


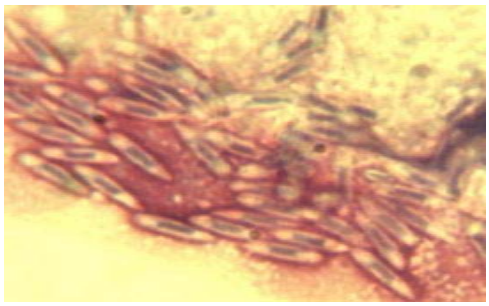

**Tawny Crazy Ant (*Nylanderia fulva*) Study**
**Balcones Canyonlands Preserve – Jollyville Unit**
**Task 5: Data analysis and Final Report**

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**Executive Summary**

*Nylanderia fulva* (tawny crazy ants) are an invasive ant species introduced from central South America that is spreading throughout the Gulf Coast region of the United States. These ants have devastating impacts on native arthropod assemblages and likely the wildlife that feeds on them. To mitigate these impacts on wildlife, particularly endangered wildlife, a means to sustainably control populations of tawny crazy ants (TCA) in natural habitats is highly desirable. A microsporidian pathogen, *Myrmecomorba nylanderiae* (Mn), discovered in 2015 presents a potential avenue for sustainable control of these ants. Herein we report the results of the second year of work on an effort to evaluate the impact of Mn on the TCA population currently invading a tract in the Jollyville Unit of the Balcones Canyonlands Preserve. The goals are: 1) Track the distribution and abundance of TCA in the preserve as well quantifying the composition of the resident ant assemblage, 2) Defining the magnitude and phenology of TCA canopy use as well as impacts upon canopy arthropod abundance in order to provide a qualitative assessment of impacts upon golden cheeked warbler (*Setophaga chrysoparia*) nesting success, 3) Track the spread of Mn through the local TCA population, and 4) Test methods for inoculating TCA with this pathogen as a means to develop this pathogen as a biological control tool as well as provide augmentative biological control for the TCA population invading the preserve. Major findings this year are: 1) TCA densities were overall much lower in the preserve in 2019 as compared to 2018, with median densities during the summer peak only 31% of their levels in 2018. This is likely a result of an extremely wet spring immediately followed by an intense, flash drought. 2) In 2019, the TCA population retracted in some areas and expanded in others. This contrasts with the uniform albeit slow expansion observed from 2017 to 2018. Also, for the first time in 2019 locations where TCA were absent were commonly found behind the leading edge of the TCA population. These absences were more common in areas infected with Mn, and may represent an interaction of drought and pathogen burden. 3) TCA began foraging in large numbers in the canopy in late May of 2019, very similar timing to that observed in 2018. This suggests that the direct impact of these ants on golden cheeked warbler nestlings and canopy arthropod prey availability may be restricted to the end of the breeding season. 4) In 2019 the area of infected TCA nests in the preserve had spread from only nests occurring on the northern, roadside preserve boundary to being prevalent in nests distributed from between 64 to 165 meters into the preserve depending on the part of the edge examined. 4) Sampling of the 2018 inoculation attempt continued through March of 2019 (8 months post inoculation) and was then discontinued due to an absence of Mn detection. However, 16 months post inoculation a positive detection of Mn was made in this area. Currently, sampling is underway to evaluate whether this represents delayed establishment. Two additional inoculation efforts in 2019 have so far yielded no evidence of establishment. Following on observations made during inoculation attempts, a behavioral experiment revealed the presence of previously unknown aggression between TCA that had been held in the laboratory under standard lab conditions and TCA workers in the field. This aggression appears to be the result of odors acquired in the lab, and may be responsible for our difficulties establishing this pathogen with previous inoculation techniques. Although not included as a deliverable for this grant, we have included a supplement at the end of this report documenting the developments in controlling a TCA population in the Convict Hill area. We include it herein as this TCA population overlaps a karst feature (Whirlpool Cave) covered by the Balcones Canyonlands Conservation Plan (BCCP).

**Project Background**

*Nylanderia fulva* (Tawny Crazy Ants) are an invasive ant species introduced from central South America that is spreading throughout the Gulf Coast region of the United States [1]. These ants invade natural habitats and have devastating effects on the arthropod assemblages of those environments [2]. By doing so, these ants reduce the available prey base for foraging birds and other vertebrates. These ants also forage in large numbers in the forest canopy and directly impact bird nesting success by irritating hatchlings to the point that they fall out of the nest (J. Giese 2015). To mitigate these impacts on wildlife, particularly endangered wildlife, a means to sustainably control populations of tawny crazy ants (TCA) in natural habitats is highly desirable.

In 2015 myself and collaborators discovered and described a microsporidian pathogen of TCA, *Myrmecomorba nylanderiae* [3]. We found this pathogen infecting a minority of TCA populations surveyed in both Texas and Florida. Subsequent work demonstrated that this pathogen has impacts on the growth and mortality of TCA nests under laboratory conditions [4], while accumulating evidence indicates that infection can reduce abundance of TCA in field populations sometimes dramatically (LeBrun unpub. data).

The larger goal of this research program is to refine the technical expertise necessary to implement and evaluate the efficacy of using this microsporidian pathogen as a biological control agent for TCA. The data from this project is part of a larger multi-site study. Herein I will be reporting only on the results from the year 1 efforts at the Collins Tract, Jollyville Unit, Balcones Canyonlands Preserve. The goals or tasks for year 1 were to:

1) Quantify the native ant assemblage within the preserve in both the invaded and uninvaded habitat adjacent to the expanding TCA population in order to better understand impacts of this invader and be able to assess the resiliency of the ant assemblage should we succeed at controlling this TCA population.
2) To assess TCA usage of the canopy in order to gauge their likely intersection with nesting golden cheeked warblers and black capped vireos. These data will also be used to support ongoing work by Travis County staff to assess how arboreal arthropod assemblages (food resources for nesting warblers and vireos) respond to TCA invasion.
3) Map the TCA population extent within the preserve.
4) Perform an inoculative release of the microsporidian into the TCA population and follow its establishment.

Prior to the initiation of this project, naturally occurring infections of *M. nylanderiae* were encountered in TCA in areas of the residential neighborhoods surrounding the preserve. In addition to the above tasks delineated in the proposal, we conducted sampling throughout the year of these areas. The results of this work are also reported herein.

**Task 1: Prepare a research plan**

Completed and submitted March 11, 2019.

**Task 2: Assessment of tawny crazy ant (TCA) densities and native-ant assemblage composition within the preserve**

*Pitfall trapping in areas invaded by TCAs as well as areas outside of the TCA colony extent*

Ant Community composition and TCA densities were assessed using pitfall traps set within the different areas of the preserve defined by the amount of time TCA had been present in the habitat. Permanent pitfall trapping stations established in 2018 in the invaded and uninvaded areas of the preserve were run in May, August, September, and October 2019. Permanent pitfall trapping stations consist of PVC sleeves set flush in the ground. Sleeves are plugged between trapping intervals and then an open tube containing a mixture of water, and soap and propylene glycol are inserted. Propylene glycol is a non-toxic preservative (approved by the FDA as a food additive) and poses no known threat to wildlife.

Three habitat areas defined by TCA occupancy were sampled: Long-term Invaded, Edge - Invaded and Out - Uninvaded. Long-term Invaded areas were in the interior of the TCA populations and have harbored TCA for several years prior to the beginning of research in fall 2017. Edge - Invaded habitat were invaded areas near the edge of fall 2017 TCA distribution in the preserve. Out – Uninvaded areas are of similar vegetation that are outside of the TCA population.

Findings

TCA densities were overall much lower in the preserve in 2019 as compared to 2018, with median densities during the summer peak only 31% of their levels in 2018 (Fig 1). This may be due to a combination of an extremely wet spring immediately followed by a flash drought that began in June of 2019 and lasted until early October. Albeit at currently lower density, TCA remain prevalent in the Long-term Invaded habitat. For the first time TCA were recovered in a substantial fraction of traps in the Edge area, however abundances were very low and most sites in this habitat remain uninvaded.

The uninvaded area of the preserve is habitat for a diverse ant assemblage. We were unable to

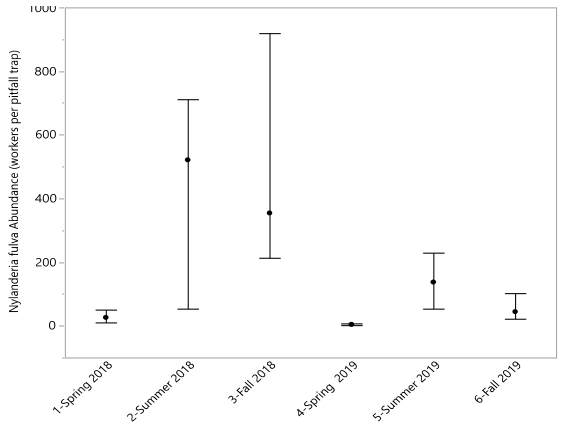



Figure 1: Abundance of *N. fulva* in areas of the site where the ants have been established since the beginning of the study (Long-term Invaded traps).

confidently determine one ant to species (*Temnothorax* sp. 2). This may turn out to be undescribed. We are reaching out to other experts to ascertain whether this ant belongs to a described taxon or is

present in other collections. The ant assemblage composition for these three areas is presented in Table 1.

**Table 1:** Ant assemblage composition 2019. Results from pitfall traps across three habitats sampled in 3 seasons. The cells report the percent of traps that contained a given species.

| Ant Species | Edge | | | Invaded | | | Uninvaded | | |
|---|---|---|---|---|---|---|---|---|---|
| | Spring (18) | Summer (11) | Fall (25) | Spring (18) | Summer (17 | Fall (33) | Spring (9) | Summer (9) | Fall (18) |
| *Aphaenogaster texana* | | 9 | | | | | | 11 | |
| *Camponotus discolor* | 11 | | | | | | | | |
| *Crematogaster laeviscula* | 11 | 27 | | | | | | | |
| *Crematogaster lineolata* | | 18 | 4 | | | | | 11 | |
| *Cyphomyrmex rimosus* | | | | | | | | 11 | |
| *Forelius pruinosus* | | | | | | | | 11 | |
| *Leptogenys elongata* | | 9 | | | | | | | |
| *Monomorium minimum* | 22 | 55 | 29 | 28 | 100 | 53 | 44 | 67 | 11 |
| *Myrmecina americana* | | | | 11 | 24 | 6 | | | |
| *Nylanderia fulva* (TCA) | | 18 | 4 | 61 | 100 | 67 | | | 11 |
| *Pheidole bicarinata* | | | | | | | | | 6 |
| *Pheidole constipata* | | | | | | | | 11 | |
| *Pheidole dentata* | 11 | | | | | | 11 | | 6 |
| *Pheidole floridana* | | | | | | | | 11 | |
| *Pheidole hyatti* | 11 | 9 | 17 | | | | | | 22 |
| *Pheidole lamia* | | | | | 6 | | | | |
| *Pheidole pelor* | | | | | | | | | 6 |
| *Solenopsis invicta* | | | | | | | 11 | 67 | 17 |
| *Solenopsis diplorhoptrum* sp. | 22 | 27 | | 17 | 18 | 3 | | 22 | |
| *Temnothorax subdivitus* | | | | | 18 | | | | |
| *Temnothorax andrei* | | 9 | | | 12 | | | | |
| *Temnothorax sp. 2* | | | | | | | | 11 | |
| *Tetramorium bicarinatum* | | 9 | | | 6 | | | | |

*Mapping the extent of the TCA population* (Fall of 2019)

TCA lack alate flights with nests reproducing though budding. These ants are also supercolonial with all nests within a population belonging to the same colony. As a result, as populations expand, they form a discrete boundary with one-side occupied by a dense network of interconnected TCA nests and the other side lacking TCA entirely. Mapping this boundary provides a way to track the spread of the invasion.

In the Fall of 2017 and the Fall of 2018, data on the extent of the TCA population within the preserve was collected to assess the extent of preserve occupied and the amount of year-to-year spread that has occurred. We continued this work in November 2019 along transects spaced at approximately 100 intervals throughout the section of the TCA population front that occurs in the preserve as well as the edges that adjoin the preserve, we assessed the presence or absence of TCA. Presence / absence was determined by visual inspection of the ant assemblage foraging near likely nesting locations and at the bases of trees. Observers identified the ants present at locations spaced every 20 m along the transects. Stations where 20 nest sites were searched, and no TCA workers were found either in the nests or foraging on the ground were considered free of TCA. Three negative stations in a row were defined as the threshold indicating the observer has passed beyond the edge of the TCA population.

Findings

In 2019, the TCA population retracted in some areas and expanded in others. This contrasts with the uniform albeit slow expansion observed from 2017 to 2018. Also, for the first time in 2019 locations where TCA were absent were commonly found behind the leading edge of the TCA population (population front) (Fig. 2). Previously TCA were uniformly present behind the population front. This occurred despite increasing the intensity of searching required before TCA was determined to be absent.

Although it is unclear what role Mn may have played in this population expansion / retraction dynamic, population retraction occurred in areas where the population front adjoined habitat where TCA harbor the disease while expansion occurred in the parts of the population front most distant from areas infected with Mn (see Figure 5). The rate of nest budding drives population expansion. Nest budding or worker emigration to nearby nests is stimulated by worker population exceeding the nest capacity. This flow of workers between nests allows for any depression in growth rates in areas adjacent to the population front to impact the rate of nest budding at the front.

Interestingly, sites behind the population front where TCA were absent occurred in many transects regardless of their proximity to infected TCA. We suspect that the TCA population suffered as a result of the intense flash drought that occurred from July to October 2019 during which essentially no rain was paired with extremely hot temperatures. It will be interesting to see if TCA population build up in the Spring of 2020 is slow as a result of starting from a lower overwintering population.

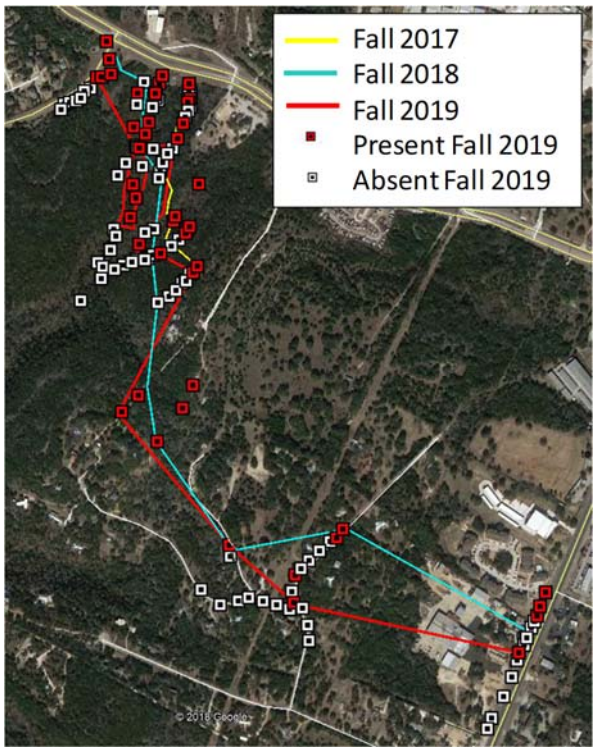



Figure 2: Edge of *Nylanderia fulva*-invaded area in Fall 2017 (yellow line), Fall 2018 (blue line), and Fall 2019 (red line). Squares show the presence (red) and absence (white) of TCA at sampling points from November 2019. Nest sites could not be exhaustively searched in sampling points outside of the City of Austin or Travis County property. Negatives in these areas (areas on west side of map) should be viewed with caution.

*Arboreal foraging density assessment* (Spring 2019)

In order to evaluate how TCA change the availability of prey for nesting golden cheeked warblers and black capped vireos, Travis County Staff performed arboreal arthropod sampling and ant foraging density assessments during the nesting season. Arthropod sampling was accomplished by standardized beating of 2 low-hanging branches per tree sampled, and foraging density measurements for TCA on trees in TCA invaded habitats. Up to four tree species were sampled depending upon their availability in the target areas.

Foraging density measurements for TCA on trees in TCA invaded habitats that were sampled for arboreal arthropods. As use of trees by TCA varies strongly both spatially and seasonally, density assessments follow a 2-tiered method. In low density foraging environments, tree trunks from the ground to 2 m high were searched for 3 min and all TCA observed foraging counted. In higher density TCA environments, the number of trails of TCA ascending into the canopy were counted, and, for each trail, a 3 min count made of the number of TCA climbing past a specific point.

This work is being led and primarily conducted by Travis County Staff. UT is providing support by identifying and counting ants, as well as processing spider and lepidopteran larvae for biomass measurements.

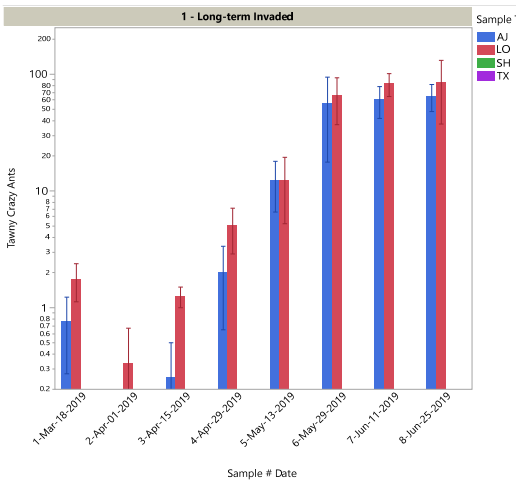
Three habitats were sampled for the arboreal arthropod surveys: Long-term Invaded, Edge - Uninvaded and Out - Uninvaded. Habitat categories were defined in the Fall of 2017 and invasion status refers to their status at that time point. Long-term Invaded areas were in the interior of the TCA populations and have harbored TCA for several years. Edge - Uninvaded habitat were in areas near but outside the Fall 2017 edge of the TCA distribution in the preserve. Out – Uninvaded areas are of similar vegetation that were well outside of the TCA population in 2017 and remain so.

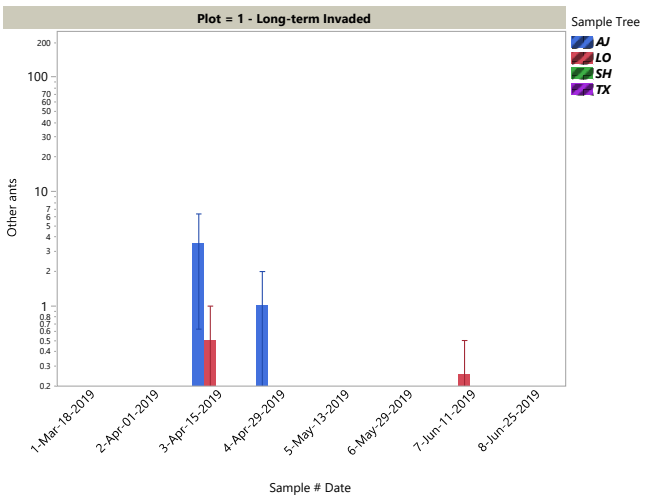
<u>Findings:</u>

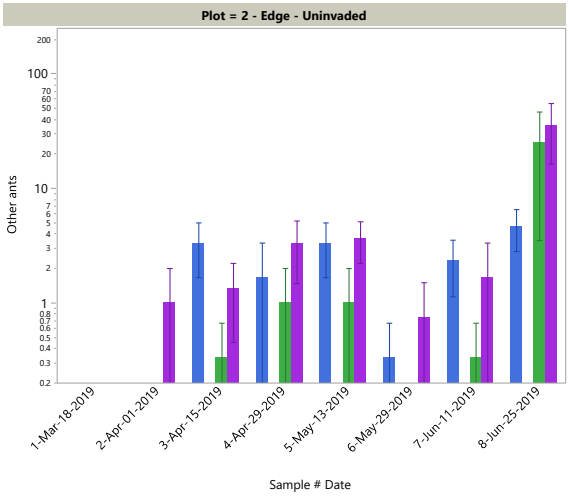
Travis County Staff are currently processing the canopy arthropod samples. When this is complete UT will count and identify the ants, and count, dry, and weigh the spiders and lepidopteran larvae.

TCA began foraging in large numbers in the canopy in late May of 2019 (Fig. 3). Prior to this numbers ascending tree trunks were quite low. Golden cheeked warblers (GCW) typically nest on the preserve from mid-March through mid-June (T. Clark pers. comm.), indicating that the direct impact of these ants on nestlings may be restricted to the end of the breeding season. The timing of intensive use of the canopy by TCA in 2019 was very similar to that observed in 2018 providing evidence that this phenology may be general. More years of data are needed to evaluate the degree to which this phenology will buffer GCW from direct negative impacts of TCA on nestlings and indirect impact on nesting success via canopy arthropod availability.

Nine ant species other than *N. fulva* are evident foraging in the canopy in the Edge and Uninvaded habitats. All but one of these species are absent in the Invaded habitat where *N. fulva* dominates the canopy (Table 2). As in 2018, the displacement of other arboreally foraging ants by TCA is again evident in 2019. Red imported fire ants (*Solenopsis invicta*), a known predator of nestling birds, are essentially absent from the canopy of this closed-canopy woodland.



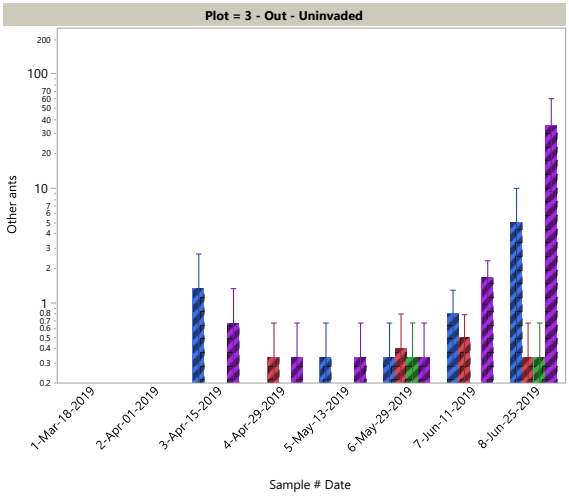




Figure 3: Ants foraging on trunks. Abundance of (A) Nylanderia fulva and (B) Other ant species on trunks of trees in the three sample areas. Average ants per tree plotted on a log scale. Error bars present standard deviations. Insufficient shin oaks or red oaks were available for sampling in the Long-term Invaded area and insufficient live oaks in the Edge-Uninvaded area.

**Table 2: Species composition and number of ants observed foraging in canopy.** Counts are totals across all sampling intervals of the number of trees where an ant species was foraging in the canopy. Insufficient shin oaks or red oaks were available for sampling in the Long-term Invaded area and insufficient live oaks in the Edge area.

| | Plot | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | EDGE | | | INVADED | | UNINVADED | | | |
| | Sample Tree | | | Sample Tree | | Sample Tree | | | |
| **Species** | **AJ** | **SH** | **TX** | **AJ** | **LO** | **AJ** | **LO** | **SH** | **TX** |
| Brachymyrmex patagonicus | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Camponotus decipiens | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Camponotus texanus | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| Crematogaster emeryana | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 |
| Crematogaster laeviscula | 4 | 1 | 8 | 0 | 0 | 2 | 0 | 0 | 3 |
| Monomorium minimum | 0 | 1 | 2 | 3 | 0 | 0 | 0 | 0 | 0 |
| Nylanderia fulva | 3 | 1 | 1 | 23 | 32 | 0 | 0 | 0 | 0 |
| Pheidole dentata | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 |
| Pheidole hyatti | 3 | 4 | 1 | 0 | 0 | 3 | 4 | 0 | 5 |
| Solenopsis invicta | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

## Task 3: Tracking the establishment of the pathogen *Myrmecomorba nylanderiae* within the TCA population in the preserve

We tracked the spread of the pathogen into the preserve from a natural acquisition in lands adjoining the preserve. We also followed the intensity of infection in areas of the TCA population that have harbored the disease since mid-2018.

*Methods for measuring infection*

To assess levels of infection intensity, we used a combination of diagnostic PCR and spore counting assays to assess how infection intensity changes over time since the original inoculation. For this purpose, all PCR positive collections made to document establishment and track rates of spread were spore counted. In addition, TCA from all pitfall traps that collected at least 20 TCA workers were spore counted, and hand collecting efforts were made in parts of the park that were not well sampled by the previous two efforts. Spore count assays were done by homogenizing the tissues of 20 workers in distilled water and concentrating that homogenate to 300 ul by centrifuging at 12000 rpm, removing the excess supernatant, and resuspending the pellet. Then 0.3 ul of that homogenate was fixed on a microscope slide and stained with Trichrome stain following protocols described in Didier et al. 1995. The number of Type I & II DK *M. nylanderiae* spores were counted across five field of view width sections of the central part of the stain using oil immersion, phase contrast microscopy at 1000x magnification. The total of these spore counts provides our measure of infection intensity for a given sample. Infection levels among individual ants within an infected nest is highly variable, which is why we rely on batch homogenizations of 20 workers. However, multiplying the total spore count values by 60 yields an approximate average for the number of spores per individual ant in the sample. We refer to

this adjusted value as the 'spore load' for a sample with the unit of spores per ant. Unadjusted measures of infection intensities are reported as 'spore counts'.

*Tracking spread of infection into TCA population from natural acquisition*

In addition to our efforts to experimentally introduce Mn into the preserve, during the fall and early winter of 2018, *M. nylanderiae* spread into the northwestern edge of the preserve from natural acquisition on nearby private lands. We have been tracking the spread of the disease into the TCA population within the preserve by sampling transects moving from the edge of the preserve into the interior every 3 to 4 weeks. Samples were collected and spore counted in the lab.

Findings

In February – March of 2019 *M. nylanderiae* infection within the preserve was restricted to areas immediately adjacent to the road on the North side of the preserve. By November, this area of infected TCA nests had spread between 64 and 165 meters into the preserve. In some areas, the extent of infection appeared to retreat between the Summer and Fall sampling intervals, disappearing entirely from one transect (Fig. 4). Figure 4 below provides a map of how the infection moved in the TCA population over 2019. Figure 5 combines the results of all *M. nylanderiae* testing conducted in 2019 excluding the sampling to test for establishment post inoculation. All positive detections occur in the zone of known expanding infection with one exception. During the November *N. fulva* edge mapping work, a single positive sample was discovered in the area of the 2018 inoculation grid (pin with spore count of 5 - Fig. 5). This 2018 inoculation was deemed to be a failure when no positives had been recovered by March of 2019, eight months post inoculation. This may have been a premature determination. We are currently conducting more extensive sampling of this area and will continue in the spring of 2020 if positives are detected.

The intensity of infection within areas of the local TCA population that have harbored the disease for the longest continued to increase from 2018 levels through June of 2019. From July through the Fall average infection intensity declined substantially (Fig. 6). The processes driving these dynamics are currently unclear.

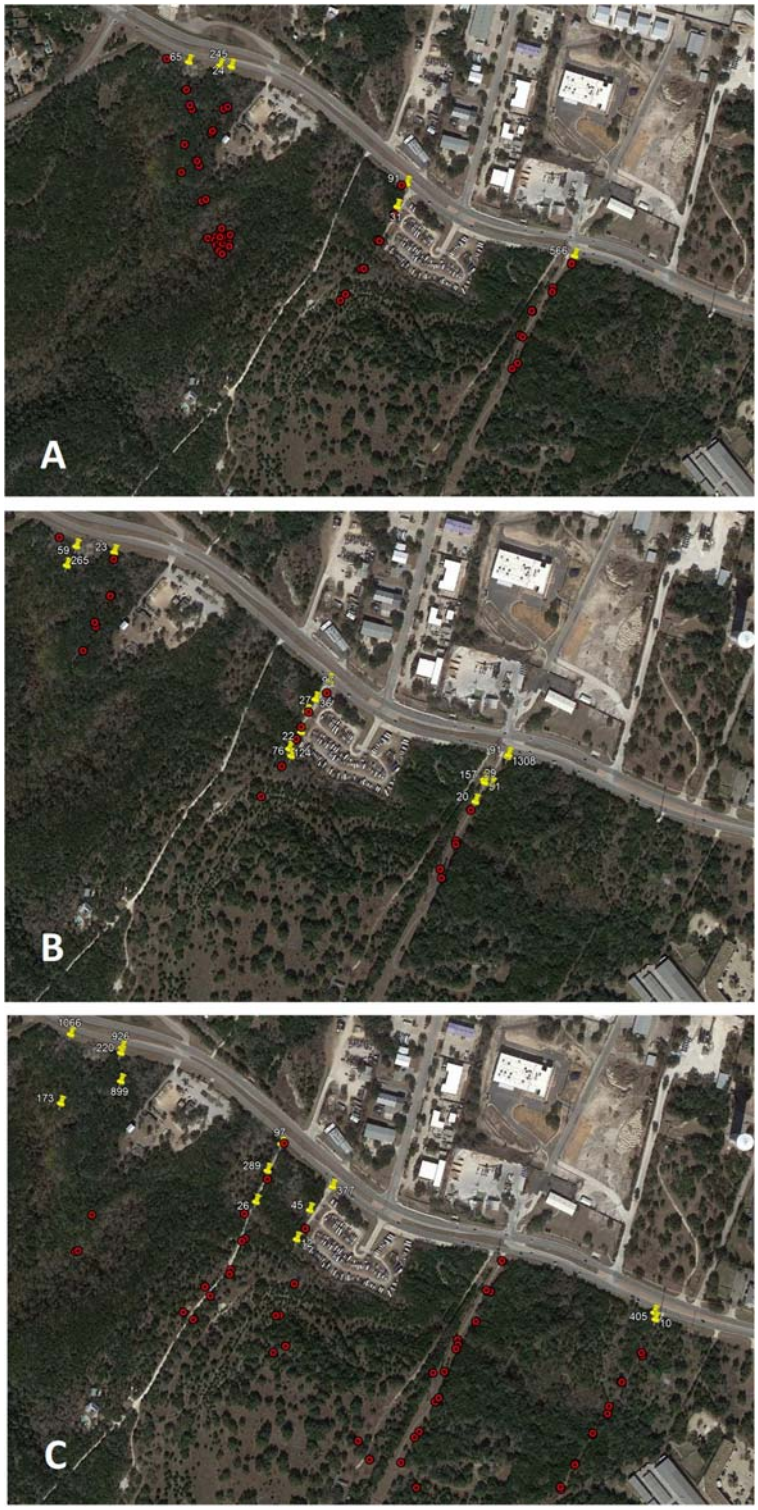



Figure 4: Maps tracking the spread of *M. nylanderiae* (Mn) infection into the preserve. Red circles indicate an *Mn*-negative sample and yellow pins indicate an *Mn*-positive sample. Positive samples are labeled with their infection intensity (spore counts). (A) Spring (February – March 2019). (B) Summer (July – September 2019). (C) Fall (October – November 2019).

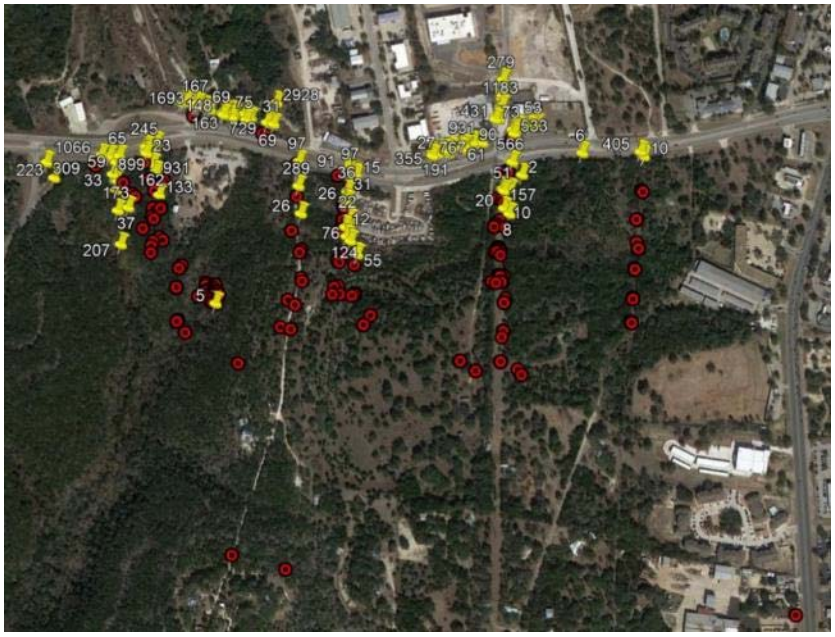



Figure 5: Map of all M. nylanderiae testing in 2019 excluding testing for establishment at 2019 inoculation sites. Red circles indicate an Mn-negative sample and yellow pins indicate an Mn-positive sample. Positive samples are labeled with their infection intensity (spore counts).

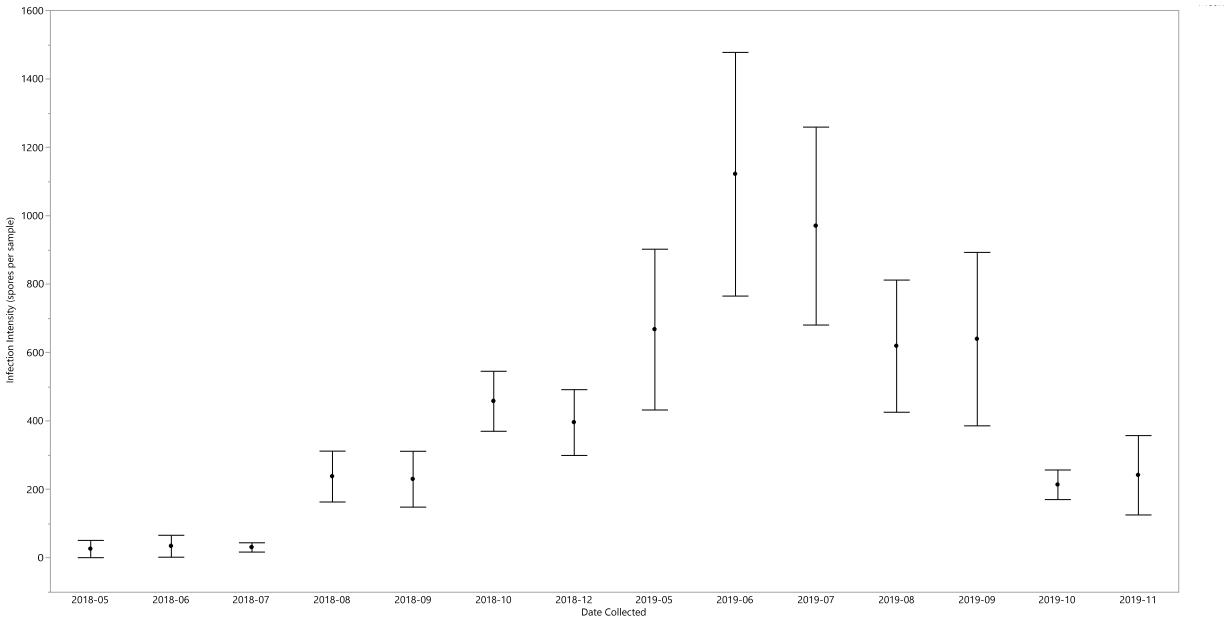



Figure 6: Intensity of *M. nylanderiae* infection intensity across time in the Anderson Mill TCA population for areas where the disease has been established the longest. Data are from sites adjacent to the preserve where the infection has been established longer.

**Task 4: Inoculation Studies**

In 2019 we conducted three sampling exercises designed to test means to establish *M. nylanderiae* in uninfected areas of the TCA population of the preserve, the goal being to develop techniques for inoculating TCA populations for biological control. The first was follow-up testing of an inoculative release test conducted in 2018. The second inoculation experiment attempted to evaluate what type of the inoculum (what castes of infected ants are included) is best suited to establish the disease. In addition, we performed a behavioral study and, based on the results of the behavioral test, and performed a pilot test of third inoculation approach indicated by the results of this behavioral assay.

*Follow-up sampling of 2018 inoculative release*

In August of 2018 we performed an inoculative release of *M. nylanderiae* (Mn) at multiple sites within a single 40 x 40 m plot within the preserve. Post inoculation spore positive ants were detected; however, detections were limited to a few locations in the inoculation zone and over a limited number of sampling intervals. In 2019 we continued sampling for the disease at this inoculation site.

Findings

We sampled ants from 29 stations in and around the 2018 inoculation site on three occasions in 2019 (January, February, and March). No spore positive samples were recovered during that period. Our conclusion was that, after initial establishment, the disease died out over the winter and ultimately the 2018 inoculation was unsuccessful. No further sampling of this site was planned. However, a single unexpected positive sample was recovered in this area during other work in November of 2019 (see below). Currently we are unsure of the actual status of this inoculation and are planning to conduct more intensive sampling in January 2020. Additional sampling will be conducted if more positive samples are encountered.

*Composition of inoculum test*

Three inoculation treatments were tested: (1) Infected workers only released onto foraging trails, (2) Infected workers plus infected brood released onto foraging trails, (3) Infected nest fragments (workers, brood, and queens) established in artificial nest sites in the field. Each had approximately 300 infected workers, 0.5 cc of mixed stage brood (if indicated by treatment), and 10 queens (if indicated by treatment).

Each treatment was replicated 8 times for a total of 24 inoculation stations. Stations were spaced a minimum of 100 m apart and a minimum of 200 m from the nearest Mn positive nest detection. All stations tested negative for *M. nylanderiae* presence prior to inoculation.

Infected ants were collected from the Convict Hill and Metcenter sites in Travis County where the TCA population is known to harbor *M*. *nylanderiae* infections. Ants were separated from nest materials in the lab at the Brackenridge Field Laboratory. Nest fragments were cultured at BFL for 14 days until inoculations were conducted. Prior to inoculation samples of 20 workers were taken from each nest box and spore counted following our batch counting protocol to assess the spore load of the inoculum. The inoculative releases occurred in May. Three weeks following the inoculation we began sampling to assess whether the TCA population acquired the infection at each inoculation site.

Findings

We recovered only one spore positive sample and a few PCR positive samples from the 24 inoculation stations sampled on 8 occasions since May (Fig. 7). No station has been repeatedly positive, suggesting that we are occasionally recovering workers from the original inoculative releases but that the disease has, so far, failed to establish at any of these sites. Given that these samples cover the 7 months post inoculation, it seems that this effort failed with respect to testing the castes necessary to facilitate establishment. We attribute this failure to the cryptic aggression between lab held and field workers discovered this past field season (see below). We will continue tracking this inoculation effort through March of 2020.




Figure 7: Map of Inoculation Methods test. Red circles indicate an Mn-negative sample and yellow pins indicate an Mn-positive sample. Positive samples are labeled with their infection intensity (spore counts).

*Behavioral test: laboratory derived aggression cues*

During the process of assembling the inoculum for a second inoculation outside of the Balcones Canyonlands Preserve system, we observed unexpected aggression between TCA workers as ants recently extracted from their natal nest material were combined with ants that had been housed in the lab for varying amounts of time up to 11 months. Previously conducted tests of recently collected TCA exposed to equivalent collection and husbandry conditions had failed to reveal aggression at any spatial scale indicating that all TCA in the Southeastern USA are unicolonial [6]. Our strategy of introducing infected ants into uninfected nests depends upon this absence of aggression to succeed. To evaluate

whether, ants housed under laboratory conditions acquire cues that lead to aggression with field collected ants, we conducted a behavioral assay.

In controlled 5 on 5 aggression assays under neutral arena conditions,) we combined ants with differing collection histories and scored the most aggressive behavior observed replicating each nest pairing 3 times (for methods see LeBrun et al. 2019). The scale used to quantify aggression (Table 3) was derived from observations of interactions in the native range in Argentina where intraspecific aggression is common [6].

We contrasted 3 treatment conditions: Field collected vs. Field collected – Different sites; Field collected vs. Lab held - Same site; and Lab held vs. Lab held – Different sites. Our hypothesis was that if lab derived cues substantively impact aggression, the Field collected vs. Lab held - Same site contrast would exhibit the highest level of aggression despite ants originating from the same local population of TCA.

**Table 3. *N. fulva* intraspecific aggression scale.**

| Score | Description of Behavior |
|---|---|
| 0 | Ants come in contact and part. |
| 1 | One or both ants antennate the other for 3 or more seconds OR trophallaxis OR allogrooming |
| 2 | One ant bites and then releases the other. |
| 3 | One ant bites and holds on for more than 3 seconds OR bites and releases two or more times consecutively. |
| 4 | One ant bites and holds the other while curling its gaster tip under its body till the gaster is parallel with substrate, acidopore pointing at its opponent. Aggressor does not spray or otherwise release formic acid. |
| 5 | One ant sprays formic acid at its opponent or both ants actively fight, typically forming a ball. |

Scale used for scoring interactions between workers in 5 x 5 neutral arena interaction assays. Scores of 3 or greater represent increasing levels of aggression.

Findings

Interactions between workers from the same site collected from the field and workers held in the lab appear more aggressive than either interactions between workers from distant sites that have been held under laboratory conditions or workers from distant sites that have been freshly collected from the field (Fig. 8). At this point our sample sizes for these contrasts are low (N=5) and, likely as a result, the contrast is not quite statistically significant ($P$=0.06). We plan to increase our sample sizes for this contrast in the coming year. Our inoculation methods rely upon introduced infected ants being rapidly integrated into local wild nests. We believe that this cryptic aggression is at least partially responsible for the difficulty in inoculating field populations.

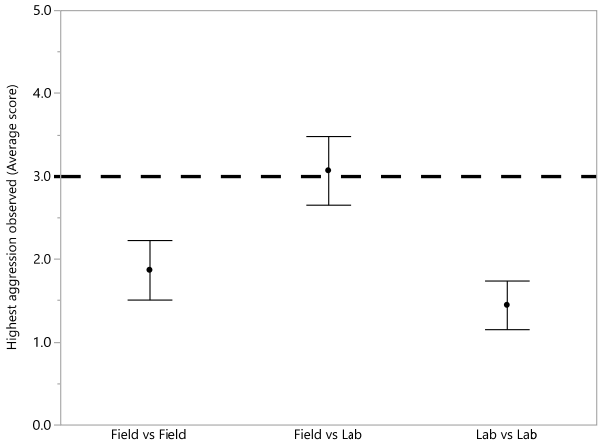



Figure 8: Results of 5x5 neutral arena aggression tests. Field ants are tested shortly after collection and held until testing in their natal nest material. Lab ants are held under standard laboratory conditions for at least 1 month prior to testing. Field vs Field are ants collected from distant local populations of TCA. Field vs Lab are ants collected from the same site on different dates. Lab vs Lab are ants collected from different local populations. The dotted line represents the level on the behavioral scale above which unambiguously aggressive interactions occur. Points are the mean of the nest pair scores for each treatment type. Nest pair scores are the average of the highest aggression score observed in each of three arenas. Bars are standard errors.

*Natal nest material inoculation*

Following our finding that TCA workers housed in the lab acquired cues eliciting aggression with TCA workers in the field, we pilot tested an inoculation approach that removes laboratory odors from the inoculum. To do this, a rotten log containing a nest of infected TCA was collected from a site in Goliad Texas. The ants plus their nest material were housed in a bucket and released at a site within an uninfected area of the Collins Tract TCA population. Ants were provided a dispensing station that provided a persistent sugar water resource to ease their transition and increase interaction with the local TCA population. The pilot release happened in late October.

Findings:

No positives have yet been recovered from this pilot test, but there has been insufficient time since the release to see a successful inoculation. We will continue testing in 2020.

**Supplementary Information on Convict Hill TCA Population**

In 2017 our group in collaboration with Travis County and City of Austin natural Resources personnel inoculated the TCA population in the Convict Hill neighborhood of Austin, TX with *M. nylanderiae.* This inoculation was part of a research effort funded by a US Fish and Wildlife Service _ Texas Parks and Wildlife Section 6 grant. The purpose was to evaluate the efficacy of controlling TCA populations that threaten endangered karst invertebrates. Inoculations were conducted in April of 2017. The microsporidian was documented to have established in TCA population in July of 2017 and since then we have continued to monitor the spread of this disease and the consequences it has had for the TCA population. This work has not been funded under the current grant, however as this site

contains Whirlpool Cave, a karst feature listed under the Balcones Canyonlands Conservation Plan (BCCP), we include some information herein on the status of this population.

Findings:

By the Fall of 2018, 19 months post inoculation, *M. nylanderiae* had spread throughout a large fraction of the local TCA population (Fig S1). TCA remained abundant and prevalent throughout. However, by the Spring of 2019, TCA were sufficiently scarce as to be difficult to sample in most parts of the previously dense population. Areas of abundant TCA were present on the northern and southern edges of the original population. By the Fall of 2019, TCA could only be reliably found on the northern edge of the area they previously infested (Fig S2). Infection by *M. nylanderiae* was present but spotty in the ants along this northern edge. In the central area of this population, over the time period since *M. nylanderiae* establishment, TCA have declined in abundance from 1000's of workers per pitfall trap to entirely absent in the Fall of 2019 sample (Figure S3).




Figure S1: Distribution of *M. nylanderiae* infection in Convict Hill TCA population at various time points. (A) July 2017, XX months, post inoculation the first establishment positives were detected. (B) Distribution of Mn within the TCA population in the Fall 2018, XX months post inoculation. Numbers beside Yellow pins indicate a sample. Samples are labeled with their infection intensity (spore counts).




Figure S2: Figure displays the infection status of the ants in the Spring and Fall of 2019. The drainage to the south was not sampled in the fall as TCA had become too scarce to reliably collect.

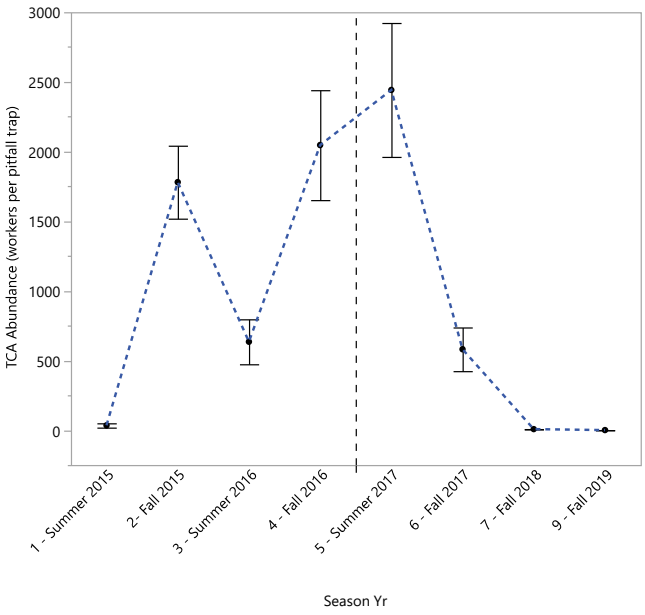



Figure S3: TCA abundance in the central area of the Convict Hill population Summer 2015 through Fall 2019. Data are the average number of TCA workers captured per pitfall trap during this period. Bars are standard errors. Dashed line represents the point at which the population was inoculated with *M. nylanderiae*.

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