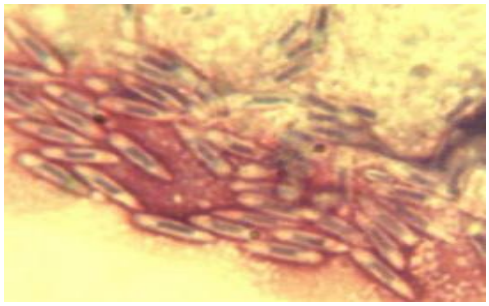




Tawny Crazy Ant (*Nylanderia fulva*) Study
Balcones Canyonlands Preserve – Jollyville Unit
Task 5: Data analysis and Final Report

Edward LeBrun, PhD – PI

Technical work: Aaron Sekula, Charlotte Nash, and Edwin Umanzor
Molecular testing: Kaitlyn Williams
Report Preparation: Edward LeBrun and Charlotte Nash

Invasive Species Research Group
University of Texas at Austin
Brackenridge Field Laboratory
2907 Lake Austin Blvd
Austin, Texas 78703

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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 the Collins tract of 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.

In 2020, we observed a widespread and substantial decline in the TCA population in the preserve and the adjoining undeveloped properties that form part of the larger study site. Abundances of TCA across the study site as a whole were only 6% of their 2019 levels and 0.2% of the levels observed during the TCA infestation peak observed in 2018. In concert with this decline, we are beginning to observe a resurgence of the ant community that was eliminated by TCA. This resurgence is still in the early stages and interpreting whether the decline of TCA is causal in this process is currently confounded by interannual variation. We look forward to quantifying it in the coming years. The decline in TCA population is coherent with the observed patterns of infection intensity and prevalence of the microsporidian pathogen *M. nylanderiae*. All available evidence indicates this disease is the causal mechanism behind this decline. An inoculation experiment conducted in 2020 at a second site infested with TCA shows early signs that the disease was transmitted to the local TCA population. However, the effort was delayed until the fall by the COVID19 pandemic making it unlikely that this incipient infection will successfully overwinter. We will continue to monitor disease prevalence at this site.

Project Background

Nylanderia fulva (Tawny Crazy Ants), an invasive ant species introduced from central South America, 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 [3]. 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* [4]. 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 [5], 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 third year of effort at the Collins Tract, Jollyville Unit, Balcones Canyonlands Preserve (BCP) and on efforts at Pace Bend Park, a Travis County park infested with TCA. The objectives for year 3 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) Map the TCA population extent within the preserve.
3) 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.
4) Tracking the establishment of the pathogen *Myrmecomorba nylanderiae* within the TCA population in the preserve
5) 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.

In 2020 our work was disrupted by the COVID19 global pandemic. All ability to conduct field work and most laboratory work was shut down from early March thru May. In May we regained our ability to conduct laboratory studies and to conduct field work, albeit at a reduced capacity. In April, as a knock-on effect of the pandemic, squatters colonized part of the study site, an undeveloped property owned by the City of Austin adjoining the Collins tract of the preserve. Under the conditions of the pandemic, the police were unable to remove the squatters from the property until September 2020. Due to safety concerns, the City of Austin denied us access to the property adjoining the preserve for this period. Unfortunately, lack of access to this tract was impactful to the project. This part of the study site is the area referred to herein as the “long-term invaded” habitat. It historically contained the densest part of the TCA population. Over the course of the 2020 field season (as described in this report), TCA progressively disappeared the study site except for in the long-term invaded habitat to which we did not have access permission.

Objective 1: 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 summer, and fall of 2020. COVID19 restrictions prevented spring sampling and lack of access prevented sampling of the Long-term Invaded plot in the summer of 2020. 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.

For assessing TCA density changes, three habitat areas defined by TCA occupancy were sampled: Long-term Invaded, Invaded Edge 2017, and 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. Invaded Edge 2017 habitat were invaded areas near the edge of fall 2017 TCA distribution in the preserve. Uninvaded areas are of similar vegetation that are outside of the TCA population and never invaded. To assess the response of native ants to the decline of TCA, areas were proscribed based on their invasion history and present invasion status. Currently Invaded habitat contained TCA in the Fall of 2020. Previously Invaded habitat consists of areas with TCA prior to 2020 but where they are now absent. Uninvaded habitat consists of areas without TCA at present or in the past.

Findings

TCA densities continued the decline which began in 2019 and were overall much less abundant in the preserve in 2020 as compared to 2019. Median densities during the fall of 2020 were only 6% of their levels in 2019 (Fig. 1), an obviously significant decline (Wilcoxon: $N = 36$, $P < 0.009$). Albeit at currently much lower abundance, TCA remain prevalent in the Long-term Invaded habitat. TCA disappeared from areas that they initially colonized in 2017, 2018 and 2019, and retreated in some other areas colonized prior to that (see next section).

Given the widespread and profound decline of TCA within the preserve, 2020 presents the first opportunity to look at the degree to which the ant assemblage may be rebounding. We lack summer 2020 data from currently invaded habitat due to lack of access, so we provide contrasts based upon fall activity for this habitat type. In Previously Invaded habitats where TCA have disappeared entirely, there has been a significant increase in the number of ants other than TCA that are present in the traps (Wilcoxon: $N = 56$, $P < 0.001$). In Currently Invaded and Uninvaded habitats a similar but non-significant trend of increasing other ant abundance over time is evident (Fig. 2). There was no significant difference in Uninvaded (Wilcoxon: $N = 27$, $P = 0.21$) or Currently Invaded (Wilcoxon: $N = 35$, $P = 0.95$) habitats, although a similar non-significant pattern of increase is evident. This indicates that at least some of the

increase in other ant abundance in the Previously invaded habitat is the result of inter-annual variation independent of TCA decline. The ant assemblage composition for these areas is presented in Table 1.

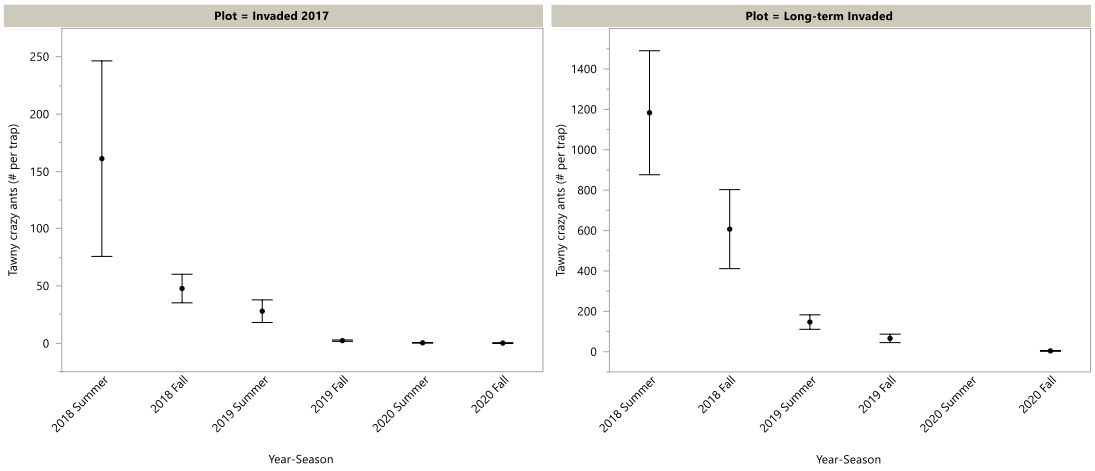


Figure 1: Abundance of *N. fulva* in areas of the site where the ants have been established since the beginning of the study. Trapping data is divided into traps in Long-term Invaded habitat, colonized prior to the initiation of the study, and Invaded 2017 habitat, colonized the year before formal sampling began.

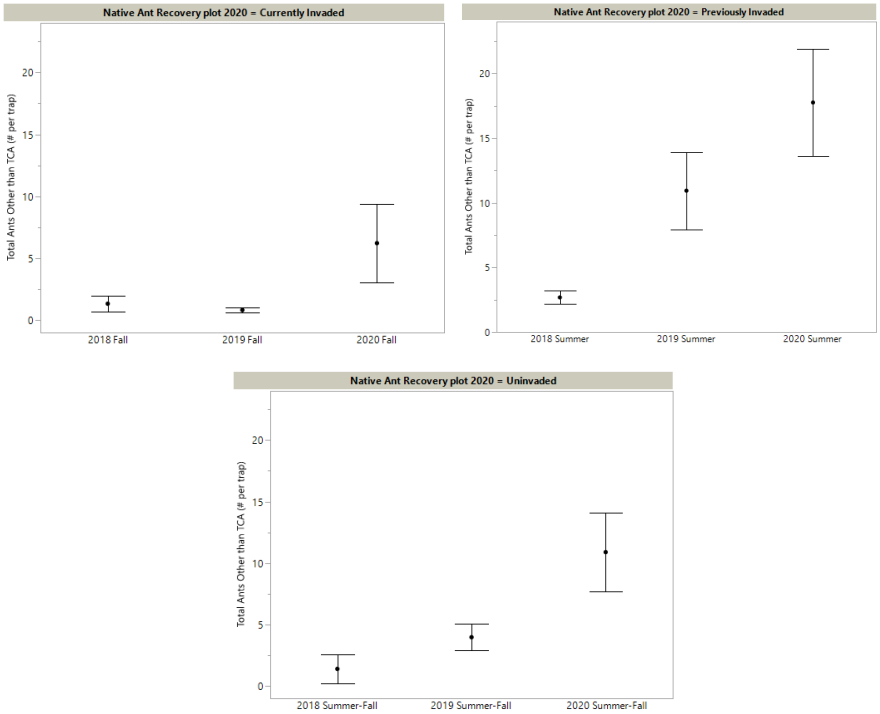


Figure 2: Average number of ants other than tawny crazy ants in habitats currently or previously invaded by TCA. Currently Invaded habitats contained TCA in the fall of 2020 at greatly reduced abundance. Previously invaded habitats contained TCA at some point prior to fall of 2020 but TCA are now absent. Uninvaded habitats never contained TCA.

Table 1: Ant assemblage composition 2020. Results from pitfall traps across three habitats sampled in summer and fall. The cells report the percent of traps that contained a given species.

Ant Species	Uninvaded		Previously Invaded		Currently Invaded	
	Summer (14)	**Fall (5)**	**Summer (16)**	**Fall (16)**	**Summer (0)[1]**	**Fall (8)**
Aphaenogaster texana	7					
Brachymyrmex patagonicus	14					
Camponotus decipiens				6		
Camponotus texanus			6			
Crematogaster laeviuscula	14		13			
Crematogaster lineolata			13			
Forelius pruinosus	29					
Monomorium minimum	50	20	81	44		38
Myrmecina americana			13	6		
Nylanderia fulva (TCA)			6			100
Nylanderia terricola sp.	21					
Pheidole constipata	21		6			
Pheidole dentata	21		6			
Pheidole hyatti	14		6			
Solenopsis diplorhoptrum sp.	21		56	6		25
Solenopsis invicta	29					
Temnothorax andrei			25			
Temnothorax subdivitus			13	6		

[1] Currently Invaded habitat was not accessible during Summer 2020.

Objective 2: Mapping the extent of the TCA population (Fall of 2020)

TCA lack alate flights with nests reproducing through 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, 2018 and 2019, 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 the fall of 2020. 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.

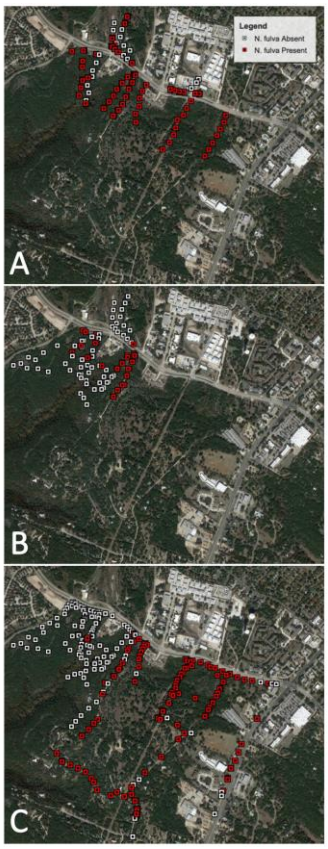


Figure 3: Maps tracking tawny crazy ant activity by season. (A) Spring (February – May 2020). (B) Summer (June – July 2020). (C) Fall (August– November 2020). Note: absence of samples on eastern part of study area in summer due to lack of access.

Findings

In 2020, TCA disappeared entirely from the heavily wooded areas of the preserve they occupied in 2019 and 2018 (NW corner of TCA population). TCA were present in this area in the spring but, except for one small area, had disappeared entirely by August of 2020 (Fig. 3). This retreat is also evident in the contrast of TCA population extent between fall of 2019 and fall of 2020 (Fig. 4). In addition to retreating substantially in the heavily wooded NE corner, the TCA population expanded somewhat in the residential areas on the southern edge of the population (Fig. 4).

Areas where TCA disappeared were areas where the TCA population was documented as having acquired the microsporidian disease, *Myrmecomorba nylanderiae* (Mn), in 2019. TCA were present at low densities in these areas in the spring of 2020 and most samples recovered from that area tested positive for Mn infection. Mn has to date never been detected in the southern portion of the population that continues to expand (see Objective 4: Findings).



Figure 4: Edges of *Nylanderia fulva*-invaded area in Fall 2017 and 2018 and Fall 2019 and 2020. Squares show the presence (red) and absence (white) of TCA at sampling points from November 2020. Top image provides TCA extent in fall of 2017 and 2018. Bottom image provides 2019 and 2020 extent. Inset map depicts a small population bubble outside of the current population retreat.

Objective 3: Arboreal foraging density assessment

Historically Travis County staff took the lead on this sampling activity, conducting all arboreal arthropod sampling as well as ant foraging counts when arboreal foraging by ants was low. UT's role was to conduct ant foraging counts when foraging activity was high (typically in the June time frame), and process lepidopteran larvae and spider collections. The COVID19 pandemic prevented Travis County staff from conducting arboreal arthropod sampling and the associated ant foraging counts. To maintain some data integrity, UT conducted a limited amount of ant foraging counts during the June (historically high-density arboreal foraging) time frame. Tree trunks from the ground to 2 m high were searched for 3 min and all ants observed foraging counted and representative specimens collected.

Findings

Twenty-four trees were sampled during June of 2020. During this time frame Long-term Invaded Habitat (LTIH) was inaccessible. By June of 2020 TCA had largely disappeared from all habitats but the LTIH. As a result, across 24 trees no TCA were observed. Only 2 ant species were observed foraging in the canopy at all. This scarcity likely reflects the depauperate nature of the ant assemblage shortly following the decline of TCA.

Objective 4: 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. 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 preserve 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 'disease burden' 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

We tracked 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

During the spring of 2020, *M. nylanderiae* occurred at high prevalence and infection intensity in the northwestern section of the study site within about 200 m of the road bounding the preserve on the north (Fig. 5A). As the year progressed into summer, this portion of the TCA population progressively collapsed, presumably because of this infection. It became increasingly difficult to locate TCA to sample. During the summer, infection prevalence and intensity among the TCA population that remained in this section of the preserve were lower. This is a pattern we have observed in other sites (see Supplementary Information on Convict Hill TCA Population). As a population collapses the last remaining nests often have low disease burdens and sometimes appear to lack the infection entirely. Despite this, the TCA population had disappeared almost entirely from this section of the preserve by the beginning of the fall (Fig. 5B). During the fall, when we once again had access to the eastern section of the study site (Long-term Invaded habitat), we observed that TCA were still prevalent and that the population up to 250 m south of the northern boundary road exhibited high infection intensity and prevalence. Farther south than this, the TCA population appeared to be uninfected at this point (Fig. 5C).

Disease burden across the time of sampling exhibits a strong pattern of oscillation between very high and low levels of infection intensity. However, the pattern of this oscillation is variable between years (Fig. 6). It is unclear whether this oscillation is driven by a seasonal dynamics or dynamics internal

to the process of population infection and collapse. The pattern in 2020 at this site is particularly difficult to interpret due to the loss of data from the Long-term Invaded Habitat (Fig. 7).



Figure 5: Maps tracking the spread of *M. nylanderiae* (Mn) infection into the preserve. Positive samples are labeled with their infection intensity (spore counts). (A) Spring (February – May 2020). (B) Summer (June – August 2020). (C) Fall (September– November 2020). Long-term invaded habitat ws inaccessible during summer. TCA had disappeared from western section by fall.

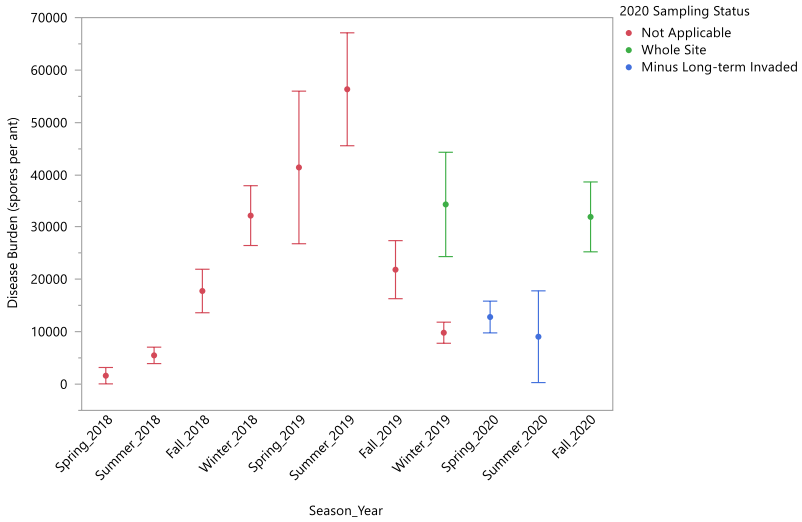


Figure 6: Average disease burden (spores per ant) of samples collected from 2018 to 2020 by season. 2020 data is coded by whether the entire site was sampled or if only those ants outside of the long-term invaded habitat (LTIH) were sampled.

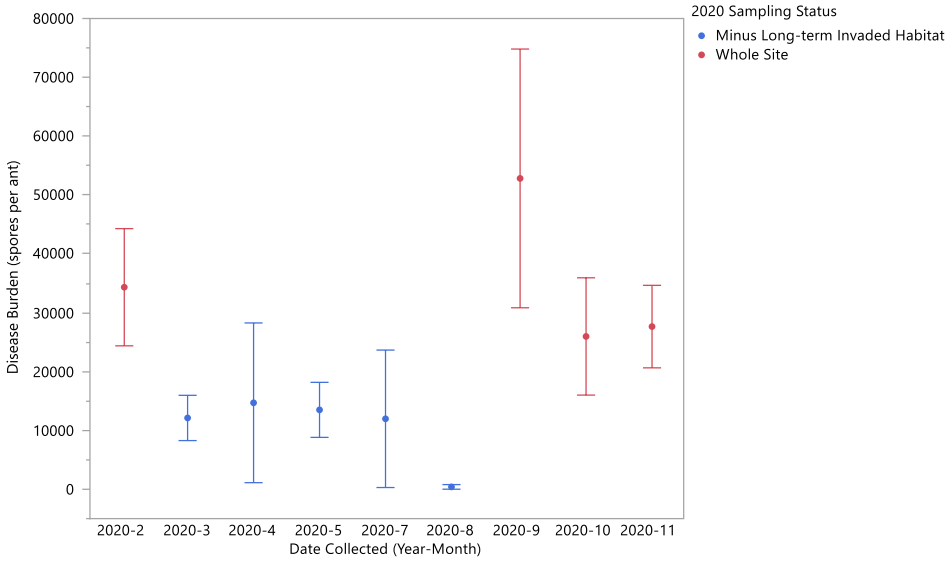


Figure 7: Average disease burden (spores per ant) of samples collected in 2020 by month. Data is coded by whether the entire site was sampled or if only those ants outside of the long-term invaded habitat (LTIH) were sampled. Lack of access permission prevented sampling the LTIH from March through August. TCA outside of the LTIH disappeared by the end of August.

Objective 5: Inoculation Study

In 2020 we introduced infected TCA into an uninfected TCA population at Pace Bend Park, the goal being to develop techniques for inoculating TCA populations for biological control described herein as the "Laboratory Nest Fusion" test. In addition, we conducted follow-up sampling of a test inoculation conducted in 2019.

In 2019, we discovered that ants held in the laboratory elicit aggressive behaviors and likely rejection from TCA in nature, confounding our inoculation attempts. This occurred regardless of whether ants were sourced from the same or different sites as the inoculation site and is not true if both colony fragments are held in the laboratory or if both are from natural nest settings. Given ant colony recognition biology, this effect arises from the acquisition of alien odor cues from ants in the laboratory setting. In 2019 we conducted a pilot test of an inoculation technique relying on fusing source site and inoculation site nests in the laboratory under conditions that minimize the addition on artificial cues to the introduced ants. In 2020 we conducted a larger scale test.

Specifically, ants were collected from an infected source site (Goliad, Texas) extracted from their nest material, to quantify amounts, and then added to nests collected from the inoculation site that

were not extracted from their nest material. Both were then introduced with the nest material they occupied.

Follow-up sampling of 2019 inoculative release: Natal nest material inoculation

In 2019, 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. This pilot release happened in late October of 2019. This was very late in the year for an inoculation attempt, therefor it was viewed as a pilot. A single sample positive for the presence of Mn DNA was recovered in December of 2019, 8 weeks post inoculation. However, there was insufficient time between the release and the onset of winter dormancy to evaluate the success or failure of the inoculation. In 2020, we continued sampling for the disease at this inoculation site. We sampled the site three times in 2020: February, March and again in October after access had been restored.

Findings

We sampled ants from 10 stations per sampling interval in and around the 2019 inoculation site on three occasions in 2019. No spore positive samples were recovered during that period. This pilot failed. The December 2019 PCR positive indicates that an incipient infection may have been achieved but failed to overwinter. Unfortunately, given very late in the year inoculation date, it is unclear if the disease was successfully transmitted prior to the winter.

Laboratory Nest Fusion Test

The likelihood of success of the 2020 inoculation effort was compromised by the COVID19 pandemic. This test was originally planned to be executed in mid-March in the long-term TCA invaded habitat adjoining the Collins Tract of the Preserve. Disruption of research effort due to the pandemic and the knock-on loss of access to the long-term invaded habitat forced us to move the inoculation experiment to Pace Bend Park. Unfortunately, the uncertainty of surrounding when access permission would be restored, and the time required to gain permission to conduct this experiment at Pace Bend Park delayed the execution of the inoculative release until August of 2020. To date, all successful inoculation attempts have been conducted in the spring. We believe that the active growth of nests during this period facilitates disease establishment. Additionally, in previous years, inoculation efforts conducted in the late summer and fall in which the disease does establish have died out over the winter.

Inoculation efforts relied upon the laboratory nest fusion technique described above. Infected ants were collected from an infected site in Goliad, Texas. Ants were separated from nest materials in the lab at the Brackenridge Field Laboratory but not removed from the separation buckets. Inoculums consisted of approximately 1100 workers, 0.375 cc of mixed brood, and 15 queens. To minimize handling, numbers of workers and amounts of brood were visually estimated. 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 August. At the time of inoculation, a second feeding treatment was imposed in which some inoculation stations were provided with 500 ml of supplementary sugar water and 50 g of supplementary protein food (dead crickets and tuna fish) to promote growth every 10 days. There were a total of 7 inoculation sites separated by a minimum of 120 meters. Four of these received supplementary food. Drought conditions prevailed in Travis County during August 2020. To address this, all inoculation stations were outfitted with 1 gallon chicken waterers that were refilled every 10 days. All inoculation stations tested negative for *M. nylanderiae* presence prior to inoculation, which is true of the Pace Bend TCA population generally. Following the inoculation, we began sampling TCA at three-week intervals at each inoculation station to assess whether the local nests acquired the infection. Four samples were collected from each inoculation station with 10 m spacing between samples.

Findings

To date, we have sampled the inoculation stations 5 times post-inoculation. Diagnostic PCR results are not yet available for the final 2 sample intervals of 2020. So far, we have detected two Mn positive samples from this inoculation (Fig. 8). Both were recovered on 11/04/2020, 11.5 weeks after the inoculation. It takes time for resident colony larvae to become infected, develop into adult workers, and mature sufficiently to take on tasks outside the nest that render them available to sample. Thus, successful inoculations typically remain cryptic for a lengthy period of time (11.5 weeks is not uncommon). Both samples were negative for Mn spores. This does not necessarily connote an absence of infection but more likely a very incipient stage of infection transmission at the colony level. Early stage Mn infections are in the meront (pre-spore formation) developmental stage and samples with only a few spores are likely to present as spore negative. One positive station received the supplemental feeding treatment and the other did not. At this point, there is no evidence that supplemental feeding influences inoculation outcome, but, given the early stage of these results we will likely carry this treatment forward to any inoculations carried out in 2021. We have collected a further two samples from December and January for which we are still awaiting molecular diagnostic PCR results. We will report these results in the first progress report of 2021. We will also conduct follow-up sampling of these sites in 2021 to assess whether this incipient infection successfully overwinters.



Figure 8: Map of Lab Fused Inoculation stations at Pace Bend Park. 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). Positive tests with 0 spore counts indicate the recovery of ants that tested positive for Mn DNA but which did not contain spores.

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. As reported previously, by the fall of 2019, TCA had been almost entirely eliminated from the site.

Findings:

In 2020, we followed the remnants of the TCA population that remained. The only substantial remaining area infested with TCA was along the hike and bike trail adjacent to the Mopac expressway. The population in this area dwindled in 2020 disappearing entirely by Fall (Fig. S1). It did so despite never testing positive in 2020 for *M. nylanderiae* (although this area was positive in previous years). This was a small area of TCA in a residential neighborhood matrix and we can only access a small fraction the site. It is possible that activities (eg. pesticide application) occurring in the yards that are the dominant component of the habitat that this TCA population can access are responsible for the decline. Elsewhere in the site, occasional positive detections of TCA at very low abundance were made. All of these samples proved to be negative for the *M. nylanderiae* infection that decimated the TCA

population that originally occupied the site. We will continue to monitor to see if any of these positive detections increase to reinfest the site.

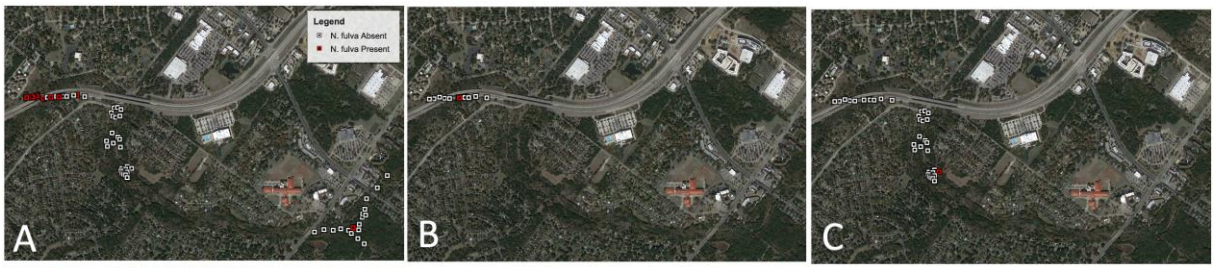


Figure S1: Distribution of TCA population at Convict Hill from (A) Spring (March - April 2020) (B) Summer (June – July 2020) (C) Fall (August-September 2020). White squares are areas sampled where TCA were absent. Red squares are sites with TCA.

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