FINAL REPORT

Controls Regulating Biological Nitrogen Fixation in Longleaf Pine Ecosystems: the role of Fire and Stand Development

SERDP Project RC-2328

AUGUST 2018

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REPORT DOCUMENTATION PAGE	*Form Approved* *OMB No. 0704-0188*

1. REPORT DATE *(DD-MM-YYYY)* 08/29/2018	**2. REPORT TYPE** SERDP Final Report	**3. DATES COVERED** *(From - To)*

4. TITLE AND SUBTITLE Controls Regulating Biological Nitrogen Fixation in Longleaf Pine Ecosystems: the role of Fire and Stand Development	**5a. CONTRACT NUMBER** W912HQ-13-C-0017
	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S) Nina Wurzburger	**5d. PROJECT NUMBER** RC-2328
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Georgia 140 E Green Street Athens, GA 30606	**8. PERFORMING ORGANIZATION REPORT NUMBER** RC-2328
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Strategic Environmental Research and Development Program 4800 Mark Center Drive, Suite 16F16 Alexandria, VA 22350-3605	**10. SPONSOR/MONITOR'S ACRONYM(S)** SERDP
	11. SPONSOR/MONITOR'S REPORT NUMBER(S) RC-2328

12. DISTRIBUTION/AVAILABILITY STATEMENT

Distribution A; unlimited public release

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Across 54 1-ha plots of longleaf pine at Fort Benning and Eglin Air Force Base representing a 227 year gradient of stand recovery, we quantified N losses from fire, patterns of N demand and availability, and quantified N fixation by legumes, soil crusts, and asymbiotic bacteria. We found surprisingly low rates of N fixation, and that the dominant contribution of N fixation differed by site (legume N fixation at Benning and asymbiotic N fixation at Eglin). This site difference appears to be driven by lower soil P availability and higher soil N availability at Eglin relative to Benning. Supporting this idea, legume N fixation increased with phosphorus (P) addition in common garden and field fertilization experiments.

15. SUBJECT TERMS

biological nitrogen, nitrogen fixation, nitrogen loss, fire, longleaf pine, Fort Benning, Eglin Air Force Base, asymbiotic bacteria, legumes, soil crusts, phosphorus, land management, soil nutrients

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Nina Wurzburger
					19b. TELEPHONE NUMBER *(Include area code)*
UNCLASS	UNCLASS	UNCLASS	UNCLASS	45	706-542-5109

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List of Acronyms

ARA: acetylene reduction activity
BNF: biological nitrogen fixation
C: carbon
C_2H_4: ethylene
DIN: dissolved inorganic nitrogen
KCl: potassium chloride
Mo: molybdenum
N: nitrogen
N_2: di-nitrogen
NO_3^-: nitrate
NH_4^+ : ammonium
P: phosphorus
RCSON: Resource Conservation Statement of Need
SERDP: Strategic Environmental Research and Development Program

Keywords

Longleaf pine, forest management, fire, disturbance, nitrogen, fixation, legumes, recovery, sand hill, phosphorus, molybdenum

Acknowledgements

We thank Stephen Hudson, James Parker, Scott Pokswinski and Brett Williams for logistical assistance and access to sites at Fort Benning and Eglin Air Force Base. We thank Joe O'Brien and Kevin Hiers for advice. This work would not have been possible without the assistance of Andres Baron, Austin Martin, Shialoh Wilson, Brian McGann, Breanna Walker, David Blount, Amy Johnson, Meryom Patillo, Jenna Peissig, Mike Ament, Julie Tierney, Grace Anne Ingham, Sumaya El Khalidi, Carly Phillips, Melanie Taylor, Rose Parham and Kelsey Morton.

Abstract

Fire is a critical force in maintaining the structure and diversity of longleaf pine ecosystems, but it also removes a substantial amount of nitrogen (N), which can limit plant growth. Biological N fixation can replenish N losses, but whether and how N fixation balances fire-induced N losses in frequently-burned longleaf pine stands has remained unknown. Nitrogen limitation may be particularly severe in longleaf stands recovering from land-use change. Across 54 1-ha plots of longleaf pine at Fort Benning and Eglin Air Force Base representing a 227 year gradient of stand recovery, we quantified N losses from fire, patterns of N demand and availability, and quantified N fixation by legumes, soil crusts, and asymbiotic bacteria. We found surprisingly low rates of N fixation, and that the dominant contribution of N fixation differed by site (legume N fixation at Benning and asymbiotic N fixation at Eglin). This site difference appears to be driven by lower soil P availability and higher soil N availability at Eglin relative to Benning. Supporting this idea, legume N fixation increased with phosphorus (P) addition in common garden and field fertilization experiments. Nitrogen fixation declined with N addition in the common garden experiment, but the degree of this response was species dependent. Although individual fire events temporarily stimulated N fixation by legumes, N fixation was insufficient to balance N losses from fire and soil N stocks declined over stand age. Progressive N loss from the ecosystem may signal a decline in resiliency and present a long-term concern for land managers. An alternative possibility is that longleaf pine ecosystems have accumulated excess N as a result of land-use change and chronic N deposition. In this case, fire may be a relief mechanism for excess N, critical for returning the ecosystem to its N-poor state. Three findings provide support for our “excess N hypothesis”. First, we found that exceptionally high N mineralization rates (12.8 to 92.3 kg N ha^{-1} year^{-1}) characterize our youngest stands, suggesting that these stands are replete with N following transitions from prior land use. Second, N demand from tree growth remains steady over stand age suggesting that forest productivity is not declining over time, as would be expected if N loss was leading to N deficiency. Third, N addition from our field fertilization experiment did not increase the growth rates of trees, even in older stands, providing additional evidence of N sufficiency. However, without historical (i.e. pre-European settlement) data on longleaf pine N stocks and fluxes, our excess N hypothesis is difficult to test. Therefore, our finding of an ecosystem N imbalance presents us with a fundamental challenge—the interaction of multiple disturbances (e.g. multiple land use changes, fire, and N deposition) may have created a novel N environment for longleaf pine ecosystems, which hinders our ability to assess its capacity to respond to a historical fire regime.

Objective

The primary objective of the work was to investigate the importance of biological N fixation in longleaf pine ecosystems influenced by land management. We expected that the soil-based process of N fixation would be central to recovery from disturbance in longleaf pine forests because they are inherently poor in N and disturbances (e.g. fire and military training) cause them to become even more N poor (Boring et al. 2004, Maloney et al. 2008, Nave et al. 2011). Biological fixation of atmospheric N_2 by soil microbes (so-called "N-fixers") offers a unique mechanism capable of rehabilitating the natural N cycle (e.g. Hendricks and Boring 1999, Hainds et al. 1999), but very little is known about these organisms and their activities in longleaf pine ecosystems.

In this project, we addressed three objectives in RCSON-13-02 relating to soil functional diversity, the role of soil organisms in facilitating recovery from disturbances and ecosystem processes that regulate soil communities in the context of longleaf pine savannas. The motivation from our works comes from previous SERDP funded projects that suggested that disturbances (e.g. fire at too high a frequency and/or military training) could lead to N limitation and unsustainable management of longleaf pine (Garten and Ashwood 2004, Garten 2006). Here, we sought to fill this knowledge gap by examining the ecology of N fixation in these ecosystems, including the patterns of fixation associated with fire and a gradient of longleaf pine stand age.

The primary objectives of our research were to:

Reduce uncertainties in our understanding of N fixation in longleaf pine ecosystems including:

1) The relative contribution of various N fixing organisms (legumes, biological crusts, and free-living bacteria) in space and time,

2) The interactions of soil nutrients (N, P and Mo) as constraints for the various types of N fixation and c) how stand age and fire influence the abundance and activity of N-fixers directly (disturbance) and indirectly (through nutrient cycling processes).

Quantify the importance of N fixation at the ecosystem level, by determining:

3) The role of fixation as a source of N relative to other ecosystem fluxes and pools and

4) Whether the mechanism of N fixation can counteract losses via fire volatilization.

We present 5 main hypotheses that we are tested in the context of these objectives:

1) *N-fixing organisms vary in the amount and timing of their N inputs to longleaf pine ecosystems. N fixation rates are: a) slow but consistent over space and time (free-living fixers in litter layer), b) moderate and patchy over space, consistent over time (biological crusts) and c) rapid and patchy over space and time (herbaceous legumes).*

2) *Abundant available nitrogen generally acts to suppress N fixation, but the soil nutrient most limiting N fixation will vary among taxonomic groups such that: Mo limits free-living N-fixers in organic soils and P limits legumes.*

3) *Disturbances have both direct and indirect effects on N-fixing organisms, where fire and stand age affect the biomass of N fixing organisms (legumes and crusts) or habitat (free-living N-fixers) and indirect effects can manifest as interactions with soil nutrient availability.*

4) *N fixation is a critical source of N for sustaining the productivity of longleaf pine ecosystems.*

5) *The potential for N inputs from N fixation to balance losses in longleaf pine ecosystems relates to the magnitude and frequency of forest disturbances (e.g. fire and stand age) and the interacting effects of soil nutrients to sustain N fixation during periods of recovery.*

Background

How longleaf pine (*Pinus palustris* Mill.) forests respond to fire and land use disturbance is an unanswered question, yet is critical knowledge for both land managers and ecologists. This question remains unanswered because of our incomplete knowledge of how disturbance events influence transformations of nitrogen (N), which constrains carbon (C) accretion during forest regeneration (Nave et al. 2014). The recovery of forest ecosystems depends on the supply of mineral N, which is often dictated by the rate at which biological N fixation introduces new N (Rastetter et al. 2001).

In temperate forests, biomass growth and associated N demands are typically greatest in early stages of development (Vitousek and Reiners 1975, Rastetter et al. 2001). Modeling efforts (Gerber et al. 2013) corroborated by observational studies (Batterman et al. 2013a) have identified ecosystem-scale synchrony among forest regrowth, vegetation N demand and N inputs from N fixation, such that fixation peaks at the time when biomass accumulates the most rapidly. Accordingly, N fixation is often restricted to early stages of ecosystem development in temperate forests and is excluded over time due to competition for light (Cleveland et al. 1999). However, in ecosystems that experience frequent perturbations from fire, N fixation may remain important as a source of new N over time and persist into late succession.

Fire-dependent longleaf pine savannas of the southeastern US are a model system in which to study interactions between ecosystem recovery, fire and N fixation. These ecosystems historically dominated the Southeast, but have been reduced to 3% of their original range due to land use change (Van Lear et al. 2005). As a result of recent restoration efforts, a significant portion of longleaf pine currently exist in early stages of recovery. Considering N dynamics is central to promoting longleaf pine restoration. These ecosystems are exceptionally N poor (Wilson et al. 1999, 2002), a condition which is exacerbated by a dependency on frequent fire (Carter and Foster 2004). Yet, longleaf pine savannas may be well-equipped to deal with N limitation imposed by fire and land use change: they support a diversity of organisms capable of fixing N, including legumes, asymbiotic N-fixing bacteria and biological soil crusts.

Frequent, low-intensity fires maintain the structure and floristic diversity characteristic of longleaf pine savannas (Glitzenstein et al. 2003, Kirkman et al. 2006, Hiers et al. 2007). However, these disturbances volatilize N stored in organic matter (Boring et al. 2004, Carter and Foster 2004, Lavoie et al. 2010). Biogeochemical theory suggests that elemental inputs (i.e. atmospheric N deposition, N fixation) must balance outputs (i.e. leaching and gaseous losses) to overcome N limitation at steady-state (Vitousek and Reiners 1975, Hedin et al. 1995, Menge et al. 2008). Indeed, researchers have hypothesized that N inputs from legumes compensate for N losses due to fire in longleaf pine ecosystems (Boring et al. 2004, Hendricks and Boring 1999, Wilson et al. 2002), however, this has yet to be rigorously quantified.

N fixation tends to be upregulated when the available N supply is low relative to demands (Vitousek and Howarth 1991), and this generally occurs in early stages of ecosystem recovery when vegetation grows rapidly (Gerber et al. 2013). Such patterns of available N supply and demand over time may dictate patterns of N fixation in recovering longleaf pine savannas. After land use transitions such as clear-cutting, there is potential for temporary, rapid mineralization of nutrients from standing detritus (Bormann and Likens 1979, Frazer et al. 1990, Li et al. 2003) but there is little vegetation to serve as an N sink. Therefore, N availability may exceed N demands very early in stand development in recovering longleaf pine savannas, but may again become limiting when biomass begins to rapidly accumulate (Bormann and Likens 1979, Vitousek and Reiners 1975). N supply typically exceeds demands as ecosystems mature and biomass accumulation slows in temperate terrestrial ecosystems (Hedin et al. 1995, Vitousek and Reiners 1975). However, the tight balance of N may persist throughout ecosystem maturity in longleaf pine savannas due to their inherently N-poor nature, thereby maintaining a niche for N fixation over time.

Longleaf pine savannas house a diversity of N-fixing organisms that may replenish N stocks after disturbance. However, the mechanisms that control legumes, asymbiotic bacteria and biological soil crusts to fix N are poorly understood, especially in the context of fire. Fire can volatilize stocks of N (Boring et al. 2004, Lavoie et al. 2010), while liberating mineral phosphorus (P) (Christensen 1977), which can limit N fixation (Vitousek and Howarth 1991). While rare, fertilization experiments have confirmed that P, more so than N or K, can limit the growth of longleaf pine trees in these P-poor soils (Lewis 1977, Dickens 2001).Whether this limitation extends to leguminous N_2-fixers in these ecosystems is less known, however. In addition to soil P, recent findings point to the potential limitation of molybdenum (Mo), a co-factor in the nitrogenase enzyme (Wurzburger and Hedin 2016), and may be in low supply in highly-weathered soils (Karimian and Cox 1979).

The physical changes caused by fire may also serve to promote or suppress N fixation. For example, fire, which reduces competition for light and water, engineers an ideal environment for herbaceous legumes, whose seeds often require scarification to germinate (Hendricks and Boring 1999). While increased light availability may similarly promote the growth of biological soil crusts, these organisms are highly sensitive to disturbance (Hilty et al. 2004, Ford and Johnson 2006), and thus may reduce their fixation-capacity post fire. Fire may also reduce N fixation by asymbiotic bacteria because these disturbances can volatilize the litter layer from which these bacteria derive their energy (Boring et al. 2004, Lavoie et al. 2010). In order to promote ecosystem recovery in longleaf pine savannas, the combined N fixation of these three groups must be adequate to balance N losses from fire and meet N demands from vegetation growth.

Our work was conducted at Fort Benning Military Installation and Eglin Air Force base, which are important for meeting the military training mission and contributing to regional conservation in the southeastern U.S. We have matched sites based on their soil texture in sandhill communites (turkey oak, pyrogenic grasses and longleaf) since they are common on military bases throughout the Southeast (Benning, Gordon, Moody, Pope, Eglin and Stewart all have substantive portions of their base dominated by sandhills). An additional reason for focusing on sandhill communities is that fire and disturbance trigger N limitation more rapidly in these coarse textured soils (Garten and Ashwood 2004) and impact the presence of legumes (Hiers et al. 2007). The motivation from our works comes from previous SERDP funded projects that suggested that disturbances (e.g. fire at too high a frequency and/or military training) could lead to N limitation and unsustainable management of longleaf pine (Garten and Ashwood 2004, Garten 2006).

Materials and Methods

Site description and study design

We conducted this research at Fort Benning Military Installation (hereafter, Benning) in southwestern Georgia and Eglin Air Force Base (hereafter, Eglin) in northwestern Florida, representing the fall-line and coastal plain ranges of sandhill longleaf pine ecosystems (Peet 2006). The climate at both sites is humid subtropical and precipitation occurs throughout the year. The 30 year (1981-2010) average annual precipitation and temperature for each site were 1260 mm and 18.0˚C at Benning and 1802 mm and 18.7 ˚C at Eglin (NCDC 2017). Both sites are the product of deep Cretaceous sand deposits with parent materials of marl, siltstone and shale at Benning, and gravel and limonite beds at Eglin (Rodgers and Provencher 1999, Dilustro et al. 2002), and we established our plots on Lakeland sands (thermic, coated Typic Quartzipsamments) at each site. Both sites are managed to promote an open-canopy longleaf pine overstory, but scrub oaks (especially *Quercus laevis* Walt.) are prevalent in the midstory. The understories consist of pyrogenic grasses and forbs, with saw palmetto (*Serenoa repens* Bart.) common at Eglin.

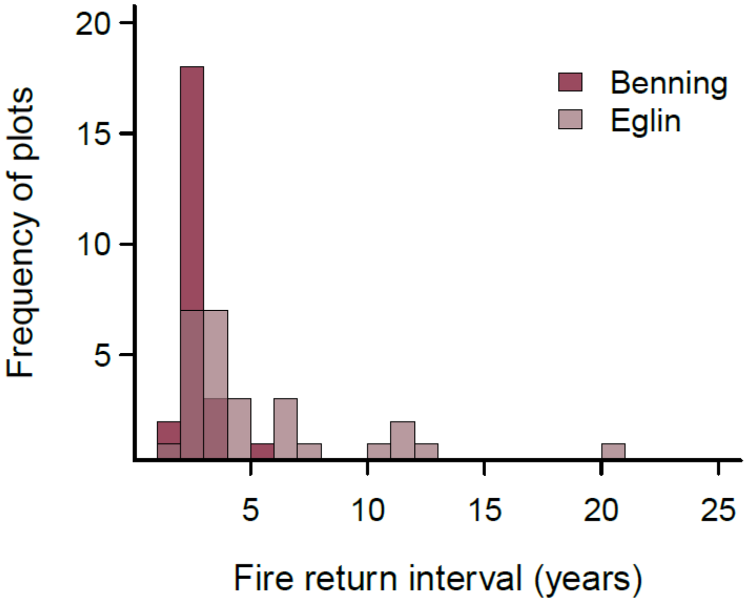

Figure 1 Frequency of our research plots across fire return intervals (years) at Benning (*n* = 24) and Eglin (*n* =27). Eight additional plots at Eglin are not included in the figure because they have no fire record - these stands are either too young (< 5 years old) and have no fuels, or they are mature but have not burned since the fire record began.

Prior to acquisition by the Department of Defense in the 1930's, land at both sites was primarily used for agriculture and grazing (Frost 1993, Rodgers and Provencher 1999). Since then, management practices such as fire suppression and disturbance from military activity have further affected these ecosystems (Maloney et al. 2008). Currently, these sites are managed with regular low-intensity fires, with plots at Benning burning on average more frequently than those at Eglin (mean ± standard error fire return intervals of 2.5 ± 0.1 and 6.2 ± 1.1 years, respectively) (Figure 1).

We established 54 1-ha plots of sandhill longleaf pine (24 plots at Benning and 35 plots at Eglin) across two gradients: stand age (2-227 years old) and fire frequency (fire return intervals of 1.5 – 20 years). These 54 plots included 37 planted stands and 17 naturally regenerated stands. Many of the plantations were previously mixed-pine forests that were harvested and site-prepped (drum-chopping sometimes paired with herbicide application) prior to planting with longleaf. We estimated ages of naturally regenerated stands by extracting tree cores of canopy longleaf and determining the maximum age with tree ring analysis. At random locations within each 1-ha plot, we established either 2, 3 or 4 - 100 m^2 subplots for the determination of soil chemical and physical properties as well as legume abundance and fine root production.

Quantifying the abundance and activity of N-fixers

To determine the abundance of legumes in each plot, we conducted censuses in each subplot at the end of the growing season (August – September) for two years (2015-2016) in which we identified legumes to species, and counted the number of individuals, stems and leaves of each species present. In June-July of 2016, we measured N fixation rates of the eight most common legume species at each site (Table 1) with acetylene reduction assays (ARAs).

We randomly selected 30-40 total individuals of target species at each plot to assess for nodulation and N fixation activity. We excavated the belowground root system of each individual (n = 1070 and n = 400 at Eglin and Benning, respectively) and collected nodules when present. Live nodules attached to fine roots were immediately placed into 250 mL gas-tight jars fit with a rubber septum, and we placed the jars in a shallow depression to maintain *in-situ* soil temperature during the incubation. We replaced 10% of the jar headspace with acetylene (generated by reacting calcium carbide with water), and mixed and sampled the headspace twice over a 30-minute period. We accounted for background levels of ethylene (C_2H_4) production by performing the same incubation procedure in jars without nodules. Subsamples (15 mL) were stored in gas-tight 20 mL vials for as long as three days, and were analyzed for C_2H_4 production using a gas chromatograph fit with a flame-ionization detector. To convert acetylene reduction rates to N fixation rates, we used a molar conversion factor of 4.8, which was determined by a greenhouse experiment of the same species conducted by our lab group (Ament et al. 2018) (see common garden experiment below).

Aboveground and belowground biomass and nodule samples of each species were collected, dried at 70 ˚C for 48 hours and weighed to the nearest milligram. We constructed site- and species-specific allometric equations to relate the number of stems or leaves of an individual to the dry mass of each biomass compartment. We expressed N fixation per mass of nodules, and we paired these measurements and allometric equations with abundance measures from the census to obtain plot-level N fixation estimates. To estimate N inputs from legumes on an annual basis (kg N^{-1} ha^{-1} $year^{-1}$), we assumed that legumes could fix N for 12 hours a day, 6 months out of the year.

We estimated N fixation by biological soil crusts (hereafter, soil crusts) by pairing abundance estimates with per-area N fixation rates. To determine the amount of soil crusts in each plot, we set up three 100 m transects in each plot and estimated the areal coverage (cm^2) of cryptobiotic crusts (conglomerates of cyanobacteria, algae, lichens and mosses at the soil surface) (Belnap et al. 2001), as well as lichens and mosses within a 1 m swath of the transects. We separated soil crusts into these three morphological groups because we expected them to differ in microbial composition and water holding capacity, both of which affects N fixation (Belnap et al. 2001, Belnap 2002).

Figure 2 Types of nitrogen-fixers in longleaf pine savannas – asymbiotic fixers in leaf litter (upper left), cryptobiotic crusts (bottom left) and herbaceous legumes, including *Baptisia* spp. (upper right) and *Tephrosia* spp. (bottom right).

In May 2016, twenty replicate samples of each soil crust type at both sites were extracted with a 4-cm diameter plastic cylinder to a depth of 1 cm and were subsequently transported back to the lab at the University of Georgia. We determined N fixation rates with ARAs as described above with an incubation time of 6 hours (Belnap 2002). Samples were kept in an incubator at 25 ˚C and lighted at 1000 μmol m^{-2} hr^{-1} on a 12-hour diurnal cycle, and fixation estimates were expressed per area of sample.

Biological soil crusts need sufficient moisture to become metabolically active, and patterns of precipitation drive patterns of N fixation (Belnap 2002, Ferrenberg et al. 2015). Therefore, we determined the potential of these organisms to fix N in the context of moisture availability. To do this, we performed ARAs for three consecutive days. On the first day, we wet the samples with 3.5 mL of deionized water to simulate a precipitation event. We allowed the samples to dry at ambient temperature in the incubator for two days, performing ARAs on the samples each day to quantify the effect cycles of precipitation cycles on nitrogenase activity. We then fit generalized least squares models with AR1 correlation for each morphological group of soil crust to quantify the decline in N fixation rates over three days after a wetting event. We obtained daily precipitation data for both sites for the years 2013-2015 (Iowa Environmental Mesonet n.d.), and identified “rainy days” as days with precipitation equal to or greater than 2.8 mm, the equivalent of the 3.5 mL water treatment. We then paired the estimates from the generalized least squares models with the precipitation time series to obtain total average fixation from soil crusts per year. To obtain plot-level N fixation estimates, we paired morphological group- and site-specific N fixation rates with abundance measures.

In July 2015, we collected random 100 cm^2 samples of organic (O)-horizon litter from each plot to determine N fixation of asymbiotic bacteria. We performed ARAs on these samples as described above with an incubation time of 6 hours, and we assumed the theoretical conversion factor of 3:1 (Hardy et al. 1973). After the ARAs, we dried the samples at 70 ˚C for 48 hours and weighed them to ± 0.01 g. We expressed N fixation rates per mass of sample, and paired these rates with estimates of total O-horizon mass per plot (methods described below).

Soil nutrient controls on N_2 fixation

Soil nutrients in-situ - We sampled mineral soils seasonally (September 2014, June-July 2015, November-December 2015 and March-April 2016) to determine N availability and mineralization rates with *in situ* buried-bag incubations and potassium chloride (KCl) extractions. In each plot, we collected six soil cores (2.5 cm diameter, 20 cm deep) from subplots. These samples were separated by depth (10 cm and 20 cm), homogenized, removed of roots and organic matter, and subsampled for extraction of the initial mineral N pools (NH_4^+ and NO_3^-). The remainder of the soil was placed in gas permeable plastic bags and buried to depths of 10 and 20 cm respective of their original depth. After the incubation period (28-35 days), soil samples were retrieved and analyzed for final pools of inorganic N. To determine inorganic N in initial and final pools, we extracted soils of approximately 30 g immediately with 100 mL of 2M KCl. Samples were shaken, exact weight of soils was determined, and extractions were filtered (Whatman No. 41 filter paper and 1µm glass fiber) and frozen until analysis by continuous flow colorimetry at the University of Georgia Stable Isotope Laboratory. Net N mineralization was calculated by the difference in initial and final dissolved inorganic N (DIN) pools per g dry weight of soil (kg N ha^{-1} yr^{-1}).

To determine total N stocks in the first 20 cm of the soil, we extracted soils in subplots, homogenized them in each plot by 10 cm increments, and picked out roots. Samples were analyzed for N content via combustion analysis as described in the methods above. All soil measurements were paired with depth- and plot-specific measurements of soil bulk density. We also quantified available phosphate using Mehlich and resin extractions (Turner and Romero 2010, Wurzburger et al. 2012).

Common garden experiments - We conducted two concurrent greenhouse experiments at the University of Georgia from March to September 2015 using seven herbaceous legume species: *Centrosema virginianum* (L.) Benth, *Chamaecrista fasciculata* (Michx.), *Desmodium floridanum* Chapm., *Lespedeza hirta* (L.) Hornem., *Lespedeza virginica* (L.) Britton, *Mimosa quadrivalvis* L., and *Tephrosia virginiana* (L.) Pers. In experiment 1, we determined if P and Mo (or their combination) constrain legume fixation, and in experiment 2 we assessed whether legumes downregulate fixation when supplied additional N and the importance of species identity in this regulation of fixation. See Ament et al. (2018) for detailed methodology on experimental procedure. Briefly, we added P, Mo and N to plants on a biweekly basis for 16 weeks in both experiments. For experiment 1, we supplied P at two levels (-P and +P, with a total application of 0 and 15.38 mg P per plant, which corresponds to 0 and 5 g P m^{-2} $year^{-1}$ as $NaPO_4$) and Mo at two levels (-Mo and +Mo, or 0 and 153 µg Mo, corresponding to 0 and 50 mg Mo m^{-2} $year^{-1}$ as Na_2MoO_4) in a complete factorial design for a total of four treatments and 10 replicates per treatment by species combination. For Experiment 2, we supplied N at three levels (-N, +N and +++N, or 0, 6.15, and 30.7 mg N per plant, and corresponding to 0, 2 and 10 g N m^{-2} $year^{-1}$ as 5 atom % labeled ^{15}N $(NH_4)_2SO_4$) with eight replicates per treatment by species combination. We destructively harvested all plants to quantify leaf, stem, root, nodule and reproductive biomass 105 -115 days after initiating nutrient treatments. During harvest, we quantified whole-plant N_2 fixation (henceforth referred to as instantaneous fixation) by performing assays of acetylene reduction activity (ARA) on all the root nodules from each plant. In parallel with ARAs, we performed $^{15}N_2$ incubations on the nodules from a subset of individuals of each species (Robertson 1999). We replaced 10% of the headspace of jars with 99 atom % $^{15}N_2$ (Sigma Aldrich; Missouri, USA), and incubated nodules for 30 minutes. Immediately following the incubation, we dried the nodules at 70 ˚C for 48 hours, ground them to a find powder with a mortar and pestle, and determined $\delta^{15}N$ of enriched and unenriched samples with isotope ratio mass spectrometry at the University of New Hampshire Stable Isotope Lab using a Costech 4010 Elemental Analyzer coupled to a Finnigan Delta Plus XP isotope ratio mass spectrometer. We also calculated fixation investment, which was the rate of fixation per unit mass of plant. For plants in experiment 2, we used the ^{15}N dilution method (Robertson et al. 1999) to calculate the percentage of plant N derived from the

Figure 3 Common garden and nutrient addition experiment. We grew seven legume species on 100% field soil from Benning and Eglin and exposed then to N, P and Mo additions.

atmosphere (%N_{dfa}) and total N_2 fixation over the lifetime of the plants (henceforth referred to as total fixation).

Field fertilization experiment - To test for N and P limitation in the field, we applied a fertilization treatment to nine of our plots at Benning and 13 plots are Eglin across age and fire frequency gradients. At each plot, one subplot was dosed with N at the equivalent rate of 50 kg ha^{-1} yr^{-1} in the form of ammonium nitrate, one subplot was dosed with P at 25 kg ha^{-1} yr^{-1}in the form of triple superphosphate, and one subplot remained as a control. Fertilizer was applied two times – once in March 2017, at the beginning of the growing season, and once in June 2017 at the peak of the growing season. In August, 2017, we quantified the number of legumes in each plot, their biomass as well as nodulation and N fixation rates using ARA (as above). Overall, we sampled 524 legumes, 339 at Eglin and 185 at Benning. Using the same N and P application rates, we fertilized individual longleaf pine trees (within a 0.5, 1 or 2 m radius of stem, depending on whether trees were < 10, 10-20 or 20 + years old), where six trees in each plot were allocated to control, +N or +P treatments. In all, we sampled 390 trees across our two sites (six trees were lost to mortality during the fertilization treatment). We measured dbh and height of all trees at the onset of the fertilization treatment, and measured trees after fertilization in December 2017 or January 2018.

N demands from biomass production

We quantified N demands from aboveground biomass production in the overstory and understory, as well as belowground fine root production. To determine overstory biomass production, we measured incremental growth (dbh or ground-line diameter and height) of a subsample of at least 20 trees in each plot for four years (2014-2017) in December or January. We collected species-specific foliage and wood samples in each plot, and these samples were analyzed for N content via combustion analysis according to the methods described above. We paired diameter and height measurements with allometric equations for pines (Samuelson et al.

Figure 4 Quantifying N demand by trees. We measured dbh and height on ~1200 trees over three years. Using allometric equations and tissue N concentrations, we quantified the annual N demand associated with tree biomass growth.

2014, 2017) and scrub oaks (Mitchell et al. 1999) and matched biomass compartments with N concentrations to determine change in aboveground biomass and associated N demands. To quantify overstory litter production and associated N demands, we collected litterfall every three months over one year with five 0.7 m^2 litter traps randomly set in each plot. We dried the litter at 70 ˚C for 48 hours, sorted it into functional groups and determined the relative contribution of each group to total litterfall in each plot. We determined plot- and functional group-specific tissue N concentrations via combustion analysis as described above, and paired N concentrations to litterfall estimates. Additionally, we collected and weighed litter every three months for one more year to obtain more rigorous estimates of litterfall.

We estimated fine root production over one year in each subplot with root ingrowth cores as described by Hertel and Leuschner (2002). In each subplot, we extracted one soil core (7.8 cm diameter**,** 20 cm depth), removed all live and dead root material, and replaced the soil. After one year, we retrieved the core, split it into 10 cm depth increments (0-10 cm and 10-20 cm) and sorted roots produced in the core into categories of size (< 1 mm, > 1 mm & < 2 mm, and > 2mm) and functional group (longleaf pine or "other"). We determined functional group- and size-specific N content of roots in each plot with combustion analysis as described above, and paired N concentrations with root production rates to obtain estimates of N demands (kg N ha^{-1} yr^{-1})

We estimated annual N demand of understory vegetation biomass as the equivalent of the late-winter understory biomass and N content as determined above. Perennial herbaceous vegetation can retranslocate approximately 50% of its aboveground N to belowground stores after a growing season (Li et al. 1992), so we assumed that the biomass N accounted for in late-winter sampling was the net annual N demand for the understory.

N losses from fire

We estimated potential N losses from fire in both the soil O-horizon and the understory. To determine the mass of the soil O-horizon (including the litter layer and partially decomposed plant material above the mineral soil) in each plot, we collected 400 cm^2 samples of O-horizon randomly placed within each subplot, dried the samples at 70 ˚C for 48 hours, and weighed them to ± 0.01 g. Samples were then ground to a fine power with a ball mill and analyzed for C and N content via combustion analysis with an elemental analyzer as described above. The masses and N content of the subsamples were averaged in each plot and normalized by sampling area to obtain plot-level estimates. Within 30 days after each fire event from 2014-2017, we resampled and processed the O-horizon according to the methods described above. We calculated the loss of N from a single fire event as the difference between ambient O-horizon mass and N content and post-burn O-horizon mass and N content.

To estimate the amount of N lost from fire in understory vegetation, we collected all aboveground biomass less than 1.3 m tall within 0.5 m^2 quadrats randomly placed in each plot. We sampled during the late winter (February 2016) because most prescribed burns occur during this time. These samples were dried, weighed and analyzed for N content as per the methods described above, and we extrapolated the biomass and N content to the plot-level. We assumed that 80% of the N in understory biomass is volatilized in a fire event (Boring et al. 2004).

Figure 5 Longleaf pine plots following a fire event. We estimated soil O-horizon N loss from 73 fire events, by quantifying the mass and N stock of the organic layer before and after fire events.

Data analysis

Abundance and activity of N fixers - We investigated N fixation dynamics over stand age with linear models testing how the interaction of stand age and site affects N fixation contributions (kg N ha^{-1} year^{-1}) from each functional group. We also fit linear models to determine how N mineralization rates and total DIN pools affect N fixation from each group. To determine how fire effects N fixation, we fit linear models testing how N fixation contributions from each three functional groups varied with fire return interval (years) and time since fire (months). To further explore short-term effects of fire, we constructed a repeated-measures ANOVA on two years of legume census data to determine differences in legume N fixation between plots that experienced fire in the past year and plots that did not (Bates et al. 2015), and performed multiple comparisons with a Tukey's post-hoc test (Hothorn et al. 2008).

Common garden experiment **-** To determine if the addition of nutrients (i.e., +P, +N, and +++N) enhanced nutrient availability to plants and affected biomass growth relative to controls, we fit linear models and used the glht function in the multcomp package to perform *a priori* contrasts with Bonferroni-corrected *P* values (Hothorn et al. 2008). In the case of Mo, we conducted t-tests to compare the foliar Mo concentration between +Mo and the control.

To analyze the effect of P and/or Mo on biomass growth (g), nodule investment (g nodule g^{-1} plant), nitrogenase activity (μmol N g^{-1} nodule hr^{-1}), instantaneous fixation (μmol N plant^{-1}

hr^{-1}), and fixation investment (μmol N g^{-1} plant hr^{-1}), we fit linear models where we treated P, Mo, species and soil origin as categorical variables. We analyzed factor effects and their interactions using a 3-way ANOVA and removed variables that were not significant. To assess significant interactions, we used the glht function in the multcomp package (Hothorn et al. 2008) to perform *post hoc* linear contrasts with Tukey correction on the interactions.

To determine species-specific fixation strategies, we assessed how N addition affected total fixation investment (mg N g^{-1} plant) with a 3-way ANOVA with N treatment (+N, +++N), species and soil origin as categorical variables, and we removed interaction terms that were not significant. Where we found a significant 3-way interaction, we analyzed the effects of N treatment and soil origin for each species separately using a linear model and a 2-way ANOVA.

***Field fertilization experiment* -** To determine if the addition of nutrients (i.e., +P, +N) affected legume nodulation, we fit generalized linear mixed models with a binomial distribution, where plots were treated as a random effect. To determine whether legume foliar nutrient concentrations changed in response to nutrient treatment, we conducted ANOVA, using factors of treatment, site and species. To determine the response of trees to nutrient addition, we calculated relative growth rates of trees and analyzed data with a linear mixed-effect model using plot as a random effect.

***Ecosystem N Balance* -** To determine if net ecosystem N inputs balance outputs, we calculated the ecosystem N balance as the difference of external inputs and outputs of N. Annual N inputs were the sum of annual atmospheric N deposition (7.0 kg N ha^{-1} $year^{-1}$, wet + dry) (NADP 2017) and plot-level total N fixation estimates, and annual N outputs were the total amount of N estimated to be lost in a single fire event in each plot divided by its fire return interval. We investigated if the N balance over time with a linear model, and included site as an interaction term to investigate whether the trajectory of the N balance differs between sites. We also determined the fraction of total ecosystem N (total soil N and vegetation biomass N) lost annually to fire at each plot. This analysis only included plots with a fire history, so three juvenile plantations that had not yet burned were excluded (n = 51).

To investigate the patterns of N demand from biomass growth over stand development, we fit a generalized additive model to test how total tree N demand (from aboveground, litter and fine root production) changes over time, using stand age as a non-parametric smoothing variable and site as a parametric effect (Hastie 2016). We chose this non-parametric approach to examine this response variable because we expected N demand from trees over time to be non-linear (i.e. Vitousek and Howarth 1975). We fit a linear model to determine how annual N demand from ground layer vegetation differs between sites and over stand development.

We estimated the available N balance for each plot as the difference between annual N mineralization rates plus annual atmospheric N deposition and the N demand from biomass growth from trees (aboveground, litter and fine root production) and ground layer vegetation. To examine how the internal N cycle changes throughout stand development, we constructed linear models testing the interaction of stand age and site on the balance of available N as well as total DIN and total soil N.

We performed ANOVAs on all linear models to analyze differences in site-level means and to obtain partial correlation coefficients for predictor variables and their interactions. If interactions were insignificant, they were removed from the models. Response variables were log-transformed to meet normality assumptions, and the normality of residuals for each model was verified using Shapiro's tests. All analyses were conducted in R (R Core Team 2016).

Results and Discussion

Quantifying N fixation

We quantified annual inputs of N from three taxonomic groups of N-fixers. We observed an unexpected and dramatic site difference where total N fixation inputs at Benning was six times greater than at Eglin (Figure 6). Further, combined estimates of N fixation were much smaller than inputs from atmospheric N deposition (Table 1). The relative contributions of each taxonomic group differed between sites, where N fixation was dominated by legumes at Benning and by asymbiotic bacteria at Eglin (Figure 6A). Across both sites, N fixation by herbaceous legumes was highly variable but was not significantly related to stand age (Figure 6B) which contrasts to many temperate forests where symbiotic N fixing plants grow exclusively in young stands (Cleveland et al. 1999). However, N fixation by legumes differed dramatically between sites, which accounted for 62% of model variance. Only 3.3% (n = 1070) of legumes surveyed at Eglin possessed nodules compared to 47.3% (n = 400) at Benning. Because of this difference in nodulation frequency, legumes contributed an order of magnitude more N at Benning than at Eglin (Table 1, Figure 6A). Despite their ubiquitous distribution and high abundance in some plots, legume N fixation did not contribute half as much N as does atmospheric deposition.

Although we found that soil crusts were actively fixing N, their contributions to the ecosystem N cycle were minimal. N fixation by soil crusts exhibited a strong site-by-stand age interaction where Benning supported elevated levels of crust N fixation in juvenile stands, but these rates rapidly declined as stands matured (Figure 6C). We found that soil crusts were most abundant and fixed the most N in juvenile stands recovering from recent disturbance, and these N inputs declined exponentially over stand age (Fig. 6C). The high-light environment typical of juvenile plantations may provide ideal growing conditions for these organisms (Belnap 2002), and litter accumulation may exclude soil crusts as stands age.

N fixation rates by asymbiotic bacteria mildly decreased with stand age, consistent with previous research in temperate forests (Distefano and Gholz 1989, Son 2001), but did not differ between sites (Table 1, Figure 6D). Compared to other ecosystems, our estimates of asymbiotic N fixation are relatively low (Reed et al. 2011). Yet, surprisingly, we found that asymbiotic bacteria contributed over 4 times more fixed N than legumes at Eglin (Table 1). While we only quantified N fixation in the soil O-horizon, asymbiotic bacteria capable of N fixation are ubiquitous in terrestrial ecosystems (Reed et al. 2011) and may also occur in the mineral soil (Distefano and Gholz 1989) or by endophytic bacteria that inhabit pine needles (Moyes et al. 2016), but all these additional sources of N fixation are likely to be modest compared to the contribution of asymbiotic bacteria in the O-horizon.

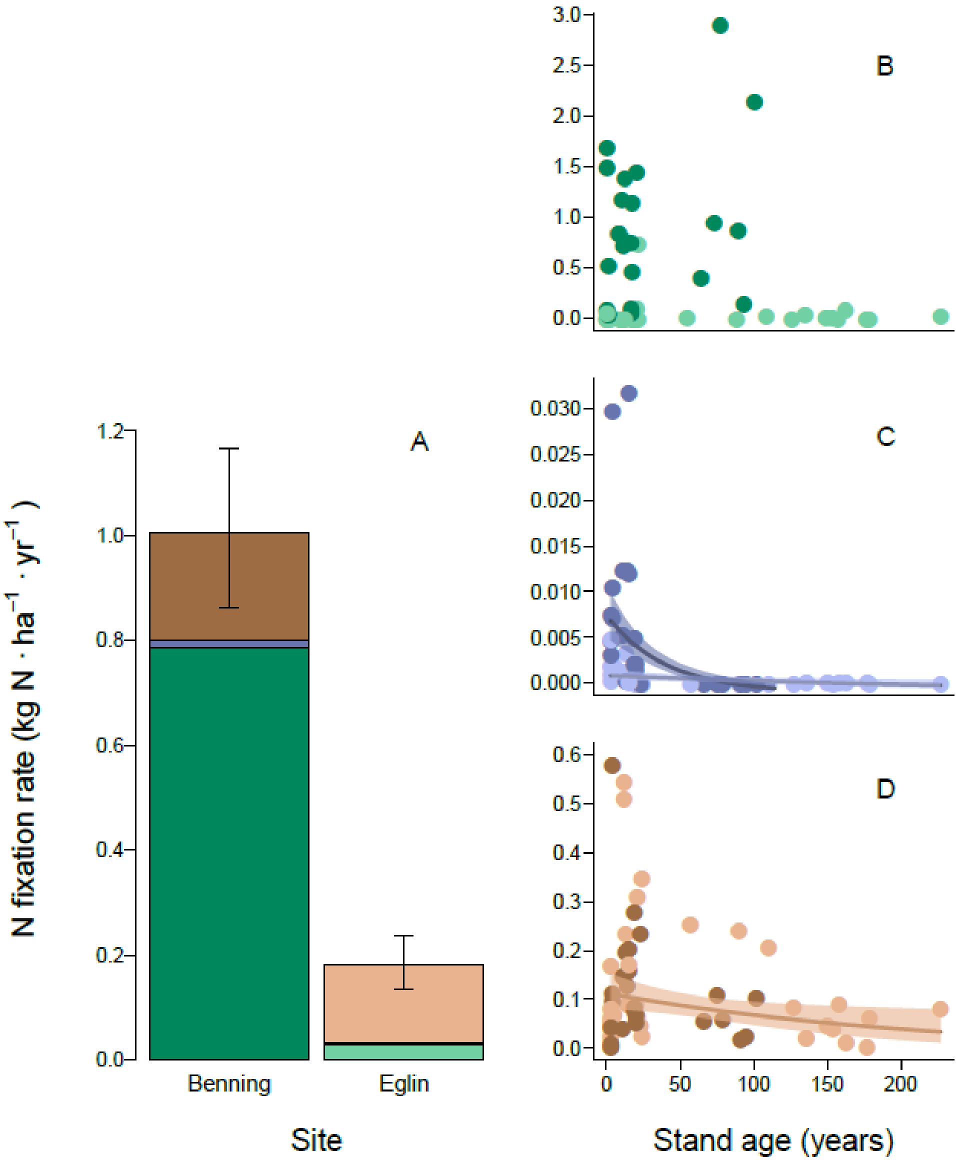

Figure 6 Total N fixation rates by site and type of N-fixer. A) the proportional contributions by legumes (green), soil crusts (purple) and asymbiotic bacteria (orange) to site-level mean total N fixation (kg N ha^{-1} yr^{-1}), and contributions by B) legumes, C) soil crusts and D) asymbiotic bacteria over stand age in longleaf pine stands at Benning (darker points) and Eglin (lighter points). Error bars represent standard errors of total N fixation estimates, lines represent model predictions and shaded areas are 95% confidence intervals.

Table 1 Site-level means of soil properties, soil N stocks and ecosystem N fluxes at Benning and Eglin. Standard errors are given in parentheses, and different levels indicate a significant difference by F-test (α = 0.05).

	Benning		Eglin	
Soil properties				
N (%)	0.04 (0.001)	a	0.03 (0.002)	b
C (%)	1.02 (0.07)	a	0.83 (0.06)	b
C:N	26.20 (1.01)	a	25.94 (0.75)	a
Sand (%)	89.60 (0.81)	a	90.26 (0.46)	a
Clay (%)	3.76 (0.49)	a	3.76 (0.25)	a
Silt (%)	6.65 (0.52)	a	5.97 (0.33)	a
pH	5.19 (0.07)	a	5.19 (0.02)	a
VWC (%)	4.30 (0.44)	a	4.79 (0.15)	a
Bulk density (g cm^{-3})	1.35 (0.05)	a	1.32 (0.03)	a
Soil N stocks (kg N ha^{-1})				
Total soil N	100795(3967)	a	79572 (4691)	b
Total DIN	0.37 (0.04)	b	0.62 (0.03)	a
O-horizon N	177.64 (65.00)	a	138.16 (25.66)	a
N fluxes (kg N ha^{-1} year^{-1})				
N loss from fire	32.97 (1.19)	a	16.89 (1.28)	
N deposition†	7		7	
Net N mineralization	31.86 (4.48)	b	38.12 (4.49)	a
Total N fixation	1.00 (0.15)	a	0.18 (0.03)	b
Legume N fixation	0.88 (0.15)	a	0.04 (0.02)	b
Soil crust N fixation	6.23E-03 (1.70E-03)	a	6.70E-04 (2.10E-04)	b
Asymbiotic N fixation	0.12 (0.02)	a	0.14 (0.03)	a

†N deposition estimates from NADP (2017).

Fire dynamics promoted legume N fixation on both short- and long-term timescales. At Benning, plots with a higher FRI supported lower rates of N fixation by from legumes ($R^2 = 0.19$, $F_{1,21} = 6.21$, $p = 0.021$), but time since fire (months) had no effect. Neither time since fire nor FRI significantly affected legume N fixation at Eglin. However, at both sites we found that plots that had experienced a fire event in the year preceding a census, supported significantly more N fixation by legumes than plots that had not burned ($F_{1,53} = 16.80$, $p << 0.001$; Figure 7). We did not observe an effect of FRI nor time since fire on N fixation by biological soil crusts or asymbiotic bacteria ($p > 0.05$ for all). Furthermore, soil N dynamics (N mineralization rates, total DIN pool, and balance of N supply and demand) did not explain rates of fixation by any group of N fixer ($p > 0.05$ for all).

The idea that fire promotes legume abundance and N fixation in longleaf pine savannas is widely-held by researchers and land managers (Hiers and Mitchell 2007). Confirming these expectations, we observed long- and short-term effects of fire on legume N fixation. At Benning, plots that burned more frequently (lower FRI) supported more N fixation from legumes. However, we did not observe a pattern of FRI and legume N fixation at Eglin. Generally, legumes fixed a negligible amount of N at this site (over half the plots did not support any N fixation by legumes), so it is unsurprising that such patterns across the gradient of FRI were undetectable. Yet, we observed short-term effects of fire disturbance on legume N fixation at both sites where plots that burned in between annual censuses supported an increase in legume N fixation (Figure 7). A single fire event can stimulate populations of legumes (Hendricks and Boring 1999), suggesting a transitory competitive advantage of fixing N post-fire (Hiers and Mitchell 2007).

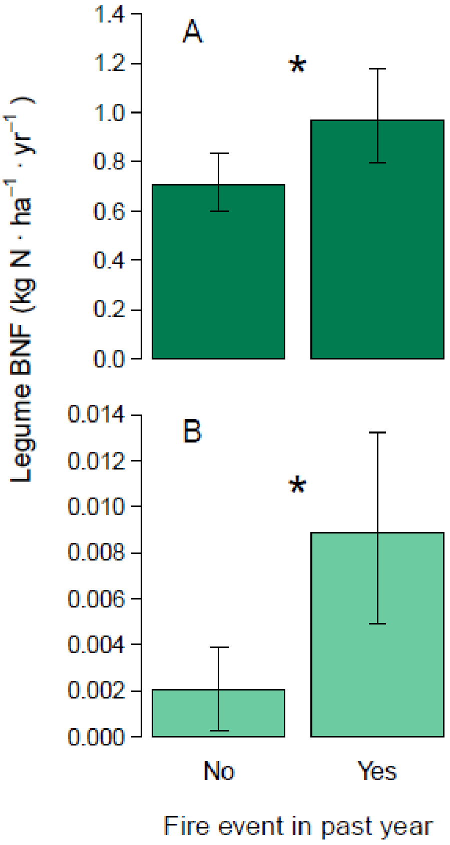

Figure 7 Estimates of legume N fixation in plots that did or did not burn in the previous year at A) Benning and B) Eglin. N fixation measurements were conducted in the summers of 2015 and 2016. Asterisks indicate a significant difference at α = 0.05 and bars represent standard errors. N fixation = biological N fixation.

Nutrient regulation of N fixation

Asymbiotic fixation - We analyzed the activity of asymbiotic fixation in the leaf litter layer of soils and determined if the activity of fixation responded to nutrient additions. In a lab experiment, we added water (control), P, Mo or both in mesocosms following the methods of Wurzburger et al. (2012). Surprisingly, we observed no response of fixation to nutrient addition, but significant differences in activity between sites ($p > 0.05$) (Figure 8). This finding suggests that P and Mo do not limit, or co-limit, N fixation by asymbiotic fixers in these longleaf pine savannas. This finding is in contrast to previous studies that demonstrate either, P, Mo or both limit asymbiotic fixation (Reed et al. 2011)

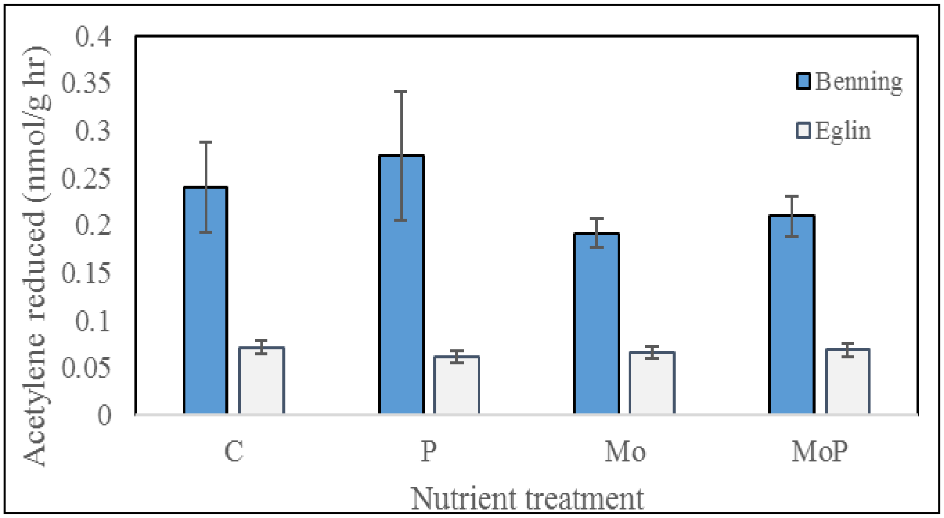

Figure 8 Asymbiotic fixation on leaf litter in response to nutrient addition experiment. Fixation did not respond to addition of P, Mo or both, relative to a control that received only water ($p > 0.05$), but was higher in Benning versus Eglin leaf litter ($p < 0.05$).

N fixation by legumes in common garden experiments - We first determined how P and Mo addition affected legume growth and fixation. P addition stimulated biomass growth, nodule investment, instantaneous fixation and fixation investment, but had no effect on nitrogenase activity (Figure 9). P addition had a stronger positive effect on biomass growth for legumes in Eglin versus Benning soil, indicating a contributing effect of soil origin. We found no support that Mo constrains legume fixation, either alone or in combination with P ($P > 0.05$ for all variables). Our findings reveal soil P as a strong constraint on N_2 fixation for all legume species in this study. The addition of P stimulated growth, instantaneous fixation, and how much plants invested in nodule biomass and fixation. The strong P effect on plant biomass growth and fixation suggests that P increased growth and thus also increased plant N demand (Perreijn 2002, Batterman et al. 2013b).

We next determined how species identity affected fixation among the herbaceous legumes of our study. To understand species-specific differences, we analyzed instantaneous fixation rates across control plants from both experiments (across seven species), and found that *Mimosa quadrivalvis* and *Tephrosia virginiana* supported the highest rates of fixation, followed by *Centrosema virginianum*, *Desmodium floridanum*, *Lespedeza virginica*, *Chamaecrista fasciculata* and *L. hirta* possessed the lowest rate ($F_{6,104} = 10.74$, $P < 0.001$) (Figure 10).

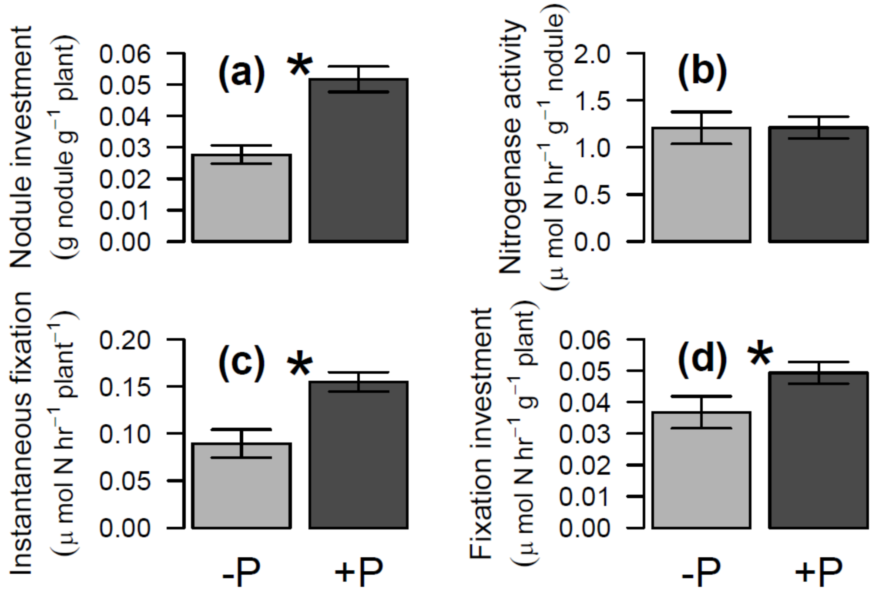

Figure 9 Growth and fixation responses of legumes to P addition in common garden experiment. Responses include a) nodule investment, b) nitrogenase activity, c) instantaneous fixation and d) fixation investment. Fixation rates were determined via ARA and conversions to N_2 and expressed on a nodule mass b), total plant c) or per plant mass d) basis. Bars represent the mean ± SE of all species across two levels of P (-P, +P), aggregated across both soil types, and asterisks denote significant response to P (α = 0.05).

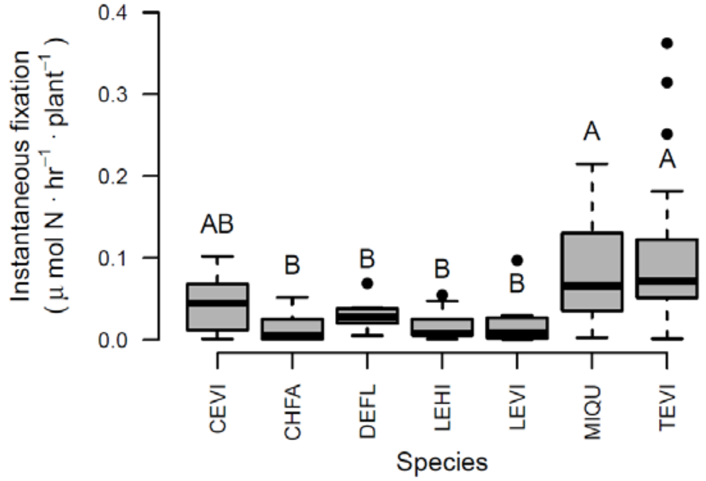

Figure 10 Species differences in instantaneous fixation rate in common garden experiment. Boxplots represent median and quartiles, and letters indicate Tukey's post-hoc comparisons (α = 0.05). Species codes: CEVI = *Centrosema virginianum*, CHFA = *Chamaecrista fasciculata*, DEFL = *Desmodium floridanum*, LEHI = *Lespedeza hirta*, LEVI = *Lespedeza virginica*, MIQU = *Mimosa quadrivalvis*, TEVI = *Tephrosia virginiana*.

Our results indicate that herbaceous legume species vary widely in their ability to supply new N to the longleaf pine savannas, corroborating previous studies (Hiers et al. 2003a, Cathey et al. 2010). Plant growth form, evolutionary history, and nodule morphology may contribute to such differences in fixation among species. In our study, a trailing vine (*Mimosa quadrivalvis*) supported high rates of instantaneous fixation relative to several prostrate species, which may reflect critical trade-offs as trailing vines do not invest in structural support for vertical growth (Pate 1996).

We then determined how N addition affected legume fixation. Species differed in their total investment in fixation and in their response to N addition (Figure 11). Four species (*Centrosema virginianum*, *Desmodium floridanum*, *Lespedeza hirta* and *L. virginica*) reduced their total fixation investment when exposed to the +++N treatment, and this response was significantly more pronounced for *C. virginianum* and *D. floridanum* when grown in Eglin soils. However, three other species (*Chamaecrista. fasciculata*, *Mimosa quadrivalvis* and *Tephrosia virginiana)* maintained fixation investment, even when exposed to higher availability of N (Figure 11). The reduction in fixation was particularly strong for *Centrosema virginianum* and *Desmodium floridanum* in Eglin soils, likely due to P constraints on growth, and hence a lower demand for N relative to Benning soils. These results suggest that some species of herbaceous legumes have the ability to down regulate fixation in response to ample soil N, while others do not. An additional possibility is that the N additions of our experiment were not enough to alleviate plant N demand.

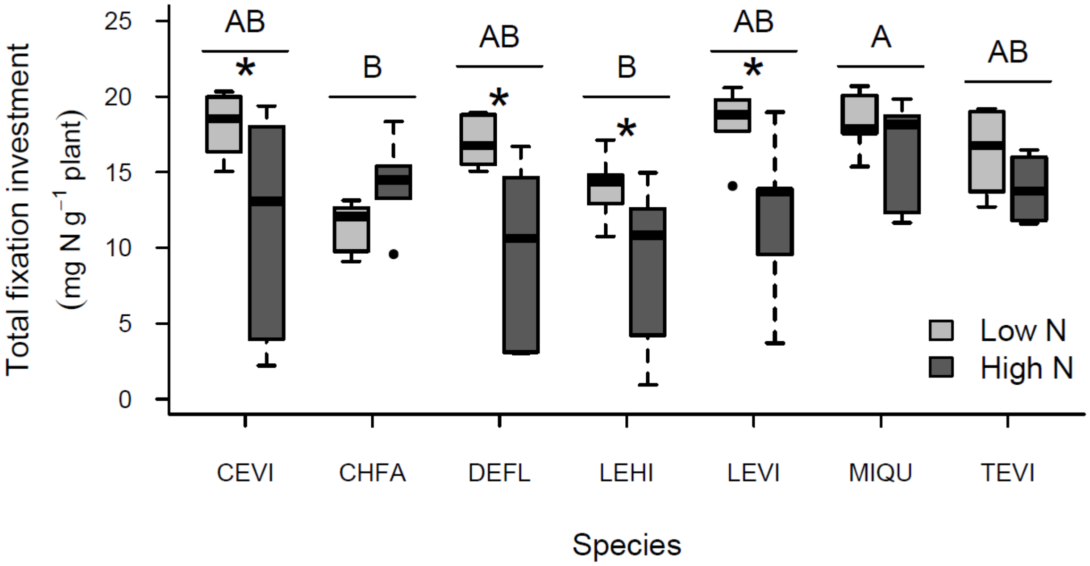

Figure 11 Species differences in total fixation investment in response to N addition in common garden experiment. Boxplots depict median values and quartiles, and letters indicate Tukey's post-hoc comparisons (α = 0.05). Fixation rates were quantified from ^{15}N dilution, where plants received either low or high additions of ^{15}N. Species codes: CEVI = *Centrosema virginianum*, CHFA = *Chamaecrista fasciculata*, DEFL = *Desmodium floridanum*, LEHI = *Lespedeza hirta*, LEVI = *Lespedeza virginica*, MIQU = *Mimosa quadrivalvis*, TEVI = *Tephrosia virginiana*.

***Field fertilization experiment* –** After one growing season of N or P fertilization, we sampled herbaceous legumes to determine the frequency of nodulation (i.e., the frequency that an individual plant contains nodules, and thus has the potential to fix N). We sampled 524 herbaceous legumes, and nodulation frequency was greater with the addition of P relative to the control ($z = 2.89$; $p = 0.003$) (Figure 12). The addition of N had no effect on nodulation, but it modestly reduced nodulation at Eglin only ($p < 0.1$). In addition, nodulation frequency was higher at Benning versus Eglin ($z = -4.33$; $p < 0.0001$), which is consistent with site differences in legume N fixation rates as reported above (see Figure 6B). In general, these findings corroborate our greenhouse experiments and demonstrate that P is a limiting nutrient for herbaceous legumes.

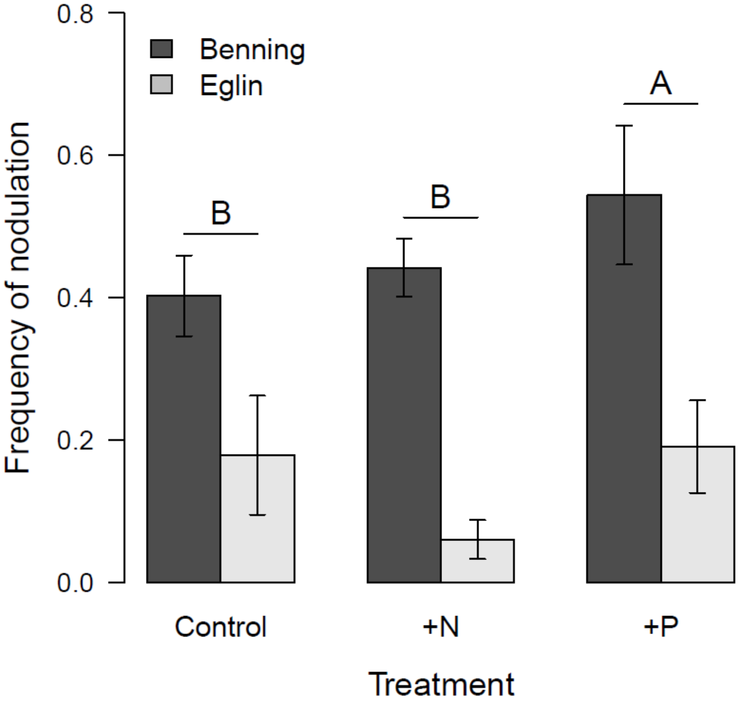

Figure 12 Frequency of nodulation among herbaceous legumes in response to applications of N and P in a field fertilization experiment. Values are means +/- standard errors. Different letters indicate significant response to +P at both Benning and Eglin, relative to +N and control.

As verification that our fertilization treatments increased soil N and P availability, we quantified foliar N and P concentrations of herbaceous legumes in our treatment plots. The addition of P increased foliar P concentrations ($F = 62.72$; $p < 0.0001$) (Figure 13). Surprisingly, foliar P also increased with N fertilization, but had no effect on foliar N concentrations. Foliar N was not affected by either nutrient treatment ($p > 0.05$), and foliar N:P declined with the addition of N and P, due to increases in P ($F = 31.66$, $p < 0.0001$) (Figure 13). The increase in foliar P concentration (Figure 13) and nodulation frequency (Figure 12) in response to the addition of P supports the idea that P limits legume growth. Further, the lack of an increase in foliar N concentrations in response to N addition supports the idea that N is not limiting legumes, which may explain why they support such a low rate of fixation (Figure 6B) and nodulation frequency (Figure 12).

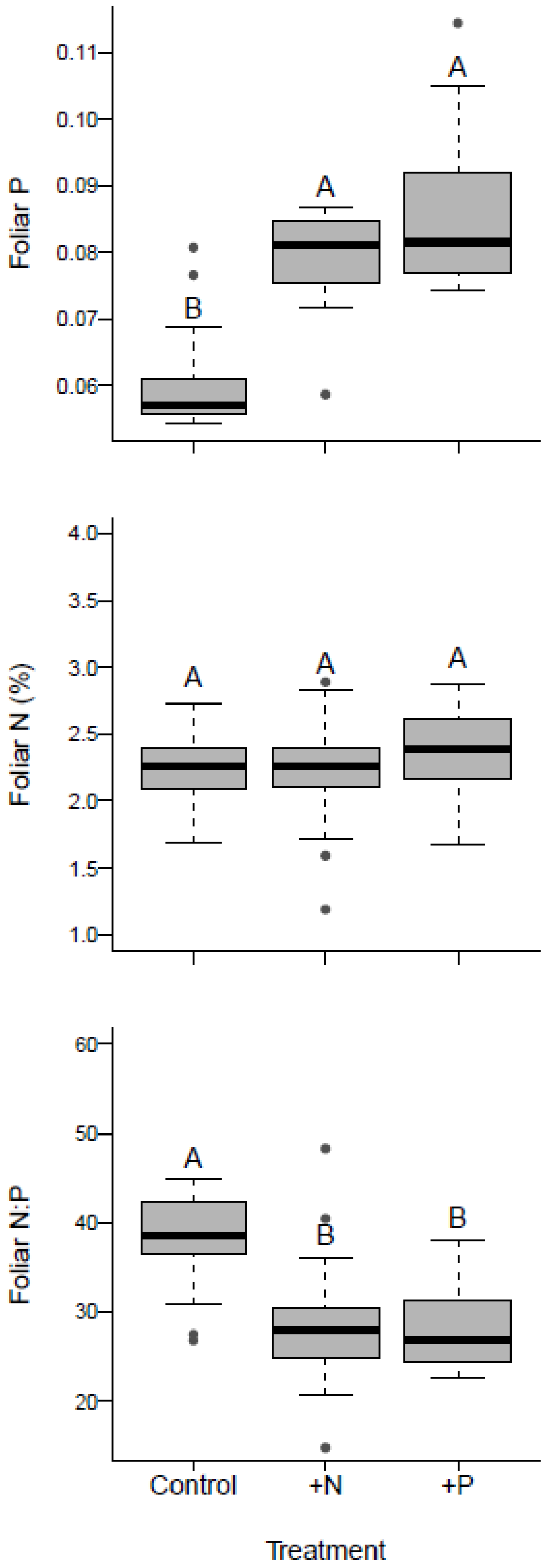

Figure 13 Foliar N, P and N:P in herbaceous legumes in response to N and P addition in field fertilization experiment. Boxplots depict median values and quartiles, and letters indicate Tukey's post-hoc comparisons (α = 0.05). Different letters indicate significant differences across treatments.

In conjunction with our field fertilization experiment, we determined if natural differences in soil nutrients explained the strong site difference in legume fixation between Benning and Eglin (Figure 6B). We quantified Mehlich III-extractable P in soils of all plots and found a dramatic difference between sites (t = -8.15, p < 0.0001) (Figure 14). Similarly, we found a difference in resin-extractable P between sites (t = -10.16, p < 0.0001) (Figure 15).

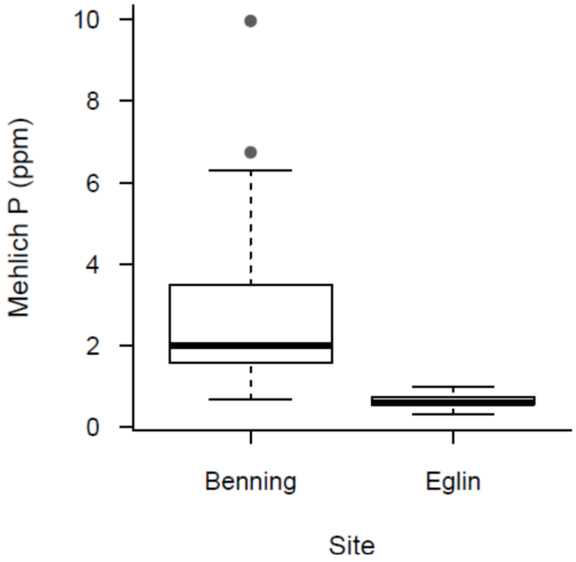

Figure 14 Mehlich III – extractable P in soils from all research plots at Benning and Eglin. Boxplots depict median values and quartiles.

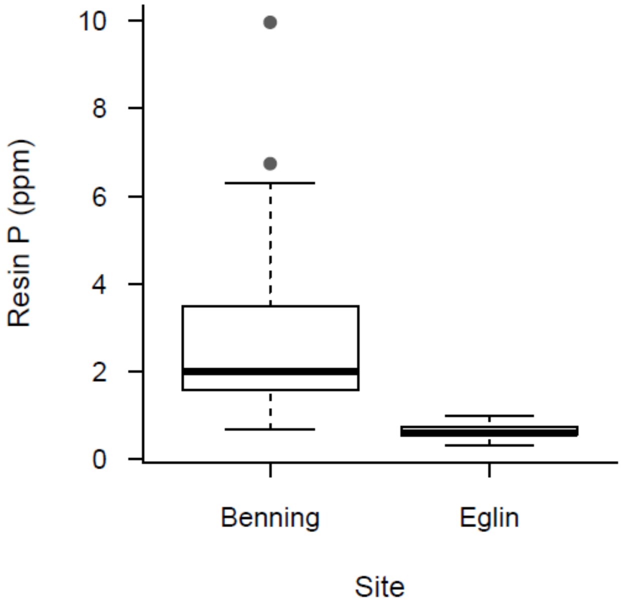

Figure 15 Resin-extractable P in soils from all research plots at Benning and Eglin. Boxplots depict median values and quartiles.

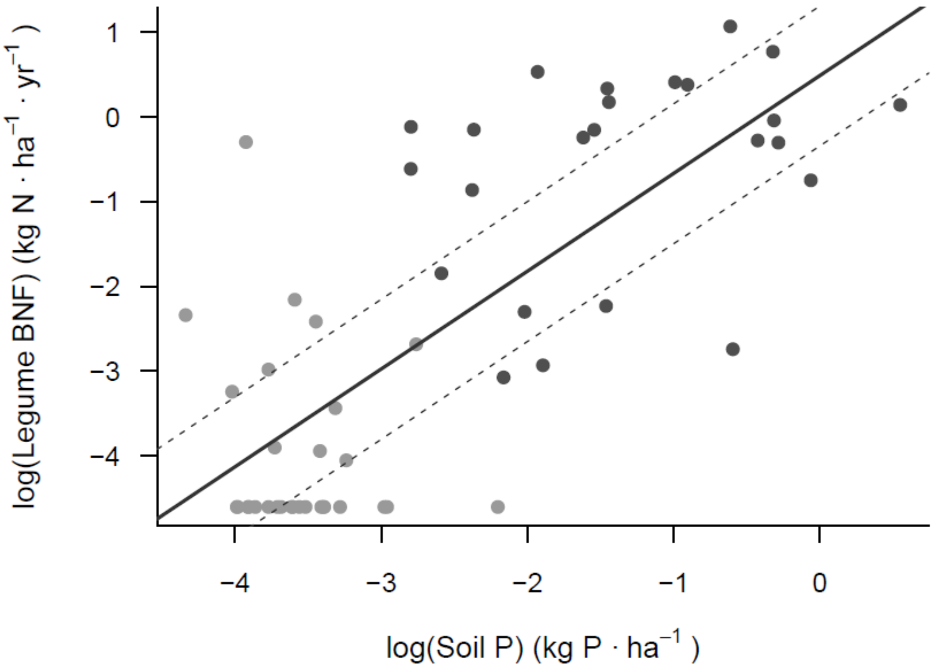

Figure 16 Relationship between plot-level legume N fixation and resin extractable P. Values are log-transformed. Plots at Eglin depicted in light grey, plots at Benning in dark grey. Dashed lines depict 95% confidence interval. BNF = biological N fixation.

These differences in bioavailable soil P may be the dominant driver of legume fixation across our study plots. To test this, we analyzed legume N fixation rate as a function of resin – extractable soil P at the plot level, and we found a positive relation between the two ($r^2 = 0.55$, $p < 0.0001$) (Figure 16), suggesting that legume fixation across the landscape is influenced by soil P availability.

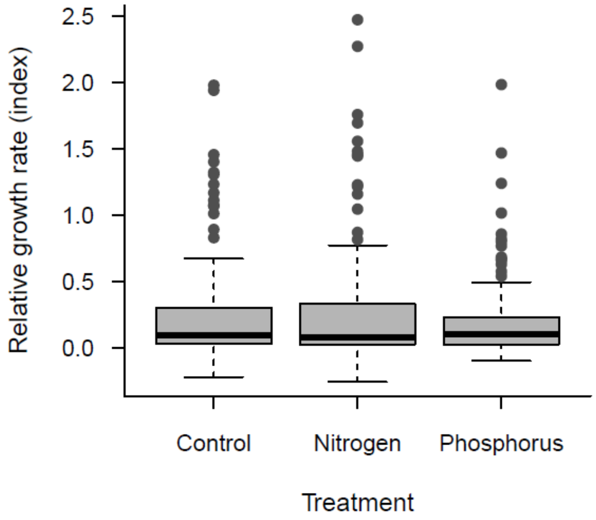

Figure 17 Relative growth rate of trees in response to field fertilization experiment. Boxplots depict median values and quartiles.

As a final part of our field fertilization experiment, we determined the growth response of trees in all plots. We measured the dbh and height of 390 trees before and after fertilization. Using allometric equations, we determined biomass growth for each individual and calculated its relative growth rate over the course of the experiment. We found no difference in the absolute growth of trees between the control and N and P addition plots (ANOVA, $F= 0.18$, $p = 0.83$). Further, we found no effect of treatments in the relative growth rate of trees (linear mixed-effects model, $t = 0.42$, $p = 0.67$; $t =-1.02$, $p = 0.3$; for N and P addition, respectively) (Figure 17), when accounting for plot as a random effect, and the decline in relative growth rate over stand age (Figure 18), we found no effect of treatment. These findings suggests that neither N nor P limit the growth of longleaf pine. It is possible that our fertilization experiment was not long enough to detect a change in tree growth, however, no response to long term N addition has been previously observed in another longleaf pine ecosystems (Kirkman et al. 2016).

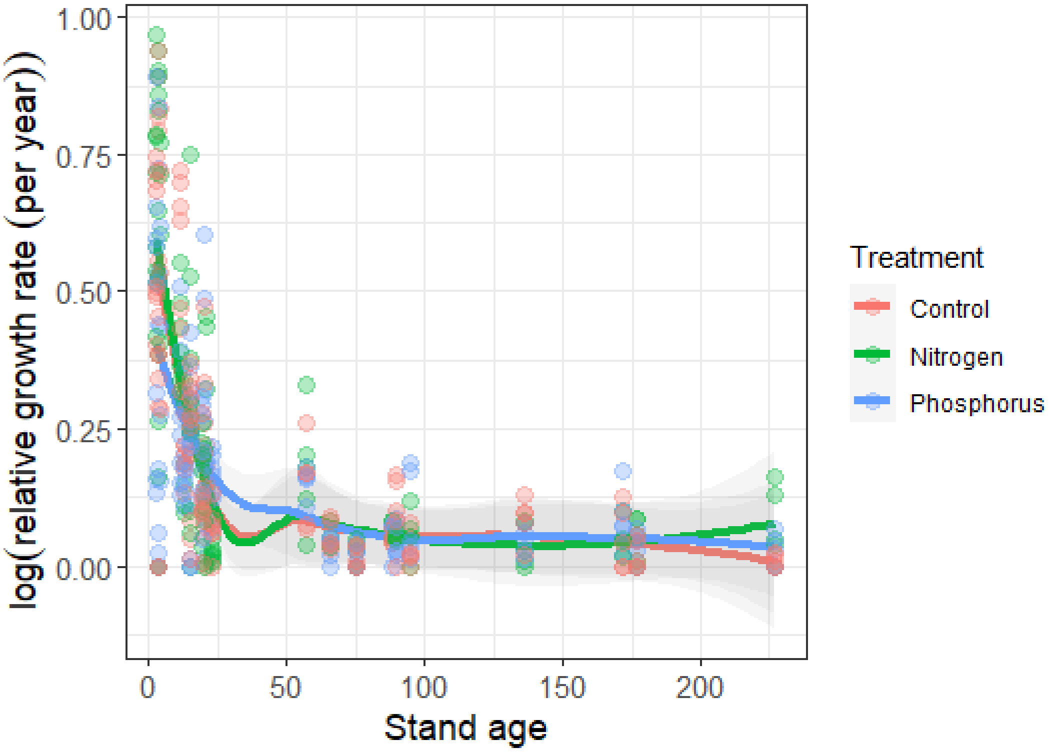

Figure 18 Tree relative growth rate declines with stand age but is unaffected by nitrogen and phosphorus addition. Each point represents a tree measured pre and post fertilization. Lines represent locally weighted averages.

N cycle and ecosystem N stocks

Despite high annual N losses to fire, mineral soils store in longleaf pine stands store a substantial amount of N (Figure 19A; Table 1). In fact, fire volatilized only 0.1% of total soil N stocks annually. However, total soil N decreased over stand age, where it declined 20% from juvenile to mature plots (max. 115 years) at Benning and 36% between juvenile and mature plots (max. 227 years) at Eglin (Table 1, Figure 19A). Similarly, N mineralization rates consistently declined with stand age at both sites, though the decline was significantly sharper at Benning (Table 1, Figure 19B) and rates ranged widely across our study plots (mean of 35.3 ± 3.2, range from 0.8 to 92.3 kg N ha^{-1} year^{-1}). Total stocks of plant-available N (NO_3^- + NH_4^+) were greater at Eglin ($F_{1,51}$ = 18.17, $p << 0.001$).

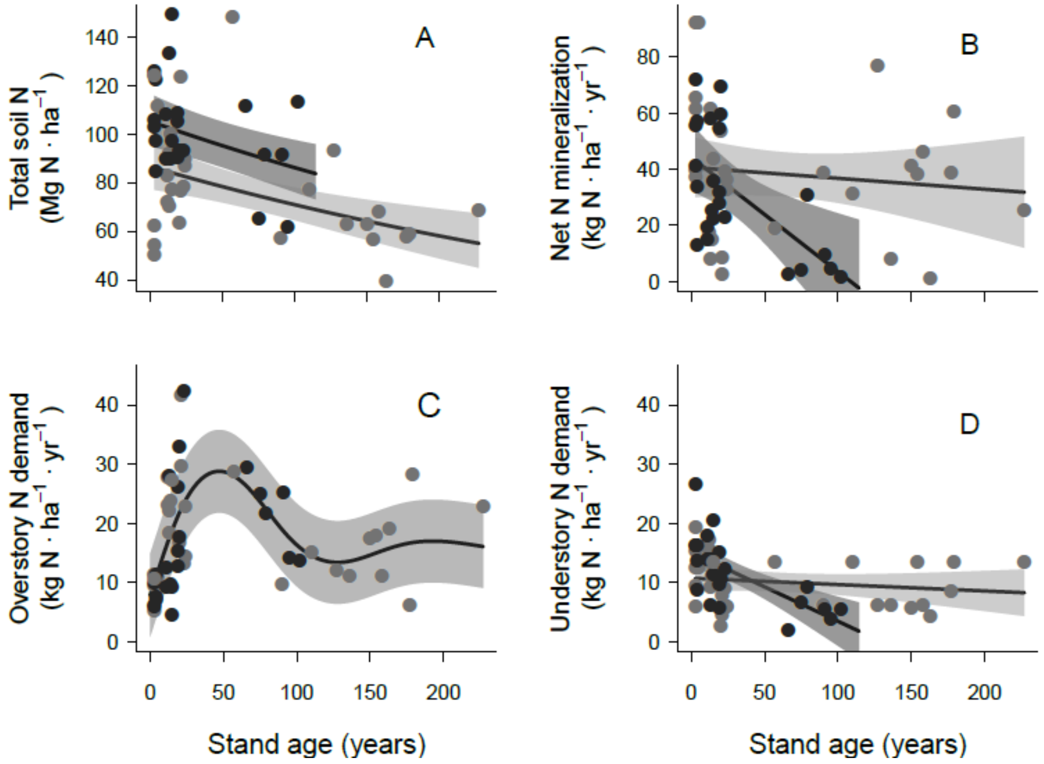

Figure 19 Ecosystem N stocks and fluxes over stand age, with A) total soil N (Mg N ha^{-1}) and B) average annual N mineralization rates (kg N ha^{-1} yr^{-1}) in the top 20 cm of soil, and C) nitrogen demand (kg N ha^{-1} yr^{-1}) from tree growth, including aboveground, fine root and litter production and D) understory annual growth across stand age at Benning (black) Eglin (gray). Lines indicate model predictions and shaded areas are 95% confidence intervals.

Site-level differences in N availability did not appear to affect N demand from trees which was consistent between sites and predictably peaked at an intermediate stand age (Figure 19C). Estimated understory N demand also did not differ between sites but decreased over stand age at Benning similar to patterns in N availability (Table 1; Figure 19D).

Ecosystem N balance

Across our plots, combined inputs from N fixation and from atmospheric deposition were insufficient to balance losses from fire (t_{50} = 29.1, p << 0.001, mean (upper 95% CI) = -16.7 (-12.1) kg N ha^{-1} year^{-1}) (Figure 20). We observed no clear trends across stand age, but notably, the ecosystem N balance was systematically more negative at Benning than Eglin (Table 1).

Fire-induced N loss was dominated by the volatilization of N in the O-horizon, which was an order of magnitude higher than volatilization from understory vegetation (means ± SE per individual fire event in 51 plots: 109.3 ± 17.3 and 8.6 ± 0.5 kg N ha^{-1}, respectively). The amount of N volatilized during a single fire event did not differ between sites ($F_{1,48}$ = 0.19, p = 0.50), however the higher fire frequency at Benning resulted in greater average annual losses ($F_{1,48}$ = 10.69, p = 0.002).

Figure 20 Ecosystem N balance. Plot-level N inputs (total BNF and atmospheric deposition; green bars) and N outputs (volatilization of ground-layer vegetation and O-horizon N; magenta bars) (kg N ha^{-1} yr^{-1}) of longleaf pine savannas at Benning (darker colors) and Eglin (lighter colors). Plots are arranged in order of continuous stand age and grouped into age classes ("mature" stands are naturally-regenerated).

Conclusions and Implications for Future Research

In longleaf pine savannas of Fort Benning and Eglin Air Force Base we found low rates of N fixation, and that the dominant contribution of N fixation differed by site (legume N fixation at Benning and asymbiotic N fixation at Eglin). This site difference appears to be driven by lower soil P availability and higher soil N availability at Eglin relative to Benning. Supporting this idea, legume N fixation increased with phosphorus (P) addition in common garden and field fertilization experiments. Nitrogen fixation declined with N addition in the common garden experiment (but not the field experiment), but the degree of this response was species dependent. Although individual fire events temporarily stimulated N fixation by legumes, N fixation was insufficient to balance N losses from fire and soil N stocks declined over stand age.

Surprisingly, we found that the long-term average loss of N from fire substantially exceeded the combined N inputs from N fixation and atmospheric deposition across gradients of stand age and fire frequency. Fire can volatilize a large portion of ecosystem N stocks (Boring et al. 2004, Lavoie et al. 2010), but inputs of N from N fixation and atmospheric deposition should compensate for these losses in ecosystems at steady-state (Vitousek and Reiners 1975). Although N fixation from herbaceous legumes is often invoked as a mechanism for N replacement after fire in longleaf pine ecosystems (Hendricks and Boring 1999, Wilson et al. 2002, Boring et al. 2004), and fixation rates of certain species can be high (e.g. 0.02 g N_2 g^{-1} plant biomass; (Hiers et al. 2003a, Ament et al. 2018), our findings do not support this mechanism at the ecosystem scale.

There are at least two potential explanations for our findings. One is that the high frequency at which these managed forests burn is causing long-term depletion of ecosystem N storage. Indeed, across 51 study plots and two sites, fire appears to volatilize more N than N fixation and atmospheric deposition can feasibly replace in the short or long term. This raises a fundamental problem because even the most frequently-burned plots adhere to the fire regime that is thought to have influenced these forests historically (1-5 year FRI; (Van Lear et al. 2005). Such frequent fires are necessary to maintain the unique plant composition and open-canopy structure that defines longleaf pine communities (Chapman 1932, Walker and Peet 1984, Glitzenstein et al. 2003, Van Lear et al. 2005). In fact, even a mild reduction in fire frequency (~7 year FRI) can initiate a shift in overstory community dynamics to favor fire-inhibiting oak species (Loudermilk et al. 2011), and less than two decades of fire suppression can induce a state change to oak-dominated forests (Mitchell et al. 2006). We are therefore left with two apparently incompatible observations: that frequent fire is needed to maintain the historical integrity of the longleaf pine ecosystem, but it also causes long-term declines in ecosystem N pools which may decrease the system's ability to respond to future disturbances.

A second possibility is that the large annual N losses are not detrimental to the recovery of these ecosystems, but instead are critical for maintaining their oligotrophic status as they recover from several transitions of prior land use. Fire-suppressed forests that previously dominated the landscape likely accumulated large stocks of N in the O horizon (Varner et al. 2005), and mechanized site preparation (i.e. drum chopping) may have further increased organic N inputs to soils (Frazer et al. 1990, Li et al. 2003) prior to longleaf restoration. In this case, fire may be serving a critical role by removing excess N as longleaf pine savannas return to their oligotrophic state. Three lines of evidence from our work provide support for this idea. First, exceptionally high N mineralization rates (12.8 to 92.3 kg N ha^{-1} $year^{-1}$; Figure 19B) characterize our youngest stands, suggesting that these stands are indeed replete with N relative to mature stands of longleaf pine, which typically have the lowest rates of N mineralization among North American forests (Reich et al. 1997, Wilson et al. 1999, 2002). Second, N demand from tree growth seems unhindered by such large annual N losses and remains steady throughout maturity (Figure 19C), suggesting that these forests are not in a state of decline. Third, N addition in our field fertilization experiment did not increase tree growth rates (Figure 17). Collectively, these findings point to the ecosystem N imbalance as evidence of N sufficiency, rather than cause for concern that fire imposes unsustainable N losses.

Another unexpected result from our study was that the principal factor determining N fixation was site. Both legumes and soil crusts provided substantially more N fixation at Benning than at Eglin (Figure 6A), a surprising result considering the similarities of the soils, vegetation and climate. While the potential for these species to fix N is high when grown as seedlings under controlled conditions (Hiers et al. 2003b, Cathey et al. 2010, Ament et al. 2018), our field populations of legumes represent a range of life history stages (including long-lived perennials), and thus, these legumes may have lower N demands. Although legume N fixation was not related to any metric of N availability in our study, we cannot exclude the possibility that low rates of N fixation are a symptom of N-sufficiency in the ecosystem; several of these species down-regulate N fixation in response to N additions under controlled conditions (Ament et al. 2018). Further, we found that Benning exhibited lower N availability and higher legume N fixation, and the opposite was true at Eglin. Although N fixation can be upregulated when N supply is low, it may be ultimately constrained by the availability of P across the landscape (Vitousek and Howarth 1991). Indeed, our greenhouse study revealed evidence of P limitation of N fixation for these legumes grown in soil from our sites, particularly for Eglin soils (Figure 9) (Ament *et al.* 2018), and our field fertilization experiment revealed that P addition stimulated nodulation of legumes (Figure 12) and increased foliar P concentrations (Figure 13). By examining legume N fixation in relation to available soil P across all plots and sites, we found a strong positive correlation (Figure 16), further supporting our idea that legume N fixation is controlled by landscape-level patterns in soil P.

Although sandhill longleaf pine stands harbor a diversity and abundance of N-fixing organisms that persist throughout stand development, their N fixation rate is insufficient to balance N losses from fire. It is likely that fixation rates of legumes, crusts and asymbiotic bacteria were higher historically, prior to anthropogenic N inputs from deposition and land-use change. Frequent prescribed fire may be critical for returning these ecosystems back to an N-poor state. Therefore, our finding of an ecosystem N imbalance presents us with a fundamental challenge—the interaction of multiple disturbances (e.g. multiple land use changes, fire, and N deposition) may have created a novel N environment for longleaf pine ecosystems, which hinders our ability to assess its capacity to respond to a historical fire regime.

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