cave, and ranged from 0.10 -221.73 ants/m^2 (*N*=5) (Fig 7B).

We examined TCA usage of Whirlpool Cave, and found that both TCA abundance and maximum distance found from the cave entrance were strongly driven by outside climatic

694 conditions. Average temperature and average relative humidity across the 14 days prior to each survey
695 date significantly predicted TCA abundance within as well as the distance they penetrated Whirlpool
696 Cave. Average daily vapor pressure deficit, a measure of the desiccating potential of the air, significantly
697 predicted abundance in the cave but provided only marginally significant predictive power for distance
698 TCA penetrated the cave (Fig. 8). Statistics are embedded in Figure 8.
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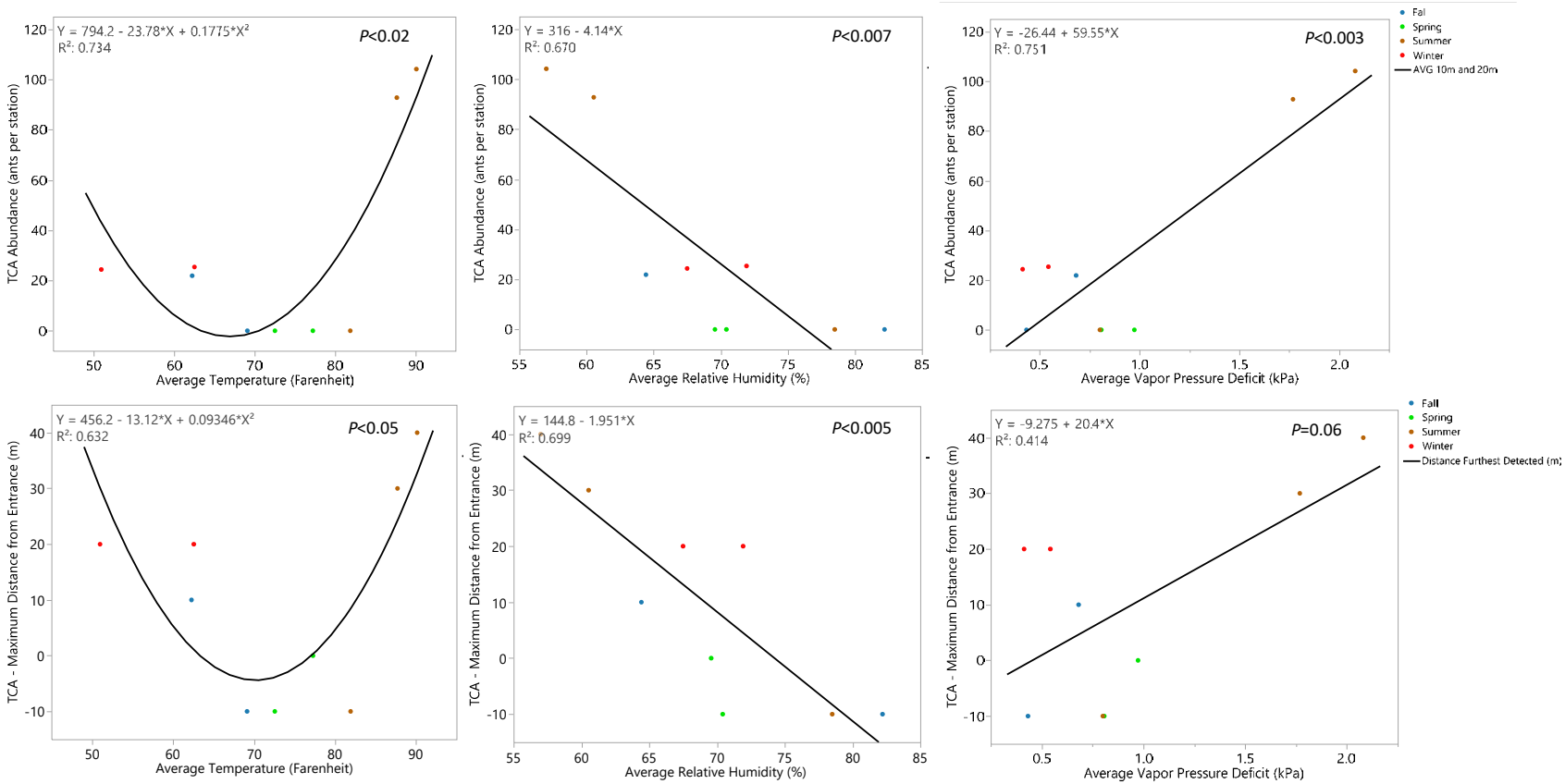


Figure 8: Usage by TCA of Whirlpool Cave for the 9 quarterly survey intervals plotted as a function of climate variables. Top panels display TCA Abundance: average number of TCA per baited tube. Bottom panels display the maximum distance TCA were observed penetrating into the cave. Maximum distance was taken from either presence in bait tubes or sampling of foraging trails. Climate variables were the averaged across the 14 days prior to the survey date. Average Temperature: average daily temperature; Average Relative Humidity: average daily relative humidity; Average Vapor Pressure Deficit: average daily vapor pressure deficit, a measure of the desiccating potential of the air.

2) An assessment of impact of TCA infestations upon karst invertebrate assemblages quantifying changes in karst invertebrate assemblage at different cave depths in parallel with TCA abundances.

Our ability to assess the degree to which TCAs impact cave arthropod faunas was severely limited by the number of currently invaded caves available to be surveyed. Only two caves were invaded during the period of this study: Whirlpool and No Rent Caves. As Whirlpool Cave was used as the site of the boric acid bait control study, only No Rent Cave provided an unambiguous test for examining impacts of TCA on karst arthropods. However, TCAs were only present at No Rent Cave for nine months, and average TCA abundance within No Rent Cave was only 28% of that observed at Whirlpool Cave during the same period. Thus, the opportunity for impacts was brief and the expected intensity of impact much lower than in higher density TCA environments.

Table 1: Results of cave cricket emergence count comparisons to TCA presence at No Rent Cave.

Cave Cricket Life-stage	TCA Status	Mean	SD	*N*	T Ratio	DF	P^2
Nymphs	Present	390	223	6	-0.25	14	0.80
	Absent	398	326	10			
Juveniles	Present	339	219	6	0.6	13	0.56
	Absent	317	390	10			
Adults	Present	349	445	6	0.93	11	0.81
	Absent	404	530	10			

2a) Impacts upon Cave Crickets: No Rent Cave

TCAs had no detectable impact upon cave crickets at No Rent Cave when comparing emergence counts performed during TCA presence versus absence at the site (Table 1.) We defined TCA presence for sampling periods when TCAs were within the cave and/or detected on the surface near the entrance to account for potential TCA impacts on cave crickets foraging outside of the cave. Our negative results were consistent for all three size classes surveyed: nymphs, juveniles, and adults. Although no TCA impact on cave crickets was detected during our study, low sample size and degree of TCA infestation at the site warrant discretion in interpretation of these results.

Table 2: Results of linear regressions relating the average normalized abundance scores from the test caves for a species during a particular sampling interval to the normalized abundance observed at No Rent cave for that same sampling interval. When test caves significantly predicted abundances at No Rent cave (bold), residuals from the relationship were used to analyze TCA impact.

Species	*N*	r^2	*P* value	m^1	*b*
Cambala speobia	15	0.53	**0.002**	0.68	0.08
Ceuthophilus cunicularis	15	0.32	**0.03**	0.82	0.07
Cicurina buwata	15	0.0	0.97	NS	NS
Cicurina varians	15	0.03	0.55	NS	NS
Helicodiscus eigenmanni	15	0.19	0.1	NS	NS
Pseudosinella violenta	15	0.19	0.1	NS	NS
Texella reyesi	15	0.27	**0.05**	0.79	-0.05

[1]Slope of relationship. NS: non-significant.
[2]Y-intercept of relationship.

2b) Impacts upon Cave Fauna: No Rent Cave

The normalized abundance scores (fraction of maximum observed abundance) from the test caves significantly predicted abundances for three species (*Cambala speobia* millipede*, Ceuthophilus cunicularis* cave cricket*,* and *Texella reyesi* harvestman) at No Rent Cave for the equivalent sampling intervals (Table 2). For these species, the residuals from these linear regressions were used to relate

754 species abundance changes with TCA presence (Fig. 9, left column). For the four remaining species, the
755 fraction of maximum observed abundance was examined (Fig. 9, right column) (see Methods).
756 Four of the seven species examined displayed the pattern of abundance changes across TCA
757 presence categories expected if TCA were negatively impacting their abundances (Fig.9). Of these four
758 species, TCA presence was significantly associated with decline in abundances of one species, *Cicurina*
759 *varians* (Table 2). Although no significant association was detected for the other species examined, the
760 generality of these results is limited due to the small number of surveys in which TCA were present in
761 the cave and the relatively low densities of TCA observed in No Rent Cave.
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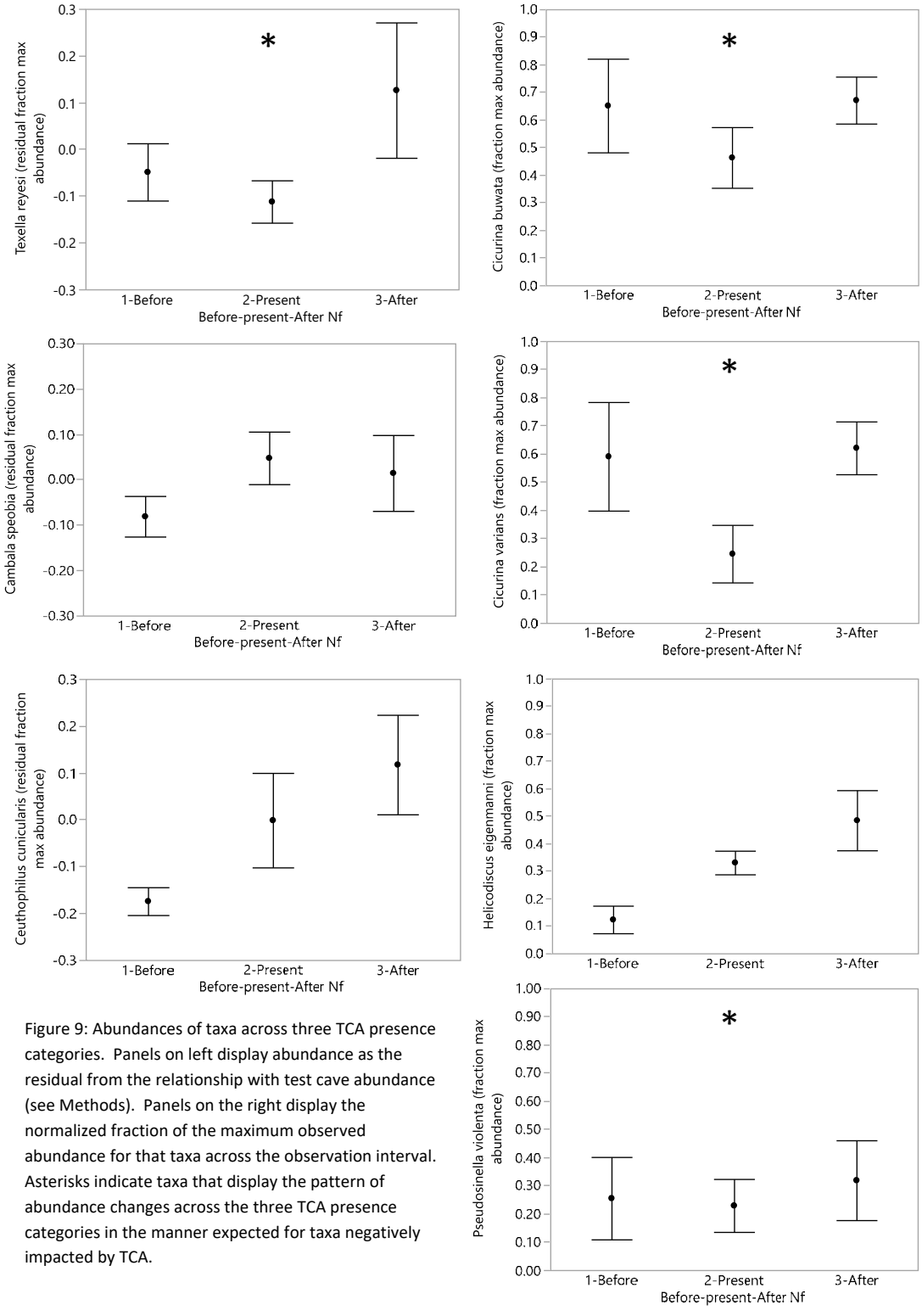


Figure 9: Abundances of taxa across three TCA presence categories. Panels on left display abundance as the residual from the relationship with test cave abundance (see Methods). Panels on the right display the normalized fraction of the maximum observed abundance for that taxa across the observation interval. Asterisks indicate taxa that display the pattern of abundance changes across the three TCA presence categories in the manner expected for taxa negatively impacted by TCA.

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Table 2: Contrasts of abundances from time periods when TCA were present at No Rent Cave with time periods when they were absent.

Species[1]	TCA Status[2]	*N*	Median (IQR) Abundance[3]	DF	X^2	*P* value[4]
Cicurina buwata - spider	Absent	10	0.75 (0.47, 0.84)	1	1.2	0.27
	Present	5	0.34 (0.28, 0.71)			
Cicurina varians - spider	Absent	10	0.55 (0.4, 0.89)	1	4.6	0.03
	Present	5	0.23 (0.05, 0.45)			
Pseudosinella violenta - springtail	Absent	10	0.21 (0.07, 0.41)	1	0.1	0.81
	Present	5	0.09 (0.07, 0.46)			
Texella reyesi - harvestman	Absent	10	0.04 (-0.17, 0.32)	1	1.5	0.22
	Present	5	-0.13 (-0.19, -0.03)			

[1]Species that showed the pattern of results expected for taxa might be impacted by TCA in Figure 9.
[2]TCA present or absent from cave. Surveys prior to TCA arrival and after their disappearance are combined as Absent.
[3] The median and interquartile range of the normalized abundance score (fraction of maximum observed abundance) for all species but *Texella reyesi*. *T. reyesi* abundance values are the residuals from the regression of No Rent and test cave abundances (see Methods).
[4] Results of a Wilcoxon rank sum test.

Control Strategy 1: Boric acid, liquid bait treatment protocol for TCAs in sensitive karst areas.

3) Formulating toxic bait: Boric Acid

(a) Aversion.

Immediately upon exposure, TCA showed a marked drop in acceptance of droplets as boric acid concentration increased (Wilcoxon: N=47, χ^2=14.1, DF=4, P<0.007). Posthoc pairwise comparisons showed the reduction in acceptance increased significantly once concentration exceeded 2% (Fig 10). On the third day of exposure to boric acid laced solutions, all solutions, including that which did not have boric acid, were accepted at an equal, and very low rate (approximately 20% acceptance) (Wilcoxon: N=47, χ^2=6.2, DF=4, P=0.18). The results from Day 2 were intermediate between Day 1 and 3.

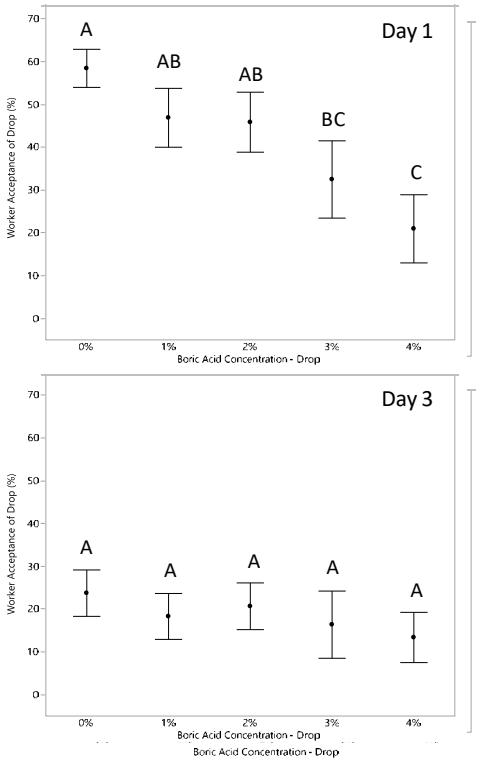


Figure 10: Percentage of workers that accept a solution for drops of varying boric acid concentration. Values are the average across fragments in acceptance rate. Bars present standard errors. Acceptance occurred when ants drank from drop for more than 3 seconds. Letters indicate significant pairwise differences. Top panel shows results of first exposure to boric acid solution (Day 1). Bottom panel shows results of third exposure to boric acid solution (Day 3).

(b) Delayed Toxicity

Time till 50% mortality of the worker population varied with the concentration of boric acid in solution (Wilcoxon: N=31, χ^2=8.8, DF=3, P<0.03) (Fig 11). Median times till 50% worker mortality were: 104.5, 55.2, 42.5, and 42.8 hours for 0.5, 1.0, 1.5, and 2% concentrations respectively. Higher concentrations were not tested as acceptance test results indicated that concentrations higher than 2% would be rejected.

(c) Macro nutrient composition: liquid protein type.

Preliminary assays of four protein sources that could be formulated into liquid baits (egg white, peanut butter, cow milk whey, bovine collagen and fish collagen), revealed that TCA workers would readily accept whey and bovine collagen. A subsequent direct comparison of these two proteins formulated in a 10% concentration solution revealed a preference of bovine collagen (whey: 12.5% acceptance, bovine collagen: 40% acceptance). Finally, an examination of preferred concentration of bovine collagen revealed a drop in acceptance at concentrations greater than 10%.

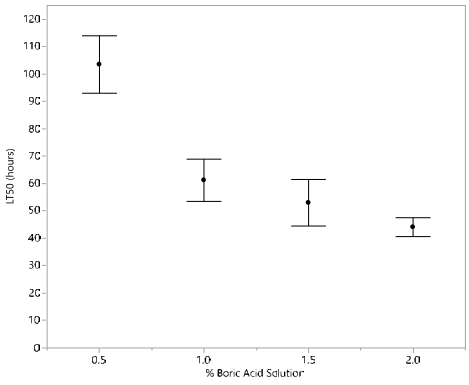


Figure 11: The time required for 50% of the workers to die (LT50) after exposure to boric acid solutions of varying concentrations. Boric acid solutions consisted of boric acid, water and 30% sucrose by weight.

(d) Macro nutrient composition: relative consumption of macronutrients by castes

Dye concentration did not affect the likelihood that workers would accept a droplet of sugar solution (Wilcoxon: *N*=63, DF=3, *P*=0.84). Thus the most concentrated, 2mM, dye solution was chosen as a tracer for examining how macro-nutrients are distributed amongst castes.

All castes consumed roughly equal amounts of both macronutrients (T-Test: Small larvae: *N*=20, *P*=0.50; Larvae-large: *N*=20, *P*=0.19; Workers: *N*=20, *P*=0.19, 0.72) (Fig. 12).

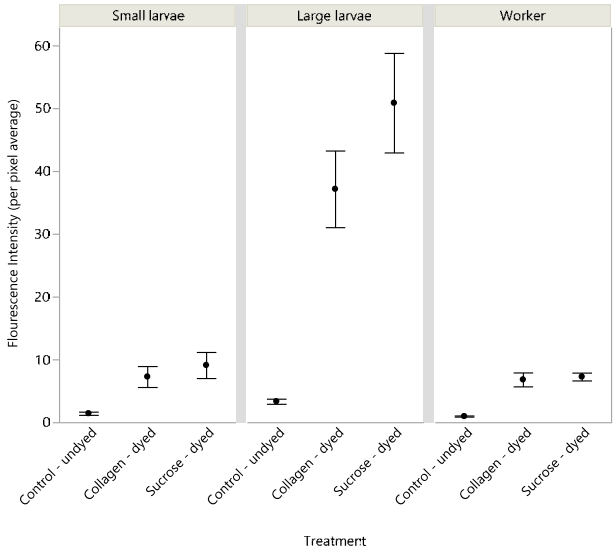


Figure 12: Results of macronutrient dye tracer study. Graphs show the mean intensity of flourescence in the 700 nm wavelength for castes exposed to differentially dyed food resources. Bars present standard errors. Fluorescence intensity is the average per pixel intensity of fluorescence. Larva-small were in the first or second developmental instar. Larvae-large were in the third or fourth developmental instars. Controls (not exposed to dye) provide a measure of autofluorescence. These were not included in analyses.

4) Quantifying the spatial scale of resource transfer among TCA nests to design spatial dispersion of bait stations.

The low-abundance TCA site had two orders of magnitude lower crazy ant abundance as measured by the number of ants caught per pitfall trap than the high-abundance site (low-abundance site: 5 ants/pitfall (3-7), high-abundance site: 565 ant/pitfall (264-1299) (median (Interquartile Range)). Dye spread farther through this low-abundance TCA population than it did through the high-abundance TCA population (low-abundance site: 45 m (41.3-56.3), high-abundance site: 32.5 m (30-38.8) (median (Interquartile Range)) (Wilcoxon: *N*=23, DF=2, *P*<0.002)(Fig. 13).

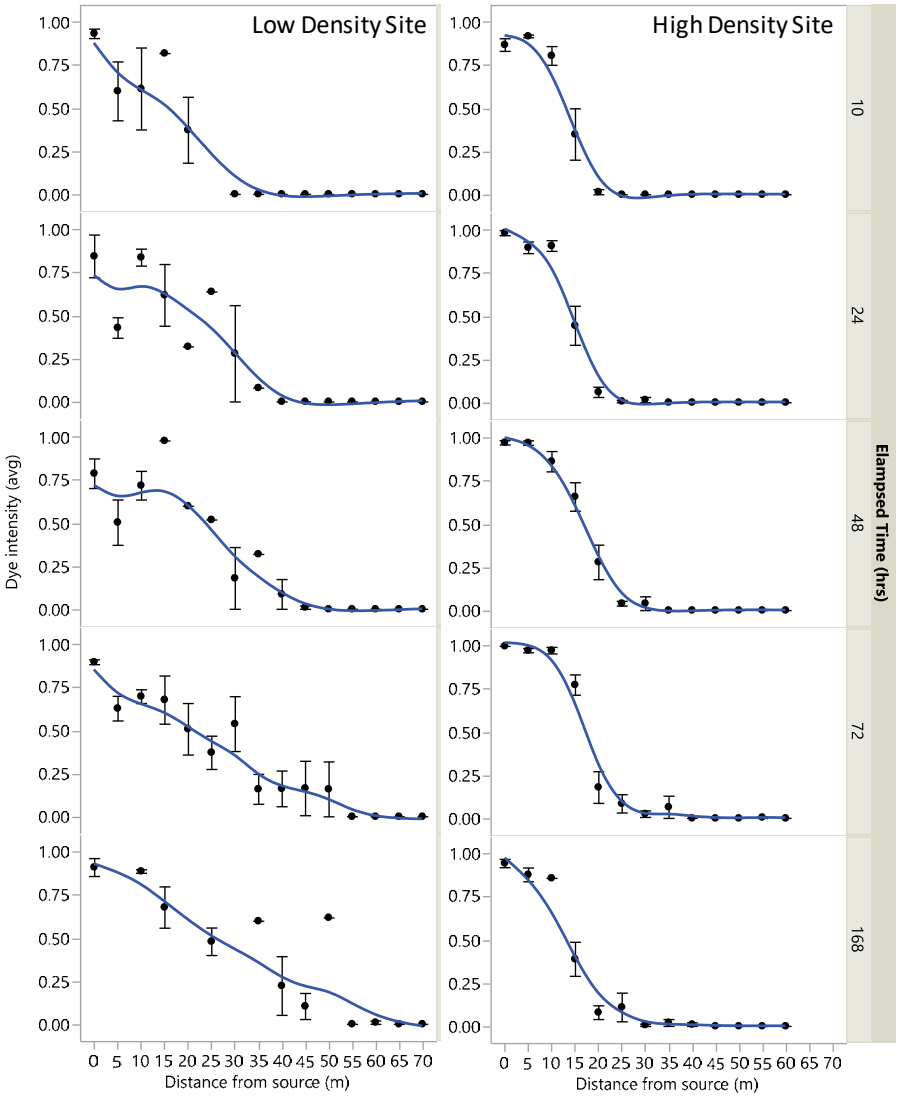


Figure 13: Results of dye movement study. Panels display the average intensity of dye in workers at sites progressively farther from the dye source. Left series presents dye dispersion at differing elapsed time intervals from the low density TCA site and right panels are from the high density TCA site.

5) Creating an inexpensive bait station designed to specifically target TCAs.

The sealed and screened boric acid bait stations described in the methods proved extremely effective at dispensing boric acid laced bait to *N. fulva* while preventing access to the bait by other arthropods. In the 672 times that individual stations were examined, only twice was an arthropod other than TCA observed feeding at the station. Both times it was the native ant *Monomorium minimum*, a species smaller in body size than *N. fulva*. The complete exclusion of other arthropods resulted from the window screening preventing access by arthropods larger than TCA but also because TCA were present foraging at the bait stations and excluding other arthropods in all but 29 of the 672 times that individual stations were checked.

6) An attempt to control a TCA population in the area around a cave entrance. We will determine the efficacy of the control attempt by measuring pre- and post-treatment TCA abundances using pitfall trap transects, as well as quantifying the duration of TCA population control.

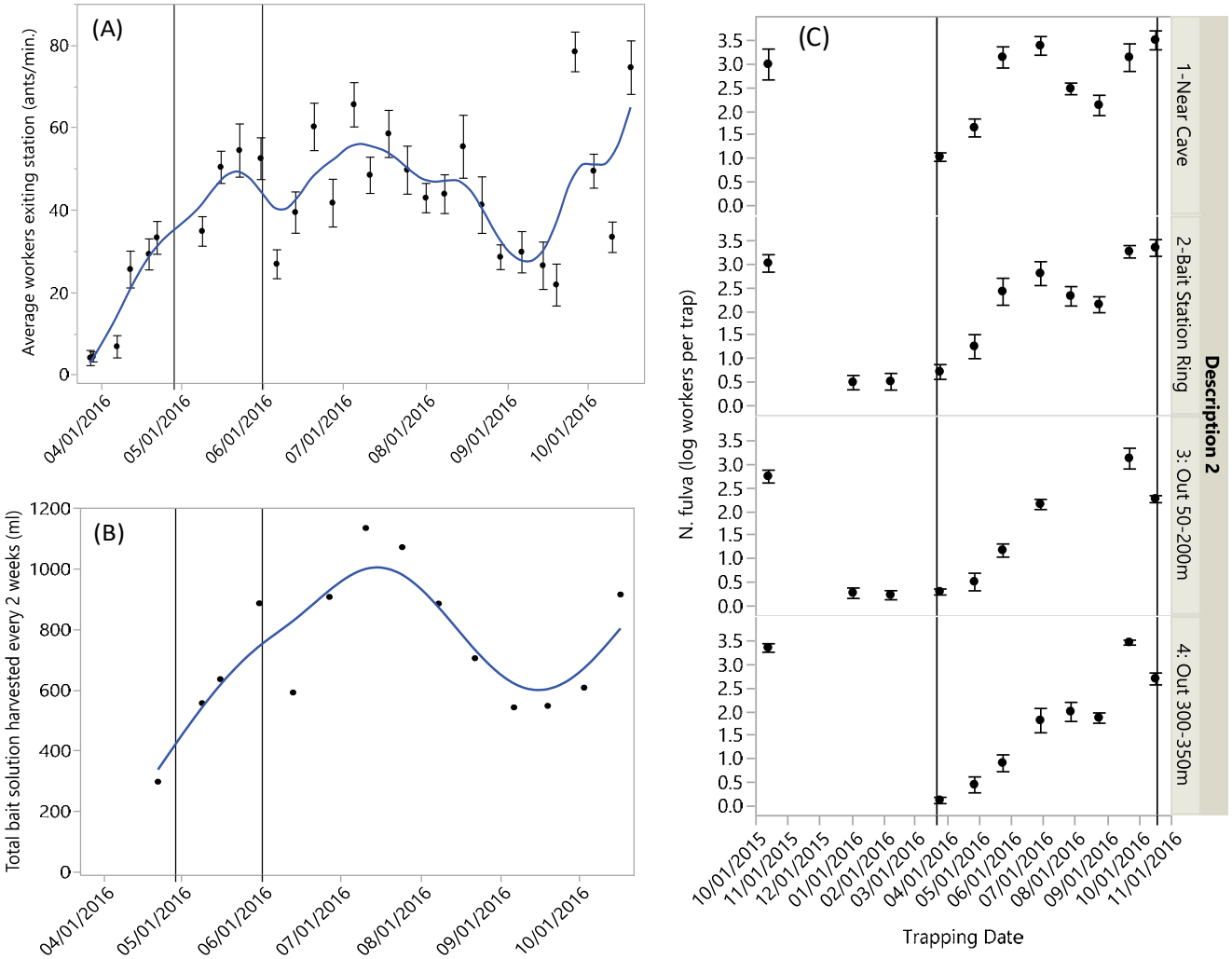


Figure 14: Results of the attempt to control a TCA population using boric acid laced sugar and protein baits. (A and B) Display the use of the bait stations by TCA during the bait dispensing interval. (C) Presents the density of the TCA population across the year. (A) Average numbers of ants exploiting a bait station across the interval bait dispensing interval. (B) Total amount of bait solution harvested from station across the bait dispensing intervals. Line 1 indicates the date that bait station number was increased from 16 to 24 stations. Line 2 indicates the date that concentration of boric acid in the bait was increased from 1 to 2%. (C) Pifall trap captures of TCA. Numbers of workers per trap are presented on a log scale. Lines indicate the beginning and end of the bait dispensing interval. 1: Near Cave Traps Bait Array Ring traps are inside the ring of bait stations and within 20 m of the cave entrance. 2: Bait Station Ring traps are interspersed among the ring of boric acid bait stations. 3: Out 50-200m traps are in transects leading away from the bait stations. 4: 300-350m traps are 300-350 meters from the boric acid bait stations.

TCA workers heavily exploited poison bait stations throughout the bait dispensing interval (Fig 14A). This exploitation translated into large amounts of poison bait being removed from the stations (Fig 14B). Despite this, there was no discernible impact of the continuous application of boric acid laced baits upon the abundance of the TCA population (Fig. 14C).

Abundances of TCA were higher near the cave/bait station ring (near cave plus bait station ring traps) than far from the bait stations (50-200 m traps plus 300-350 m traps) in March immediately before the boric acid treatment interval (Near bait stations: 7 (2-15) ants/trap; Far from bait stations: 0 (0-2.25) ants/trap median (interquartile range)) (Wilcoxon: N=49, DF=1, P<0.0001). In September, when TCA population abundances in all areas were at their peak and shortly before the end of the bait deployment interval, there was no significant difference in TCA population abundance between these two areas (Near bait stations: 2279 (916-4116) ants/trap; Far from bait stations: 3058 (1232-3397) ants/trap) (Wilcoxon: N=26, DF=1, P=0.61).

7) An assessment of non-target impacts of boric acid bait.

Tissues of cave crickets kept in the laboratory and fed a diet laced with 1% boric acid until death contained an average of 685 ± 225 (µg/g) ^{11}boron (mean ± SD) with a minimum of 359 µg/g. In comparing arthropods sampled before bait dispensing stations were opened with the same taxonomic group sampled after bait dispensing stations were closed, with the notable exception of juvenile cave crickets, significant or marginally significant increases in boron concentration were seen for all invertebrates sampled from the cave. Further wolf spiders, collected on the surface, also showed significant elevation in boron concentrations in their tissues at all collection locations. This included spiders collected more than 300 m from the boric acid dispensing bait stations. The amount that boron concentration was elevated in wolf spiders declined with the distance the spiders were collected from the bait dispensing stations. In no group did boron concentration approach the level seen in cave crickets killed by boric acid poisoning (Table 3).

Table 3: Results of ICPMS analysis of the ^{11}Boron content of insect tissues before opening (Pre) and after closing (Post) the boric acid laced bait stations.

Common Name	Scientific Name	Area[1]	Time Period	*N*	[^{11}B] Average (SD) (µg/g)[2]	[^{11}B] % Change[3]	[^{11}B] Max. Observed (µg /g)[4]	[^{11}B] % Lethal - Max. Observed[5]	*P*-value T-test
Cave crickets - adults	*Ceuthophilus cunicularis*[6]	Cave	Pre	6	18.2 (10.1)	**166%**	**45.6**	**7%**	**0.04**
			Post	7	30.3 (8.4)				
Cave crickets - juveniles	*Ceuthophilus sp*[7]	Cave	Pre	10	14.0 (7.2)	110%	**39.8**	**6%**	0.72
			Post	10	15.4 (9.6)				
Cobweb spiders	*Cryptachaea porteri*	Cave	Pre	10	1.7 (1)	**298%**	**10.8**	**2%**	**0.002**
			Post	10	5.2 (2.6)				
Greenhouse millipedes	*Oxidus gracilius*	Cave	Pre	10	4.2 (1.1)	**128%**	**7.3**	**1%**	**0.057**
			Post	8	5.4 (1.3)				
Cellar spiders	Pholcidae	Cave	Pre	10	2.4 (0.9)	**287%**	**11.7**	**2%**	**0.0005**
			Post	10	6.8 (2.7)				
Wolf spiders	Lycosidae	Near Cave	Pre	7	25.5 (7)	**264%**	**102.4**	**15%**	**0.003**
			Post	7	67.3 (24.2)				
		Bait Ring	Pre	7	26.6 (10.6)	**397%**	**156.9**	**23%**	**<0.0001**
			Post	6	105.6 (39.5)				
		Far	Pre	7	27.1 (11.4)	**197%**	**90.3**	**13%**	**0.01**
			Post	7	53.5 (19.6)				

[1] Location insects were collected. Cave: inside of cave; Near Cave: within 20 m of entrance of cave; Bait ring: within 20 m of a bait dispensing stations; Far: 300 to 350 m from the nearest bait dispensing station.

[2] The average and standard deviation of ^{11}boron concentration in units of micrograms per gram.

[3] The change in boron concentration observed between the pre and post samples expressed in percent of the pre sample.

[4] The maximum observed ^{11}boron concentration for individuals in the post sample.

[5] The percentage of the average concentration of ^{11}boron in the tissues of cave crickets intentionally poisoned with boric acid present in the individual with the greatest concentration of ^{11}boron observed in the group

[6] *Ceuthophilus cunicularis* cricket adults are considered troglophiles, and do not leave caves to forage.

[7] Juvenile *Ceuthophilus* crickets are not readily identified to species, but collections were made from cave ceilings to ensure samples were of trogloxenic species that forage outside of caves.

Control Strategy 2: An evaluation of the biological control potential of a microsporidian pathogen of TCA.

8) An assessment of whether the microsporidian parasite of TCA also infects native ant species.

8a) Occurrence of M. nylanderiae in ants other than N. fulva that are collected at sites in contact with infected N. fulva populations.

In surveys of ants collected at the edges of two TCA populations that exhibited very high levels of *M. nylanderiae* infection prevalence, no ants other than *N. fulva* tested positive for *M. nylanderiae* infection (Table 4).

Table 4: Results of efforts to detect *M. nylanderiae* in ants other than *N. fulva*.

Species Tested	Site[1]	Tested (#)[1]	Positive (#)[2]	Positive (%)
Nylanderia fulva	ABNP	**21**	**18**	**86**
Nylanderia fulva	BSP	**28**	**27**	**96**
Aphaenogaster texana	ABNP	9	0	0
Brachymyrmex patagonicus	ABNP	1	0	0
Camponotus pennsylvanicus	BSP	4	0	0
Camponotus planatus	ABNP	17	0	0
Camponotus sayi	BSP	2	0	0
Camponotus sp.	ABNP	1	0	0
Camponotus texanus	BSP	1	0	0
Crematogaster laeviuscula	ABNP / BSP	12	0	0
Cyphomyrmex rimosus	ABNP	1	0	0
Hypoponera opacior	ABNP / BSP	7	0	0
Leptogenys elongata	BSP	7	0	0
Monomorium minimum	BSP	10	0	0
Nylanderia terricola	ABNP / BSP	24	0	0
Pheidole dentata	ABNP / BSP	9	0	0
Pheidole sp.	ABNP / BSP	6	0	0
Ponerinae sp.	BSP	1	0	0
Pseudomyrmex sp.	ABNP	1	0	0
Solenopsis invicta	ABNP / BSP	28	0	0

[1]ABNA = Armand Bayou Nature Preserve; BSP = Buescher State Park
[2]Specimens were considered positive if *M. nylanderiae* spores were visible under phase contrast microscopy.

8b) Laboratory attempt to infect the native congener of TCA: Nylanderia terricola.

Attempts to artificially infect the native ant species, *Nylanderia terricola*, a congener of TCA were largely unsuccessful. However, this method of artificial transmission, feeding various diets laced with *M. nylanderiae* spores, also failed to generate infection in *N. fulva* (Table 5).

Table 5: Results of efforts to artificially transmit *M. nylanderia* to a native congener of TCA by feeding fragments infected TCA tissue.

Species	Infected Brood Tested (#)[1]	Infected Brood Positive (#)[2]	Infected Brood Positive (%)	Infected Workers Tested (#)[3]	Infected Workers Positive (#)[2]	Infected Workers Positive (%)
N. fulva	26	0	0.0	19	0	0
N. terricola	73	1	1.4	22	0	0

[1]Fragments fed dead, infected brood. Tested individuals were callow workers or pupae that completed their entire larval developmental period under the dietary regime.
[2]Specimens were considered positive if *M. nylanderiae* DNA was detected by PCR. *M. nylanderiae* spores are uncommon in infected pupae or callow workers.
[3] Fragments fed a paste of homogenized infected TCA workers and cricket tissue. Tests were of callow workers or pupae that developed as larvae under dietary regime.

9) An understanding of the temporal and spatial dynamics of the parasite's prevalence in TCA populations allowing for the design of efficient inoculation regimes.

Within infected populations, infection intensity did not vary significantly with season in either population examined (Kruskal-Wallis: East Columbia: *N*=121, DF=2, *P*=0.15; UH Coastal Center: *N*=55, DF=2, *P*=0.98)(Fig.15). Further, the non-significant differences between seasons that were observed were not consistent across the two populations (Fig.15).

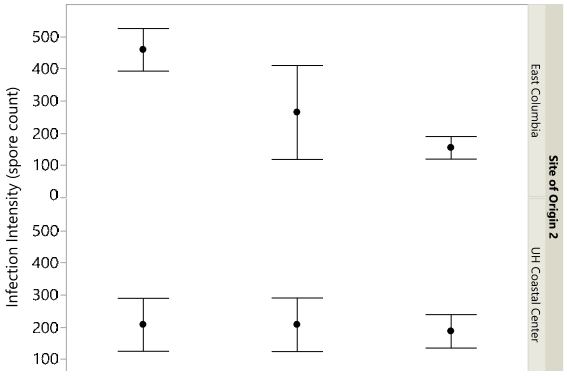


Figure 15: Variation in infection intensities across season for two populations. Within populations sampling sites are separated by 200 m. Infection intensity is the result of a spore count of the homogenized tissues of 20 workers.

The two populations that in fall of 2015 contained areas with high prevalence *M. nylanderiae* among nests and areas in which nests exhibited no evidence of *M. nylanderiae* infection demonstrated very divergent patterns of infection spread over the next two years. In one, East Columbia, infection remained spatially stable with stations that tested positive for *M. nylanderiae* remaining positive, and stations that tested negative generally remaining negative. In the second population, Iowa Colony, over the course of 2 years the infection spread throughout all monitoring stations within the population. Analytically, this idiosyncratic behavior can be seen in the dependence of frequency of station infection transition categories (eg: positive to positive, negative to positive, ect.) on site identity (Chi-squared Independence test: X^2=10.5, *N*=63, DF=3, *P*<0.02)(Fig, 16).

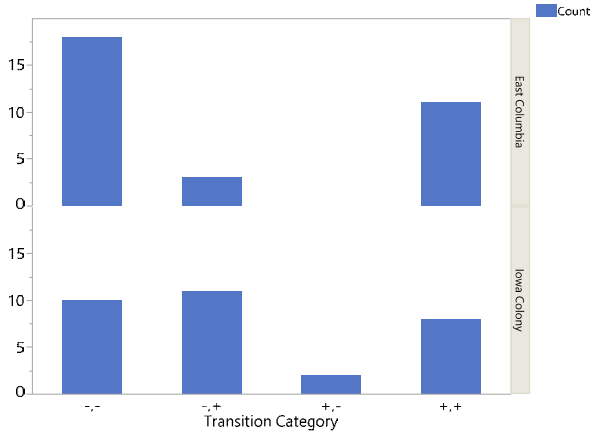
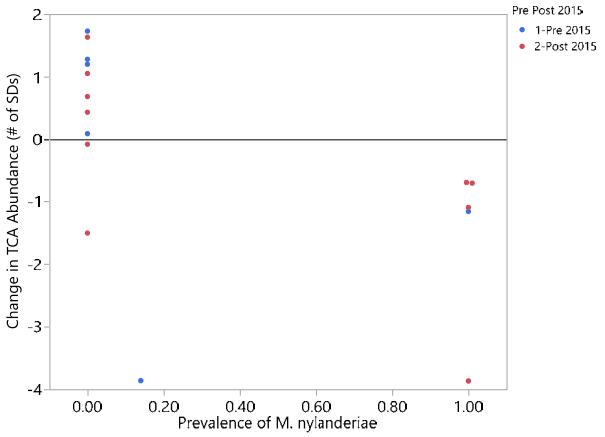


Figure16: Variation in station infection transition category for two populations harboring *M. nylanderiae* at some but not all stations. Within populations sampling sites are separated by 200 m. -,-: Uninfected sample 1 and 2; -,+: Uninfected sample 1, Infected sample 2; +,-: Infected sample 1, Uninfected sample 2; +,+: Infected sample 1 and 2. Samples from stations were separated by 4 to 6 months.

10) An assessment of whether high prevalence of this parasite reduces local abundances of TCA.

Comparisons of changes in peak fall population abundances of TCA (abundance in year 1 minus abundance in year 2) reveal that high prevalence of *M. nylanderiae* infection are associated with declines in *TCA* abundance. Comparing inter-year abundance changes where *M. nylanderiae* was highly prevalent in year 2 (greater than 50% prevalence) to inter-year changes where prevalence in year 2 was less than 50%, high prevalence of *M. nylanderiae* was associated with a decline in TCA abundance of -1.67 ± 1.62 standard deviation units, while the absence of infection or low prevalence infection was associated with an increase in abundance of 0.35±1.44 standard deviation units (mean±SD) (Fig.17).



Fig

the prevalence of *M. nylanderiae* infection within the population. Inter-year change in abundance is the difference between the Year 1 and Year 2 peak fall abundances of a particular TCA population. This change is expressed in units of the pooled standard deviation of all pitfall traps from both years. The prevalence of *M. nylanderiae* is the fraction of sites within a population that tested positive for *M. nylanderiae* in fall of the second year. Points below the 0 reference line declined in TCA abundance between year 1 and year 2. Data points in red were collected, at least in part, during the funding period of this grant. Blue points were not.

11) An evaluation of methods for inoculating uninfected TCA populations with the parasite.

11 a) *Assessing how* M. nylanderiae *is transmitted within colonies of* N. fulva

The only common way that *M. nylanderiae* infection was transmitted within colony fragments was from infected workers to uninfected developing larvae. Batch tests of pupae from all experimental worker-to-larvae transmission replicates were universally infected (Table 6). No infected pupae were produced in controls. Twenty to 83% of the individual pupae produced in these colony fragments with infected workers tested positive for *M. nylanderiae* (mean of 57%) (*N*=7). Table 6 summarizes the per replicate infection status for all experiments.

Larva-to-worker transmission occurs only rarely. None of the adult

Table 6: Results of intracolony transmission tests. Data summarizes infection status of replicates tested on the basis of homogenates of multiple individuals. Prevalence of infection among individuals within replicates summarized for efficient transmission pathways in results.

Transmission Pathway	Median Infection Inocula (IQR)[1]	Test Caste	Category	Ratio Infected, (% Infected) – End[3]
Worker-to-larva	86 (34-314)	Pupae	Treatment	9/9, (100)
			Control	0/7, (0)
Larva-to-worker	228 (123-421)	Worker	Treatment	0/7, (0)
			Control	0/7, (0)
Worker-to-worker	92 (43-170)	Worker	Treatment	2/9, (22)
			Control[4]	1/5, (20)
Environmental Acquisition	301 (202-587)	Pupae	Treatment	1/14, (7)
			Control	0/14, (0)
		Worker	Treatment	0/14, (0)
			Control	0/14, (0)

[1] The median plus interquartile range for relative infection intensities (spore counts for a group of 20 workers) present in the material used to inoculate treatment replicates. For the larva-to-worker test these counts are from workers belonging to the fragment from which the infected brood was harvested.

[2] Infection status of each replicate at end of observation interval.

[3] Positive tests results in these manipulation controls result from marked, infected workers losing their markings and being included in the test.

workers from any of the transmission replicates harbored infection at the end of the transmission interval, however a single positive sample was found in a mid-interval test (Table 6). Worker-to-worker transmission is also rare, if it occurs, as infected workers occurred at equal frequency in the experimental boxes as in the manipulation controls (Table 6). A limited amount of transmission was observed in the environmental acquisition manipulation with none of the larvae or queens but 7% of the worker samples from the experimental replicates testing positive at the end of the observation interval. No infection was observed arising in control replicates (Table 6).

Role of queens in transmission

Only 5% of queens from nests with infected workers in the field tested positive for *M. nylanderiae* DNA (42 queens collected from 25 nests). Further, a comparison of spore numbers in 20 queens and 20 workers from the same infected colony fragment revealed that 65% of individual workers harbored the infection with a median of 17,368 spores per spore positive individual, while only 10% of queens had a median of 334 spores per spore-positive individual. No other spore types were observed in these individuals.

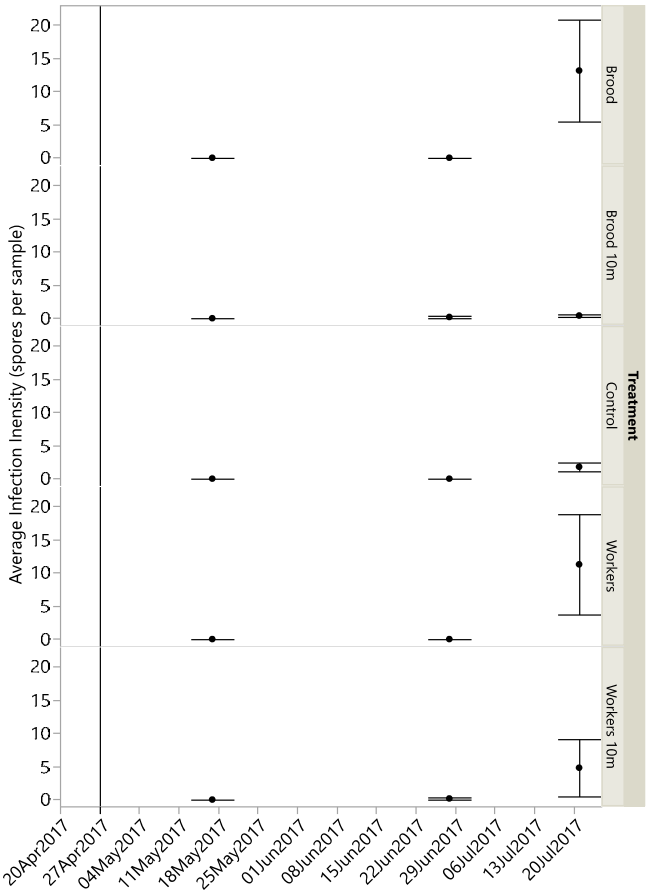


Figure 18: Results of Field inoculation releases. Brood = infected brood release sites. Brood 10m = Sites 10 meters from Brood release sites. Control = sites where uninfected workers were released. These sites were 20 m from both the infected brood and infected worker release sites. Workers = infected worker release sites. Workers 10m = Sites 10 meters from Workers release sites.

11b) *Evaluating methods for inoculating uninfected N. fulva nests with M. nylanderiae*

We succeeded in inoculating field nests of *N. fulva* with *M. nylanderiae* using both small amounts of infected brood and small amounts of infected workers. Both treatments exhibited a substantial and similar lag time before infection became evident at the site of release. The first positive detection of field infection occurred 64 days after initial inoculation and infection was not common at release points until 86 days post inoculative release (Fig. 18). As of 86 days post inoculation, there was no difference in prevalence (Positive release sites: Brood = 57%, Worker = 40%) (Chi-square: X^2=0.85, *N*=29, DF=1, *P*=0.47) or

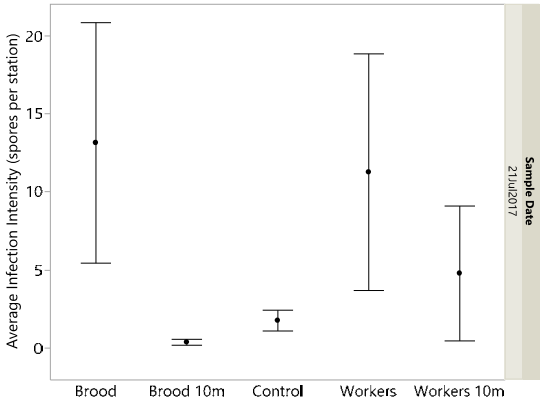


Figure 19: Intensity of infection at field inoculation release sites 86 days after inoculative release.

intensity (Average spore count: Brood = 13, Workers = 11) of infection (Wilcoxon: X^2=0.01, *N*=29, DF=1, *P*=0.92) between infected brood and infected worker release points (Fig.19). The low spore count numbers indicate that these still represent very early stage infections within the nests nearest to these release sites. Simultaneously with the first detection of infections at the release sites, we also detected infections at sites 10 meters from both the brood and worker inoculation points (Fig.19).

Discussion

Cave usage by TCA

TCA usage of the cave was strongly driven by outside climatic conditions. When surface conditions were stressful, either cold or desiccating (hot and dry), TCAs invaded the cave in moderate to high numbers. When surface conditions were favorable, TCAs were entirely absent from the cave. Ants are present in caves during the winter but only at moderate densities, likely because TCA populations are generally low in the winter. High TCA densities occur in caves when high summer population densities coincide with hot, dry conditions. These episodic, high density incursions will likely have the greatest impacts on cave arthropod faunas.

Impacts upon karst arthropods

Our ability to assess the degree to which TCAs impact cave fauna was severely limited by the number of currently invaded caves available to be surveyed. Whirlpool and No Rent Caves were already known to have been invaded before our study began, but we also expected a third cave, Weldon Cave, to be invaded during the study period, as its entrance was <200 m from No Rent Cave and TCA populations have been documented to spread an average of 200 m per year (Myers 2008). This anticipated spread to Weldon Cave did not occur, therefore limiting our ability to assess TCA impacts at different cave depths, as No Rent Cave is much smaller and shallower than Weldon Cave. Beyond lack of spread, the TCA population also disappeared from around the No Rent Cave area in the Summer of 2016. This population tested negative for the microsporidian and local property managers confirmed that pesticide use had not increased (W. Stewart, pers. comm. 2017). Therefore, the cause of reduction in the TCA population is unclear, but resulted in a lower intensity of impacts at No Rent Cave.



Figure 20: TCA preying on cave invertebrates in Whirlpool Cave. Top: *Cryptachaea porteri*. Bottom: *Oxidus gracilius*.

Four of the seven karst invertebrates sufficiently common to test showed the pattern of response expected if TCA presence depressed their abundances. However, the depression in abundance associated with TCA presence was only significant for one of these species, the troglophilic spider *Cicurina variens*. Thus, the

magnitude of TCA impact on karst invertebrates in No Rent Cave was not large. However, because this level of impact arises from only a very limited invasion of the cave by TCA over a very short period of time, we expect that it is also not generalizable to TCA invasions of other caves. Had the invasion at No Rent Cave been of similar magnitude as that at Whirlpool Cave, impacts upon karst invertebrates would have been substantially larger.

We were not able to assess TCA impacts upon karst invertebrate assemblages at Whirlpool Cave due to boric acid entering the cave ecosystem as a result of our control study. Any results indicating a decline were confounded by the possibility that the boric acid contributed to that decline. However, during Whirlpool cave fauna surveys we did observe TCAs preying upon karst arthropods including *Ceuthophilus* cave crickets, *Cryptachaea porteri*, Lycosidae spiders, and *Oxidus gracilius* millipedes (Fig. 20). We suspect that, despite the episodic nature of TCA incursions into the cave system, further study of additional infested caves would likely demonstrate negative impacts to cave species. Further, we expect these impacts to be greatest on species similar to *Cicurina variens*: troglophilic or troglobitic cave floor-dwelling species, which include endangered species such as *Texella reyesi*.

Control Strategy 1: Boric acid, liquid bait treatment protocol for TCAs in sensitive karst areas

The goals of the boric acid laced liquid bait station control study were to achieve a reduction in the abundance of the TCA population surrounding caves without introducing pesticide into the cave environment. Ultimately our attempt to control a TCA population using persistent, boric acid-laced bait stations was unsuccessful. Over the course of the bait delivery season, TCA population abundances near the bait stations and cave entrance fluctuated more than those far from the stations. However, despite deploying boric acid laced bait for the entire season of high ant activity and these stations being heavily exploited by the ants continually, by the Fall TCA population peak, there was no significant difference in the population abundance of ants far from the bait stations as compared to close to the bait stations and cave. Both areas had very high abundances of TCA. This occurred despite increasing the number and proximity of bait stations in the ring around the cave, as well as increasing the concentration of boric acid in the bait to the point just before behavioral avoidance of boric acid laced sugar solutions by the ants.

Why did we fail to measurably reduce TCA abundances? The primary reason is probably simply the magnitude of the ant population. At the start of the boric acid bait trial, we observed an average of 10 TCA per 24 hour pitfall around the cave entrance. Two months later that number had grown to 1900 ants per trap. This very rapid increase was seen throughout the site. It may be that in this enormous ant population the poison bait consumed by the TCA was diluted by trophallaxis (sharing with other ants) below the threshold of toxicity over a short distance, allowing workers to immigrate into the area around bait station from areas outside the zone of toxicity as fast as they died from boric acid poisoning. Another possible factor is the increase in behavioral avoidance of boric acid laced baits over time seen in the lab trials. Efficacy of the bait may have been reduced by ants that fed and survived avoiding the bait in the following days. However, TCA foraging at the bait stations, and depletion by the ants of the toxic bait therein remained at high levels that reflected overall ant abundance throughout the period of bait delivery.

The second goal of the boric acid bait control study, to not introduce pesticide into the cave, was also not met. Despite the buffer zone without bait stations around the cave, there was evidence

that the pesticide penetrated the cave ecosystem. All populations of cave invertebrates tested with the exception of juvenile cave crickets had significantly higher levels of ^{11}boron in their tissues by the end of the bait deployment interval as compared to prior to bait deployment. The highest of these levels were only a small fraction of those observed in cave crickets fed boric acid laced food until death, indicating that secondary ingestion of boric acid by cave invertebrates was well below the lethal threshold. To our knowledge this is the first test of whether pesticide treatments outside of a cave infiltrate into the karst system.

Surface arthropods also showed evidence of ingestion of boric acid. Wolf spiders, showed significant increases in boron in their tissues at all distance intervals examined, including 350 m from the bait stations. This was surprising. Wolf spiders are very large-bodied, top-predators of the leaf-litter arthropod community. The small-bodied TCAs are unlikely to comprise a large fraction of their diet, although they are expected to prey on animals preying on TCA. Further, in the dye dispersion assay, used to roughly quantify the expected distance that a liquid bait would spread through the network of TCA nests, visible evidence of liquid bait ingestion by ants disappeared by 30 to 55 m from the bait station depending upon ant abundance. Based on this, our expectation was that wolf spiders distant from the bait stations would not show an increase in boron levels. That they did indicates either the operation of an unknown mechanism of boron increase independent of our introduction of boric acid and one for which we did not sufficiently control, or that the boron in the boric acid ingested by the TCA spreads very widely through the arthropod community through pathways of primary and secondary consumption. The decline in boron concentration in wolf spiders with distance from the boric acid bait stations suggests that the latter is correct.

Based on the above results, we do not think that any alterations in the design of the boric acid-laced bait station approach would achieve the desired result of reducing TCA populations without introducing unacceptable amounts of pesticide into the cave. It is clear that achieving TCA population reductions would require a much greater number of boric acid-laced bait stations. This more intensive release of pesticides would necessarily increase the amount of pesticide already entering the cave. Although pesticide levels entering the cave system appear to be well below the lethal threshold for cave crickets, we do not know what long-term, low-level exposure to boric acid would do to karst arthropod populations. Nor do we know if other types of karst arthropods are more susceptible to boric acid toxicity than cave crickets. We cannot recommend this approach for controlling well established TCA populations in the area around the cave entrance. Pesticides may be the best tool for addressing establishing TCA populations which it is possible to control without treating near the cave.

Control Strategy 2: An evaluation of the biological control potential of a microsporidian pathogen of TCA

Our tests to date indicate that *M. nylanderiae* is a highly specific pathogen of *N. fulva* (TCA). In sampling 141 colonies from 18 different non-TCA ant species found at the edges of infected TCA populations, we did not find any non-TCA colonies infected by *M. nylanderiae.* Further, we attempted to forcibly transmit *M. nylanderiae* infection to a native, close relative of TCA (*N. terricola*) by feeding colony fragments spore contaminated material without success. Feeding on infected TCA brood or dead, infected TCA workers is the most likely mechanism by which non-TCA ant species would contract *M. nylanderiae*. Neither *N. terricola* colony fragments nor TCA fragments contracted infection by this

means, indicating that this is not a viable means of transmission. No amount of sampling can completely rule out the possibility that some infection of non-TCA ant species occurs at the edges of TCA populations where infected TCA overlap native ant species. However the data indicate that if this occurs, it does not appear to lead to persistent, self-sustaining infections in populations of ants other than TCA. Thus *M. nylanderiae* meets a critical criterion for use as a biological control agent. It is highly host specific.

The factors that govern the course of *M. nylanderiae* infection at the population scale remain mysterious. Across populations, there was no evidence of a consistent seasonal signal to infection intensity. Further, in the two populations where infection was patchy, the dynamics of infection spread through the populations were highly idiosyncratic. One population retained a stable pattern of infection with some areas of the population consistently harboring the microsporidian at high infection intensities while adjoining areas remained uninfected for long periods of time. In the other population, infection rapidly swept through the entire population with all previously uninfected stations harboring the infection by the end of the study. It is unclear at present what drives these differences.

Despite uncertainties about what drives *M. nylanderiae* infection to high prevalence in some TCA populations and what prevents it from reaching high prevalence in others, an emerging pattern is that highly prevalent infections by *M. nylanderiae* are devastating for TCA populations. Relating infection prevalence to the year-on-year changes in TCA abundance reveals that in all five available contrasts stemming from three TCA populations in which *M. nylanderiae* infection was highly prevalent by the end of the second year, TCA abundances dropped precipitously from year-1 to year-2. Declines of similar magnitude occurred in one uninfected population and one population that exhibited a lower prevalence infection in year-2. In this second population, prevalence increased to 100% in the following year and the population continued to collapse. In the other nine uninfected contrasts, TCA abundances remained stable or increased substantially. Two of these TCA populations with highly prevalent *M. nylanderiae* infections are no longer available for future contrasts as their TCA populations have declined to the point that we are no longer able to find them at the study sites.

11) An evaluation of methods for inoculating uninfected TCA populations with the parasite.

11a) Efficient Modes of Intra-nest Transmission

N. fulva in Texas exhibit a supercolonial form of social organization in which workers from distant nests are not aggressive to each other. As a result, the simplest scenario for inoculating an uninfected population is to introduce infected ants in a manner that they are adopted into nests of uninfected ants. But understanding what castes and developmental stages to introduce requires an understanding how *M. nylanderiae* is transmitted among the various castes and developmental stages of a TCA nest. Unlike most ants where modes of inter-colony transmission govern the spread of a pathogen at the landscape scale, the supercolonies of *N. fulva* are open systems with respect to the movement of workers between nests, thus the dominant forms of intra-nest transmission will be of primary importance in governing pathogen prevalence within the local worker population as well as the rate at which an infection spreads within supercolonies.

Infected workers transmit *M. nylanderiae* reliably to uninfected larvae. No other pathway of transmission was observed with frequency. Further, queens from nests with infected workers are very rarely infected with this microsporidian, reducing the functional importance of intracolony transmission pathways involving the queen with respect to determining disease prevalence (Fig. 21). As ants infected as larvae remain infected as pupae and emerge as infected adult workers, worker-to-larva transmission is sufficient to ensure that infection will cycle and grow within the worker population of a nest and spread between nests within a supercolony via worker migration. This would suggest that inocula containing workers only should be effective in transmitting infection to adoptive nests. However, ants structure colony work using temporal polyethism in which the tasks that individual workers perform change with age. Brood care is typically carried out by the youngest workers. Thus it is unclear if workers introduced into a population that succeed in joining a resident nest will engage in brood care and thus have the opportunity to transmit the pathogen. On this basis, we implemented a field trial of inoculation testing the efficacy of introducing infected workers or infected brood into an uninfected population.

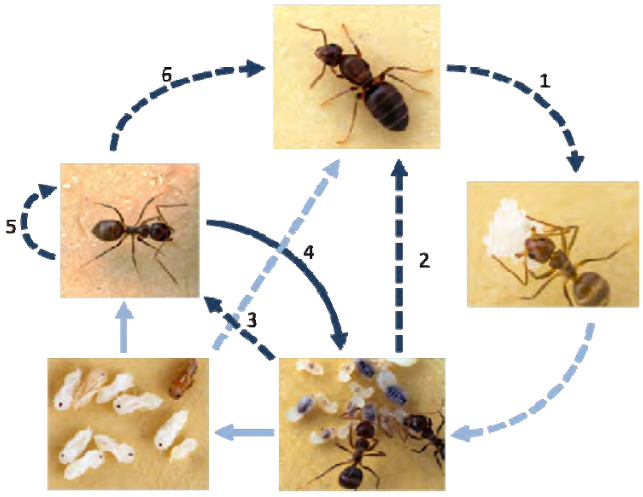


Figure 21: Potential routes of intracolony transmission for the microsporidian pathogen *Myrmecomorba nylanderiae*. Dark arrows indicate inter-individual transmission pathways. Light arrows represent transition of individuals between *Nylanderia fulva* developmental stages. Solid-line arrows indicate routes of transmission, or maintenance of infection between developmental states, confirmed to occur at high frequency. Dashed-line arrows indicate potential pathways that lead to transmission or transition of the pathogen only rarely if at all.

Why infection appears rare in queens but common in workers is puzzling. However, from the perspective of designing inocula for field populations, queens do not seem to be an important component. This is helpful as they are difficult to collect in quantity and inoculations can be undertaken with nest fragments that do not increase the reproductive capacity of the target population.

11b) *Evaluating methods for inoculating uninfected* N. fulva *nests with* M. nylanderiae

Our field trial attempting to introduce *M. nylanderiae* into an uninfected population succeeded. Both infected brood and infected workers succeeded in transmitting the infection to the local nests at their site of introduction. However, it took three months for this transmission to be detectable, so we are still in an early stage of evaluating the relative efficacy of these two modes of inoculation. The early data suggest that both strategies are equally efficient in transmitting the pathogen locally, but (although significant differences are so far lacking) that the infected worker inoculations lead to more rapid spread from the site of initial inoculation. These conclusions are very preliminary and subject to change as we continue to collect and test ants from these sites of inoculation. However, infected workers are by far the easiest component of the colony to collect in quantity, and, since they are effective in transmitting infection to uninfected nests under field conditions, they will be the central element in any program of widespread inoculation.

M. nylanderiae is patchily distributed within the North American range of *N. fulva* with surveys of populations finding that a minority of populations harbor this microsporidian (Plowes et al. 2015). Thus using *M. nylanderiae* as a tool in both traditional and augmentative biological control seems a feasible and worthwhile prospect.

Recommendations

With respect to protecting karst and other sensitive environmental features from long-term degradation by TCA infestations, we recommend that TPWD, USFWS, and other public entities support the following efforts:

1) Due to its short duration and the vagaries of TCA infestation dynamics, this study was unable to satisfactorily quantify the impacts of TCA on sensitive karst fauna. We recommend that invertebrate surveys at caves likely to be invaded or re-invaded by TCA as well as nearby, uninvaded caves continue in order to better quantify TCA impacts and evaluate how resilient karst fauna is to episodic, high-density incursions by TCA.

2) At this point, using *M. nylanderiae* as a biological control tool seems a feasible and worthwhile prospect. However much remains to be learned. Research is required to refine inoculation techniques and understand the time frame required for small inocula to spread. We fundamentally do not understand what governs the transition from a system characterized by prolonged low prevalence of *M. nylanderiae* to one where the pathogen actively spreads to infect essentially every nest in the population. Basic monitoring efforts of density and infection prevalence and intensity of extant infected populations need to continue, research into the impacts of this pathogen on host physiology is needed, and contributing environmental factors need to be tested. Research is also needed into whether augmentative biological control inoculations can shift populations from a low to a high prevalence state.

3) We also recommend pro-actively inoculating TCA populations that currently overlap or are likely to spread into sensitive karst features or other habitats containing sensitive organisms or high diversity arthropod assemblages generally.

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