Successional dynamics of nitrogen fixation and forest growth in regenerating Costa Rican rainforests

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Abstract. Regenerating tropical forests have an immense capacity to capture carbon and harbor biodiversity. The recuperation of the nitrogen cycle following disturbance can fuel biomass regeneration, but few studies have evaluated the successional dynamics of nitrogen and nitrogen inputs in tropical forests. We assessed symbiotic and asymbiotic nitrogen fixation, soil inorganic nitrogen concentrations, and tree growth in a well-studied series of five tropical forest plots ranging from 19 yr in age to old-growth forests. Wet-season soil inorganic nitrogen concentrations were high in all plots, peaking in the 29-yr-old plot. Inputs from symbiotic nitrogen fixation declined through succession, while asymbiotic nitrogen fixation peaked in the 37-yr-old plot. Consequently, the dominant nitrogen fixation input switched from symbiotic fixation in the younger plots to asymbiotic fixation in the older plots. Tree growth was highest in the youngest plots and declined through succession. Interestingly, symbiotic nitrogen fixation was negatively correlated with the basal area of nitrogen-fixing trees across our study plots, highlighting the danger in using nitrogen-fixing trees as a proxy for rates of symbiotic nitrogen fixation. Our results demonstrate that the nitrogen cycle has largely recuperated by 19 yr following disturbance, allowing for rapid biomass regeneration at our site. This work provides important insight into the sources and dynamics of nitrogen that support growth and carbon capture in regenerating Neotropical forests.

Key words: asymbiotic nitrogen fixation; forest regeneration; Neotropics; nitrogen; succession; symbiotic nitrogen fixation; tropical forests.

Introduction

Increases in human land-use change in the tropics over the last half-century have dramatically increased the global extent of regenerating tropical secondary forests (Houghton 1994, Taubert et al. 2018) such that secondary forests now make up more than one-half of the world's tropical forests (FAO 2010). Secondary tropical forests are increasingly being recognized for their critical role in capturing carbon (Pan et al. 2011, Poorter et al. 2016), cycling water and nutrients (Powers and Marín-Spiotta 2017), harboring biodiversity (Finegan 1996, Chazdon et al. 2009), and supporting local economies (Brown and Lugo 1990). The ability of tropical secondary forests to serve these roles is largely dependent on their regeneration rates, but the controls over tropical forest regeneration are not well understood.

In addition to the effects of previous land use (Powers and Marín-Spiotta 2017) and landscape context (Chazdon et al. 2007), tropical forest regeneration is often limited by the availability of soil nutrients, especially nitrogen (N; Erickson et al. 2001, Davidson et al. 2004, 2007, Davidson and Martinelli 2009, Batterman et al. 2013a). Although primary tropical forests are typically thought to be relatively N rich (Hedin et al. 2009, Brookshire et al. 2012), substantial N losses during vegetation clearing and land use (Kauffman et al. 1995, McGrath et al. 2001, Davidson et al. 2007) can result in low N availability early in tropical secondary succession. Despite the recognition that N dynamics are an important potential control over tropical forest regeneration rates, characterizing these N dynamics has been hampered by the large biogeochemical heterogeneity across secondary tropical forests (Townsend et al. 2008) and the limited, but growing (Powers and Marín-Spiotta 2017), number of studies on the subject.

One of the main ways N is brought back into regenerating tropical forests is via biological N fixation: the

conversion of N_2 gas into bioavailable forms, which occurs asymbiotically by free-living bacteria, symbiotically by certain legumes and their endosymbiotic bacteria, and in other symbioses as well. Symbiotic N fixation (SNF) from legumes and their endosymbiotic bacteria has an exceptionally large potential to bring new N into tropical forests, with measured rates of SNF exceeding 100 kg N·ha^{-1}·yr^{-1} (Binkley and Giardina 1997). By contrast, asymbiotic N fixation (ANF) rates typically have lower maxima, but are less variable across the landscape (Cleveland et al. 2010, Reed et al. 2011). Despite the large discrepancy in potential N inputs via these two N-fixation pathways, the relative importance of ANF vs. SNF for actual N inputs into tropical secondary forests is poorly understood. While SNF plays a key role in meeting the external N demands of some regenerating tropical forests (Batterman et al. 2013a), the absence of N-fixing trees in some tropical forests (Vitousek 2004) and the downregulation of SNF by many tropical N-fixing trees suggests that ANF can be the dominant N input into many tropical forests (Reed et al. 2011, Sullivan et al. 2014).

Ecosystem theory may help form predictions about the relative importance of N inputs from ANF and SNF during tropical forest succession. Theoretical models generally show that SNF is cost-effective relative to soil N uptake for plants in young forests, but becomes increasingly cost-ineffective as soil N accumulates through succession (Vitousek and Field 1999, Rastetter et al. 2001). Changes in the cost-effectiveness of N acquisition strategies predict that SNF rates peak early in succession and decline to near 0 in older forests. Asymbiotic N fixation is rarely incorporated into these models explicitly and may not experience the same successional cost–benefit dynamics. Free-living N-fixers can only access soil N that is directly adjacent to their cell walls, and this highly localized soil N pool might be more variable across small spatial scales than through successional time. Free-living N fixers also require litter or soil C, which can increase through succession. Therefore, ANF might increase in importance relative to SNF in later successional stages.

Theoretical models of successional dynamics strongly suggest that total fixation N inputs and the relative importance of ANF vs. SNF during tropical succession depend largely on the environmental factors that regulate each N-fixation pathway and how those environmental factors change as forests regrow. Asymbiotic N fixation rates can be controlled by such environmental factors as temperature (Cassar et al. 2012), moisture (Reed et al. 2007), N availability (Crews et al. 2000, 2001, Barron et al. 2009), phosphorus availability (Crews et al. 2000, 2001, Reed et al. 2007, Wurzburger et al. 2012), molybdenum (Barron et al. 2009, Wurzburger et al. 2012) or other micronutrient (Crews et al. 2000, 2001) availability, patterns of canopy litterfall (Reed et al. 2008), and asymbiotic N-fixing bacteria

taxonomy (Reed et al. 2010). While all of these factors likely change during succession, the only studies (to our knowledge) that explicitly measured the dynamics of ANF during secondary succession in tropical forests found decreases (Sullivan et al. 2014) or no change (Winbourne et al. 2018a) in ANF from secondary to primary forests.

Symbiotic N fixation is typically considered to be more variable across secondary forests than ANF (Reed et al. 2011, Sullivan et al. 2014), with a variety of environmental factors believed to regulate this variability. Tropical SNF can be regulated by resources such as soil N (Barron et al. 2011, Batterman et al. 2013b), phosphorus (Crews 1993, Batterman et al. 2013b, Nasto et al. 2014), and light (McHargue 1999, Myster 2006, Taylor and Menge 2018). Of the studies that have assessed SNF, directly or indirectly, during tropical forest succession, all but one show that SNF rates decline through succession (Pearson and Vitousek 2001, Gehring et al. 2005, Batterman et al. 2013a, Sullivan et al. 2014, Bauters et al. 2016). Winbourne et al. (2018a) found no significant successional change in SNF rates in regenerating Brazilian Atlantic forests, which they suggest may be due to an N cycle that fully recovered prior to the age range they assessed. Some evidence suggests that SNF rates increase slightly from older secondary forests to primary forests (Batterman et al. 2013a) as gap dynamics become more prominent (Barron et al. 2011), but few data explicitly test this.

The paucity of data on tropical secondary forest N cycling limits our understanding of what controls tropical forest regeneration rates and hinders our ability to predict the function of these forests in the global biosphere. To improve this understanding, we measured soil inorganic N, ANF, SNF, and tree biomass in a chronosequence of humid tropical forest plots, asking (1) How do soil inorganic N, ANF, and SNF change through forest succession and relative to tree biomass and tree growth? (2) What is the relative contribution of ANF vs. SNF to ecosystem N inputs at each stage of succession? and (3) How are changes in N-fixing tree abundance through succession related to changes in SNF? Based on available theory and limited data, we predicted that tree biomass and soil inorganic N would accumulate through succession, that SNF would decline as soil inorganic N accumulates and shading from neighboring trees intensifies during succession, but that ANF rates would remain relatively constant across our age gradient. These predicted N fixation dynamics led us to further predict that SNF would be a much larger source of N than ANF early in succession, but that these two fixation pathways would be relatively similar in primary forests as has been demonstrated empirically (Sullivan et al. 2014). Finally, because tropical N-fixing trees have high potential SNF rates, we predicted that the prevalence of N-fixing trees would be positively correlated with SNF at our study sites.

Study site and plot design

Our study took place in humid tropical rainforests in the Caribbean lowlands of northeastern Costa Rica, in and around La Selva Biological Station (10.4233° N, 84.022° W). These forests receive approximately 4,500 mm/yr of rainfall, have a relatively constant mean annual temperature of 25°C, and are positioned on soils primarily composed of weathered ultisols (McDade and Hartshorn 1994).

We measured tree growth, soil inorganic N concentrations, and symbiotic and asymbiotic N fixation in five 1-ha (50 × 200 m) plots, which are each subdivided into 100 10 × 10 m subplots. Four of the plots range in age from 19 to 37 yr since pasture abandonment, and one is in old-growth forest that has no history of disturbance for at least 200 yr. Putative N-fixing species comprise between 24% and 33% of total tree basal area in these plots (Appendix S1: Table S1; Menge and Chazdon 2016, Taylor et al. 2017). All plots are within 15 km of each other and are similar in elevation (5–220 m), topography, soil type, and climate. These plots are a subset of an eight-plot chronosequence described in detail elsewhere (Chazdon et al. 2005), including studies on the demographics and neighborhood interactions of N-fixing vs. non-fixing trees (Menge and Chazdon 2016, Taylor et al. 2017).

Sampling for tree growth, soil inorganic N concentrations, and N fixation

Within each plot, all adult trees ≥5 cm diameter at breast height (DBH) were tagged, identified to species, measured for DBH, and mapped onto a plot-level X, Y coordinate system. Our analyses use data from censuses in 2013 and 2014 to calculate tree basal area and annual basal area increment (the change in basal area from one census to the next, which accounts for growth, recruitment, and mortality). Based on species identification, each tree was categorized as a putative N fixer if it was listed or was a congener (fixation is thought to be primarily conserved at the genus level, Sprent et al. 2017) of an N-fixing species in Sprent (2009). We calculated basal area and basal area increment for all trees, N fixers only, and non-fixers only for each plot (Appendix S1: Table S1).

We sampled soil inorganic N concentrations using both plot- and tree-based approaches. Plot-based samples were taken in 10 locations in each plot spread evenly across the subplot grid in July 2015 (Appendix S1: Fig. S1). Tree-based samples were taken around 20 randomly selected N fixers in each plot in July 2017. These two separate sampling dates help capture small temporal variation (i.e., from recent weather events) but because both sampling dates were during the wet season, they do not capture seasonal variation in soil inorganic N concentrations (see *Discussion*). Three individual samples were taken at each sampling location resulting in 90 individual samples at 30 sampling locations in each of the five plots. Samples were taken in a triangular configuration with individual samples 1 m apart for plot-based sampling and 2.8 m apart for tree-based sampling (to allow sufficient distance from the tree base). For each sample, soil was extracted using an 8-cm diameter soil core to a depth of 15 cm, homogenized in a plastic bag, and approximately 5 g of soil was massed and immediately placed in 30 mL of 2 mol/L KCl extractant. Soil was immediately mixed thoroughly in the KCl extractant then incubated for approximately 8 h unagitated and finally placed on a reciprocating shaker for 3 h prior to filtering. Following incubation and filtering, we analyzed KCl extractions for inorganic N (NH_4^+ and NO_3^-) concentrations on a Smartchem 170 Discrete Analyzer (Westco Scientific Instruments, Brookfield, Connecticut, USA) at Columbia University.

We measured ANF in the leaf litter at eight locations distributed evenly across each plot (Appendix S1: Fig. S1) in July, 2017. At each sample location, we removed all leaf litter and fine woody debris from a 50.24-cm^2 area of the forest floor, placed it in an airtight glass container, and incubated the sample for 24 h in an atmosphere where 49% of the natural N_2 was replaced with isotopically labeled $^{15}N_2$ (Sigma-Aldrich, St. Louis, Missouri, USA, and see Dabundo et al. 2014 for discussion of contamination error in $^{15}N_2$ sources). Samples were incubated in situ to approximate ambient environmental conditions as well as possible. We then calculated ANF rates using the deviation of each sample's isotopic signature (percentage of atoms that were ^{15}N as opposed to ^{14}N) from the natural isotopic signature in the environment (Appendix S2).

We estimated SNF inputs by pairing estimates of nodule biomass with per-nodule-biomass N fixation rates. We measured nodule biomass within each plot using both a plot- and tree-based sampling approach similar to that used for soil inorganic N sampling (Appendix S1: Fig. S1). Plot-based nodule sampling took place at 20 evenly distributed locations in each plot in July 2015 using an 8-cm diameter soil core sampled to a depth of 15 cm. Nodulation around specific N-fixers (tree-based approach) was measured on the same samples taken for tree-based soil inorganic N measurements in July 2017. This resulted in 80 individual samples (20 plot-based, 60 tree-based) taken at 50 sampling locations per plot (Appendix S1: Fig. S1). Each soil sample was hand-searched for nodules on the same day as collection, and all nodules were dissected to verify N-fixation activity. All nodules in an individual sample were dried at 60°C for at least 3 d and massed (Data S1).

Per-nodule-biomass fixation rates were measured using $^{15}N_2$ incubations similar to those described above for ANF. Although nodule biomass was sampled within each plot, to minimize destructive sampling within the plots, in July 2017 we measured per-nodule N fixation

rates on nodules from 11 *Pentaclethra macroloba* trees (which comprise 69% of the N-fixing tree abundance in these plots; Menge and Chazdon 2016) surrounding and adjacent to each plot rather than inside each plot. For each tree, nodules were unearthed by hand and separated from the tree, leaving approximately 5 cm of proximate root tissue attached. Nodules were then placed in an airtight chamber containing an atmosphere of 20 atom % ^{15}N and incubated for 30 min. N fixation rates were calculated using the deviation from natural N isotope ratios (we used a natural abundance atom % ^{15}N value of 0.3663) in a similar manner to the ANF calculations (Appendix S2). Although there is slight variation in natural abundance atom % ^{15}N, this variation is small compared to enriched isotope values (Menge et al. 2015). We ran Monte-Carlo simulations incorporating uncertainty in the natural abundance atom % ^{15}N and in the proportion of the incubation chamber replaced with enriched ^{15}N gas. These simulations indicated that the average error that uncertainty in our N_2 method could create is approximately ±6%, which is small compared to the error associated with nodule biomass sampling (Winbourne et al. 2018*b*). For both ANF and SNF, our use of $^{15}N_2$ incubations obviates the need for the acetylene reduction method, which is cheaper but a source of much more uncertainty (Anderson et al. 2004). Therefore, although our nodule incubation sample size is lower than some studies that use ARA, our approach yields more robust results. Both SNF and ANF rates were scaled to the plot level using conversions described in Appendix S2.

Statistical analyses

While our analyses all involved regression-style tests, we implemented slightly different model approaches based on the underlying structure of different data sets. To address successional dynamics of soil inorganic N, ANF, SNF, and tree growth using individual core-, focal tree-, or subplot-scale data, we used a maximum-likelihood framework to compare null (no change), linear (or exponential if the response variable was log-transformed), and Gaussian fits between the response variable and stand age. In some cases, additional models were also fit (Appendix S1: Table S2). Data for soil inorganic N and ANF inputs were natural-log transformed for statistical analyses to meet parametric assumptions. Because data for SNF (both SNF inputs and nodule biomass) were zero-inflated lognormally distributed, we used a two-part model that allowed us to test for successional changes in both the probability of finding nodules and the biomass of nodules (or SNF rates) when they occurred (see Appendix S2). For statistical tests on SNF data, we used geometric means (median of the lognormal distribution) accounting for zero inflation (Appendix S2) because this allowed us to use appropriate statistical tests while staying true to the structure of our data. For presentation of the data in figures, however, we used arithmetic means, which give a better approximation of the ecosystem-level fluxes. Please see a comparison and discussion of the geometric vs. arithmetic means in Table 1 and Appendix S3.

Although the old-growth forest plot has no recorded history of disturbance, for regression purposes, we assumed an age of 100 yr. Assuming different ages for the old growth plot (up to 400 yr) did not qualitatively change any of our results for successional trends. For each response variable, models were compared using differences in the corrected Akaike information criterion (ΔAIC_c). We interpreted models with $\Delta AIC_c < 2$ to be similar fits ($\Delta AIC_c = 2$ is the approximate equivalent of $P = 0.05$, Anderson 2008) and report results for the model with the lowest AIC_c along with ΔAIC_c for the next best-fit model. All models and ΔAIC_c values can be seen in Appendix S1: Tables S2–S4. For plot-scale analyses that used plot means as individual data points, we used ordinary least squares regression models with $\alpha = 0.05$. All analyses were done in the base and bbmle

TABLE 1. Metrics of N cycling and tree biomass recuperation for our five successional study plots.

Plot	Age (yr)	Basal area (m²/ha)		N fixation (kg N·ha⁻¹·yr⁻¹)					
		Total	N-fixer	Soil inorganic N	ANF	SNF 0-inflated geometric mean	SNF arithmetic mean	p†	v†
BEJ	19	27.69	7.20	25.56 (1.28)	2.57 (0.5)	3.01 (2.00–4.52)	10.57 (4.85)	0.20	1.83
JE	19	20.43	4.96	26.40 (1.17)	1.90 (0.4)	5.50 (3.69–8.18)	13.10 (6.18)	0.15	1.64
LSUR	29	27.60	8.06	49.82 (2.18)	3.39 (0.74)	7.60 (5.96–9.70)	8.21 (1.58)	0.05	0.38
LEPS	37	32.13	10.73	29.85 (2.27)	8.65 (1.48)	0.97 (0.55–1.72)	1.84 (0.74)	0.08	0.99
LEPP	OG	31.60	9.88	23.04 (1.42)	2.82 (0.48)	0.56 (0.24–1.28)	2.33 (0.96)	0.11	1.23

Notes: ANF, asymbiotic N fixation; SNF, symbiotic N fixation. Plot age is reported in years since agricultural abandonment, and the age "OG" for our LEPP plot signifies old growth forest with no recorded history of disturbance. Values in parentheses are SE or SE range. See Appendix S3 for a discussion of the relative merits of different metrics of the SNF central tendency. The parameter v is the standard deviation of nodule biomass divided by the mean of nodule biomass for cores that contained nodules (both p and v are unitless).

† Metrics calculated or estimated from Winbourne et al. (2018*b*) for estimation of SNF sampling error; p is the proportion of soil cores containing nodules.

packages of R statistical software version 3.4.3 (Bolker and R Development Core Team 2017, R Core Team 2017).

RESULTS

How do soil inorganic N, ANF, and SNF change through succession relative to tree biomass and growth?

Our data showed a hump-shaped trend of total soil inorganic N through succession, with a peak in the 29-yr-old forest (Fig. 1a; $\Delta AIC_c = 85.3$; Table 1; Appendix S1: Table S2). Ammonium (NH_4^+) made up the vast majority (90–96% for plot-level means; Appendix S1: Fig. S2) of soil inorganic N at these sites. Due to the large contribution of ammonium to total soil inorganic N, the successional pattern of ammonium largely mirrored that of total soil inorganic N: a hump-shaped trend through succession ($\Delta AIC_c = 219.2$; Appendix S1: Table S3). Soil nitrate (NO_3^-) also followed a hump-shaped relationship through succession ($\Delta AIC_c = 82.0$; Appendix S1: Table S3). Due to general decreases in ammonium and increases in nitrate with forest age, we found the percent contribution of nitrate to total soil inorganic N was higher in the older plots than in the youngest plots and peaked in the 37-yr plot. ($\Delta AIC_c = 40.38$; Appendix S1: Table S3 and Fig. S2b).

Asymbiotic N fixation also changed through succession in a hump-shaped fashion ($\Delta AIC_c = 21.5$; Table 1, Appendix S1: Table S2; Fig. 1b), peaking in the 37-yr-old plot, where N inputs from ANF were ~3.9 times greater than the 19-yr plots. This variation in ANF came primarily from variation in asymbiotic fixation rates per gram of leaf litter rather than variation in the amount of leaf litter per ground area (Appendix S1: Fig. S3). Our best-fit model for asymbiotic fixation rates per gram of litter was a hump-shaped trend through succession, similar to the dynamics of total ANF ($\Delta AIC_c = 22.2$; Appendix S1: Table S3, Fig. S3b), whereas our best-fit model for leaf litter mass per ground area showed no successional change ($\Delta AIC_c = 2.1$; Appendix S1: Table S3, Fig. S3a).

Symbiotic N fixation decreased in an exponential fashion through succession in our sites (Fig. 1c). The geometric mean of SNF (gray triangles in Fig. 1c) was almost seven times greater in the 19-yr plots than in the old-growth plot, and the arithmetic mean SNF (black dots in Fig. 1c) was over five times greater in 19-yr plots than in old-growth forest. Our best-fit model indicated no successional change in the probability of SNF occurring within a core (probability of finding active nodules), but an exponential decrease in SNF through succession for cores that contained nodules ($\Delta AIC_c = 1.46$; Appendix S1: Table S2). The two primary sources of
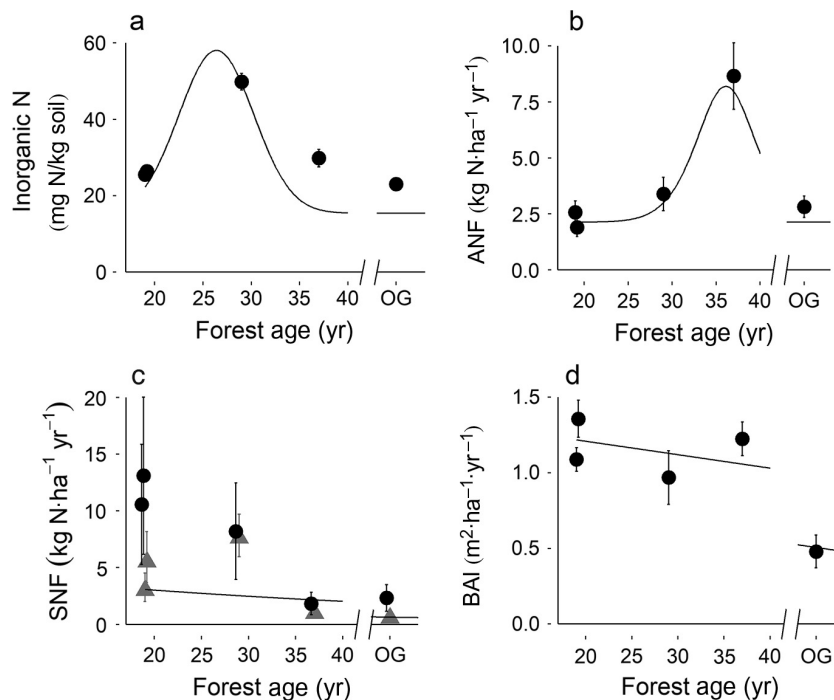

FIG. 1. Dynamics of (a) soil inorganic N, (b) asymbiotic N fixation, (c) symbiotic N fixation, and (d) tree growth (basal area increment, BAI) across forest succession in our five study plots. Each point represents the plot-level mean with error bars representing ± SE. In panel c, gray triangles represent geometric means for which we could run appropriate statistical tests while black dots represent arithmetic means, which are a better representation of ecosystem-level symbiotic N fixation (SNF) fluxes. Curves represent the best-fit models across forest age (Appendix S1: Table S2). Both left-hand points represent 19-yr forests but are jittered for viewing purposes. OG indicates our old-growth plot that has no recorded history of disturbance.

variation in SNF are variation in nodule biomass and in fixation rates per gram of nodule biomass. Variation in nodule biomass was the primary driver of the successional dynamics in total SNF in our plots (Appendix S1: Figs. S4, S5). Nodule biomass declined in an exponential fashion through succession ($\Delta AIC_c = 1.46$; Appendix S1: Table S3), such that geometric mean nodule biomass was ~6.8 times greater in 19-yr plots than at the old-growth forest site. Conversely, our best-fit model for fixation rates per gram of nodule biomass was a quadratic relationship where per-nodule fixation rates were intermediate in the youngest plots, declined in mid-successional forests, and were greatest in the old-growth forest plot ($\Delta AIC_c = 38.01$; Appendix S1: Table S3 and Fig. S4). Within individual soil cores, we found significantly positive but noisy relationships between SNF and total inorganic N ($\Delta AIC_c = 2.03$; Appendix S1: Table S3, Fig. S6e; $R^2 = 0.09$) and between SNF and soil ammonium ($\Delta AIC_c = 2.05$; Appendix S1: Table S3, Fig. S6c; $R^2 = 0.095$), but no relationship (non-significant negative trend) between SNF and soil nitrate ($\Delta AIC_c = 1.8$; Appendix S1: Table S3, Fig. S6a; $R^2 = 0.01$). These relationships between SNF and forest age and soil N are based on the zero-inflated lognormal geometric mean of SNF for each plot. The arithmetic means (black dots in Figs. 1c, 2c), which better reflect ecosystem-level fluxes but are not as amenable to statistical tests, showed higher total SNF estimates but visually similar trends (Table 1; Appendix S3: Figs. S1–S3).

Plot-level tree growth rates declined significantly through succession ($\Delta AIC_c = 8.65$; Fig. 1d; Appendix S1: Tables S1, S2; Fig. S7). Despite declines in both SNF and tree growth through succession, we found no significant relationship between SNF and tree growth at either the individual-tree scale or the plot scale (Appendix S1: Fig. S8). At the individual-tree level, our best-fit model showed that SNF in the cores taken around a focal N-fixer did not correlate to the growth of that N fixer ($\Delta AIC_c = 2.18$; Appendix S1: Table S4). At the subplot-scale, we also found that our best-fit models described no relationship between total basal area change or basal area change in N fixers and SNF inputs in the subplot ($\Delta AIC_c = 2.33$ and $\Delta AIC_c = 1.85$ for total basal area change and N-fixer basal area change, respectively; Appendix S1: Table S4). This was also true at the plot-scale where we found no significant relationships between total basal area change or N-fixer basal area change and SNF inputs ($P = 0.541$ and $P = 0.116$, respectively). We did, however, find a significant positive relationship between total plot-level tree growth and combined N inputs from ANF and arithmetic mean SNF ($P = 0.038$; Fig. 2a).

What is the relative contribution of ANF vs. SNF at each stage of succession?

When combining all plots across our chronosequence, SNF (arithmetic mean 6.05 kg N·ha^{-1}·yr^{-1}) contributed 41% more to total N inputs than ANF (mean
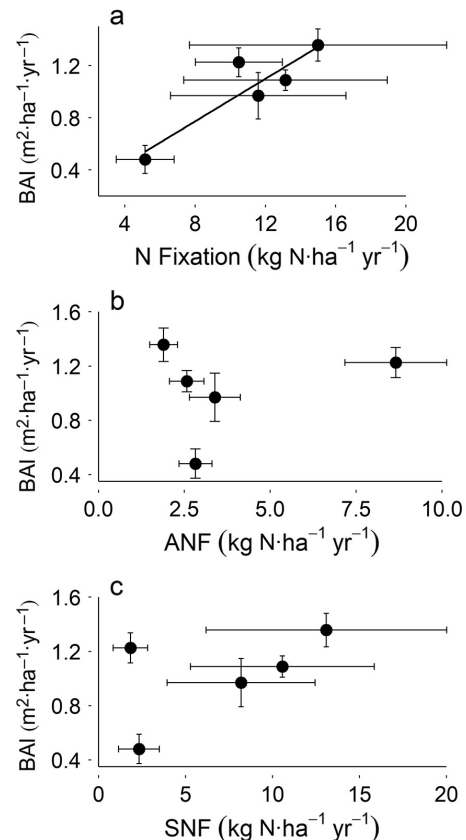

Fig. 2. Plot-level tree growth (basal area increment) is positively related to (a) total N inputs from fixation (SNF + ANF) but not (b) inputs from asymbiotic nitrogen fixation (ANF) or (c) inputs from SNF in our plots. Points represent each of our five 1-ha study plots. BAI is the mean ($\pm$SE) of BAI in each of 100 10 × 10 m subplots scaled to 1 ha area. SNF is the arithmetic mean for each plot, but geometric means for SNF can be found in Appendix S3: Fig. S1.

4.27 kg N·ha^{-1}·yr^{-1}), an effect that was primarily driven by younger plots. The dominant N input from fixation changed from early- to late-successional forests (Fig. 3). In the 19-yr-old forests, arithmetic mean N inputs from SNF were 5.3 times that of ANF. Symbiotic N fixation also dominated N inputs in the 29-yr plot, where SNF was 2.4 times greater than ANF. However, ANF was 4.7 times greater than SNF in the 37-yr plot and 1.2 times greater than SNF in the old-growth plot. Results for geometric means of SNF can be seen in Appendix S3: Fig. S2.

How are changes in N-fixing tree abundance through succession related to changes in SNF?

To better understand both localized and stand-level patterns in the relationship between N-fixer abundance and SNF, we assessed this relationship at the core, tree, and plot level. At the individual core level, we did not find a significant relationship between SNF rates and
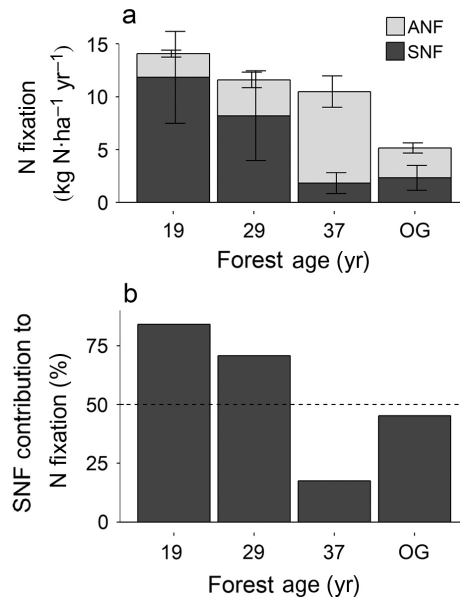
FIG. 3. The (a) relative contributions of asymbiotic N fixation (ANF) and symbiotic N fixation (SNF) and (b) percent contribution of SNF to total N fixation inputs measured in forests from 19 yr old to old-growth (OG). Bars for 19-yr forests represents the mean rates of our two 19-yr forest plots. All bars represent arithmetic means and error bars represent ±SE. Comparisons of ANF and the geometric mean of SNF can be found in Appendix S3: Fig. S2. The dashed line in panel b indicates the 50% line such that bars above the line represent plots where SNF is the dominant N input and ANF is the dominant N input for bars below the line. The dominant N input measured switched from SNF in young plots to ANF in older forests.

the crowding of N-fixers around the location of the core sample ($\Delta AIC_c = 3.38$; Appendix S1: Table S4). We also found no relationship between the crowding of other N fixers around a focal N-fixing tree and SNF estimates for that tree ($\Delta AIC_c = 2.79$; Fig. 4a; Appendix S1: Table S4). When analyzing plot-level arithmetic means, we found a significant negative relationship between the basal area of N fixers in a plot and SNF inputs in that plot ($P = 0.004$; Fig. 4b). When analyzing plot-level geometric means, the trend in this relationship was negative but nonsignificant ($P = 0.23$, Appendix S3: Fig. S3). There was also no correlation between mean SNF inputs and the stem density of N fixers ($P = 0.345$) or the ratio of N-fixer to non-fixer stems ($P = 0.227$) at the plot level for either arithmetic (Fig. 4) or geometric (Appendix S3: Fig. S3) mean SNF.

DISCUSSION

Combining measurements of soil inorganic N availability, ANF, SNF, and tree growth, our study provides a rare assessment of multiple facets of N-cycle recovery and tree growth in regenerating tropical forests. We found neither systematic increases nor decreases in soil inorganic N availability or ANF inputs across the

successional range we studied, but we did find significant declines in SNF from the youngest (19-yr) plots to the old-growth forest site and successional declines in tree growth rates, as has been previously reported for these plots (Chazdon et al. 2007, Menge and Chazdon 2016, Taylor et al. 2017). These findings support our hypotheses that SNF rates will decline, tree biomass will accumulate, and ANF rates will not vary systematically with increasing forest age. However, the data contradict our hypothesis that soil inorganic N would accumulate across the successional gradient we measured. Declines in SNF through succession led to a successional switch in the dominant N-fixation input that we measured. As hypothesized, SNF dominated inputs in the youngest forest sites but ANF and SNF were similar in the old-growth plot. We also found that SNF rates were negatively related to N-fixing tree basal area, directly contradicting our hypothesis. Together, these results indicate that N-fixer abundance is a poor predictor of SNF rates, that ANF makes an important (and sometimes dominant) contribution to N inputs from fixation, and that N cycling has largely recovered in these forests by 19 yr following disturbance.

Recovery of the N cycle during tropical forest succession

The recovery rate of the N cycle represents a critical, but poorly understood, aspect of tropical forest succession. Our results indicate that the N cycle has largely recovered in the forests we studied prior to 19 yr post-disturbance (Fig. 1a) and that our sites exhibit high soil inorganic N availability compared to many other Neotropical forest sites (Matson et al. 1987, Piccolo et al. 1994, Silver et al. 2001, Davidson et al. 2007, Sullivan et al. 2014). Given that these sites have land-use histories that are thought to create large N losses (McGrath et al. 2001, Davidson and Martinelli 2009), it seems likely that N inputs from fixation and N deposition (~6 kg N·ha^{-1}·yr^{-1} in our study region; Galloway et al. 2004) drove rapid and substantial recuperation of N cycling.

Soil inorganic N is often highly temporally variable in response to recent weather events and seasonal changes in climate, calling for cautious interpretation. The fact that soil inorganic N data for each plot was consistent across our two sampling times (July 2015 and July 2017) is encouraging but does not provide information on seasonal variation. Previous work at different plots near our site shows that soil inorganic N concentrations do vary seasonally, but these seasonal variations vary in parallel across intact and disturbed forests (Vitousek and Denslow 1986). If our plots exhibit similar seasonal variation in soil inorganic N concentrations, then the successional patterns in soil inorganic N concentrations we observed should be similar during other seasons. Regarding the magnitude of soil inorganic N concentrations we measured, previous work indicates that soil inorganic N concentrations in this site are lowest during
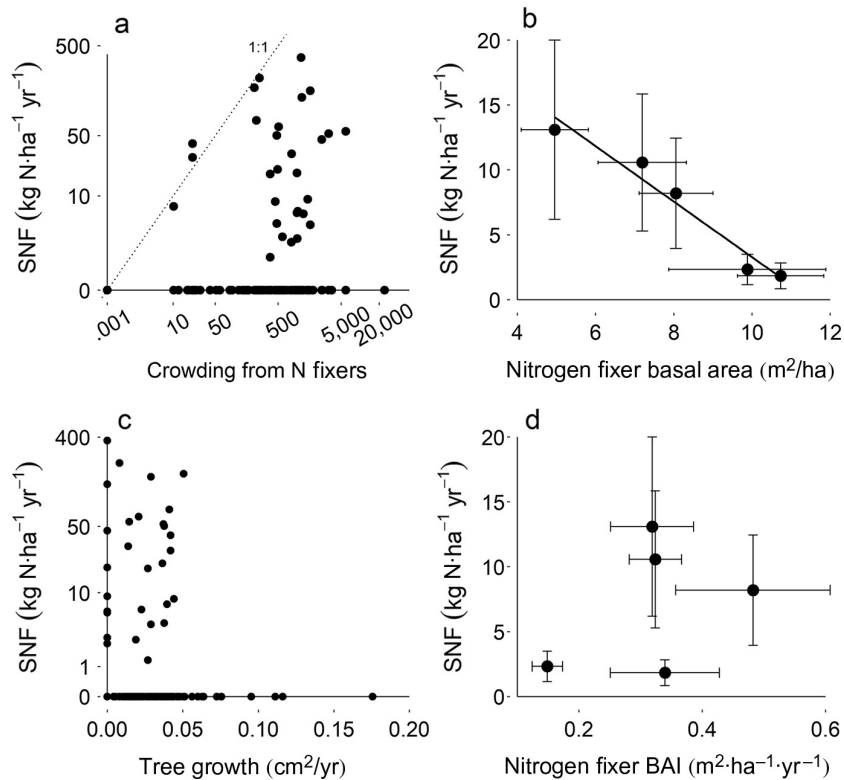
FIG. 4. The relationship between symbiotic N fixation (SNF) and N-fixer prevalence at the (a) individual tree, (b) N-fixer basal area at the plot scale, (c) N-fixer tree growth at the individual tree scale, and (d) N-fixer basal area increment at the plot scale. The vertical values of points in panels a and c represent SNF rates sampled from three cores each for 100 N-fixing trees. The horizontal values in panel a, "crowding from N fixers," were measured as the neighborhood crowding index (which is unitless) from neighboring N fixers for each focal tree. Points in panels b and d represent plot-level arithmetic means for SNF and N-fixer basal area and N-fixer BAI (±SE), respectively. Versions with geometric mean SNF can be found in Appendix S3: Fig. S3. The line in (b) represents a significant negative linear relationship between SNF and N-fixer basal area. Both axes in panel a and the vertical axis in panel b are presented on log scales with linear (untransformed) values. Panels a and c plotted with linear axes are presented in Appendix S1: Fig. S8. We found no relationship between SNF and crowding from N fixers at the tree scale.

the wet season (the season sampled in this study; Vitousek and Denslow 1986), so the concentrations we report, which are higher than many Neotropical forests, are likely at the low end of the annual range for our study site.

Recently, Winbourne et al. (2018a) reported rapid (faster than 20 yr) recovery of N cycling in Brazilian Atlantic Forests, similar to the rates found at our study site. These rapid rates contrast with estimates of 30–70 yr for N-cycle recovery in Panama (Batterman et al. 2013a) and the Brazilian Amazon (Davidson et al. 2007). While many differences exist between these study sites, one potentially important difference may be N-fixer abundances. Both fast-recovery sites (ours and Winbourne et al. 2018a) exhibit high N-fixer abundances (~30% of forest basal area), whereas N fixers only comprise 6–14% of the basal area at the Panama site and in the Brazilian Amazon region (ter Steege et al. 2006). Although N-fixer abundance is not a good predictor of actual localized SNF rates (Fig. 4a, b), N-fixer abundances do indicate the maximum potential SNF

rates for a forest. Thus, sites with high N-fixer abundances may experience especially high SNF rates in the earliest years of forest regrowth, allowing the N cycle to recover more rapidly than in forests with lower N-fixer abundances. Because our youngest plots were 19 yr old when N-fixer abundances and SNF were both assessed, it is possible that N fixers drove large N inputs (and potentially a positive relationship between N-fixer abundance and SNF) at our sites in the earliest years of secondary succession prior to our initial sampling.

Importance and regulation of SNF during tropical succession

Because of the large potential for SNF to bring new N into regenerating tropical forests, the environmental factors that regulate SNF rates are important for both N cycling and biomass recovery dynamics. Ecosystem theory predicts that SNF is downregulated as it becomes energetically unfavorable relative to soil N uptake as succession proceeds (Rastetter et al. 2001, Menge et al.

2008). Our data do show a successional trend in SNF inputs (Fig. 1c), but this trend was not negatively related to soil inorganic N concentrations (Appendix S1: Fig. S6b, d, f), suggesting that other resources might play a stronger role. Recent data for the most common N fixer in our plots, *P. macroloba*, demonstrate that light availability can be a strong driver of SNF rates (Taylor and Menge 2018). A pattern of progressively reduced light availability following canopy closure (Denslow and Guzman G 2000, Guariguata and Ostertag 2001) leading to lower SNF rates matches the successional SNF pattern found in our study. Other environmental factors such as soil phosphorus, moisture, and pH may also influence successional SNF dynamics.

We found that the abundance of N-fixing trees was negatively related to SNF rates in our plots, contrary to our predictions. This negative relationship has at least two important implications. First, as our study and other recent studies have shown (Batterman et al. 2013a, Sullivan et al. 2014), SNF rates in mature tropical forests are often low, even in forests that have many N fixers. Our data add to a growing body of evidence that early estimates based on high N-fixer abundances overestimate tropical SNF inputs (Vitousek et al. 2013), especially in intact old-growth tropical forests (Gehring et al. 2005, Batterman et al. 2013a, Sullivan et al. 2014, Bauters et al. 2016). The second implication of our data goes beyond the concept that "N fixers do not always indicate SNF" to show that, in our plots, the relative abundance of N fixers is actually negatively correlated with SNF (Fig. 4b), with N-fixer abundance increasing, but SNF decreasing, through succession.

The most likely explanation for the unexpected negative relationship between N-fixer abundance and SNF is that environmental changes during forest succession influence N-fixer success and SNF inputs in fundamentally different ways. Some combination of seed dispersal characteristics (Wilcots et al. 2018), leaf traits (Gei et al. 2018), and demographic traits of N fixers in our plots make them increasingly successful in later successional stages (although this is not true in all tropical forests; Gei et al. 2018). However, successional changes in soil nutrients and light availability may mean that per-fixer SNF rates decline through succession strongly enough to create low N-fixer abundances and high SNF rates early in succession, but high N-fixer abundances and low SNF later in succession. This negative relationship between N-fixer abundance and SNF rates highlights the potential danger of using local N-fixer abundances as a proxy for SNF inputs when estimating N cycling in tropical forests, particularly when it is conflated with successional trends. As we continue to improve our estimates of N-fixer abundances over large areas of the tropics (ter Steege et al. 2006, Menge et al. 2017), it may be tempting to assume that areas with many N fixers experience high SNF rates that could fuel rapid forest growth. Our data indicate that this assumption is not valid and could mislead our understanding of how N and C cycles are coupled in regenerating tropical forests.

Symbiotic N fixation at the ecosystem scale is notoriously difficult to measure, due in part to its patchy and non-normal spatial distribution (Winbourne et al. 2018b). Therefore, it is important to be cautious when interpreting ecosystem-scale fluxes of SNF and how they change along environmental gradients. Despite this general caution, multiple lines of evidence suggest that we should be cautiously optimistic about our confidence in the trends we report. First, the characteristics of our plot sampling (sample size, fraction of cores containing nodules, and variation in nodule biomass among cores with nodules; Table 1) suggest that our estimates fall within the range of estimation error recommended as a reasonable target in Winbourne et al. (2018b). Specifically, a relatively large fraction of our cores contained nodules. Second, qualitatively similar results for relationships between SNF and BAI, N-fixer abundance, and soil inorganic N at both small (individual core or tree) and large (plot) spatial scales suggest that our results are not primarily driven by errors in scaling SNF up to the ecosystem level. Third, although any given SNF estimate is rather uncertain, all three studies of SNF through secondary succession in Neotropical rainforests (Batterman et al. 2013a, Winbourne et al. 2018a, and our study) show qualitatively similar declines in SNF through secondary succession. Together, this evidence suggests that the uncertainty inherent to SNF estimates does not preclude our ability to demonstrate successional trends.

It is important to note that substantial differences exist between different calculations for estimating SNF. We present results for both the zero-inflated geometric mean and the arithmetic mean because they are both informative. The zero-inflated geometric mean allows us to conduct statistical tests that make the proper assumptions about the structure of our data, but is relatively insensitive to rare, large values that can comprise a large portion of actual plot-scale SNF (Winbourne et al. 2018b). The arithmetic mean is a better representation of actual ecosystem-level SNF fluxes precisely because it does a better job of incorporating these rare, large values, which is why we present it as our primary estimate of flux values. However, the arithmetic mean is not appropriate for statistical tests because it violates basic assumptions of data structure for zero-inflated lognormal data. Both metrics exhibit the same qualitative successional patterns (Table 1, Fig. 1c). Please see Appendix S3 for a detailed discussion of different SNF calculations.

Importance and regulation of ANF during tropical succession

Our data suggest that ANF is an important source of N for forest regeneration at our study site. In the older (37-yr and old-growth) plots, ANF represented the dominant or codominant N input from fixation, supporting

the assertion that ANF is the largest N input via fixation in some, especially mature, tropical forests (Reed et al. 2011, Sullivan et al. 2014). It is important to note that Batterman et al. (2013a) did not estimate ANF in their study and reported relatively low SNF rates in late-successional forests, allowing for the possibility that ANF may also be the dominant N input in later stages of succession at their site in Panama and other sites throughout the tropics. Further, our study only measured ANF in the leaf litter. Other important sources of ANF not measured in our study, such as the mineral soil, phyllosphere (Fürnkranz et al. 2008, Reed et al. 2011), or decaying wood (Matzek and Vitousek 2003), suggest that we are underestimating total ANF, and that this may be an even more important ecosystem N input at our site than our data indicate. Additionally, if there are N fixation inputs in our sites from other symbioses and associations such as lichens (Benner et al. 2007) and bryophyte-associated cyanobacteria (Matzek and Vitousek 2003), the overall N fixation input would be even larger.

Given the important contribution that ANF can have to total N inputs, understanding the drivers of variation in ANF can provide important insight into successional N dynamics in regenerating tropical forests. The two previous studies assessing ANF dynamics in successional tropical forests reported either a reduction in leaf-litter ANF from secondary to primary forests (Sullivan et al. 2014) or no successional trend in ANF (Winbourne et al. 2018a). This paucity of data inhibits any broad empirical consensus about the successional trajectory of ANF in tropical forests. However, our results do provide important insight that per-litter-biomass fixation rate (rather than litter mass itself) can be the primary driver of variation in leaf-litter ANF. Current evidence suggests that tropical ANF rates increase with the availability of litter phosphorus (Thompson and Vitousek 1997, Crews et al. 2000, Reed et al. 2008, 2010, Cusack et al. 2009), molybdenum (Barron et al. 2009), moisture (Reed et al. 2007, Cusack et al. 2009), and C quality (Thompson and Vitousek 1997, Vitousek and Hobbie 2000), but decline with litter N concentrations (Thompson and Vitousek 1997, Cusack et al. 2009). Although many of these environmental factors change through succession, their effects on ANF may interact and counteract one another, currently inhibiting our ability to make general predictions of how ANF rates change during tropical forest succession.

Implications for tropical forest N dynamics

The successional dynamics of soil inorganic N, SNF, and ANF reported in this study can also provide important context to our broader understanding of N richness in tropical forests. A fundamental question in tropical ecosystem ecology asks why many lowland, humid tropical forests often export large amounts of inorganic N (Brookshire et al. 2012) given that the largest potential

N input in these forests, SNF, should cease once N limitation has been relieved (Rastetter et al. 2001, Menge et al. 2015). Hedin et al. (2009) provide a potential resolution, proposing that SNF shuts off under N saturated conditions but that other sources of N fixation that are not tied to ecosystem N richness (such as ANF) sustain high N exports by continuing to bring N into mature N-saturated tropical forests. Our findings show mixed evidence for this proposed resolution. We found that SNF does downregulate during succession, but we did not find that SNF completely ceased in mature tropical forests. However, we did find that ANF continues to bring significant N into mature tropical forests regardless of high soil inorganic N availability, lending support to the assertion by Hedin et al. that total biological N fixation represents a "leaky nitrostat" in mature tropical forests. Other Neotropical forest sites indicate similar patterns of incomplete downregulation of SNF through succession (Batterman et al. 2013a, Sullivan et al. 2014, but see Winbourne et al. 2018a) and sustained inputs from ANF in N-saturated primary tropical forest sites (Sullivan et al. 2014, Winbourne et al. 2018a) indicating that the "leaky nitrostat" model of Hedin et al. is a likely contributor to the observed N richness of many tropical forests sites.

CONCLUSIONS

Understanding the successional N dynamics of regenerating tropical forests is critical for well-informed modeling efforts and management practices. Our results demonstrate rapid recovery of the N cycle during tropical forest succession and that SNF and ANF play important roles bringing N into tropical forests at different stages of succession. The rapid recovery of N cycling and corresponding accumulation of biomass in these forests suggest that the biomass resilience seen in many tropical secondary forests (Poorter et al. 2016) may be at least partially attributable to successional N dynamics. That both N and C pools in our plots recovered from disturbance within 20–40 yr without human intervention also qualitatively support recent studies showing that natural forest regeneration is an effective management strategy in many tropical forests (Crouzeilles et al. 2017, Meli et al. 2017). Together, these data suggest that the dynamics of N cycling are critical to the C-capturing potential of secondary tropical forests, and that current and future studies on these N dynamics will prove useful for global models that predict how tropical forests will respond to future environmental and land-use changes.

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SUPPORTING INFORMATION

DATA AVAILABILITY