

COOPERATION UNDER CHANGING CONDITIONS:
TESTS OF MUTUALISM THEORY IN LEGUME-RHIZOBIUM SYSTEMS

Mackenzie Allen Caple

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Doctoral Committee

Jennifer A. Lau, Ph.D.

Jay T. Lennon, Ph.D.

Irene L. G. Newton, Ph.D.

Heather L. Reynolds, Ph.D.

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Cooperation between leguminous plants and nitrogen-fixing rhizobium bacteria is a critical component of global nitrogen cycling. However, evolutionary and mutualism theory predict that increased soil nitrogen will disrupt this mutualism. I explored the effects of soil nitrogen on legume-rhizobium mutualism with a combination of greenhouse and field studies. First, I grew field-collected plants and soil microbes from across a natural soil nitrogen gradient with three levels of nitrogen fertilizer to study how soil nitrogen contributes to local adaptation. Although plants from high-nitrogen sites were more plastic in their allocation of resources to rhizobia than plants from low-nitrogen sites, I only found local adaptation of rhizobia to high-nitrogen sites; there was no evidence for plant local adaptation to N. Second, I used a field experiment to study the effects of the 2021 emergence of Brood X cicadas, which should result in a natural nitrogen pulse, on wild legumes through changes in maternal effects and soil microbial communities. I found that decaying cicadas affected multiple generations of plants: seeds from plants amended with cicadas were more likely to germinate, and soil microbial communities from cicada-addition plots accelerated early seedling growth. Finally, I experimentally evolved soil microbial communities in the greenhouse to investigate the direct and indirect (light and host availability) pathways by which nitrogen fertilization of plant communities can lead to a decline in microbial mutualism, and whether the mutualism decline observed in the field can be reversed by ceasing fertilization. I found that no single factor caused strong mutualism decline, but that any combination of two or three factors caused soil microbes

to be less beneficial to plant growth. However, soil microbes from nitrogen-addition field plots did not become more beneficial to plants after evolving in low-nitrogen greenhouse conditions. Together, these results demonstrate how the complexities of real-world conditions complicate the predictions of simple theoretical frameworks and highlight the importance of considering the broader biotic community context in studies of evolutionary ecology.

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CHAPTER 1:

ASYMMETRIC LOCAL ADAPTATION TO NITROGEN IN A PLANT-MICROBE SYMBIOSIS

ABSTRACT

Local adaptation is widely documented, but selective agents contributing to local adaptation are rarely identified. Soil nitrogen (N) availability and microbes, including N-fixing rhizobia, have large effects on plant traits and may contribute to plant local adaptation. We grew twelve *Amphicarpaea bracteata* populations and four microbial communities from across a naturally occurring soil N gradient in three levels of contemporary N to investigate whether N and/or soil microbes contribute to plant local adaptation. We found that plants with a history of high N shifted investment from mutualism to reproduction when grown in high N while plants from low-N sites did not, suggesting that mutualism-related traits have diverged across the N gradient, but we detected no evidence for plant local adaptation favoring sympatric partners. In contrast, rhizobia exhibited local adaptation to high N: under high contemporary N, rhizobia from high-N sites formed greater nodule mass than rhizobia from low-N sites. However, rhizobium local adaptation did not translate into microbe-mediated plant adaptive plasticity, as plant fitness was not affected by microbial N history. This study shows that despite theory leading us to expect adaptation, soil N and its effect on mutualism may not always be a contributor to plant local adaptation, although N may affect interactions between plants and microbes.

INTRODUCTION

Local adaptation (when local genotypes have higher fitness than non-local genotypes) has long been a dominant paradigm in plant evolutionary ecology. Despite hundreds of studies testing for local adaptation with classic approaches (e.g., reciprocal transplant experiments where genotypes are transplanted between sites, and common gardens, where genotypes from multiple sites are grown in a common environment; reviewed in Briscoe Runquist et al., 2020; Hargreaves et al., 2020; Leimu & Fischer, 2008), most studies are unable to identify the selective agents that cause local adaptation because many possible selective agents covary across sites. As a result, we have only a limited understanding of the ecological forces responsible for the vast diversity of plant form and function observed across populations in nature, though geographic variation in both abiotic and biotic factors undoubtedly contributes. Early studies documented plant local adaptation to sites strongly differing in key abiotic factors, such as elevation (Clausen et al., 1941), soil type (Kruckeberg, 1951), or heavy metals (Shaw et al., 1987). However, most studies, even recent studies, do not disentangle the abiotic environment from biotic factors that covary with abiotic conditions (Hargreaves et al., 2020), despite the fact that biotic factors can act as strong selective agents driving local adaptation on their own or may interact with the abiotic environment to enhance or inhibit local adaptation (Briscoe Runquist et al., 2020).

Microbes may be particularly strong, yet cryptic, biotic mediators and drivers of plant local adaptation (Bolin & Lau, 2024). Microbial communities and populations often vary across the same abiotic environmental gradients that many evolutionary biologists might assume drive plant local adaptation (e.g., Johnson et al., 2010). Further, microbial communities and populations of key symbionts can have large effects on plant fitness, and plants can be adapted or maladapted to local microbial populations, such as specific pathogens (e.g., Parker, 1985) or

mutualists (e.g., Parker, 1995; Wilkinson et al., 1996). As a result, reciprocal transplant experiments across sites that differ in abiotic conditions actually might be documenting adaptation to local microbial populations rather than to the more obvious abiotic environmental factor (e.g., Sherrard & Maherali, 2012). In addition to potentially being a hidden factor driving local adaptation, microbes also may mediate plant local adaptation to abiotic conditions through at least two mechanisms. First, plant genotypes can select for particular rhizosphere microbial communities by promoting the growth of certain microbial taxa through exudates or chemical signaling (Huang et al., 2014), and these selected root microbiomes might be the trait (or, in this case, the extended phenotype) underlying local adaptation. For example, plant-recruited microbes facilitate local adaptation to drought in pinyon pines: when microbes are absent, “drought-tolerant” genotypes are no more fit than “drought-intolerant” genotypes in a simulated drought in a common garden (Gehring et al., 2017). Second, the microbial community might shift in response to environmental variation in ways that maximize plant fitness in that environment, reducing differential selection between environments and essentially eliminating the need for plant evolutionary adaptation; these microbial shifts can cause local adaptation-like patterns of plant fitness in the absence of plant genotypic variation if local microbes are more effective partners than non-local microbes (microbe-mediated adaptive plasticity; Petipas et al., 2021). Microbes can, therefore, either promote or limit plant local adaptation.

Interactions between plants and their belowground resource mutualists like mycorrhizae or rhizobia provide particularly strong examples of how microbes can influence plant adaptation to the abiotic environment, especially to spatial variation in nutrient availability (e.g., Johnson et al., 2010; Kiers et al., 2002; Porter et al., 2011; Schultz et al., 2001). First, sympatric microbial mutualists may promote plant local adaptation because coevolved microbial genotypes can be

better at acquiring resources for their hosts. For example, sympatric combinations of mycorrhizae and big bluestem interacted more intensely (more arbuscules formed, more resources exchanged) and increased plant fitness and acquisition of limiting resources more than allopatric combinations (Johnson et al., 2010), although other studies have found no differences in benefit from sympatric vs. allopatric microbes (Barrett et al., 2012; Harrison et al., 2017; Heath, 2010). Second, extensive mutualism theory predicts that increased nutrient availability will affect the evolution of both plants and microbial partners, selecting for plants that more weakly associate with resource mutualists in high nutrient environments (Akçay & Simms, 2011; Schwartz & Hoeksema, 1998) and for microbes that provide reduced benefits to the host plant (Akçay & Simms, 2011; West, Kiers, Pen, et al., 2002; West, Kiers, Simms, et al., 2002). Such evolutionary shifts in mutualism-related traits may lead to plant adaptation to local soil nitrogen (N) conditions that is largely due to how the benefits and costs of associating with mutualists are affected by nutrient availability rather than due to the direct effects of nutrients on plants.

Leguminous plants associating with N-fixing rhizobium bacteria are an ideal system for investigating how diverse soil microbial communities and specific symbionts may contribute to plant local adaptation. This is a tractable system to test ecological and evolutionary questions about the mechanisms of microbe-mediated plant local adaptation across resource gradients because resources and plant-microbe combinations are readily manipulated in the greenhouse. Furthermore, past work suggests that plants are sometimes locally adapted to sympatric partners (Parker, 1995; Wilkinson et al., 1996), that N can influence the abundance and evolution of rhizobium symbionts (Weese et al., 2015), and that plant fitness depends on both rhizobium genotypes as well as N availability (Heath, 2010; Van Cauwenberghe et al., 2016). These results suggest that both N and mutualists have the potential to contribute to plant local adaptation. It is

also possible that rhizobium populations are locally adapted to their host plants (e.g. Murray-Stoker & Johnson, 2024; Van Cauwenberghe et al., 2016, but see Heath, 2010).

Here, we investigate whether local adaptation of a native legume is driven by soil N and/or soil microbial communities and whether plant investment in rhizobium mutualists varies with evolutionary history. We grew plant populations originating from across a natural N gradient with diverse soil microbial communities collected from sympatric and allopatric sites and manipulated N fertilization to assess plant adaptation and responses to soil N, as well as how microbial communities may influence these responses. We hypothesized that plants would exhibit local adaptation to soil N because resources levels are expected to be particularly important selective agents for resource mutualists (Thrall et al., 2007), that plant adaptation to N would be stronger (greater fitness benefit of growing in N conditions similar to the sites where the plants originated) in the presence of live microbes because biotic interactions generally strengthen local adaptation (Briscoe Runquist et al., 2020, but see Hargreaves et al., 2020) and because stressful environments are predicted to increase the benefits of mutualism (Johnson et al., 2010; O'Brien et al., 2018; Thrall et al., 2007), and that plant fitness would be maximized when microbe N history and contemporary N match because well-adapted partners can provide greater benefits (Johnson et al., 2010). Based on classic theory (Akçay & Simms, 2011; West, Kiers, Simms, et al., 2002), we also hypothesized that plants from high-N environments have reduced participation in mutualism such that plants originating from high-N sites invest relatively less in rhizobium symbionts. Finally, we investigated whether plants and/or rhizobia are locally adapted to their symbiotic partners. We hypothesized that reciprocal coevolution in traits influencing the likelihood of association would increase compatibility between mutualist partners with a history of sympatry (Parker, 1995; Wilkinson et al., 1996).

MATERIALS AND METHODS

Overview

To test whether plants have adapted to historical N conditions, whether microbes contribute to plant local adaptation, and whether plants and rhizobia are co-adapted to local N conditions and to each other, we grew plant populations from sites that varied in soil N content in combination with soil microbial communities collected from a subset of these sites in the greenhouse under three levels of N fertilization.

Natural history and site information

Amphicarpaea bracteata (American hog-peanut; *Amphicarpaea* hereafter) is an annual vining legume native to forest understories of eastern North America that produces chasmogamous (outcrossing) aboveground flowers and cleistogamous (self-fertilized) above- and belowground flowers (Schnee & Waller, 1986). In the field, the large majority of seeds produced are from cleistogamous flowers, and populations are generally highly inbred with low genetic variation (Parker, 1986; Schnee & Waller, 1986). The low genetic variation and gene flow in wild *Amphicarpaea* populations make these populations ideal for testing interpopulation variability and local adaptation because genetic variation is likely greater between than within populations (Parker, 1985). *Amphicarpaea* associates with *Bradyrhizobium spp.* bacteria (Parker & Kennedy, 2006), which form nodules on the plants' roots and fix atmospheric N into ammonia in exchange for photosynthetically derived carbon.

Belowground seeds were collected in 2011 from 12 sites across southwest Michigan that varied in soil N content from 3.47-19.97 $\mu\text{g N/g soil}$ (Table S1, Figure S1; Suwa 2016). Soil N was measured at each site in two consecutive years and did not vary significantly between years

(Suwa, 2016). The range of soil N levels at our sites is similar to the average difference between fertilized and unfertilized field plots in a long-term N addition experiment (mean total N in unfertilized plots: 4.7 $\mu\text{g N/g soil}$; fertilized plots, 14.2 $\mu\text{g N/g soil}$; Gross & Lau, 2022) that demonstrated effects such as plant diversity loss (Dickson & Gross, 2013) and a reduction in rhizobium quality (Weese et al., 2015). Each plant ‘population’ was a discrete patch (approximate order of magnitude 100 m^2); there were often several populations located within one km of each other separated by unoccupied spaces larger than the occupied patches. Field-collected seeds were grown in the greenhouse for one generation to propagate seeds and reduce maternal effects. Aboveground seeds produced by self-fertilized flowers in this initial greenhouse generation were used for this study.

Greenhouse experiment

Soil collection

On 4 January 2019 we collected approximately 250 mL of soil at a depth of 10-20 cm directly beneath each of three individual *Amphicarpaea* plants growing in each of four sites in southwest Michigan and kept them refrigerated until use (one month). Three of these soil collections were from three of the plant collection sites described above, while the fourth was collected from a nearby *Amphicarpaea* population (within 1 km; see Table S1, Figure S1) because other populations could not be found or were inaccessible due to flooding.

Seed preparation and greenhouse conditions

We surface-sterilized all seeds by rinsing in bleach (5.25% NaClO) for one min and in distilled water for ten min. We then scarified each seed to break dormancy and placed them on damp filter paper in Petri dishes to germinate in the dark. On 31 January 2019 (6-10 d after

scarification), we planted 60 germinants from each plant population individually into 10 cm diameter round terra cotta pots filled with sterilized potting mix (3:3:3:1 compost:peat:sand:perlite). This was a low-nutrient potting mix, so to avoid trace nutrient deficiency, we applied diluted N-free trace element fertilizer (50 mL of 0.19 mg/L Peters Professional S.T.E.M.) to each plant on 7 March 2019. After seedling emergence, we added a three-foot bamboo stake (sterilized with Physan 20 {Maril Products Inc, Tustin, CA}) to each pot so plants could climb, and all pots were watered as needed; not all plants were given the same amount of water, but plants varied enormously in biomass and this was preferable to some plants being overwatered while others became drought-stressed. Supplemental lighting providing approximately 450 $\mu\text{mol}/\text{m}^2/\text{s}$ PAR (photosynthetically active radiation) was set to a 14 hr day. *Amphicarpaea* at field sites in southwest Michigan naturally experience PAR levels ranging from 10 to >1300 $\mu\text{mol}/\text{m}^2/\text{s}$ (Suwa, 2016).

Greenhouse treatments

To manipulate soil microbiota, we prepared live soil inoculants from each of the four soil collection sites by combining 10 mL field soil (~5g dry mass) with 40 mL distilled water to create a slurry. We prepared a sterile control by mixing 2.5 mL soil from each field site, autoclaving the mixture (120°C for two 2-hr periods with 24 hr rest between), and combining the 10 mL sterilized soil with 40 mL distilled water to create a slurry. We inoculated each pot with either 2 mL of one of the four live soil inoculates on 7 February 2019 or with 2 mL sterilized soil slurry on 8 February 2019. To manipulate soil N, we treated each plant with 4 mL distilled water (“low contemporary N” treatment), 4 mL of 200 mg N/L solution (571 mg ammonium nitrate/L; “medium contemporary N” treatment), or 4 mL of 400 mg N/L solution (1143 mg ammonium nitrate/L; “high contemporary N” treatment) on 4 March, 20 March, and 11 April 2019.

Hereafter, we will use “contemporary N” to refer to the N fertilization manipulation in the greenhouse, and “N history” or “historical N” to refer to the soil N levels measured at the field sites where seeds and soils were collected. Although we did not add any N fertilizer to our low N treatment, we chose to call this treatment “low N” rather than “no N” because the plants were not grown in N-free media—plants had access to limited N from the compost included in our potting media. These N fertilizer quantities were chosen to mirror the range of historical soil N concentrations found in our field sites (Table S1) and were sufficient to significantly affect plant growth (see Results): in total, we added 8.3 $\mu\text{g N/g}$ soil to the medium contemporary N treatment and 16.6 $\mu\text{g N/g}$ soil to the high contemporary N treatment. We did not measure the N content of the soil collected to make our microbial inoculants, but the amount of N that plants received from the slurries was negligible compared to our contemporary N fertilizer treatments: based on site soil N measurements, the slurries added approximately 0.1-0.3 $\mu\text{g N/g}$ soil to each pot. Although this means that plants inoculated with soil from high-N sites received more than twice as much N from the slurry than plants inoculated with soil from low-N sites, the N received from the soil slurry was an order of magnitude less than our fertilizer manipulations. We planted four replicates per treatment combination in a fully factorial design: N = 5 inoculants (4 live soil microbial communities + sterilized control inoculant) \times 12 plant populations \times 3 levels of contemporary N fertilization \times 4 replicates = 720 plants in 180 treatment combinations, which included 48 different combinations of plant populations and microbial communities (Table S2). Plants were placed randomly on greenhouse benches in a non-blocked design.

Fitness and resource allocation measures

Plant fitness: We collected aboveground fruits as they matured. As plants began to senesce, we harvested above- and belowground biomass (24 Apr-9 Aug 2019, 90-197 d since

scarifying). There was large variation in when plants were harvested, but because *Amphicarpaea* is an annual and we harvested plants when they began to senesce, our data captures total lifetime fitness of each plant and growth time should therefore be considered a response rather than a predictor. Nevertheless, we investigated whether accounting for growth time variation affected our results. Date of harvest did not vary significantly with plant ($p = 0.46$) or microbe ($p = 0.54$) N history and including growth time as a covariate in our statistical models did not qualitatively affect our results. Belowground biomass was stored at 4 °C until processed (roots washed to remove potting media, belowground seeds counted, nodules counted and collected). We weighed above- and belowground biomass after drying for 3 d at 60 °C. We measured three components of plant fitness: survival, seed production, and biomass. There was high mortality in our plants (see Results), but survival was not significantly affected by plant or microbe N history, so we present results of survival analyses only in the Supplemental Information (Appendix A). We present seed production as our main proxy of plant fitness and report biomass results only in the Supplemental Information because seed production is likely a better fitness metric for *Amphicarpaea* (which is an annual and does not reproduce vegetatively) and because total biomass and total seeds were positively correlated ($\rho = 0.65$) with only minor qualitative differences in analysis results.

Rhizobium fitness: As plants were harvested, we counted and weighed fresh root nodules. We measured the strength of interactions between plants and rhizobium mutualists by using nodule presence, nodule number, and mean nodule mass as proxies of rhizobium fitness. We used a hurdle model to analyze nodule traits, but nodule presence rarely differed between treatments, so nodule presence results are reported only in the Supplemental Information (Tables S3-S5, Figure S2). Because we waited until plants began to senesce to collect nodules, some nodules

also had begun to senesce. We counted all nodules but calculated mean nodule mass from intact nodules only. Total nodule mass was calculated by multiplying mean nodule mass by nodule number to estimate the total mass of nodules before senescence. Our nodule number data are therefore more reliable than our nodule mass data, so we used nodule number as our primary measure of mutualism interaction. Nodule number and total nodule mass were highly positively correlated; however, the correlation was stronger in low ($\rho = 0.72$) and medium ($\rho = 0.78$) contemporary N than in high ($\rho = 0.29$) contemporary N. Analyses using total nodule mass had few qualitative differences from our nodule number results (see Tables S3-S4).

Resource allocation: We investigated how historical and contemporary N affects plant resource allocation by calculating root:shoot ratios (relative investment in nutrient foraging vs. growth and reproduction: dry belowground biomass / dry aboveground biomass), nodules:root biomass (relative investment in mutualism vs. nutrient foraging: nodule number / g dry belowground biomass), and seeds:nodule number (relative investment in reproduction vs. mutualism: total seed count / nodule number). For nodules:root biomass and seeds:nodule we saw few qualitative differences if nodule number was replaced with total nodule mass (Tables S3-S4, Figure S2).

Statistical analyses

Overview

Due to high mortality (275: 39.2%; Table S3, Figure S2) and contamination (17: 18.7% of plants inoculated with sterilized control inoculant formed nodules), our final dataset included 428 plants unevenly distributed across treatment combinations (Table S2). The majority of mortality occurred after treatment application began, so treatments could not be reassigned among surviving plants to even out replication between treatments. Control plants that formed

nodules indicate that there was some cross-contamination of live microbes in our experiment, but contaminated control plants formed fewer nodules than nodulating plants that were inoculated with live microbes (control: 1-11, mean 2.8; live inoculants: 1-1245, mean 57.3). Pot treatments were randomly spatially assigned, so microbial cross-contamination among pots would make our results more conservative by homogenizing microbial communities among treatments. To ensure results were not driven by a few non-representative individuals (especially for ratios such as root:shoot and seeds:nodule, where extreme values are magnified) we removed statistical outliers (more than three times interquartile range below the first quartile or above the third quartile) from each response variable before analysis. Removing outliers had only minor qualitative effects on results.

Our plant populations spanned a wide range of soil N, so we modelled plant history as a continuous variable. Our microbial communities spanned a smaller range of soil N (Table S1) and were cleanly grouped into three discrete levels, so we explored modeling microbe N history as a categorical variable. However, treating microbe N history as categorical did not have a qualitative effect on any of our outcomes of interest. Therefore, we also treated microbe N history as a continuous variable. Contemporary N was modeled as a categorical variable.

All analyses were performed in R v.4.2.2 (R Core Team, 2020). We fit models using the *glmmTMB* package (Brooks et al., 2017) and assessed significance using Type III Wald chi-squared tests with the `Anova` function from the *car* package (Fox & Weisberg, 2019). We checked model fits using the `simulateResiduals` function from the *DHARMA* package (Hartig, 2022), calculated estimated marginal means using the *emmeans* package (Lenth, 2022), and used *ggplot2* (Wickham et al., 2024) to create figures.

Are plants locally adapted to soil N conditions, do plants from different evolutionary histories invest differently in microbial mutualists, and do microbes contribute to plant acclimation?

To test for plant local adaptation to soil N, we fit generalized linear mixed models (GLMMs) with plant N history, contemporary N, and their interaction as fixed effects and microbial community and maternal line (nested within plant population) as random effects to account for variation between plant populations beyond that explained by differences in contemporary or historical soil N (model structure: $\text{response} \sim \text{plant N history} * \text{contemporary N} + (1|\text{microbial community}) + (1|\text{plant population/maternal line})$). A significant plant N history \times contemporary N interaction, such that plant fitness is maximized when historical plant N and contemporary N match, would indicate plant local adaptation. Similarly, a significant plant N history \times contemporary N interaction for nodule-related traits would indicate that plant evolutionary history affects the plasticity of plant investment in rhizobium resource mutualists.

Plant local adaptation may result from variation among plant populations in how they interact with locally adapted soil microbes or with microbes in general (rather than specific microbial communities). To test whether plant local adaptation to N depended on microbial communities that also were adapted to local soil N, we fit GLMMs with plant N history, microbe N history, contemporary N, and all interactions as fixed effects; we also included microbial community and maternal line (nested within plant population) as random effects (model structure: $\text{response} \sim \text{plant N history} * \text{microbe N history} * \text{contemporary N} + (1|\text{microbial community}) + (1|\text{plant population/maternal line})$). To test whether plant local adaptation was dependent on the presence of live microbes rather than specific microbial communities, we fit GLMMs as above but replaced microbe N history with a binary variable indicating whether live soil microbes were added (model structure: $\text{response} \sim \text{plant N history} * \text{microbe presence} *$

contemporary N + (1|plant population/maternal line)). We omitted the random effect of microbial community in these models because microbial community identity is fully confounded with microbe presence. If plant local adaptation to N largely results from variation in plant traits that mediate interactions with microbes, then we expect a significant three-way interaction between microbe presence or N history, plant N history, and contemporary N, with plant fitness maximized by the presence of live soil microbes or microbes that match plant historical N and contemporary N conditions.

To test for microbe-mediated adaptive plasticity, we fit GLMMs with microbe N history, contemporary N, and their interaction as fixed effects and microbial community and maternal line (nested within plant population) as random effects (model structure: response ~ microbe N history * contemporary N + (1|microbial community) + (1|plant population/maternal line)). Microbe-mediated adaptive plasticity would be indicated by a significant interaction between microbe N history and contemporary N, with plant fitness maximized when contemporary and historical N conditions match.

Are plants and/or microbes locally adapted to their sympatric partners?

To test whether plants and microbes were locally adapted to each other, we restricted our data to plants and microbes from the three sites where we collected both seeds and soils, so each population and community had a sympatric partner (3 sites, N = 82 plants; Table S2). We fit GLMMs with plant population, microbial community, contemporary N, and the interaction between plant population and microbial community as fixed factors, and maternal line as a random effect (model structure: response ~ plant population * microbial community + contemporary N + (1|maternal line)). Interactions with contemporary N were not included in these analyses due to insufficient statistical power. Evidence for local adaptation to mutualist

partners would be indicated by a significant interaction between plant population and microbial community, with sympatric combinations of symbiotic partners having higher fitness than allopatric combinations. We then further explored plant population interactions with different soil microbial communities ($G \times G$ interactions) more generally by running the same models on the full data set (Table S2).

RESULTS

Are plants locally adapted to soil N conditions, do plants from different evolutionary histories invest differently in microbial mutualists, and do microbes contribute to plant acclimation?

Plant local adaptation: Although plant populations varied in every trait measured (Table S5a), we found no evidence for plant local adaptation to N, regardless of the presence or evolutionary history of microbes (no significant plant N history \times contemporary N {Table 1, Figure 1}, plant N history \times microbe presence \times contemporary N {Table S6, Figure S4}, or plant N history \times microbe N history \times contemporary N {Table S7, Figure S5} effects on plant fitness metrics: all $p > 0.2$).

Plant investment in mutualism: Investment in mutualism may contribute to local adaptation to N because investment in N-fixing mutualists has a large carbon cost (Kaschuk et al., 2009). Consistent with predictions, we found a trend for high N history plants to form fewer nodules relative to root mass when grown in high compared to low contemporary N (plant N history \times contemporary N: nodules:root mass, $\chi^2 = 4.9$, $p = 0.09$, Table 1, Figure 1). In contrast, low N history plants did not alter their investment in rhizobia in response to contemporary N. This difference was not due to high N history plants forming fewer but larger nodules: mean

nodule mass did not vary with plant N history or contemporary N (plant N history: $\chi^2 = 2.44$, $p = 0.12$, contemporary N: $\chi^2 = 3.97$, $p = 0.14$, plant N history \times contemporary N: $\chi^2 = 3.78$, $p = 0.15$, Table 1, Figure 1). Plant and rhizobium fitness were generally weakly but positively correlated (total nodule mass vs. total seeds: low N, $\rho = 0.05$; med N, $\rho = 0.17$; high N, $\rho = 0.19$; nodule number vs. total seeds: low N, $\rho = 0.10$; med N, $\rho = 0.27$; high N, $\rho = 0.11$), indicating that investment in mutualism benefitted plants across environments. However, relative investment in rhizobia depended on microbe N history and contemporary N (microbe N history \times contemporary N: seeds:nodule, $\chi^2 = 10.37$, $p < 0.01$, Table 2, Figure 2). When inoculated with low N history microbes, plants invested relatively more in reproduction than mutualism when grown in high contemporary N (i.e., higher seeds:nodule) and invested more in rhizobia when grown in low contemporary N (i.e., lower seeds:nodule). However, plant relative investment was unaffected by contemporary N when plants were inoculated with high N history microbes (microbe N history \times contemporary N: seeds:nodule, $\chi^2 = 10.37$, $p < 0.01$, Table 2, Figure 2).

Microbe-mediated adaptive plasticity: One way microbes might inhibit local adaptation is if local microbes better maintain plant fitness in that particular environment. Although we detected no evidence for microbe mediation of plant fitness in our system (no significant microbe N history \times contemporary N effects on plant fitness metrics: all $p > 0.2$, Table 2, Figure 2), relative plant allocation to nodulation vs. root growth was highest when microbe N history and contemporary N matched (microbe N history \times contemporary N: nodules:root mass, $\chi^2 = 10.48$, $p < 0.01$, Table 2, Figure 2).

Rhizobium local adaptation: While we did not detect evidence for plant local adaptation to N, our results are consistent with local adaptation of rhizobia to high-N conditions. Under high contemporary N, plants inoculated with high N history microbes produced more, larger nodules

than plants inoculated with low N history microbes, while under low contemporary N plants produced similar nodule number and average mass regardless of microbe N history (microbe N history \times contemporary N: nodule number, $\chi^2 = 8.98$, $p < 0.05$; mean nodule mass: $\chi^2 = 8.83$, $p < 0.05$; Table 2, Figure 2).

Are plants and/or microbes locally adapted to their sympatric partners?

Despite limited evidence for plant local adaptation to N, in our full analysis of all plant populations and microbial communities (12 plant populations \times 4 microbial communities = 48 combinations, Table S2), fitness alignment and plant population differences in traits related to plant investment in mutualism depended on the microbial community the plants were inoculated with (plant population \times microbial community: seeds:nodule, $\chi^2 = 68.41$, $p < 0.0001$; nodules:root biomass, $\chi^2 = 79.18$, $p < 0.0001$; Table 3a, Figure S10). Rhizobium fitness proxies also varied across specific plant population-microbial community combinations (plant population \times microbial community: total nodule mass, $\chi^2 = 47.91$, $p < 0.05$, Table 3a, Figure S9), but plant growth and fitness traits did not (no significant plant population \times microbial community interactions on seed number, biomass, or root:shoot ratios: all $p > 0.5$; Table 3a, Figure S8).

In our more restricted dataset of three plant populations growing with sympatric vs. allopatric microbial communities, we did not find evidence that plants were locally adapted to sympatric partners (plant population \times microbial community: seeds, $\chi^2 = 1.92$, $p = 0.75$; biomass, $\chi^2 = 2.33$, $p = 0.68$; Table 3b, Figure S8). However, there was a trend for one population of rhizobia (site NCD) to benefit more (higher nodule number and marginally more nodules per gram of roots) when grown with sympatric plants than with allopatric plants (plant population \times microbial community: nodule number, $\chi^2 = 9.62$, $p < 0.05$; nodules:root biomass, $\chi^2 = 7.85$, $p > 0.1$; Table 2b, Figures S9-10).

DISCUSSION

Although theory predicts that soil nitrogen (N) is a key factor in the ecology and evolution of plant populations and determines the outcome of legume-rhizobium interactions, our results imply that the effect of N on plant local adaptation may be weaker or more nuanced than often assumed. Specifically, we found that the evolutionary effect of N on plants is limited, primarily influencing the evolution of plant traits that reduce allocation to rhizobium mutualists under high N but not translating into plant local adaptation. We hypothesized that plant populations and rhizobium communities from across a naturally occurring soil N gradient would exhibit local adaptation to soil N levels, that plant local adaptation would be mediated by belowground microbial communities, and that both plants and rhizobia would benefit from sympatric mutualist partners. We found that plant populations from across the historical N gradient varied in plasticity of resource allocation to rhizobium mutualists, with populations that had evolved in high-N conditions investing relatively less in rhizobium mutualists when fertilized. We also detected some evidence for local adaptation of rhizobia such that rhizobium populations from high historical N had higher fitness than rhizobia from low historical N only in high contemporary N. However, this rhizobium adaptation did not translate to effects on plant local adaptation: no evidence for plant local adaptation was detected regardless of microbe presence or microbial N history. Finally, we found no consistent patterns suggesting that coevolution between mutualist partners increased partner fitness.

No evidence for plant local adaptation to N, but local adaptation of rhizobia at some sites

Despite observing effects of plant N history on traits mediating allocation to rhizobia, these trait differences did not translate into effects on plant fitness: we did not detect evidence for plant local adaptation regardless of the presence or evolutionary history of soil microbial

communities. Several mechanisms can inhibit local adaptation. First, just as microbial populations can promote local adaptation, they can also inhibit it if they reduce the fitness effects of the environmental factor that could otherwise drive differential selection and result in local adaptation (Petipas et al., 2021). We detected no evidence for this in our system; microbial communities from across the historical N gradient did not alter plant fitness responses to contemporary N. Second, gene flow from other populations swamping locally adapted phenotypes is often cited as a reason for a lack of local adaptation despite seemingly sufficient selection (reviewed in Tigano & Friesen, 2016), but is unlikely to be a factor in our system because *Amphicarpaea* is largely selfing and gene flow between wild populations is extremely low (Parker, 1986). Instead, our populations may have low opportunity for selection due to the low rate of outcrossing and low genetic variation of *Amphicarpaea* lineages in the field (Parker, 1986). Unfortunately, we cannot explicitly evaluate opportunity for selection within our populations because our study was designed to maximize the number of populations to better capture the natural N resource gradient rather than family level replication within populations.

In contrast to plants, rhizobia from high historical N produced more and larger nodules than rhizobia from low historical N when grown in high contemporary N, suggesting that rhizobia may be locally adapted to high N conditions. Greater nodule number and mass for high N history microbes in high contemporary N indicate that these rhizobia are well-adapted to elicit plant investment even when soil N is abundant. Our results are consistent with a previous study that also found that local adaptation in rhizobia was stronger than in their clover hosts, with sympatric pairings of plants and rhizobia outperforming allopatric pairings in relative investment in rhizobia (nodules:root length) but not plant biomass, especially when N-fertilized (Murray-Stoker & Johnson, 2024). Similarly, mycorrhizae along the Pacific coast of North America were

locally adapted to pine populations, while pines were locally adapted to edaphic factors correlating with elevation rather than to soil biota (Hoeksema & Thompson, 2007). This asymmetry in the strength of local adaptation between host plants and microbial symbionts could be due to the greater potential for rapid evolution in microbes than in plants due to extremely large population sizes and short generation times or could be due to differences in selection (Kaltz & Shykoff, 1998; Kawecki & Ebert, 2004). For example, the strongest selection on rhizobia could be from soil N altering plant investment in mutualists, while the strongest selection on plants may be from an unrelated environmental factor (e.g., in this system soil moisture seems to be a key selective agent promoting plant local adaptation; Suwa, 2016). Symbiosis may be a stronger selective force for rhizobia than for plants because rhizobia receive large fitness increases from successful nodulation (Denison, 2000). However, plants gain little or no benefit from investing in symbiosis when soil N is abundant or light (carbon) availability is low (Heath et al., 2020; Lau et al., 2012; Regus et al., 2017) and host plants often have strong control over rhizobia (Kiers & Denison, 2008; Oono et al., 2009). For example, plants reduce investment in rhizobia when carbon is limited or N is abundant (Friel & Friesen, 2019) and maintain selection for beneficial rhizobia despite prolonged N enrichment (Wendlandt et al., 2022). However, the observed rhizobium adaptation to N did not translate into plant fitness effects, as microbial N history did not affect plant fecundity.

Plant evolutionary history only predicts plant allocation to rhizobia in high nutrient environments

The effects of N enrichment on plant community composition are well-characterized (reviewed in Cleland and Harpole 2010), but there is still little empirical work evaluating N effects on natural selection and the evolution of plant traits within a species (but see Petipas et

al., 2020; Waterton et al., 2022). Historical soil N explained little of the variation between our plant populations, indicating that soil N has not been an important driver of the evolution of the plant traits we measured in the populations we sampled. The one exception is that plants that evolved in high-N soils invested less in rhizobia relative to root mass when fertilized, while plants from low-N sites did not alter resource investment in response to contemporary N. This difference in plasticity suggests that plants in high-N soil experience selection to only invest in rhizobium mutualists when they are most beneficial (i.e., in low N conditions), but plants evolving in low-N soils experience little or no selection on control over nodule provisioning. If soil N is always low, investing in N-fixing rhizobia is likely consistently beneficial and high investment in rhizobium mutualists could become canalized in low-N plant populations. Therefore, plants from low-N environments may be less plastic in resource investment, especially if there is a cost to plasticity. This same general pattern of reduced investment in resource mutualists in plant populations from high nutrient sites was also observed in mutualism between mycorrhizae and big bluestem, where plant ecotypes from higher-nutrient sites had reduced investment in AMF, although for bluestem the reduced investment was consistent when grown in high or low nutrient environments (Schultz et al., 2001).

No strong evidence for coevolution in mutualistic interactions

We found that plant and rhizobium fitness is positively, but weakly, aligned, suggesting that symbiotic coevolution in our system is mutualistic rather than antagonistic, consistent with previous studies (e.g., Friesen, 2012). However, we did not find local adaptation of plants and soil microbes to sympatric partners. This lack of mutual adaptation may be partially explained by our finding that under high contemporary N, rhizobia from high historical N elicit strong investment from plants across populations while, conversely, plants from high historical N limit

investment in rhizobium symbionts across microbial communities. This suggests that evolutionary conflict between plants and rhizobia may prevent mutual adaptation to high-N soils despite both partners benefitting from symbiosis. Our finding that plants did not generally benefit from sympatric microbes is inconsistent with previous work that found *Amphicarpaea* had higher fitness when grown with local than with foreign rhizobia (Parker, 1995; Wilkinson et al., 1996). The *Amphicarpaea* populations studied by Parker and Wilkinson originated from three different states, with sites separated by as much as 1000 km (Wilkinson et al., 1996), but the sites in this study have a much more localized distribution, with the furthest sites separated by less than 50 km (Table S1; Suwa, 2016). Although previous studies have found population differentiation for growth traits in sites separated by as little as 30 m (Parker, 1986), and although we also find population differentiation even at relatively small spatial scales (~300 m), the scale of local adaptation to soil microbes in particular may be larger than the scale of this study. For example, the *Amphicarpaea* populations studied here may not be as divergent in symbiotic compatibility traits that are predicted to coevolve, such as genes underlying partner recognition. However, our findings are also consistent with prior studies that found no local adaptation of legume hosts to rhizobium symbionts across scales of hundreds of km (Harrison et al., 2017) as well as smaller scales (Heath, 2010; Heath et al., 2010).

Our results are also consistent with Thompson's geographic mosaic theory of coevolution, which posits that coevolutionary interactions vary in strength and outcome across the landscape (i.e., coevolutionary 'hot' spots where reciprocal selection shapes interactions and 'cold' spots where reciprocal selection is weak or absent; Thompson, 1999). We detected interpopulation variation in symbiosis traits in both plants and rhizobia, and rhizobia from one site interacted more strongly with sympatric partners, possibly indicating that this site may be a

‘warmer’ site for coevolution. Theory suggests that low-nutrient sites are more likely to promote the evolution of strong mutualisms (Thrall et al., 2007), but we found no benefit of sympatry at our lowest-N site. Ultimately, despite theoretical predictions that legumes and rhizobia should experience strong pairwise coevolution, our study mainly found coevolutionary ‘cold’ spots rather than a mix of hot and cold.

Conclusions and future directions

Based on mutualism theory, we predicted that soil N and symbiotic interactions would be strong selective forces influencing local adaptation and coevolution in legumes and rhizobia. We investigated both biotic and abiotic factors potentially driving plant local adaptation and found that although soil N history of both plants and microbes affected relative plant investment in mutualism, the effects of N and microbes on plant evolution are not universal and are not always adaptive. Identifying the drivers of local adaptation is especially challenging when multiple potential drivers exist, particularly when these drivers interact with each other or when abiotic factors are mediated through cryptic biotic factors like belowground microbial communities and symbionts. Future theoretical and empirical work should go beyond testing for the presence of local adaptation and coevolutionary patterns and focus on identifying specific biotic and abiotic factors that make local adaptation and coevolution more likely to develop over ecological and evolutionary time.

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TABLES AND FIGURES

Table 1. Values shown are Type III Wald chi-squared tests for each fixed factor included in the mixed model ANOVA; p-values are shown in parentheses. Plants inoculated with sterilized control were excluded from this analysis. Nodule number, nodule mass, nodules:root biomass, and seeds:nodule analyses include only plants with at least one nodule present. Significant predictors are indicated by bold type and asterisks ($p < 0.001^{***}$, $p < 0.01^{**}$, $p < 0.05^*$, $p < 0.1^+$). Model structure: response \sim plant N history * contemporary N + (1|microbial community) + (1|plant population/maternal line). See Table S3 for additional response variables.

	<i>Df</i>	<i>Total seeds</i>	<i>Root: shoot</i>	<i>Nodule number</i>	<i>Mean nodule mass (g)</i>	<i>Nodules: root mass (g)</i>	<i>Seeds: nodule</i>
Plant N history	1	0.13 (0.717)	0.32 (0.573)	0.36 (0.550)	2.44 (0.119)	0.00 (0.964)	0.03 (0.857)
Contemp. N	2	33.49*** (<0.0001)	13.44** (0.001)	3.7 (0.157)	3.97 (0.137)	0.24 (0.888)	1.39 (0.500)
Plant N hist. × contemp. N	2	0.09 (0.958)	4.61+ (0.100)	1.51 (0.471)	3.78 (0.151)	4.94+ (0.085)	2.28 (0.320)

Table 2. Values shown are Type III Wald chi-squared tests for each fixed factor included in the mixed model ANOVA; p-values are shown in parentheses. Plants inoculated with sterilized control were excluded from this analysis. Nodule number, nodule mass, nodules:root biomass, and seeds:nodule analyses include only plants with at least one nodule present. Significant predictors are indicated by bold type and asterisks ($p < 0.001^{***}$, $p < 0.01^{**}$, $p < 0.05^*$, $p < 0.1+$). Model structure: response \sim microbe N history * contemporary N + (1|plant population/maternal line). See Table S4 for additional response variables.

	<i>Df</i>	<i>Total seeds</i>	<i>Root: shoot</i>	<i>Nodule number</i>	<i>Mean nodule mass (g)</i>	<i>Nodules: root mass (g)</i>	<i>Seeds: nodule</i>
Microbe N history	1	1.89 (0.169)	1.41 (0.236)	0.20 (0.657)	1.37 (0.242)	0.25 (0.616)	2.24 (0.135)
Contemp. N	2	18.77*** (<0.0001)	3.02 (0.221)	5.85+ (0.054)	11.3** (0.004)	16.22*** (<0.0001)	18.15*** (<0.0001)
Mic. N hist. × contemp. N	2	0.27 (0.875)	0.50 (0.777)	8.98* (0.011)	8.83* (0.012)	10.48** (0.005)	10.37** (0.006)

Table 3. Values shown are Type III Wald chi-squared tests for each fixed factor included in the mixed model ANOVA; p-values are shown in parentheses. Plants inoculated with sterilized control were excluded from these analyses. Nodule number, nodule mass, nodules:root biomass, and seeds:nodule analyses include only plants with at least one nodule present. Significant predictors are indicated by bold type and asterisks ($p < 0.001^{***}$, $p < 0.01^{**}$, $p < 0.05^*$, $p < 0.1+$). Model structure: response \sim plant population * microbial community + contemporary N + (1|maternal line). See Table S5 for additional response variables.

a. All populations/communities							
	<i>Df</i>	<i>Total seeds</i>	<i>Root: shoot</i>	<i>Nodule number</i>	<i>Mean nodule mass (g)</i>	<i>Nods:root biomass</i>	<i>Seeds: nodule</i>
Plant population	11	20.42* (0.04)	52.95*** (<0.0001)	71.79*** (<0.0001)	24.43* (0.011)	48.32*** (<0.0001)	62.61*** (<0.0001)
Microbial community	3	2.51 (0.474)	0.16 (0.984)	6.73+ (0.081)	0.12 (0.989)	5.88 (0.118)	6.85+ (0.077)
Contemporary N	2	132.96*** (<0.0001)	16.88*** (<0.0001)	6.99* (0.030)	3.77 (0.152)	9.07* (0.011)	15.23*** (<0.0001)
Plant pop. \times microbial comm.	33	21.55 (0.937)	28.45 (0.693)	93.28*** (<0.0001)	39.49 (0.203)	79.18*** (<0.0001)	68.41*** (<0.0001)
b. Only populations/communities with a sympatric partner							
	<i>Df</i>	<i>Total seeds</i>	<i>Root: shoot</i>	<i>Nodule number</i>	<i>Mean nodule mass (g)</i>	<i>Nods:root biomass</i>	<i>Seeds: nodule</i>
Plant population	2	0.34 (0.844)	15.9*** (<0.0001)	24.39*** (<0.0001)	0.86 (0.652)	22.13*** (<0.0001)	30.13*** (<0.0001)
Microbial community	2	0.47 (0.791)	0.02 (0.99)	0.97 (0.616)	2.25 (0.325)	0.54 (0.762)	3.11 (0.211)
Contemporary N	2	12.05** (0.002)	0.17 (0.916)	7.34* (0.025)	11.56** (0.003)	0.19 (0.911)	0.88 (0.644)
Plant pop. \times microbial comm.	4	1.92 (0.751)	1.33 (0.856)	9.62* (0.047)	2.34 (0.673)	7.85+ (0.097)	13.25* (0.01)

Figure 1: Plant local adaptation to N (plant N history × contemporary N).

Plant fitness did not correlate with plant N history (a). Root:shoot ratios of plants from low historical N were marginally more responsive to contemporary N (b) and there was a trend for plants from high historical N to decrease nodules:root mass (nodulation relative to plant size) in response to high contemporary N (e). Total nodule mass, mean nodule mass, and seeds:nodule (relative investment in reproduction vs mutualism) did not vary with historical or contemporary N (c, d, f). Dashed lines indicate marginally significant plant N history × contemporary N interactions; dotted lines indicate nonsignificant interactions. Filled points are plants inoculated with sympatric microbes. Note: vertical axes are presented on a log scale.

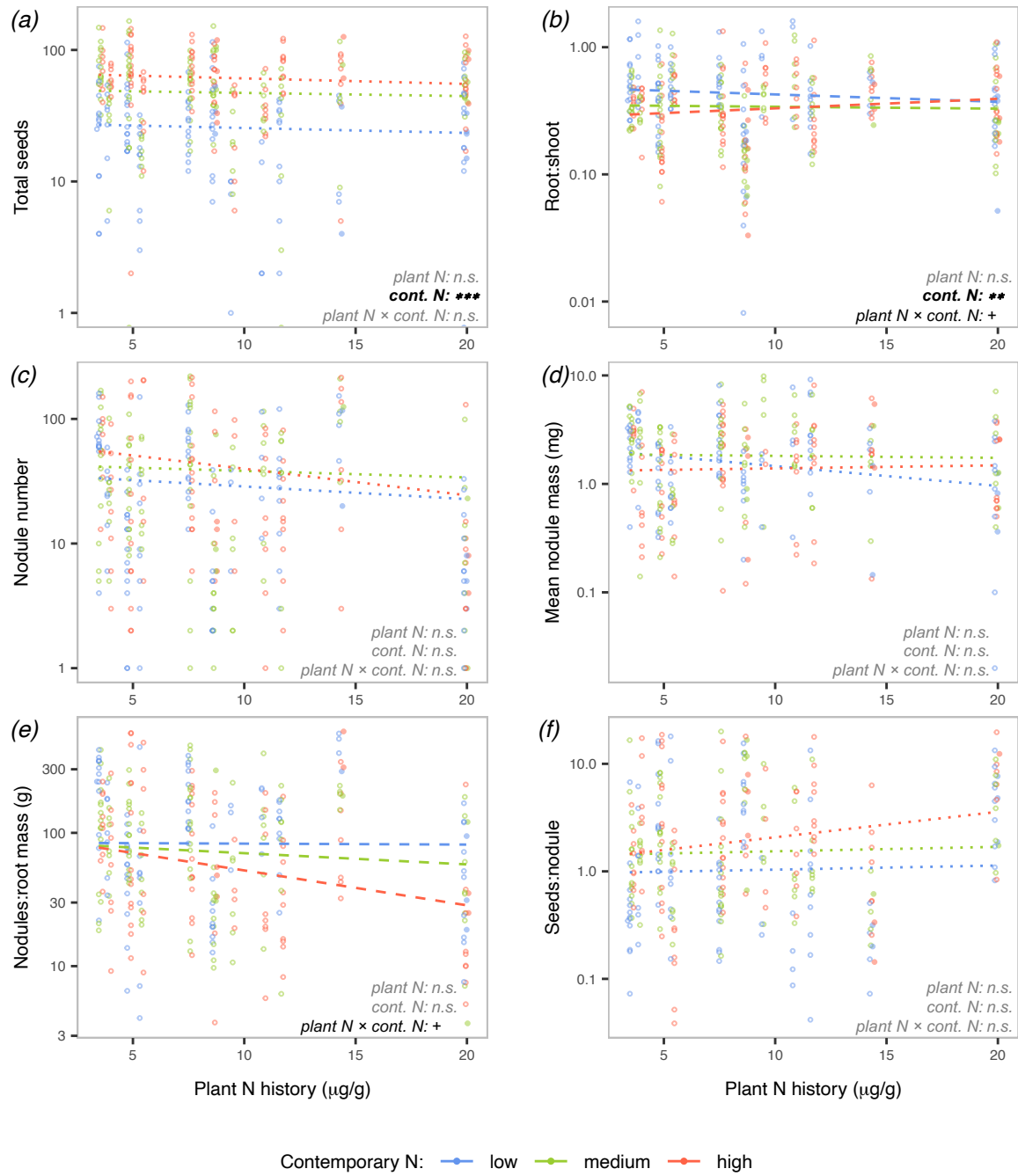


Figure 1.

Figure 2: Microbe-mediated adaptive plasticity (microbe N history × contemporary N).

Plant fitness and root:shoot ratios did not vary with microbial N history (a-b), but microbial communities from different N histories had different effects on traits related to nodule investment in different contemporary N environments (c-f): plants inoculated with microbes from high-N sites produced more nodule mass than plants inoculated with microbes from low-N sites when contemporary N was also high (c). Mean nodule mass was positively correlated with microbe N history in high contemporary N but negatively correlated in low or medium contemporary N (d). Plants reduced allocation to root growth (e) and seeds (f) relative to nodules in high contemporary N when inoculated with microbial communities from high N sites. Larger points in panels on left side of (a-b) show estimated marginal means \pm SE of plants inoculated with sterile control instead of live microbes. Solid lines indicate significant microbe N history \times contemporary N interactions; dotted lines indicate nonsignificant interactions. Filled points are plants inoculated with sympatric microbes. Note: vertical axes are presented on a log scale.

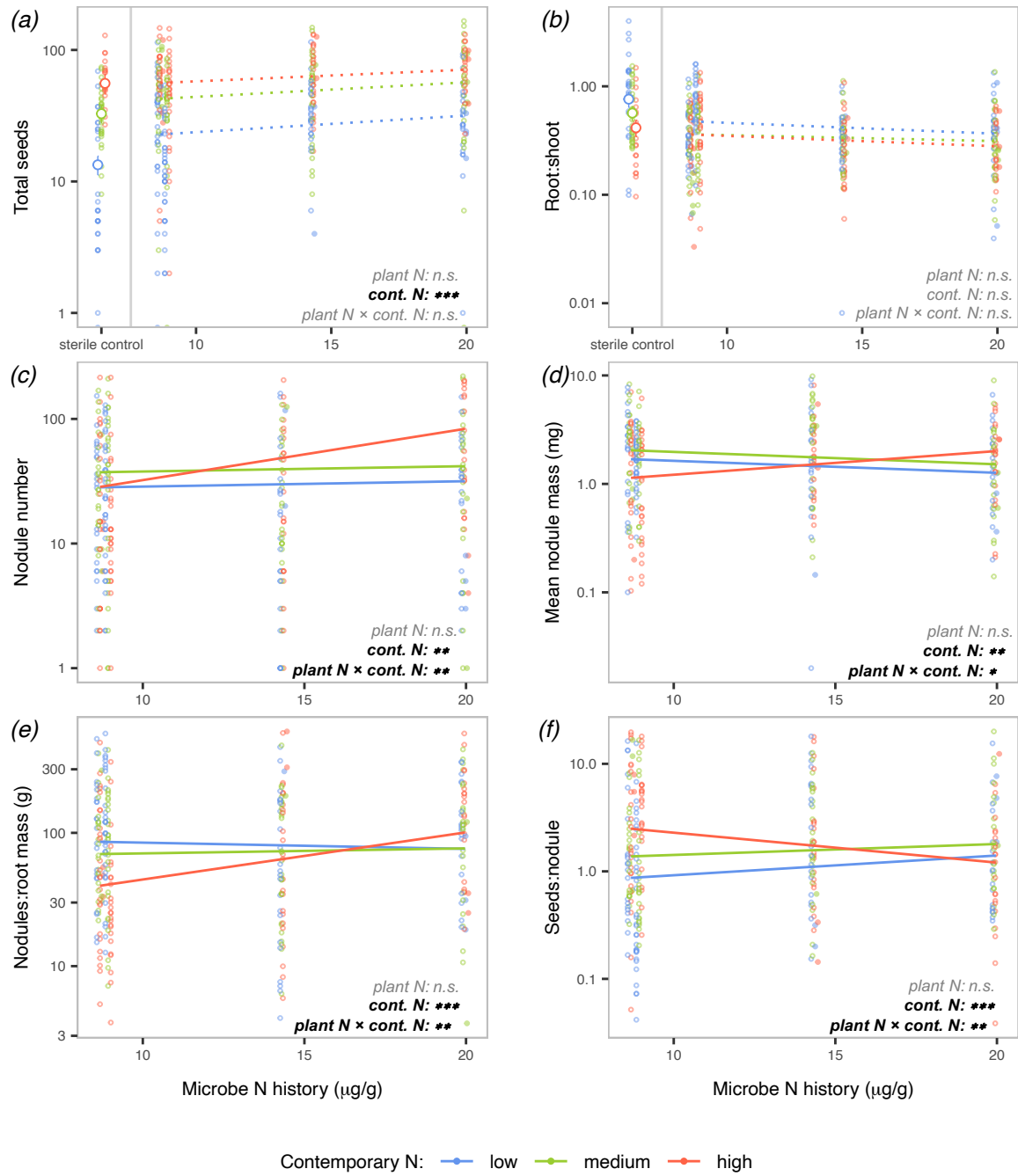


Figure 2.

CHAPTER 2:

CICADA RESOURCE PULSE HAS TRANSGENERATIONAL EFFECTS ON PLANT GROWTH VIA BOTH MATERNAL EFFECTS AND SOIL MICROBES

ABSTRACT

The emergence and subsequent death of periodical cicadas causes a pulse of nutrients, including nitrogen, into soils. Nutrient enrichment has immediate direct effects on plant growth but also may have lasting impacts via maternal effects and soil microbial legacies. For example, increased resource availability is expected to contribute to maternal effects via seed provisioning, and nitrogen enrichment can alter soil microbial community composition, including the abundance of resource mutualists. We capitalized on the summer 2021 emergence of 17-year Brood X periodical cicadas and performed a field experiment adding or removing cicada carcasses from forested plots to study the effect of cicada litterfall on understory plants. We predicted that cicada addition would increase plant growth in the field and alter soil microbial communities, subsequently increasing growth and survival of the following plant generation via maternal effects and changes in soil microbes. To test this, we collected seeds of the native annual legume *Amphicarpaea bracteata* from each plot, planted them in the greenhouse, and inoculated them with microbes from the addition and removal plots. Cicada addition increased plant cover in the field and affected offspring plants through both maternal effects and soil microbes: offspring from cicada addition plots were more likely to germinate and produced more seeds but were less likely to survive, and soil microbes from cicada addition plots accelerated early plant growth. Overall, our results suggest that cicada litterfall affects plant growth via multiple mechanisms and that these effects can last beyond a single generation of plants.

INTRODUCTION

Terrestrial ecosystems are often limited by nutrients, particularly nitrogen (N). As a result, nutrient enrichment has wide-ranging effects on natural ecosystems (Vitousek et al., 1997). Resource-addition experiments have revealed that nutrient enrichment commonly increases plant productivity and can alter plant competition and community composition (Dickson & Gross, 2013; Tilman, 1987; Wedin & Tilman, 1993) as well as plant-herbivore (Burney & Jacobs, 2013; Strauss & Agrawal, 1999) and plant-microbe interactions (Friel & Friesen, 2019; Johnson, 1993; Simonsen et al., 2015; Weese et al., 2015). Many resource-enrichment experiments are press experiments where resources are added annually for years to decades to mimic effects of anthropogenic environmental changes. However, many natural fertilization events are resource pulses. These pulses are common in nature but differ widely in effect, duration, and regularity (Yang et al., 2010). Some resource pulses have abiotic causes, such as El Niño rains (Grant et al., 2000; Polis et al., 1997). Others are biotic, such as acorn masting (Bogges et al., 2021; Wolff, 1996) or insect outbreaks (Carlton & Goldman, 1984; Haney, 1999). For example, periodical cicadas (*Magicicada spp.*) are long-lived insects that spend 13 or 17 years belowground before emerging synchronously in massive densities to reproduce (Williams & Simon, 1995). Most adult cicadas escape predation and die naturally, and cicada litterfall can increase soil N (Yang, 2004), CO₂ flux (Beverly et al., 2024), and stream metabolism (Menninger et al., 2008).

Although brief, resource pulses can have longer-lasting effects that persist after the pulse. First, pulses can have lasting effects on populations. For example, in a classic study, Gibbs and Grant (1987) showed that intense selection from a severe El Niño event (where intense rainfall increased food availability) caused the evolution of reduced body size in Darwin's finches

despite larger body sizes being favored most years. Similarly, resource enrichment can affect subsequent generations through maternal effects (Yang et al., 2008). In plants, maternal effects can be caused by changes in maternal seed provisioning (Herman & Sultan, 2011) and increased N can lead to larger seeds with a higher concentration of protein (Roach & Wulff, 1987). These better-provisioned seeds may be more likely to survive and reproduce in the next generation. Second, resource pulses can shift community composition. For example, a single intense pulse of N and phosphorous increased the prevalence of invasive plants in patches of intermountain prairie for at least five years following the disturbance (Besaw et al., 2011) and amendment with a pulse of dissolved organic matter rapidly increased the prevalence of certain microbial taxa in rainforest soils (Cleveland et al., 2007). Similarly, litterfall from periodical cicadas shifted detritivore community composition and increased detritivore activity, which in turn increased decomposition rates and thus accelerated nutrient cycling (Yang, 2006).

While resource pulses can cause longer-term effects both through transgenerational effects (like evolutionary changes or maternal effects) and by altering the composition of interacting species (like plant competitors or belowground microbes), these processes also can interact with one another or with other environmental factors that also respond to the resource pulse. First, resource pulse-induced maternal effects can depend on biotic context. For example, preferential herbivory of maternal plants with high leaf nutrient content (Knops et al., 2000) could erase any benefits of nutrients on seed provisioning. Alternately, herbivory sometimes causes plants to increase rather than decrease seed mass by allocating more resources to reproduction under stress (Agrawal, 2001; Roach & Wulff, 1987). In such a case, a resource pulse and herbivory could have a synergistic effect on seed provisioning. Second, soil microbes and other interacting species can shift which plant traits are favored during stressful conditions

(Fitzpatrick et al., 2019), which could enhance or counteract the benefits of maternal effects. Similarly, microbes can positively or negatively affect seed germination (Keeler & Rafferty, 2022; Miller et al., 2019). Beneficial microbes that increase germination could nullify the benefit of maternal seed provisioning if they counteract the deleterious effects of poor provisioning, while microbes that delay or decrease germination or growth could increase the importance of maternal effects to plant growth and reproduction. However, these interactions have been tested in only a few studies. For example, De Long and coauthors (2019) found that droughted soils increased the shoot biomass of plants whose mothers were not exposed to drought, but soil drought legacy did not affect the biomass of plants whose mothers experienced drought. In other words, drought soil legacies were only apparent in the absence of drought-induced maternal effects. Similarly, Xue and coauthors (2022) found that parental effects of shade on *Hydrocotyle* offspring were mostly negative, but soils from shaded parents mitigated these effects when offspring were grown in ambient light. In both of these examples, it is not clear whether the soil legacy effects result from microbial community responses or soil abiotic (chemical/structural) responses. Therefore, although we might expect beneficial soil microbes to decrease the relative benefit of well-provisioned seeds, it is still largely unknown how maternal effects and changes in soil microbial communities interact to influence subsequent generations of plants.

We capitalized on the dramatic 2021 emergence of Brood X periodical cicadas to explore multigenerational effects of a nutrient pulse on a native legume via maternal effects and changes in soil microbial communities (Figure 1), as well as how biotic context (ungulate browsing) changes these transgenerational and microbial legacy effects. We chose a legume as our focal species because symbiosis with rhizobium bacteria may make legumes particularly sensitive to N-induced changes in soil microbes (e.g., Weese et al., 2015). We manipulated deer presence

because mammalian herbivory can reduce or negate effects of N addition on plant communities (Knops et al., 2000), may be particularly detrimental to N-fixing legumes (Knops et al., 2000), can elicit maternal effects (Aguirrebengoa et al., 2018), and can alter plant-microbe interactions (Andriuzzi & Wall, 2017; Bardgett et al., 1998). As a result, herbivory may influence the effect of the cicada resource pulse on maternal effects and microbial legacy effects. We hypothesized that increased resource availability caused by cicada litterfall would increase plant growth in the field (Figure 1a), enabling maternal plants to produce higher-quality seeds that would be more likely to germinate, grow quickly, and survive (Figure 1c). Furthermore, we hypothesized that this effect would depend on whether maternal plants were protected from deer because preferential herbivory of larger plants could negate any growth and reproductive benefits of the resource pulse (Knops et al., 2000) or could increase plant investment in reproduction (Agrawal, 2001; Roach & Wulff, 1987). Finally, we hypothesized that cicada litterfall would reduce the plant growth benefits of soil microbial communities (Figure 1b/d) because long-term N enrichment typically decreases the abundance of resource mutualists while increasing pathogen abundance (Lekberg et al., 2021) and can reduce rhizobium mutualist quality (Weese et al., 2015), although increases in plant growth benefits are also possible given that short-term multi-nutrient fertilization can increase the benefits provided by rhizobia (Simonsen et al., 2015) and cicada litterfall can increase soil microbial biomass (Yang, 2004).

MATERIALS AND METHODS

Overview

We conducted a field experiment testing the legacy effects of a periodical cicada resource pulse on a native legume by manipulating the presence of cicada carcasses in forested plots that

were either protected or unprotected from deer. We collected seeds from naturally occurring plants in our field experiment, then grew these seeds in the greenhouse and inoculated them with soil microbial communities collected from each plot in a factorial design. This factorial design allowed us to test the direct effects of the cicada resource pulse on plant maternal effects, how the resource pulse alters microbial communities in ways that affect plant growth of the subsequent generation, as well as how cicada resource pulse effects (via both maternal effects and microbial community responses) are influenced by exposure to deer herbivory.

Study system

Periodical cicadas (*Magicalcada spp.*) are long-lived hemipteran insects that spend 13 or 17 years belowground as nymphs before crawling aboveground and emerging synchronously in massive numbers as adults to reproduce (Williams & Simon, 1995). Areas with high brood density may have over 500 cicadas m⁻² emerge, and litterfall densities can reach more than 1800 carcasses m⁻² (Yang, 2004). This high litterfall occurs because mass emergence reduces predation pressure by causing predator satiation (Karban, 1982). Bloomington, IN is near the center of Brood X 17-year cicadas and has a high density of cicadas (Kritsky et al., 2005). Brood X cicadas began emerging in Bloomington in late April 2021 and were active until late June, when they died en masse resulting in locally high densities of decaying cicada carcasses (pers. obs.).

Amphicarpaea bracteata (American hog-peanut, hereafter *Amphicarpaea*) is an herbaceous annual vining legume native to forest understories in eastern North America, including Indiana, that associates with N-fixing *Bradyrhizobium* bacteria. It is the most common legume at our field site and the only legume present in our sampling quadrats. *Amphicarpaea* plants in our area were well-established (multiple mature leaves) by May 2021, well before adult cicadas began to die off in June (pers. obs.).

Field experiment

This study was conducted at the Indiana University Research and Teaching Preserve Kent Farm site (Monroe County, IN: 39°19' N, 86°25' W). Our field plots were located in the dry uplands of a broadleaf deciduous forest. During the 2021 emergence, cicadas were abundant in suburban areas of Bloomington but relatively scarce at Kent Farm (pers. obs.). Trees near our plots had a density of ~10 emergence holes m⁻², and soil CO₂ flux was higher when emergence holes were present (Beverly et al., 2024).

Plot treatments

We manipulated the presence of cicada carcasses in paired 10 × 10 m field plots (Figure S1). We used a split-plot design where cicada presence was manipulated at the whole plot scale by scattering cicada carcasses throughout the plot (Table S1), and deer herbivory was manipulated at the subplot scale by enclosing half of each plot with fencing to exclude deer. We established four 2 m × 1 m quadrats within each subplot and tagged individual plants to monitor growth and reproduction (Figure S1). We set up seven plot pairs (14 plots total, 7 each cicada addition/removal). Ten of the plots contained *Amphicarpaea* plants, but *Amphicarpaea* produced aerial seeds in only seven plots (Table S2).

Cicada-addition plots received a total of approximately 172 cicada carcasses m⁻². This density aligns with natural densities observed in many regions and with a previous study that showed significant effects of 120-240 cicada carcasses m⁻² on soil N, plants, and soil microbes (Yang, 2004), but is higher than the densities observed at our field site (see above). Carcasses were collected from across Bloomington, Indiana, USA (primarily the Indiana University, Bloomington campus), homogenized, measured volumetrically, and scattered in each addition plot. We spread cicadas on the plots over five addition days (8 June, 11 June, 16 June, 22 June,

and 2 July 2021). Addition days differed in the number of cicada carcasses added, but on each of the five addition days we visited all addition plots and added an equal volume of carcasses to each (Table S1). Although we did not see an increase in available soil N in our field soil analyses (see Results), we estimate that the carcasses resulted in a total of 2.62 g N m⁻² added to the soil in the cicada-addition plots. We based this estimate on a mean cicada carcass dry mass of 0.132 g (averaged from 19 cicada carcasses oven-dried at 65 °C) with 11.5% N content (19 oven-dried cicada carcasses ground, homogenized, and measured using triplicate samples run on a Costech Elemental Analyzer). We removed any cicada carcasses found in control plots but found very few (46 total across 7 plots: average of 0.07 cicada carcasses m⁻²). Therefore, we refer to plots without cicada carcass addition as “ambient” plots rather than “removal” plots.

Plant and soil field data

We identified and tagged focal plants in our field plots after cicada additions were completed in July 2021. We monitored up to 32 *Amphicarpaea* plants in each quadrat and measured total leaf number as a proxy for growth; since *Amphicarpaea* is a twining vine, it was infeasible to accurately measure plant length without disturbing the plants. In some plots, there were very few individuals growing in the sampling quadrats, so some plants growing outside the designated quadrats were tagged and monitored. We collected mature fruits of tagged plants as they appeared. At the end of the growing season, we collected aboveground biomass of each tagged plant. On 11-15 Oct 2021 (over three mo since the final cicada addition) we estimated percent cover of all herbaceous species in the quadrats to see whether cicada addition or deer exclusion affected overall plant cover and plant community composition, and whether our focal legume (*Amphicarpaea*) benefitted relatively more or less than other plant species.

On 23 July 2021 we collected soil samples from each plot to compare the soil nutrient content of cicada-addition and ambient plots. This sampling period was ~1 mo after most cicada carcasses had been applied to correspond to peak soil N levels observed in past studies (Yang, 2004). We took soil cores (2.5 cm diameter × 6-10 cm deep) 1 m in from each corner of each subplot (Figure S1) and the resulting eight cores from each plot were homogenized so that we had one soil sample from each plot. Organic matter was cleared from the soil surface before coring. Between samples, we cleaned the soil corer with 95% ethanol. Each soil sample was air dried for one week and shipped to Brookside Laboratories, Inc. (New Bremen, OH) for analysis.

Greenhouse experiment

We scarified seeds on 11 March 2022 by nicking with a razor blade. To examine the effect of seed surface microbes, a subset of seeds were surface sterilized by soaking in a 33% commercial bleach (5.25% NaClO) solution for 1 min and rinsing for 10 min in distilled water. Surface sterilization did not significantly affect germination ($p = 0.95$) or survival ($p = 0.28$), so is not discussed further (Table S3, Figure S3). All seeds were germinated in covered containers on filter paper in the dark for 13 d. On 24 March 2022, germinants were transplanted approximately 2.5 cm deep into 656 mL Deepots (Stuewe and Sons, Tangent, Oregon, USA) filled with a steam-sterilized 1:1:1 mix of sand, calcined clay, and commercial potting media (Metro-Mix 360, Sun Gro Horticulture, Bellevue, WA, USA). Pots were spaced apart to minimize microbial cross-contamination and treatments were randomly assigned to pots so that any cross-contamination that did occur would not bias results. We planted 145 germinants, but only 62 survived until the end of the experiment. This high mortality and the limited number of seeds produced in the field (Table S2) caused uneven sample sizes in our final dataset (Table S4).

Soil microbial treatments

On 28 March 2022 we collected soil from each plot where *Amphicarpaea* grew during the previous season. We collected soil microbes in early spring following our field experiment rather than at the end of the first growing season because spring is when the next generation of *Amphicarpaea* germinates and begins interacting with microbes. We removed the litter layer, then collected a 10 cm × 1.6 cm soil core from the corner of each subplot 1 m in from each edge to minimize edge effects (Figure S1). The four soil cores from each subplot were homogenized together to create one soil sample per subplot (two samples per plot) for a total of 16 different soil microbial communities. We mixed 10 mL of each soil sample with 40 mL distilled water to create soil slurries to inoculate our plants with soil microbial communities from each field treatment. To create a control inoculant, we homogenized equal volumes of soil from each subplot, autoclaved at 120 °C for 60 min, then combined 10 mL of the sterilized soil mixture with 40 mL water. Each plant was inoculated with 2 mL live soil slurry on 28 March 2022 or 2 mL sterilized control slurry on 30 March 2022. Each seed treatment group was divided as evenly as possible into microbial treatments, but microbial treatments had uneven replication due to uneven production of seeds in the field. Each live inoculant was applied to at least four individuals (min 4, max 12, mean 9), and 13 plants received sterilized control inoculant.

Greenhouse conditions and data collection

All plants were watered as needed. Natural lighting was supplemented with artificial light (*ca.* 450 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation) when needed to simulate a 16-hr day. We monitored plants for leaf and fruit production. We harvested all plants ~8 mo after planting (3-10 Nov 2022). We separated above- and belowground biomass, counted and weighed root nodules, and counted belowground seeds. Biomass was weighed after drying at 60 °C for 3 d.

Statistical analysis

We calculated absolute percent cover by averaging percent cover estimates of *Amphicarpaea* in the four sampling quadrats (Figure S1) to obtain one measure per subplot. We calculated relative cover by dividing *Amphicarpaea* percent cover by the total cover of non-woody species in each subplot. To test whether *Amphicarpaea* cover differed between cicada-addition and control plots, we fit a generalized linear mixed model (GLMM) with cicada treatment (whole plot level), deer exclusion treatment (subplot), and their interaction as fixed effects and plot (nested within plot pair) as a random effect (model structure: percent cover \sim cicadas * deer + (1|plot pair/plot)). We log-transformed the data to fit model assumptions of normality and test for multiplicative statistical interactions. Because we had to remove zeros to allow log transformation, we also fit a model with the same structure and a binomial distribution to a binary variable indicating if *Amphicarpaea* was present or absent in each subplot. To test whether cicada addition or deer exclusion affected the growth and fitness of *Amphicarpaea* in the field, we fit models for plant traits (leaf count, aboveground biomass, and seed production) with cicada treatment, deer exclusion treatment, and their interaction as fixed effects and subplot (nested within plot within plot pair) as a random effect (model: response \sim cicadas * deer + (1|plot pair/plot/subplot)).

To test the effect of cicada addition on the germination of seeds in the subsequent generation, we fit a GLMM with a binomial distribution with cicada_{seed} (maternal plant grew in a cicada-addition plot or an ambient plot) and deer_{seed} (maternal plant grew within a deer enclosure or not) as fixed effects and the subplot (nested within plot) where the maternal plant grew as a random effect (model: germination \sim cicada_{seed} * deer_{seed} + (1| plot_{seed}/subplot_{seed})). Microbe source was not included because microbial treatments were applied after seeds had germinated.

To test the transgenerational and indirect effects of cicadas on plant growth via maternal effects and soil microbes, we fit GLMMs with $cicada_{microbes}$ (soil microbes collected from a cicada-addition plot or an ambient plot), $cicada_{seed}$, $deer_{seed}$, and all interactions as fixed effects and $subplot_{microbes}$ (nested within $plot_{microbes}$) and $subplot_{seed}$ (nested within $plot_{seed}$) as random effects ($response \sim cicada_{microbes} * cicada_{seed} * deer_{seed} + (1|plot_{microbes}/subplot_{microbes}) + (1|plot_{seed}/subplot_{seed})$). Our response variables were survival to harvest, days until the plant formed its first true leaf, total number of seeds (aerial + subterranean), total dry biomass (g), root:shoot ratio (belowground / aboveground biomass), nodule presence, nodule number, and total nodule mass (g, fresh). To examine the mutualist quality of rhizobia, we fit an additional model for seed production that also included nodule number as a fixed effect ($seeds \sim nodule\ count * cicada_{microbes} * cicada_{seed} * deer_{seed} + (1|plot_{microbes}/subplot_{microbes}) + (1|plot_{seed}/subplot_{seed})$). A significant nodule count \times $cicada_{microbes}$ interaction would indicate that per-nodule fitness benefits differ across cicada treatments. Because interactions reduce statistical power and our data set was small (Table S4), we removed interactions when they were not significant and removal did not harm model fit. Except for nodule presence, analyses involving nodules only included plants that formed at least one nodule. Plants inoculated with sterile controls were excluded from analyses that included microbial treatment; means for all response variables in the sterile controls were calculated and are indicated on each figure to show the overall effect of live vs. sterile inoculant.

We conducted all analyses with R version 4.2.2 (R Core Team, 2020). We fit models with the *glmmTMB* package (Brooks et al., 2017) and checked model fits using the ``simulateResiduals`` function from the *DHARMA* package (Hartig, 2022). Models for binary variables were fit using a binomial distribution, models for count data fit using a negative

binomial distribution, and continuous variables were log-transformed and models were fit using a gaussian distribution. We assessed significance using Type III Wald chi-squared tests with the `Anova` function from the *car* package (Fox & Weisberg, 2019). We calculated estimated marginal means (EMMs) using the *emmeans* package (Lenth, 2022) and created figures with *ggplot2* (Wickham et al., 2024).

RESULTS

Effects of cicada addition on field plots

Cicada addition did not affect ammonium, nitrate, or organic matter in our soil samples (NH₄N, $\chi^2 = 0.01$, cicada: 7.06 ppm \pm 0.92, ambient: 6.90 ppm \pm 1.49; NO₃N, $\chi^2 = 0.04$, cicada: 4.17 ppm \pm 1.36, ambient: 3.91 ppm \pm 1.40; organic matter, $\chi^2 = 0.46$, cicada: 5.75% \pm 0.22, ambient: 5.57% \pm 0.27; all $p > 0.4$, Table S2). However, cicada carcass addition increased soil N flux in our plots (Phillips, pers. comm.), suggesting that the cicada resource pulse did alter N cycling. Protection from deer increased total plant cover by 50%, but cicada addition had no effect (deer: $\chi^2 = 6.42$, $p < 0.05$; cicadas: $\chi^2 = 0.18$, $p = 0.67$, Table 1, Figure S2a).

Cicada addition increased relative *Amphicarpaea* cover from 6% to 19% and percent *Amphicarpaea* cover from 0.7% to 3.4% (relative cover, $\chi^2 = 6.72$, $p < 0.01$; percent cover, $\chi^2 = 6.95$, $p < 0.01$; Table 1, Figure 2a, Figure S2b). Protection from deer herbivory reduced the benefit of cicada addition on *Amphicarpaea* relative cover and tended to reduce the benefit of cicadas on percent cover (cicadas \times deer: relative cover, $\chi^2 = 4.13$, $p < 0.05$, cicada benefit 487% and 85% in unprotected and protected subplots, respectively; percent cover, $\chi^2 = 3.59$, $p < 0.1$, cicada benefit 910% and 146% in ambient and cicada addition plots, respectively; Table 1,

Figure S2b). However, cicada addition and protection from deer had no effect on *Amphicarpaea* presence, leaf count, biomass, or seed production in the field (all $p > 0.2$; Table 1, Figure S4).

Maternal effects of cicada addition

Although individual plant fitness in the field was not affected by cicada addition, seeds produced by plants in cicada-addition plots were 25% more likely to germinate than seeds from plants in ambient plots ($\chi^2 = 7.01$, $p < 0.01$; Table 1, Figure 2b). Protection from deer did not result in a maternal effect on germination ($\chi^2 = 0.26$, $p = 0.6$; Table 1, Figure 2b) or influence the benefit of cicadas on germination (cicada_{seed} × deer_{seed}: $\chi^2 = 0.26$, $p = 0.6$; Table 1, Figure 2b). Overall germination was high (80%).

Offspring of plants from cicada-addition plots were 53% less likely to survive than offspring of plants from control plots ($\chi^2 = 6.44$, $p < 0.05$, Table 2, Figure 3a) but produced 16% more seeds ($\chi^2 = 5.75$, $p < 0.05$, Table 2, Figure 3b). This seed production result should be interpreted cautiously: although interactions between treatments were only marginally significant (Table 2), this result appears to be driven by only one treatment group (ambient microbes, ambient seeds, unprotected from deer) that produced especially few seeds and had only four surviving individuals (Table S4). Cicada addition also resulted in offspring with 21% higher root-shoot ratios and tended to increase offspring biomass, but only when maternal plants were protected from deer (cicada_{seed} × deer_{seed}: root:shoot $\chi^2 = 9.76$, $p < 0.01$, Table 2, Figure 4a; total biomass $\chi^2 = 3.00$, $p < 0.1$, Table 2, Figure 3c). This result also should be interpreted cautiously, as survival was particularly low for offspring of maternal plants from this treatment (seeds from the deer-protected subplots of cicada-addition plots; Table S4).

Soil microbial legacy effects

Transgenerational effects resulting from microbial legacy effects of cicada addition were typically weaker than cicada-induced maternal effects and were rarely statistically significant. Microbes from cicada-addition plots had minimal effect on the expression of cicada- or ungulate-induced maternal effects (no significant $\text{cicada}_{\text{microbes}} \times \text{cicada}_{\text{seed}}$ or $\text{cicada}_{\text{microbes}} \times \text{deer}_{\text{seed}}$ interactions: all $p > 0.09$, Table 2). Microbial communities from different cicada treatments did not significantly affect nodule presence, number, or total mass (all $p > 0.05$, Table 2, Figure 4). However, microbes from cicada-addition plots decreased the time that plants took to form their first true leaf by 6% ($\chi^2 = 4.25$, $p < 0.01$, Table 2, Figure 3d) and tended to increase seed production by 227% ($\chi^2 = 3.66$, $p < 0.1$, Table 2, Figure 3b).

Plants grown with microbes from cicada-addition plots also produced more seeds per nodule at low nodule numbers, but seed production increased more steeply with increased nodule number when plants were inoculated with ambient microbes, which might suggest greater per-nodule fitness benefits (nodule number \times $\text{cicada}_{\text{microbes}}$: total seeds, $\chi^2 = 3.94$, $p < 0.05$, Table 2, Figure S5). However, these results appear to be driven by a small number of plants that produced large numbers of both seeds and nodules and there was no significant main effect of microbe source, so we did not find compelling evidence that cicada addition increased or decreased rhizobium mutualist quality. Plant and rhizobium fitness were largely aligned, but more tightly with ambient microbes: seed number was positively correlated with total nodule mass (cicada microbes, $\rho = 0.34$; ambient microbes, $\rho = 0.93$) and nodule number (cicada microbes, $\rho = 0.50$; ambient microbes, $\rho = 0.86$).

DISCUSSION

The emergence of periodical cicadas is expected to produce a resource pulse with potentially lasting effects on plants through two mechanisms: maternal effects and changes in soil microbial communities. We found that the offspring of maternal *Amphicarpaea* plants amended with Brood X cicada carcasses were more likely to germinate and tended to produce more seeds and biomass, but were less likely to survive, while microbial legacy effects of cicada addition were rarely significant but also tended to increase seed production and early growth. Together, these results demonstrate that despite their brief duration, resource pulses can impact future plant generations through multiple mechanisms.

Cicada amendment was largely beneficial to plants

We found that offspring of maternal plants amended with cicadas tended to have higher fitness (higher germination, tendency for higher seed production and biomass). These maternal effects are most likely due to better-provisioned seeds because maternal plants had increased access to nutrients. We did not measure seed size, but seed provisioning effects of increased N availability also can result in seeds with higher nutritional quality, not just increased mass (Roach & Wulff, 1987). Maternal effects also can change germination likelihood by affecting dormancy, generally through changes in seed coat hardness or permeability (Roach & Wulff, 1987), but this is unlikely to be a factor in our experiment because we physically scarified the seeds to ensure that water could enter the seed coat and induce germination.

Despite increases in other metrics of plant fitness, we unexpectedly found that cicada amendment decreased the survival of offspring plants. Prior work has also shown that maternal effects of nutrient supplementation can lead to decreased survival when seedlings are deprived of nutrients (Aarssen & Burton, 1990), possibly due to increased metabolism. Our greenhouse

plants, which were grown in a low-nutrient potting media, may be showing a similar effect.

However, we did not manipulate the offspring nutrient environment, so could not test whether the maternal effect we observed on survival also depended on the offspring environment.

Cicada amendment largely benefitted our focal legume in both the maternal and offspring generations, but prior long-term studies have found that legumes decline in abundance after fertilization (Dickson & Gross, 2013; Suding et al., 2005; Tilman, 1987). This difference in outcome for legumes may be a function of time, intensity, or ecosystem context. First, our study was a relatively brief and mild pulse of nitrogen (N) in a single growing season. We added all cicada carcasses within a one-month period, whereas many studies documenting diversity declines span many years. Second, we added less than a third as much N as seminal studies that have shown a decline in legume abundance following fertilization (Dickson & Gross, 2013; Tilman, 1987). Although a prior meta-analysis did not find an effect of duration or amount of N on legume decline (Suding et al., 2005), we may have seen different effects in this study due to it being a pulse, rather than press, experiment. Finally, most previous studies occurred in relatively densely vegetated plots, such as grasslands (Suding et al., 2005), many of which are dominated by tall, rhizome-forming perennials (e.g., Dickson et al. 2014). In contrast, our plots were sparsely vegetated overall, nearly devoid of grasses, and dominated by canopy trees. This sparse overall vegetation likely prevented N from causing an increase in competition for light, which may be the main driver of legume decline under N enrichment (Hautier et al., 2009). Even if N fertilization did increase competition for light, *Amphicarpaea* is a shade-tolerant forest understory legume species and may therefore be less sensitive to a reduction in light availability than grassland legumes. In the absence of grasses or rhizomatous forbs, the legumes in our plots may not have experienced differences in interspecific competition between our treatments.

Overall, these results demonstrate that the effects of resource enrichment on plant populations and communities likely depend on the duration and intensity of the resource pulse, specific natural history of the plant species, and environmental and community context. Therefore, predictions of plant responses to future disturbances cannot be easily extrapolated from past studies conducted in disparate ecosystems.

Cicada amendment made microbial communities more beneficial

Cicada amendment tended to cause soil microbial communities in our plots to be more beneficial to offspring plants, although soil legacy effects were rarely significant. Because cicada amendment increased *Amphicarpaea* cover in our plots, this could be due to increased overall investment in rhizobia and other mutualists, although we did not observe any effects on offspring nodule numbers so changes in rhizobium abundance is unlikely. Additionally, increased resource availability may alter the way plants interact with soil microbes (e.g., investment in resource symbionts, root exudates, etc.; West et al. 2002b, a; Tao et al. 2024), and changes in these interactions may alter the soil microbial community in ways that feed back and affect the growth of the next generation of plants (biotic plant-soil feedback; Bever et al. 1997; Smith-Ramesh and Reynolds 2017). For example, previous short-term N fertilization studies have shown increases in soil mutualist quality after fertilization (Simonsen et al., 2015), and here we also found that fertilization with cicada carcasses altered the relationship between plant fitness and investment in rhizobium mutualists. Comparisons with our sterilized control inocula suggest that our soil microbial communities were dominated by mutualists rather than pathogens (i.e., plants generally performed better with live rather than sterilized inocula). However, N enrichment also could contribute to overall less harmful soil microbial communities if elevated N allows bolstered plant defenses and a subsequent reduction in pathogen prevalence (Smith-Ramesh &

Reynolds, 2017), instead of increased pathogen abundance as is sometimes observed under nutrient enrichment (Lekberg et al., 2021).

Biotic context rarely shifted maternal effects

We found few strong instances where biotic context (soil microbes or exposure to deer herbivory) shifted the expression of maternal effects, indicating that biotic context typically has only weak effects on the outcomes of maternal effects. The one exception is that offspring plants had increased root:shoot ratios when maternal plants were amended with cicada carcasses and protected from deer; however, our sample sizes were low for some treatment combinations, so this result should be interpreted cautiously. Increased root:shoot ratios are associated with higher tolerance of herbivory (increased regrowth capacity from nutrients stored in roots and more ability to reacquire nutrients quickly; Strauss & Agrawal, 1999) and can benefit plants growing in low resource (nutrient or water) conditions (Gruber et al., 2013; Wilson, 1988). Therefore, the root:shoot ratio responses we observed are unlikely to be adaptive. More generally, interactions between maternal effects and soil microbes or herbivory may be rare and weak, although, as discussed in the Introduction, few empirical studies have tested for such interactions.

Caveats

Our experimental setup may have obscured some of the transgenerational and legacy effects caused by cicadas and deer. We saw stronger effects on early growth than on overall fitness, which is common for many maternal effects (Roach & Wulff, 1987). A more stressful abiotic environment or the presence of competitors may have resulted in persistence of early growth effects because competition for space and light can magnify differences in early growth (Ross & Harper, 1972), such that slow early growth makes it difficult for plants to become established and compete effectively. Therefore, the early growth differences caused by both

maternal and soil legacy effects may have more extensively affected plant fitness if we had conducted this experiment in a complex, competitive field environment rather than in a benign greenhouse environment without competitors.

Conclusions

Overall, we saw that the addition of hundreds of decomposing 17-year Brood X periodical cicadas influenced current and future generations of legumes via multiple mechanisms and was largely beneficial to our focal plant species. We saw stronger effects from maternal effects than from changes in soil microbial communities and did not find strong evidence that biotic context shifted the outcomes of plant maternal effects. Our results demonstrate that although resource pulses are brief and may happen only rarely, they can have impacts on populations that last beyond a single generation.

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TABLES AND FIGURES

Table 1. Mixed-model ANOVA results testing for the effects of cicada carcass amendment ('Cicada') and protection from deer herbivory ('Deer') on total plant cover and traits of *Amphicarpaea bracteata* ('A.b.') in field plots. Values shown are Type III Wald chi-squared tests for each fixed factor included in the mixed-model ANOVA; Df = 1. Significant predictors are indicated by bold type and asterisks ($p < 0.001^{***}$, $p < 0.01^{**}$, $p < 0.05^*$, $p < 0.1^+$). Percent and relative cover models included only subplots with *Amphicarpaea* present. Model structures: presence/relative cover/percent cover ~ cicadas * deer + (1| plot pair / plot); leaves/biomass/seeds ~ cicadas * deer + (1| plot pair / plot / subplot); germination ~ cicada_{seed} * deer_{seed} + (1| plot_{seed} / subplot_{seed}).

	<i>Total cover</i>	<i>A.b. presence</i>	<i>A.b. rel. cover</i>	<i>A.b. % cover</i>	<i>A.b. leaf number</i>	<i>A.b. biomass (g)</i>	<i>A.b. seed number</i>	<i>A.b. germ.</i>
<i>n</i>	28	28	16	16	829	412	78	236
Cicada	0.18	0.00	6.72**	6.95**	0.15	0.43	0.18	7.01**
Deer	6.42*	0.00	1.37	4.58*	0.17	0.06	0.61	0.26
Cicada × deer	1.34	0.00	4.13*	3.59+	0.02	0.01	1.09	0.26

Table 2. Mixed-model ANOVA results testing for the effects of cicada carcass amendment ('Cicada') and protection from deer herbivory ('Deer') on plant offspring traits via maternal effects ('seed') and changes in soil microbial communities ('microbes'). Values shown are Type III Wald chi-squared tests for each fixed factor included in the mixed-model ANOVA; Df = 1. Significant predictors are indicated by bold type and asterisks ($p < 0.001^{***}$, $p < 0.01^{**}$, $p < 0.05^*$, $p < 0.1+$). Interactions were excluded when not significant and exclusion did not harm model fit. Full model structure: response ~ cicada_{microbes} * cicada_{seed} * deer_{seed} + (1| plot_{microbes} / subplot_{microbes}) + (1| plot_{seed} / subplot_{seed})

	<i>Survival</i>	<i>Seed number</i>	<i>Total biomass (g)</i>	<i>Days to first leaf</i>	<i>Root: shoot</i>	<i>Nodule presence</i>	<i>Nodule number</i>	<i>Total nod. mass (g)</i>
<i>n</i>	145	62	62	54	62	62	32	32
Cicada _{microbes}	0.37	3.66+	2.62	4.25*	0.13	2.02	0.07	0.36
Cicada _{seed}	6.44*	5.75*	0.40	0.08	4.49*	0.75	0.02	0.58
Deer _{seed}	2.21	8.53**	0.33	0.18	0.56	0.01	0.00	0.05
Cicada _{microbes} × cicada _{seed}	--	2.81+	2.11	--	--	--	--	--
Cicada _{microbes} × deer _{seed}	2.73+	2.54	2.92+	--	--	2.11	--	--
Cicada _{seed} × deer _{seed}	--	3.78+	3.00+	--	9.76**	--	--	--
Cicada _{seed} × cicada _{mic} × deer _{seed}	--	2.50	--	--	--	--	--	--

Figure 1. We predicted that the resource pulse caused by decaying periodical cicada carcasses would (a) increase plant growth and (b) shift soil microbial community composition, leading to (c) maternal effects and (d) soil legacy effects on the next generation of plants; soil legacy effects may be directly caused by cicada carcasses or may be indirect and plant-mediated (e.g., changes in plant investment in root mutualists). (e) Preparing cicada carcasses for spreading on the field plots. (f) An *Amphicarpaea bracteata* plant growing in the field.

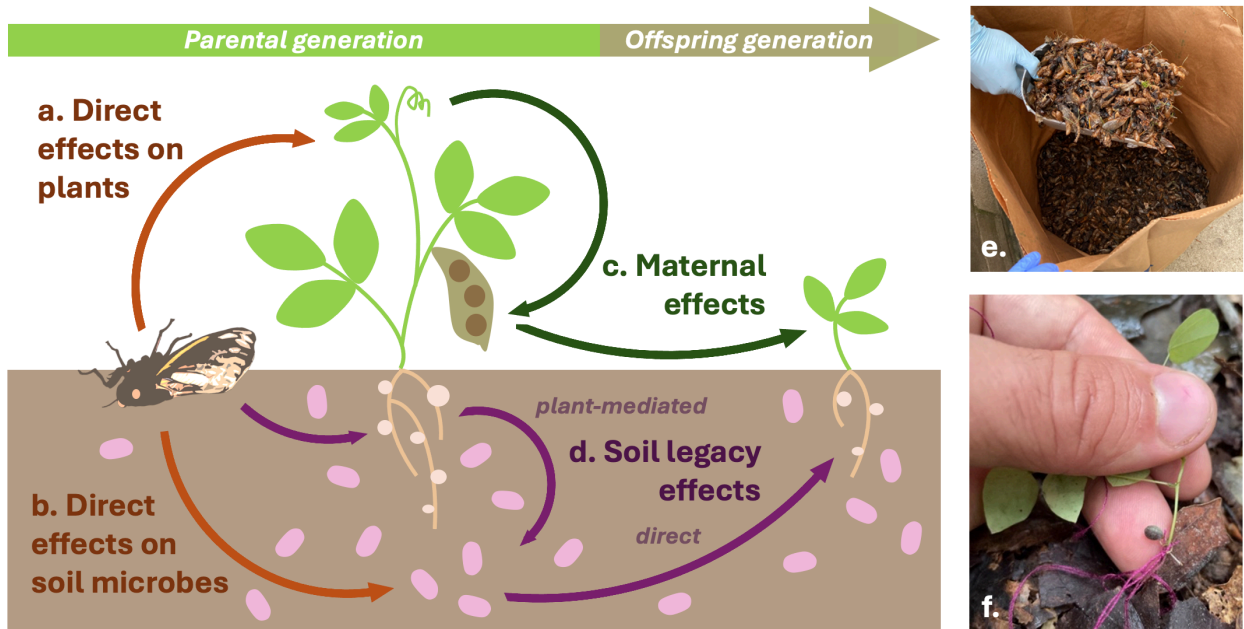


Figure 2. Addition of cicada carcasses (a) increased *Amphicarpaea* relative cover ($p = 0.010$), especially in the presence of deer (cicada \times deer: $p = 0.042$) and (b) increased likelihood of offspring seeds germinating ($p = 0.008$). Larger points: EMMs \pm SE.

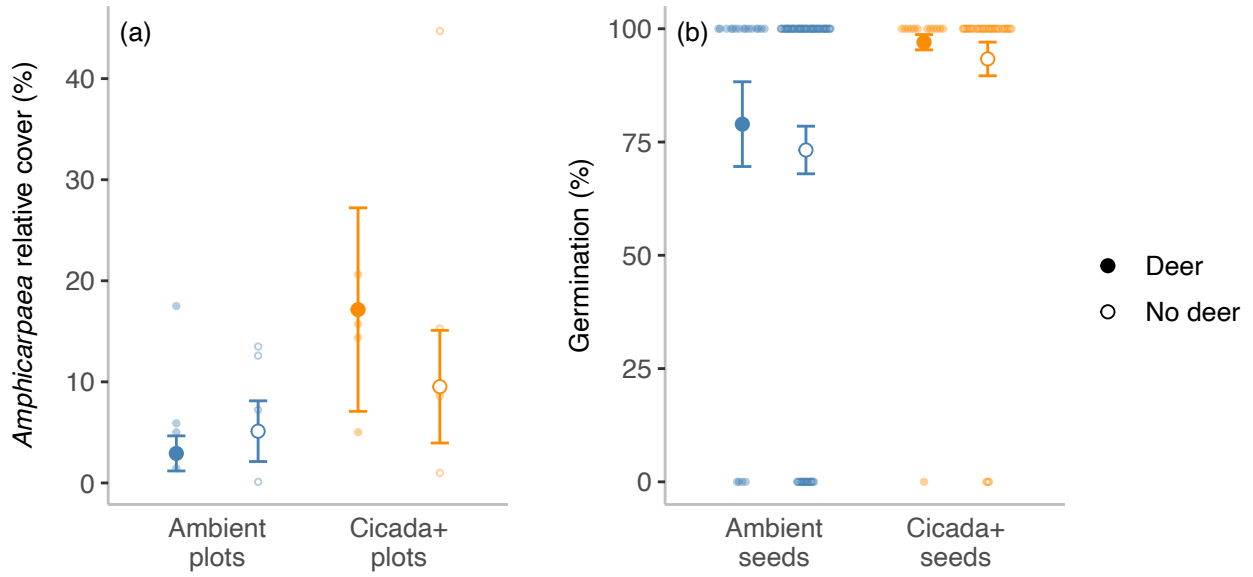


Figure 3. Cicada-induced maternal effects (a) reduced survival ($p = 0.011$) but (b) slightly increased seed number ($p = 0.016$) and (c) tended to increase biomass when maternal plants were protected from deer ($cicada_{seed} \times deer_{seed}$: $p = 0.083$). (d) Microbes from cicada-addition plots accelerated leaf phenology ($p = 0.039$). Larger points: EMMs \pm SE. Dashed lines: mean trait values for plants inoculated with sterilized soil slurry (all seed treatments pooled due to low replication). Three data points were cropped from (c) for clarity but not omitted from analyses.

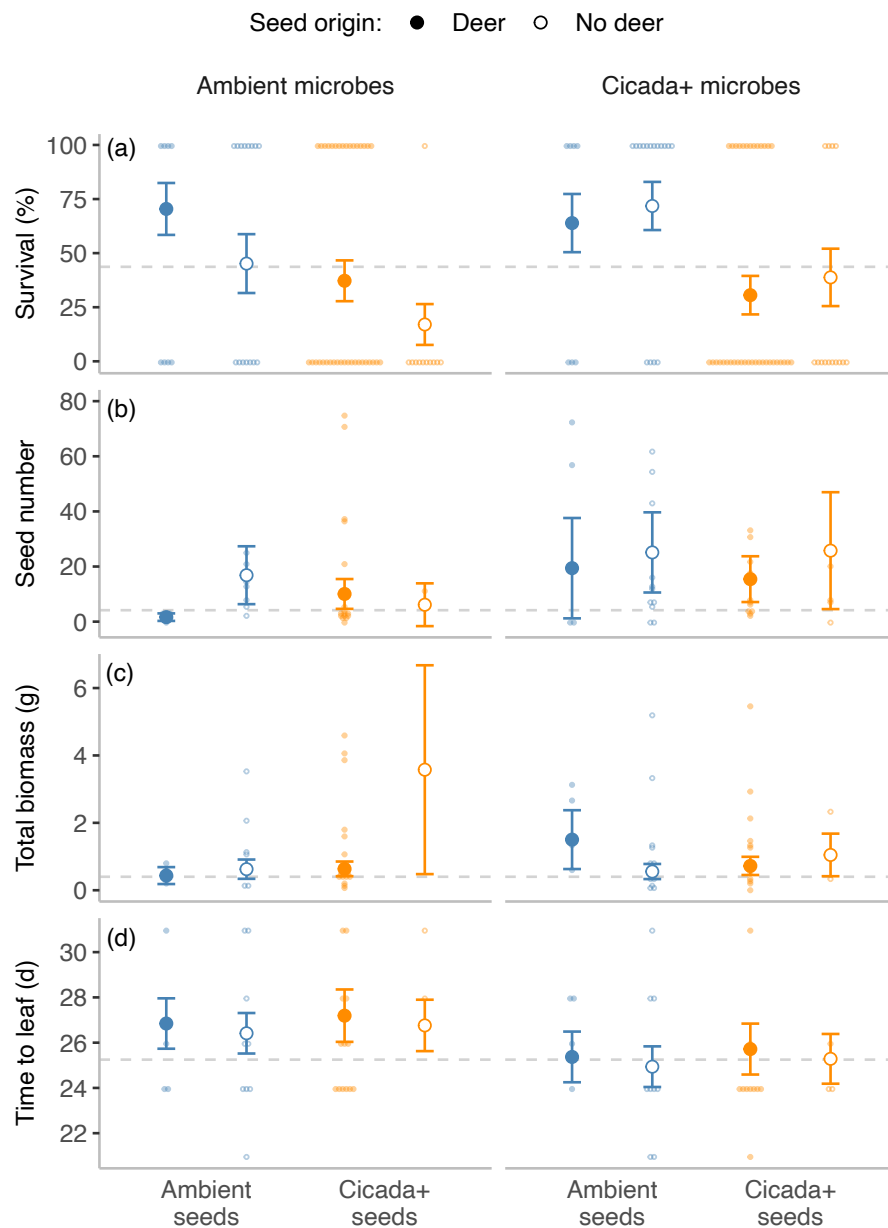
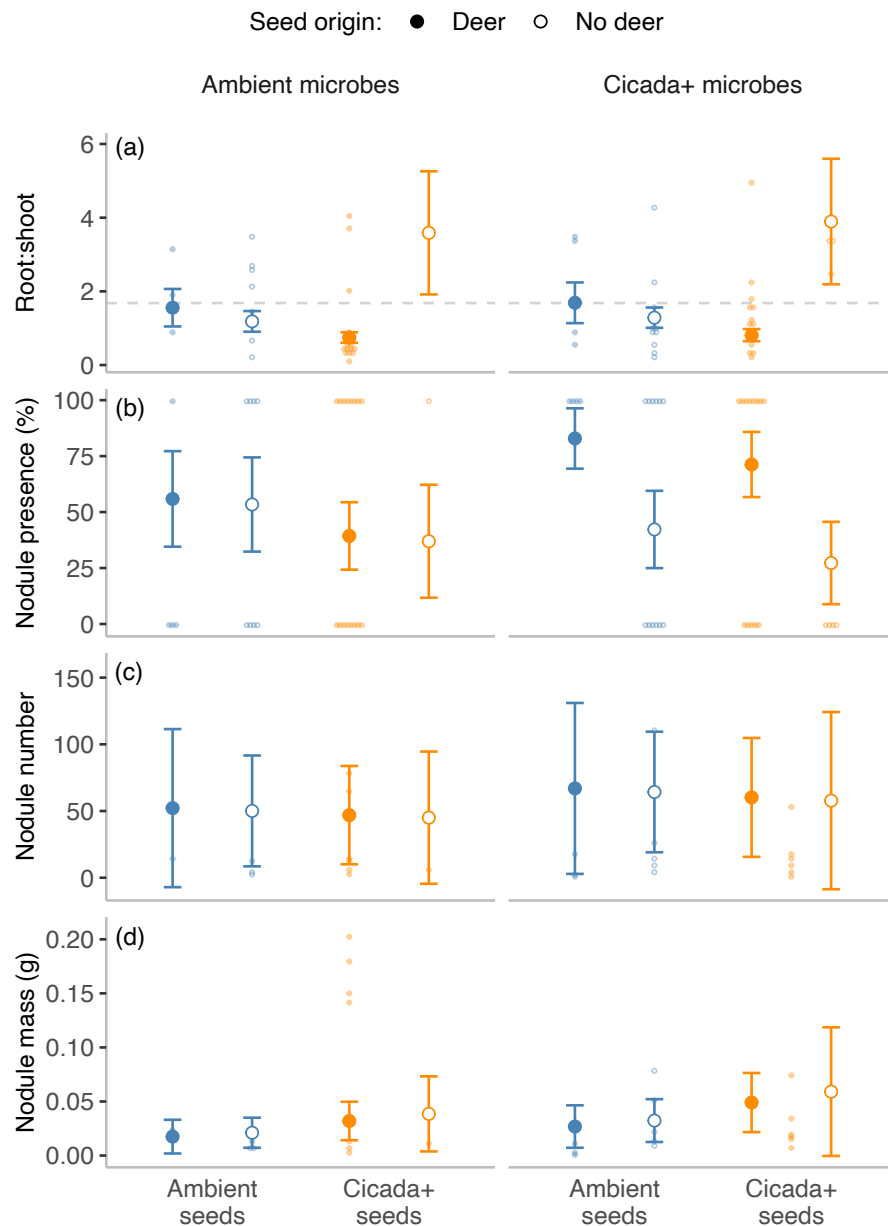


Figure 4. (a) Offspring of maternal plants from the fenced portions of cicada-addition plots had higher root:shoot ratios (cicada_{seed} × deer_{seed}: $p = 0.002$). Neither seed nor microbial origin affected (b) nodule presence, (c) nodule number, or (d) nodule mass (all $p > 0.1$). Larger points: EMMs ± SE. Dashed line: mean trait value for plants inoculated with sterilized soil slurry (all seed treatments pooled due to low replication). Two data points from (a), seven from (c), and five from (d) were cropped for clarity but were not omitted from analyses.



CHAPTER 3:

NITROGEN FERTILIZATION CAUSES MUTUALISM DECLINE BY ALTERING LIGHT AVAILABILITY AND HOST DENSITY

ABSTRACT

In addition to increasing soil nitrogen (N), fertilization also reduces light availability and legume density, and biological market theory predicts that any of these changes could lead to the evolution of reduced mutualist quality. Here, we experimentally test the relative contributions of increased N, reduced light, and reduced legume density to declines in microbial mutualist quality and whether the decline is reversible. We inoculated greenhouse mesocosms with field soil and factorially manipulated contemporary N fertilization, light availability, and legume density to investigate their interacting effects on rhizobium evolution. We inoculated additional mesocosms with soil from 33-year-old N-addition plots to test whether rhizobium quality recovers after cessation of N enrichment. Our one-year greenhouse experiment recapitulated a decline in mutualist quality of soil microbes found in a two-decade field experiment. Light had the strongest effect on microbial mutualist quality, but multiple drivers (e.g., both low legume density and high N) were required to cause mutualism decline. Although we recapitulated the mutualism decline observed in a long-term field experiment, we did not see an evolutionary reversal: microbes from high-N field plots did not regain plant benefits after evolving in conditions simulating cessation of N fertilization. Overall, we found that indirect effects of N enrichment synergistically caused a rapid decline in microbial mutualism and that mutualist quality cannot be recovered as easily as it can be lost.

INTRODUCTION

Evolutionary responses to environmental change often depend on the broader ecological community (Lau & terHorst, 2020; Lawrence et al., 2012). For example, microbial communities can help plants acclimate to drought stress (e.g., Lau and Lennon, 2012; Gehring et al., 2017), potentially reducing selection on plants. Conversely, plant adaptation to tolerate an invasive competitor was stronger when invasive herbivores were also present (Lau, 2008), and *Daphnia* only adapted to warmer temperatures in the presence of a predator (Tseng & O'Connor, 2015). Because the biotic community can act synergistically or antagonistically with abiotic change to affect evolution in ways that can be difficult to predict from single-species studies, understanding how community context affects evolution is important if we are to understand and predict evolutionary outcomes in nature.

Mutualisms are ubiquitous (Boucher et al., 1982; Bronstein, 1994) and underlie key steps of global nutrient cycling (Cleveland et al., 1999; van der Heijden et al., 2015) but may be particularly sensitive to global change because negative effects on one partner can create fitness feedbacks with other partners (Kiers et al., 2010). For example, the legume-rhizobium resource mutualism is based on the exchange of fixed nitrogen (N) by rhizobium bacteria and carbon (C) provided by leguminous plants via photosynthesis (Denison, 2000). However, N deposition is increasing globally (Vitousek et al., 1997), reducing the plant benefit of investing in rhizobia (Regus et al., 2017), and long-term N enrichment can lead to the evolution of reduced mutualist quality of rhizobium populations (Weese et al., 2015). Theory supports multiple plant-mediated pathways for this evolution to occur. These pathways are both direct and indirect because in addition to elevating soil N, fertilization also changes plant community productivity and composition, among other effects (reviewed in Cleland and Harpole, 2010). First, abundant soil

N is theorized to directly reduce plant investment in rhizobium mutualists because it may be less energetically costly for plants to forage for N in the soil by investing C in root growth rather than investing C in rhizobia (Akçay & Simms, 2011; Voisin et al., 2002; West et al., 2002). Second, N enrichment causes an increase in primary productivity, particularly favoring tall, rhizomatous perennial species (Dickson et al., 2014), which reduces light—and therefore C—availability for short-statured plants (Tilman, 1987). Biological market theory predicts that change in the availability of either traded resource in a nutritional mutualism will alter trade dynamics between mutualist partners (Schwartz & Hoeksema, 1998), so both increased N (direct effect of N fertilization) and decreased C (indirect effect via light availability) should favor reduced plant investment in rhizobia because both increase the relative cost of rhizobium-fixed N compared to investing C in plant growth. Third, N enrichment can shift the outcome of interspecific competition and cause dramatic reductions in the density of leguminous plant species that form symbioses with N-fixing rhizobia (Bobbink et al., 2010; Dickson & Gross, 2013; Suding et al., 2005). A reduction in the frequency of partnerships between plants and rhizobia either because of individual plant-level reduction in investment in symbiosis (as occurs with high soil N and reduced light) or because of an overall reduction in legume population density decreases plant selection for high-quality mutualists and also results in rhizobia spending more time in soil rather than within host environments. Rhizobia that spend relatively more time living saprophytically rather than in symbiosis with host plants are expected to become less effective mutualists (West et al., 2002), potentially because of trade-offs between traits favored in soil environments and mutualism-related traits (Sachs et al., 2011, but see Denison and Kiers, 2004).

Although legumes are particularly affected by the pairwise mutualisms they form with symbiotic rhizobium bacteria, plant fitness is also strongly affected by non-rhizobial members of

the soil microbial community. There is ample evidence that N fertilization affects microbial community composition (e.g., Leff et al., 2015) and functioning (e.g., Ramirez et al., 2012), but evidence is mixed as to whether these changes to microbial communities benefit or harm plant growth. Conceptual frameworks support either an increase or decrease in soil microbial community growth benefits to plants under nutrient enrichment depending on factors such as plant defense responses (i.e., defense vs. tolerance) or relative responses of mutualists vs. pathogens (Smith-Ramesh & Reynolds, 2017). A variety of experiments have demonstrated a reduction in plant growth benefits of fertilized soil microbial communities (Johnson, 1993; Lekberg et al., 2021; Simonsen et al., 2015; Weese et al., 2015), but other experiments have shown the opposite: for example, the decay of periodical cicadas (which causes a pulse of N and other nutrients into soil) increased the early plant growth benefits of soil microbes (Caple, in review {Ch. 2}). However, these studies have largely focused on symbiotic microbes and many were conducted in legumes, which are particularly reliant on mutualists.

Here, we used experimental evolution to disentangle three hypothesized drivers of reduced microbial mutualist quality under elevated N (repeatability of evolution) and investigate whether reduced N would improve mutualist quality (reversibility of evolution). To test the repeatability of evolution and identify the ecological factors driving this evolution, we inoculated plant communities in greenhouse mesocosms with whole soil microbial communities and factorially manipulated N fertilization, light availability, and legume host density. To test the reversibility of evolution, we inoculated additional mesocosms with soil microbes from a long-term N-addition field experiment and grew them in low and high N conditions. After one year, we used a common garden design to evaluate the plant phenotypic effects of the soil microbial communities that were conditioned by different greenhouse and field conditions. Throughout this

manuscript we loosely use the term “evolution” to refer to changes in our microbial communities because of the extensive body of mutualism theory predicting specific evolutionary changes in rhizobia due to soil N enrichment. In our experiment we cannot fully disentangle within-taxa evolution due to shifts in allele frequencies from shifts in microbial community composition, and our results are likely due to a combination of both community changes and strict-sense evolution, but we describe additional analyses that help provide evidence for the relative roles of microevolution, changes in rhizobium abundance, or other shifts in microbial community composition.

MATERIALS AND METHODS

Overview

In this study, we tackled two parallel aims. First, to investigate the repeatability and drivers of the evolution of reduced mutualist quality, we evolved a common soil microbial community in factorial combinations of low or high N, high or low light, and high or low host density. Second, we investigated the reversibility of reduced mutualist quality by evolving two different soil microbial communities—one from high-N soils and one from low-N soils—under low or high N. All mesocosms used to investigate evolutionary reversal had high light availability and low host density, because this most closely simulates the field conditions that would occur immediately following cessation of fertilization: light availability would recover quickly as productivity dropped, but there would be a time lag before legume hosts recolonized the plots to former densities. This design resulted in ten different treatment combinations. Eight treatment combinations were used to evaluate the repeatability of evolution, and four treatment combinations were used to evaluate evolutionary reversal; two treatment combinations were used

for both sets of analyses (Figure 1a). After conditioning soil microbial communities with these ten different treatment combinations for five simulated growing seasons (~one year), we inoculated individual plants with each experimental community to assess the plant phenotypic effects of each community.

Field soil collection

On 21 Dec 2021 we collected field soil from a long-term N-addition experiment in the early successional treatment plots at the Kellogg Biological Station Long-Term Ecological Research Site (KBS). Briefly, this N-addition experiment has added ammonium nitrate or urea to six replicate early-successional old field plant communities annually since 1989 (for full details, see Dickson & Gross, 2013). The plant communities in the N-addition plots have declined in diversity, with legumes now rare and tall rhizomatous perennials (e.g., *Solidago*) dominant (Dickson & Gross, 2013). We collected soil cores to a depth of ~15 cm from each of the six replicate field plots using a 5 cm diameter soil corer. One “N-addition” core was collected from each 5 m x 5 m N-addition experimental subplot and the six cores were homogenized together to create a single “N-addition” inoculant. While there is a formal 5 m x 5 m “control” experimental subplot paired with each N-addition subplot, we needed more “control” soil than “N-addition” soil (one soil core per N-addition plot and six cores per control plot; see below) and we were not able to collect such a large soil volume from these long-term experimental subplots without risking damage to the subplots. Therefore, instead of collecting “control” soil from the formal control subplots, we collected six “ambient” cores from throughout the unfertilized surrounding area, which is treated identically to the control subplots. All “ambient” soil cores were collected at least 2 m from the edge of any subplots that received other treatments (e.g., N addition, tilling). The 36 “ambient” cores (six cores from each of the six replicate field plots) were

homogenized together to create a single “ambient” inoculant. Organic matter was removed from the surface of the soil before sampling and the soil corer was sterilized using 95% ethanol between samples. Samples were sieved (4 mm) to remove rocks and homogenized within treatment, creating two soil microbial communities to evolve in the greenhouse: one from ambient soil and one from N-addition subplots. Soils were kept at 4 °C until use (23 d).

Greenhouse mesocosms

Each experimental mesocosm was a 9 L round plastic pot filled with a steam-sterilized 1:1:1 mixture of commercial potting media (Metro-Mix 360, Sun Gro Horticulture, Bellevue, WA, USA), sand, and calcined clay. To provide each mesocosm with a live, diverse soil microbial community, we sprinkled 250 mL “ambient” or “N-addition” soil across the surface of each mesocosm on 13 Jan 2022, the day before planting the first seeds.

Because microbial communities in the field are exposed to a diverse array of plant species rather than only to a single legume species, we simulated the early successional plant communities found at KBS in our mesocosms. Each pot contained 22 individuals from eight species commonly found in the KBS early-successional plots (Robertson & Snapp, 2020). Our host legume was Alsike clover, *Trifolium hybridum*, which forms mutualistic symbioses with *Rhizobium leguminosarum*. The plant communities also contained four grass species (tall oat grass, *Arrhenatherum elatius ssp. elatius* ‘Ruffner’; brome, *Bromus inermis*; orchard grass, *Dactylis glomerata* ‘Potomac’; timothy, *Phleum pratense* ‘Climax’) and three forb species (common yarrow, *Achillea millefolium*; Queen Anne’s lace, *Daucus carota*; Canada goldenrod, *Solidago canadensis* PA ecotype) (Table S1, Figure S2). All seeds were purchased from Ernst Conservation Seeds, Inc. (Meadville, PA).

Evolution phase

Clover density: To vary host legume density, we created two different plant community types. Each community contained the same number of total individuals so that plant competition would vary as little as possible, but the two communities had a different proportion of clover. The low-clover-density communities each contained one clover individual and three individuals of each of the other seven species, while the high-clover-density communities each contained eight clover individuals and two individuals of each of the other seven species (Figure S1).

N fertilization: During each fertilizer application, 100 mL N fertilizer solution (6 g ammonium nitrate {2.1 g N} / L) or 100 mL tap water was applied to each pot; this fertilizer level was chosen to closely mimic the amount applied to N-addition subplots at KBS (Dickson & Gross, 2013). Mesocosms were fertilized three times each ‘season’: three, four, and five weeks after seeds were planted (Table S2).

Light availability: We built cubic shade shelters out of 2 m PVC pipes covered in 60% shade cloth and placed them over the mesocosms assigned to low-light treatments. The entire greenhouse area received ambient sunlight supplemented with artificial light as needed to simulate a 16-hr day. In the absence of sunlight, supplemental lighting supplied approximately 450 $\mu\text{mol}/\text{m}^2/\text{s}$ PAR (photosynthetically active radiation).

The evolution phase had ten different treatment combinations. Eight treatments (factorial combinations of low/high N, low/high light, low/high clover density; all inoculated with ambient soil) were used to investigate the repeatability of microbial evolution and four (factorial combinations of low/high N and ambient/N-addition microbes; all high light and low clover density) were used to investigate the reversibility of microbial evolution; two treatments (low/high N with high light, low clover density, ambient microbes) were used for both questions

(Figure 1a). Each treatment combination was applied to ten replicate experimental mesocosms for a total of 100 mesocosms. Pots were arranged in a split-block design (Figure 1b). We created five blocks that each contained a shaded and unshaded half-block. We randomly determined which half-block would be on the left or right of the greenhouse bench, and N, host density, and microbial community treatments were distributed randomly within each half-block. All pots were watered as needed using drip lines.

Soil microbes evolved in the mesocosms for five simulated ‘growing seasons’ (Table S2). At the beginning of each ‘season’, all plants were planted from seed (Figure S1). Seeds were monitored for emergence from the soil; extra seedlings were thinned or additional seedlings transplanted in so that each mesocosm contained the target plant community composition. After growing for six weeks, all plants were harvested at the soil level, and then all mesocosms were left to fallow (un-watered) for three weeks so that root nodules would senesce and rhizobia would return to the soil. At the end of the fallow period, all soil and belowground biomass was removed from the pots. 4 L fresh sterilized media was then placed in the bottom of the pot, and 4 L homogenized soil from the previous ‘season’ placed on top so that germinating seeds would immediately be in contact with soil microbes. At the end of the fifth ‘season’, we collected soil from each mesocosm to evaluate the plant phenotypic effects of each microbial community. Fifth-season soils were collected 20-21 Feb 2023 and kept at 4 °C until use (3-4 d). Additional soil samples were saved for molecular analysis of the microbial communities. All tools, hands, and work surfaces were cleaned with 80% ethanol between each mesocosm during plant harvesting, biomass removal, soil replacement, and soil collection to minimize cross-contamination of microbial communities between mesocosms.

Test phase

We grew individual clover plants in a common greenhouse environment to assess the differential effects of soil microbes conditioned by different treatments during the evolution phase. On 24 Feb 2023 we sowed clover seeds into conetainers (164 mL Ray Leach Conetainers, Stuewe & Sons, Tangent, OR) filled with sterilized media prepared as in the evolution phase (see above); after emergence, we thinned seedlings so each conetainer contained a single individual.

We created one soil slurry from each of the 100 experimental mesocosms by mixing 10 mL mesocosm soil (~5 g dry mass) with 40 mL distilled water and shaking to homogenize. To evaluate the effects of each live soil microbial community relative to a low-microbe background, we inoculated control plants with distilled water or with a slurry created by combining equal volumes of each of the 100 live slurries and autoclaving at 120 °C for 60 min. We inoculated 2.5 mL of each live soil slurry onto five replicate clover plants and 2.5 mL of water or sterilized slurry onto 26 plants each on 24 Feb and 3 March 2024 (N = 5 reps × 100 mesocosms + 52 controls = 552 plants). Conetainers were arranged in 46 trays of 12 plants each; each tray contained at least one control. Microbial treatments were randomly assigned to each conetainer and controls were randomly arranged within each tray. Plants were watered as needed using drip lines and provided with ambient sunlight and supplemental lighting (approximately 450 $\mu\text{mol}/\text{m}^2/\text{s}$ PAR in the absence of sunlight) as needed to simulate a 16-hr day.

We counted the number of opened leaves on each plant on 3 Apr 2023 to gauge treatment effects on early growth, and measured leaf chlorophyll content using a SPAD meter (Konica Minolta, Inc., Osaka, Japan) on 4 Apr 2023. We harvested plants 17-30 Apr 2023, separated above- and belowground biomass at the soil surface, removed all potting media from the roots, and counted root nodules on each plant. We weighed biomass after drying for 3 d at 60 °C.

Statistical analyses

To identify the drivers of reduced mutualist quality, we fit generalized linear mixed models (GLMMs) with N fertilization, light availability, host density, and all interactions as fixed factors, and tray (test phase) and mesocosm (evolution phase; nested within split-block nested within block; see Figure 1b) as random effects (model structure: response \sim N * light * clover + (1|tray) + (1|block/split-block/mesocosm)). The model for nodule number also included the person who harvested the roots as a fixed effect because nodule counts can vary between observers, especially when nodules are small and numerous as tends to occur with low-quality rhizobia (model structure: response \sim N * light * clover + harvester + (1|tray) + (1|block/split-block/mesocosm)).

To investigate possible mutualism recovery, we fit GLMMs with greenhouse N fertilization, field N fertilization (microbe source) and their interaction as fixed factors, and tray (test phase) and mesocosm (evolution phase; nested within block) as random effects (model structure: response \sim N * microbe source + harvester + (1|tray) + (1|block/mesocosm); harvester was included only for the nodule number model).

We calculated log response ratios (LRRs) of estimated marginal means (EMMs) for each treatment combination relative to the baseline treatment to assess the relative and combined effects of each ecological factor that we manipulated. The LRR for each treatment group was calculated as $LRR = \ln(\text{treatment EMM} / \text{baseline treatment EMM})$; the baseline group was the treatment that most closely corresponded to conditions in ambient field plots (low N/high light/high clover for the subset of data examining repeatability of evolution; low N/ambient microbes for the subset of data examining reversibility of evolution). We calculated standard error for LRRs following Hedges et al. (1999).

We performed all analyses in R v.4.2.2 (R Core Team, 2020). We fit GLMMs using the *glmmTMB* package (Brooks et al., 2017), assessed significance using Type III Wald chi-squared tests with the `Anova` function from the *car* package (Fox & Weisberg, 2019), and checked model fits using the `simulateResiduals` function from the *DHARMA* package (Hartig, 2022). We calculated EMMs and assessed pairwise differences between treatment groups using the `emmeans` and `pwpm` functions from the *emmeans* package (Lenth, 2022). To ensure results were not driven by spurious data points, we excluded statistical outliers from each analysis; we considered a data point an outlier if the value was higher than the third quartile or lower than the first quartile by more than three times the interquartile range (three data points were excluded for shoot:root ratio and three for nodule number; all outliers were high values and had little qualitative effect on analysis results). We created figures using *ggplot2* (Wickham et al., 2024).

RESULTS

Rapid repetition of evolution

Our one-year greenhouse experiment rapidly recapitulated the results of a multi-decade field experiment. Compared to the treatment simulating ambient field conditions (low N, high light, high clover), the combined effect of the three manipulated factors, which simulated N-addition field conditions, resulted in microbial communities that reduced plant biomass, chlorophyll, early growth, shoot:root ratios, and probability of nodulation (pairwise comparisons, all $p < 0.01$; Table S3, Figure 2; for full ANOVA results see Table 1), but did not affect nodule number ($p = 0.78$, Table S3, Figure 2). The mutualism decline was caused by the combination of multiple ecological factors: N fertilization, decreased light availability, and reduced host density acted synergistically to select for less beneficial microbial communities (N \times light \times clover:

biomass, $\chi^2 = 14.93$, $p < 0.001$; chlorophyll, $\chi^2 = 8.96$, $p < 0.01$; early leaf count, $\chi^2 = 2.85$, $p = 0.09$; Table 1, Figure 2).

Light had the largest impact on microbial effects on plant growth, and a reduction in light availability was sufficient on its own to cause mutualist decline when measured by chlorophyll content, early leaf count, or nodule presence, but not biomass or nodule number (chlorophyll, $\chi^2 = 8.75$, $p < 0.01$; leaf count, $\chi^2 = 4.98$, $p = 0.03$; shoot:root, $\chi^2 = 5.81$, $p = 0.02$; nodule presence, $\chi^2 = 6.87$, $p < 0.01$; biomass, $\chi^2 = 2.29$, $p = 0.13$; nodule number, $\chi^2 = 1.29$, $p = 0.26$; Table 1, Figure 2). Neither increased soil N nor reduction in clover host availability were sufficient to cause mutualist decline alone (pairwise differences, all $p > 0.25$; Table S3, Figure 1), but both N and clover density contributed to decline in microbial benefits to plant fitness when combined with a reduction in light (N \times light: biomass, $\chi^2 = 5.32$, $p < 0.05$; chlorophyll, $\chi^2 = 2.97$, $p < 0.1$; nodule number, $\chi^2 = 11.55$, $p < 0.001$; light \times clover: biomass, $\chi^2 = 9.61$, $p < 0.01$; chlorophyll, $\chi^2 = 4.47$, $p < 0.05$; Table 1, Figure 2) or with each other (N \times clover: biomass, $\chi^2 = 15.56$, $p < 0.0001$; chlorophyll, $\chi^2 = 6.77$, $p < 0.01$; nodule presence, $\chi^2 = 9.85$, $p < 0.01$; Table 1, Figure 2).

No evolutionary reversal

Although we were able to recapitulate the decline in mutualism observed in the field, microbial communities that had experienced three decades of N fertilization did not recover their plant growth benefits after one year of conditioning in low N (Table 2, Figure 3). Microbes from ambient field plots exhibited mutualist decline (i.e., became less beneficial to plant growth) when grown under high N greenhouse conditions; however, microbial communities from N-addition field plots did not become more beneficial when grown in low N greenhouse conditions (greenhouse N \times microbe source: biomass, $\chi^2 = 4.59$, $p < 0.05$; Table 2, Figure 3), and early leaf count and chlorophyll content showed similar trends (greenhouse N \times microbe source:

chlorophyll, $\chi^2 = 3.27$, $p = 0.07$; leaf count, $\chi^2 = 3.50$, $p = 0.06$; Table 2, Figure 3). Neither nodule presence nor number differed between any microbial treatment groups (all $p > 0.3$; Table 2, Figure 3).

Cross-contamination between plants

18 of 52 control plants in the common garden phase formed root nodules, indicating that there was cross-contamination of soil microbes between our treatments. Nodulating control plants formed fewer nodules (1-105, mean 25) than nodulating plants inoculated with live microbes (1-390, mean 79). Since inoculant treatments were placed randomly, such contamination would make our results more conservative by making soil microbial communities in the common garden containers more similar to each other.

DISCUSSION

Rapid decline in microbial mutualist quality

Our one-year greenhouse experiment rapidly recapitulated the decline in mutualism observed in a two-decade field experiment and provides evidence that the evolution of reduced microbial mutualism under N fertilization largely results from plant community responses to N, namely reduced light availability and reduced legume density. These results provide empirical support for a combination of multiple pathways all theorized to contribute to mutualism decline and implies that mutualism decline observed in the field by Weese and coauthors (2015) may have occurred within the first few years of the experiment rather than over decades. Our experiment demonstrates the necessity of simultaneously and factorially manipulating theorized drivers of evolution, because we found many non-additive effects that would be impossible to

predict from studies of single ecological factors. It also illustrates the importance of considering ecological context in evolutionary studies, because we saw the strongest effects on microbial changes from indirect ecological effects mediated by the plant community response to N.

While N and legume density on their own did not drive the evolution of less beneficial microbial communities, shade tended to result in microbial communities that provided reduced plant benefits. This may result because light (the source of plant C) is a particularly important resource for the mutualism. Fixation of atmospheric N is energy-intensive, and host plants allocate as much as 14% of recently-fixed C to support N-fixing rhizobia (Kaschuk et al., 2009). When light is abundant, symbiotically-fixed N allows plants to increase photosynthetic rates to offset the C provided to rhizobia (Kaschuk et al., 2009), but when light is limited plants do not have sufficient C to support N fixation and investing in rhizobia no longer provides a fitness benefit (e.g., Lau et al., 2012). However, it is also possible that these findings resulted because our shading treatment was severe. Although our light reduction (60% shade cloth) was a similar intensity to light reductions experienced by understory plants in fertilized plots at KBS (~47%, K. Gross, *unpub.*) and in a similar experiment in Swiss grasslands (~62%, Hautier et al., 2009), we shaded entire mesocosms, which reduced light availability for overstory plants as well as the understory. This could affect the soil microbial community by altering the root exudates of overstory plants (Huang et al., 2014; Wall & Moore, 1999) which were C limited in this study but not in field studies.

One caveat of our study is that we cannot disentangle the effects of rhizobium evolution from the effects of rhizobium abundance or changes in other microbial community members because we used whole-soil microbial inoculations. In real-world systems microevolution and shifts in microbial community composition happen simultaneously and both contribute to plant

fitness, but it is worth further consideration because implications are slightly different if our results are driven by evolution of rhizobia or simply a decline in abundance or a shift in microbial community composition. We did not observe differences in relative abundance of rhizobia between treatments (S. Bedwell, *in prep*), but shade, high N, and reduced legume density decreased plants' likelihood of nodulation. This implies that the declines in mutualism we observed likely were due to a decrease in the average mutualist quality of rhizobia—which could include a decline in proportion of strains able to nodulate—rather than simply due to declines in rhizobium abundance. Non-rhizobium microbes also may have contributed to an overall decline in the plant growth benefits of soil microbial communities. In particular, our shading treatment significantly altered microbial community composition (16S sequences; S. Bedwell, *in prep*) and these severe shading treatments may have contributed to a proliferation of pathogens in low-light environments (Smith-Ramesh & Reynolds, 2017). However, theoretical predictions on how non-mutualist microbes should shift in response to elevated N are equivocal. Plant-soil feedback literature predicts that N may either suppress pathogens if plant defenses are bolstered by high resource availability or promote pathogen proliferation if plants invest in growth rather than defense (Smith-Ramesh & Reynolds, 2017). Similarly, a decrease in clover abundance could lead to a decrease in the abundance of host-specific pathogens or mutualists (Bever et al., 1997), which could make microbial communities either more or less beneficial to plant growth. We did not see significant effects of N or clover density on microbial community composition (16S sequences; S. Bedwell, *in prep*), but undetected changes in non-rhizobium microbes may have contributed to our observed declines in microbial plant growth benefits.

No evolutionary reversal

This experiment demonstrates that it is easier to shift soil microbes to decreased than increased mutualism. If the decline is driven by evolutionary changes in rhizobium populations as observed in the field (Weese et al. 2015), then this could be due to at least three factors, including lack of genetic variation, insufficient host selection, or low rhizobium population sizes.

First, if beneficial rhizobium strains were rare in the N-addition field inoculant, plants may have had only a slim chance of encountering a high-quality rhizobium strain. N fertilization could have eroded genetic variation for rhizobium quality in N-fertilized field plots by selection on standing genetic variation, gain/loss of plasmids, or *de novo* mutations. KBS rhizobium populations maintained a high level of diversity after twenty years of fertilization (Weese et al., 2015), and we did not see differences in *Rhizobium* 16S Shannon diversity between any of our treatments (S. Bedwell, *in prep*), so loss of strain-level rhizobium genetic variation is unlikely to have caused our results. However, many of the *Rhizobium* genes necessary for symbiosis and N fixation are housed on plasmids (Reeve et al., 2010). Differences in these ‘pSym’ plasmids contribute to rhizobium quality differences, and N fertilization reduced nucleotide variation of several key pSym genes (Klinger et al., 2016). We did not see differences in nodule presence or number between field soil or greenhouse fertilization treatments, so overall pSym prevalence is unlikely to explain our results. Instead, our observed mutualism decline could be due to changes in the relative abundance of low- and high-quality pSyms. Plasmids can be gained or lost through horizontal gene transfer (HGT) (Scott & Ronson, 1982; Vereau Gorbitz et al., 2024), but if high-quality pSyms were absent in high-N soils there would be no way for even strong host selection and frequent HGT to increase their prevalence. In the field, it’s likely that high-quality pSyms would eventually re-colonize areas where they were lost because pSyms can be transferred

between populations separated by large distances (Geniaux et al., 1993), but cross-mesocosm contamination in our experiment may have been infrequent enough to prevent this from occurring during our relatively short experiment, or conditions may not have favored HGT of plasmids (Ling et al., 2016; Wardell et al., 2022). Alternately, if changes in rhizobium genetic variation for partner quality were due to microevolution and *de novo* mutations rather than changes in strain frequencies and HGT of plasmids, then it is unsurprising that we would see more rapid decline than recovery of mutualist quality because most mutations are deleterious (reviewed in Bataillon and Bailey, 2014). However, evolution occurs more rapidly through standing variation than by new mutations (Innan & Kim, 2004; Prezeworski et al., 2005), so although our year-long experiment could have included many rhizobium generations, mutation accumulation is also not likely to be a major factor in our relatively short experiment.

Second, even if rhizobium genetic variation remained high, there may have been low selective pressure from host plants on rhizobium quality because the mesocosms used to test for evolutionary reversal only contained one clover plant each. Even if each plant preferentially rewarded the most beneficial rhizobium strains that it associated with, this host selection may not have been strong enough to counteract selection occurring in the soil. A higher density of clover or more ‘seasons’ of plant selection may have increased the likelihood that plants would encounter high-quality strains that still remained in the community, so a longer experiment with more host plants may have caused us to show a recovery of mutualist quality.

Finally, as in the first section of the experiment, our results confound effects of rhizobium abundance and evolution. Rhizobium abundance was likely low in the microbial inoculants created from N-addition field soil, as posited by Weese and coauthors (2015) and observed along a naturally-occurring N gradient (Thrall et al., 2007). Low population densities might prevent

mutualism recovery because selection is less likely to outweigh drift in small populations (Wright, 1931), and because the opportunities for partnership between plants and rhizobia would be rare and thus the opportunity to select for higher mutualism quality limited. However, we did not find differences in rhizobium relative abundance or diversity (based on 16S sequences; S. Bedwell, *in prep*) or in nodule presence or number between treatments. Therefore, differences in overall rhizobium abundance are unlikely to be the main factor underlying our results because the opportunities for interactions between plants and rhizobia as a whole were likely similar between treatments.

Our experiment also shows that legacies of prior selection can be important, because the two microbial communities we used as starting inoculants responded differently to our experimental N addition: ambient microbes became less mutualistic while N-addition field microbes did not respond. Previous studies have shown lasting effects of short-term plant selection (vs. plant-free soil) on soil microbes' effects on the growth of the following 'generation' of plants (Burghardt et al., 2019). Our experiment supports and expands these previous results by demonstrating that differences between soil microbial communities that have undergone differential selection can persist even after a year of selection (five 'seasons' of plant selection), showing that these effects can be very long-lasting. Legacies of past selection may therefore be an important consideration when predicting how microbial communities in the field may respond to environmental change.

Conclusion

Overall, this experiment demonstrates the importance of biotic community context on microbial responses to global change. Specifically, we show that the evolution of reduced cooperation under elevated soil N is largely mediated through ecological factors that change due

to plant community responses to N: light and host legume availability. Additionally, we found that mutualist quality is more readily lost than gained. More broadly, we demonstrate that while the general course of microbial evolution may be predictable and repeatable, the specific drivers of this evolution are complex and have non-additive effects when combined that would be difficult or impossible to predict from experiments involving single selective factors or without a realistic community context.

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TABLES AND FIGURES

Table 1. ANOVA results of experimentally manipulated N fertilization, light availability, and clover density on the plant growth effects of soil microbial communities. All predictors in the models are ecological factors applied to microbial communities in the greenhouse; all plants were measured under identical abiotic conditions, differing only in the evolutionary history of the microbes they were inoculated with. Plants inoculated with sterilized control were excluded. Nodule number analysis includes only plants with at least one nodule. Values shown are Type III Wald chi-squared tests for each fixed factor included in the mixed model; p-values are given in parentheses. Significant predictors are indicated by bold type and asterisks ($p < 0.001^{***}$, $p < 0.01^{**}$, $p < 0.05^*$, $p < 0.1^+$). Model structure: response \sim N \times light \times clover + harvester + (1|tray) + (1|block/split-block/mesocosm); harvester was included only for nodule number.

	<i>Total biomass (g)</i>	<i>Chlorophyll</i>	<i>Leaf count</i>	<i>Shoot:root</i>	<i>Nodule presence</i>	<i>Nodule number</i>
N fertilization	0.00 (0.982)	0.02 (0.879)	0.09 (0.766)	0.05 (0.815)	0.00 (1.00)	1.28 (0.259)
Light level	2.29 (0.130)	8.75** (0.003)	4.98* (0.026)	5.81* (0.016)	7.09** (0.008)	1.29 (0.256)
Clover density	0.00 (0.949)	0.00 (0.992)	1.18 (0.277)	0.58 (0.448)	0.20 (0.657)	0.19 (0.661)
N \times light	9.82** (0.002)	6.29* (0.012)	2.01 (0.156)	3.19+ (0.074)	0.00 (1.00)	11.55*** (0.001)
N \times clover	2.29 (0.131)	2.62 (0.105)	1.00 (0.318)	0.09 (0.766)	0.00 (1.00)	0.20 (0.652)
Light \times clover	9.53** (0.002)	4.48* (0.034)	0.13 (0.718)	0.30 (0.583)	0.88 (0.347)	0.00 (0.970)
N \times light \times clover	14.93*** (<0.001)	8.96** (0.003)	2.85+ (0.091)	1.63 (0.202)	0.00 (1.00)	3.68+ (0.055)

Table 2. ANOVA results of contemporary/short-term ('N fertilization') and historical/long-term ('Microbe source') N enrichment on the plant growth effects of soil microbial communities. All predictors in the models are ecological factors applied to microbial communities in the greenhouse; all plants were measured under identical abiotic conditions, differing only in the evolutionary history of the microbes they were inoculated with. Plants inoculated with sterilized control were excluded. Nodule number analysis includes only plants with at least one nodule. Values shown are Type III Wald chi-squared tests for each fixed factor included in the mixed model; p-values are given in parentheses. Significant predictors are indicated by bold type and asterisks ($p < 0.001^{***}$, $p < 0.01^{**}$, $p < 0.05^*$, $p < 0.1^+$). Model structure: response \sim N \times microbe source + harvester + (1|tray) + (1|block/mesocosm); harvester was included only for nodule number.

	<i>Total biomass (g)</i>	<i>Chlorophyll</i>	<i>Leaf count April 3</i>	<i>Shoot:root</i>	<i>Nodule presence</i>	<i>Nodule number</i>
N fertilization	5.02* (0.025)	4.77* (0.029)	1.49 (0.223)	0.58 (0.446)	1.06 (0.303)	0.63 (0.426)
Microbe source	1.58 (0.209)	4.86* (0.028)	1.86 (0.173)	3.77+ (0.052)	0.00 (1.00)	1.77 (0.183)
N \times microbe source	4.59* (0.032)	3.27+ (0.071)	3.50+ (0.061)	0.11 (0.741)	0.46 (0.498)	0.41 (0.521)

Figure 1. Graphical depiction of mesocosm treatments used for each subset of data analysis (repetition of evolution, reversal of evolution) (a). Layout of greenhouse mesocosms (b) showing levels of replication in the evolution phase.

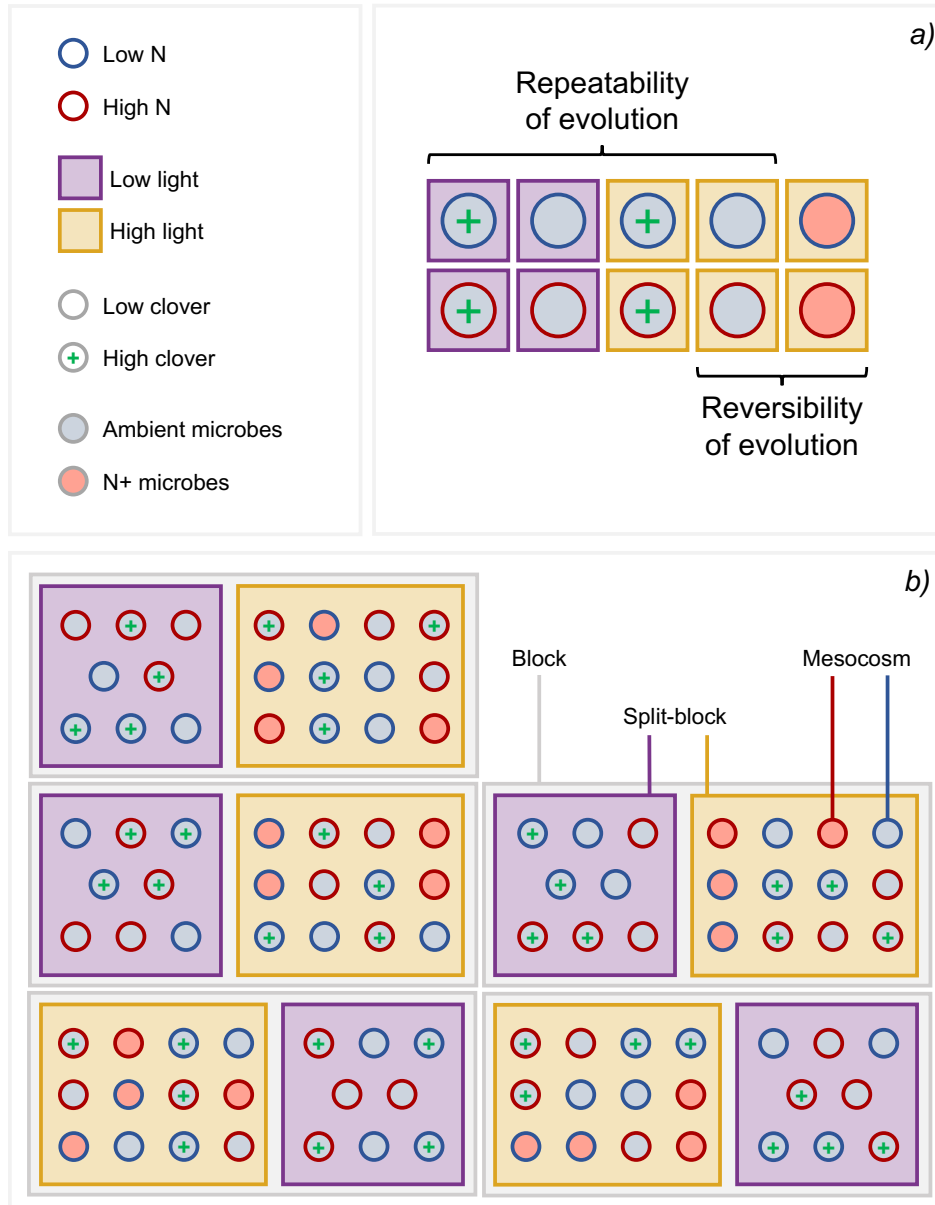


Figure 2. The combination of all three manipulated factors (a-d) reduced the benefit of microbial communities to plant fitness and growth and (e) reduced the prevalence of nodulation but (f) not the number of nodules formed. Light alone altered microbial communities in ways that affected (b) plant chlorophyll content, (c) early growth, (d) shoot:root ratio, and (e) likelihood of nodulation, but not (a) biomass or (f) nodule number. Most combinations of increased N, decreased light, and decreased clover density also reduced the mutualist quality of microbial communities. The conditions of ambient field plots most closely correspond to the ‘ambient’ treatment (low N, high light, high clover density) while the conditions of N-fertilized field plots most closely correspond to the ‘+N, -L, -C’ treatment (high N, low light, low clover density). Log response ratios (LRRs) \pm SE; asterisks indicate treatments that significantly differed from the ‘ambient’ treatment ($p < 0.001$ ***, $p < 0.01$ ** , $p < 0.05$ *, $p < 0.1$ +). LRRs are calculated relative to the ‘ambient’ treatment; grey shaded regions correspond to the SE of the ‘ambient’ treatment.

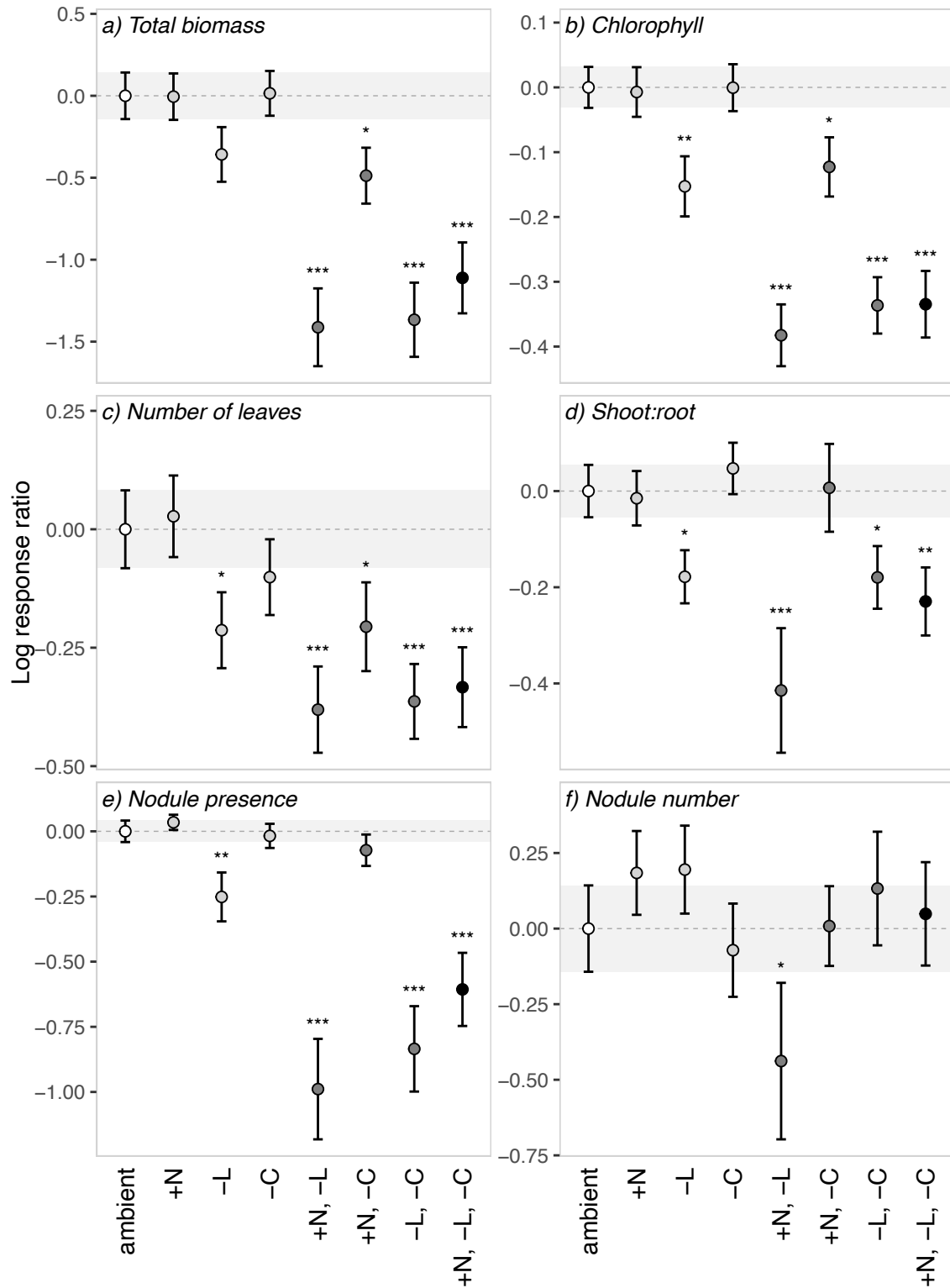


Figure 2.

Figure 3. (a, b) The plant growth benefits of ambient field soil microbial communities declined after being conditioned by high N in the greenhouse, but conditioning in low N did not make soil microbes from N-addition field plots more beneficial to plant growth. Neither greenhouse nor field microbial N history affected (c) early growth or (e) the likelihood of plants to form nodules. Plants had (d) lower shoot:root ratios and (f) formed more nodules when inoculated with microbes from N-addition field soil than with microbes from ambient soil but (d) shoot:root ratio and (f) nodule number were not affected by greenhouse mesocosm N treatment. LRRs \pm SE; asterisks indicate treatments that significantly differed from the baseline treatment of low N/ambient microbes ($p < 0.001^{***}$, $p < 0.01^{**}$, $p < 0.05^*$, $p < 0.1+$). LRRs are calculated relative to the low N/ambient microbes treatment; grey shaded regions correspond to the SE of this treatment.

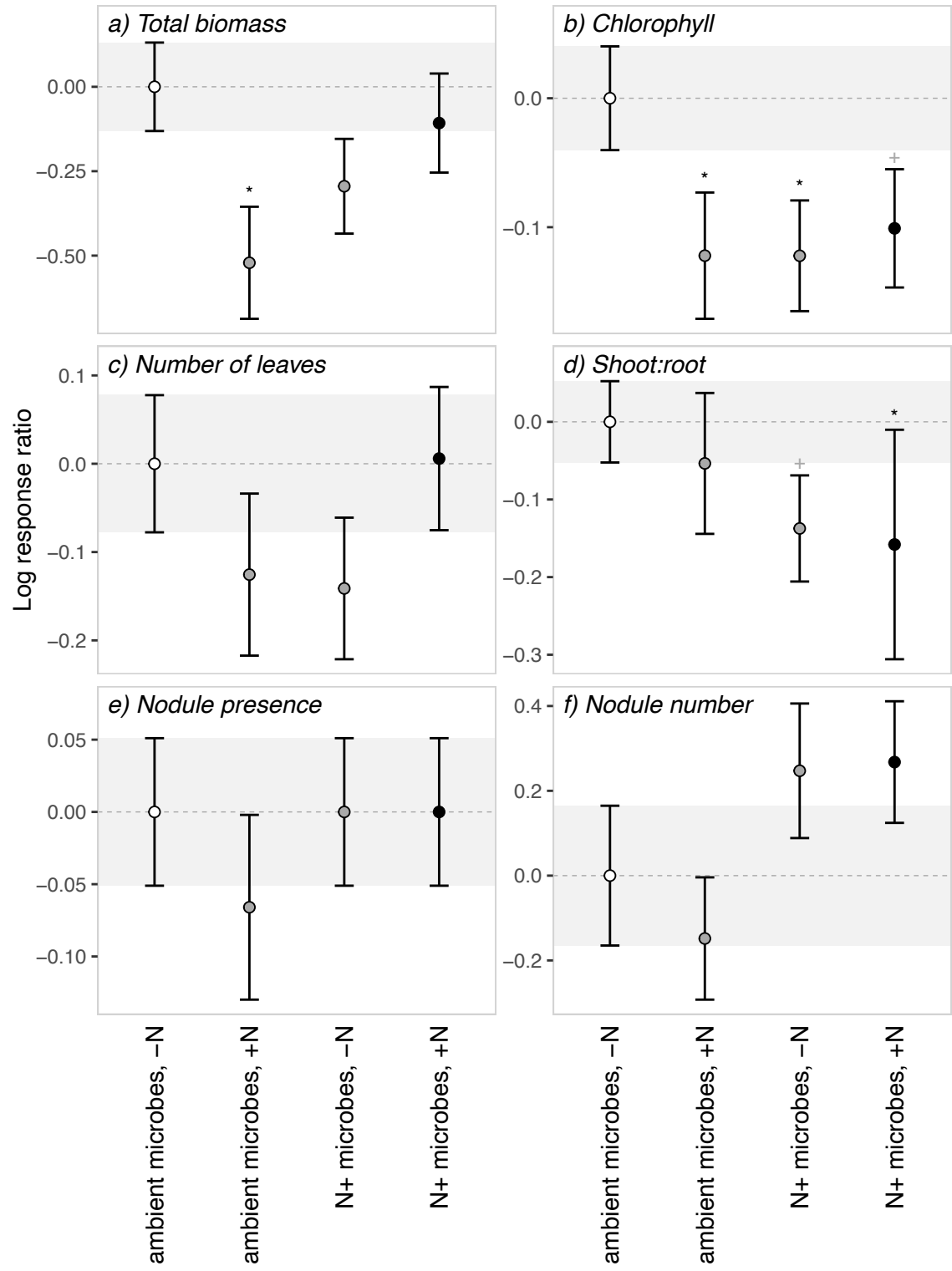


Figure 3.

APPENDIX A:

SUPPLEMENTAL INFORMATION FOR ‘ASYMMETRIC LOCAL ADAPTATION TO NITROGEN IN A PLANT-MICROBE MUTUALISM’

Table S1. Soil nitrogen (NH₄ and NO₃), soil moisture (gravimetric water content, GWC), and PAR of each site, measured by T. Suwa; see (Suwa, 2016) for full methods. Briefly, five soil samples per site were homogenized, extracted in KCl, and analyzed with an Alpkem/OI Analytic Flow Solution IV analyzer (Model 3550). GWC was measured by comparing wet and dry weights of these same soil samples and was correlated with volumetric water content (VWC) measurements taken repeatedly in the field using HydroSence II (Campbell Scientific Inc., North Logan, Utah). Bold: sources for soil microbial communities. Italics: source of microbial communities but not seeds.

<i>Site</i>	<i>Total soil N (µg/g)</i>	<i>Soil H₂O (%)</i>	<i>Lat.</i>	<i>Long.</i>	<i>Families</i>	<i>Seeds</i>	<i>Seeds/family</i>
Brooke Lodge A (BLA)	3.47	14.78	42.3560	-85.3804	5	3	1-3
Fort Custer H (FCH)	3.56	15.31	42.2975	-85.3227	37	10	2-5
Brooke Lodge B (BLB)	3.93	4.17	42.3583	-85.3769	29	9	1-7
Carter Lake B (CLB)	4.83	11.29	42.6743	-85.3004	57	9	3-8
Pierce Cedar Creek I (CCI)	5.39	9.80	42.5343	-85.2907	35	10	1-6
Brooke Lodge E (BLE)	7.57	6.04	42.3649	-85.3734	51	10	2-7
Luxe Arbor E (LAE)	8.68	10.47	42.4788	-85.4600	55	10	4-7
<i>Nature Center C (NCC)</i>	<i>8.93</i>	<i>37.94</i>	<i>42.3614</i>	<i>-85.5784</i>	<i>0</i>	<i>0</i>	<i>0</i>
Nature Center A (NCA)	9.48	20.28	42.3616	-85.5794	18	8	1-4
Pierce Cedar Creek B (CCB)	10.87	30.09	42.5428	-85.2992	24	7	2-5
Pierce Cedar Creek C (CCC)	11.66	24.14	42.5441	-85.2937	35	10	1-6
Nature Center D (NCD)	14.36	39.10	42.3644	-85.5793	27	10	1-6
Luxe Arbor A (LAA)	19.97	26.88	42.4817	-85.4640	55	10	4-7

Table S2. Surviving plants in each treatment combination (from 4 replicates planted). Bold italics: restricted dataset examining sympatric/allopatric combinations of plants and microbes. Plant populations and microbial communities are listed in order of increasing historical soil N (see Table S1).

Mic. community	LAE			NCC			NCD			LAA			Control		
	low	med	high	low	med	high	low	med	high	low	med	high	low	med	high
BLA	0	0	1	0	1	0	0	2	0	1	0	0	0	0	0
FCH	3	3	2	4	3	2	2	3	2	4	3	2	1	1	2
BLB	1	2	3	3	2	2	2	2	2	1	2	1	1	4	1
CLB	4	4	4	4	4	4	4	4	4	4	4	4	4	3	2
CCI	2	1	2	2	2	2	4	3	2	2	2	3	2	4	2
BLE	4	4	3	4	4	4	3	4	3	2	4	4	2	3	3
LAE	3	4	4	4	3	3	4	4	4	4	4	4	3	4	3
NCA	1	2	3	2	0	2	0	2	2	1	2	0	0	1	0
CCB	1	2	2	3	1	2	0	2	3	0	2	1	3	2	0
CCC	4	2	2	2	2	3	2	2	3	2	2	2	3	2	2
NCD	2	0	2	2	3	2	2	1	2	0	1	3	3	2	2
LAA	4	4	4	4	4	4	4	4	4	4	3	3	4	3	2

Table S3 (extension of Table 1). We fit models for six additional response variables not described in the main text. Plant survival and nodule presence essentially act as the first step in hurdle models, because all other analyses were performed only on plants that survived until harvest and all analyses involving nodule traits were performed only on plants that formed at least one nodule. Here, we also compare results of analyses on total nodule mass (and ratios thereof) with analyses on nodule number (and ratios thereof). Plant populations did not exhibit local adaptation to soil N (i.e., no significant plant N history \times contemporary N interactions affecting plant fitness components). Values shown are Type III Wald chi-squared tests for each fixed factor included in the mixed model ANOVA; p-values are shown in parentheses. Plants inoculated with sterilized control were excluded from this analysis. Significant predictors are indicated by bold type and asterisks ($p < 0.001^{***}$, $p < 0.01^{**}$, $p < 0.05^*$, $p < 0.1+$). Model structure: response \sim plant N history * contemporary N + (1|microbial community) + (1|plant population/seed family).

	<i>Df</i>	<i>Survival</i>	<i>Total biomass (g)</i>	<i>Nodule presence</i>	<i>Total nodule mass (g)</i>	<i>Nodule mass: root mass</i>	<i>Seeds:nodule mass (g)</i>
Plant N history	1	0.39 (0.530)	1.63 (0.201)	0.18 (0.669)	1.39 (0.238)	0.62 (0.432)	0.72 (0.397)
Cont. N	1	2.44 (0.295)	37.86*** (<0.0001)	2.18 (0.336)	0.30 (0.861)	0.22 (0.897)	1.72 (0.422)
Plant N hist. \times cont. N	2	2.85 (0.241)	2.11 (0.348)	2.14 (0.342)	0.24 (0.885)	0.58 (0.748)	0.33 (0.848)

Table S4 (extension of Table 2). We fit models for six additional response variables not presented in the main text. Plant survival and nodule presence essentially act as the first step in hurdle models, because all other analyses were performed only on plants that survived until harvest and all analyses involving nodule traits were performed only on plants that formed at least one nodule. Here, we also compare results of analyses on total nodule mass (and ratios thereof) with analyses on nodule number (and ratios thereof). Values shown are Type III Wald chi-squared tests for each fixed factor included in the mixed model ANOVA; p-values are shown in parentheses. Plants inoculated with sterilized control were excluded from this analysis. Significant predictors are indicated by bold type and asterisks ($p < 0.001^{***}$, $p < 0.01^{**}$, $p < 0.05^*$, $p < 0.1+$). Model structure: response \sim microbial N history * contemporary N + (1|microbial community) + (1|plant population/seed family).

	<i>Df</i>	<i>Survival</i>	<i>Total biomass (g)</i>	<i>Nodule presence</i>	<i>Total nodule mass (g)</i>	<i>Nodule mass: root mass</i>	<i>Seeds:nodule mass (g)</i>
Microbe N history	1	3.02+ (0.073)	0.65 (0.421)	0.50 (0.48)	0.54 (0.463)	1.23 (0.268)	<i>NaNs</i>
Cont. N	1	1.19 (0.551)	15.63*** (<0.0001)	0.78 (0.678)	11.13** (0.004)	24.93*** (<0.0001)	<i>NaNs</i>
Mic. N hist. × cont. N	2	1.82 (0.403)	2.90 (0.235)	1.00 (0.607)	11.55** (0.003)	19.85*** (<0.0001)	<i>NaNs</i>

Table S5 (extension of Table 3). We fit models for six additional response variables not presented in the main text. Plant survival and nodule presence essentially act as the first step in hurdle models, because all other analyses were performed only on plants that survived until harvest and all analyses involving nodule traits were performed only on plants that formed at least one nodule. Here, we also compare results of analyses of total nodule mass (and ratios thereof) with analyses of nodule number (and ratios thereof). When all plant populations and microbial communities were considered (a), nodule number (but not presence) differed with different combinations of populations and communities, but this effect was only marginally significant when only plant populations and microbial communities that had a sympatric partner were included in the analysis (b). We did not find local adaptation of plants or microbes to sympatric partners: neither plants nor rhizobia maximized fitness when grown with sympatric mutualist partners (Figs. S6-S9). Values shown are Type III Wald chi-squared tests for each fixed factor included in the mixed model ANOVA; p-values are shown in parentheses. Plants inoculated with sterilized control were excluded from this analysis. Nodule presence was analyzed using adjusted data to allow comparisons between plant populations that had 100% of plants form nodules (see Figure S11). Total nodule mass and nodule number analyses include only plants with at least one nodule. Some interactions are omitted because our dataset was not large enough to run such complex models and analyses that included the interaction returned NaNs. Significant predictors are indicated by bold type and asterisks (**p < 0.001*****, **p < 0.01****, **p < 0.05***, p < 0.1+). Model structure: response ~ plant population * microbial community + growth time + (1|seed family).

Table S5.

a. All populations/communities							
	<i>Df</i>	<i>Survival</i>	<i>Total biomass (g)</i>	<i>Nodule presence</i>	<i>Total nodule mass (g)</i>	<i>Nod mass:root mass</i>	<i>Seeds:nod mass (g)</i>
Plant population	11	20.47* (0.039)	14.83 (0.190)	29.41** (0.002)	45.27*** (<0.0001)	39.98*** (<0.0001)	48.96*** (<0.0001)
Microbial community	3	0.44 (0.933)	1.04 (0.791)	1.64 (0.651)	2.02 (0.567)	1.62 (0.654)	5.70 (0.127)
Contemporary N	2	0.36 (0.836)	135.31*** (<0.0001)	1.07 (0.586)	2.58 (0.275)	6.87* (0.032)	12.85** (0.002)
Plant pop × mic comm	33	11.36 (1.00)	30.81 (0.577)	--	47.91* (0.045)	49.54* (0.032)	59.8** (0.003)
b. Only populations/communities with a sympatric partner							
	<i>Df</i>	<i>Survival</i>	<i>Total biomass (g)</i>	<i>Nodule presence</i>	<i>Total nodule mass (g)</i>	<i>Nod mass:root mass</i>	<i>Seeds:nod mass (g)</i>
Plant population	2	14.66*** (0.001)	1.14 (0.565)	1.40 (0.496)	5.13+ (0.077)	6.32* (0.043)	9.38** (0.009)
Microbial community	2	1.34 (0.512)	1.37 (0.504)	0.36 (0.836)	1.36 (0.506)	1.12 (0.570)	2.37 (0.306)
Contemporary N	2	2.88 (0.237)	17.13*** (<0.0001)	0.11 (0.948)	6.48* (0.039)	6.67* (0.036)	1.9 (0.387)
Plant pop × mic comm	4	--	2.33 (0.676)	--	0.35 (0.987)	1.84 (0.765)	7.90+ (0.095)

Table S6. The presence of a live, diverse soil microbial communities (i.e., compared to sterilized control inoculant) did not contribute to plant local adaptation to soil N. Microbial effects on local adaptation would be indicated by a significant three-way interaction between plant N history, microbe presence, and contemporary N. Values shown are Type III Wald chi-squared tests for each fixed factor included in the mixed model ANOVA); p-values are shown in parentheses. Significant predictors are indicated by bold type and asterisks ($p < 0.001^{***}$, $p < 0.01^{**}$, $p < 0.05^*$, $p < 0.1+$). Model structure: response ~ plant N history * microbe presence * contemporary N + (1|plant population/seed family).

	<i>Df</i>	<i>Survival</i>	<i>Total seeds</i>	<i>Total biomass (g)</i>	<i>Root:shoot</i>
Plant N history	1	3.59+ (0.058)	0.5 (0.478)	3.76+ (0.052)	0.02 (0.895)
Presence of live microbes	1	2.6 (0.107)	3.66+ (0.056)	1.79 (0.181)	1.84 (0.174)
Contemporary N	2	3.32 (0.190)	9.5** (0.009)	1.74 (0.420)	2.49 (0.288)
Plant N history × live microbes	1	2.93+ (0.087)	0.22 (0.636)	1.36 (0.243)	0.6 (0.440)
Plant N history × contemporary N	2	1.62 (0.445)	0.77 (0.681)	1.95 (0.376)	1.48 (0.477)
Live microbes × contemporary N	2	1.78 (0.410)	0.63 (0.731)	1.67 (0.435)	0.5 (0.780)
Plant N history × live microbes × contemporary N	2	2.2 (0.333)	0.59 (0.745)	2.93 (0.231)	2.63 (0.269)

Table S7. ANOVA results for the full model, including three-way interactions. The N history of live soil microbial communities did not contribute to plant local adaptation to soil N (no significant three-way interactions between plant N history, microbe N history, and contemporary N). Values shown are Type III Wald chi-squared tests for each fixed factor included in the mixed model ANOVA; p-values are shown in parentheses. Plants inoculated with sterilized control were excluded from this analysis. Analyses involving nodule number or mass include only plants with at least one nodule present. Significant predictors are indicated by bold type and asterisks (**p** < **0.001*****, **p** < **0.01****, **p** < **0.05***, p < 0.1+). Model structure: response ~ plant N history * microbial N history * contemporary N + growth time + growth time + (1|microbial community) + (1|plant population/seed family).

Table S7.

	<i>Df</i>	<i>Survival</i>	<i>Total seeds</i>	<i>Total biomass</i>	<i>Root: shoot</i>	<i>Nodule presence</i>	<i>Nodule number</i>	<i>Total nod mass</i>	<i>Mean nodule mass (g)</i>	<i>Nods: root mass</i>	<i>Nod mass: root mass</i>	<i>Seeds: nodule</i>	<i>Seeds: nod mass</i>
Plant N hist	1	2.46 (0.117)	0.00 (0.984)	0.02 (0.875)	0.83 (0.363)	0.16 (0.685)	1.29 (0.256)	0.13 (0.721)	0.00 (0.959)	0.16 (0.688)	0.26 (0.612)	0.08 (0.782)	0.13 (0.720)
Microb e N hist	1	0.37 (0.543)	1.34 (0.247)	1.46 (0.227)	1.85 (0.173)	1.00 (0.317)	3.01+ (0.083)	0.40 (0.525)	0.00 (1.00)	0.07 (0.798)	0.13 (0.716)	0.13 (0.717)	0.01 (0.912)
Cont N	2	0.08 (0.963)	5.12+ (0.077)	9.02* (0.011)	4.96+ (0.084)	1.65 (0.439)	3.68 (0.159)	5.11+ (0.078)	3.27 (0.195)	2.56 (0.279)	5.37+ (0.068)	4.88+ (0.087)	8.06* (0.018)
Plant N hist × mic N hist	1	2.55 (0.110)	0.06 (0.802)	0.83 (0.362)	0.49 (0.484)	0.62 (0.432)	3.29+ (0.070)	1.51 (0.220)	0.62 (0.430)	0.32 (0.570)	1.26 (0.261)	0.34 (0.56)	1.30 (0.254)
Plant N hist × cont N	2	0.38 (0.826)	0.04 (0.982)	1.79 (0.408)	2.55 (0.279)	1.08 (0.584)	1.93 (0.382)	1.63 (0.443)	0.38 (0.826)	0.19 (0.908)	0.87 (0.648)	0.21 (0.902)	1.42 (0.491)
Mic N hist × cont N	2	0.68 (0.713)	0.14 (0.932)	5.29+ (0.071)	1.51 (0.469)	0.82 (0.664)	5.20+ (0.074)	5.59+ (0.061)	2.05 (0.359)	3.53 (0.171)	5.16+ (0.076)	4.90+ (0.086)	7.34* (0.025)
Plant N hist × mic N hist × cont N	2	0.76 (0.685)	0.07 (0.963)	3.05 (0.218)	1.20 (0.549)	0.33 (0.846)	2.77 (0.251)	2.12 (0.347)	1.59 (0.452)	0.11 (0.946)	0.92 (0.631)	0.78 (0.676)	1.78 (0.410)

Table S8. The presence of a live soil microbial community (i.e., compared to sterilized control inoculant) increased plant seed production, particularly in high contemporary N environments, and decreased root:shoot ratios but did not affect plant biomass. Live microbes also eliminated the effect of contemporary N on plant survival. Values shown are Type III Wald chi-squared tests for each fixed factor included in the mixed model ANOVA); p-values are shown in parentheses. Significant predictors are indicated by bold type and asterisks ($p < 0.001^{***}$, $p < 0.01^{**}$, $p < 0.05^*$, $p < 0.1^+$). Model structure: response ~ microbe presence * contemporary N + growth time + (1|plant population/seed family).

	<i>Df</i>	<i>Survival</i>	<i>Total seeds</i>	<i>Total biomass</i>	<i>Root:shoot</i>
Presence of live microbes	1	0.00 (0.954)	22.69*** (<0.0001)	0.33 (0.565)	28.95*** (<0.0001)
Contemporary N	2	9.00* (0.011)	61.05*** (<0.0001)	29.64*** (<0.0001)	19.78*** (<0.0001)
Live microbes × contemporary N	2	7.48* (0.024)	7.27* (0.026)	0.51 (0.775)	5.87+ (0.053)

Figure S1. Location of field sites in Barry and Kalamazoo counties in southwest Michigan. Green: sites where seeds were collected. Bold text, triangles: sites where soils were collected. *Italicized, brown:* site where soil was collected but not seeds. See Table S1 for additional site details. Maps created in R using the *osmdata* (Padgham et al., 2023), *mapdata* (Brownrigg et al., 2022), *tigris* (Walker, 2024), and *ggspatial* (Dunnington, 2023) packages.

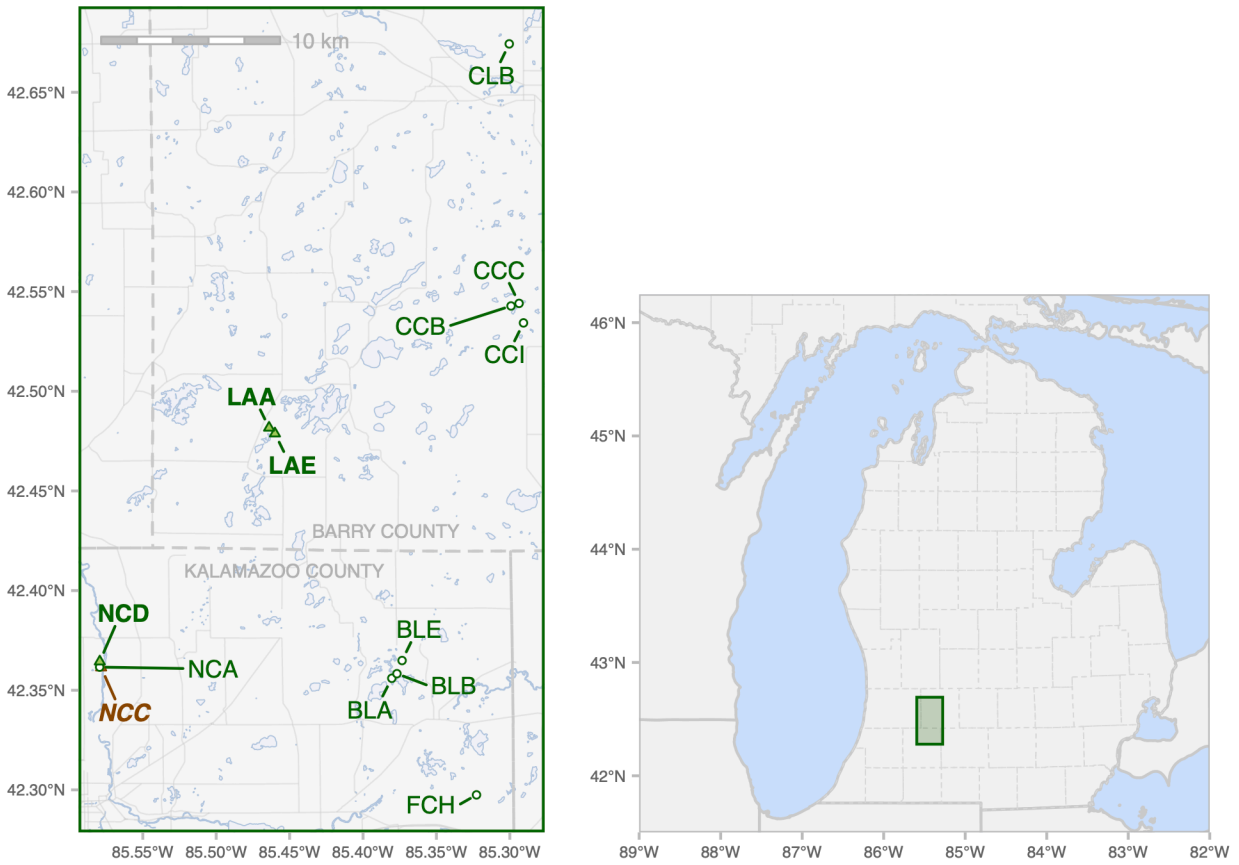


Figure S2 (extension of Figure 1). Plant soil N history did not affect plant fitness (a-b) or nodule traits (c-f). Dotted lines indicate nonsignificant plant N history \times contemporary N interactions. Note: vertical axes for (b) and (d-f) are presented on a log scale.

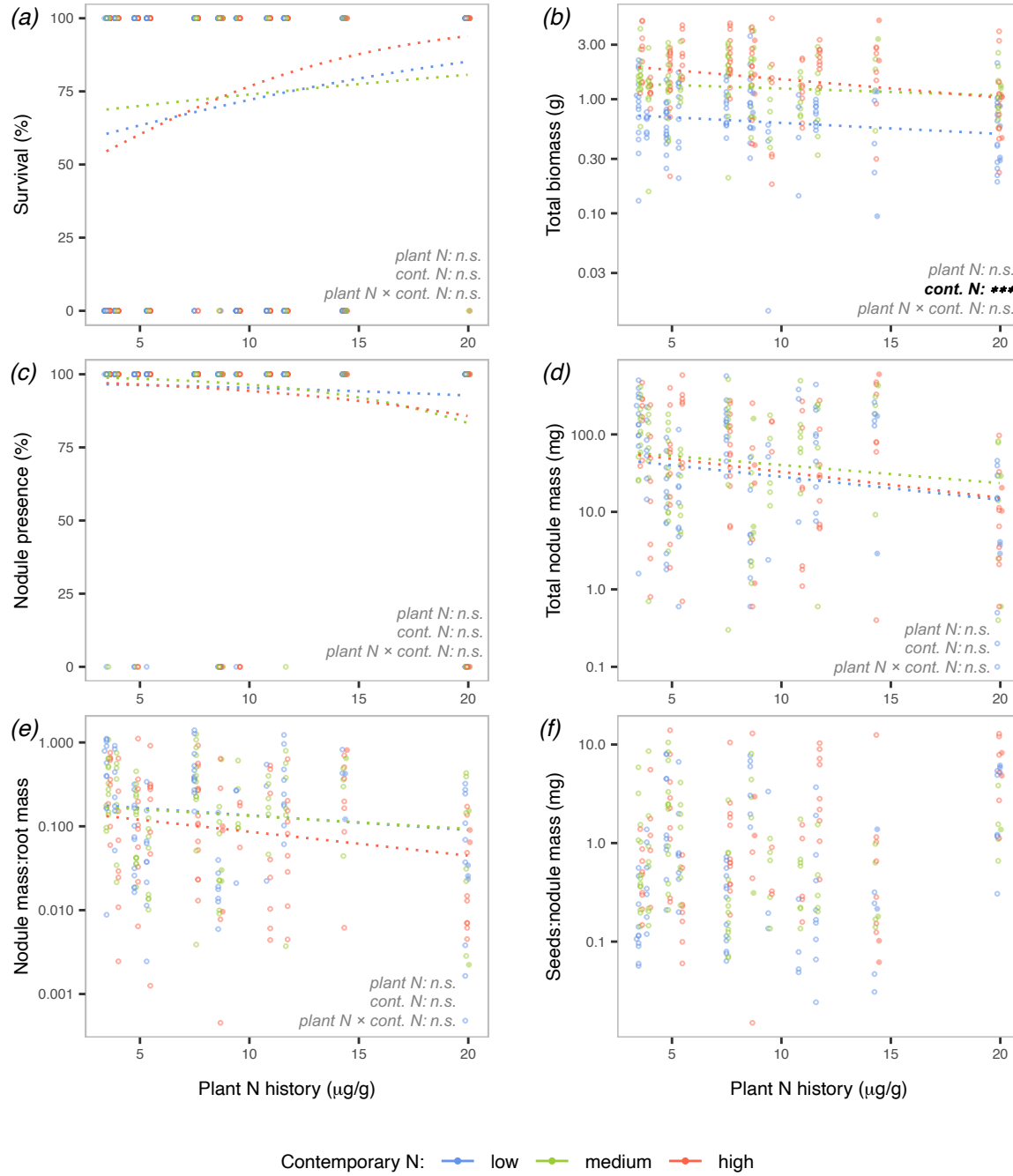


Figure S3 (extension of Figure 2). There was a trend for microbes from high soil N to decrease plant survival, but microbe N history did not affect plant biomass (b) or likelihood of nodulation (c). Plant nodule traits varied with microbe N history only when grown in high contemporary N (d-e). Panels on left sides of (a-b) show estimated marginal means (EMMs) \pm SE of plants inoculated with sterile control. Note: vertical axes for (b) and (d-f) are presented on a log scale.

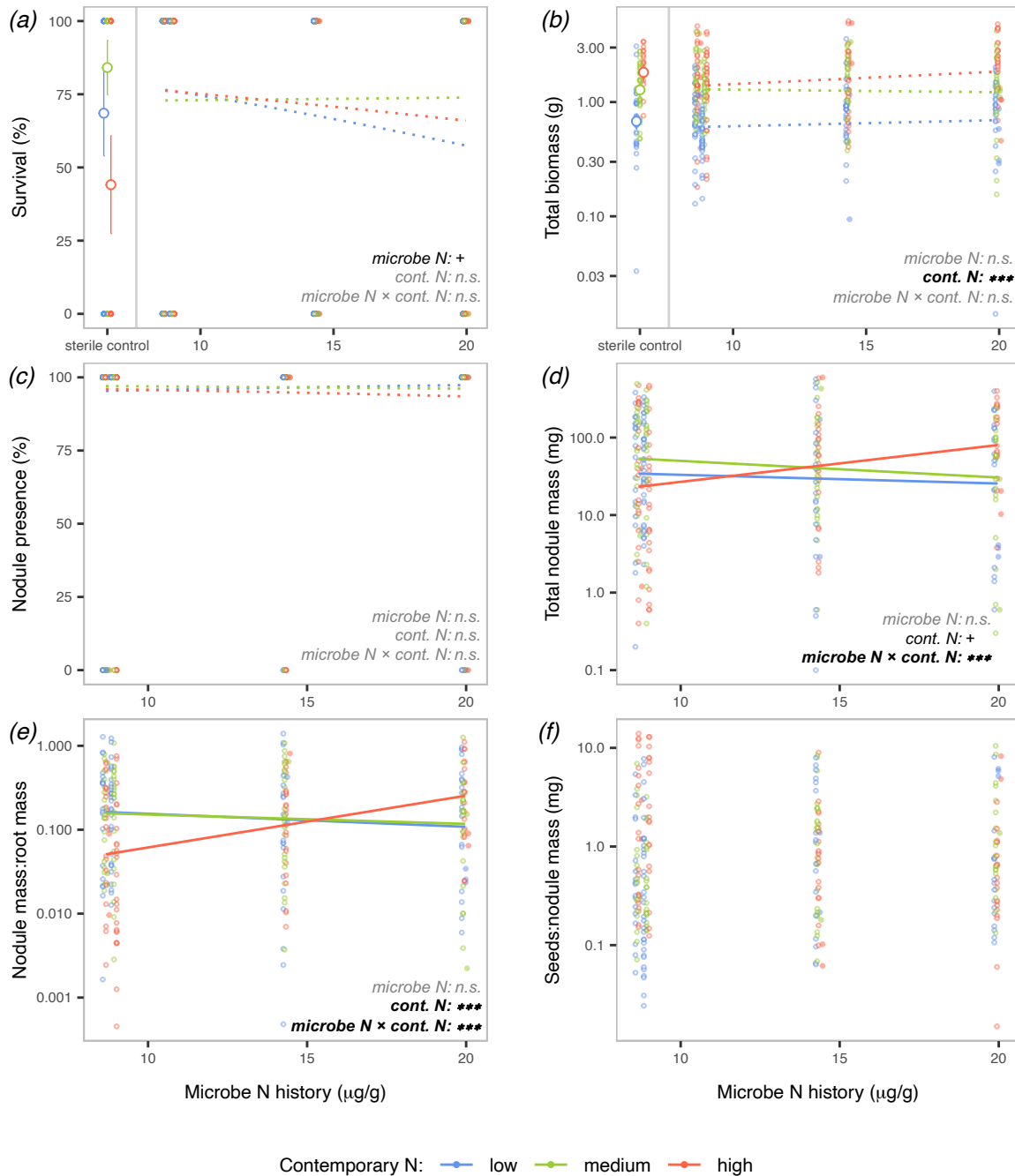


Figure S4. We found no evidence of microbe-mediated adaptive plasticity to N: the presence of a live soil microbial community did not affect plant local adaptation to N. We found no significant interaction between historical and contemporary N regardless of microbe presence (Table S6). Note: vertical axes for panels (b-d) are presented on a log scale.

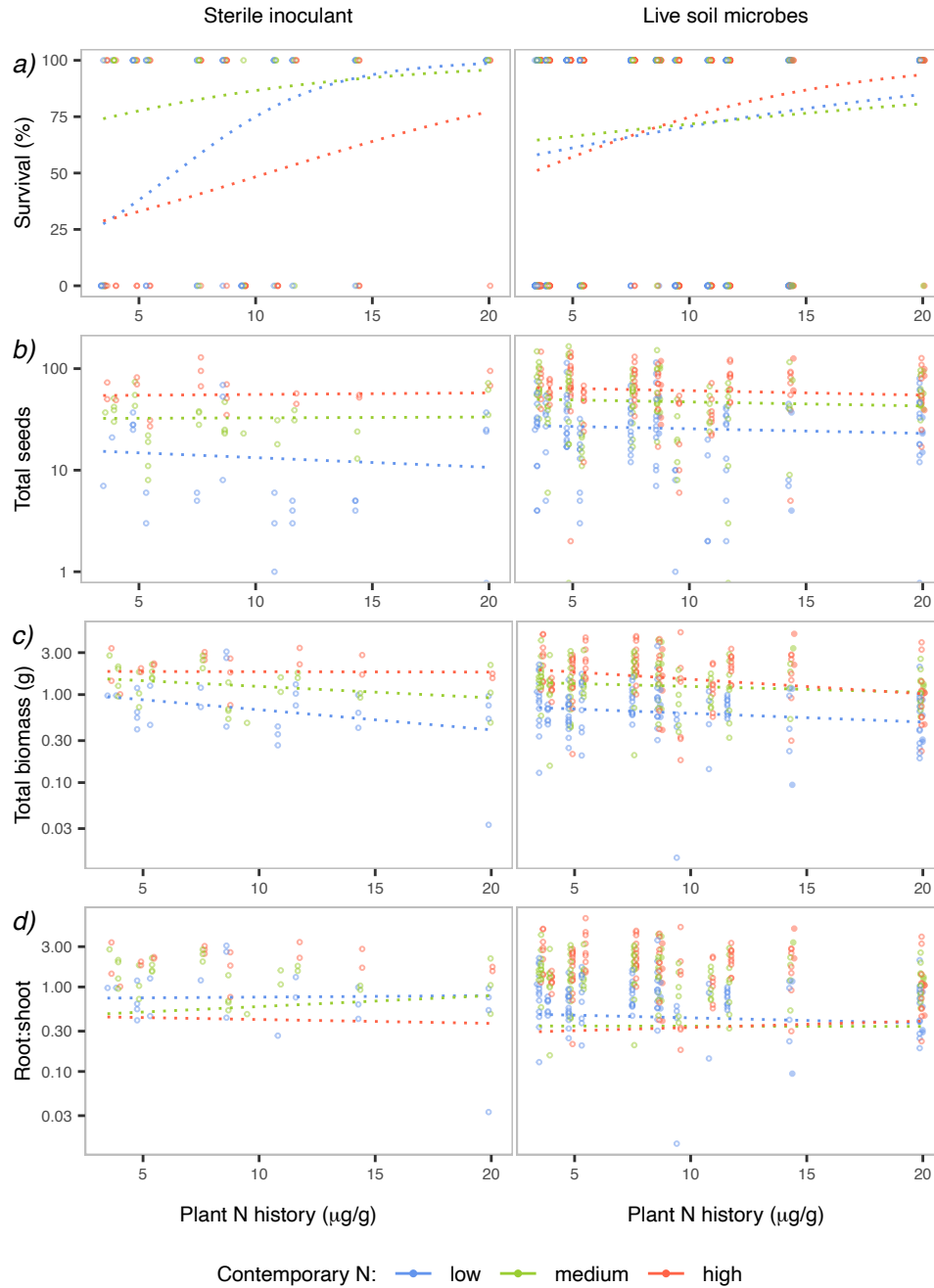


Figure S5. We found no evidence for local adaptation between plants and rhizobia from matching N environments (no significant 3-way interactions between plant N history, microbe N history, and contemporary N; Table S5). Note: vertical axes for (b-d) are presented on a log scale.

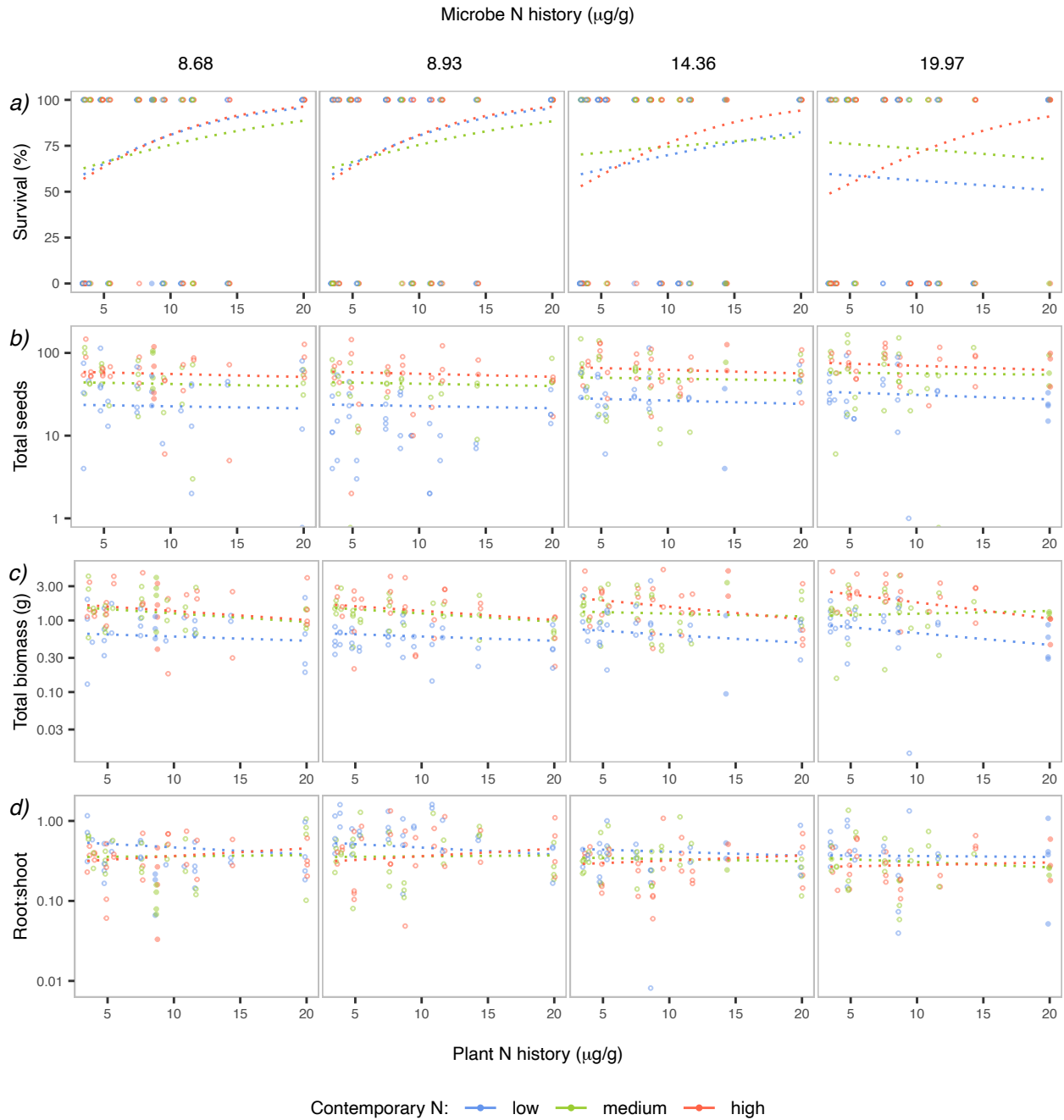


Figure S6. We found no evidence for local adaptation between plants and rhizobia from matching N environments (no significant 3-way interactions between plant N history, microbe N history, and contemporary N; Table S5). Note: vertical axes for (b-d) are presented on a log scale.

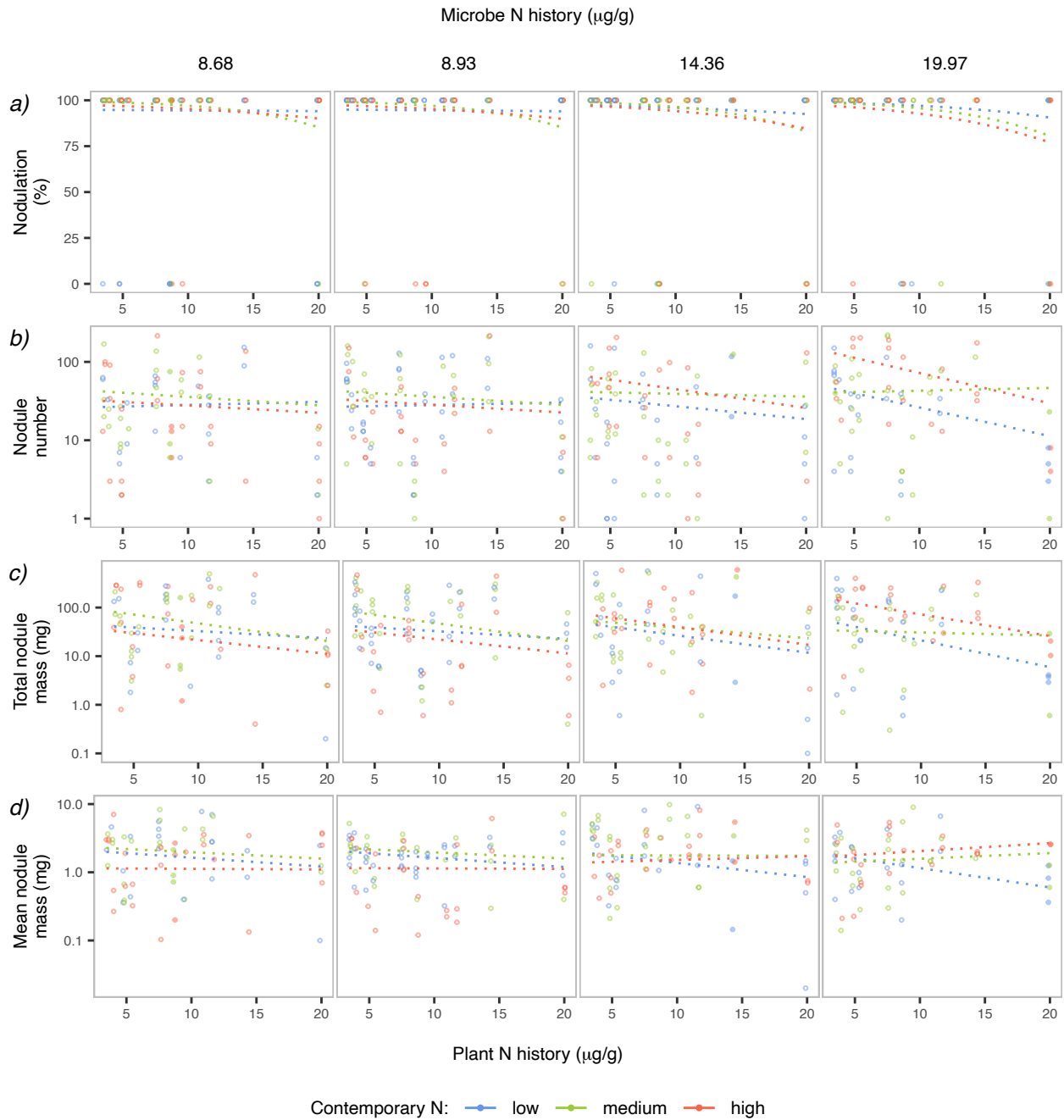


Figure S7. We found no evidence for local adaptation between plants and rhizobia from matching N environments (no significant 3-way interactions between plant N history, microbe N history, and contemporary N; Table S5). Note: vertical axes for (b-d) are presented on a log scale.

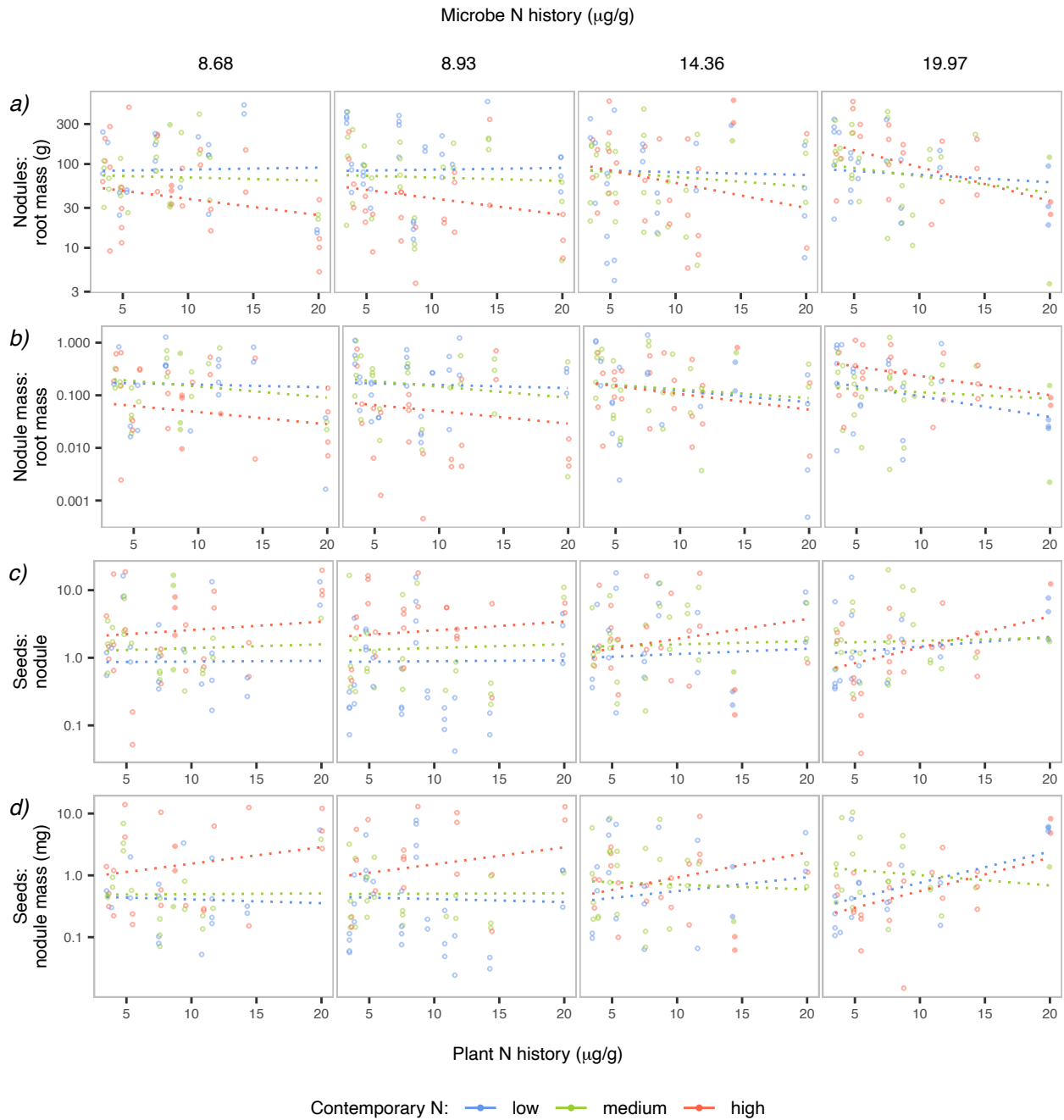


Figure S8. We did not find plant adaptation to local microbial communities (no significant plant population \times microbial community interactions on plant fitness proxies, Table 2a, Table S4a).

Filled points: sympatric combinations; open points: allopatric combinations. Black points: EMMs \pm SE. Note: vertical axes for panels (b-d) are presented on a log scale.

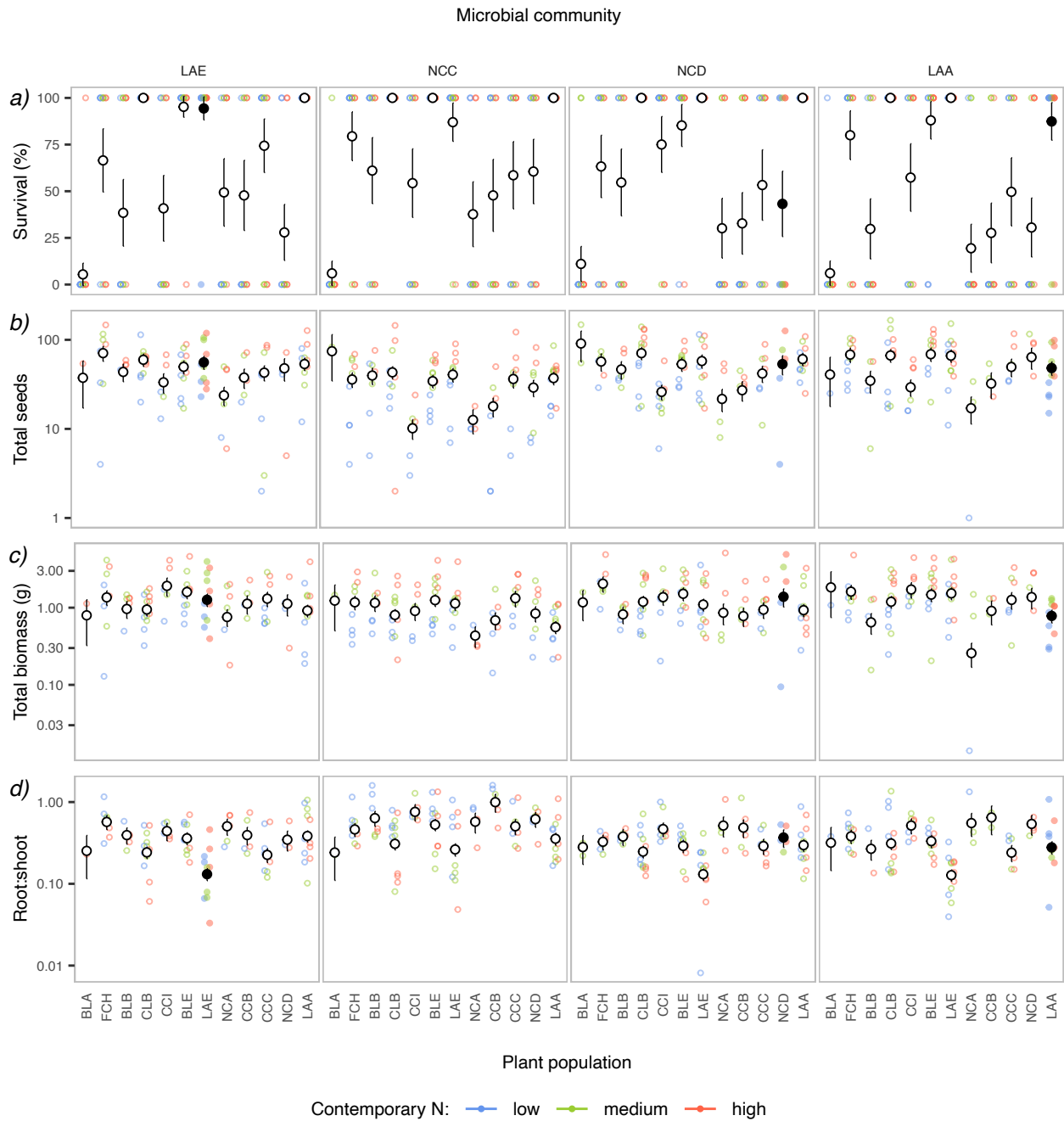


Figure S9. We found variation in plant-microbe interactions (nodule number (c), nodules:root biomass (d) and seeds:nodule (e)) and a trend in rhizobium fitness (b), but we did not find a general signature of co-adaptation (Tables 2a, S4a). Filled points: sympatric combinations; open points: allopatric combinations. Black points: EMMs \pm SE. Note: vertical axes for panels (b-d) are presented on a log scale.

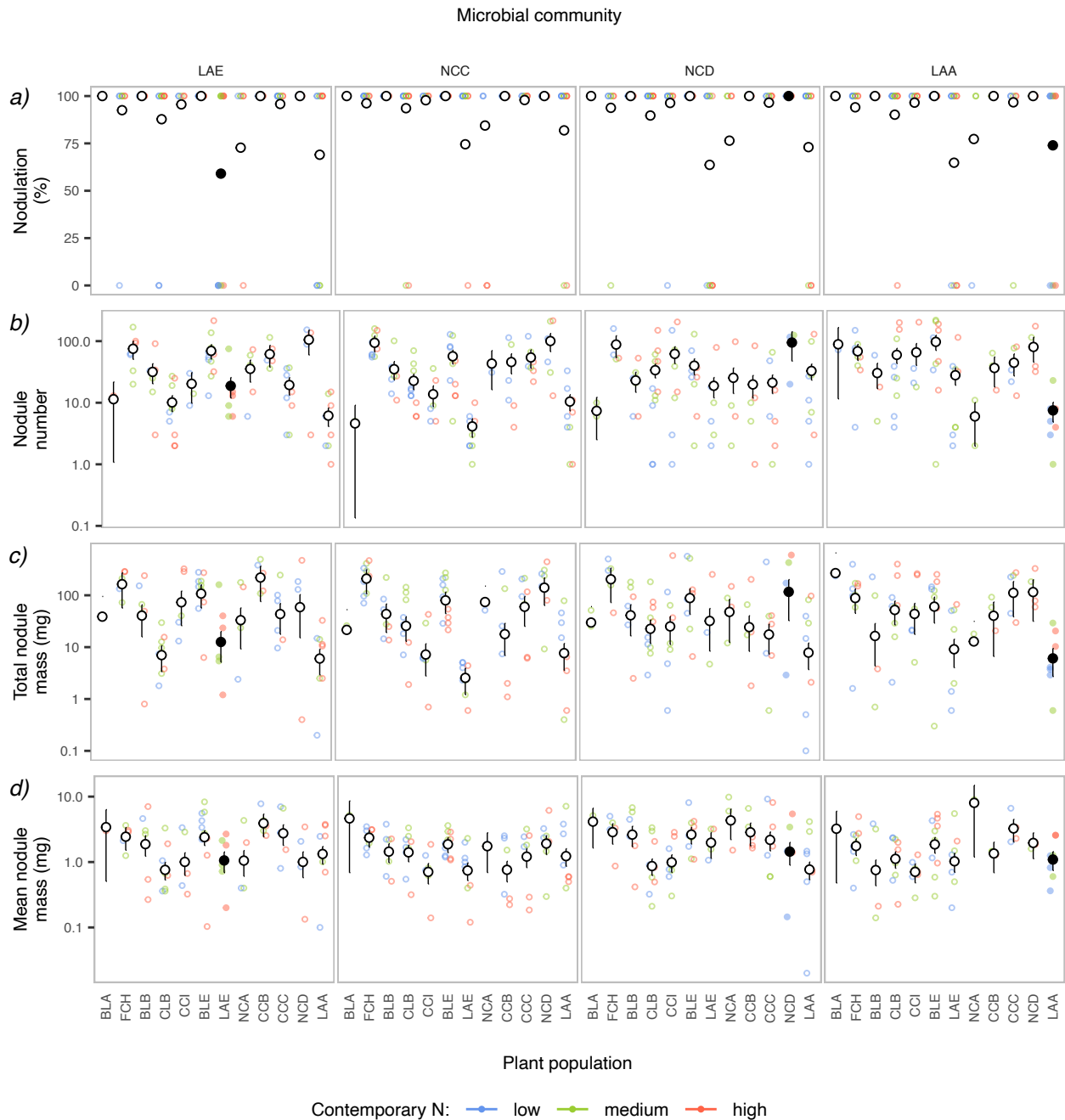


Figure S10. We found variation in plant-microbe interactions (nodule number (c), nodules:root biomass (d) and seeds:nodule (e)) and a trend in rhizobium fitness (b), but we did not find a general signature of co-adaptation (Tables 2a, S4a). Filled points: sympatric combinations; open points: allopatric combinations. Black points: EMMs \pm SE. Note: vertical axes are presented on a log scale.

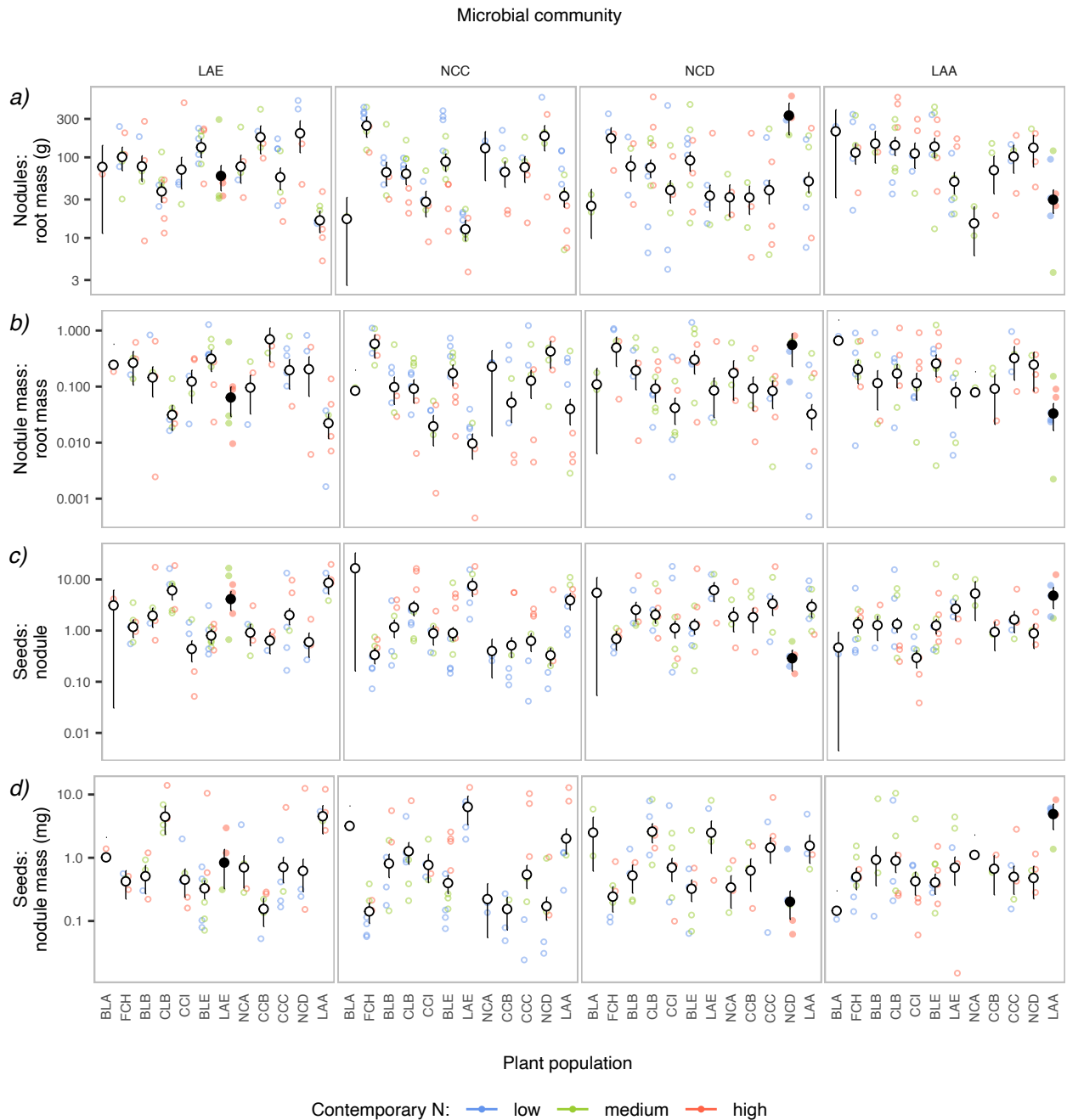


Figure S11. Live microbes increased seed production (especially in low N), decreased root:shoot ratios, and only increased survival in high contemporary N. EMMs \pm SE. Note: vertical axes for panels b-d are presented on a log scale.

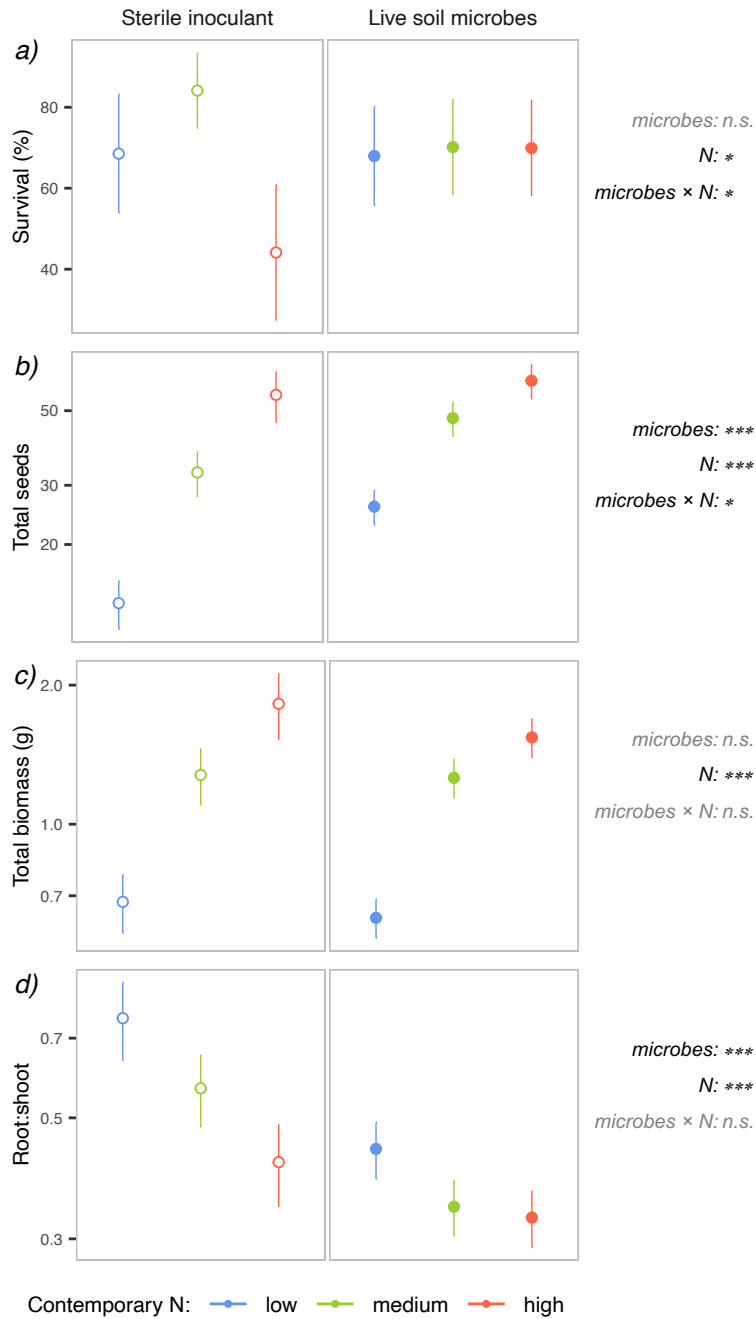
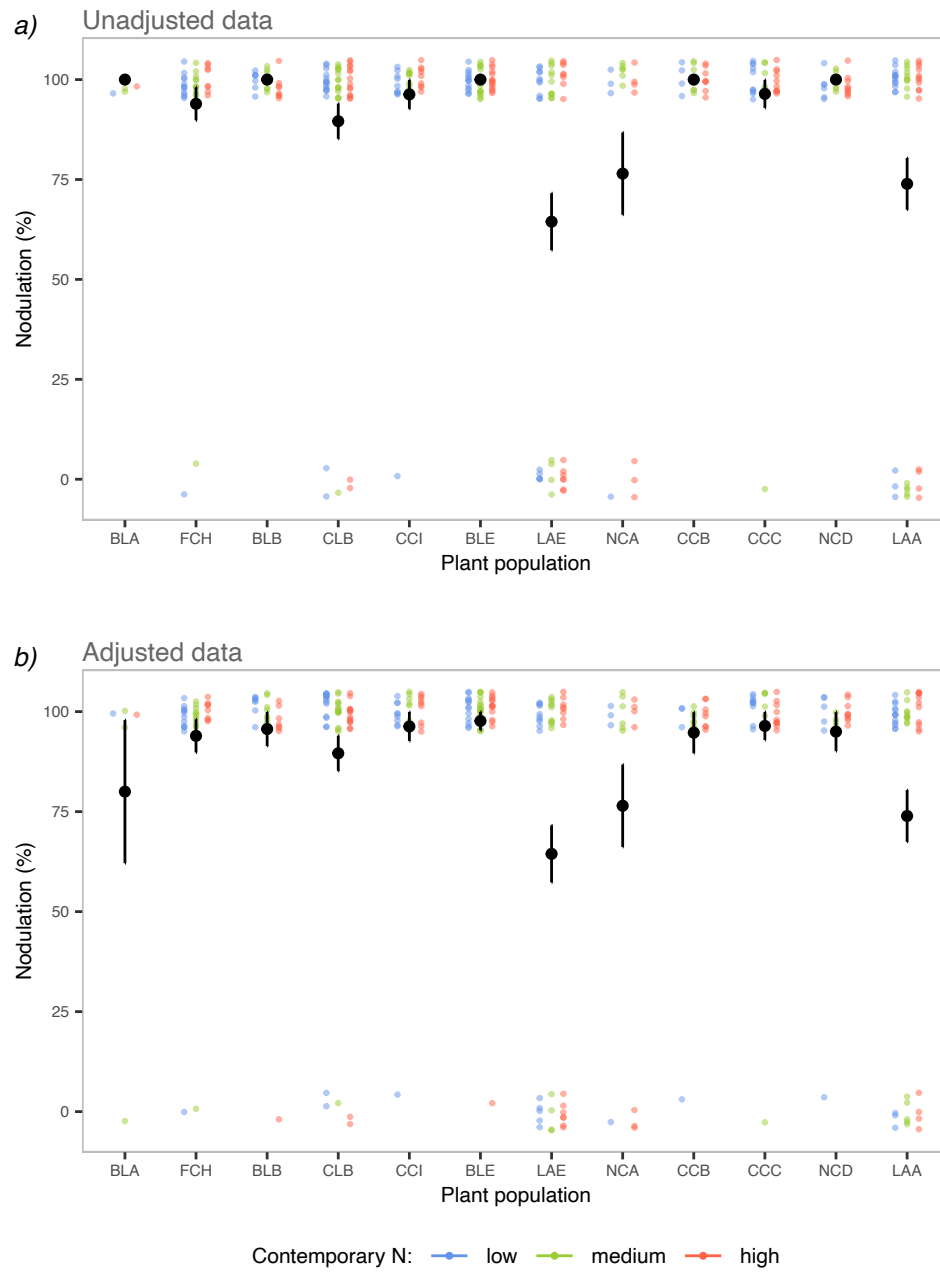


Figure S12. (a) Unadjusted and (b) adjusted data for nodule presence. In each population with 100% nodulation, one randomly selected data point was changed from 1 to 0 to allow statistical comparisons. Adjusted data was used for analysis comparing nodule presence between plant populations (Table S5a); unadjusted data was used for all other analyses. Black points: population means \pm SE.



APPENDIX B:

**SUPPLEMENTAL INFORMATION FOR ‘CICADA RESOURCE PULSE HAS
TRANSGENERATIONAL EFFECTS ON PLANT GROWTH VIA BOTH MATERNAL
EFFECTS AND SOIL MICROBES’**

Table S1. Dates and amounts of cicada carcasses and estimated nitrogen added to plots. Cicada carcasses averaged 0.132 g dry mass with 11.5% N.

<i>Date</i>	<i>Cicadas / plot</i>	<i>Cicadas / m²</i>	<i>Cicada mass (g) / m²</i>	<i>N (g) / m²</i>
08-Jun-2021	1036	10.36	1.37	0.16
11-Jun-2021	1088	10.88	1.44	0.17
16-Jun-2021	4096	40.96	5.41	0.62
22-Jun-2021	6072	60.72	8.02	0.92
02-Jul-2021	4959	49.59	6.55	0.75
Total	17246	172.46	22.76	2.62

Table S2. Cicada treatments applied to each field plot and the number of *Amphicarpaea* seeds collected from each plot and planted in the greenhouse experiment. Bold: number of seeds from each plot. Parentheses: number of dams, average number of seeds per dam.

<i>Plot pair</i>	<i>Plot</i>	<i>Treatment</i>	<i>Number of seeds collected</i>	<i>Number of seeds planted</i>
K03K04	K03	Ambient	42 (13, 3.2)	20 (12, 1.7)
K03K04	K04	Cicada addition	26 (11, 2.4)	20 (10, 2)
K09K10	K09	Ambient	0 (0, 0)	0 (0, 0)
K09K10	K10	Cicada addition	100 (24, 4.2)	70 (24, 2.9)
K11K12	K11	Ambient	18 (5, 3.6)	11 (5, 2.2)
K11K12	K12	Cicada addition	12 (5, 2.4)	11 (5, 2.2)
K13K14	K13	Ambient	30 (15, 2)	20 (12, 1.7)
K13K14	K14	Cicada addition	8 (5, 1.6)	6 (4, 1.5)

Table S3. Surface-sterilization did not affect seed germination or survival. Due to the low number of seeds collected from all other plots (Table S2), only seeds from plot K10 were used to test the effect of surface sterilization, and as a result, we could not test for interactions between seed surface-sterilization and cicada addition. In this subset, only two seeds (2%) failed to germinate. Plot K10 was a cicada-addition plot. Values shown are Type III Wald chi-squared tests for each fixed factor included in the mixed-model ANOVA; n = 100 seeds. Significant predictors are indicated by bold type and asterisks ($p < 0.001^{***}$, $p < 0.01^{**}$, $p < 0.05^*$, $p < 0.1+$). Model structure: germination \sim sterilization + deer_{seed} + (1| subplot / dam).

	<i>Germination</i>	<i>Survival</i>
Seed surface sterilization	0.00	1.17
Deer protection	0.00	0.02
Sterilization \times deer protection	0.00	0.13
Cicada microbes	--	1.81

Table S4. Number of plants that survived to the end of the experiment from each treatment combination. Parentheses: number of plants that formed root nodules.

		Ambient microbes		Cicada microbes	
		Unfenced	Fenced	Unfenced	Fenced
Ambient seed	Unfenced	1 (1)	3 (3)	2 (2)	2 (2)
	Fenced	3 (3)	5 (5)	6 (5)	6 (1)
Cicada seed	Unfenced	9 (4)	7 (4)	6 (5)	7 (3)
	Fenced	0 (0)	1 (1)	2 (0)	2 (0)

Table S5. Nodule number and seed production were positively correlated, indicating general alignment between plant and rhizobium fitness. Values shown are Type III Wald chi-squared tests for each fixed factor included in the mixed-model ANOVA; n = 32 plants. Significant predictors are indicated by bold type and asterisks ($p < 0.001^{***}$, $p < 0.01^{**}$, $p < 0.05^*$, $p < 0.1+$). There were too few data points to fit models that included additional interactions, and a similar model with total nodule mass instead of nodule number failed to converge. Model structure: seeds ~ nodule number * cicada_{microbes} + cicada_{seed} + deer_{seed} + (1| plot_{microbes} / subplot_{microbes}) + (1| plot_{seed} / subplot_{seed}).

	<i>Total seeds</i>
Nodule number	11.12***
Cicada microbes	0.52
Cicada seed	0.71
Deer seed	2.26
Nod number × cicada mic	3.94*

Figure S1. Field plot layout. Plots were constructed in pairs; we added cicada carcasses to one plot of each pair and removed any carcasses found in the other. Black dashed line: deer enclosure fence. Grey region: subplot protected from ungulate herbivory. White region: subplot unprotected from herbivory. Lettered rectangles: quadrats for plant monitoring and percent cover estimation. Stars: soil core sampling locations.

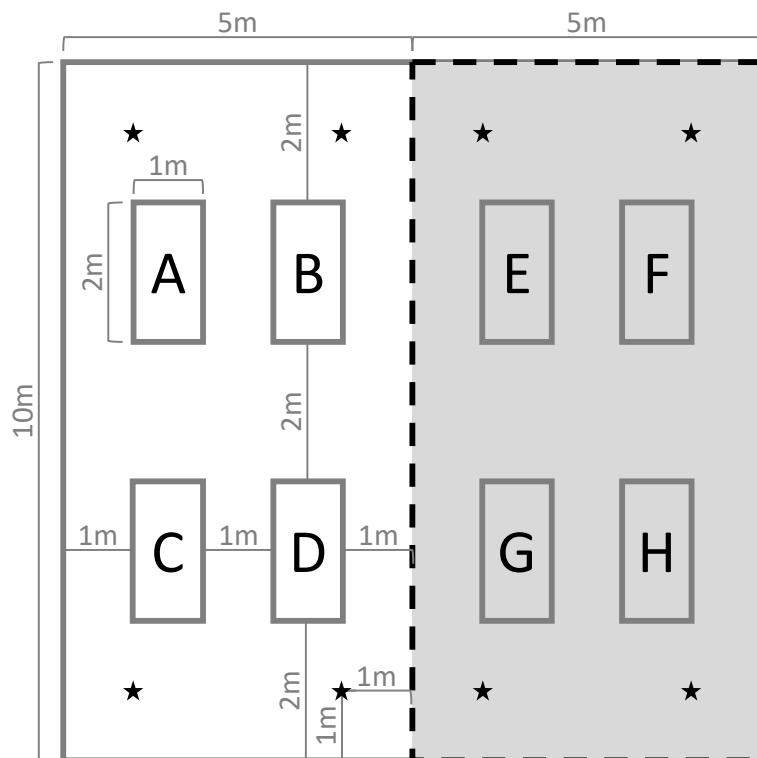


Figure S2. (a) Protection from deer increased total plant cover ($p = 0.011$). (b) Cicada carcasses increased *Amphicarpaea* percent cover ($p = 0.008$), and there was a trend for this increase to be weaker in plots protected from deer (cicada \times deer: $p = 0.058$). Larger points: EMMs \pm SE.

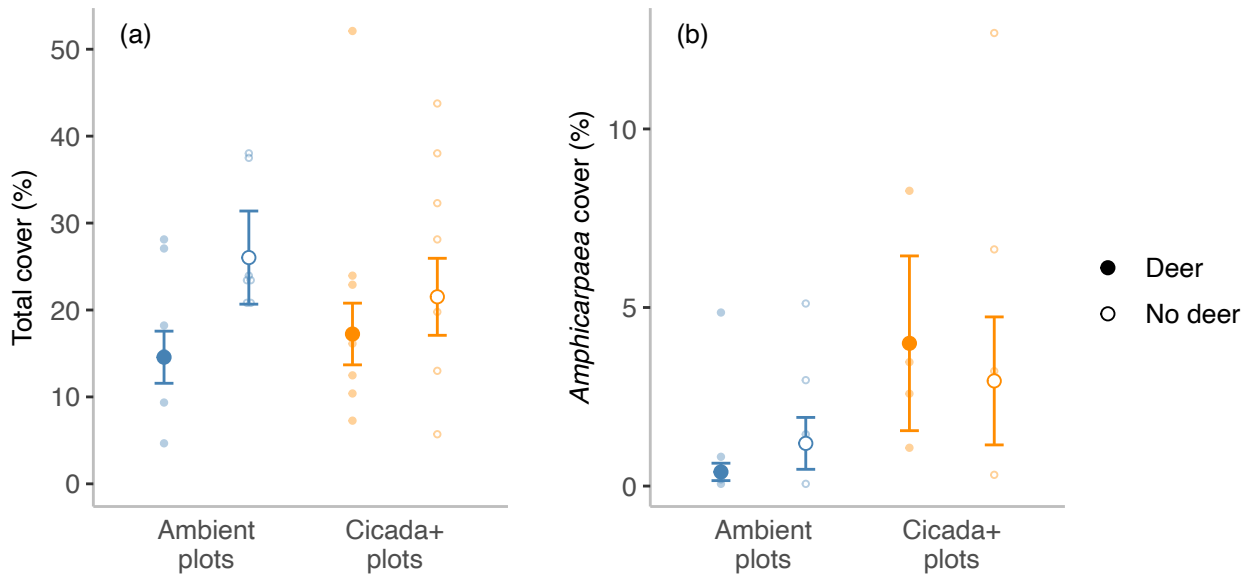


Figure S1. Neither surface-sterilization ($p = 0.948$) nor protection from deer ($p = 0.999$) affected germination. Larger points: EMMs \pm SE.

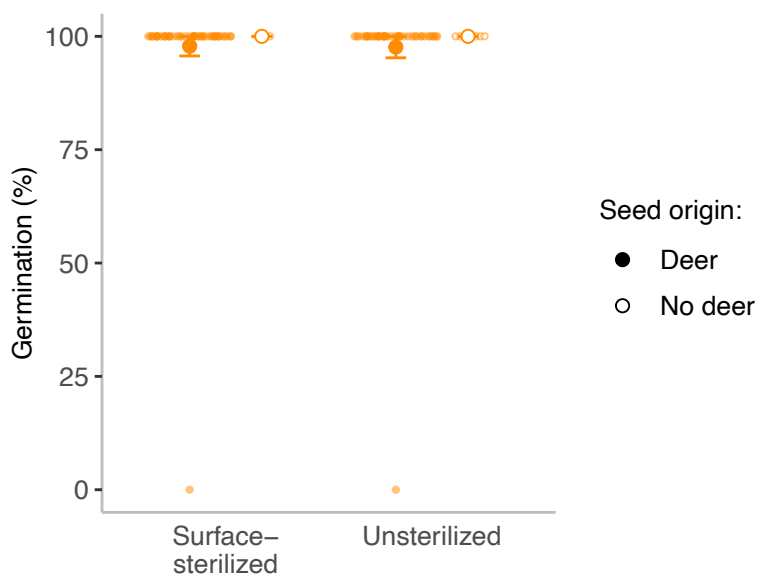


Figure S4. Neither cicada addition nor protection from deer affected *Amphicarpaea* (a) presence (cicada: $p = 1$, deer: $p = 1$, cicada \times deer: $p = 1$), (b) leaf count (cicada: $p = 0.914$, deer: $p = 0.896$, cicada \times deer: $p = 0.958$), (c) biomass (cicada: $p = 0.514$, deer: $p = 0.800$, cicada \times deer: $p = 0.942$), or (d) seed production (cicada: $p = 0.669$, deer: $p = 0.436$, cicada \times deer: $p = 0.296$) in field plots. Leaf count, biomass, and seed production are per plant. Larger points: EMMs \pm SE.

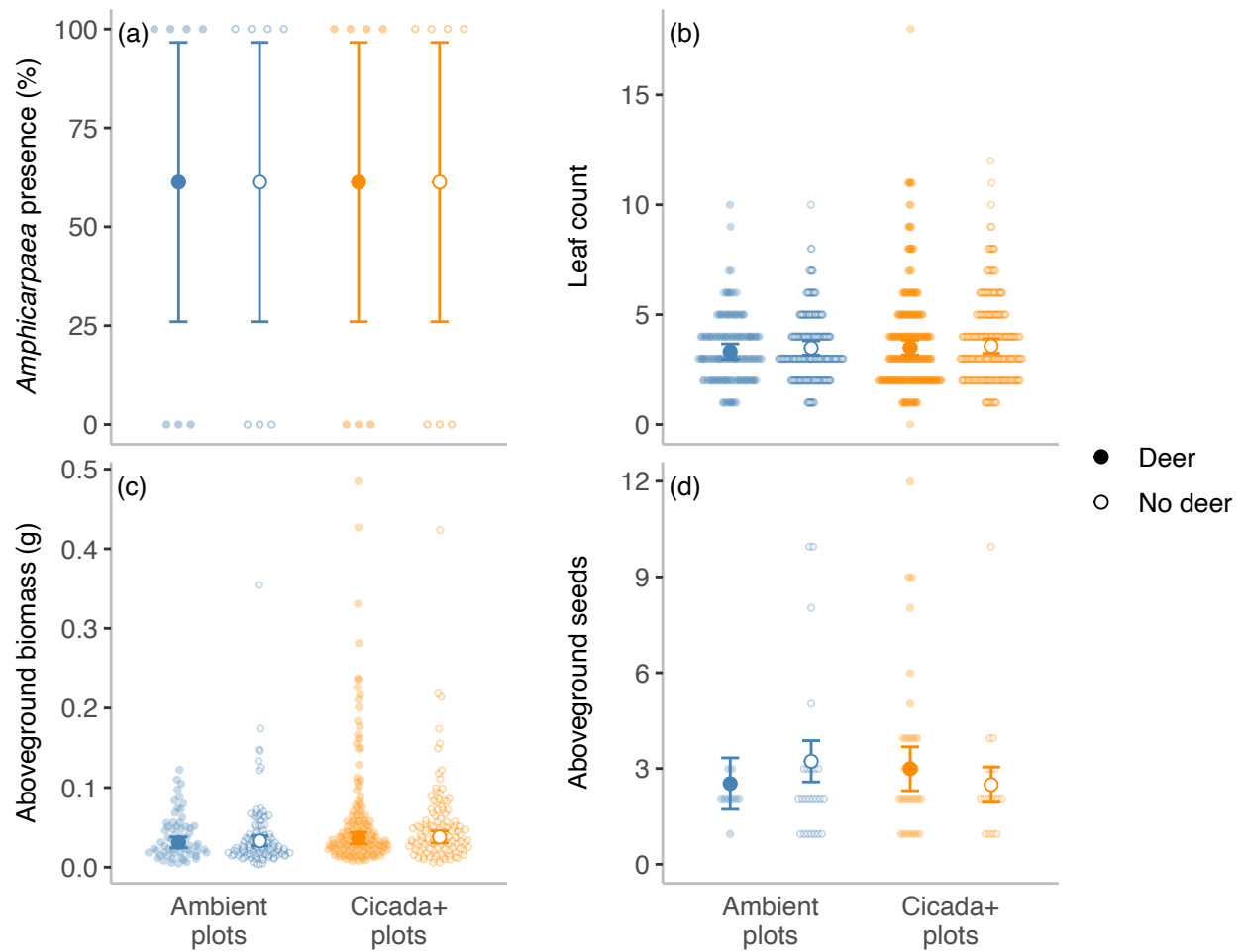
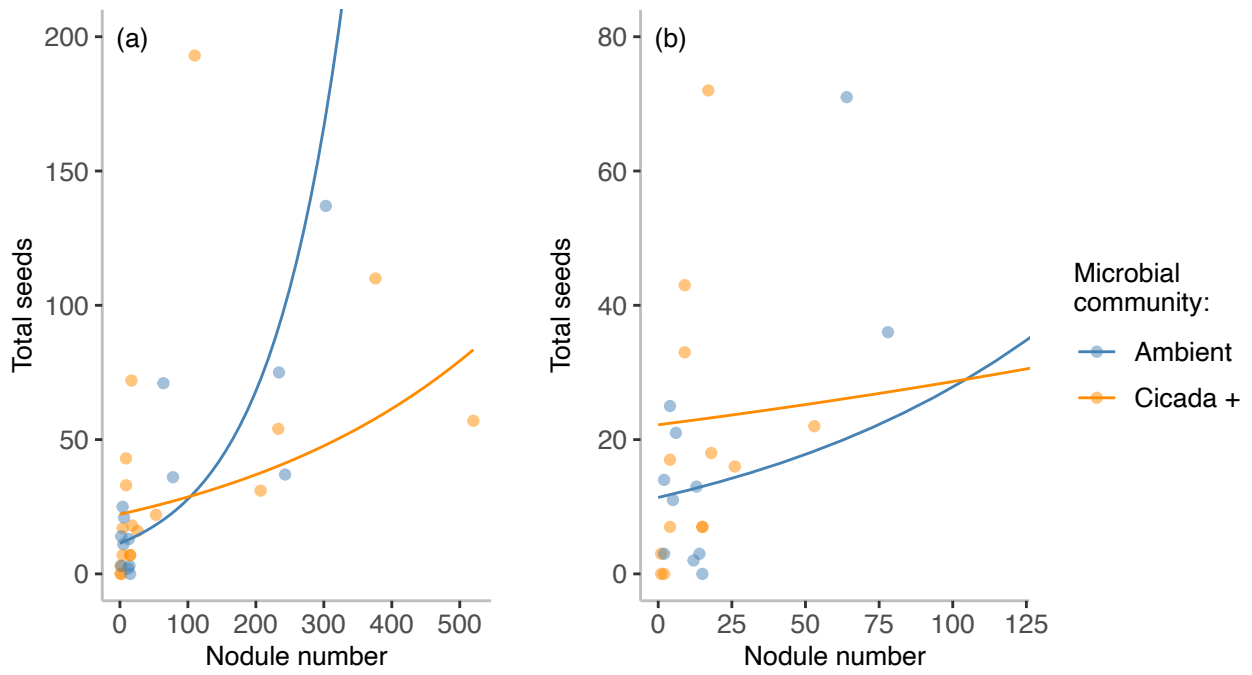


Figure S5. Plant and rhizobium fitness were largely aligned: seeds and nodules were positively correlated ($p = 0.0009$). (a) Plants benefit from increased nodule number more when inoculated with microbes from ambient plots ($p = 0.047$), but (b) at low nodule numbers, plants benefit more from microbes from cicada addition plots. Lines: GLMM described in Table S6. Axis ranges in (a) taken from the ranges of seeds (0-193) and nodule numbers (0-520) seen in our experiment. Axis ranges in (b) are cropped for increased clarity at low nodule numbers.



APPENDIX C:

SUPPLEMENTARY INFORMATION FOR ‘NITROGEN FERTILIZATION CAUSES MUTUALISM DECLINE BY ALTERING LIGHT AVAILABILITY AND HOST DENSITY’

Table S1. Species planted in the mesocosms used to condition the soil microbial communities during the evolution phase of the experiment.

Species	Common name	Functional group
<i>Achillea millefolium</i>	common yarrow	forb
<i>Arrenatherum alatum</i> ssp. <i>elatius</i> ‘Ruffner’	tall oat grass	C3 grass
<i>Bromus inermis</i>	smooth brome	C3 grass
<i>Dactylis glomerata</i> ‘Potomac’	orchard grass	C3 grass
<i>Daucus carota</i>	Queen Anne’s lace	forb
<i>Phleum pratense</i> ‘Climax’	timothy	C3 grass
<i>Solidago canadensis</i>	Canada goldenrod	forb
<i>Trifolium hybridum</i>	Alsike clover	legume

Table S2. Dates for each phase of the experiment.

‘Season’	Pots inoculated	Seeds planted	Fertilized	Harvested	Roots removed
1	13 Jan 2022	14 Jan 2022	3 Feb 2022, 10 Feb 2022, 17 Feb 2022	16 Mar 2022- 22 Mar 2022	6 Apr 2022- 8 Apr 2022
2	6 Apr 2022- 8 Apr 2022	8 Apr 2022	29 Apr 2022, 6 May 2022, 13 May 2022	6 June 2022- 8 June 2022	29 June 2022- 30 June 2022
3	29 June 2022- 30 June 2022	1 Jul 2022	22 Jul 2022, 29 Jul 2022, 4 Aug 2022	24 Aug 2022- 26 Aug 2022	13 Sep 2022- 14 Sep 2022
4	13 Sep 2022- 14 Sep 2022	17 Sep 2022	7 Oct 2022, 14 Oct 2022, 21 Oct 2022	9 Nov 2022- 11 Nov 2022	30 Nov 2022- 3 Dec 2022
5	30 Nov 2022- 3 Dec 2022	3 Dec 2022	26 Dec 2022, 2 Jan 2023, 9 Jan 2023	30 Jan 2023- 1 Feb 2023	20 Feb 2023- 21 Feb 2023
Common garden	24 Feb 2023 3 Mar 2023	24 Feb 2023	n/a	17 Apr 2023- 30 Apr 2023	n/a

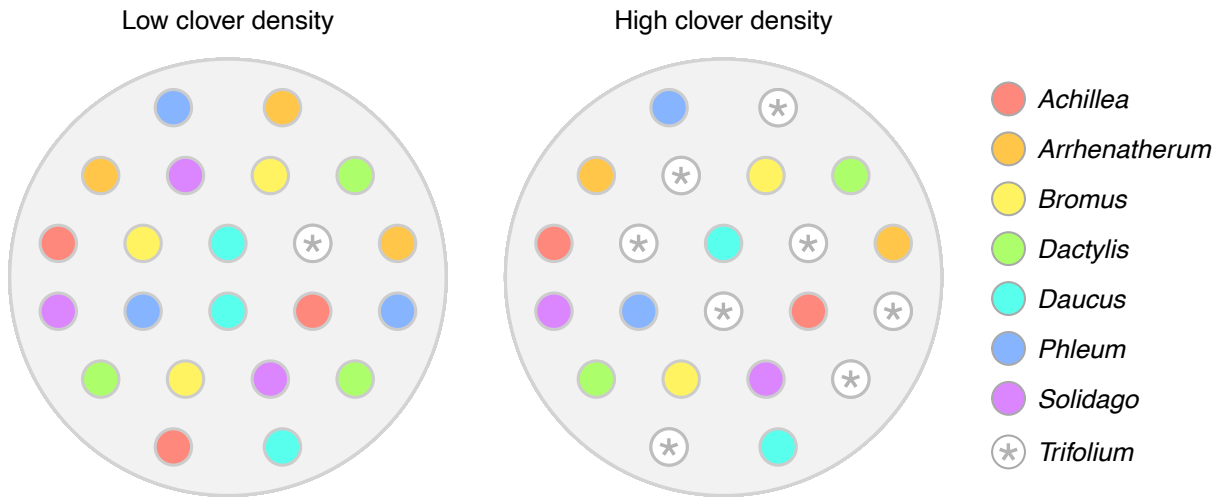
Table S3. P values for pairwise comparisons of EMMs of each treatment combination with the ‘ambient’ treatment of low N, high light, high clover density. Treatments are described relative to the ambient treatment.

	<i>Total biomass (g)</i>	<i>Chlorophyll</i>	<i>Leaf count</i>	<i>Shoot:root</i>	<i>Nodule presence</i>	<i>Nodule number</i>
+N	0.982	0.879	0.766	0.814	0.999	0.259
-light	0.131	0.003**	0.026*	0.016*	0.008**	0.256
-clover	0.949	0.992	0.277	0.448	0.657	0.661
+N, -light	<.0001***	<.0001***	0.0001***	<.0001***	<.0001***	0.018*
+N, -clover	0.038*	0.016*	0.029*	0.916	0.175	0.959
-light, -clover	<.0001***	<.0001***	0.0002***	0.015*	<.0001***	0.468
-light, -clover, +N	<.0001***	<.0001***	0.0006***	0.002**	0.0001***	0.779

Table S4. P values for pairwise comparisons of EMMs of each treatment combination with the ‘ambient’ treatment of ambient microbes and low N. Treatments are described relative to the ambient treatment.

	<i>Total biomass (g)</i>	<i>Chlorophyll</i>	<i>Leaf count</i>	<i>Shoot:root</i>	<i>Nodule presence</i>	<i>Nodule number</i>
+N	0.026*	0.030*	0.223	0.447	0.303	0.426
N+ microbes	0.211	0.029*	0.173	0.054+	1.00	0.183
+N, N+ microbes	0.647	0.069+	0.954	0.026*	1.00	0.149

Figure S1. Layout of plant species in mesocosms used to condition the microbial communities in the evolutionary phase of the experiment.



MACKENZIE ALLEN CAPLE

Department of Biology
Indiana University, Bloomington, IN
mcaple@iu.edu

EDUCATION

- 2025 **Ph.D., Indiana University**
April *Advisor:* Dr. Jennifer A. Lau
 Major: Evolution, Ecology, and Behavior
 Minor: Genetics
- 2013 **B.S., University of Michigan**
May *Majors:* Plant Biology, Chinese Studies

PROFESSIONAL & RESEARCH EXPERIENCE

- 2018 – **Microbial Ecology Section Member**
present Ecological Society of America
- 2014 – **Project Coordinator**, NSF Collections in Support of Biological Research Museums
2018 University of Michigan Herbarium
 PI: Paul Berry
- 2013 – **Electronic Imaging Technician**
2014 University of Michigan Herbarium
 PI: Timothy James
- 2018 **Field Research Technician**
2013 **EEB 300 Research**
 University of Michigan
 PI: Robyn Burnham
- 2012 **REU Student**, Biosphere/Atmosphere Interactions in a Changing Global Climate
 University of Michigan Biological Station
 PI: Valeriy Ivanov
- 2010 **REU Student & Research Assistant**
 Kellogg Biological Station
 PI: Jeffrey Conner

PUBLICATIONS & PRESENTATIONS

- Caple, M.** and Lau, J. Nitrogen causes mutualism decline primarily through indirect effects via light and host availability. *In prep.*
- Caple, M.** and Lau, J. Cicada resource pulse has transgenerational effects on plant growth via both maternal effects and soil microbes. *In review.*
- Caple, M.** and Lau, J. Asymmetric local adaptation to nitrogen in a plant-microbe symbiosis. *In revision.*
- Caple, M.** and Lau, J. Nitrogen causes mutualism decline primarily through indirect effects via light and host availability. *Oral presentation:* Ecological Society of America Annual Meeting, 2024 Aug 4-9; Long Beach, CA.
- Caple, M.** Plant community and functional responses to nitrogen and diverged soil microbial communities. *Oral presentation:* weekly seminar for Genetics and Eco-Evolution of Multiscale Symbioses (GEMS), 2024 Feb 12; virtual.
- Caple, M.** and Lau, J. Cicada litterfall indirectly affects plant growth through multiple mechanisms. *Oral presentation:* Ecological Society of America Annual Meeting, 2023 Aug 6-11; Portland, OR.
- Caple, M.** and Lau, J. Experimentally disentangling multiple ecological effects of nitrogen enrichment. *Poster presented at:* Changing Microbiomes Symposium, Penn State University, 2022 May 31-Jun 3; Boalsburg, PA.
- Caple, M.** and Lau, J. Ecological drivers of rhizobium evolution: nitrogen, light, host density, and voracious herbivores. *Oral presentation:* weekly seminar for Genetics and Eco-Evolution of Multiscale Symbioses (GEMS), 2022 Mar 28; virtual.
- Caple, M.** and Lau, J. Host and nutrient availability interact to influence microbial communities' effects on plant fitness. *Oral presentation:* Ecological Society of America Annual Meeting, 2021 Aug 2-5; virtual.
- Caple, M.** Intraspecific variation in a legume-rhizobium mutualism across a natural nitrogen gradient. *Oral presentation:* weekly seminar for Genetics and Eco-Evolution of Multiscale Symbioses (GEMS), 2020 Nov 2; virtual.
- Caple, M.** and Lau, J. Does nitrogen influence intraspecific variation in a legume-rhizobia mutualism? *Poster presented at:* Ecological Society of America Annual Meeting, 2019 Aug 11-16; Louisville, KY.
- Charbonneau, A; Tack, D; Lale, A; Goldston, J; **Caple, M**; Conner, E; Barazani, O; Ziffer-Berger, J; Dworkin, I; Conner, J. (2018) Weed evolution: Genetic differentiation among wild, weedy, and crop radish. *Evolutionary Applications*, 11(10):1964-1974.
<https://doi.org/10.1111/eva.12699>
- Caple, M.** and Williams, B. (2017). The Houghton Geological Survey and the First University Collections, pp 23-30 in Kirsten Barndt and Carla Sinopoli (editors), *Object Lessons and the Formation of Knowledge: The University of Michigan Museums, Libraries & Collections, 1817–2017*. University of Michigan Press. Ann Arbor, Michigan.

GRANTS & AWARDS

2025	William R. Ogg Final Year Fellowship (\$12,500)
2024	IU Executive Dean's Travel Award for Women in Science (\$600)
2022 – 2024	Genetics & Eco-Evolution of Multiscale Symbioses Institute Project Grant (\$184,846)
2023	IU Provost's Travel Award for Women in Science (\$650)
2022	GEMS Institute Summer Seed Grant (\$9,583)
2021	IU Research and Teaching Preserve Student Grant (\$3,000)
2021	IU Floyd Memorial Fund in Plant Sciences Summer Fellowship (\$1,617)
2020	IU Floyd Memorial Fund in Plant Sciences Summer Fellowship (\$3,234)
2020	NSF Graduate Research Fellowship Program (Honorable Mention)
2019	IU Floyd Memorial Fund in Plant Sciences Summer Fellowship (\$617)

UNDERGRADUATE MENTORSHIP

2022 – 2025	K. K. NSF RAPID Grant REU IU Drs. Sidney and Becca Fleischer Research Scholarship
2023 – 2024	K. R. NSF-GEMS Project Grant
2022 – 2024	M. M. NSF-GEMS Seed Funding
2022 – 2023	M. Y. NSF RAPID Grant REU
2023	A. M. IU Undergraduate Research Summer Research Program D. H. IU Women in STEM D. S. IU Undergraduate Research Summer Research Program L. B. IU Louis Stokes Alliances for Minority Participation P. L. IU Undergraduate Research Summer Research Program S. C. IU Undergraduate Research Summer Research Program
2022	E. S. IU Science, Technology, and Research Scholars
2021	A. M. IU Louis Stokes Alliances for Minority Participation C. N. IU Louis Stokes Alliances for Minority Participation G. B. IU Women in STEM E. D. IU Women in STEM

SERVICE

2019 – 2025	EEB Organization Representing Graduate Students <i>Founding member / president</i> Advocate for student concerns and facilitate faculty-student communication; compile resources and foster cross-lab communication to help graduate students understand departmental requirements and navigate the hidden curriculum
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2021 – 2023 **EcoLunch Committee**
Weekly graduate student-led forum for presenting and discussing research, career paths, etc.

Reviewer for: *American Journal of Botany* (2)
Ecology & Evolution (1)
Evolutionary Applications (1)
Journal of Applied Ecology (1)
Journal of Ecology (2)
Oecologia (1)
Plant Communications (1)
PLOS ONE (1)
Soil Biology & Biochemistry (1)

TEACHING

2022 **BIOL-L113: Biology Laboratory**
Associate Instructor

2020 **BIOL-X325: Field Ecology and Evolution Research Lab 2**
Arts and Sciences Undergraduate Research Experience (ASURE)
Associate Instructor

2020 **BIOL-X150: Field Ecology and Evolution Research Lab 1**
Arts and Sciences Undergraduate Research Experience (ASURE)
Associate Instructor

2019 **COLL-C104: Observations and Experiments in Science**
Arts and Sciences Undergraduate Research Experience (ASURE)
Associate Instructor

2018 **BIOL-L11: Foundations of Biology: Diversity, Evolution, and Ecology**
Associate Instructor