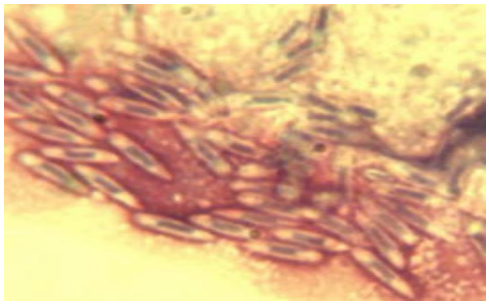
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Tawny Crazy Ant (*Nylanderia fulva*) Study
Balcones Canyonlands Preserve – Jollyville Unit
Task 5: Data analysis and Final Report

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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 supercolonies 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 the TCA supercolony currently invading tracts of the Balcones Canyonlands Preserve (BCP), and to explore using this microsporidian as a control agent.

By the fall of 2022 most of the BCP and allied TCA invaded sites had been infected with *Mn*. In 2023, we observed widespread declines of TCA populations at many BCP and allied sites as a result. Our inoculation efforts in 2023 were highly successful, establishing *Mn* in what, at that time was the only remaining BCP site invaded by an uninfected TCA population. In 2024, TCA were found at an additional two sites within the BCP: the Lime Creek Tract and Sandy Creek Park. At sites where the TCA populations have previously gone extinct, native ants are resurging including those species most closely related to TCA. The recovery of species related to TCA corroborates laboratory tests that indicated a high level of specificity for this pathogen. Major developments in 2025 include:

1. Following our 2022 – 2023 inoculation efforts, the TCA supercolony at the McNeil Tract of the BCP has entered the collapse phase of the *M. nylanderiae* epizootic. TCA abundances are only 4.5% of what they were in the fall of 2024 and TCA are no longer present throughout the tract. This is the site of two caves known to contain federally endangered species. No TCA were found in these caves in 2025.
2. Our April 2025 inoculation effort at the Lime Creek and Wilcox Cave Preserves has led to the successful establishment of the *Mn* pathogen with the TCA supercolony that spans these sites. Both sites contain caves with federally listed endangered species. Further these sites lie within Buttercup Creek Cave Complex, an area that contains many karst features known to harbor endangered species. Establishing *Mn* within this TCA supercolony was one of our highest priorities in 2025.
3. Mn has spread throughout the extant Pace Bend TCA supercolony which showed no signs of expansion in 2025. We anticipate its further decline in 2026.
4. In post-TCA collapse sites, as more sites reach 5+ years of recovery a trend towards recovery sites reaching higher levels of native ant diversity and lower levels of imported fire ant abundance compared to similar never invaded contrast sites is emerging. This is

an unexpected result. More sites with long recovery windows are needed to evaluate the significance of these patterns.

5. As TCA have spread in collaboration with City and County staff we have added another invaded cave to our study of TCA impacts on karst systems. The data to date have yielded a clear seasonal pattern of use of cave features by TCA where crazy ants are present in caves at high abundances in summer and winter. This supports previous work done in 2016 – 2017 where available data was limited.
6. The TCA supercolony first found impacting Sandy Creek Park in late 2024 is much larger than the preliminary edge mapping we conducted in 2024 indicated. In actuality, it penetrates deeply into several adjoining tracts of the BCP. We have found spotty, low-intensity, naturally acquired *Mn* infections within this expansive supercolony. For reasons we do not understand, naturally acquired infections can take considerably longer than inoculative releases before the pathogen becomes widespread and the supercolony declines. We are recommending an inoculative release into this TCA supercolony be conducted in 2026.
7. In the cool season of 2025, SINV-3 prevalences were very low. Only 1.5 percent of imported fire ant colonies sampled tested positive for infection. Evidence indicates that a substantial decrease in the prevalence of SINV-3 has occurred since 2016 in central Texas.

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). At a local scale, following introduction, the ants spread by nest budding, maintaining connections between neighboring nests, eventually forming an interconnected web of nests that can span many square kilometers. Herein we will refer to these interconnected groups of nests within a site as a supercolony although in fact they are fragments of a much larger supercolony with a highly fragmented distribution. 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 and directly predating them (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 supercolonies 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 supercolonies 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 supercolonies combined with experimental introductions of this pathogen into uninfected TCA supercolonies 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).

A 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 we report on the results from Balcones Canyonlands Preserve (BCP) tracts impacted by TCA including, the Collins Tract BCP, the Whirlpool Cave Tract BCP, Lime Creek Preserve BCP, Sandy Creek Park BCP, McNeil Tract BCP, Lime Creek Tract BCP, Trails End Tract BCP, and Wheless Tract BCP as well as 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. Types of investigations at sites are determined by the TCA invasion site status and the *Mn* infection status of the local TCA supercolonies within a site (Table 1). This report is also organized based on these factors.

The objectives for 2025 were to:

1) Assess tawny crazy ant (TCA) densities, and distributions – currently invaded sites.
2) Assess whether tawny crazy ant (TCA) super colonies are infected with *M. nylanderiae* (Mn); quantify disease extent, prevalence, and intensity if infected – currently invaded sites.
3) Biological control of TCA using the pathogen: *M. nylanderiae*
   a) Perform inoculative releases of the microsporidian pathogen *Mn*.
   b) Quantify pathogen establishment post inoculation and track the spread of the pathogen once established.

4) Quantify ant assemblage and monitor for TCA at Collapsed sites where TCA have been observed within the past year – post TCA collapse sites.
5) Evaluate recovery of native ant assemblage at TCA collapse sites.
6) Evaluate TCA impacts on karst invertebrates.
7) Evaluate the prevalence of *Solenopsis invicta* virus 3 (SINV-3) during the cool season in central Texas.
8) Attempt to increase the SINV-3 viral loads of imported fire ant colonies by holding them under laboratory conditions designed to maximize viral transmission.
9) Use imported fire ants highly infected with SINV-3 to make a bait to transmit the virus to uninfected colonies.

Table 1: TCA Research sites 2025. Sites within the BCP or sites of special concern with extant or recently collapsed TCA supercolonies. Previously collapsed sites are sites previously invaded by TCA where TCA declined below the level of detection prior to fall 2022. This table reflects the status of these sites during the time of research activity planning: February 2025.

TCA / *Mn* Status	**TCA Infected with *Mn*[2]**	**TCA Uninfected with *Mn***
Active TCA Supercolony - Site	Pace Bend Park	Lime Creek Preserve (BCP) - Wilcox Cave Preserve
	McNeil Tract - BCP	Town Lake Metropolitan Park[3]
	Sandy Creek Park -Trails End - Wheless Tracts – BCP[4]	
Collapsed TCA Supercolony - Site	Collins Tract BCP – Old Water Treatment Plant 4	
	Whirlpool Cave Tract- BCP	
	Emma Long Park	
Native Ant Recovery Control Sites[6]	Dick Nichols Park	
	New Life Tract - BCP	
	Blackmore Tract - BCP	

[1] TCA no longer present at site.; [2] Infection status of collapsed sites determined by status at time of collapse.; [3] Newly reported infestation in 2025. [4] *Mn* infection at low levels and restricted to small areas (see report). [5] *Mn* infection status has not yet been extensively tested. [6] These sites, used to evaluate the recovery of the native arthropod fauna serve as contrasts to post TCA collapse sites. They have never been invaded by TCA.

Objective 1&2: Within currently invaded sites: 1) Assess tawny crazy ant (TCA) densities, and distributions; 2) Assess whether tawny crazy ant (TCA) supercolony is infected with *M. nylanderiae*, and quantify disease extent, prevalence, and intensity.

Since the project began following the invasion of the Collins Tract of the BCP by TCA in 2018, additional areas of the BCP have been invaded and additional properties of importance for constraining TCA spread regionally have also become established. TCA continue to colonize sites in the Travis County area. These sites provide opportunities to accelerate our knowledge gain and better protect BCP sites. Some sites are also near BCCP protected areas and thus the TCA in them present a direct risk of spreading into areas of the BCP. All sites have the potential to be source populations for future BCP infestations.

TCA supercolony densities peak in the late summer and fall. In these seasons we conducted a variety of assays described below to quantify the density of the TCA supercolony, its extent, the infection status of the TCA supercolony, and the disease intensity of the infected supercolonies.

At currently infested sites, we conducted the following sampling activities:
(For a list of currently infested sites see Table 1.)

Quantify TCA Density using Pitfall traps:

Permanent pitfall trapping stations, consisting of PVC sleeves set flush in the ground, have been established in invaded and uninvaded areas of the preserve. An open tube containing a mixture of water, soap, and propylene glycol is inserted. Propylene glycol is a non-toxic preservative (approved by the FDA as a food additive) and poses no known threat to wildlife. Tubes were left open for 24 hours to collect ants. Between trapping intervals, sleeves were plugged.

Map the extent of the TCA supercolony:

TCA supercolonies expand radially from the point of initial introduction through the process of large nests dividing into smaller adjoining nests. Throughout this process nest networks remain interconnected. As a result, TCA supercolonies tend to saturate a contiguous area of habitat. Visual inspections of the presence or absence of TCA on the ground allow for a rapid assessment of the amount of habitat occupied by a given supercolony. Year-to-year increases or decreases in the spatial extent of the supercolony provide another measure of the health of the TCA supercolony and the likelihood that it will impact nearby sensitive habitats or features.

In the Fall of 2025, areas that contained continuous infestation of interconnected nests were mapped for TCA presence - absence. Spatial sampling designs were opportunistic and, where possible, structured around existing path networks and public rights-of-way where appropriate. Presence / absence was determined by visual inspection of the foraging ant assemblage as well as examinations of likely nesting locations (under rocks and rotten logs). Stations where non-TCA, ant workers were encountered at 3 locations, and no TCA were found or where 15 likely nest locations do not reveal TCA were considered free of crazy ants. Three negative stations in a row indicated that the observer has passed beyond the edge of TCA occupied habitat.

Assess whether tawny crazy ant (TCA) supercolony is infected with M. nylanderiae, and quantify disease extent, prevalence, and intensity:

At all extant TCA supercolonies, we tested ants to assess whether the supercolony harbors the pathogen *M. nylanderiae*. Those sites that do harbor the disease were sampled more intensively to assess the extent of the pathogens spread through the TCA supercolony and the intensity of infection present. These factors help to assess whether the TCA supercolony is on a trajectory of disease induced collapse or if an intervention via inoculation of the disease is warranted.

Approach:

Sampling followed the strategies used from 2018 to 2024. Sites were assessed for infection status by testing *N. fulva* workers from every pitfall trap for the pathogen. Further, for all TCA supercolonies, workers from three points near the edges of the TCA supercolony along the TCA mapping transects were collected and tested for infection ensuring that we were likely to detect an incipient infection if it is not yet widespread. At every point where infection status was tested, DNA was extracted from a batch of 20 workers, and pooled DNA was tested with diagnostic PCR (7).

For TCA supercolonies that previous PCR tests have revealed harbor the disease, aliquots from tissue homogenates of 20 workers were fixed on slides, stained, and *M. nylanderiae* spores counted at 1000x magnification (8). These samples provide a measure of the prevalence and intensity of *Mn* infection. In infected supercolonies, we tracked the prevalence and intensity of infection by the *Mn* pathogen with both Spring and Fall sampling. For all sampling efforts, worker ants were collected off baits, foraging, trails or from nest sites.

McNeil Tract of the BCP

TCA History:

McNeil Tract is a part of the BCP containing two caves with endangered karst invertebrates: No Rent and Weldon caves. The current infestation at the McNeil site was first reported in the spring of 2022. Subsequent testing revealed it to be uninfected with *Mn*. We conducted an inoculation in August of 2022. Post inoculation testing in November, did not find evidence of disease establishment. A second inoculation was performed in the spring (2023). By Fall of 2023 evidence of pathogen establishment was found at both 2022 and 2023 inoculation stations. The local TCA supercolony was abundant in 2023 and much more so for most of 2024.

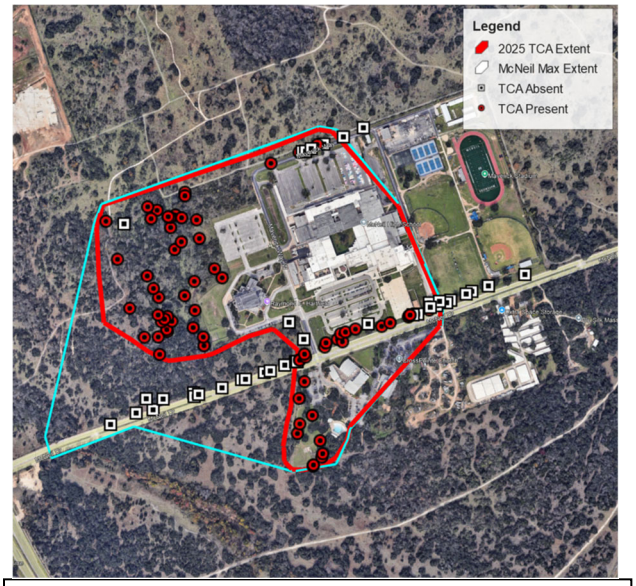


Fig. 1: McNeil Caves Tract of BCP – Map of TCA extent. Red circles - TCA present. White circles - sampled and TCA absent. Red polygon - 2025 TCA population edges. The extent polygon approximates the minimum supercolony extent as the northern and southern supercolony edges cannot be located due to private property access restrictions.

2025 Findings:

The TCA supercolony at the McNeil tract is in a state of collapse. This collapse is evident in both the area infested by the supercolony and by the pitfall trap data on TCA abundance. The western edge of the TCA supercolony retreated 628 meters from its 2024 maximum extent (Fig. 1). Lack of access to private properties to the north and south prevent making an accurate assessment of extent in these directions and also prevent measures of changes in the area occupied by the supercolony.

Pitfall traps also show this collapse. We recovered TCA in only 42% of the pitfall traps set at the site with an average of only 8.6 workers per trap when TCA were present (Fig. 2). The precipitous decline from the universal prevalence and high abundance of 2024 is stark. Based on declines observed at other sites, complete

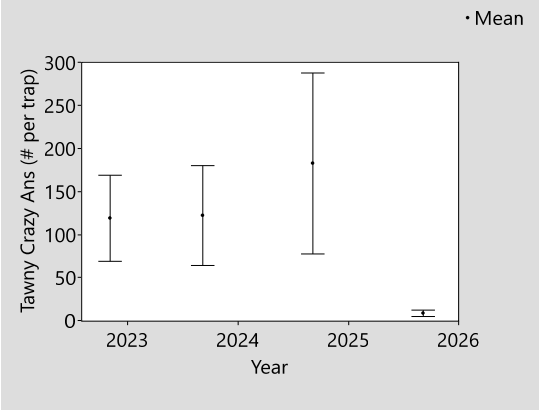


Fig. 2: TCA density McNeil Caves Tract BCP. Results of pitfall trapping. Number of ants captured in traps left open for 24-hours. Supercolony collapse occurred over the 2026 field season.

eradication of TCA from the site is likely to occur in 2026.

We anticipated the collapse of this supercolony in the fall of 2024 based on the universal prevalence and high microsporidian infection intensities seen at that time (see 2024 report). This is one of the fastest inoculation efforts we have conducted to go from inoculation, to pathogen establishment, to collapse of the local TCA supercolony.

We will continue to track TCA abundance at this site in 2026. Although this site is not formally a part of our native ant recovery study due to the lack of access to similar habitat locally, we will continue to track the response of karst invertebrates to the elimination of tawny crazy ants from this environment. (For data on *Myrmecomorba* infection in 2025 see Objective 3 - post inoculation establishment tracking of *Mn*).

Pace Bend Park

TCA History:

TCA first invaded Pace Bend Park as the leading edge of a much larger infestation spreading south from an initial introduction in the community of Briar Cliff (2011). The expanding TCA supercolony reached Pace Bend Park in the Spring of 2019. Following inoculation efforts in 2020 and 2021, we successfully established the pathogen in the park. By Fall of 2022 *Mn* was widespread in the supercolony, and by Spring of 2023 decline in TCA abundance was evident, and, by the end of 2023, the TCA supercolony in the original area of the park infested by TCA had essentially collapsed to extinction. This area is where the inoculation and monitoring stations are located. However, an offshoot of the original infestation survived and, at the end of 2023, showed no evidence of infection by *Mn*.

2025 Findings:

The Pace Bend tawny crazy ant supercolony retracted in 2025 (Fig. 3 right panel). The total area infested by the supercolony decreased from 89 acres in 2024 to 72 acres in 2025. This contrasts with the year of maximum extent, 2022, when the supercolony infested 317 acres within the park and a much larger area on private lands outside the park to the southwest (Fig. 3 left panel). This is the first year when no edge within the supercolony has exhibited substantial expansion. This retraction is likely the result of the combined influence of an increase in the prevalence of *Myrmecomorba* among TCA nests (see next section) and the drought conditions experienced in the summer and fall of 2025. We do not have data on changes in TCA abundance within the extant portion of the TCA infestation at Pace Bend. The pitfall trapping stations established in 2018, 2019, and 2021 that were originally used to quantify changes in TCA abundance. These traps are located in an area where the TCA

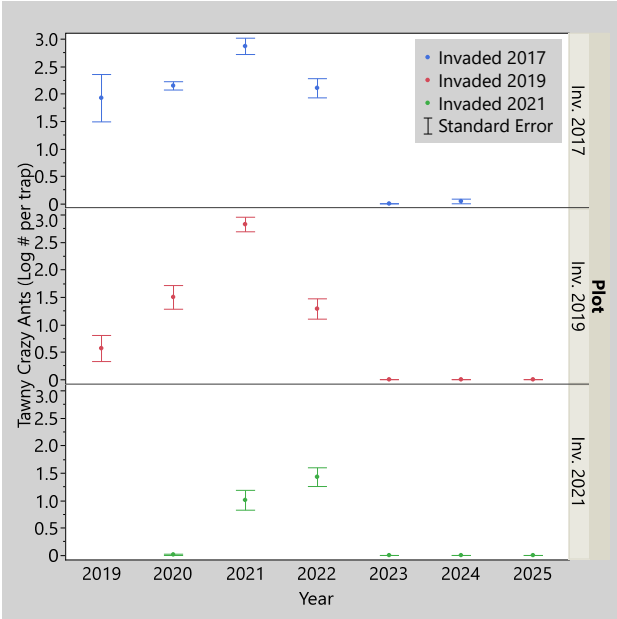


Fig. 4: TCA density Pace Bend Park. Results of pitfall trapping. Number of ants captured in traps left open for 24-hours. Data is split across the three plots of traps run at Pace Bend. Each plot is characterized by a different year when the expanding edge of the tawny crazy ant supercolony reached the plot.

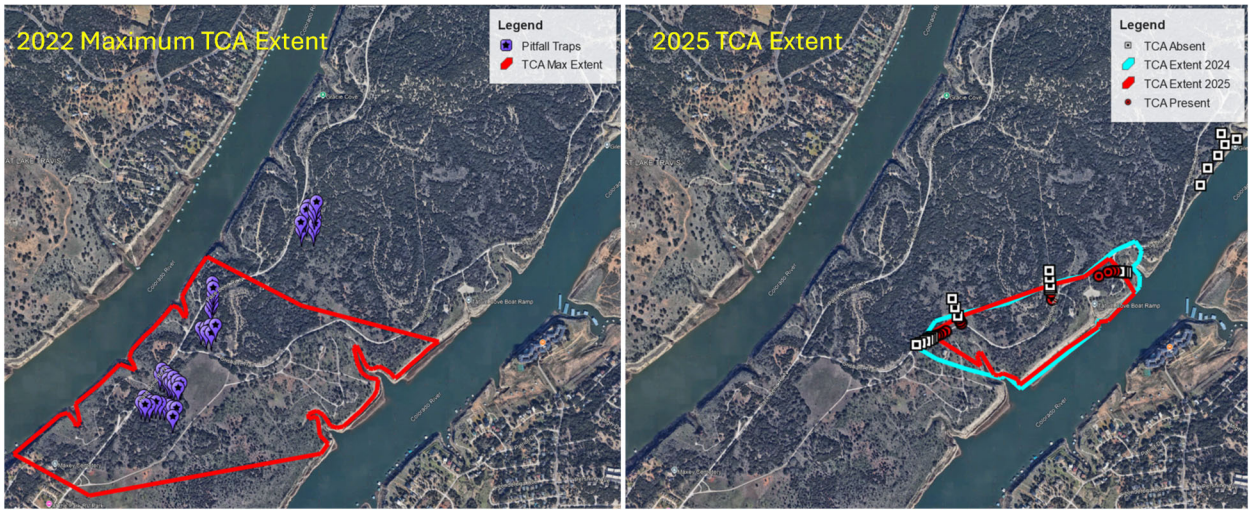


Fig. 3: Pace Bend Park TCA Extent. Red circles - TCA present. White circles - searched and TCA absent. (Left) 2022 - Year of maximum extent. Red polygon - 2022 TCA population edges. (Right) 2025 TCA Extent. Blue polygon – 2024 TCA extent. Red polygon - 2025 TCA extent.

supercolony collapsed in 2023 and do not reflect the TCA abundance in this expansion of the originally infested area. For the third year in a row, the pitfall traps in the original area of infestation intercepted no TCA (Fig. 4). All evidence indicates that the supercolony has been eliminated from this area.

Following reports from park staff of suspected TCA presence outside of the known infested area, we conducted some scouting. No TCA were found. TCA absent pins to the north east of the infestation document this effort (Fig. 3 right panel).



Fig. 5: Lime Creek Preserve – Wilcox Cave Preserve TCA supercolony extent 2025.

Lime Creek Preserve – Wilcox Cave Preserve

In the Lime Creek Preserve area, TCA were first reported in 2024 by Texas Cave Management Association staff as this supercolony invaded the adjoining Wilcox Cave Preserve. In collaboration with City of Austin Water staff and Texas Cave Management staff, we completed an initial extent mapping and infection testing of this supercolony in August and September of 2024.

As of fall 2024, this supercolony was impacting Whitewater, Wilcoxon 1, and Broken Arrow Caves. Its continued spread threatens the entire Buttercup Cave Complex which includes 63 named karst features.

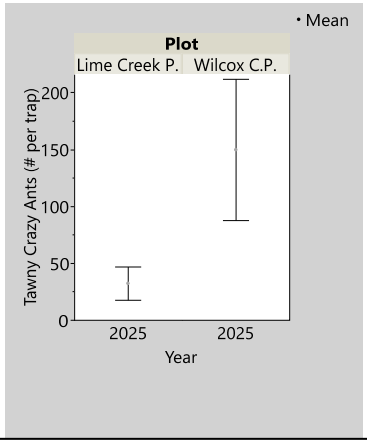


Fig. 6: Tawny crazy ant abundance Lime Creek Preserve and Wilcox Cave Preserve. Both sites are impacted by single TCA supercolony. Results of pitfall trapping. Number of ants captured in traps left open for 24-hours.

2025 Findings:

The Lime Creek TCA supercolony expanded in 2025. The expansion was most pronounced to the west into the Lime Creek Preserve tract of the BCP and to the southeast. The expansion of the southeastern boundary has the limit of the supercolony in this direction now including TWAS Cave and Buttercup Dome Cave. Although we do not have firsthand reports from inside these caves, these features are now likely impacted. This expansion

occurred despite the drought conditions of the summer and fall of 2025. Expansion in other directions, mostly in areas of residential housing, was more limited or did not occur. Areas of residential development are impossible to survey exhaustively due to limited access, making supercolony limits in these areas less clear. In addition, the general lack of appropriate nest sites for TCA (rotten logs, large rocks, ect.) in residential settings and the ongoing pesticide applications in these areas can slow supercolony spread (Fig 5).

We ran pitfall traps for the first time in 2025 to assess the abundance of TCA in the two sites that are the focus of our inoculation efforts: Lime Creek Preserve BCP, and Wilcox Cave Preserve. Currently, TCA are denser at the Wilcox Cave Preserve than in the Lime Creek Tract (Fig. 6). These sites were the focus of our inoculation efforts in 2025 (see next section).

Objective 3: Biological control of TCA using the pathogen: *M. nylanderiae* - (a) Perform inoculative releases of the microsporidian pathogen *Myrmecomorba nylanderiae*. (b) Quantify pathogen establishment post-inoculation and track the spread of the pathogen once established.

At sites with uninfected TCA supercolonies, we have been introducing the pathogen *M. nylanderiae.* This pathogen is now a proven and highly effective biological control agent for TCA. Its establishment at high rates of nest prevalence leads to complete collapse of the TCA supercolony (9). In 2025, inoculations were conducted in the Lime Creek Preserve and the nearby Wilcox Cave Preserve. Establishment sampling was carried out at these sites, while spread and intensity sampling was carried out at Pace Bend Park and the McNeil Cave Tract – BCP, sites where *Mn* inoculations have been conducted in previous years and the TCA supercolonies remain extant.

<u>*Myrmecomorba nylanderiae* Surveys Approach:</u>

Mn establishment, *Mn* spread and infection intensity sampling were conducted at: Pace Bend Park (Mn spread and infection intensity), the McNeil Caves Tract of the BCP (Mn spread and infection intensity), and at the Lime Creek – Wilcox Cave Preserve sites (Mn Establishment). *Mn* establishment sampling involved intensive sampling of areas immediately surrounding current year inoculation stations. *Mn* spread and infection intensity samping involved sampling a grid predetermined stations spread across the accessible areas of the local supercolony combined with opportunistic sampling and testing of ants in the course of carrying out other sampling activities such as TCA supercolony edge mapping.

Quantification of *Mn* infection was conducted by hand sampling ants from disturbed nests. Samples were tested for *Mn* DNA using diagnostic PCR. Infection intensities of PCR

positive samples were quantified by homogenizing batches of 20 ants from a nest and counting the number of Type II DK *Mn* spores present in a fixed, stained, standardized aliquot of tissue homogenate.

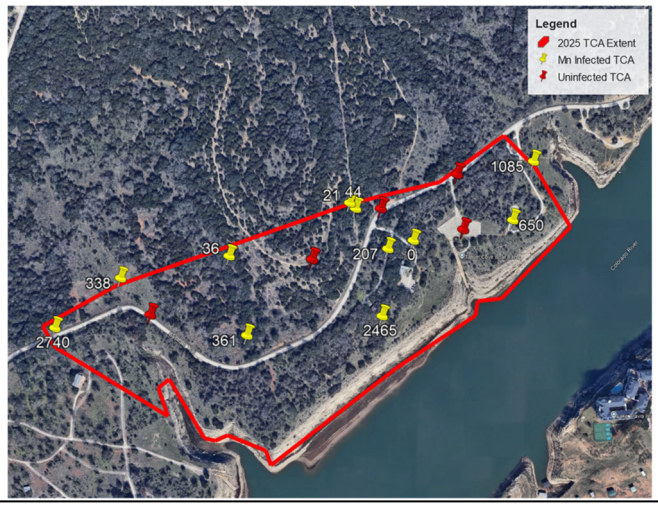


Fig. 7: *M. nylanderiae* infection extent – Pace Bend Park 2025. Yellow pins - Mn presence. Number labels show infection intensity as spore count values. Red circles - uninfected TCA. Red polygon - approximate limits of TCA super colony. (See Fig. 3 for TCA extent at time of inoculation.)

Pace Bend Park

Following inoculation efforts in 2020 and 2021, we successfully established the pathogen in the park. By Fall of 2022 *Mn* was widespread in the supercolony. By the end of 2023, the TCA supercolony in the original area of the park infested by TCA had essentially collapsed to extinction. This area is where the inoculation and monitoring stations are located. However, an offshoot of the original infestation survived and, at the end of 2023, showed no evidence of infection by *Mn*. In 2024, *Mn* infections emerged in the area still invaded by TCA .

2025 Findings:

In 2025, *Mn* infection spread throughout the area occupied by TCA (Fig. 7). Infection intensities increased relative to those seen in the fall of 2024 (mean = 77 spores per infected nest test) with an average of 900 spores per infected nest test in 2025. Infection prevalence among nests in fall 2025 did not reach saturation with 61% of sampled nests testing positive for *Mn*.

We anticipate the continued decline of this supercolony in 2026. There no longer appears to be a section of the supercolony free from *Mn* infection and thus able to expand in its absence. It remains unclear how it will take for this decline to play out.

McNeil Caves Tract of the BCP

In 2022 and 2023 we conducted inoculations at the McNeil Caves BCP site. Establishment of the *Mn* pathogen was demonstrated by the Fall of 2023. In 2024, *Mn* spread from localized infections near multiple inoculation sites to widespread, almost universal infections across the entire extent of the supercolony within the preserve and the accessible

areas on the far side of the McNeil Drive. In this year, among infected nests, over the course of the warm season, infection intensities per nest increased 5-fold presaging the collapse of the TCA supercolony observed in 2025.

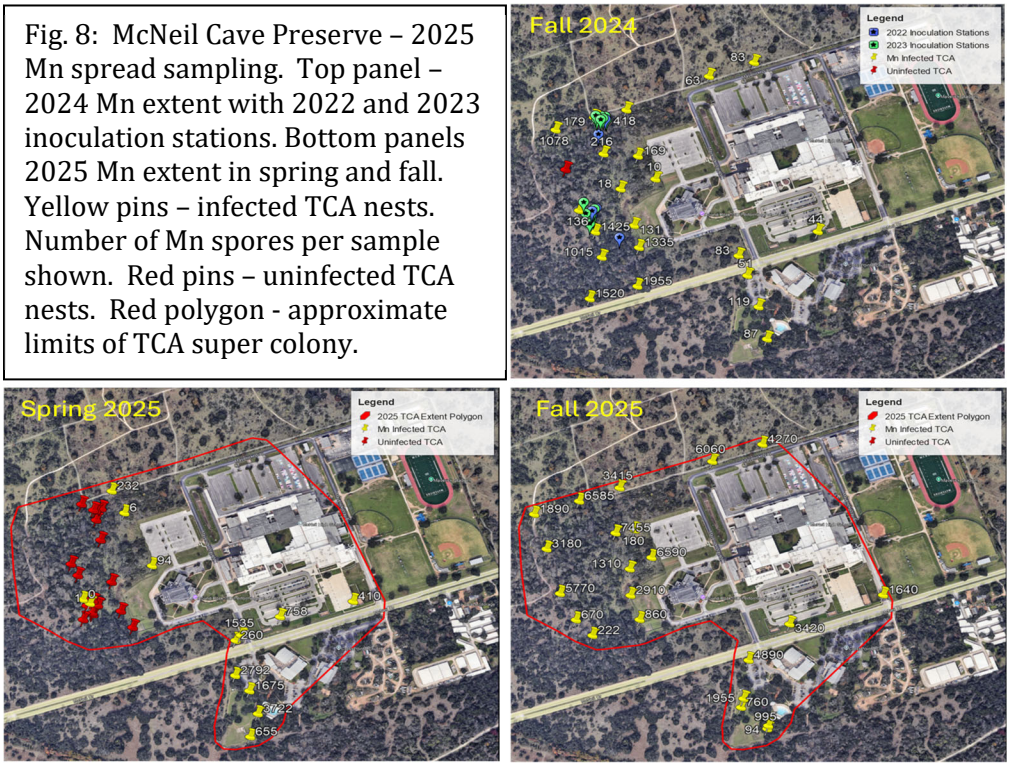


Fig. 8: McNeil Cave Preserve – 2025 Mn spread sampling. Top panel – 2024 Mn extent with 2022 and 2023 inoculation stations. Bottom panels 2025 Mn extent in spring and fall. Yellow pins – infected TCA nests. Number of Mn spores per sample shown. Red pins – uninfected TCA nests. Red polygon - approximate limits of TCA super colony.

2025 Findings:

Despite the dramatic decline in TCA abundance in 2025, TCA nests were still sufficiently common to sample and test for *Mn* infection. In 2025, *Mn* infection prevalence declined over the winter from near universal nest infection in fall 2024 to 42% nest infection in spring of 2025. This decline was steepest near to the original inoculation stations where infection intensities were highest in the fall of 2024 (Fig. 8 bottom left). By the fall of 2025, infection prevalences had resurged to near universal prevalence and infection intensities had increased to very high levels again (Fig. 8).

From observations at other sites, this appears to be a cycle typical of infected TCA local-supercolonies. Infected workers and highly infected nests in the fall tend to die out over the winter, leading to a partial clearing of the infection from the supercolony by the spring. However, the infection persists at lower intensities, sometimes below the threshold for our sampling methods to detect. As the warm season progresses, infection resurges and over the following winter either the cycle repeats or the supercolony dies out entirely.

Lime Creek Preserve – Wilcox Cave Preserve

<u>Inoculative releases of *Myrmecomorba nylanderiae* approach:</u>

We carried out inoculations in April of 2025 at two sites within the TCA supercolony that spans Lime Creek Preserve - BCP and the Wilcox Cave Preserve. Six TCA nests with high intensity *Mn* infections (mean infection intensity = 436 spores per nest sample) were collected from the Boggy Creek TCA supercolony. Ants were separated from nest materials with heat while minimizing exposure to laboratory derived odors and unnatural food sources. Infected ants were combined and then split into 12 colony fragments each containing approximately 1750 workers, 1.3 cc of mixed developmental stage brood, and 5 queens. Infected ants were taken to the field and inoculations were conducted following two protocols.

At the higher TCA density Wilcox Cave Preserve, TCA nests were easily located so infected ants were introduced directly into disturbed nests. TCA nests were located by turning rocks and logs. Once found the structure of the nest was disrupted using a shovel or hatchet. Then ants were dumped from the tube into the disrupted nest. Laboratory work indicates that disease transmission occurs more easily when colony fragments must relocate or reorganize in the presence of immigrating infected workers (10).

At the lower TCA density Lime Creek Preserve, we were not always able to find nests into which to introduce the ants. Anticipating this challenge, all ants were introduced in inoculation nest boxes. Nest boxes were 15cm x 15 cm plastic tubs with an exit hole. Inside were nest chambers for the ants, a 50 ml centrifuge tube with water stoppered with cotton for humidity, and 15 ml tube with a 20% sucrose solution stoppered with cotton for food. Inoculation nest boxes were either placed next to a disturbed TCA nest or TCA foraging trail.

2025 Findings:

Inoculations were conducted successfully in April of 2025.

Establishment testing in August did not return reliable signs of infection. Establishment testing 6 weeks later in September also recovered no *Mn* positive samples. However, testing in December recovered 14 infected samples that spanned both the Lime Creek Preserve inoculation stations and the Wilcox Cave Preserve inoculation stations (Fig 9). Molecularly positive samples proved highly infected with an average of 402 spores pers sample. This large number of highly infected samples gives us confidence that we have successfully established *M. nylanderiae* infection within this TCA supercolony.

Objective 4: Quantify ant assemblage and monitor for TCA at Collapsed sites where isolated TCA have been observed within the past year – post TCA collapse sites.

At sites where TCA supercolonies have collapsed in the recent past, there is a continuing need to assess whether a resurgence of TCA is occurring.

Approach:

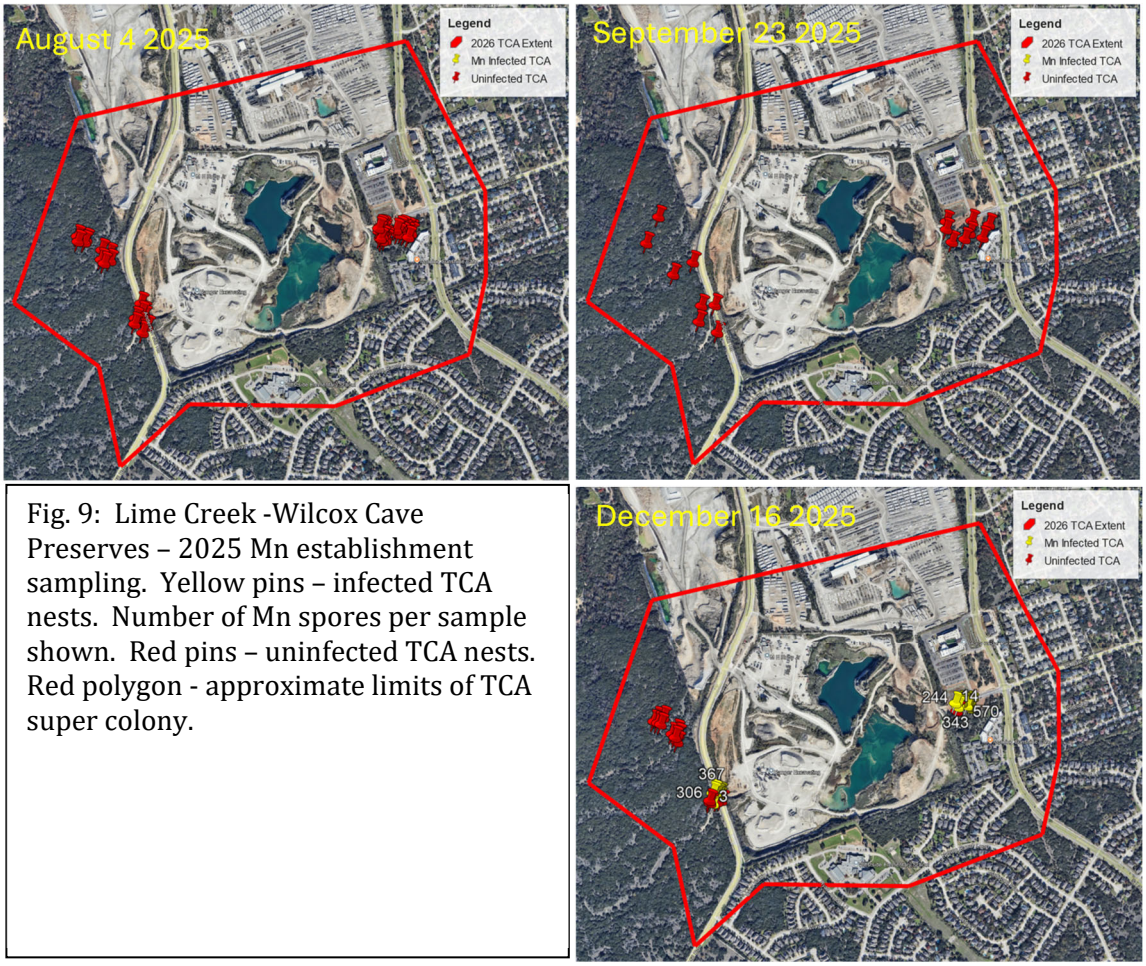


Fig. 9: Lime Creek -Wilcox Cave Preserves – 2025 Mn establishment sampling. Yellow pins – infected TCA nests. Number of Mn spores per sample shown. Red pins – uninfected TCA nests. Red polygon - approximate limits of TCA super colony.

Our protocol is to visit all locations where TCA were found in the preceding year at 3-month intervals to assess whether TCA resurged. If no TCA were found at a location for 1 year, it was dropped from the survey list. Per established protocol, previously executed quarterly confirmations of TCA absence at Collins Tract of the BCP – Old Water Treatment Plant 4, Emma Long Park, and Walnut Creek Park were discontinued in 2025. By Spring of 2025, at all these sites it had been more than a year since any TCA were found.

2025 Findings:

At all other post collapse sites (Table 1), it has also been longer than a year since any TCA have been seen at the site. We are continuing to sample some of these sites (Emma Long, Collins Tract of the BCP – Old Water Treatment Plant, Whirlpool Cave Tract) to monitor the post collapse recovery of the native ant community (see Native Ant Recovery). This effort will provide an additional longer-term assessment of TCA status.

Objective 5: Evaluate recovery of native ant assemblage at TCA collapse sites.

As TCA declines 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 happens in the context of an environment that has been saturated with spores of *Mn* left behind by the dying TCA supercolony. To date, all data we have gathered indicates that this pathogen is specific to TCA. Quantifying the recovery of native ant assemblage is an important act of due diligence as it ensures that we know that our laboratory-based prediction that the introduction of *Mn* will not cause unintended consequences is borne out in nature. Quantification of the native ant assemblage as ants are generally regarded as a taxonomically tractable group that provides insight into the health of the terrestrial arthropod community more generally (11-13).

<u>Approach:</u>

Pitfall traps were used to assess native ant community recovery. Pitfall trapping followed the methods outlined in the above section on tracking TCA densities.

2025 Findings:

Our effort to follow the recovery of sites previously invaded by TCA continues to reveal interesting patterns. In 2025, we sampled 8 sites. We now have enough information about later stages of the recovery process to see trends. As discussed in previous reports, early-stage recovery is dramatic and rapid. The decline of TCA at a site is arbitrarily set as the point where TCA are absent from 50% of the pitfall traps. Ecosystem transition appears to start before that

point. By 1 year post, imported fire ant assemblage dominance has reached the level of uninvaded sites (Fig 10 left panel). By 1 year post native ant abundance is equivalent to controls (Fig 10 middle panel). By 3 years following this point, native ant species density is equivalent to controls (Fig 10 right panel).

Interestingly, the assemblages appear to be diverging again later in the recovery process. Imported fire ant dominance appears to be decreasing below that of the controls in years 6 and 7 (Fig 10 left). While native ant abundance and native ant species density are exceeding the levels of paired, never-invaded control sites in years 6 and 7 (Fig 10 middle and right). These late-stage recovery patterns are unexpected, and ultimately, we need more sites to reach this stage of recovery to properly evaluate their validity. However, potentially these patterns may be driven by changes in the imported fire ant populations. As we have reported previously, imported fire ant population in recovery sites are more dominated by monogyne colonies as compared to uninvaded controls. Monogyne fire ant populations are considered less environmentally damaging. Alternatively, changes in the dynamics of interspecific competition among the native ants could be driving these late recovery changes. Future, more in depth analyses with data from more sites with late-stage results, will allow for an assessment of these processes.

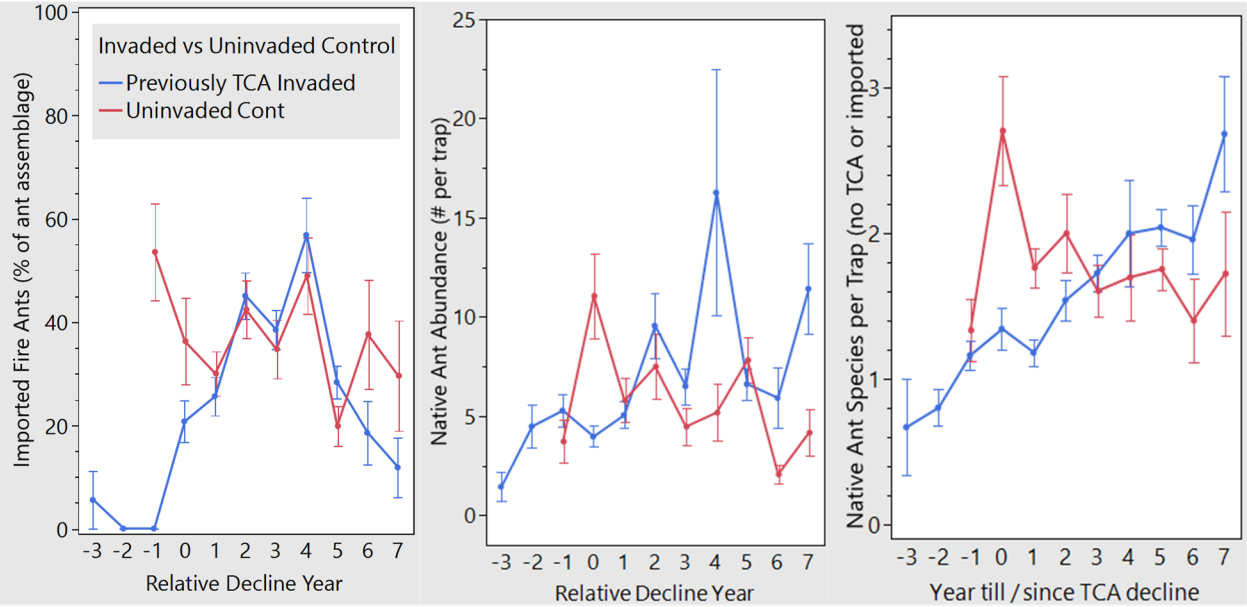


Fig. 10 – Results of native ant assemblage recovery study to date: Left: Imported fire ant ecological dominance, Middle: Native ant abundance, Right: Native ant species density. All sites are areas previously invaded by TCA populations that collapsed and disappeared following *M. nylanderiae* infection. Response variables plotted versus the number of years since or till the collapse of the TCA population. The year of collapse (year 0) determined as the first year that TCA prevalence in pitfall traps dropped below 50%. Control sites are nearby never invaded sites with similar vegetation characteristics.

A more rigorous analysis that considers canopy cover at each sampling station is needed to resolve whether this variation is a result of differences in fire ant population growth or site-specific habitat factors. Canopy cover measures were made in 2025 and will be incorporated into future analyses of these data. Table 2 reports all ants captured in these traps in 2025.

Table 2: Ant species found in pitfall traps at all sites sampled in 2025.

Site	Site Category	*Nylanderia fulva* (TCA)	*Solenopsis invicta*	*Brachymyrmex patagonicus*	*Crematogaster laeviscula*	*Crematogaster minutissima*	*Forelius damiani*	*Forelius pruinosus*	*Hypoponera opacior*	*Hypoponera punctithsima*	*Monomorium minimum*	*Myrmecina americana*	*Nekamyrmex pilosus*	*Nylanderia terricola*	*Pheidole constipata*	*Pheidole metallescens*	*Pheidole dentata*	*Pheidole floridana*	*Pheidole hyatti*	*Pheidole pelor*	*Pheidole tetra*	*Solenopsis cf. texana*	*Temnothorax andersoni*	Species Richness (Total Species)
Lime Creek/WCP	Active TCA	1488	2	4	0	0	0	7	0	0	17	0	0	0	15	0	0	1	0	0	0	3	0	8
McNeil	Active TCA	69	33	0	2	1	21	0	0	0	169	0	0	0	6	0	5	0	0	0	0	23	0	9
Emma Long	Native Ant Recovery	0	127	4	0	0	1	0	0	0	4	0	1	0	17	3	0	0	1	0	0	18	0	9
New Life	Native Ant Recovery	0	29	0	67	0	0	0	0	1	14	0	0	4	8	0	43	0	1	0	31	30	1	11
Pace Bend	Native Ant Recovery	0	61	0	13	0	1	0	1	0	27	2	0	0	9	0	137	0	0	13	7	107	0	11

Objective 6: Evaluate TCA impacts on karst invertebrates.

Two areas of the BCP containing caves with federally protected endangered species are being impacted by TCA: the McNeil Tract, with two impacted caves (No Rent and Weldon Caves) and the Lime Creek Tract with one impacted cave (Broken Arrow). Based upon spread data, the TCA supercolony at Lime Creek likely reached Broken Arrow Cave in the fall / winter of 2024. Seasonal surveys with an added TCA quantification component began nearly simultaneously in January of 2025. The invasion of these caves creates the opportunity to enhance our understanding of the impacts of TCA on karst invertebrates. In 2015 to 2017, University of Texas, Travis County, and City of Austin staff collaborated on a study of the impacts of TCA on karst invertebrates and mitigation measures (6). However, at that time, there were insufficient caves invaded by this ant to be able to draw general conclusions from the work. Current sampling employs the methods of this previous study to collect additional

data from No Rent, Weldon, and Broken Arrow Caves to increase our understanding of how TCA impact karst invertebrates.

Approach:

Travis County and City of Austin staff quantified TCA occurrence and cave use by setting bait stations outside cave entrances and at set distances inside the caves. For the larger Weldon cave, bait stations inside the cave were placed every 10 m and extended 40 m into the cave. Both No Rent and Broken Arrow are smaller caves (10 – 15 m accessible depth), bait stations were deployed every 5 m to the back of the cave. Bait stations consisted of two pairs of 15 ml centrifuge tubes one containing protein (a hot dog slice) and one containing sugar (a cotton ball soaked in 30% sucrose solution). Bait tube pairs were placed 2 m apart from each other. Bait stations were collected after one hour and sealed after removing and releasing all non-ant species. UT Austin staff counted, identified, and recorded all ants collected in each tube. TCA abundances were quantified as the average of the total ants captured in each pair of bait tubes for a given distance from the entrance.

2025 Findings:

Reflecting the pitfall trap data for McNeil Tract presented earlier, in 2025 TCA have been eliminated from No Rent and Weldon Caves. In contrast, in Broken Arrow Cave, invaded at the end of 2024, TCA penetrated all accessible depths of the cave at high to moderate abundance TCA (Fig. 11).

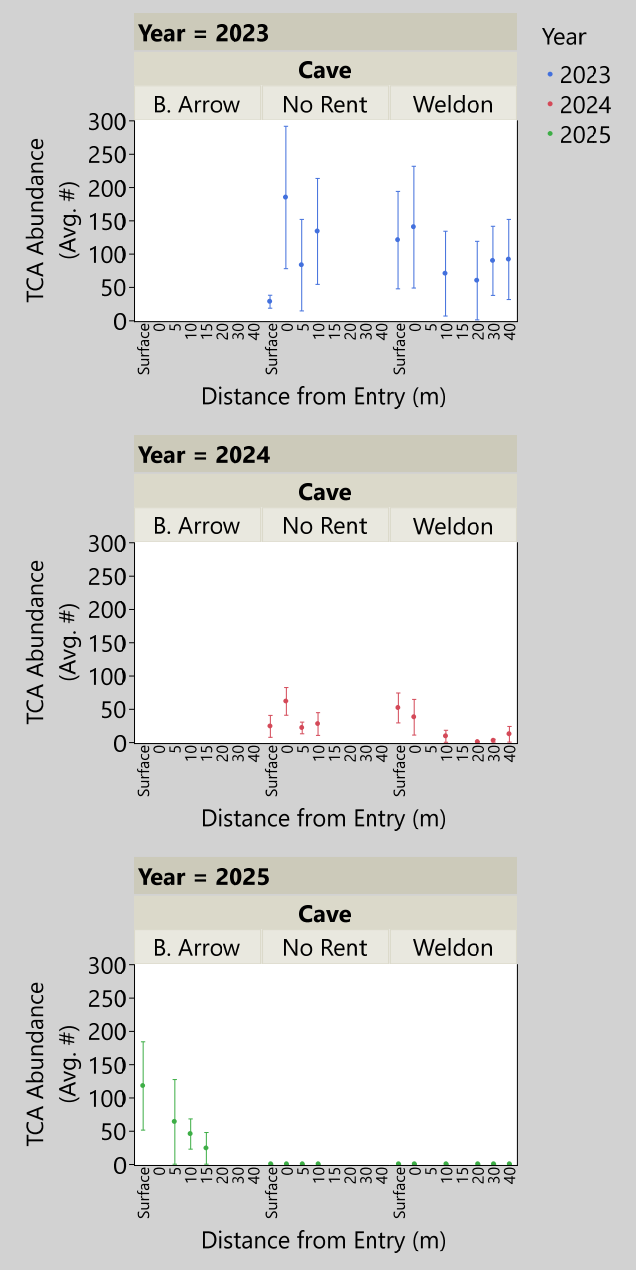


Fig. 11: TCA cave occupancy by distance from cave entrance. Surface stations are immediately outside cave entrance. Figure presents average number of ants captured in bait tube stations across all 4 seasonal samples. Bars are standard errors. Data from the three caves being actively monitored for TCA presented: Broken Arrow, No Rent, and Weldon Caves.

There is a distinct seasonal pattern to cave occupancy by TCA. The ants are typically present inside of caves at abundances in excess of those found at the surface in late summer through late winter and are largely absent from cave interiors in spring through mid-summer. This activity reflects seasonal patterns of dry and hot or cold weather when surface conditions are less conducive to nesting than cave interiors (Fig. 12).

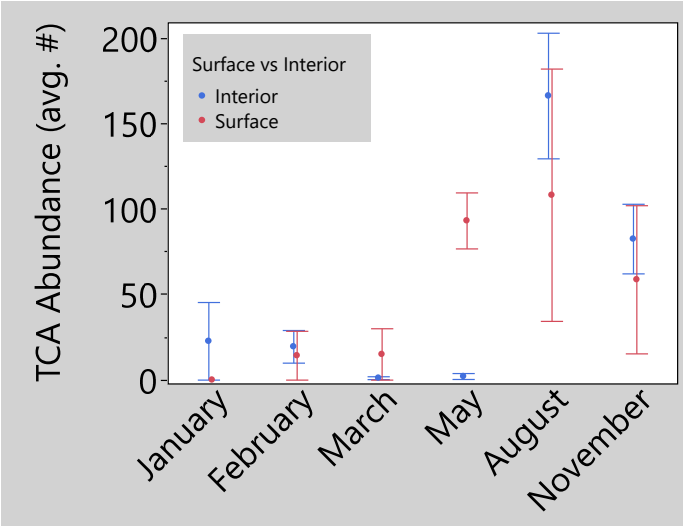


Fig. 12: Season patterns of TCA cave occupancy. Figure presents average number of ants captured in bait tube stations across all 3 caves: Broken Arrow, No Rent, and Weldon Caves. Only data from years where TCA were present on surface were included in figure. Bars are standard errors.

Within these caves, and others, Travis County and City of Austin Staff are collecting a long-term data set on karst arthropod richness and abundance. Once 2 years of post-TCA recovery have occurred at some survey caves, we will initiate a larger scale data analysis geared towards a publication on TCA impacts on karst invertebrate fauna. We are in discussion with County and City on timing. These data will be combined with similar data collected in 2015-2017 to produce a more general examination of evidence of impact on karst invertebrates.

TCA Infestations Threatening High Conservation Value Habitat

Sandy Creek Park – Wheless – Trails End

Travis County Staff reported an infestation of TCA at Sandy Creek Park to us in September of 2024. In 2024, a preliminary mapping of the supercolony was completed. In addition, in 2024 a limited area of *Mn* infection (2 nests) was found. Given that information, we recommended mapping and tracking this population in 2025 but not conducting an inoculation.

2025 Findings:

The preliminary mapping done in late fall of 2024 was flawed. The apparent edge to the north of Sandy Creek Park we found was inaccurate. This TCA supercolony extends considerably farther north and is impacting three separate BCP tracts: Wheless, Trails End, and Lime Creek Tracts. In total, it currently covers 337 acres (Fig. 13).

In 2025, we confirmed the observation that *Mn* had naturally colonized this local supercolony but is restricted in its extent. We found two areas with evidence of *Mn* infection one in the southeast corner and one at the confluence of Lime Creek and Lake Travis. For reasons we do not fully understand, naturally acquired infections take longer to lead to supercolony collapse than do established inoculative releases (see following section on Onion Creek Park Area). Given this, and given the size of this local-supercolony, the limited nature of *Mn* infection within it, and the high conservation value of the habitat it is impacting, we plan to recommend a supplemental inoculation with *Mn* in the spring of 2026.

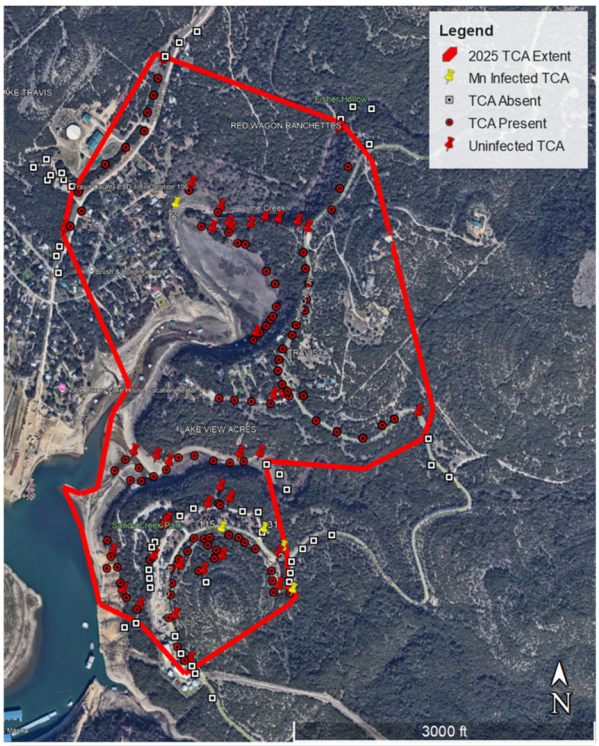


Fig. 13: Sandy Creek Park – Wheless – Trails End TCA infestation.

TCA Populations Near Areas of Conservation Interest

Onion Creek Park Area

Since 2021, we have been tracking the expansion of a TCA supercolony that is along Boggy Creek near its confluence with Onion Creek and Onion Creek Park. In December of 2023 we found that *Mn* had colonized this TCA supercolony naturally. In 2024 the area of infected nests expanded to include most of the area accessible to sample, and infection intensities had increased.

2025 Findings:

Testing from spring – summer of 2025 largely reflected findings at the end of 2024 with *Mn* infection widespread through the area. However, fall sampling results were surprising. The infection appears to have greatly decreased in prevalence over the summer with nest cohort infection intensities also decreasing (Fig. 14). In areas sampled in both seasons, prevalence decreased from universal (100%) in the spring to 38% infection in the fall. Similarly, infection intensity decreased from an average of 377 spores per infected nest to 23. These changes are particularly surprising as site level *Mn* infection prevalences and intensities typically peak in the fall.

These results suggest a potential increase in resistance within this supercolony or some other unusual aspect of internest disease transmission dynamics. We have observed similar, albeit not as extreme behavior, in other naturally infected TCA local-supercolonies. Specifically, it frequently takes longer for the pathogen to spread throughout local-supercolonies in naturally acquired infections as compared to local-supercolonies where we have introduced the pathogen. For example, at a similarly sized site, it took approximately 1.75 years for the TCA supercolony we inoculated at the McNeil Tract to go from *Mn* establishment to TCA supercolony collapse. This contrasts with Boggy Creek where 2 years post discovery of pathogen establishment, the TCA colony has not decreased in extent and infection prevalences are low. In a more comprehensive analysis, local-supercolonies distributed across Texas that naturally acquired infections required 4 to 7 years to decline to extinction while inoculations took 2 (9). This contrast highlights the potential need for inoculative releases even in cases where localized infections may already occur depending on the conservation importance of the impacted site.

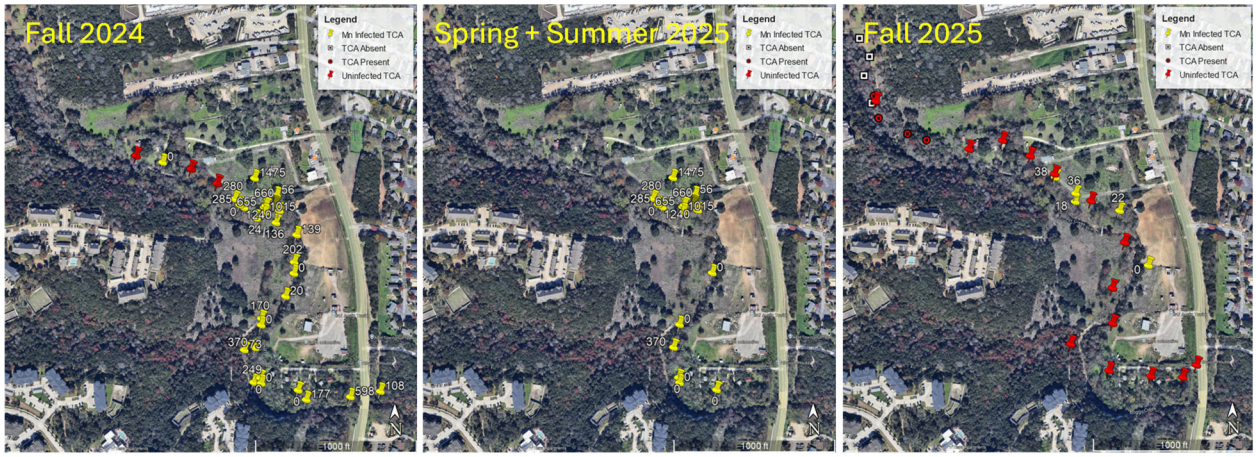


Fig. 14 – 2025 TCA extent and prevalence of Mn infection in the Boggy Creek watershed near Onion Creek Park. Left: 2024 sampling; Center: Spring -Summer 2025 samples; Right: Fall 2025 samples.

Lady Bird Johnson Lake Park

A new infestation of TCA was reported this year downtown along Town Lake Metropolitan Park (Fig. 15). This is not an area of high conservation value. However, it is an area of high public use and has the potential to be the source of new infestations locally. It is also close to endangered salamander habitats at Barton Springs. Although TCA are unlikely to directly impact salamanders, an infestation could indirectly harm underground aquatic ecosystems through pesticide runoff.



Fig. 15 – Preliminary extent mapping of TCA infestation along Town Lake Metropolitan Park – summer 2025.

Evaluating SINV-3 as a tool for control of imported fire ants in sensitive habitats

In 2024, we began a research program to evaluate whether the fire ant virus SINV-3 can provide sustained, localized control of imported fire ants around sensitive environmental features such as endangered species caves and endangered species nesting grounds. In its native range in South America, 17 viruses have been found infecting red imported fire ants (RIFA) *(Solenopsis invicta*) (14), far more than are known for any other ant. Four of these viruses are found commonly in introduced populations of RIFA, including those in Texas (15). One of these, *Solenopsis invicta* virus 3 (SINV-3) is highly pathogenic to RIFA colonies in both the field and laboratory settings. Infection almost always leads to death in a laboratory setting but field colonies can recover (16). This virus is also highly specific to *S. invicta*, infecting no other insects, including fire ants in the North American species group (16). This combination of pathogenicity and specificity make this virus a very attractive biological control agent.

SINV-3 is already widespread in North American imported fire ant populations including in Texas, so there is no expectation that its further release will lead to landscape scale changes in RIFA abundance. However, it remains to be tested whether intensive inoculations on a local scale can suppress RIFA abundances for a protracted period. Only a single, small field study of the ability of this virus to suppress a RIFA population has been conducted. They observed limited suppression but a surprisingly long-term persistence (22 months) of elevated SINV-3 prevalence in the treatment area after inoculation (17).

In 2024, we developed the ability to perform a variety of quantitative assays of viral load. We also made progress in transmitting the virus via viral particle laced food created in the lab. However, we found that very highly infected colonies were needed for this lab culture to produce highly infected imported fire ant colonies. We had limited success locating highly infected colonies in nature.

Approach:

Field Surveys for SINV-3

Using sterile techniques, we collect 100 fire ants from all mounds within a local area and preserve them in 95% ethanol. We record the GPS locations of each mound and mark them with biodegradable flags made from bamboo skewers and paper tape. In the laboratory, we extract RNA from all samples, convert it to cDNA, and perform diagnostic PCR on the cDNA to test for infection by SINV-1, SINV-2, and SINV-3.

Laboratory Ramp-Up SINV-3 Viral Load

Ant colonies utilize the spatial structure of their nests to isolate infected individuals from those that are uninfected. This behavior can prevent low-level infections from becoming acute and may allow colonies to eliminate infections altogether. Our goal is to determine whether we can disrupt this process to create highly infected colonies suitable for bait production.

We will collect colonies that test moderately positive for SINV-3 (containing hundreds to thousands of viral particles per ant) by digging up the nest mound and the top 10 cm of soil. The ants will be separated from the soil using a drip flotation method. After processing, the ants will be held in trays for nine weeks. They will be tested weekly using qPCR to monitor their viral loads.

Laboratory Transmission: producing a SINV-3 culture

No fire ant colonies heavily infected with SINV-3 (100,000+ viral particles per ant) were found in field surveys in 2025. The infection intensities of colonies brought into the laboratory never increased to this minimum threshold to use as a bait for a transmission assay test. As a result of this lack of suitable material, we were unable to perform a transmission test in 2025.

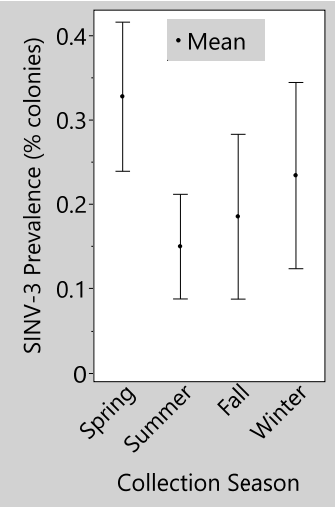


Fig. 16 – Seasonal cycle of SINV-3 infection in central Texas.

2025 Findings:

Consistent with suggestions in the literature, SINV-3 infection is most common in central Texas in the winter and

early spring months (Fig. 16). SINV-3 prevalence increases during the cooler weather in the fall through spring. When temperatures climb in the summer, SINV-3 prevalences decline precipitously. This could be either due to imported fire ant colonies having more success at clearing the virus during hot temperatures (eg. behavioral fever) or the virus being more pathogenic at higher temperatures, thereby killing off infected colonies.

In 2025, we concentrated our efforts on sampling imported fire ants during the cool season (January – March). We collected ants from a total of 200 imported fire ant colonies across 5 local sites (Table 3). Of these 200 colonies, only 3 tested positive for SINV-3. We had thought that shifting the collecting season would allow us to locate sufficient highly infected imported fire ant colonies to make culturing the virus viable. Unfortunately, it did not.

Our preliminary conclusion from 2024 that the SINV-3 prevalence in central Texas has declined since our lab last worked on this system, appears to be correct (Fig. 17 left panel). In both warm and cold seasons, SINV-3 prevalences have dropped substantially since the 2016 to 2021 era. This decline has been most precipitous in the prevalence of imported fire ant colonies that exhibit high intensity SINV-3 infections (Fig. 17 right panel).

Table 3: SINV-3 site surveys. As qPCR was only performed on highly infected colonies and in on samples from our attempts to culture the virus in the lab, infection intensities are derived from diagnostic PCR band strengths. qPCR results indicate that gel band strength is qualitatively reflective of colony infection intensity (viral particle load) in this reaction.

Year	Site	Negative	Weak +	Moderate +	Strong +	Grand Total
2024	**2024 Total**	**223**	**10**	**10**	**1**	**244**
	Circle C	7	1	0	0	8
	Elgin/Webberville	42	9	7	0	58
	Indian Grass	49	0	2	1	52
	Karst Preserve	19	0	0	0	19
	Stengl Lost Pines	19	0	0	0	19
	Wildflower	47	0	0	0	47
	Wildflower Center	40	0	1	0	41
2025	**2025 Total**	**197**	**0**	**2**	**1**	**200**
	Barton Creek	59	0	0	0	59
	Bauerle Park	74	0	0	0	74
	Elgin/Webberville	6	0	0	0	6
	Indian Grass	41	0	0	0	41
	Stengl	17	0	2	1	20
Grand Total		**582**	**16**	**24**	**39**	**664**

All three SINV-3 positive colonies found in 2025 were collected from the field, brought into the laboratory to see if we could ramp up the levels of viral infection under lab conditions. The goal of this effort was to produce *S. invicta* workers high viral loads and use this material to transmit SINV-3 to other colonies.

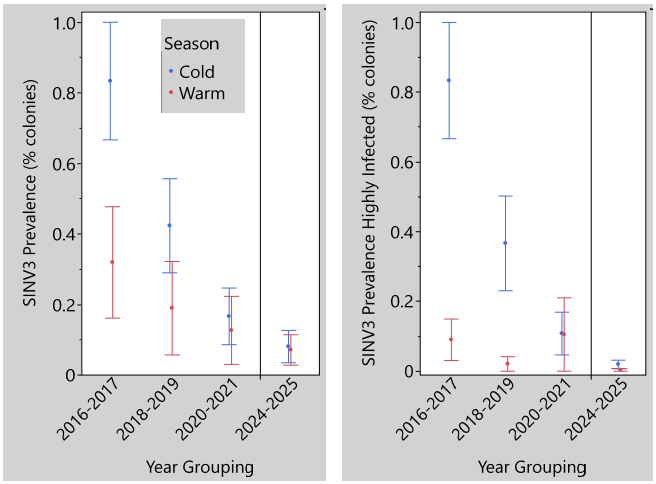


Fig. 17 – Prevalence of SINV-3 viral infection in imported fire ant colonies from sites near Austin across time. Historical lab data displayed with data collected under this contract (right side of line). Left panel: Prevalence of all SINV-3 infection. Right panel: Prevalence of the highly infected colonies needed to seed lab cultures.

In contrast with 2024, none of the infected colonies brought into the laboratory and held under conditions designed to maximize viral transmission increased in viral load (Fig 18 lower panels). Due to a lack of infected colonies in the field, we were forced to collect all positive colonies irrespective of their viral load. However, initial viral loads were only slightly lower for the 2025 colonies. We are not sure what drove this difference in behavior. One possibility is that in 2004 colonies were unintentionally held at room temperature (22˚ C) rather than the planned 27˚ C . These cooler temperatures may have favored viral transmission and primed the spike in viral load we observed in 2024.

It may be possible to culture highly infected ants in the laboratory from ants, like these, with much lower viral loads. However, doing this reliably will require developmental effort. Evaluating the role of temperature in favoring viral ramp up would potentially be a productive avenue.

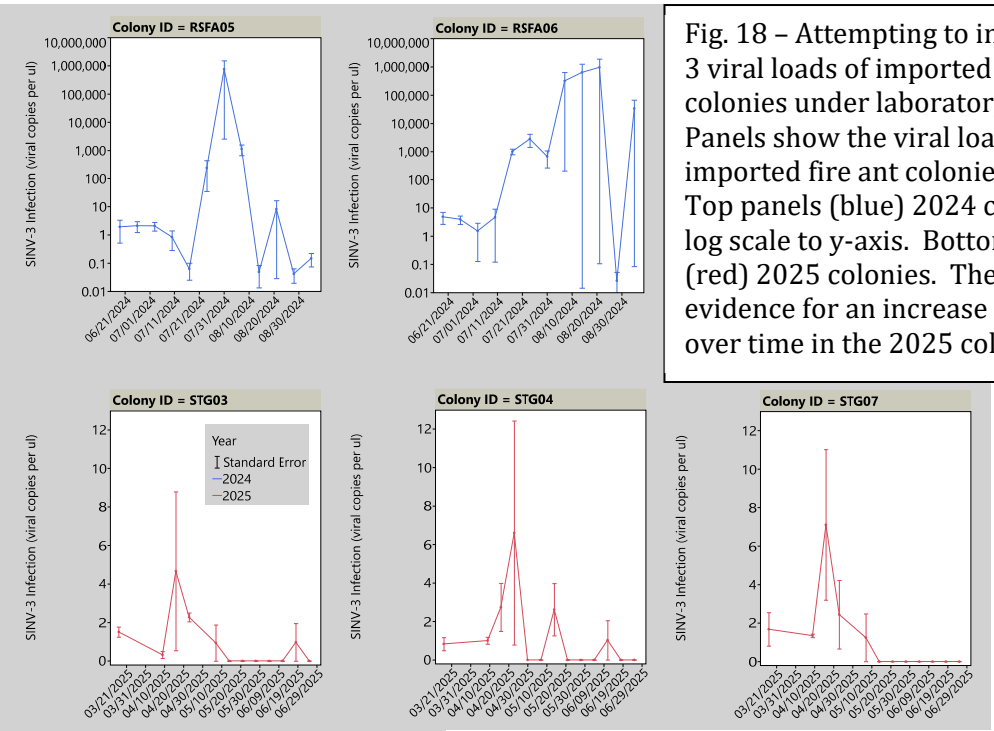


Fig. 18 – Attempting to increase SINV-3 viral loads of imported fire ant colonies under laboratory conditions. Panels show the viral load of infected imported fire ant colonies over time. Top panels (blue) 2024 colonies. Note log scale to y-axis. Bottom panels (red) 2025 colonies. There was no evidence for an increase in viral load over time in the 2025 colonies.

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