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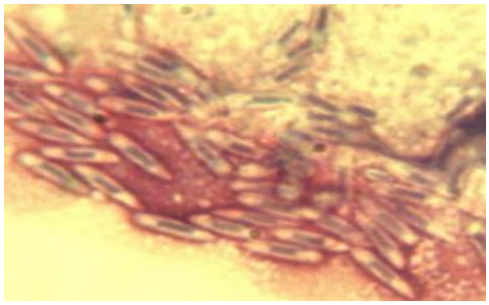

Tawny Crazy Ant (*Nylanderia fulva*) Study
Balcones Canyonlands Preserve – Jollyville Unit
Task 5: Data analysis and Final Report

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Executive Summary

Nylanderia fulva (tawny crazy ants) are an invasive ant species introduced from central South America 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 work on an effort to evaluate the impact of TCA population currently invading tracts of the Balcones Canyonlands Preserve, and to explore using this microsporidian as a control agent.

In 2022, we documented the near elimination of the TCA population at the Collins Tract of the BCP following the establishment of Mn. Only one individual TCA was found across the numerous methods we use to locate and track TCA. At Pace Bend Park, we recorded the outward spread of Mn throughout the park from our inoculation stations started in 2020 and 2021. We also introduced Mn to the area surrounding No Rent Cave, a high-priority endangered species cave and part of the karst system, using our proven inoculation technique. Inoculative success appears contingent upon introducing infected ants into high-density TCA populations, like the one located at No Rent Cave. Data on arboreal foraging by TCA, changes in the ant assemblage following TCA elimination, and monitoring of TCA at other sites are also presented.

Project Background

Nylanderia fulva (Tawny Crazy Ants), an invasive ant species introduced from central South America, are spreading throughout the Gulf Coast region of the United States (1). Current evidence indicates that all tawny crazy ants (TCA) within North America are members of the same supercolony, likely descended from a single successful introduction (2, 3). These ants invade natural habitats and have devastating effects on the arthropod assemblages of those environments (4). 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 (5). In addition, TCA penetrate karst environments to a depth of up to 40 m from the entrance in one impacted cave. TCA prey on karst invertebrates and a trend of declining karst invertebrate abundance in impacted caves has been documented. The strength of this trend is unclear as the study was hampered by intermittent TCA site occupancy (6). However, all available data indicate that populations of endangered karst invertebrates are threatened by this ant. 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* (7). 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 (8). Longitudinal field studies of infected populations combined with experimental introductions of this pathogen into uninfected TCA populations reveal that this disease is capable of causing TCA infestations to collapse to local extinction (9). Testing of native ants naturally in contact with *M. nylanderiae* spores and laboratory efforts to artificially transmit this

disease to the close relatives of TCA indicate that this pathogen is a highly specific natural enemy of TCA (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 on the results from the Collins Tract, Jollyville Unit, Balcones Canyonlands Preserve (BCP), Pace Bend Park, and other parklands infested with TCA. Work at sites other than those on the BCP is conducted with goal of improving our ability to control infestations within the preserve, and prevent the ants from colonizing preserve land from other infested public lands. The objectives for 2022 were to:

1. Assess tawny crazy ant (TCA) densities, distribution, and native-ant assemblage composition within the Collins Tract of the BCP Preserve and the Old Water Treatment Plant 4 Site
2. Continue the canopy arthropod prey availability and ant arboreal foraging study
3. Quantify spread of the pathogen *M. nylanderiae* within the Collins Tract - Old Water Treatment Plant 4 TCA population
4. Follow native ant assemblage recovery at TCA decline sites
5. Test the thresholds for detection of the pathogen *M. nylanderiae* and track its spread at Pace Bend Park
6. Conduct and track inoculative releases microsporidian pathogen Myrmecomorba nylanderiae
7. Monitor TCA populations at various sites within Travis County

Objective 1: Assessment of tawny crazy ant (TCA) densities, distribution, and native-ant assemblage composition within the Collins Tract of the BCP Preserve and the Old Water Treatment Plant 4 Site

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 spring and fall of 2022. 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 are inserted.

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 outside of the TCA population and were 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 2022. Previously Invaded habitat consisted of areas with TCA prior to 2022 but where they are now absent. Uninvaded habitat consists of areas without TCA at present or in the past.

Mapping of the TCA population extent within the preserve has occurred every fall since 2017. We continued this effort in 2022 to assess the extent of preserve occupied and the

amount of spread / retraction that has occurred in the intervening year. We focused on (1) areas in and adjoining the preserve where TCA were still present in a continuous infestation of interconnected nests, and (2) we explored areas disjoint from the original areas of infestation to ensure that we are not missing infested areas.

Findings

TCA densities continued their long-term decline within the preserve and were almost entirely absent. In the spring of 2022, TCA were found at only one monitoring site where they were previously present. As well, only 1 worker was found across all pitfall traps compared to approximately 1200 workers per trap at the peak of the invasion. TCA were not collected at any of the long-term abundance monitoring sites in the fall.

In July, we scouted areas of the preserve near the originally invaded area to see if any additional area of infested habitat could be found, but no TCA were located. Similarly, we found no TCA in our fall mapping survey both on the city owned land and in the residential neighborhood south of the Old Water Treatment Plant 4 and north of the preserve boundary. Tawny crazy ants have effectively been eliminated from the area (Fig. 1).

It appears that over time both the diversity and abundance of native ants has increased in the long-term invaded, now TCA free habitat, relative to uninvaded habitat. The ant assemblage composition for these areas is presented in Table 1. A more detailed analysis of the ant community response is presented in the cross site Native Ant Recovery section.

Figure 1: Inter-annual change in TCA distribution within the area of the Collins tract of the BCP. Left, middle and right panels provide the same information from 2020, 2021 and 2022 respectively. Red squares indicate positive TCA detections during. White squares indicate areas sampled where TCA were not encountered.

Table 1: BCP Collins Tract ant assemblage composition 2022. Results from pitfall traps across three areas defined by tawny crazy ant invasion status. Cells report the present of traps that contain a given species. Currently invaded includes traps where TCA was found in Spring 2022; no TCA were found in Fall 2022.

Ant Species	Never Invaded	Previously Invaded		Currently Invaded	
	Fall (18)	**Spring (9)**	**Fall (30)**	**Spring (1)**	**Fall (1)**
Aphaenogaster texana	6		3		
Brachymyrmex depilis			10		
Brachymyrmex patagonicus		0			
Camponotus texanus			3		

Forelius pruinosus	11	0	7	100	100
Labidus coecus	6				
Monomorium minimum	28	1	50	100	100
Nylanderia fulva (TCA)				100	
Nylanderia terricola sp.	6		3		
Myrmecina americana	6	0			
Pheidole constipata	11	0	7	100	100
Pheidole dentata		0	13		
Pheidole floridana			3		
Pheidole hyatti complex	6				
Pheidole lamia			3		
Pheidole pelor		0	3		
Pheidole tetra		0	3		
Solenopsis diplorhoptrum sp.	33	0	37	100	100
Solenopsis invicta	17	1	17		

Objective 2: Canopy arthropod prey availability and ant arboreal foraging study

Since 2018 UT staff have been supporting an ongoing golden-cheeked warbler impact study conducted by Travis County staff. The original design of the study was to examine changes in arboreal invertebrate prey availability as TCA invaded previously unoccupied habitat. The rapid spread and impact of the *M. nylanderiae* pathogen mean that TCA never strongly impacted the areas that were sampled ahead of the TCA front. This study was continued to evaluate how arboreal prey availability responds to the disappearance of TCA from the habitat.

Arthropod sampling was accomplished by a standardized beating over a collection sheet of 2 low-hanging branches for every tree sampled. The sampling was spread across four tree species (ashe juniper, live oak, shin oak and Texas oak) in three habitats determined by TCA invasion status in the Fall of 2017 (In: invaded areas that harbored TCA for several years prior to 2017, Edge: uninvaded habitat near but outside the Fall 2017 TCA distribution edge of the preserve, Out: uninvaded areas well outside of the TCA population in 2017.)

As well, the arboreal foraging density of both TCA and native ants was assessed with timed counts of all ant species entering the tree in the three different invasion area categories. Surveys occurred during the golden-cheeked warbler's peak nesting season. Travis County and subcontractors are in the process of identifying and counting the arthropods collected during sheet beating sampling. Herein we will report only on the arboreal ant foraging results.

<u>Findings:</u>

Matching the declines observed throughout the preserve, TCA were absent in the arboreal survey in 2022 (Fig. 2A). *Monomorium minimum,* one of the only species not displaced by TCA during invasions, was responsible for the majority of arboreal foraging in the previously invaded (In) habitat (Table 2). Following TCA decline, *Solenopsis invicta* (red imported fire ants (RIFA))

are returning to the habitat and were seen for the first time in the surveys of trees in previously invaded habitat (Fig. 2B).

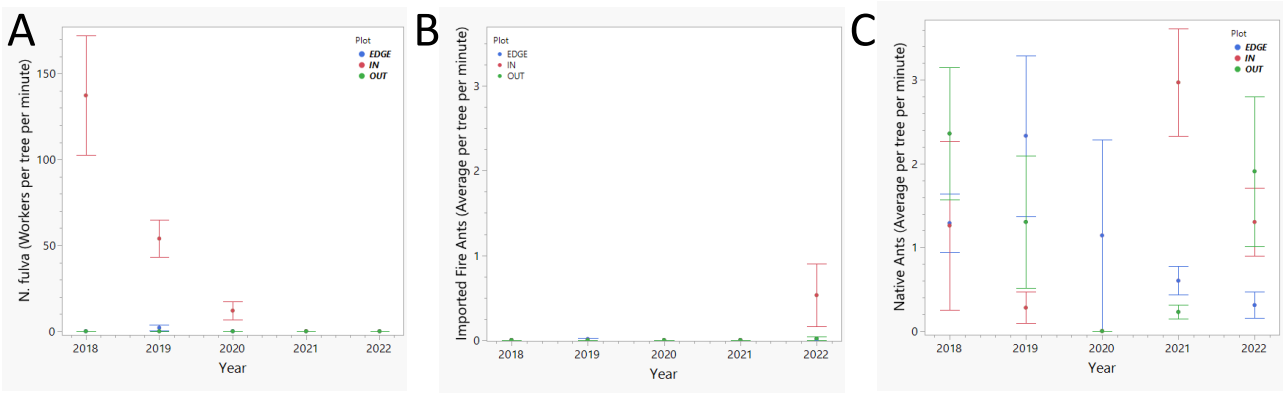

Figure 2: Arboreal foraging activity of **A)** *Nylanderia fulva* (TCA), **B)** *Solenopsis invicta* (imported fire ant), and **C)** native ants across three habitats with different invasion histories from 2018-2022 at the BCP Collins Tract**.**

Table 2: Species composition of ants observed foraging in tree canopies at the Collins Tract in 2022. Counts of the number of ants are the total observed across all sampling intervals and are broken down by the tree species observed on (AJ = Ashe Juniper, LO = Live Oak, SH= Shin Oak, TX = Texas Oak) and by sampling location (EDGE= edge of TCA invaded area, IN = inside invaded area , OUT = outside of TCA invaded area).

Species	EDGE				IN		OUT				Total
	AJ	LO	SH	TX	AJ	LO	AJ	LO	SH	TX	
Nylanderia fulva - TCA	0	0	0	0	0	0	0	0	0	0	0
Solenopsis invicta - RIFA	0	0	0	0	0	35	0	0	2	0	37
Brachymyrmex patagonicus						4					4
Camponotus decipiens				2		3	1	1			7
Crematogaster isolata				1		4				2	7
Crematogaster laeviuscula			9	2		8	19	3	88	64	193
Monomorium minimum	12				17	48					77
Nylanderia terricola					2						2
Pheidole dentata							8				8
Pheidole hyatti			6				15	4		5	30

Objective 3: Quantifying spread of the pathogen *M. nylanderiae* within the Collins Tract - Old Water Treatment Plant 4 TCA population

M. nylanderiae (Mn) is a microsporidian pathogen of unknown origin that infects TCA populations. This pathogen can be devastating to TCA populations and can lead to the extinction of local TCA populations (supercolonies) in 4 to 7 years following pathogen acquisition (9). By an unknown route, this pathogen colonizes uninfected TCA supercolonies in Texas and Florida, and likely throughout its North American range. The Collins Tract TCA supercolony was colonized by this pathogen in 2018. By fall of 2021, the pathogen had spread to all TCA in the nearby area.

In an effort to track the prevalence and intensity of Mn infection, we attempted to collect TCA workers in the spring and fall. Infection intensity can be determined by testing samples of 20 TCA workers with diagnostic PCR. However, TCA were almost entirely absent throughout 2022 and not enough workers were present for infection testing to occur. Only one singular TCA worker was found in the spring of 2022.

Objective 4: Native Ant Assemblage Recovery

As TCA decline at sites due to infection by *M. nylanderiae*, the native ant assemblage can rebound. It is important to document this process to see if the assemblage succession is on a trajectory to return to the pre-invasion state. This recovery will happen in the context of an environment that has been saturated with Mn spores left behind by the declining TCA population. To date, all data we have gathered indicates that this pathogen is specific to TCA. Following the recovery of native ant assemblages provides an additional opportunity to evaluate this hypothesis.

Pitfall trapping at 4 sites where TCA are Mn infected and currently declining or absent were used to assess native ant community recovery. The native ant densities at these recovery sites were compared to those at similar nearby sites that were never TCA invaded. Native ants were also tested for Mn infection as potential non-target impacts of the pathogen. Pitfall trapping followed the methods outlined in the Objective 1. Sites included the Emma Long Metropolitan Park, Pace Bend Park, Walnut Creek Greenbelt, and the Collins, New Life and Blackmore tracts of the BCP.

To evaluate which species may recolonize post TCA decline, malaise traps were deployed at the Collins tract and the nearby uninvaded New Life tract. As well, hand sampling of fire ant colonies was used to assess the social form composition of recolonizing fire ant populations.

<u>Findings:</u>

This is a longer-term project. Currently we have two sites within the BCP that are at least 2 years post TCA collapse and thus have enough data to show initial changes: Whirlpool Cave and Collins tract. To evaluate recovery, sampling years have been transformed into years relative to TCA decline. The year of TCA decline was determined as the first year that TCA populations declined to the point of 50% prevalence across the pitfall traps.

At both sites, two years following decline red imported fire ants (RIFA) begin a rapid increase in prevalence and relative abundance (Fig. 3). So far, the abundance of fire ants at these sites remains below the levels seen at paired uninvaded comparison sites (controls). As reported previously, these resurging fire ant populations are heavily biased towards monogyne colonies as compared to control sites. This is expected as monogyne fire ants can colonize remote habitats through winged dispersal and independent founding. Monogynes populations tend to be lower density fire ant populations than polygynes, so we hope these will remain at or rapidly decline to lower fire ant abundance as compared to control sites. At both sites, starting prior to TCA declining to 50% prevalence, native ants began to rebound and continue to increase in both abundance and species density 3 years following TCA decline. We will continue

to follow these trends until fire ant and native ant abundances reach an equilibrium. As previous data indicated, no native ants recolonizing the site have tested positive for Mn infection (Table 3).

Table 3: Tests for Mn from ants other than TCA in environments where they have exposure to the pathogen. (# positives : # of tests).

Ant Species Category	**Infected TCA Present**	**1-3 Years Post TCA Decline**	**Total Tests**		**Infected TCA Present**	**1-3 Years Post TCA Decline**	**Total Tests**
Formicine species	12	27	39		0 : 12	0 : 27	0 : 39
Nylanderia - native species	13	34	47		0 : 13	0 : 34	0 : 47
Other Invasive Ants	32	62	94		0 : 32	0 : 62	0 : 94
Other Native Ant	30	138	168		0 : 30	0 : 138	0 : 168
Total Species Tested	17	28	32		0 : 17	0 : 28	0 : 32
Total Tests	87	261	348		0 : 87	0 : 261	0 : 348

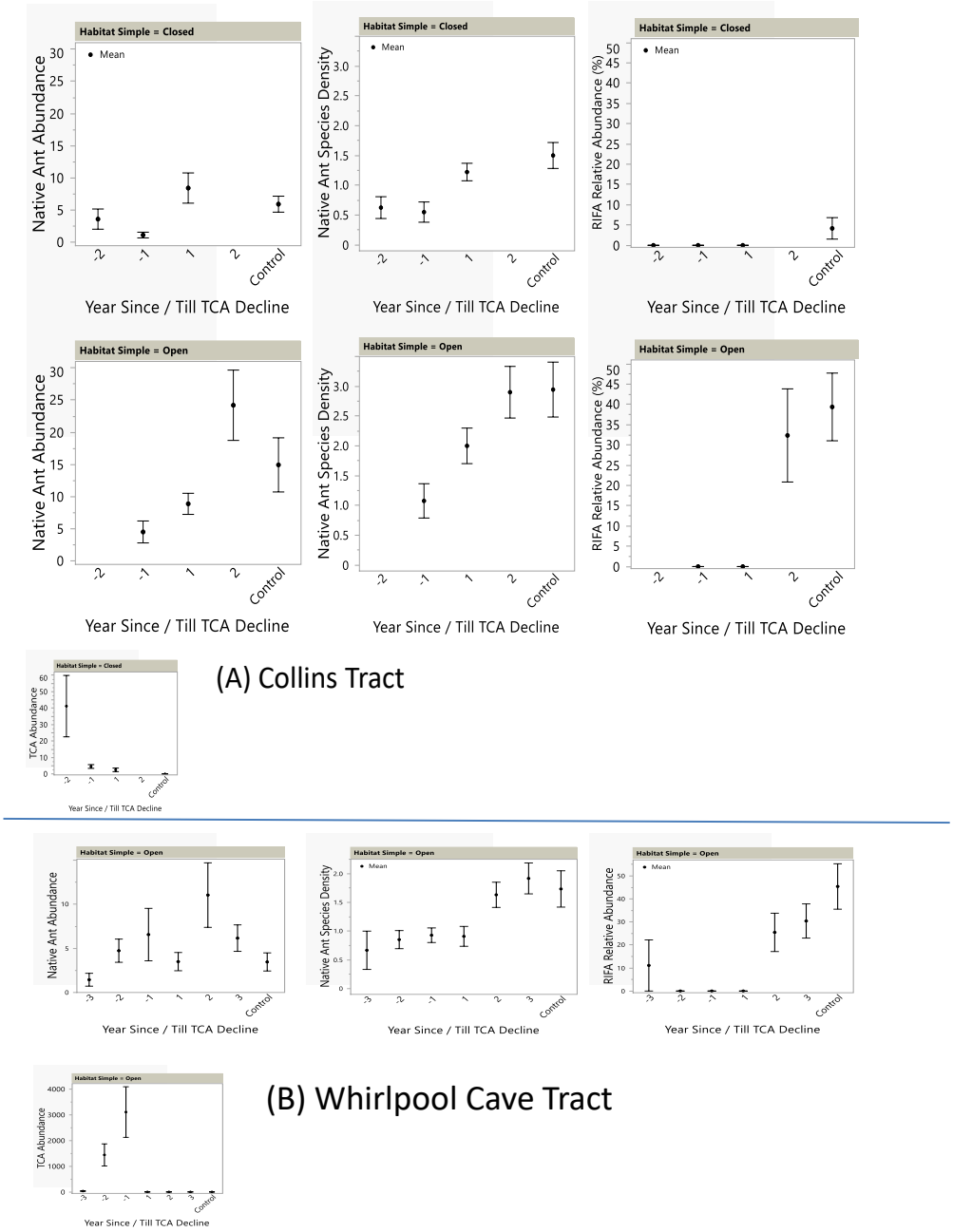

Figure 3: Recovery of native ant community over time at two BCP sites: (A) Collins Tract and (B) Whirlpool Cave Tract. X axis is the number of years before or since the decline of the TCA population. Year 0 on this axis was defined as the year that the TCA population declined such that only 50% of the pitfall traps contained TCA. RIFA – red imported fire ants. Native Ant Abundance – average number of workers per trap from all species other than TCA and RIFA. Native Ant Species Density – average number of ant species per trap excluding TCA and RIFA. RIFA Relative Abundance – average fraction of the ants in a pitfall trap that were red imported fire ants. Bottom left panel shows TCA abundance through time for reference. No spring pitfall trap data is available for year 0 at either site.

Objective 5: Testing the thresholds for detection of the pathogen *M. nylanderiae* and tracking its spread: Pace Bend Park

In 2020 and 2021, we performed inoculative releases within the Pace Bend Park supercolony using the 'lab-fused nest approach'. Post release, we have detected the pathogen several times at several inoculation sites and have detected pathogen spread away from the inoculation stations at three. We have some confidence that the pathogen is now well established within the Pace Bend supercolony. Previously, recently established inoculations have died out over the winter, but we feel that this site is beyond this stage and expect to see the pathogen spreading in 2022.

Testing the threshold of detection of Mn and assessing it as a tool for detecting establishment

Post inoculative release, sampling at the inoculation stations at Pace Bend Park frequently alternated between spore positive and spore negative samples. Some inoculation stations that behaved in this manner died out over the winter of 2021-2022 while other remained negative for a protracted period and then the infection resurged. We will continue a sampling exercise begun in Fall of 2021 to determine at what threshold of infection populations are likely to establish.

In November of 2021, we collected 500 workers from each of the inoculation stations. In the lab we tested these workers in subsets of 10 repetitively for Mn infection using diagnostic PCR. Using statistical models commonly employed in epidemiological studies, these data allowed us to calculate the frequency of individual ant infection at the inoculation station at the time of sampling (10). We resampled these stations again in March and May to assess whether the pathogen overwintered successfully at each inoculation station. Relating these variables will allow us to test for the average level of individual ant infection that leads to station-level pathogen establishment. We can then determine the depth of sampling required to test for this level of individual infection. We hope that this will eliminate the need for protracted sampling of inoculation sites in future inoculation efforts.

Findings:

To date, inoculation efforts have been characterized by a high failure rate as measured on a per inoculation station basis. We have generally overcome this through intensive efforts where inoculations have been conducted at many stations simultaneously. Even when successful positive detections at inoculation stations have been sporadic, often punctuated by long periods of negative testing. The threshold of detection experiment has clarified why. Stations that frequently tested as negative for the disease using standard detection assays, commonly tested as having the disease present but at very low prevalence within the ants in that area. Epidemiological statistics for calculating individual prevalence from pooled samples found positivity rates as low as 2 ants per 1000 workers (Table 4). This the lowest level of prevalence that our sampling design was capable of detecting. As expected, these low prevalence infections were associated with high rates of negative testing during that period.

Spring of 2022 testing revealed that the pathogen appeared to disappear from some stations that tested positive but at very low prevalence in the fall 2021 and its prevalence increased at others that had exhibited higher disease prevalence the preceding fall (Fig. 4A).

The likelihood function relating the estimated infection prevalence in the fall of 2021 to whether or not a station tested positive for the pathogen the following spring showed a strong inflection point at a fall prevalence of 9 infected ants per 1000 ants (Fig. 4B). All stations that had an infections of this level in the fall either remained constant in the fraction of pooled worker tests that were positive for the pathogen or increased markedly in this quantity by the following spring (Fig. 4A).

From a practical perspective, testing at a level of sufficient depth to detect a pathogen prevalence of 9 ants per 1000 workers is appropriate to detect inoculations that are sufficiently established that they are likely to increase and spread in the next year. This requires testing more than 111 ants per inoculation station. We will target establishment tests of at least 150 ants per inoculation station going forward. Rapid spread of the disease out from inoculation stations was evident in the spring and fall from inoculation stations that demonstrated intermediate to high per worker pathogen prevalence levels in the fall of the preceding year Fig. 4 and Fig. 5). This suggests that for the disease to reach the point of an epizootic outbreak, local disease prevalence must be above a minimum threshold. These epizootic outbreaks are what lead to TCA population collapse. Potentially intensive releases of infected ants into a single area may be a superior approach to reaching population control than many inoculative releases spread over a wide geographic area.

Table 4: Results of fall 2021 threshold of detection sampling. Detection probability – 20 ants: the likelihood that an individual standard establishment sample would detect the infection at the estimated per ant infection prevalence measured. Establishment Testing: % positive tests (Fall – Winter 2021) – the fraction of 20 ant pooled tests that returned positive results between September and December 2021.

Inoculation Station	Inoculation Year	Estimated Infection Prevalence (per ant)	Detection Probability - 20 ants	Establishment Testing: % positive tests (Fall - Winter 2021)
PB INOC 1	2020	0.002	4%	6%
PB INOC 2	2020	0.002	4%	0%
PB INOC 3	2020	0.010	21%	18%
PB INOC 4	2020	0.006	12%	18%
PB INOC 5	2020	0.006	12%	0%
PB INOC 6	2020	0.141	25%	65%
PB INOC 7	2020	0.002	4%	0%
PB_INOC 1_21	2021	0.020	25%	24%
PB_INOC 2_21	2021	0.013	25%	18%
PB_INOC 3_21	2021	0.000	0%	0%
PB_INOC 4_21	2021	0.004	8%	12%
PB_INOC 5_21	2021	0.000	0%	6%

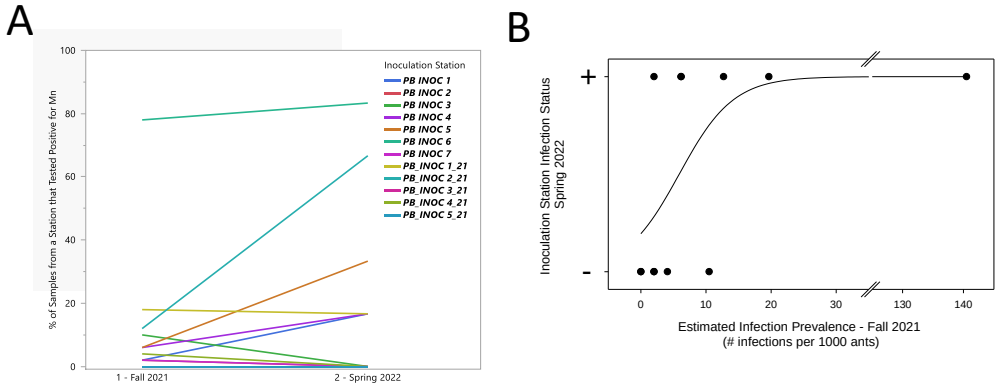

Figure 4: Relationships between infection levels at inoculation stations in the fall of 2021 and spring of 2022. (A) The percentage of pooled samples that tested positive during the two time intervals. (B) Logistic regression describing how the likelihood that a station is positive in the spring changes as a function of disease prevalence in the preceding fall.

Tracking the Spread of Mn Infection in the Pace Bend TCA Supercolony

We followed the spread of the pathogen within and outside of the park as it moves outward from inoculation sites. In past inoculation efforts we have observed periods of very rapid pathogen spread and periods where pathogen distribution appeared to remain relatively fixed. An open question is the degree to which the pathogen can establish in areas well ahead of the region of high pathogen prevalence. Such a pattern could be driven by long-distance migration of infected workers among the interconnected nests of the population, or potentially dispersal of infected males during alate flights. We will focus our efforts on determining the frequency of long-distance dispersal of infection. Long-distance dispersal is one of the few mechanisms with the potential to explain very rapid pathogen spread.

At 8- week intervals beginning in April, we sampled ants from widely spaced predetermined stations outside of areas of known infection. Any positive detections will be followed by sampling around the area of the positive detection to assess whether the infection is connected to a larger area of infected nests. In addition, two transect based sampling efforts in April and November were completed to determine the limits of high infection prevalence habitat.

<u>Findings:</u>

Long-distance, jump dispersal of the disease appears to be common. In the April, June, and August sampling intervals, positive disease detections were made far from areas of known *M. nylanderiae* infection. See isolated yellow pins in Figure 5. As there was no seasonal pattern to the appearance of these positives, most likely these arise as a result of infected workers dispersing widely amongst the network of interconnected nests. Populations of organisms in which long-distance dispersal is common spread radically faster than populations without this pattern (11). This pattern of spread likely explains how *M. nylanderiae*, once established, can

spread throughout a TCA population in a 1 to 2 years (9). By fall of 2022, *M. nylanderiae* was well established throughout much of the TCA population within Pace Bend Park (Fig. 6). The TCA population itself at Pace Bend Park showed limited expansion in 2022 (Fig. 7)

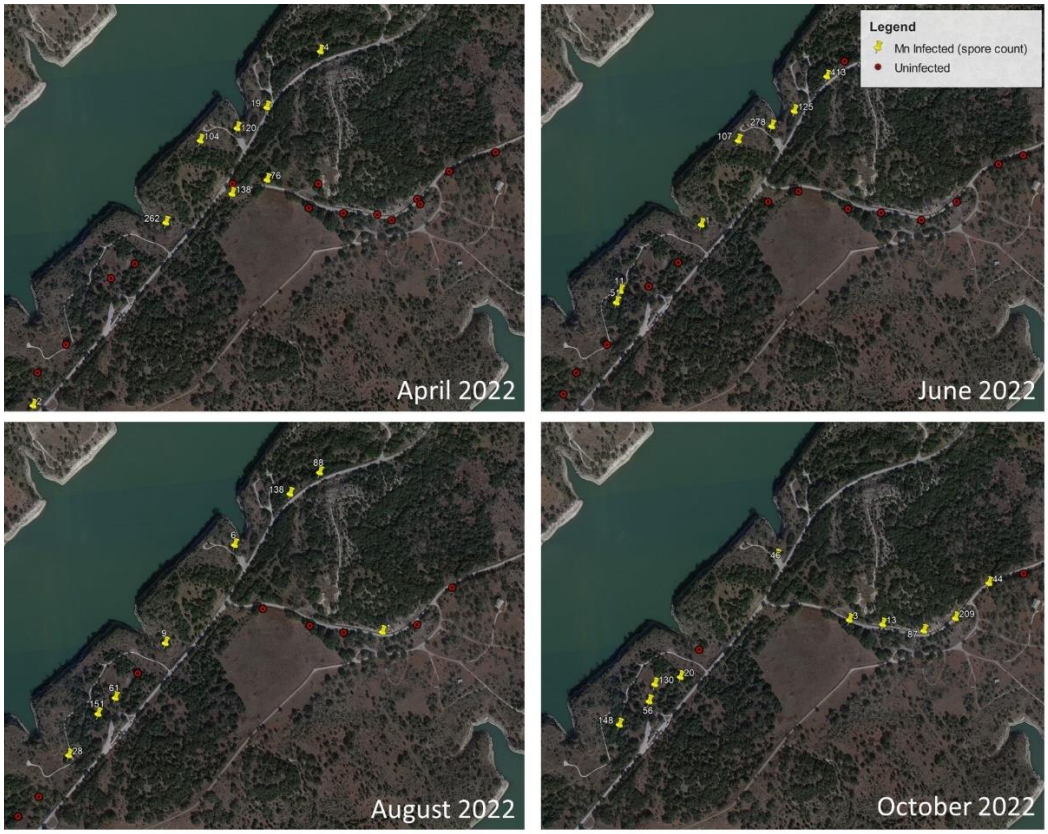

Figure 5: Long distance dispersal detection array sampling.

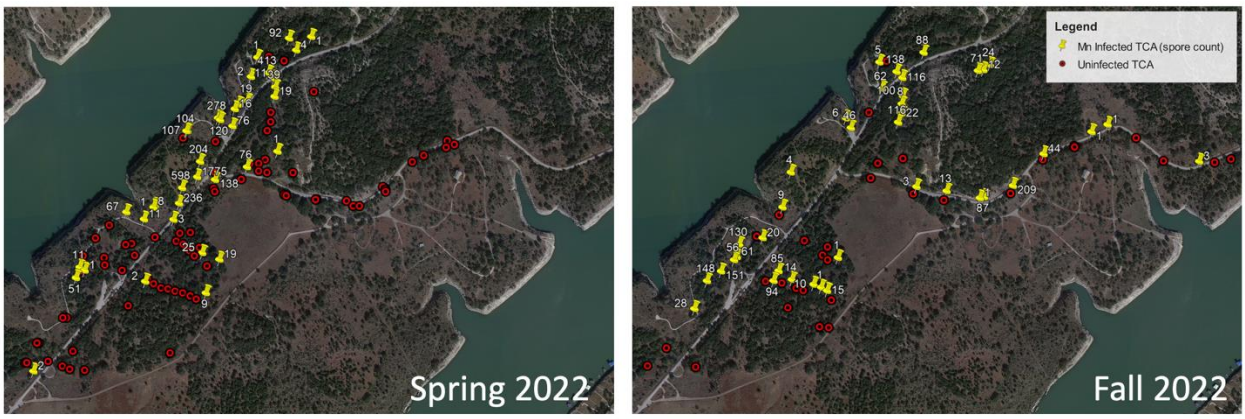

Figure 6: Maps tracking the spread of *M. nylanderiae* (Mn) infection throughout Pace Bend Park

in the Spring (March-June) and Fall (August-November) of 2022. Samples that were positive for Mn infection are labeled with their infection intensity (spore counts).

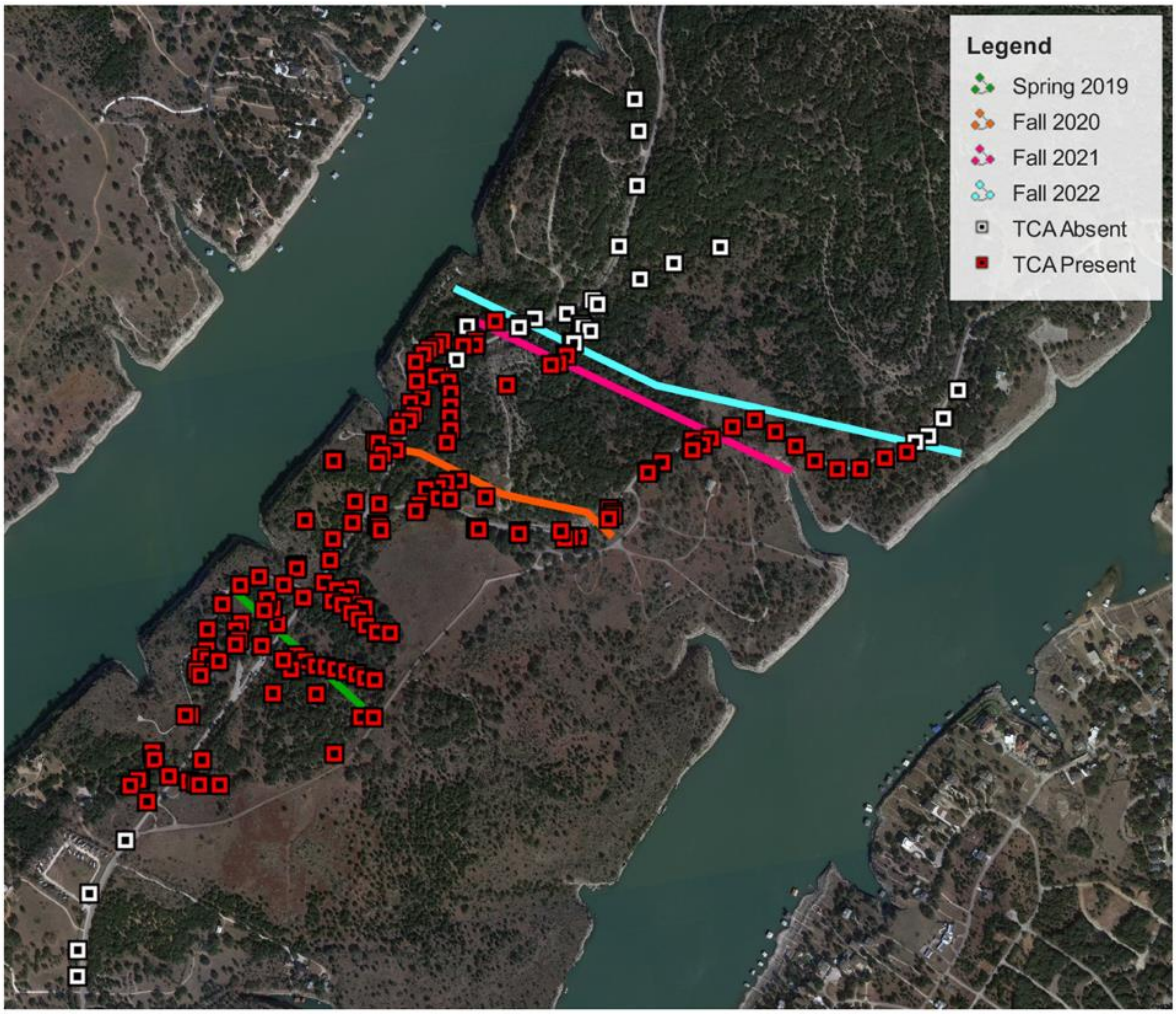

Figure 7: Progression of edges of TCA-invaded area from Spring 2019-Fall 2022 at Pace Bend Park. Squares show the presence (red) and absence (white) of TCA at sampling points from Fall 2022.

Objective 6: Inoculative releases of the microsporidian pathogen Myrmecomorba nylanderiae

In addition to Pace Bend (discussed above), inoculative releases were conducted at Roy Guerrero Park in 2021. TCA population density is low at Roy Guerrero, and historically inoculations into low density populations have not succeeded. No positives were recovered from Roy Guerrero in 2021, but resampling was conducted in the early winter and spring of 2022 to confirm that the pathogen did not take from the previous year's releases.

Instead of attempting to inoculate the low-density Roy Guerrero population again, inoculative releases were completed at the very high-density No Rent Cave site. In August 2022, five inoculative releases were conducted in areas spaced at least 100 m from each other. Because of the summer drought, a new technique was employed wherein nest boxes

containing sugar and water tubes and nesting material were buried at Emma Long Metropolitan Park, where the entire TCA population is now Mn infected. TCA moved into these boxes where the conditions were more tolerant than the outside aridity. Next these boxes containing infected TCA were transported to No Rent Cave and reburied near large TCA foraging trails. Hopefully the nest box resources will keep uninfected TCA near and increase interactions between the populations, thus increasing the chances that the infection will take hold.

Findings:

Inoculation station sampling at Roy Guerrero Park in the early winter and spring of 2022 showed no signs Mn establishment, with all samples testing negative (Fig. 8). However, invaded habitat at Roy Guerrero Park was mapped in summer of 2022, and TCA continued to retract. TCA could not be located near the inoculation stations anymore and were only found at one location near a creek bed. The extremely sandy soil at Roy Guerrero may not have provided sufficient voids for the ants to find refuge from the summer drought or the hard freeze in the preceding winter. By the fall of 2022, overall TCA abundance had returned to near but still below fall 2021 levels (see below).

The nest box approach at No Rent Cave appeared to work well, and TCA were observed swarming around the nest boxes, which provided good nesting habitat during the drought. However, no positive samples have yet to be recovered and, so far, there is no evidence of disease establishment (Fig. 9). In past successful inoculations it has taken up to 9 months to recover evidence of pathogen establishment. Follow-up sampling will continue in 2023.

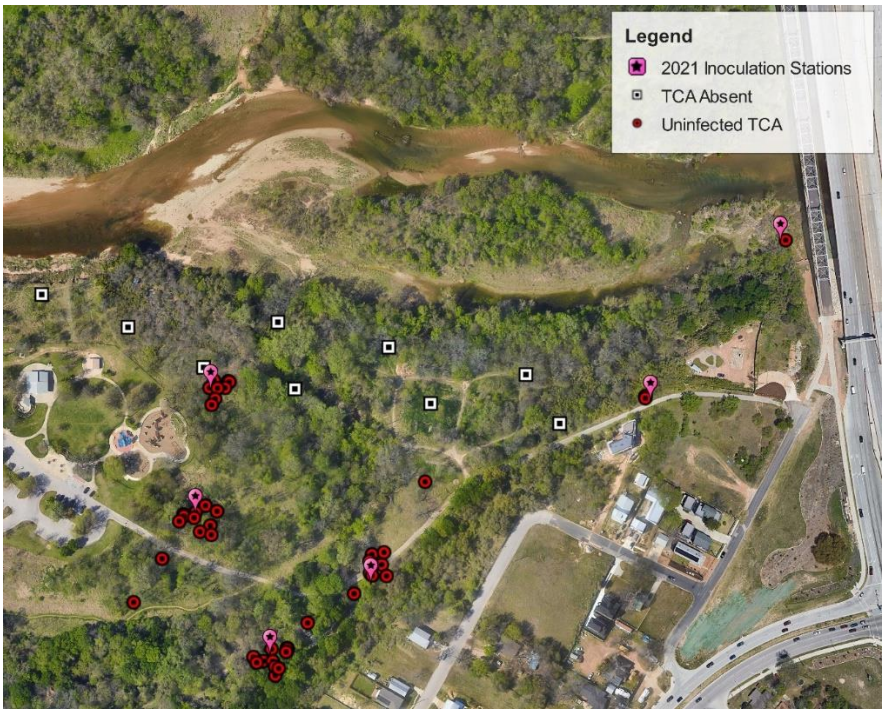

Figure 8: Roy Guerrero 2022 inoculation station locations and sampling (January – July 2022). All TCA sampled were Mn-negative (red circles).

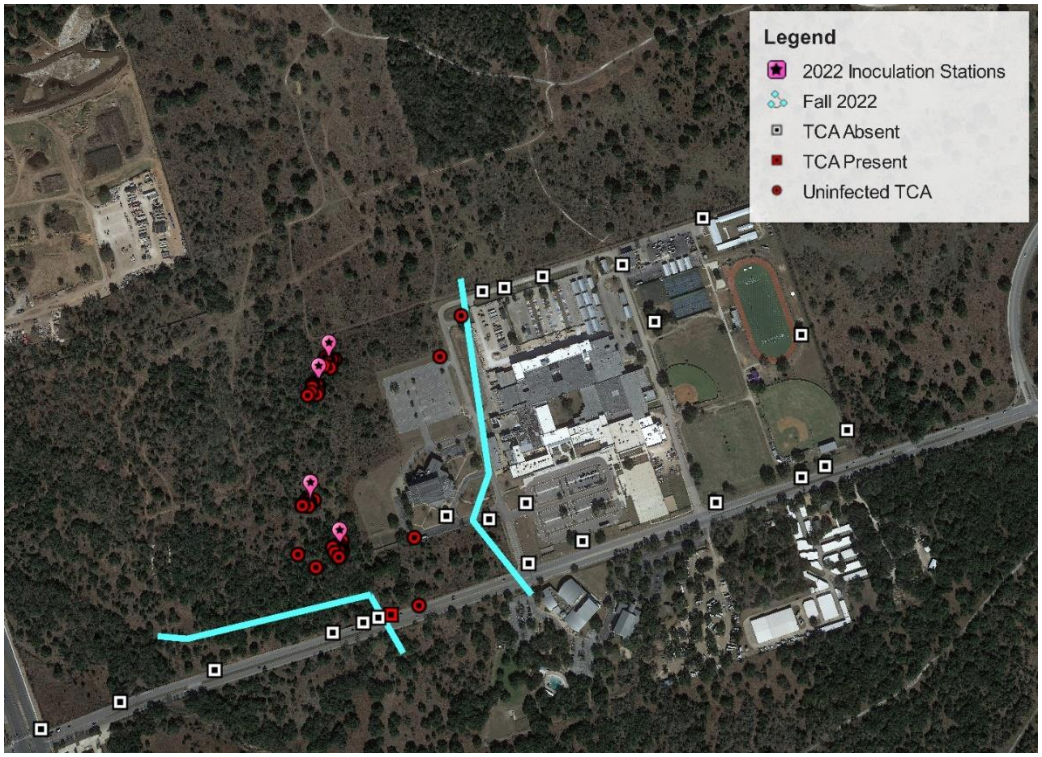

Figure 9: No Rent Cave 2022 inoculation station locations and sampling (August – November 2022) and TCA population extent mapping (July 2022). Squares show the presence (red) and absence (white) of TCA at sampling points. All TCA sampled were Mn-negative (red circles).

Objective 7: Monitoring TCA Populations at Other Sites

TCA continue to colonize sites in the Travis County area. These sites provide opportunities to accelerate our knowledge and better protect BCCP sites. As well, since some invaded sites are near BCCP protected areas, they present a direct risk of spread into the BCCP. All sites have the potential to be source populations for future BCCP infestations.

Active TCA populations at five sites were monitored: Pace Bend Park, Southern Walnut Creek Greenbelt, Roy Guerrero Park, Emma Long Metropolitan Park, and the McNeil Tract of the BCP. All sites were pitfall trapped in early fall to assess TCA density population pathogen burden and in the case of Mn infected populations. A minimum of 10 pitfall trapping spaced at least 50 m apart were be used to monitor TCA density changes and infection levels. In the fall, limits of invaded habitat were mapped with presence/absence sampling. Areas of reinfestation within the Whirlpool Cave Preserve area were monitored and delimited on a 3-month interval.

<u>Findings:</u>

TCA abundances as measured by pitfall traps decreased at Emma Long, Roy Guerrero, Walnut Creek, and Pace Bend (Fig. 10). These declines were potentially exacerbated by the summer

2022 drought, as TCA are highly susceptible to drought. At both Emma Long and Walnut Creek, the TCA population retracted such that the pitfall trap plots are no longer in invaded habitat. As a result, although these sites still contain active TCA infestations, no TCA were captured in the traps.

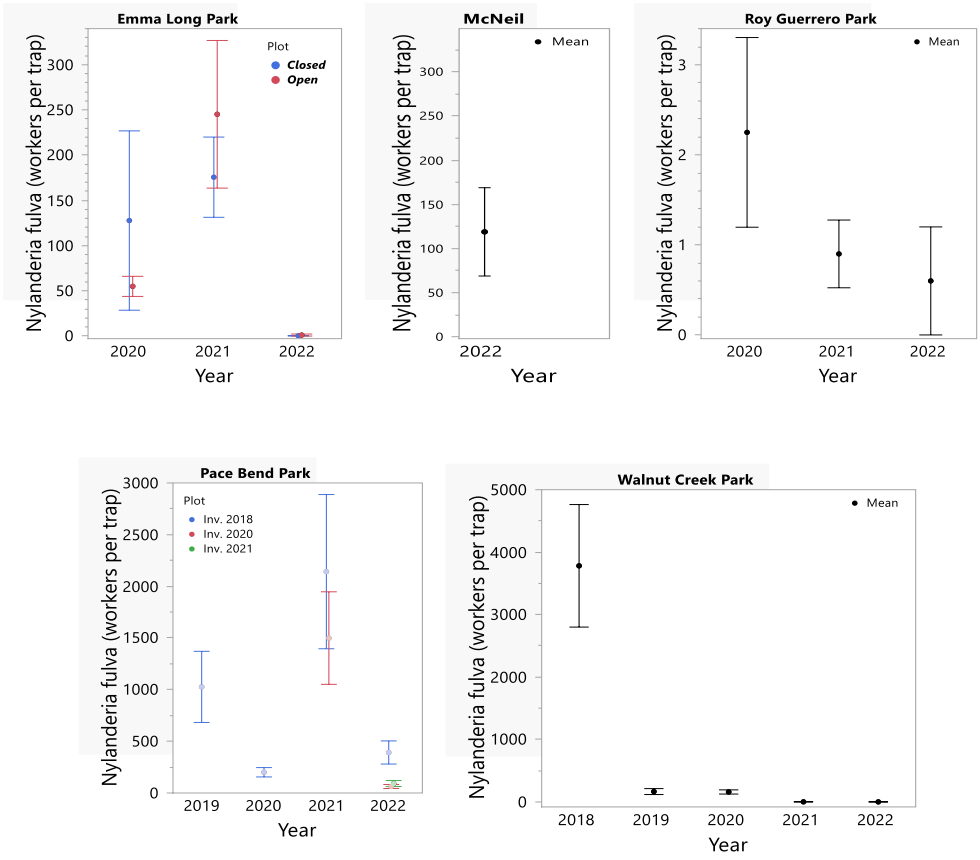

Figure 10: *Nylanderia fulva* (TCA) abundance across years as measured by 24-hr pitfall traps at sites with active infestations.

Generally, in 2022 TCA populations tended to expand only a small amount or retract depending upon their infection status. The TCA population at Emma Long Park retracted substantially in 2022 (Fig. 11). Given that *M. nylanderiae* infection had become essentially universal in 2021 a decline was expected. This decline was also likely exacerbated by the drought conditions. The population at Pace Bend expanded only marginally in 2022 (shown previously Fig 7). Widespread infection by Mn was achieved this year at this site. It is unclear what role this played in inhibiting population spread. The population at Roy Guerro has diminished to the point that it is only present in localized areas. The TCA population in the Southern Walnut Creek Greenbelt expanded in some directions and retracted in others (Fig. 12). Conversations with the rugby club at Walnut Creek Park indicate that the retraction in the area near the rugby pitch, where the pitfall trap plot is located, may have been exacerbated by

pesticide application by the club. Mn infection is now widespread in this population making population decline in the coming year likely.

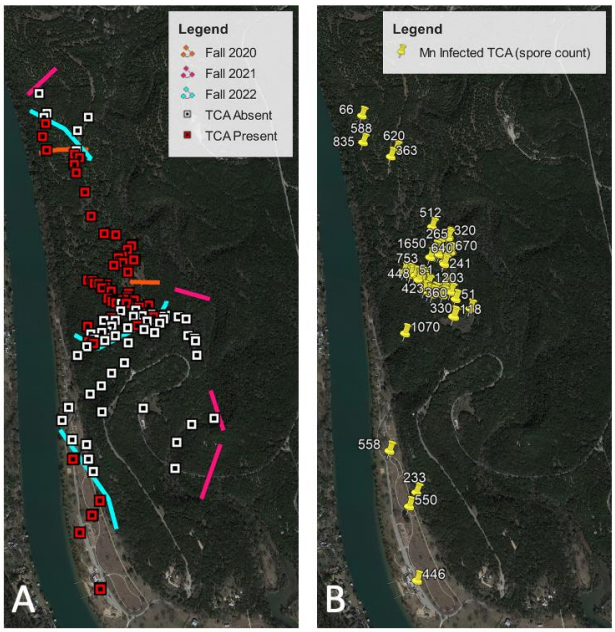

Figure 11: TCA at Emma Long Metropolitan Park in 2022. A) Edges of TCA-invaded area at from Fall 2020 to Fall 2022. Squares show the presence (red) and absence (white) of TCA at sampling points in Fall 2022. B) All TCA collected were Mn infected and are labeled with their infection intensity (spore counts).

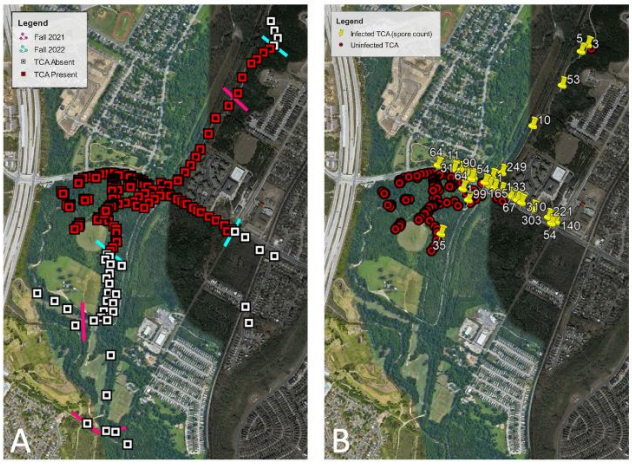

Figure 12: TCA at Southern Walnut Creek Greenbelt in 2022. **A)** Edges of TCA-invaded area at from Fall 2021 to Fall 2022. Squares show the presence (red) and absence (white) of TCA at sampling points in Fall 2022. **B)** Red circles indicate an Mn-negative sample and yellow pins indicate an Mn-positive sample. Positive samples are labeled with their infection intensity (spore counts)

Reinfestations at the Whirlpool Cave Tract

The resurgent TCA infestations present in 2021 in the Whirlpool Cave Tract died out over the course of 2022. These emergent TCA populations were all infected with the Mn disease at a very small size relative to what we typically see in other contexts (9). This supports the hypothesis that environments that were occupied by TCA that collapse due to Mn infection remain resistant to reinvasion due to the presence of Mn spores in the environment. This hypothesis requires further study in the laboratory to assess the ease of environmental disease transmission in Mn and the longevity of viable spores in the environment. In 2022, TCA were spotted in only one area in February, but were not seen anywhere during the summer or fall (Fig. 13).

Figure 13: Resampling of resurgent areas of TCA invasion at Whirlpool Greenbelt. TCA were only present once and were Mn infected. The yellow pin represents their infection intensity (spore count).

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