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RESEARCH ARTICLE

Cold adaptations along a range limit in an obligate symbiosis

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Abstract

1. Symbionts can have profound effects on host fitness, adaptations and range distributions. Stress-induced evolution is difficult to show in obligate symbioses; however, adaptive evolution within an obligate symbiosis can be investigated experimentally or by correlating trait variation with stress along an ecological cline (i.e. temperature-stress gradient).
2. We investigated the cold tolerance of the fungus-growing ant *Trachymyrmex septentrionalis* by performing cold tolerance assays comparing two populations collected from either the southernmost range of their distribution (Bastrop, Texas) or from a site that is approximately 600 km further north (Norman, Oklahoma). We first compared isolated fungal symbionts grown on artificial media to determine cold tolerance of fungus alone. Subsequently, we conducted cross-fostering experiments between northern and southern host and symbionts to test for synergisms between the partners in generating adaptations of cold tolerance.
3. Ants of the northern fungal populations were more cold adapted than southern fungal populations. Northern nests were deeper and northern colonies initially rejected fungi from the southern population. The cross-fostering experiments demonstrated that only one partner must be cold tolerant to confer maximum cold tolerance to the ant–fungus symbiosis, because northern ants growing southern fungus under cold stress performed just as well as northern ants growing northern fungi.
4. Our results suggest that cold stress has been an important selective factor during the migration of this ant–fungus symbiosis into northern latitudes during the last 10,000 years, and that cold tolerance likely is an energetically demanding trait that may be traded off with other aspects of the symbiosis' life history. The symbiosis also appears to have evolved several additional adaptations that increase survival in cold environments, such as building deeper nests that insulate the fungi from cold surface.

KEYWORDS
Attina, cold tolerance, fungus-growing ants, *Leucocoprinus*, symbiosis, *Trachymyrmex*

1 | INTRODUCTION

Symbioses exist among almost every organism on the planet and typically consist of two or more organisms that are often in persistent, physical and mutually beneficial contact with one another (Borror & White, 1998; Bronstein, 2015; Kattes, 2009). Such mutualistic symbioses between microbial symbionts and eukaryotic hosts have generated diverse ecological and evolutionary important transitions (Douglas, 2010, 2015; Russell & Moran, 2006; Szathmáry, 2015). Symbionts can have profound effects on the fitness, adaptation and range distribution of its host (Hofmann & Todgham, 2010; Márquez et al., 2007; McFall-Ngai et al., 2013; Wernegreen & Wheeler, 2009). A key advance in recent years is appreciating that symbioses are composed of multiple moving parts; the outcome of the interaction can be determined by the performance of the component parts that may vary in their susceptibility to environmental stress (Márquez et al., 2007; Russell & Moran, 2006; Silverstein et al., 2012; Wernegreen & Wheeler, 2009) to the extent that extinction of the interaction could result if environmental extremes exceed the tolerance of one or more partners (Rafferty et al., 2015; Warren II & Bradford, 2014).

In addition to short-term plastic responses, host–symbiont relationships may respond evolutionarily when one or both partners are subjected to ongoing environmental stress (Harmon et al., 2009; Mueller et al., 2011). However, stress-induced evolution is often more difficult to show in obligate symbioses compared to facultative symbiont reassociation, since many obligate symbionts are not free-living or able to be cultured in the laboratory. Fungus-gardening ants cultivate an external fungal symbiont as their primary food source (Hölldobler & Wilson, 2011; Schultz et al., 2015; Schultz & Brady, 2008). Attine ants present a unique study system to investigate co-evolution in two or more organisms because the fungal symbiont can be cultured in the laboratory and ants and fungi can be switched experimentally, making it possible to disentangle and observe consequences of novel host–symbiont pairings on performance and behaviour (DeMilto et al., 2017; Mehdiabadi et al., 2006; Seal et al., 2014; Seal & Mueller, 2014; Seal & Tschinkel, 2007a). Although fungus-gardening ant symbioses have been very successful at colonizing the temperate zone from tropical origins (Branstetter et al., 2017; Farji Brener & Ruggiero, 1994; Mueller et al., 2011; Senula et al., 2019), there have been very few studies that have investigated the adaptive evolution with respect to cold tolerance experimentally. For example, fungi from the northernmost populations of *Atta texana* had increased survivorship when exposed experimentally to cold temperatures compared to fungal symbionts found in southern populations (Mueller et al., 2011). The evolution of a cold-tolerant fungal cultivar and the translocation of fungal gardens to deeper chambers within the ant mound allowed this attine species to expand their ecological niche northward into previously uninhabitable habitats. Cold-tolerant fungal cultivars likely increase colony fitness and garden productivity, resulting in longer fungus-growing seasons or increased garden output under colder temperatures (Mueller et al., 2011). Although it seems intuitive that northward expansion was accompanied by increased cold tolerance, no studies have examined the cold tolerance of attine ants or tested for significant ant–fungal interactions, especially because ants are likely exposed to greater temperature fluctuations than the fungus growing in temperature-buffered underground chambers.

Trachymyrmex is a northern subtropical to temperate genus with all species found in southern North America (Guatemala to US; Solomon et al., 2019). North American *Trachymyrmex* species are thought to have been originally adapted to survive in dry, arid seasonal environments (Branstetter et al., 2017; Rabeling et al., 2007; Seal & Tschinkel, 2006; Seal & Tschinkel, 2010), with one species, *T. septentrionalis*, expanding well into temperate northeastern North America (>30°N) and thus experiences a wetter and cooler climate compared to those found in the arid southwest (Mikheyev et al., 2008; Seal et al., 2015; Senula et al., 2019). *Trachymyrmex septentrionalis* is found from central Texas to Illinois, along the coast of the Gulf of Mexico to Florida, and along the Atlantic coast as far north as Long Island, New York (approx. 40°N; Rabeling et al., 2007). *Trachymyrmex septentrionalis* is a widespread species; their biomass can be considerable in sandy soil of pine forests, with over 1,000 nests present in a single hectare moving significant amounts of soil annually (Seal & Tschinkel, 2006; Tschinkel & Seal, 2016). Ecological modelling indicated that the range of *T. septentrionalis* is limited by cold temperatures (Senula et al., 2019).

In this study, we investigate the cold tolerance of *Trachymyrmex septentrionalis* by performing cold-stress assays comparing two populations collected from a southern site located near the southernmost range of the species distribution (Bastrop, Texas) and from a northern site that is approximately 650 km further north (Norman, Oklahoma) and relatively close to the northern range limit at that longitude. First, we wanted to determine cold tolerance of fungal cultivars in artificial culture in the absence of ants. We predicted that fungus from the northern collection site would be more cold tolerant compared to fungus from the southern collection site. Second, we tested whether there exists an ant–fungal synergism in cold tolerance by conducting a fully balanced, cross-fostered experiment that consisted of either northern or southern ants growing either northern or southern fungus. We predicted that ant presence would have an interactive effect on the cold tolerance of the entire symbiosis, where northern ants and northern fungus would be the most tolerant to cold, southern ants and southern fungus would be the least tolerant, and the cross-fostered groups (northern ants with southern fungus and southern ants with northern fungus) would have an intermediate tolerance to cold.

2 | MATERIALS AND METHODS

2.1 | Colony collection and maintenance

Live *Trachymyrmex septentrionalis* colonies were collected between April and June 2017 using the methods of Seal and Tschinkel (2006, 2008). Colonies were collected in two different

sites: Norman, Oklahoma (35°12′36.22″N, 97°30′09.34″W) on 22–24 May 2017 (colonies from northern population), or Bastrop, Texas at the University of Texas' Stengl 'Lost Pines' Biological Station (30°5′13.1″N, 97°10′25.5″W) on 6–8 April 2017 (colonies from southern population). Permits were not required at these locations. Norman, Oklahoma is near the northeastern range limit of *T. septentrionalis*, which is likely in sand prairies of north central Oklahoma and southeastern Kansas (DuBois, 1981, 1985). Likewise, the southern population is near the southwestern range limit of *T. septentrionalis* (Seal et al., 2015). The southern location is located on the western edge of the southeastern coastal plain, a global biodiversity hotspot, in a mixed loblolly pine *Pinus taeda* and post oak *Quercus stellata* savanna. The northern population is located along sandy ridges adjacent to the Canadian River in the southern Great Plains, and the habitat is characterized by post oaks and eastern redcedar *Juniperus virginiana*. Although *T. septentrionalis* is not unknown in the Great Plains, it appears to be patchily distributed and associated with isolated sand ridges or prairies throughout Oklahoma, south-east Kansas, Missouri, northeastern Arkansas and southern Illinois. The linear distance between the northern and southern populations is approximately 650 km. We aimed to excavate complete ant colonies, obtaining all ants, all fungus gardens (typically 2 or 3 gardens) and queen(s). Colonies were collected by excavating a small pit approximately 30 cm from the nest entrance. Tunnels and fungal garden chambers were found by carefully removing soil from the side of the pit towards the ant nest with a trowel, putty knife and kitchen spoons. All tunnels were traced to collect all ants (Seal & Tschinkel, 2007a, 2007b, 2008). Because both mean and variation in temperature decrease with depth and the location of chambers likely reflects suitable fungiculture conditions (Seal & Tschinkel, 2006), measurements were made of each chamber's depth by one investigator. Only nests where we were reasonably confident we had collected all ants (including the queen) and found all chambers were analysed. To increase our sample size of chamber depths, we included colonies that were additionally collected in 2015. The depth at which a colony occurs in the spring reflects the depth at which the colony excavated its chamber in the preceding fall; subsequent nest depth increases result from activity in the late spring and summer (Seal & Tschinkel, 2006). As a result, the depths observed in this study were reflective of the depth at which the colony spent the preceding winter. Nest depths were analysed using one-way ANOVA. Upon collection, ants were placed in plaster-lined trays coated in Fluon© (Northern Products) to prevent escape. Ants were kept in collection trays for 1–2 days to allow ants to clean debris from the fungus. Once the fungal garden was cleaned, all fungus and ants were moved into 7.5 × 7.5 × 3 cm plastic containers lined with water-saturated dental plaster on the bottom and a plastic lid to prevent ants from escaping. Foraging containers of the same dimensions were connected using plastic tubing. Fungal chambers were added as needed to allow for colony growth. All colonies were fed weekly with a mixed diet of polenta, rose petals or oak catkins. Although diet was variable, colonies always received the same type and approximately amount of food on feeding days. Distilled water was added to fungal boxes as needed to prevent colonies from desiccation. While the ants and fungi were treated humanely throughout the study, approval from an animal ethics committee was not required.

2.2 | Fungal isolation for cold tolerance assay

To obtain fungal isolations, sterile forceps were used to take a small part of the fungal garden from the colony and placed into a small petri dish. The fungus was then further separated into smaller pieces and put onto sterile PDA agar plates. The plates were wrapped in parafilm and placed into an incubator at 22.5°. Plates were checked daily for growth. If plates were contaminated, they were discarded immediately, unless the fungal sample was salvageable. This was performed until pure fungal strains were isolated from each collected colony. These isolates were kept in the incubator at 22.5° until they attained growth of about 1 cm in diameter. In total, eight fungal cultures from Norman, Oklahoma (Northern population) and nine fungal cultures from Bastrop, Texas (Southern population) were isolated.

2.3 | Fungal cold tolerance assay

To determine fungal garden cold tolerance, we examined cold tolerance of the fungal symbiont by quantifying viability and survivorship after being exposed to gradually decreasing temperatures (Mueller et al., 2011). Each colony fungal sample from each collection site was subcultured onto sterile plates with 10 ml of PDA agar in each plate. Only one piece of similar sized fungus was placed onto each plate. Fungal isolates from each colony from both Northern and Southern collection sites were subcultured onto 30 separate small agar plates. Each plate was wrapped in parafilm and placed into the incubator 22.5° for 6 weeks to grow to a size of approximately 1 cm in diameter.

For each fungal isolate, the 30 plates were split between two box freezers (Insignia NS-CZ35WH7) to control for slight differences in humidity or air flow. Each freezer was temperature controlled by its own Inkbird Itc-308 Digital Temperature Controller Outlet Thermostat and was monitored by its own Elitech RC-5 USB Temperature Data Logger. Freezer (or cold incubator) temperatures were monitored daily so ensure they were staying at a consistent temperature.

Cold tolerance assays repeated the method of Mueller et al. (2011) that examined the impact of reducing temperatures from ambient to 2°C over the course of 42 days. Accordingly, temperatures were gradually decreased by increments of 4°C for the first half of the experiment to imitate the gradual cooling from Fall to Winter. This temperature range includes what southern colonies likely experience during the winter (Figure S1). On Day 0 of the experiment, 15 plates for each colony were moved into either cold incubator (set at 10°C) to start the regime of the decreasingly colder temperatures. The temperature was set to 10°C and was kept at

this temperature for the first 4 days of the experiment. On day 4, the temperature was then lowered by 4°C and stayed at this temperature for another 4 days (10°C for 4 days, then 6°C for 4 days). On day 8, the temperature was lowered again by 4°C to 2°C and then stayed at this temperature for 16 days, totalling four 4-day cycles at a constant temperature of 2°C. The total process took 24 days to complete and there were six 4-day cycles. At the end of each 4-day cycle, before lowering the temperature, two plates from each colony from each freezer were randomly chosen to be moved into a flow hood for subculturing isolates onto a corresponding set of 'recovery plates'. Subculturing involved cutting a 3 mm by 5 mm strip radially into the fungal mycelium, to ensure that both young and old mycelia were transferred onto the recovery plate. During the recovery stage, the plates were kept at room temperature (23°) to allow fungal mycelium recovery. At this time, pictures were taken once a week to account for weekly growth and recovery rate. This was done for a total 6 weeks after each set of recovery plates was made. Area (mm^2) of fungal culture was measured for each sample using ImageJ (Rueden et al., 2017). Total number of fungal plates measured from each population in each temperature regime varied, as some recovery plates were contaminated, and measurements were not taken after contamination was noted.

2.4 | Ant–fungal cross-fostering experiment

To determine the proximate contribution of each member of the ant–fungal symbiosis (and possible ant–fungal interactions) to the cold tolerance in the overall symbiosis, we conducted a cross-fostering experiment. We performed this experiment by creating small colonies of 15 workers and small fungus garden fragments to place into the cold-temperature incubators. These experiments were conducted during the period of this seasonal species when gardens are largest and the ants most active (June/July; Seal & Tschinkel, 2006, 2008). We created four types of colonies reflecting all possible combinations: northern ants and northern fungus ($n = 18$), southern ants and southern fungus ($n = 18$), northern ants and southern fungus ($n = 18$), and southern ants and northern fungus ($n = 18$). Initially, there were 20 colonies of southern ants growing northern fungus, but two of which were outliers and had exceptionally high residual values; thus, these were excluded from analysis. Thus, the design was balanced. Replicates were created for each subcolony (one for each freezer). We used ants from nine colonies from the southern population and nine colonies from the northern population. We used fungal clones from one colony from the northern population but two from the southern population. Two source colonies of southern fungus were used because northern colonies unexpectedly rejected the southern fungus over the period of 8 days such that our initial fungus supply had been exhausted (see Results). As a result, we used fungus from another southern colony as source fungus. All colonies in the cross-fostering experiment received a fungus from the B4 clade using the terminology of Luiso et al. (2020). Fungal lineage was not replicated since suitable molecular markers that could differentiate within clade diversity of fungi are lacking. For example, all of the B4 fungi collected in Oklahoma and Texas have identical ITS sequences (Luiso et al., 2020).

Ants and gardens were physically separated, then reassembled by treatment, following an established cultivar switch procedure (Seal & Mueller, 2014; Seal & Tschinkel, 2007a). On 21st May 2018, workers were taken from queenright colonies that had at least 100 individual ants and placed into plaster-lined petri dishes that had a smaller unlined petri dish lid in the centre, without fungus. Ants were monitored for 48 hr to make sure the ants did not retain any of their native fungus or possess brood items. If there was any evidence of fungus or brood, these items were immediately discarded. Colonies were watered with a few drops of distilled water every 2–3 days during the entirety of the experiment. Fresh food (polenta, catkins, frass, rose petals) was provided every 2–3 days. All colonies were given approximately the same amount of food and water. Colonies were cleaned before every feeding session by removing old food and cleaning the small plastic petri dish with 70% ethanol solution. If ants rejected the fungus, the fungus was removed and new fungus provided until the ants accepted the cultivar.

Once subcolonies were established, they remained at room temperature for 3 weeks to allow ants and fungus to become acclimated. On 14th June 2018, duplicate sets of subcolonies were separated into two chest freezers described above in the fungal cold tolerance experiment, as to control for slight differences in humidity or air flow. On Day 0 of the experiment, the temperature was set to 10°C and was kept at this temperature for the first 4 days of the experiment. The decreasing temperature regime was identical to the fungal cold tolerance experiment, except the colonies were only at 2°C for 12 days (10°C for 4 days, 6°C for 4 days, 2°C for 12 days). The total process took 20 days to complete. Unlike the fungal cold tolerance, all subcolonies remained in the freezers for the entire duration of the cold temperature regime. Water and food were provided, though ants became decreasingly inactive as the temperatures decreased.

Subcolonies were taken out of the cold temperatures on 4th July 2018 and allowed to become reacclimated to room temperature. On July 5th, the number of dead ants was recorded. Ant survival was recorded daily for 2 months. Fungal-garden size was also measured weekly for 2 months. The area (mm^2) of the fungal garden was measured using ImageJ (Rueden et al., 2017). If a subcolony lost its garden, they were not given new fungus and garden size was measured as 0. All colonies received the same amount of distilled water and food regardless of garden size and health.

2.5 | Data analysis

For the isolated fungal growth curves, the data were fit using a linear mixed growth model with the LME4 package on R (Bates et al., 2015). The fixed effects of the model consisted of time, temperature, population and all interactions of the three. Random effects were added to the time factor using the interaction of colony and freezer. The model was optimized to fit a growth transformation, where

the transform of growth is equal to $\log_{30}(\text{growth} + 30)$. After fitting the model, estimated marginal means were calculated for each population and temperature interaction levels (emmeans). The estimated marginal means were then compared pairwise using a *t*-test; the Tukey method was used to adjust the *p* values for multiple comparisons.

The performance (net growth) of fungus was analysed in the cross-fostering experiment using a factorial ANOVA containing the main effects of freezer, fungal origin, ant origin and an interaction term between fungal and ant origin. While the full model was a nested factorial with fungal and ant origin nested within freezer, the effect of freezer was not significant and pooled in all subsequent analyses. Fungal garden sizes were transformed by the following: $\ln(\text{garden} + 1)$. Each level combination of each factor was compared post-hoc using the Tukey method.

The performance of ants was quantified using Kaplan–Meier survival estimators. Individual survival curves were then compared pairwise using a log-rank test. *p* values were adjusted for multiple comparisons using the false discovery rate (FDR) adjustment method. Survival analysis was done with the SURVIVAL package on R (Therneau, 2022).

2.6 | Genetic identification of fungal symbionts of *T. septentrionalis*

Since *T. septentrionalis* is known to associate with a diverse group of fungal symbionts, we genotyped fungi collected at both northern and southern locations. DNA extraction and sequencing followed established protocols (Luiso et al., 2020). Fungal samples (clusters of hyphae) were collected using flame-sterilized forceps from laboratory colonies and placed in 200 µl of a 20% Chelex (Sigma-Aldrich) solution. Samples were incubated for 1.5 h at 60°C followed by 15 min at 99°C. The supernatant was then diluted 10-fold. Primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) were used to amplify ~600 bp of the internal transcribed spacer (ITS; White et al., 1990). The resulting sequences from this study were genotyped and published previously and deposited on Genbank under accession numbers: MK1424008–MK142436, MK142441–142445 and MK142395–MK142396 (Luiso et al., 2020). Data have been posted on Dryad Digital Repository (Senula et al., 2022).

3 | RESULTS

3.1 | Fungal cold tolerance

Growth curves were generated for fungi from the northern and southern site according to each temperature treatment. Each growth curves were composed of seven measurements, recorded first when fungus was placed on the recovery plate (to serve as zero growth), and then once weekly for 6 weeks after the fungus was taken out of cold incubator and placed on the recovery plate. When northern and southern fungus was exposed to 10°C for 4 days, there was no difference in growth between these two types of fungi during the recovery phase ($p = 0.2536$, $t = 2.69$, $df = 68.5$; Figure 1a). Fungus that was exposed to 10°C for 4 days and 6°C for 4 days both had a significant decrease in growth during the recovery phase when compared to fungus exposed to 10°C for 4 days: north fungus ($p < 0.0001$, $t = 6.13$, $df = 2672.7$) and south fungus ($p < 0.0001$, $t = 25.304$, $df = 2672.7$; Figure 1b). Furthermore, northern fungus and southern fungus differed after this temperature exposure ($p < 0.0001$, $t = 7.908$, $df = 61.4$), with southern fungus growing less (Figure 1b). When fungus was exposed to 10°C for 4 days, 6°C for 4 days and 2°C for 4 days, there was no decrease in recovery growth by northern fungus ($p = 0.2501$, $t = -2.654$, $df = 2670.9$); however, southern fungus had a significant decrease in recovery growth; in fact, no southern fungus was able to grow beyond this point (10°C for 4 days, 6°C for 4 days and 2°C for 4 days) in the experiment growth ($p < 0.0001$, $t = -9.455$, $df = 61.5$; Figure 1c). While growth decreased in northern fungi in progressively colder temperature regimes (Figure 1d–f), northern fungus nevertheless grew more than southern fungi 10°C for 4 days, 6°C for 4 days and 2°C for 8 days ($p < 0.0001$, $t = 6.097$, $df = 2670.9$; Figure 1d), 10°C for 4 days, 6°C for 4 days and 2°C for 12 days,($p < 0.0001$, $t = 7.47$, $df = 2671$; Figure 1e), and 10°C for 4 days, 6°C for 4 days, and 2°C for 16 days ($p < 0.0001$, $t = 12.18$, $df = 2670.7$; Figure 1f).

3.2 | Ant–fungal cross-foster experiments: Tests for ant–fungal synergism

Fungal growth did not vary between the freezers used for experiments ($F_{1,65} = 0.018$, $p = 0.895$; Freezer A growth 215.45 ± 236.56 mm vs. Freezer B172.55 ± 183.65 mm); consequently, the data from the two freezers were pooled in all subsequent analyses. After being exposed to the cold regime, gardens in the combination of southern ants with southern fungus gardens were significantly smaller than all other subcolonies ($F_{1,68} = 44.5$, $p < 0.0001$). All but two subcolonies from the southern ants with southern fungus had fungal gardens that perished after exposure to the cold-temperature regime. Two months post-cold-incubation, subcolonies with northern ants and northern fungus did not differ in fungal garden size from subcolonies with northern ants with southern fungus or from subcolonies of southern ants with northern fungus (Figure 2). The low growth of southern ants and southern fungi when exposed to cold appears to be driving significant ant and fungal population effects ($F_{1,68} = 27.18$, $p < 0.0001$) or fungal origin ($F_{1,68} = 74.2$, $p < 0.0001$, $p < 0.0001$), respectively. In summary, the main statistical effect was driven by the low growth rates of southern ants growing southern fungus (Figure 2).

Similar to the measures of garden size above, survival of ants in subcolonies with southern ants and southern fungus was the lowest in the manipulated temperatures relative to all other ant–fungal combinations (Kaplan–Meier Test $p < 0.001$, Figure 3). Subcolonies

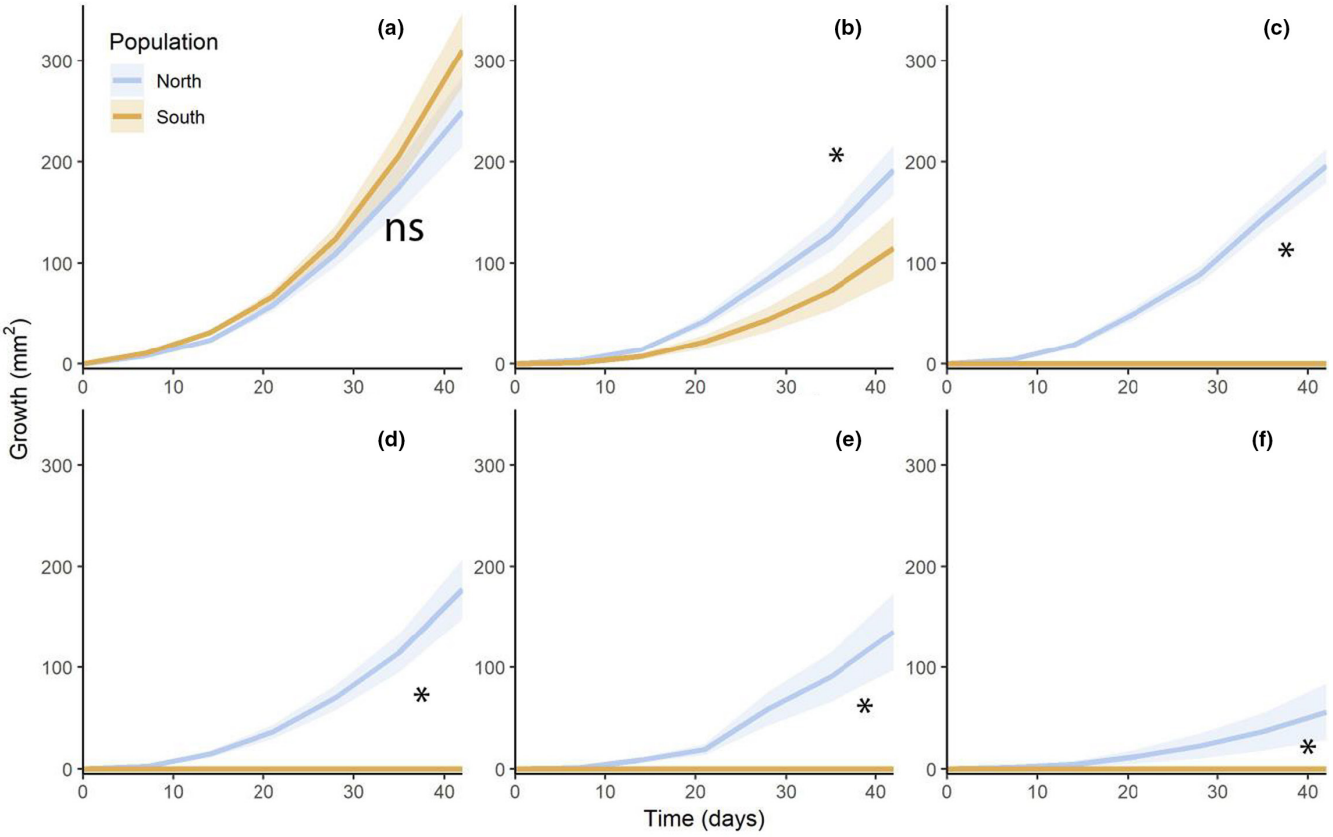

FIGURE 1 Fungal symbionts from the northern population exhibit higher cold tolerance than those from southern populations. Growth curves represent Northern and Southern populations of fungus after exposed to a cold temperature regime: (a) 10°C for 4 days, (b) 10°C for 4 days, 6°C for 4 days, (c) 10°C for 4 days, 6°C for 4 days, 2°C for 4 days, (d) 10°C for 4 days, 6°C for 4 days, 2°C for 8 days, (e) 10°C for 4 days, 6°C for 4 days, 2°C for 12 days, (f) 10°C for 4 days, 6°C for 4 days, 2°C for 16 days. Cold stress increased from a to f. Curves depict growth after fungus was taken out of cold temperature regime and placed on recovery plate at room temperature. Asterisks indicate significant differences ($\alpha < 0.0001$) in fungal growth between populations during the recovery phase.

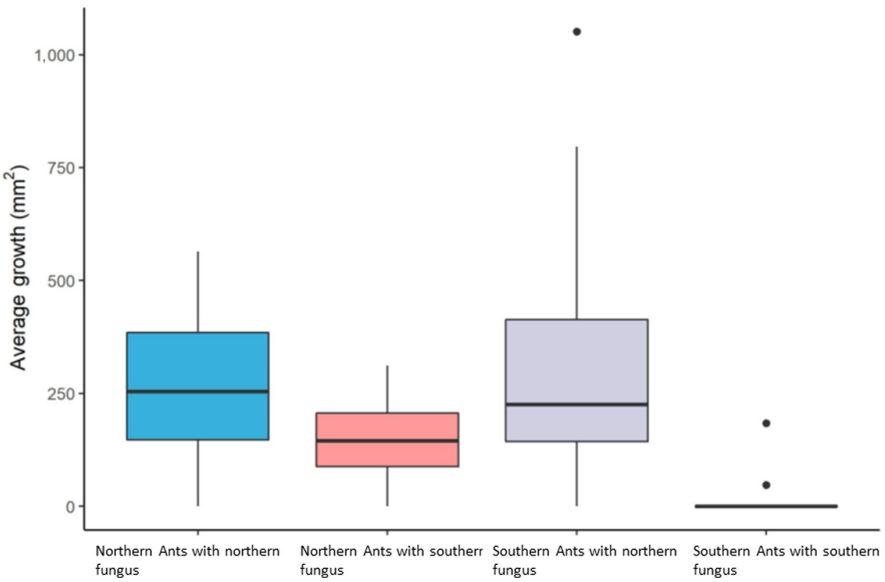

FIGURE 2 Average area of fungus gardens (mm^2) in subcolonies 2 months after being exposed to cold temperatures. Fungus gardens were the smallest in southern ant colonies growing southern fungi when exposed to cold. Asterisks (*) indicate significant differences in subcolony fungus garden size.

composed of northern ants and northern fungus did not differ in ant probability of survival from subcolonies with northern ants and southern fungus ($p = 0.88$) or from subcolonies with southern ants and northern fungus ($p = 0.61$). Subcolonies with northern ants and southern fungus also did not differ from subcolonies with southern ants and northern fungus ($p = 0.61$).

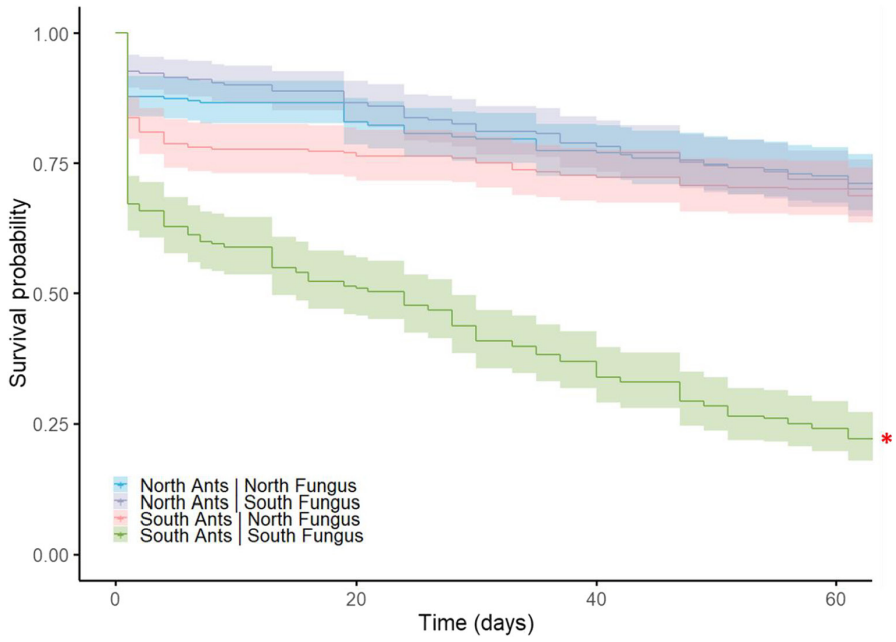

FIGURE 3 Colony survivorship after being exposed to cold temperatures. Ant survival was recorded for 64 days once taken out of cold incubators. Asterisks (*) indicate differences in ant survival probability.

TABLE 1 Numbers of colonies that initially accepted or rejected the novel fungus. Most colonies accepted the novel fungi regardless of source, except that most northern colonies initially rejected fungus from the southern population.

Combination	Accepted	Rejected	Chi-square	*p*-value
Northern Ants/Northern Fungus	16	2	0.222	<0.637
Northern Ants/Southern Fungus	**6**	**12**	**8**	**<0.005**
Southern Ants/Northern Fungus	18	0	0	1
Southern Ants/Southern Fungus	16	4	0.8	<0.371

Bold values indicate significant of *p*-values < 0.05.

3.3 | Behavioural differences of acceptance by ant population

While most colonies accepted the new fungus in the cross-fostering experiment, colonies from the north population rejected the fungus from the southern population (Table 1). All colonies accepted the provided garden material within 7 days of the start of the manipulations.

3.4 | Regional differences in Nest depth

Colonies in the two locations had two different architectures. Northern colonies had significantly deeper fungus gardens spread across fewer chambers, whereas those in the southern location had chambers closer to the surface. Northern colonies contained two chambers, whereas southern colonies had three chambers during the collection period. For example, the first chamber in the north was significantly deeper than the first chamber in the south [18.58 ± 6.04 (*SD*) vs. 13.15 ± 3.51; $F_{1,23} = 7.77$, $p = 0.0.01$; Figure 4]. Similarly, the second chamber was also significantly deeper in northern populations than southern populations [36.5 ± 7.69 (*SD*) vs. 27.23 ± 6.63; $F_{1,19} = 8.58$, $p = 0.009$; Figure 4]. Five of the 13 southern colonies also had a third chamber that was either in the process of being excavated or that also contained fungus. This third chamber was approximately the same depth as the second chamber of northern colonies (>31 cm). None of the northern colonies were observed to have more than two chambers.

3.5 | Fungal genotyping

The vast majority of *T. septentrionalis* colonies used in this study were found growing 'B4' fungi, which is the most common fungal clade found in North American *Trachymyrmex* species (Luiso et al., 2020). The two exceptions were two colonies growing 'B5' fungi at the northern population. While B5 fungi exhibited lower performance than the B4 fungi at some of the cold treatments, this was not true for all cold treatments (Figure S2). Similarly, the inclusion of the 'B5 colonies' (Colonies JNS170523-7 and SS170523-5; ants that were found growing B5 fungi at the northern site) in the cross-fostering experiment did not appear to influence the outcome on the growth of the fungus garden. For instance, gardens of the northern ants

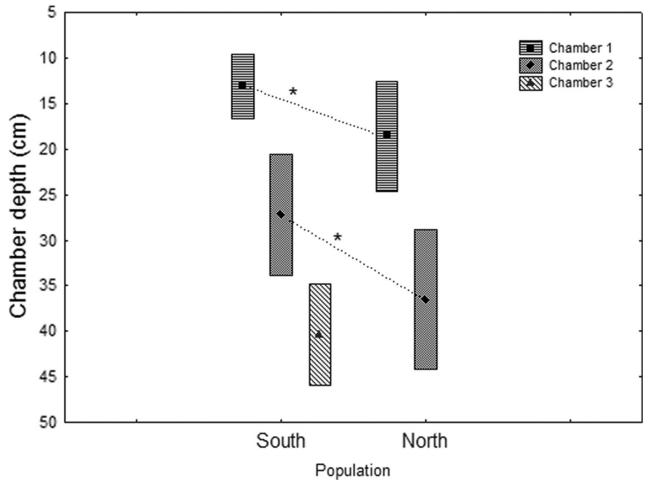

FIGURE 4 Mean nest depth (cm) in a northern and a southern population of *Trachymyrmex septentrionalis*. *T. septentrionalis* constructs a series of chambers at increasing depths, and hatched lines indicate comparable chambers in the chamber series of the two populations. Northern fungus-garden chambers are significantly deeper than southern fungus-garden chambers. Northern populations lacked a third chamber (Chamber 3) but northern second chambers were at a depth similar to the third chamber of southern colonies. Boxes correspond to one standard deviation of the mean. Asterisks indicate significant differences ($\alpha = 0.05$).

growing northern B4 fungus were not significantly larger than the gardens of these ants growing southern B4 fungus [137.45 ± 56.1 cm vs. 194.4 ± 86.3 (mean ± 1 *SD*); $F_{1,6} = 1.23$, $p = 0.31$].

4 | DISCUSSION

The overarching aim of this study was to investigate the cold tolerance of partners comprising the obligate symbiosis, *T. septentrionalis* ants and their cultivated fungi, comparing populations collected near the southernmost range of their distribution (Bastrop, Texas) and populations collected from a site that is approximately 600 km further north (Norman, Oklahoma). The goal was to determine whether cold tolerance was a function of ant or fungal traits, or arose from a synergistic interaction between ants and fungus. The most significant finding is that cold tolerance arises from the interaction so that only one cold-adapted partner would appear to be required for the symbiosis to survive cold conditions. When southern fungus was grown by northern ants and then subjected to cold temperatures, we found that the southern fungus survives the entire duration of cold and then grows during the recovery phase similar to ants growing northern fungus. This could indicate behavioural differences between northern and southern ant populations. Similarly, we found southern ant survival while growing northern fungus is on par with that of northern ants growing either northern fungus or southern fungus. Additionally, deeper nests and increased behavioural preference in northern latitudes may be important in a cold environment, should an ant colony or fungus end up with a non-cold-adapted partner.

By examining the cold tolerance and thus adaptive evolution of this symbiosis, we may be able to predict how other ectotherms may adapt and respond to global climate change. Many studies have addressed climate change responses in symbioses, though terrestrial ectotherm symbioses studies are limited. For the first time, we were able to disassemble a fungal gardening ant symbiosis and observe cold tolerance in two populations from areas that differ in an average low temperature by about 5°C. We have documented that when grown without the presence of ants, northern populations of fungus are more cold tolerant than southern populations of fungus. Furthermore, we have shown that there is an ant effect on fungal cold tolerance and survivability, and likewise, there is a fungus effect on ant survivability when exposed to cold temperatures. More work is needed to understand why certain populations are more cold tolerant than others, such as physiological or behavioural differences.

The precise mechanisms that are involved in cold tolerance of ants or fungi are largely unknown. Studies of polar fungi indicates that fungi may employ a variety of strategies that include the secretion of antifreeze compounds (alcohols, proteins, enzymes) or avoidance strategies such as spore formation during winter (Robinson, 2001; Tsuji et al., 2013) or psychrophilic metabolism (Leung et al., 2011). Since attine fungi are rarely known to produce spores, it seems that any adaptations to cold would likely involve physiological modifications crucial to growth in colder conditions. Although ant fungi likely originated in warm, lowland neotropics (Branstetter et al., 2017; Mueller et al., 2001; Nygaard et al., 2016), the ant fungi are known to be temperature sensitive, with temperatures around 25°C appearing to be optimal to the extent that the ants go to great lengths to maintain suitable conditions for their symbiont (Bollazzi et al., 2008; Bollazzi & Roces, 2002; Mueller et al., 2011; Powell & Stradling, 1986; Seal & Tschinkel, 2006). Moreover, temperatures above 25°C appear to be more stressful and even lethal than cooler temperatures (Bollazzi & Roces, 2002; Powell & Stradling, 1986). Future work will need to examine whether the fungal symbionts known to associate with *T. septentrionalis* (Luiso et al., 2020) along northern range margins are similarly cold adapted or may persist if their ant hosts are cold adapted as the results of this study predict.

It is well established that cold temperatures have been a significant selective force in ants. Cold tolerance as a strategy may require acclimation (Modlmeier et al., 2012) and apparent trade-offs, since many species exhibit increased cold tolerance in northern latitudes (Heinze et al., 1998; Nguyen et al., 2019; Stanton-Geddes et al., 2016; Tonione et al., 2020). Some species appear to withstand cold by with social traits either by sharing food (Heinze et al., 1996) or thermoregulatory behaviours (Kadochová et al., 2017). Consequently, cold has been an important ecological factor that can influence distributions (Bishop et al., 2017; Warren II & Chick, 2013). Unknown is how cold but above freezing temperatures can suppress growth. For example, cool yet above freezing temperatures not only inhibit foraging yet can cause death within a short period (Brightwell et al., 2010; Porter, 1988;

Tschinkel, 2006). This suggests that metabolism requires temperatures well above freezing to function adequately. Populations in mountains appear to have a higher metabolic rate than those in lower elevations (Lytle et al., 2020). Insects are known to employ a variety of strategies that range from extreme cases where insects can tolerate freezing for several months of the year [e.g. the arctic moth *Gynaephora groenlandica* (Wocke 1874)] to where some life-history stages escape freezing by migration or the synthesis of antifreeze compounds (e.g. monarch butterflies *Danaus plexippus* Linnaus 1758 or the moth *Epiblema scudderiana* Clemens, 1860) (Bale, 1987; Bale, 1993; Bale & Hayward, 2010; Chown & Nicholson, 2004; Storey, 1990). Unlike most insects that can survive cold weather by modifications of one life-history stage, ant colonies are perennial and are often composed of several life-history stages at once. Unknown are biochemical adaptations used by *Trachymyrmex septentrionalis*. Winter (November through March, depending on latitude), dormant behaviour consists of ants occupying a chamber they excavated the previous fall and ants forming a ball surrounding a highly reduced fungus garden (Seal & Tschinkel, 2006, 2008).

One of the more surprising findings was the apparent rejection of southern fungus by northern ants. The pattern of rejection behaviour of ants tearing apart the novel fungi was very similar to that of incompatibility reactions that have been proposed to maintain specificity (fungal monocultures within each colony) in the ant–fungal symbiosis (Bot et al., 2001; Poulsen et al., 2009; Poulsen & Boomsma, 2005). Curiously, forcing *T. septentrionalis* to grow distantly related fungi from 'Clade A' [a fungus this species does not grow in nature (Mueller et al., 2018)] did not result in similar incompatibility reactions (Seal & Mueller, 2014; Seal & Tschinkel, 2007a); rather specificity appears to be maintained via a mechanism that allows for temporary polycultures in *T. septentrionalis* as colonies gradually revert back towards their original fungus (Seal et al., 2012). The apparent higher degree of preference of northern ants towards northern fungus could be a safety mechanism to avoid ants from cultivating a fungus that might not tolerate cold conditions as are more frequent in northern latitudes, though it remains to be determined experimentally that the cues the ants are using are directly related to cold tolerance.

Another possible behavioural safeguard to cold suboptimal conditions is the excavation of deeper nests in northern populations. Chambers in which *T. septentrionalis* overwinter are likely constructed the previous fall (Seal & Tschinkel, 2006, 2008); thus, the chamber depths we observe were not likely an artefact of observing nests at different times (northern colonies were collected approximately 1 month after the southern colonies). Other factors that could influence nest depth are grain size that will influence heat loss and gas exchange. Interestingly, nests in this study were still shallower than those reported in northern Florida (Seal & Tschinkel, 2006, 2008) where the first chamber is typically deeper than 30 cm, which is close to the deepest chambers in Texas and Oklahoma; while this could be due to the younger sands in Florida (quaternary origin vs. tertiary origin of soils in central Texas and Oklahoma [Brown et al., 1990; Diggs et al., 2006]) and larger sand grains, a recent distribution model did not support a significant impact of soil type in explaining *T. septentrionalis* distributions (Senula et al., 2019). It is possible that behavioural plasticity is sufficient in this species to build nests that are influenced more by climate than soil.

Frequently in east and central Texas, we have found *T. septentrionalis* nests nearly exposed to the surface, the first chamber can be very shallow and covered by leaf-litter rather than soil. Colonies in such an environment might benefit from exposure to heat and accelerate growth, but at the same time might expose them to extreme temperatures and desiccation [*T. septentrionalis* is not known to be desiccation resistant (Hood & Tschinkel, 1990)]. Typically, this top most chamber contains fungus only during the spring (March–mid-May in central Texas) when it is warm and humid but not yet hot (daytime temperature < 32°C, unpublished observations). Some of this pattern might be driven by the more continental climate of Oklahoma—long winters and abrupt transitions to summer from winter or spring so that a period where colonies may 'bask' in warm humid air may short if not lacking altogether, whereas the southern population is <250 km from the Gulf of Mexico and consequently experiences gradual transitions from winter to spring. Generally, there might be trade-offs between temperature, humidity and growth rate, among other factors (Bollazzi & Roces, 2010; Roces & Kleineidam, 2000) that could drive variation in nest architecture and location of the fungus garden (Seal & Tschinkel, 2006). Future work might want to examine trade-offs between cold and heat tolerance and desiccation and growth in northern and southern populations, an understanding of which will be crucial in understanding how the flora and fauna of the southeastern US may respond to climate change (Warren II & Chick, 2013).

AUTHORS' CONTRIBUTIONS

K.K., J.N.S. and U.G.M. conceived the ideas and designed the methodology; S.S.F., K.K. and J.N.S. designed the experiments; S.S.F. collected the data; S.S.F. and J.T.S. analysed the data; K.K. and S.S.F. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data are available from the Dryad Digital Repository https://doi.org/10.5061/dryad.c866t1g8p (Senula et al., 2022).

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SUPPORTING INFORMATION

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