BIOLOGICAL NITROGEN FIXATION DYNAMICS DURING ECOSYSTEM RECOVERY IN LONGLEAF PINE SAVANNAS

by

JULIE ANNE TIERNEY

(Under the Direction of Nina Wurzburger)

ABSTRACT

Biological nitrogen fixation (BNF) can alleviate nitrogen (N) deficiencies that inhibit ecosystem recovery, yet how BNF achieves this under frequent fire disturbance is unclear. We investigated BNF's capacity to provide N to restored longleaf pine savannas, how it is organized and regulated over time, and how it responds to fire. We conducted this study in 54 1-ha plots of longleaf pine across gradients of stand age and fire frequency at two sites in the southeastern US. We determined BNF by three functional groups of N-fixers (legumes, soil crusts, and asymbiotic bacteria), and quantified components of the N cycle. We found that BNF was maintained over time, and fire enhanced BNF from legumes. Despite this, BNF was generally insufficient to balance N losses from fire. Unexpectedly, differences between the two sites described the most variation in BNF, highlighting that landscape-level drivers can have dramatic influences on BNF.

INDEX WORDS: Nitrogen fixation, Disturbance, Fire, Ecosystem recovery, Longleaf pine savannas

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B.S., The University of North Carolina at Asheville, 2013

A Thesis Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTERS OF SCIENCE

ATHENS, GEORGIA
2017

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August 2017

DEDICATION

This thesis is dedicated to Dorothy Oliver, who advised me to always paddle my own canoe.

ACKNOWLEDGEMENTS

I thank my advisor, Nina Wurzburger, who has been an invaluable mentor and role model over the past two years. I also thank my committee members, Joe O’Brien and Miguel Cabrera, for their guidance and logistical support, as well as Lars Hedin and Erik Hobbie for their contributions to the longleaf pine project. This project required many hands and minds, and I am grateful to everyone in the Wurzburger Lab: Andrés Barón-Lopez (who I thank especially for his hardcore field and lab skills, and for many long hours in the car listening to audiobooks), Mike Ament, Carly Phillips, Jeff Minucci, Kelsey Morton, Sumaya El-Khalidi, Tyler Ellison, Jenna Peissig, David Blount, and Breanna Walker. I would also like to thank the personnel at Fort Benning and Eglin Air Force Base, the staff at the Holiday Inn Express in Niceville, FL and Columbus, GA, the pancake machine at continental breakfast, La Mexicana de Columbus for post-field work tamales, and the Classic City Rollergirls for keeping me sane in Athens. Our field vehicle, Air Force One, also deserves my gratitude and respect. Finally, I would like to thank my family and friends for their endless support throughout graduate school, especially Carly Phillips, Paige Miller and Jake Syersak.

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CHAPTER 1

INTRODUCTION

The response of temperate forests to disturbance remains one of the greatest unknowns in the land carbon (C) sink (Gerber et al. 2013). This uncertainty results from our incomplete knowledge of how disturbance events influence transformations of nitrogen (N), which constrains C accretion during forest regeneration (Nave et al. 2014). Ecosystem recovery depends on the supply of mineral N, which is often dictated by the rate at which biological nitrogen fixation (BNF) introduces new N to the ecosystem (Rastetter et al. 2001). But how BNF achieves this in ecosystems prone to frequent fire is unclear.

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. 2013) have identified ecosystem-scale synchrony among forest regrowth, vegetation N demand and N inputs from BNF, such that BNF peaks at the time when biomass accumulates the most rapidly. Accordingly, BNF is often restricted to early stages of ecosystem development in temperate forests and is excluded over time (Cleveland et al. 1999). However, in ecosystems that experience frequent perturbations from fire, BNF may remain important as a source of new N over time and persist into late succession.

Fire-dependent longleaf pine (*Pinus palustris* Mill.) savannas of the southeastern US are a model system in which to study interactions among ecosystem recovery, fire and BNF. 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, with new plantations likely increasing by 4.6 million ha in the next 15 years (America's Longleaf 2009). 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 maintained 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, BNF) must balance outputs (i.e. leaching and gaseous losses) to overcome N deficiency 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 not been rigorously quantified.

BNF 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 BNF 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 BNF over time.

Longleaf pine savannas house a diversity of N-fixing organisms that may replenish N stocks after disturbance. However, the mechanisms that control the ability of 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), creating an ideal nutrient niche for BNF (Vitousek and Howarth 1991). However, the physical changes caused by fire may also serve to promote or suppress BNF. 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 BNF-capacity post fire. Fire may also reduce BNF 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 BNF of these three groups must be adequate to balance N losses from fire and meet N demands from vegetation growth.

Here, we examined BNF dynamics over the course of ecosystem recovery in longleaf pine savannas. We sought to understand the role of BNF in the context of the N cycle, and the mechanisms that control BNF's ability to replenish N after disturbance. We conducted this research at two sites in the southeastern US (Fort Benning, GA and Eglin Air Force Base, FL) and established study plots across gradients of age (2-227 years) and fire frequency (fire return intervals of 1.5 – 20 years). We hypothesized that:

(**1**) Ecosystem N inputs (from BNF and atmospheric deposition) will balance outputs (from fire disturbance).

(2) Over time, the available N supply will diminish relative to N demand such that it approaches a balance at stand maturity.

(**3**) BNF will respond to changes in N supply and demand over time, such that it will be the greatest in early stages of stand development, but will persist due to frequent N losses from fire.

(**4**) Fire will have differential effects on the three types of N-fixers such that more frequent fire may reduce BNF by asymbiotic fixers and biological soil crusts but increase BNF by legumes.

Understanding BNF's role in ecosystem recovery is particularly important in this vulnerable ecosystem, and is critical for isolating the mechanisms by which N controls the land C sink.

CHAPTER 2

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 for both sites is humid subtropical, with precipitation occurring throughout the year. The 30 year (1981-2010) average annual precipitation, average annual temperature, and average annual minimum and maximum temperatures for each site were: 1260 mm, 18.0 ˚C, 11.3 ˚C, 24.7 ˚C at Benning (NCDC 2017, station GHCND:USC00092159), and 1802 mm, 18.7 ˚C, 12.6 ˚C, 24.8 ˚C at Eglin (NCDC 2017, station GHCND:USC00086240).

Sandy, xeric entisols characterize both sites. Eglin is dominated by Lakeland sands (Rodgers and Provencher 1999), while soil series at Benning include mostly Lakeland sands (thermic, coated Typic Quartzipsamments) as well as Cowarts (fine-loamy, kaolinitic, thermic Typic Kanhapludults), Nankin (fine, kaolinitic, thermic Typic Kanhapludults) and Troup (loamy, kaolinitic, thermic Grossarenic Kandiudults) series (Dilustro et al. 2002). Both sites are the product of deep Cretacous 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). 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.

Prior to acquisition by the Department of Defense in the 1930's, land at both sites was primarily used for agriculture, silviculture and grazing (Frost 1993, Rodgers and Provencher 1999). Since then, management practices such as fire exclusion 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).

We established 54 1-ha plots of sandhill longleaf pine (24 plots at Fort Benning and 30 plots at Eglin AFB) 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.

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 and N_2 carrier gas (SRI Instruments, Torrance, CA, USA).

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), and incubated nodules for 30 minutes. Immediately following the incubation, we dried the nodules at 70 ˚C for 48 hours, ground them to a fine 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 (Costech 4010 Elemental Analyzer coupled to a Finnigan Delta Plus XP isotope ratio mass spectrometer). However, due to the small mass of the samples, the results of this analysis were unreliable. Instead, we used a conversion factor of 4.8 μmol C_2H_4 : μmol N_2, which was determined by a greenhouse experiment of the same species conducted by our lab group (Ament 2016).

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 BNF from 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).

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 2017), 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 μmol C_2H_4 : μmol N_2 (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).

N losses from fire

We estimated potential N losses from fire in both the soil O-horizon and the understory. To determine the ambient load 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).

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. 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.

Soil N measurements

We sampled mineral soils seasonally (March-April 2016, June-July 2015, September 2014, November-December 2015) 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 (Alpkem) at the University of Georgia Stable Isotope Laboratory (Furman and Walker 1976). 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.

Data analysis

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 BNF 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 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 (R package: gam; 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. Site-level means of the metrics used to calculate the available N balance are available in the appendix. 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 investigated BNF dynamics over stand age with linear models testing how the interaction of stand age and site affects BNF 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 BNF from each group. To determine how fire impacts BNF, we fit linear models testing how BNF 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 BNF between plots that experienced fire in the past year and plots that did not (R package: lme4; Bates et al. 2015), and performed multiple comparisons with a Tukey's post-hoc test (R package: multcomp; Hothorn et al. 2008).

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).

Table 1. Species of legumes sampled for N fixation assays at Fort Benning, GA and Eglin Air Force Base (AFB), Florida. Species common at both sites are indicated with an asterisk.

Fort Benning	**Eglin AFB**
Centrosema virginianum (L.)	*Chamaecrista fasciculata* (Michx.)*
Chamechrista fasciculata (Michx.)*	*Galactia regularis* (L.)*
Desmodium strictum (Pursh)	*Lupinus diffusus* (Nutt.)
Galactia regularis (L.)*	*Mimosa quadrivalvis* (L.)
Lespedeza hirta (L.)	*Rhynchosia cyticoides* (Bertol.)
Lespedeza virginica (L.)	*Rhynchosia reniformis* (DC)*
Rhynchosia reniformis (DC)*	*Stylosanthes biflora* (L.)
Tephrosia florida (F. Dietr.)	*Tephrosia chrysophylla* (Pursh.)
Tephrosia virginiana (L.)*	*Tephrosia virginiana* (L.)*

CHAPTER 3

RESULTS

Ecosystem N balance

Overall, the combined inputs from atmospheric deposition and BNF did not balance losses from fire. Total BNF at Benning (sum of legumes, soil crusts and asymbiotic fixers) was an order of magnitude higher than at Eglin (1.08 ± 0.15 and 0.18 ± 0.03 kg N ha^{-1} $year^{-1}$ respectively; Table 2), but these estimates were much smaller than inputs from atmospheric N deposition (7 kg N ha^{-1} $year^{-1}$). When we subtracted plot-level N losses from N inputs, we observed an overall N deficit throughout the chronosequence, but this balance was highly variable over stand age ($\beta = 0.00$, $p = 0.68$; Fig. 1A).

Total ecosystem N losses were driven by the volatilization of N in the O-horizon, which was generally an order of magnitude higher than estimated losses from ground-layer vegetation (means ± standard errors of 88.3 ± 12.2 and 8.4 ± 0.5 kg N ha^{-1}, respectively). On average, 81.2 ± 3.2% of N in the O-horizon was lost from fire. The amount of N volatilized during a single fire event did not differ between sites ($F_{1,47} = 0.004$, $p = 0.95$; data not shown), however, the higher fire frequency at Benning resulted in greater average annual losses.

Although the means for ecosystem N balance indicated that both sites were deficient in N, Benning's deficiency was larger than Eglin's ($F_{1,48} = 1.33$, $p = 0.006$). It is possible that inputs of N balance losses at Eglin: the upper 95% confidence interval (CI) on the mean N balance at this site barely surpassed zero (1.1 kg N ha^{-1} $year^{-1}$; Fig. 1B). However, because of the magnitude of the N deficiency at Benning, we can infer that stands at this site progressively lose N (upper 95% CI on the mean = -21.6 kg N ha^{-1} $year^{-1}$, Fig. 1B).

Despite this deficiency in the ecosystem N balance, mineral soils at Benning stored significantly more N than Eglin ($F_{1,51} = 5.52$, $p = 0.02$; Fig. 2A), and these stocks decreased over stand age at both sites ($\beta = 0.22$, $p = 0.003$). However, the fraction of total ecosystem N lost annually to fire was twice as large at Benning than Eglin (means ± standard error of 0.16 ± 0.04 and 0.08 ±0.02 %; $F_{1,49} = 4.20$, $p = 0.05$; Fig. 2D). Although fire volatilized less than 1% of total ecosystem N stocks annually, this can lead to the depletion of N stocks over time if N inputs are not sufficient to balance losses.

N supply and demands

We found that N supply was generally sufficient to meet N demands (overall mean balance ± 95% CI = 14.78 ± 6.89), but this balance decreased with stand age ($\beta = 0.01$, $p = 0.005$). Furthermore, we observed a significant site by stand age interaction where N became deficient as stands mature at Benning ($\beta = 0.11$, $p = 0.01$; Fig. 2D), suggesting intensifying N limitation over time. However, N supply consistently exceeded N demand at Eglin (Fig. 2D).

N demand from tree growth peaked at an intermediate stand age and did not differ between sites ($R^2 = 0.38$, Fig. 2A). Understory N demand decreased over the chonosequence ($\beta = 0.11$, $p << 0.001$), but more sharply at Benning ($\beta = 0.18$, $p < 0.001$; Fig. 2B) than Eglin. Overall, mean understory N demand did not differ between sites ($F_{1,52} = 1.23$, $p = 0.80$).

We observed considerable variation in average annual N mineralization rates, which ranged from 0.8 to 92.3 kg N ha^{-1} $year^{-1}$ across plots. Trends in net N mineralization decreased with stand age at both sites ($\beta = 0.05$, $p << 0.001$), however, N mineralization declined more sharply with age at Benning than Eglin ($\beta = 0.21$, $p = 0.002$; Fig. 2C). Mean N mineralization rates marginally differed between sites ($F_{1,52} = 3.66$, $p = 0.061$), with average rates of 30.7 ± 4.3 and 38.1 ± 4.5 kg N ha^{-1} $year^{-1}$ at Benning and Eglin, respectively. Furthermore, soil DIN pools were greater at Eglin ($F_{1,53} = 18.70$, $p < 0.001$; Fig. 2B).

BNF dynamics throughout stand development

Surprisingly, we observed no effect of stand age on total BNF contributions (Table 3, Fig. 3A), but instead observed a dramatic site difference where Benning supported more BNF than Eglin (Table 3, Fig. 3B). The relative contributions of each functional group to total BNF also differed between sites. Total BNF was driven by legume BNF at Benning and asymbiotic BNF at Eglin (Table 2).

N fixation by herbaceous legumes remained constant over stand age (Table 3, Fig. 3C). We observed a dramatic difference in legume BNF between sites, which accounted for 62% of the variance in the linear model between age and site (Table 3). Only 3.3% of legumes surveyed at Eglin possessed nodules ($n = 1070$) compared to 47.3% at Benning ($n = 400$).

Estimates of N fixation rates from ARAs and field censuses indicate that legumes contribute an order of magnitude more N at Benning than at Eglin (Table 2, Fig. 3D).
Soil crusts contributed significantly more BNF at Benning (Table 3, Fig. 3F). However, this group contributed an order of magnitude less BNF than from legumes at both sites (Table 2). BNF estimates from soil crusts exhibited a strong site-by-stand age interaction (Table 3) where Benning supported elevated levels of BNF from soil crusts in juvenile stands. But, these BNF rates exponentially decreased as stands matured (Fig. 3E).

BNF from asymbiotic bacteria mildly decreased with stand age, but did not differ between sites (Table 3, Figs. 3G and 3H). The decrease in BNF from this group over stand age was driven by a similar decrease in per-mass nitrogenase activity rates (data not shown).

Surprisingly, we found that asymbiotic bacteria contributed over 4 times more fixed N than legumes at Eglin (Table 2).

Drivers of BNF

Soil N dynamics (N mineralization rates, total DIN pool, and balance of N supply and demand) did not affect BNF contributions from any group of N fixer ($p > 0.05$ for all). However, fire dynamics promoted legume BNF on both short- and long-term timescales. Because BNF from legumes was so different between Eglin and Benning, we split the data by site to better discern how fires drive patterns of legume BNF. At Benning, FRI had a significant negative effect on legume BNF ($R^2 = 0.19$, $F_{1,21} = 6.21$, $p = 0.021$; Fig. 4), but the effect of time since fire (months) was not significant. Neither time since fire nor FRI had a significant effect on legume BNF at Eglin. However, at both sites, we found that plots that had experienced a fire event in the year preceding the censuses supported significantly more BNF from legumes than plots that had not burned ($F_{1,53} = 16.80$, $p << 0.001$; Fig. 5). Legumes were the only group of N fixer that responded to fire dynamics. We did not observe an effect of FRI nor time since fire on BNF from biological soil crusts or asymbiotic bacteria ($p > 0.05$).

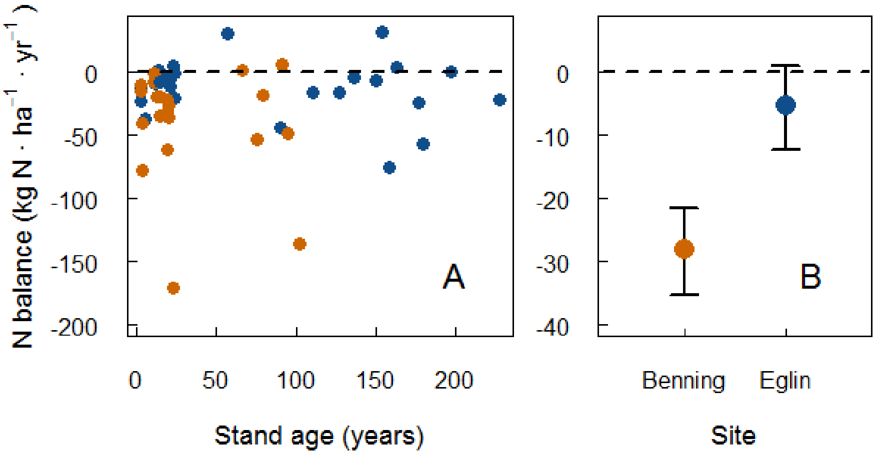

Figure 1. A) Ecosystem balance of N inputs (total BNF and atmospheric deposition) and N outputs (volatilization of ground-layer vegetation and O-horizon N) (kg N ha^{-1} yr^{-1}) across stand age (years) and B) site-level means and 95% confidence intervals of the ecosystem balance of N inputs and outputs at Fort Benning and Eglin AFB. Benning is represented in orange and Eglin is represented in blue, and the dashed lines indicate an equilibrium N balance of 0.

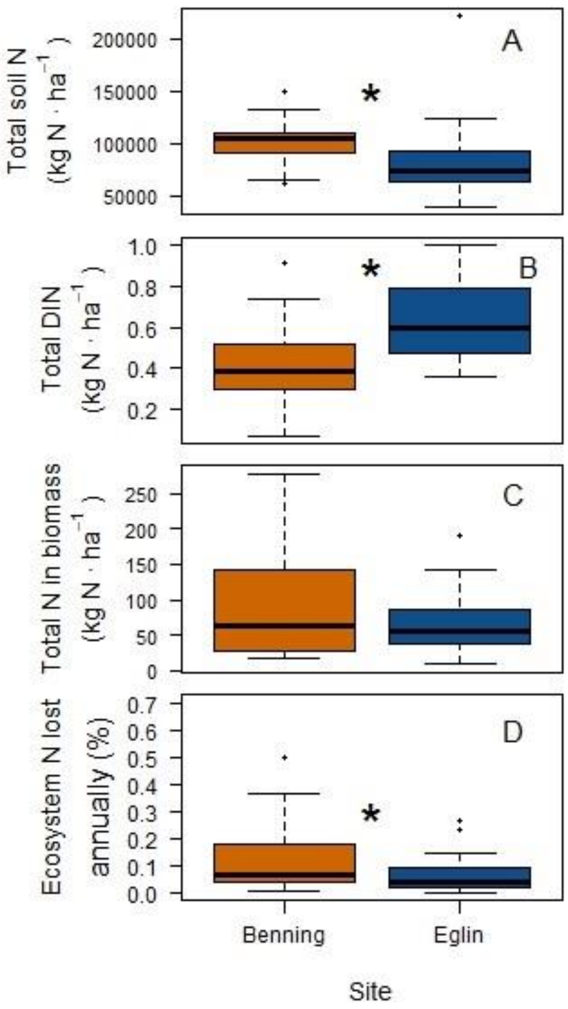

Figure 2. Ecosystem stocks of A) total soil N, B) total DIN, C) vegetation biomass N and D) the fraction of total ecosystem N stocks lost annually to fire at Fort Benning and Eglin AFB. Boxplots represent median and quartiles, and significant differences (α = 0.05) between sites are indicated with an asterisk.

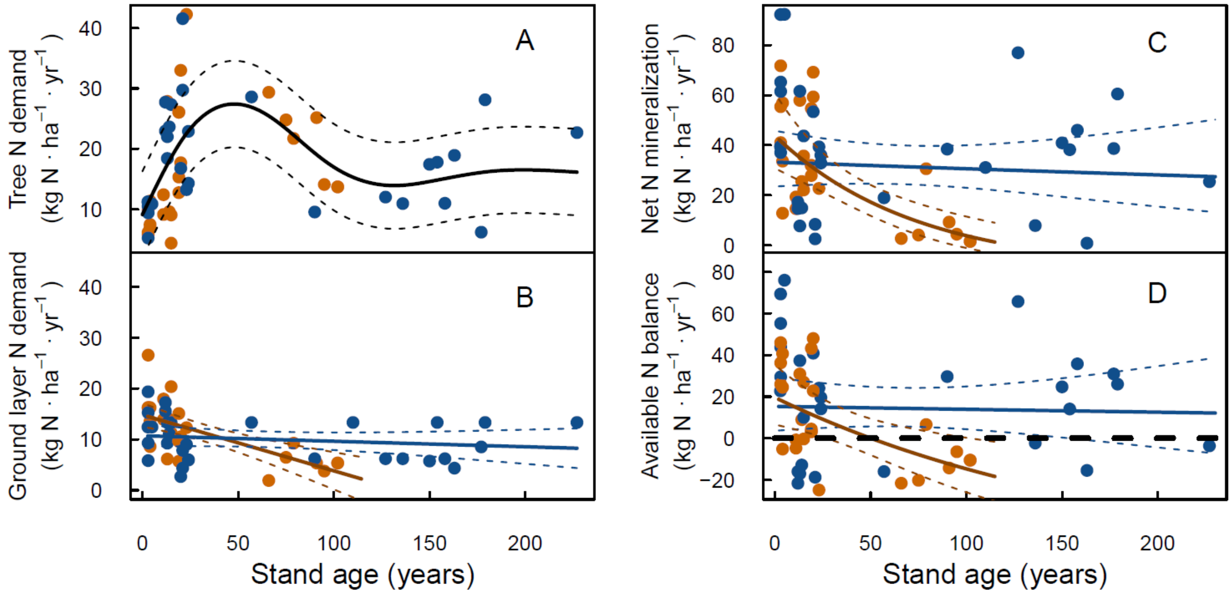

Figure 3. Nitrogen demand (kg N ha^{-1} yr^{-1}) from A) tree growth, including aboveground, fine root and litter production and B) ground-layer annual growth; C) net N mineralization (NH_4^+ and NO_3^-) (kg N ha^{-1} yr^{-1}) and D) the available N balance (N mineralization + N deposition – N demand from biomass growth, kg N ha^{-1} yr^{-1}) across stand age (years) at Eglin AFB (blue) and Fort Benning (orange). Lines indicate model predictions and 95% confidence intervals (A: R^2 = 0.39; B: R^2 = 0.25, $F_{3,52}$ = 7.27, $p < 0.001$; C: R^2 = 0.26, $F_{3,52}$ = 7.59, $p < 0.001$; D: R^2 = 0.12, $F_{3,52}$ = 3.41, $p = 0.02$). The black dashed line in (D) indicates an available N balance of 0.

Table 2. Site-level means ± standard errors of BNF inputs from legumes, soil crusts and asymbiotic bacteria at Fort Benning and Eglin AFB.

BNF inputs	Site	
	Benning	Eglin
Legumes	0.93 ± 0.15	0.039 ± 0.024
Soil crusts	0.022 ± 0.006	0.0025 ± 0.0008
Asymbiotic	0.12 ± 0.02	0.16 ± 0.03
Total	1.08 ± 0.15	0.18 ± 0.03

Table 3. Results from linear models evaluating BNF (kg N ha^{-1} yr^{-1}) from legumes, soil crusts and asymbiotic bacteria across stand age and between sites at Fort Benning and Eglin AFB, including partial correlation coefficients (β), p values from t-tests (stand age, continuous) and F-tests (site, categorical) for explanatory variables. Significant models and variables (at $\alpha = 0.05$) are shown in bold, and non-significant (n.s.) interactions were removed from models.

BNF	Stand age		Site		Stand age*Site		Model		
	β	p	β	p	β	p	R^2	$F_{2,52}$	p
Legumes	0.00	0.54	0.62	**<<0.001**	n.s.	n.s.	0.61	**43.76**	**<<0.001**
Soil crusts	0.35	**<<0.001**	0.09	**<0.001**	0.21	**<<0.001**	0.63	**31.68**	**<<0.001**
Asymbiotic	0.10	**0.005**	0.04	0.10	n.s.	n.s.	0.12	**4.63**	**0.01**
Total	0.09	0.54	0.47	**<<0.001**	n.s.	n.s.	0.55	**34.17**	**<<0.001**

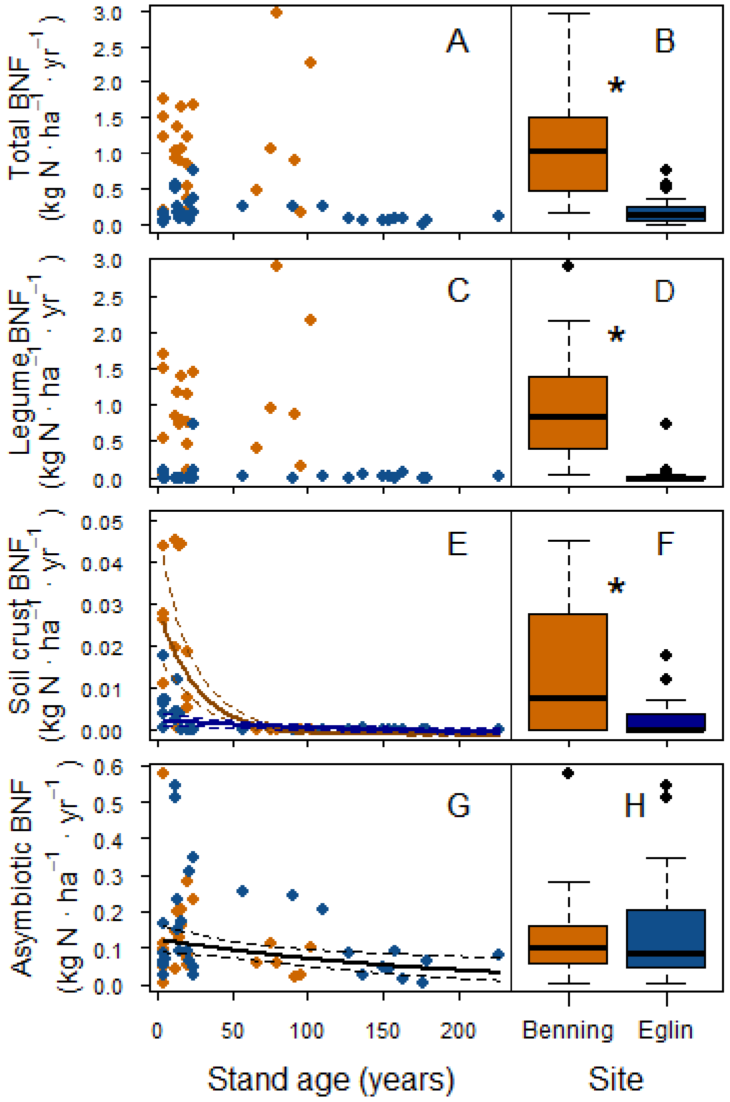

Figure 4. A) Total BNF (kg N ha^{-1} yr^{-1}) (contributed by legumes, soil crusts, and asymbiotic bacteria) over stand age and B) between sites; C) BNF contributed by legumes over stand age and D) between sites; E) BNF contributed by soil crusts over stand age and F) between sites; BNF contributed by asymbiotic bacteria G) over stand age and H) between sites at Fort Benning (orange) and Eglin AFB (blue). Lines represent model predictions and 95% confidence intervals. Boxplots represent median and quartile estimates, and differences in means at $\alpha = 0.05$ are indicated with an asterisk.

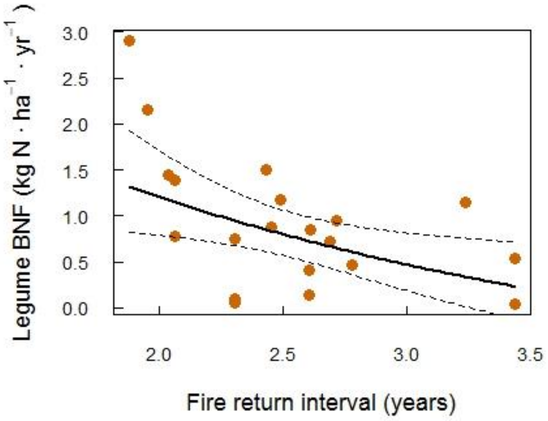

Figure 5. Relationship of legume BNF (kg N ha^{-1} yr^{-1}) with fire return interval (years) at Fort Benning. Lines indicate model predictions and 95% confidence intervals.

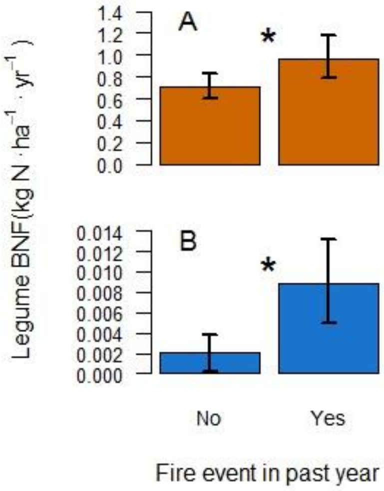

Figure 6. Mean estimates of legume BNF (kg N ha^{-1} yr^{-1}) in plots that had not burned and plots that had burned in the year preceding the censuses in 2015 and 2016 at A) Fort Benning and B) Eglin AFB. Asterisks indicate a significant difference at $\alpha = 0.05$, and bars represent standard errors.

CHAPTER 4

DISCUSSION

We investigated how BNF serves as a mechanism of ecosystem recovery in longleaf pine savannas, and specifically, how BNF is organized over time in the context of land use change and fire. Although longleaf pine ecosystems contain a high abundance and diversity of organisms capable of fixing N, we found that N losses from fire generally exceeded N inputs from BNF. Furthermore, we found that BNF was relatively constant throughout stand development, despite the paradigm that BNF is greatest in early stages of ecosystem recovery (Cleveland et al. 1999, Vitousek et al. 2013, Gerber et al. 2013). Unexpectedly, we observed strong site differences in N cycling and BNF dynamics, despite similarities in soil, vegetation and land-use history. Our findings highlight the importance of landscape-level differences in parent material and land-use history that may determine how BNF facilitates ecosystem recovery.

Methodological considerations

Many difficulties arise in describing the N economy of an ecosystem, not only from identifying all potential stocks and fluxes, but also from compounded measurement uncertainty. For example, our calculations of the balance of N supply and demand are estimated from buried-bag incubations and KCl extractions, which serve as a proxy for N mineralization rates and tend to over-estimate DIN concentrations (Wilson et al. 2002) yet under-estimate rhizosphere-induced N mineralization (Herman et al. 2006). Furthermore, our estimates of N fixation from legumes may be conservative despite the large sample size. Although we attempted to extract the entire root systems of individuals, the belowground systems of these perennial legumes are extensive and deep, and it is possible that we did not completely represent nodulation. Our estimates of ecosystem N losses may also be conservative because we do not account for N leaching, which may be considerable due to the sandy soils and frequent precipitation. N can also be lost through denitrification, but this flux is likely meager in xeric savannas (Sanhueza and Crutzen 1998).

Finally, there are temporal and spatial discrepancies in our sampling strategy. Many of the measurements used to construct the N balance were taken in two to four 100 m^2 subplots within the 1-hectare plots, and this area may not have been adequate to capture ecosystem variability or burn heterogeneity (Hiers et al. 2009). This study provides estimates of N losses from 73 fire events (from 2014-2017) across the 54 plots, but environmental variables such as weather and soil moisture at the time of burning as well as the time passed since the previous fire can result in heterogenous fire effects across the landscape and between individual burning events (Hiers et al. 2009, Whelan et al. 2013). Furthermore, although seasonal measurements were used to estimate N mineralization, the weak temporal resolution of these measurements may have prevented us from detecting any influence of N availability on BNF. Nonetheless, these measurements and back-of-the-envelope calculations reveal important patterns in the N cycle. Attempts to quantify the N budgets of fire-dependent ecosystems are rare and provide contradictory results (Cook 1994, Sanhueza and Crutzen 1998, Boring et al. 2004, López-Hernández et al. 2006, López-Hernández 2013), and this research contributes to our understanding of N cycling in these ecosystems, and how they recover from disturbance.

Ecosystem N balance

Interestingly, we found N losses from fire generally exceeded the combined N inputs from BNF and atmospheric deposition. Although fires can volatilize significant portions of ecosystem N stocks (Boring et al. 2004, Lavoie et al. 2010), at steady-state, inputs of N from

BNF and atmospheric deposition should compensate for these losses. In fact, N fixation from herbaceous legumes are often invoked as a mechanism for N replacement after fire in these ecosystems (Hendricks and R. Boring 1999, Wilson et al. 2002, Boring et al. 2004), but our findings do not support this assumption.

Benning incurred larger annual N losses than Eglin (Fig. 1B). The amount of N lost from a single fire event was similar between sites (overall mean of 99 kg N ha^{-1}), but average annual N losses were higher at Benning where plots burned more frequently (mean± se FRI of 2.5 ± 0.1 years, compared with 6.2 ± 1.1 at Eglin). N inputs at this site were also greater: Benning supported six times more BNF than Eglin (Table 2). Proportionally, however, N inputs compensated for more N losses at Eglin. On average, N fixation and atmospheric N deposition replaced 58% of N lost due to fire annually at Eglin but only 47% at Benning.

That longleaf pine savannas at Benning incur progressive N loss suggests that N storage at this site may be unstable over the long term (McLauchlan et al. 2014). In support of this idea, we found that Benning loses more N annually in proportion to total ecosystem N stocks (Fig. 2D), and total soil N declines over time at both sites. Curiously, total soil N stocks are larger at Benning (Fig. 2A). Although Benning loses more N annually, differences in climate and soil texture may slow microbial mineralization of soil organic matter (Burke et al. 1997, Six et al. 2002). Still, the higher frequency of fire at this site appears to volatilize more N than BNF and atmospheric deposition can feasibly replace.

The general management protocol of longleaf pine calls for a high frequency of prescribed burning (1-5 year FRIs) that mimic historical fire regimes and promote a floristically diverse understory and an open-canopy structure (Chapman 1932, Walker and Peet 1984, Glitzenstein et al. 2003, Van Lear et al. 2005), but our data suggest that these high fire frequencies may lead to the instability of ecosystem N stocks in the long term. While the vegetative communities of longleaf pine savannas are resilient to, and evolved with, frequent fire disturbance, our results imply a potential decoupling of vegetative and biogeochemical resilience in response to frequent fire (Smithwick 2011). It is also possible that the large annual N losses at Benning ensure the oligotrophic status that these ecosystems are adapted to. Indeed, both fire exclusion and N additions can decrease species richness in longleaf pine savannas (Kirkman et al. 2016). However, if such progressive N losses continue over time, these high FRIs may exhaust N stocks in the long term and result in decreased resilience to subsequent disturbances (Garten and Ashwood 2004, Garten 2006).

N supply and demand

We observed divergent temporal patterns of N supply and demand between sites (Fig. 3D). At Eglin, the balance between N supply and demand remained positive and relatively consistent throughout stand development, indicating that there may be surplus mineral N relative to demands at this site. At Benning, there was surplus available N in early stages of development, but as stand age progressed, N supply declined more relative to demands. In fact, our estimates indicate that there is not sufficient available N to meet demands from biomass growth in mature stands (> 60 years old) at Benning.

Although this negative balance initially seems improbable, similar conclusions have been reached from analogous methods in mature longleaf pine ecosystems (Wilson et al. 2002) and other xeric savannas (López-Hernández 2013). A negative balance of N supply and demand indicates that plants may be able to access N that is not measured by our methods, such as deep sources of mineral N (Wilson et al. 2002) or rhizosphere-induced N mineralization (Herman et al. 2006). Furthermore, longleaf pine trees may exploit organic sources of N to supplement their demands. These trees associate with ectomycorrhizal fungi, which can extract mineral N directly from soil organic matter and may contribute substantially to C and N cycling in these ecosystems (Hendricks et al. 2016).

The available N balance was driven by patterns in N mineralization (Figs. 2C and 2D). N mineralization was higher at Eglin, but these rates declined over stand age at both sites (Fig. 2C). The elevated rates of N mineralization in juvenile stands likely results from a higher availability of substrate from standing detritus in plantations that have been previously clear-cut (Frazer et al. 1990, Li et al. 2003). N mineralization rates sharply declined over stand age at Benning, while the higher rates of N mineralization persisted over time at Eglin. Climatic differences between sites could promote more rapid N cycling at Eglin, where elevated temperature and moisture may increase microbial activity (Burke et al. 1997). Despite the higher N availability at Eglin, N demand from tree growth did not differ between sites (Fig. 2A), suggesting that tree growth is not limited by N. Indeed, increased N availability does not necessary increase aboveground net primary productivity (ANPP) in these ecosystems (Mitchell et al. 1999), and N additions over a decade in another longleaf pine savanna produced no change in overstory ANPP (Kirkman et al. 2016).

Although N limitation can manifest from competition for mineral N, it can be maintained by the loss of organic N (Hedin et al. 1995, Menge 2011). Here, we see the progressive loss of organic N from fire disturbance at Benning reflected in the declining availability of mineral N over time (Figs 1B and 2D). If BNF tracks patterns of N availability and demands over time and space, we would expect BNF to be higher at Benning and increase with stand age. Although we did find Benning to support more BNF, it doesn’t appear to be sensitive to changes in N availability over time. Instead, BNF may ultimately be regulated by landscape-level differences in P availability.

BNF dynamics

In temperate forests, BNF is typically highest in early phases of secondary ecosystem development (Cleveland et al. 1999, Rastetter et al. 2001, Gerber et al. 2013). We hypothesized that BNF would peak early on in stand development and be maintained over time due to chronic N losses from fire. Contradicting our expectations, we found no relationship with total BNF and stand age at either Benning or Eglin (Fig. 3A). However, BNF from legumes, soil crusts and asymbiotic bacteria exhibited distinct patterns over time and between sites.

BNF from legumes remained constant over stand age (Fig. 3C). These legumes were abundant in all stages of stand development, contrary to most other temperate forests where symbiotic N fixing plants grow exclusively in young stands (Cleveland et al. 1999). Furthermore, the abundance of legumes even in the youngest plantations (2 – 5 years old) suggests that these plants can establish quickly after land use change disturbance. Although some species of legumes may be more vulnerable to disturbance (Kirkman et al. 2004), our results demonstrate that their functional contribution to nutrient cycling remains relatively constant throughout ecosystem recovery.

Legumes are assumed to have a significant role in N cycling in longleaf pine savannas because they are ubiquitously distributed across the landscape (Hainds et al. 1999) and demonstrate a high capacity for N fixation (Hiers et al. 2003). BNF from these legumes have been estimated at 7-9 kg N ha^{-1} year^{-1} in more mesic longleaf pine types (Hendricks and R. Boring 1999), though previous estimates of legume BNF at Benning have been as low as 0.077 kg N ha^{-1} year^{-1} (converted from 2.648 g N m^{-2} h^{-1}) (Lajeunesse et al. 2006). Our plot-level estimates of legume BNF ranged from 0 – 3 kg N ha^{-1} year^{-1}, with site-level means of 0.93 and 0.039 kg N ha^{-1} year^{-1} at Benning and Eglin, respectively. Although legumes are abundant in sandhill longleaf pine systems, their capacity for fixation may be lower relative to other soils with more water-holding capacity. Water stress has been shown to be the primary factor controlling N fixation of legumes in longleaf pine savannas (Hendricks and R. Boring 1999). Other studies that have demonstrated high N fixation potential for these species have been from seedlings grown under controlled conditions (Hiers et al. 2003, Cathey et al. 2010, Ament 2016), where experimental plants had ample access to water. Furthermore, these greenhouse and garden experiments only assessed the N fixation potential of seedlings, whereas we assessed plants in all stages of their life histories. BNF may be more active in seedlings of perennial herbaceous legumes compared to older individuals, which can live up to 15 years.

Although soil disturbance can have long-term negative impacts on communities of soil crusts (Evans and Belnap 1999, Ferrenberg et al. 2015), we found these organisms were most abundant and fixed the most N in juvenile stands recovering from recent land use change, and these BNF inputs decreased exponentially over stand age (Fig. 3E). The high-light environment and greater area of interplant spaces typical of juvenile plantations may provide ideal growing conditions for these organisms (Belnap 2002), and litter accumulation may exclude soil crusts as stands age. The presence of N fixing soil crusts in longleaf pine savannas has been previously acknowledged (Wilson et al. 2002), yet this is the first study that quantifies BNF from this group. Soil crusts have been studied mostly in desert ecosystems, where they contribute significantly to nutrient cycling and can fix up to 13 kg N ha^{-1} year^{-1} (Belnap 2002). Although we found that soil crusts were actively fixing N, their contributions to the N cycle in this ecosystem were minimal.

At their highest abundance, we estimated them to fix only 0.004 kg N ha^{-1} $year^{-1}$, with inputs approaching zero in stands >20 years of age.

BNF from asymbiotic bacteria in the O-horizon gradually decreased with stand age (Fig. 3G), consistent with previous research in temperate forests (Distefano and Gholz 1989, Son 2001). This pattern was driven by a decrease in mass-based nitrogenase activity over stand age, possibly due to increased substrate quality in younger stands. On average, we estimated these asymbiotic bacteria to fix about 0.15 k g N ha^{-1} $year^{-1}$. While we only present estimates of BNF in the soil O-horizon, asymbiotic bacteria capable of BNF are ubiquitous in terrestrial ecosystems (Reed et al. 2011). Asymbiotic BNF may also be substantial in the mineral soil (Distefano and Gholz 1989) or in endophytic bacteria that inhabit pine needles (Moyes et al. 2016).

Unexpectedly, the principal factor determining BNF was site. Both legumes and soil crusts provided substantially more BNF at Benning than at Eglin (Figs. 3D and 3F). Indeed, a previous greenhouse study found that legumes grown in Benning soil exhibited enhanced growth and N fixation rates compared with those grown in Eglin soil (Ament 2016). Such strong differences were surprising considering the similarities of the soils and vegetation at these sites. However, these differences could be driven by landscape-level characteristics such as parent material (Peet 2006). Specifically, these factors can determine the availability of P, which stimulates the growth and fixation of legumes found at these sites (Ament 2016), and can benefit communities of soil crusts (Schmidt et al. 2012).

Drivers of BNF

Although we observed distinct patterns in BNF over stand age and between sites, the direct mechanisms that regulate BNF in these three functional groups were more obscure. While there is evidence that additions of mineral N decrease growth and BNF of legumes typical of these study sites (Ament 2016), we could not statistically detect a pattern between N availability and BNF in any group of N-fixer. Instead, these patterns were more apparent at the site-level: Benning exhibited low N availability and high BNF, and the opposite was true at Eglin. Although BNF can be upregulated when N supply is low, it may ultimately be constrained by the availability of P across the landscape (Vitousek and Howarth 1991). In this case, P availability, driven by differences in parent material (Peet 2006), may be more important in dictating patterns of BNF.

That fire promotes legume abundance and BNF in longleaf pine savannas is a widely-held assumption by both researchers and land managers (Hiers and Mitchell 2007). Confirming these expectations, we observed long-term effects of fire return interval and short-term effects of a single fire event on BNF. At Benning, plots that burned more frequently supported more BNF from this group (Fig. 4). However, we did not observe a pattern of FRI and legume BNF at Eglin. Generally, legumes fixed a negligible amount of N at this site (over half the plots did not support any BNF by legumes), so it is not surprising that patterns of legume BNF across the gradient of fire frequency were undetectable. Yet, short-term effects of fire disturbance on legume BNF were present at both sites. When we compared plots that had burned in the year preceding the assays to those that did not, we found that plots that had burned supported significantly more legume BNF (Fig. 5). A single fire event can substantially stimulate populations of legumes (Hendricks and Boring 1999), which may gain a competitive advantage in post-fire communities (Hiers and Mitchell 2007). Regular burning has been shown to maintain populations of herbaceous legumes in southeastern forests (Robbins and Myers 1992), however, the effects of a single fire on these populations may be short-lived (Hendricks and Boring 1999).

Fire frequency did not affect BNF inputs from soil crusts or asymbiotic bacteria. Although fire has been shown to negatively impact communities of soil crusts (Hilty et al. 2004), the high light environment engineered by fire disturbance may benefit these organisms. Further, these organisms may benefit from post-fire deposits of mineral P (Carter and Foster 2004, Schmidt et al. 2012). While we expected that greater frequency of fire would decrease O-horizon mass and thereby asymbiotic N fixation, both remained static over the gradient of fire frequency. Regardless of chronic perturbations from fire, these N-fixing microbes proved to be a reliable source of new N at both sites.

Conclusions

Although sandhill longleaf pine savannas harbor a diversity and abundance of N-fixing organisms, their ability to balance N losses from fire depends on site, and may be insufficient to promote sustainable recovery from land use change. While theory points to BNF as a mechanism for replenishing N losses (Vitousek and Howarth 1991), what allows for the maintenance of BNF over time but prevents it from alleviating N depletion in these ecosystems? We found that BNF was generally insensitive to within-site changes in N availability, and landscape-level drivers of BNF (i.e. P availability) need further investigation to understand controls of BNF in longleaf pine savannas.

Frequent fire maintains the structure of these ecosystems and provides habitat for endangered species (Kirkman et al. 2006), and fire exclusion for as little as 4 years can decrease biodiversity and allow for hardwood encroachment (Way 2006). However, these chronic perturbations may undermine the biogeochemical resilience of longleaf pine savannas also recovering from land use change disturbance. One destructive disturbance can substantially decrease the ability of ecosystems to recover from subsequent disturbances, and their interactions can alter ecosystem function in synergistic ways (Paine et al. 1998, Simard et al. 2011, Buma 2015). As such, the combination of land use change and frequent fire may trigger cascading interactive disturbance effects, with the potential to decrease ecosystem resilience and even initiate retrogression (Smithwick 2011, McLauchlan et al. 2014). After a single disturbance event, N stocks in temperate forests generally recover to pre-disturbance levels after a few decades (Maloney et al. 2008, Smithwick et al. 2009). However, if N fixation is so limited that it cannot replace losses, such disturbances can trigger a decrease in long-term N stocks and constrain the extent of C storage (McLauchlan et al. 2014). Our research highlights how the interaction between land use change and fire can shape the recovery of longleaf pine savannas, however, the potential mechanisms by which these disturbances interact to affect biogeochemical cycling warrants further investigation.

Longleaf pine savannas can exhibit vastly different biogeochemical functionality across the landscape, and management strategies may need to consider these differences to promote sustainable ecosystem recovery. Furthermore, the interactive effects of land use change and fire disturbance may provide novel challenges to remediating biogeochemical cycling in recovering ecosystems, and how these disturbances interact to constrain BNF may have cascading effects on ecosystem resilience and the land C sink.

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APPENDIX

Appendix 1. Table of site-level means (± standard errors) of metrics used in the calculation of the available N balance (kg N ha^{-1} $year^{-1}$): net N mineralization, atmospheric N deposition (dry + wet)[†], aboveground overstory N demand, overstory litter N demand, fine root N demand, and understory N demand. Site-level means are derived from plot (1 ha) means at Fort Benning (*n* = 24) and Eglin AFB (*n* = 30), and all units are kg N ha^{-1} $year^{-1}$.

	Benning	Eglin
N supply		
Net N mineralization	30.7 (4.3)	38.4 (4.6)
Atmospheric N deposition[†]	7.0	7.0
N demand		
Aboveground overstory production	3.1 (0.5)	1.9 (0.3)
Overstory litter production	9.1 (1.4)	8.9 (0.9)
Fine root production	4.6 (0.5)	7.2 (0.7)
Understory production	11.2 (1.1)	9.9 (0.8)

† Data obtained from National Atmospheric Deposition Program (2017). Standard errors not available.