PNAS RESEARCH ARTICLE | APPLIED BIOLOGICAL SCIENCES

Pathogen-mediated natural and manipulated population collapse in an invasive social insect

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Edited by Daniel Simberloff, University of Tennessee, Knoxville, TN; received August 6, 2021; accepted February 7, 2022

Boom-bust population dynamics are a recurrent, widespread, and typically unexplained property of many species invasions. Declines also occur in invasive social insects from unknown causes. Nevertheless, social insects have proved intractable to biological control. Tawny crazy ants, an environmentally damaging invasive pest in several countries globally, are spreading in North America. Examining 15 local populations spanning 9 y, we document both the collapse of local populations of this ant in North America and a strong association of collapse with infection by the microsporidian pathogen, *Myrmecomorba nylanderiae*. Over the observation period, all longitudinally sampled local populations that harbored the pathogen declined, with 62% of these populations disappearing entirely. We test the causality of this relationship by introducing this pathogen into two local populations. At both sites, within 7 mo the pathogen was nearly universally prevalent, and within 2 y, tawny crazy ants were eliminated. In contrast, uninfected populations showed no tendency to decline over a similar period. Concurrent laboratory studies indicate that colony fragments died out because infected workers do not survive long enough to bridge the gap created by normal, winter cessation of immature ant production. Population-level collapse occurred because the pathogen spread faster than colony fragments declined, eliminating the density-dependent regulation seen with many pathogens. Invasive species beset by such pathogens may collapse if factors favoring transmission, like genetic homogeneity, high population density, or socially facilitated intragroup transmission, allow virulent pathogens to spread widely before disease impacts occur. These invasive species may be susceptible to boom-bust dynamics and pathogen-driven local extinction.

biological control | invasive ant | natural enemy | *Nylanderia fulva* | microsporidia

Significance

Invasive social insects are among the most damaging of invasive organisms and have proved universally intractable to biological control. Despite this, populations of some invasive social insects collapse from unknown causes. We report long-term studies demonstrating that infection by a microsporidian pathogen causes populations of a globally significant invasive ant to collapse to local extinction, providing a mechanistic understanding of a pervasive phenomenon in biological invasions: the collapse of established populations from endogenous factors. We apply this knowledge and successfully eliminate two large, introduced populations of these ants. More broadly, microsporidian pathogens should be evaluated for control of other supercolonial invasive social insects. Diagnosing the cause of unanticipated population collapse in invasive organisms can lead to applied solutions.

The invasion of one biogeographic region by a species native to another is a dramatic and repeated feature of life on Earth (1). Human activities have elevated the rate of this natural, but naturally infrequent, process to such a degree that invasive species do significant harm to regional biodiversity and ecosystem integrity (2). In natural systems, species invasions are characterized by an alien species spreading into new environments, growing exponentially to extreme local abundance followed by a protracted period of high abundance (3). On longer time scales, invasiveness and the abundance of the invader may gradually decline, typically through a mixture of adaptive and compositional changes in the natural enemy and competitor assemblage of the invaded ecosystem (4, 5). Other invaders resist this "naturalization" process with no evidence of declining abundance or impact several decades after introduction (6, 7).

In rare cases, after an initial period of expansion and explosive growth, invasive species rapidly decline even to local extinction without human intervention (8, 9). These collapses, termed boom-bust dynamics, occur in plants, insects, mollusks, birds, mammals, and amphibians in both terrestrial and aquatic environments. With few published experimental or quantitative examinations, boom-bust collapses are understudied and their causes are typically mysterious (8). Understanding mechanisms driving boom-bust population dynamics presents a persistent and important puzzle in biological invasions. Such an understanding could improve predictions of whether invaders are likely to exhibit these dynamics, and potentially enhance the elimination or naturalization of invasive species. Achieving this understanding for any invader requires large, long-term data sets and clear definitions of the process under study (9).

Invasive social insects are among the most damaging category of terrestrial invasive species (10, 11). They are also one of the most intractable to control. Notably, despite intensive effort, there are no documented examples of successful biological control of any social insect at population scale (12, 13). Surprisingly, given this, invasive social insect populations sometimes exhibit boom-bust dynamics. In particular, populations of a variety of globally significant invasive ant species have exploded and then declined steeply (14). The most mysterious declines occur when once dense populations of an

Author contributions: E.G.L. designed research; E.G.L., M.J., and R.M.P. performed research; E.G.L. analyzed data and wrote the paper; E.G.L and R.M.P. created figures; R.M.P. and L.E.G. discussed results and contributed to final manuscript; and L.E.G. supervised the project.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2114558119/-/DCSupplemental.

Published March 28, 2022.

invasive social insect collapse to local extinction. The collapse of Argentine ant (*Linepithema humile*) populations in New Zealand but not in other areas (15, 16) and the collapse of some populations of yellow crazy ants (*Anoplolepis gracilipes*) in Australia (17) provide examples. Natural enemies, particularly pathogens, have been invoked as likely causes in these declines. However, to our knowledge, no study has through quantitative observation or experimental manipulation identified the cause of and documented the characteristics of population collapse in an invasive social insect.

Invasive species generally may be more vulnerable to pathogens due to facilitated transmission from high population densities (3) and low genetic diversity arising from introduction bottlenecks (18). In invasive social insects, a supercolonial social organization, in which behavioral boundaries between nests do not exist, has been speculated to further enhance susceptibility to pathogens (19). This social organization creates genetically and behaviorally homogenous introduced populations that are highly interconnected, with workers moving between nests and engaging in regurgitative food sharing, potentially eliminating barriers to between nest pathogen transmission. Most globally significant, ecologically damaging invasive ant species exhibit a supercolonial social structure in their introduced range (20).

Tawny crazy ants (*Nylanderia fulva*) are an invasive ant species of emerging importance. These ants, native to east-central South America (21), have been introduced into Columbia (22, 23), Cuba (24), the US Virgin Islands (25), the southeastern United States (26), and Ecuador (27). *N. fulva* infestations reach extreme densities. Multiple, several centimeter-wide trails of ants stream up most tree trunks and every possible nest site (any preexisting cavity typically under rocks, in logs, or other ant nests) is occupied by these ants and their brood. At these densities, tawny crazy ants have substantial ecosystem impacts. Most native ant species are rapidly eliminated, and arthropod abundance and diversity is depressed (28–31). Although quantitative studies of population impacts on vertebrates do not exist, anecdotal reports of the blinding of small burrowing mammals (thought to result from spraying acid in the eyes of developing young), loss of ground-nesting vertebrates like lizards and snakes, and the destruction of canopy nesting birds are common (31–33).

Populations of *N. fulva* also have a biogeographic history of collapse. Introduced into Columbia prior to 1971, isolated, dense populations became established in low to mid-elevation regions throughout Colombia (23). Since introduction, many of these populations have disappeared (22). In addition, evidence of *N. fulva* population collapse exists from St. Croix, US Virgin Islands, where ants were absent in areas densely occupied by *N. fulva* 11 y prior (34). No mechanism was suggested for the decline.

N. fulva are distributed throughout the Gulf region of the southeastern United States and Texas in a constellation of disconnected infestations typically separated from their nearest neighboring infestation by many kilometers (26, 35). Areas occupied by these ants are commonly kilometers in diameter (*SI Appendix*, Table S1) with densely distributed nests that, as is common in supercolonial ants (36), are interconnected, sharing workers and food. Behavioral and genetic data indicate that all these infestations are disjoint parts of the same supercolony (37–39). Because nests of *N. fulva* within an area comprise interconnected entities lacking contact with other parts of the southeastern United States supercolony, we use the term local population to refer to these geographically proscribed infestations.

In 2015, a previously undescribed microsporidian pathogen (*Myrmecomorba nylanderiae*) was found infecting *N. fulva* in Texas, Florida, and St. Croix (40). Microsporidians are intracellular, spore-forming pathogens related to fungi (41). *M. nylanderiae* has been found infecting all castes of *N. fulva*; however, infection of queens is uncommon. *N. fulva* larvae appear to be the only caste susceptible to acquiring infection with intracolony transmission restricted to infected workers passing the pathogen to developing larvae (42). There are no records of this pathogen in the native range of *N. fulva*. How this pathogen came to infect local populations of *N. fulva* in North America is unknown.

Herein, utilizing a combination of long-term longitudinal studies, laboratory studies of colony fragments, and the experimental inoculation of uninfected local populations, we document the decline and collapse of local *N. fulva* populations in North America. We demonstrate that a principal causative agent is the microsporidian pathogen *M. nylanderiae*.

Results and Discussion

Naturally Infected Local Populations. From 2009 to 2020, we located and sampled 24 *N. fulva* local populations to evaluate the prevalence of *M. nylanderiae* at a landscape scale. Of these, 15 were sampled repeatedly over a 9-y window to assess annual disease prevalence, intensity, and *N. fulva* abundance (Fig. 1). Abundance was assessed with pitfall traps set during periods of peak activity and left open for 24 h. Disease status was assessed from pitfall or hand-collected ants using a combination of diagnostic PCR and counting of microsporidian spore density from tissue homogenates of batches of 20 workers.

At the first sample, 36% of *N. fulva* local populations were found infected with *M. nylanderiae*. Among initially

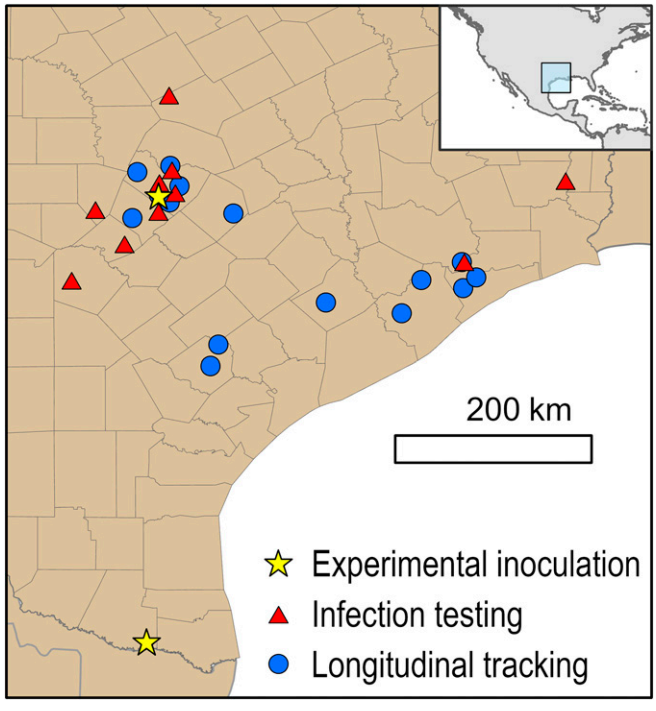

Fig. 1. Map of study sites. Sites are categorized by the type of data collected. Longitudinal tracking sites: *N.* fulva abundance and infection status tracked over multiple years. Infection testing sites: tests from a single sampling event of *N. fulva* for the microsporidian pathogen *M. nylanderiae*. Experimental inoculation: sites where *M. nylanderiae* was intentionally introduced into the *N. fulva* population. See *SI Appendix*, Fig. S3 for South American sampling locations.

uninfected, longitudinally sampled sites ($n = 8$), 75% acquired the pathogen without apparent means of introduction. Pathogen acquisition occurred from 5 to 114 mo after sampling began. Populations that were infected or acquired the infection tended to be larger, although not significantly so, than those that did not (maximum observed extent mean [SD]: 3,238 [1,750] m for infected vs. 1,802 [767] m for uninfected) (*SI Appendix*, Table S1). The biogeographic source of this pathogen and how it colonizes distant sites remains unknown (*SI Appendix*, *Supplemental Text*).

Longitudinal sampling of local populations of tawny crazy ant documents a repeated pattern of population decline and a clear association between decline and infection with the microsporidian pathogen, *M. nylanderiae*. *N. fulva* abundance varies widely between years due to environmental conditions. However, comparing abundance in 1 y to the following, on average, infected local populations decreased substantially (median of −86% [interquartile range (IQR), −98 to −35%] per year) while, on average, uninfected local populations varied substantially but changed little (11% [−35 to 320%] per year) (Wilcoxon: $X^2 = 5.2$, $n = 28$, $P < 0.02$). Consequently, on average, infected *N. fulva* local populations declined in abundance from very high levels to local extinction over 4 to 7 y from pathogen acquisition (Fig. 2 and *SI Appendix*, Table S2). Among the monitored local populations infected at some point with *M. nylanderiae* ($n = 13$), *N. fulva* has disappeared entirely or become scarce (less than one ant per sample station) from 10. These were previously extremely dense local populations spanning many square kilometers (*SI Appendix*, Table S1). Declines have occurred in uninfected local populations but none of this magnitude.

The quantitative details of the association between infection and tawny crazy ant population collapse suggest this pathogen may be a significant causal factor in these declines.

Infection prevalence typically increased rapidly from first detection, often reaching universal prevalence. Across 11 naturally infected sites, in 24 of 27 annual fall-to-fall transitions, infection prevalence among sampling stations either increased or remained fixed at 100%. Prior to infection prevalence reaching 100%, site infection prevalence increased an average (median) of 30 (IQR, 13 to 43%) annually. Disease prevalence was strongly and positively associated with the average disease burden of nestmate ants. As site-level disease prevalence increased from 0 to 100%, the average disease burden of nestmate ants increased exponentially across four orders of magnitude (Fig. 3*A* and *SI Appendix*, Table S2). Further, a strong, negative relationship exists between site-level tawny crazy ant abundance and the disease burden of local nests (Fig. 3*B* and *SI Appendix*, Table S2).

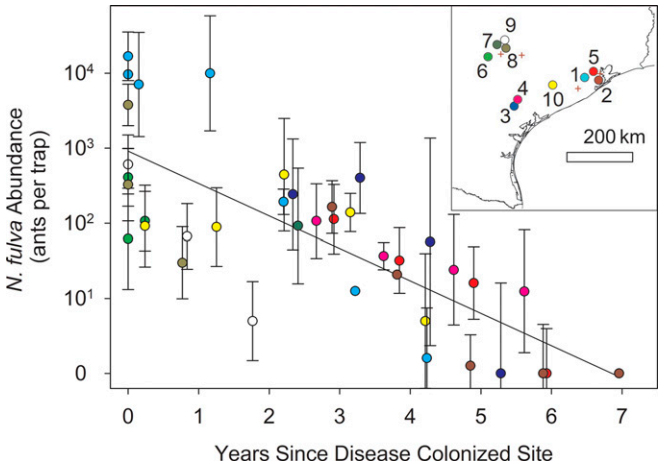

Fig. 2. Infected tawny crazy ant population decline. Data from *N. fulva* local populations infected with *M. nylanderiae* where time of pathogen colonization is known or could be estimated. Data at 0 on the x-axis are from time points prior to *M. nylanderiae* presence. Bars provide SDs. *Inset* map: Plus symbols indicate excluded infected sites where the date of pathogen colonization is unknown. Tawny crazy ants also declined at these sites. Numbers identify sites in *SI Appendix*, Table S1. Line provides overall decline trend [abundance (log): $n = 39$, $R^2 = 0.60$, $P < 0.0001$] (*SI Appendix*, Table S2).

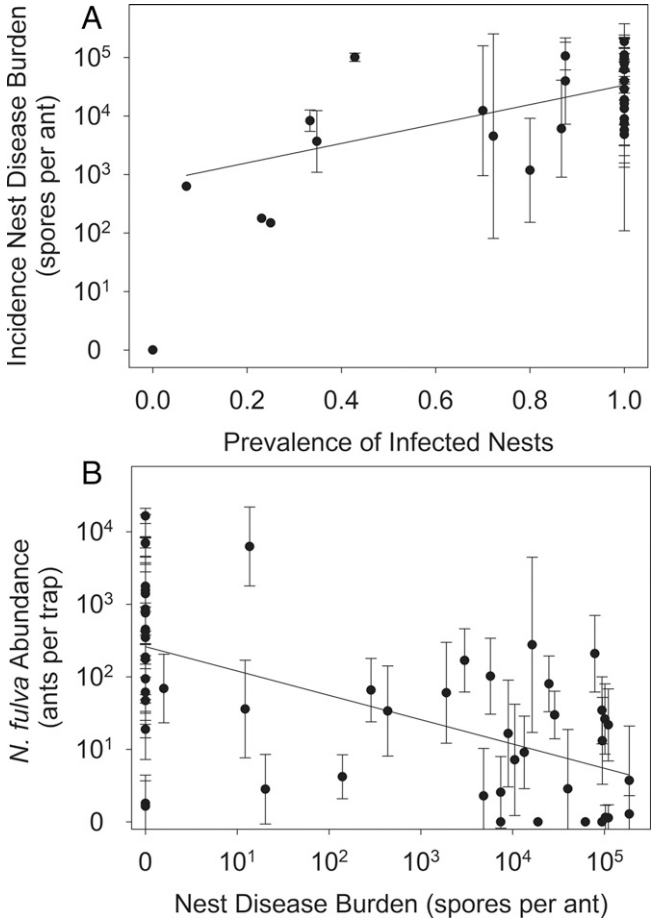

Fig. 3. Relationships between site-level disease states and host abundance. Each point presents a site average for a sampling interval. (*A*) Relationships between site-level disease prevalence of *M. nylanderiae* infection and incidence disease burden: the average disease burden of *N. fulva* workers from infected nests. Only nonzero data are fitted (linear regression: disease burden [log]: $n = 28$, $R^2 = 0.37$, $P < 0.0006$) (*SI Appendix*, Table S2). (*B*) Average disease burden of workers from all nests and the local abundance of *N. fulva* [linear regression: abundance (log): $n = 52$, $R^2 = 0.37$, $P < 0.0001$] (*SI Appendix*, Table S2). Bars provide SDs.

Experimentally Inoculated Local Populations. Experimental inoculations of two local populations of *N. fulva* with this pathogen were undertaken to test whether this pathogen causes tawny crazy ant populations to collapse. Ants were sampled by hand and with pitfall traps from both sites at multiple occasions prior to inoculation. Ants from both sites tested negative for infection at the beginning of preinoculation sampling. At inoculation site 1, some ants collected from pitfall traps run prior to inoculation were later found to harbor low-intensity infections. At inoculation site 2, the pathogen was never found prior to inoculation despite sampling ants from 106 nests on 28 occasions spanning 3 y.

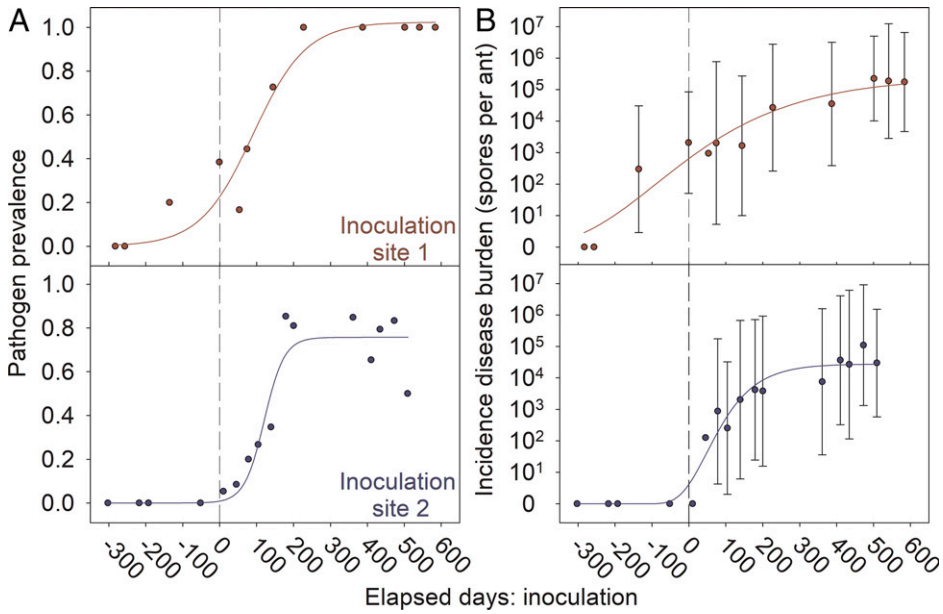

Fig. 4. Rise in disease intensity over elapsed time since inoculation. Dashed lines indicate inoculation day. Bars display SDs. For curve fits, see *SI Appendix*, Table S3. (*A*) Pathogen prevalence: fraction of nests infected with *M. nylanderiae*. (*B*) Incidence disease burden: average disease burden (spores per ant) for all nests testing positive for infection. Zero values indicating no positive nests are not fitted.

Because all *N. fulva* in Texas share the same supercolonial identity (37, 38), when ants from distinct local populations are brought into contact, they are nonaggressive, allowing for the use of live infected ants to inoculate uninfected local populations with the pathogen. Inoculations were conducted by introducing infected workers and brood collected from a distant infestation into the inoculation sites. Infected ants were introduced in nest boxes or directly onto foraging trails. For 2.5 y following inoculation, ant abundance, pathogen spread, and disease burden were quantified. The spread of the pathogen and the change in ant abundance were assessed over a 91-ha area at site 1 and a 12-ha area at site 2.

Despite different-sized study areas, both local populations exhibited similar patterns of pathogen spread and tawny crazy ant population decline. At inoculation site 1, 8 wk elapsed between the inoculation effort and the first recovery of infected workers from an inoculation station. Infected workers were consistently recovered from that station thereafter. At that time, disease burden declined significantly with increasing distance from the successful inoculation station (*SI Appendix*, Fig. S1*A*). Although the 95% CI for peak infection intensity overlapped the inoculation station, areas of the site distant from inoculations also exhibited elevated infection intensities (*SI Appendix*, Fig. S1*B*). In sum, at inoculation site 1, the widespread establishment of the pathogen likely resulted from a combination of the inoculation and the locally acquired infection present at inoculation. In contrast, at inoculation site 2, despite intensive sampling, *M. nylanderiae* was never detected prior to inoculation. Across the 15 inoculation stations at inoculation site 2, the pathogen established in a median of 85 d (IQR, 62 to 133 d).

The pathogen spread very rapidly in both populations, likely a result of the interconnected nature of local *N. fulva* supercolonies facilitated by their genetic homogeneity (38). At both inoculation sites, prevalence of the infection across the sampling areas increased in a sigmoidal fashion. Although sites differed in observation area, the disease reached its maximum prevalence levels throughout both sites in about 200 d (Fig. 4*A*), becoming universally prevalent by this time at site 1. At both inoculation sites, average disease burden of infected nests increased exponentially throughout the period of pathogen spread and *N. fulva* population decline (Fig. 4*B*). At both inoculation sites, *N. fulva* populations declined to local extinction following widespread pathogen establishment. After 11 to 15 mo, *N. fulva* had declined to 50% of their preinoculation abundance levels (site 1: 328 d, site 2: 464 d). By 2 to 2.6 y postinoculation, *N. fulva* disappeared entirely from the study sites (site 1: 2.6 y, site 2: 2.0 y) (Fig. 5*A*). At both inoculation sites, steep declines occurred over the winters of the first and second years postinoculation and tawny crazy ant abundances remained comparatively stable over the summers. Infected *N. fulva* populations generally exhibited this trend, with abundance in the spring a median 59.7 (IQR, 8.5 to 392.6) times lower than the preceding fall while over the growing season abundance increased by a factor of 8.6 (IQR, 0.95 to 11.5). Tawny crazy ant abundance across an array of uninfected sites observed for a similar length of time exhibited no overall tendency to decline over time (linear regression: $n = 26$, $P = 0.87$), and no individual site declined to scarcity (Fig. 5*B*).

Mechanisms Driving Population-Level Decline. The growth and survivorship of tawny crazy ant colony fragments provides insight into why *M. nylanderiae* infection causes tawny crazy ant population collapse. Tawny crazy ant nests collected from the field from 2015 through 2020 were held in the laboratory at 26 °C on a 12-h light cycle and regularly censused for size and brood composition.

Explaining the over-winter decline in worker abundance observed in all populations, regardless of infection status, colony fragments collected in winter had very little or no brood (eggs + larvae + pupae) (mean 0.009 cm^3 per 100 workers). Brood levels dropped to this low level in September and resurged in April (Fig. 6). During the warm seasons, infected and uninfected colony fragments differed in brood composition. Infected fragments primarily contained eggs (61%) while pupae comprised the largest fraction of brood in uninfected fragments (49%) (likelihood ratio $X^2 = 13.9$, $n = 80$, $P <$

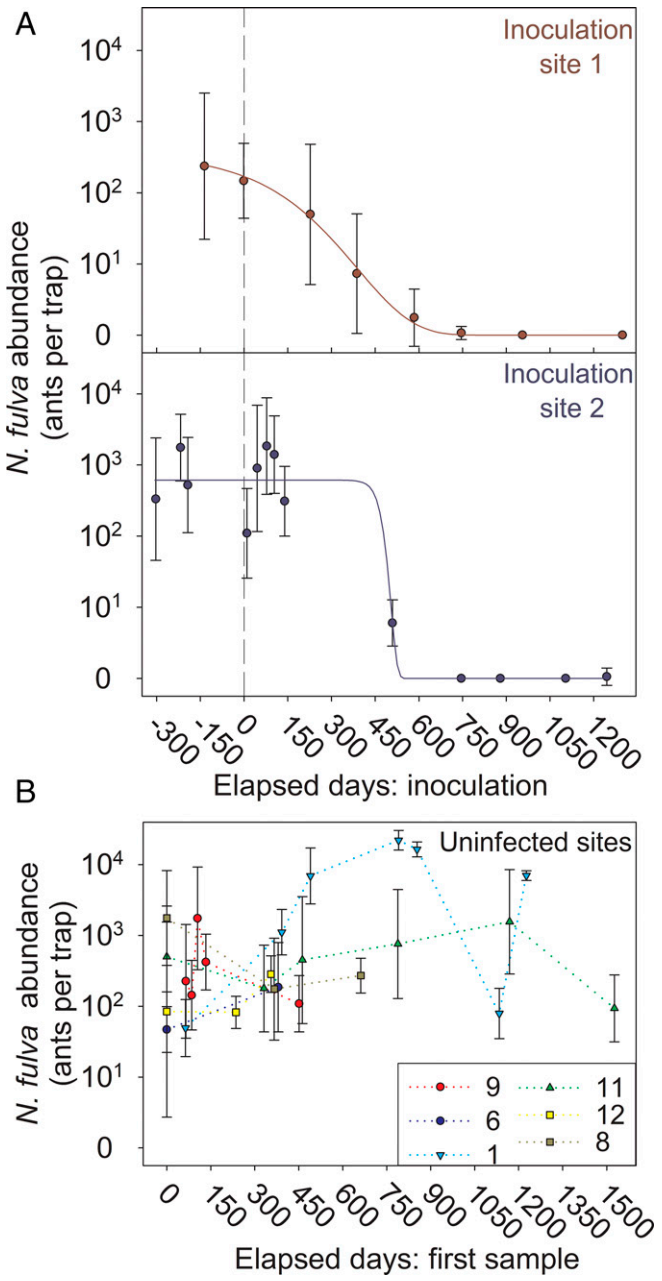

Fig. 5. Tawny crazy ant abundance over time. (*A*) *N. fulva* abundance (average number of *N. fulva* workers per pitfall trap) at inoculation sites across time since inoculation. Dashed lines indicate inoculation day. For curve fits, see *SI Appendix*, Table S3. (*B*) Abundance of *N. fulva* at uninfected sites across elapsed population monitoring time. Numbers identify sites in *SI Appendix*, Table S1. Bars display SDs.

0.0009) (Fig. 6). This difference reflects the reduced developmental success of infected brood (42) and may explain why infected local populations do not grow explosively during the warm season as do uninfected local populations (28).

Declines in both experimentally inoculated local populations occurred primarily over the winter. Perhaps population collapse arises because the lifespan of the infected worker population is insufficient to bridge the gap in winter brood production. The decline of colony fragments in the laboratory supports this hypothesis. Unsurprisingly, infected colony fragments decline more rapidly than uninfected fragments (median [IQR]: 84 [53 to 120] d for infected vs. 207 [109 to 380] d for uninfected) (Kaplan-Meier survival time, log-rank: $X^2 = 28.5$, $n = 102$, $P < 0.0001$). More interestingly, the rate at which infected colony fragments decline depends upon the season in which nests were collected. Rate of decline was independent of season for uninfected colony fragments. However, infected colony fragments collected during the fall-winter period declined almost twice as fast as those collected during the spring-summer (68 [47 to 100] d vs. 126 [84 to 154] d, respectively) (Fig. 7). The rate of fall-winter worker mortality observed in the laboratory, a 75% decline in worker population in only 1.5 mo, would likely prevent infected colony fragments from bridging the 7-mo gap in winter brood production, leaving queens in spring with insufficient workers to rear the first generation of brood. Further research is needed to determine whether natural conditions alter this over-winter worker mortality rate.

This mechanism for population decline proposes that population-level virulence arises from an interaction of climate, social biology, and disease pathology. Such mechanisms, if common features of social insect–pathogen interactions, will lead to high levels of condition dependence in the collapse of invasive social insect populations. This type of interaction may be partially responsible for the geographic contingency seen in the population dynamics of other invasive ants (15, 16).

Boom-Bust Dynamics and Biological Control in Invasive Ants. Acquisition of the pathogen, *M. nylanderiae*, by tawny crazy ants results in extremely dense local populations crashing to extinction. This is surprising, as natural enemies do not typically drive their host to extinction. In tawny crazy ants, very rapid pathogen spread is the proximate cause for this extreme result. In naturally infected and inoculated populations, the disease reached near universal prevalence prior to a notable decline in abundance. Essentially, very fast transmission rates overwhelmed the density-dependent feedbacks that typically stabilize host–pathogen interactions (43). Factors such as genetic homogeneity (18), high population density (3), and interconnected networks (20) can facilitate rapid disease transmission in this and other invasive species populations. Whether invasive species populations are prone to collapse (8, 9) when beset by virulent pathogens may hinge on if the invader's population ecology amplifies the transmission rate above the rate of disease induced decline. Enhanced transmission allows these populations to temporarily support more virulent pathogens than would otherwise be possible. High-density, genetically homogenous, highly interconnected invasive species populations may be prone to boom-bust population dynamics and vulnerable to pathogen-driven local extinction.

These results point to a larger potential opportunity to use microsporidian pathogens to control the most damaging invasive ant species. Microsporidian pathogens of insects range from extreme generalists to those that appear species specific with the majority attacking a limited range of related hosts (44). The restricted specificity of microsporidians creates the opportunity to seek these pathogens in both invasive ants and in their close relatives, as supercoloniality may reduce the need for pathogens to have host-specific adaptations. Once inside an ant supercolony, a pathogen does not require host-specific adaptations for dispersal to and infection of new colonies. Intracolony transmission suffices to be successful at large geographic scales. In contrast, pathogen persistence in nonsupercolonial, native ant populations requires adaptations for long-distance transmission and penetration of colony defenses. Thus, attempts to use microsporidian pathogens adapted to related hosts to control invasive supercolonial ants might be both more likely to succeed and less likely to cause unintended to harm than it might appear.

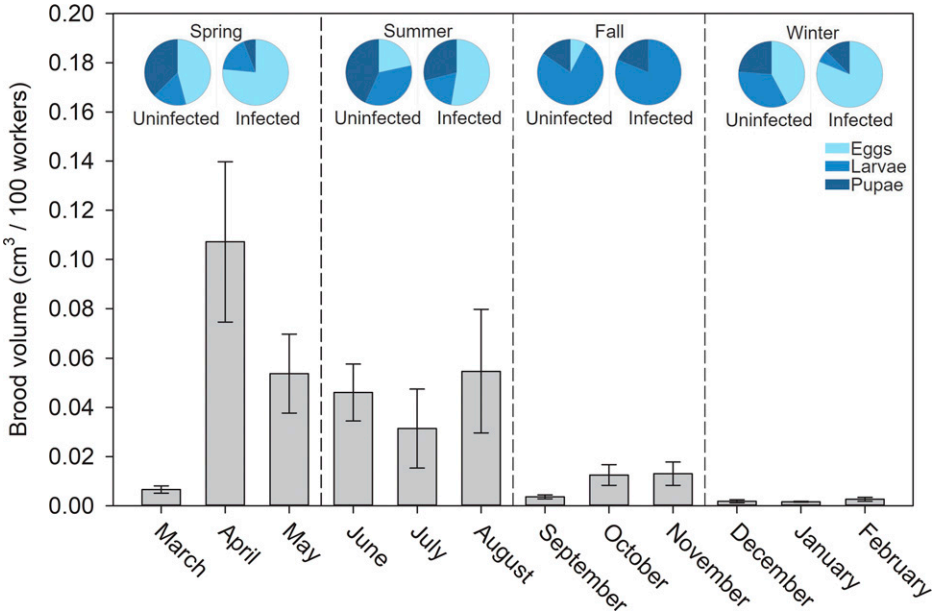

Fig. 6. Brood production of tawny crazy ant colony fragments. Brood production for all colony fragments regardless of infection status by month of year. Months divided into northern hemisphere seasons by vertical dashed lines. Bars provide SE. *Inset* pie diagrams display the brood composition of *N. fulva* colony fragments infected with *M. nylanderiae* ($n = 68$) and uninfected ($n = 79$) by season of collection.

Currently, *N. fulva* exist in the southeastern United States as a scattered set of local populations, colonizing new environments via human transport, expanding for years to tens of years, and then frequently declining to local extinction as they acquire the microsporidian *M. nylanderiae*. These population-level extinction events provide a dramatic proof of the concept that supercoloniality, a social organization shared by most globally invasive ant species, enhances vulnerability to pathogens. Like the unitary organisms they resemble, supercolonies possess a shared fate. It has been suggested that the best management approach to invasive species that exhibit boom-bust dynamics like these is to "do nothing" (9). Contrarily, research into the causal agent of this boom-bust cycle has created the opportunity to utilize this microsporidian as a tool to interrupt the dynamic of episodic and profound ecological disruption.

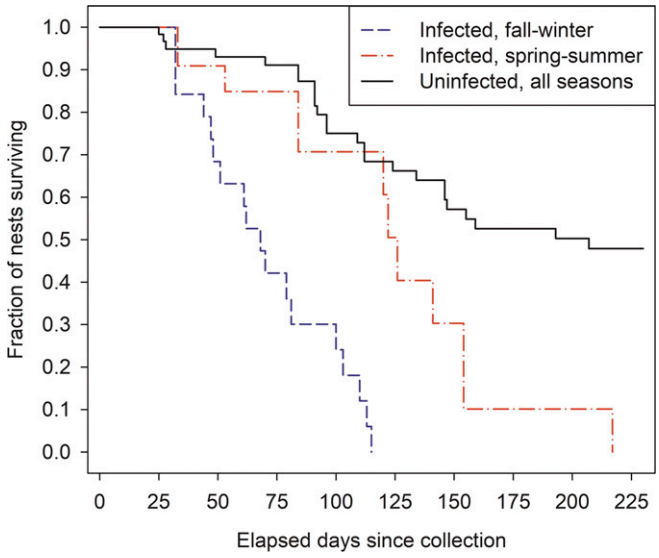

Fig. 7. The seasonally dependent fate of laboratory-held tawny crazy ant nest fragments. Failure is defined as decline to 25% of original nest fragment size. Uninfected nests did not differ in their decline trajectory based upon season of collection and are displayed as a group (Kaplan-Meier survival time, log-rank: $X^2 = 0.61$, $n = 61$, $P < 0.43$). Infected fragments decline faster if collected in fall-winter than in spring-summer (Kaplan-Meier survival time, log-rank: $X^2 = 15.5$, $n = 41$, $P < 0.0001$).

Materials and Methods

Study Sites. Newly identified *N. fulva* local populations (24 in total) were assessed for *M. nylanderiae* infection by sampling workers from 8 to 15 nests separated by at least 20 m. Fifteen sites were repeatedly sampled over 9 y (2012 to 2020) to quantify disease status and impacts on local *N. fulva* supercolonies. All sites sampled longitudinally had *N. fulva* invading wildland habitats: forest, open woodland, and grassland environments. At two additional sites, we experimentally inoculated uninfected local populations (Fig. 1). The extent of *N. fulva* infestations was periodically assessed by locating the limit of the infested habitat in three to four directions using standardized, visual surveys of the ground foraging ant assemblage (*SI Appendix, Supplemental Methods* and Table S1).

***N. fulva* Abundance.** At all sites, to avoid the rapid changes in abundance associated with initial invasion, sampling stations were placed in areas where *N. fulva* were known to have been present for a year or were at least 100 m from the nearest edge of the *N. fulva* infestation (28). We quantified *N. fulva* abundance using pitfall traps. Pitfall traps, a standard ant sampling technique, provide good measures of relative abundance during periods of similar foraging activity. Annual maximum abundance measures were made by trapping all sites in the fall between September and November on days without rain close to the calendar date of preceding samples. To quantify intra-annual variation, some sites were trapped in the spring and summer after local high temperatures were above 32 °C. These conditions captured periods of highest *N. fulva* activity. Pitfall traps, 50-mL plastic centrifuge tubes with 2.7-cm-diameter openings, were installed in polyvinyl chloride pipe sleeves set flush with the ground. Between trapping intervals, trap sleeves were plugged. Traps were charged with odorless detergent and water and left open for 24 h. Traps were sorted and ants were counted in the laboratory (*SI Appendix, Supplemental Methods*). To increase our threshold of detection at sites with low *N. fulva* density, the areas within 5 m of sampling stations were searched for *N. fulva*. Herein, local extinction is a decline in abundance so profound that this intensive sampling fails to detect *N. fulva*. *N. fulva* is scarce when less than one ant is recovered per pitfall trap, an abundance reflective of uncommon native species trapped with these methods.

Disease Assessment. Tawny crazy ants were tested for *M. nylanderiae* infection using diagnostic PCR and spore counting. Both diagnostic PCR and spore counting were based on batches of 20 homogenized workers. This averaged across variation in individual infection present among workers in a nest. Workers were homogenized and DNA extracted following rinsing to remove external spores and venom gland products to improve DNA amplification (42) (*SI Appendix, Supplemental Methods*). Diagnostic PCR amplified a 600-bp amplicon of *M. nylanderiae* 16S rDNA (40) (*SI Appendix, Supplemental Methods*).

Disease burden was assessed by spore counting. Tissue homogenates were concentrated to 300 μL and a 0.3-μL aliquot was fixed with ethanol and stained using trichome blue (45). Type 2 DK spores, the predominant spore type, were counted under 1,000× magnification (40) (*SI Appendix*, Fig. S2). To calculate an approximate average number of spores per ant in the sample, total spore count values were multiplied by a correction factor (62.5) to adjust for the number of workers in the sample, and the size and fraction of the aliquot was counted (*SI Appendix, Supplemental Methods*). We refer to this adjusted value as the disease burden of a nest, which has a natural unit of spores per ant.

Impact of Naturally Acquired Infection on Local Populations. An array of 15 sampling stations were installed per site, each separated by a minimum of 50 m. For some sites, annual tracking of *N. fulva* abundance began prior to discovering *M. nylanderiae* (40). Samples from these traps, stored in 95% ethanol at −20 °C, were assessed for infection with diagnostic PCR. Ethanol denatures tissue proteins causing spores to clump so disease burden was not quantified in older samples.

The course of disease development and relation to *N. fulva* population decline was assessed by the relationships between disease prevalence, incidence disease burden, and *N. fulva* abundance. Incidence disease burden is the average of the number of spores per ant summing across only infected nests. It and disease prevalence are the orthogonal components of our measure of population-level disease intensity. At some infected sites, *N. fulva* was too scarce by the final fall to measure infection levels. For these site-by-year samples ($n = 5$), disease parameters for the year prior were used.

The rate at which infected *N. fulva* populations decline was assessed by plotting *N. fulva* abundance against the estimated or observed time since the pathogen first colonized the site. Estimates of time since pathogen colonization were made for sites where the pathogen was present but not universally prevalent at the first sample. The median rate of increase in pathogen prevalence (30% per year) was calculated for all sites and used to estimate the time the pathogen had been present at a site prior to sampling. Data from three sites where disease prevalence was at 100% at the first sampling event were excluded.

Experimentally Inoculated Local Populations. At two sites, we intentionally introduced the infection into local populations of *N. fulva*. Ants were sampled on multiple occasions prior to inoculation. Ants from both sites tested negative for infection at the beginning of preinoculation sampling. At site 1, some *N. fulva* from pitfall traps run prior to inoculation were found to harbor low-intensity *M. nylanderiae* infections. At inoculation site 2, the pathogen was never found prior to inoculation despite sampling on 28 occasions across 3 y.

Because all *N. fulva* in Texas share the same supercolony identity, when ants from distinct local populations are brought into contact in the laboratory, they are nonaggressive (37–39). This allows for using live infected ants to inoculate uninfected local populations with the pathogen. Infected source nests were collected near East Columbia, Texas (29.1318°, −95.6228°). Inoculations were conducted by introducing infected workers and brood in nest boxes or directly onto foraging trails (*SI Appendix, Supplemental Methods*). At site 1 (Estero Llano Grande State Park), 3,200 infected workers and 1.33 cc of brood were introduced in eight nest boxes at two locations separated by 400 m and allowed to integrate into the local population. At site 2 (Austin, TX), 250 infected workers and 0.25 cc of brood were released directly onto foraging trails at 15 locations spread over 4.5 ha.

Following inoculation, at 4-wk intervals, ants collected off baits were tested for infection at inoculation stations plus additional stations within 10 m of the inoculation stations. To account for recovering released infected workers, a pattern of positive results determined establishment: the first of three out of four consecutive *M. nylanderiae*–positive samples marked establishment. Recovery of released workers was uncommon, as the first 7 wk after inoculation all stations tested negative.

Pathogen prevalence, intensity, and *N. fulva* abundance were assessed in areas more than 100 m from the edge of the *N. fulva* infestation. Sampling stations spanned a 91-ha area at site 1 and a 12-ha area at site 2. Pathogen prevalence and intensity were assessed by testing ants sampled from a combination of pitfall traps, nests, and baits. At both sites, samples were spaced a minimum of 40 m apart and spanned the full extent of the assessment areas (*SI Appendix, Supplemental Methods*). Because all ants foraging at a particular point come from a single nest or a group of neighboring, interconnected nests, we refer to these samples as nest samples. At inoculation site 1, *N. fulva* abundance was assessed using 26 pitfall traps spread over the central 63 ha of the site. At inoculation site 2, 24 pitfall traps were spread over the entire 12-ha site.

At inoculation sites, changes in disease characteristics and ant abundance were nonlinear. All response variables were expected to asymptote at both ends (either at 0 or a variable equilibrium value). To describe these responses, three-parameter sigmoid functions that fit this a priori biological expectation were fitted to the data with nonlinear regression. The specific sigmoid function chosen was based upon minimization of the Akaike information criterion (*SI Appendix*, Table S2).

For evaluating changes in *N. fulva* abundance, long-term, uninfected monitoring sites provided noninoculated, comparison sites. To create a comparable data set, we included *N. fulva* abundance surveys from uninfected sites taken during the period which inoculation sites were sampled (mid-May to October). Data from all sites observed as uninfected for at least 1 y were included. All data collected during the length of time the experimental sites were monitored (1,500 d) were included. If sites acquired the pathogen after 1 y, subsequent data were not included. Sampling methods were the same for infected and uninfected sites.

Laboratory-Held Nest Fragments. Tawny crazy ant nests were collected throughout the year from 2015 through 2020. Nests, in rotting logs, were extracted from the wood in the laboratory using heat. Colony fragments were held at 26 °C, on a 12-h light cycle, given ad libitum access to sucrose, and fed crickets and a protein supplement three times per week (*SI Appendix, Supplemental Methods*). Colony fragments were censused at the time of collection and once per month thereafter. The number of workers was visually estimated by summing groups of 10 workers across the surface of the container and nest, the number of queens was counted, the amount of brood was categorized by visually comparing the brood pile to a set of volumetric standards, and the composition of the brood pile was described by the volumetrically predominant developmental state present (eggs, larvae, or pupae).

Colony fragments were included in survivorship analyses if they contained at least one queen at time of collection and if no ants were removed for at least 30 d following collection. Fragments were disposed of when worker numbers reached low levels; so for survivorship analysis, colony fragments failed (died) when worker numbers fell below 25% of their size at collection. Colony fragments for which ants were removed after 30 d but before declining to 25% of their original size were right censored in the analysis. This yielded 126 colony fragments, 39 of which were right censored.

Data Availability. All study data are included in the article, *SI Appendix*, and Datasets S1–S5.

ACKNOWLEDGMENTS. Sam Gallagher, Michael Mueller, Charlotte Nash, Alejandro Santillana, Aaron Sekula, Edwin Umanzor, April Kwan, Kaitlyn Williams, Shawn Evenson, and Nathan Jones provided technical assistance. Todd Bayless, Mark Sanders, Travis Clark, and Javier DeLeon provided logistical support. David Holway provided manuscript feedback. Martin Bollazzi and Patricia Folgarait provided logistical support for work in South America (*SI Appendix, Supplemental Methods*). This work was funded by the Lee and Ramona Bass Foundation (E.G.L., R.M.P., and L.E.G.), Texas Parks and Wildlife Department Grant TX T-163-R-1 (E.G.L., R.M.P., and L.E.G.), U.S. Fish and Wildlife Service Grant F17AF01069 (E.G.L., R.M.P., and L.E.G.), the Travis County Natural Resources Division (E.G.L.), and the Austin Water Wildlands Conservation Division (E.G.L.).

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