# Estimating Ecosystem Nitrogen Addition by a Leguminous Tree: A Mass Balance Approach Using a Woody Encroachment Chronosequence

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# Estimating Ecosystem Nitrogen Addition by a Leguminous Tree: A Mass Balance Approach Using a Woody Encroachment Chronosequence

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## ABSTRACT

Difficulty in quantifying rates of biological N fixation (BNF), especially over long time scales, remains a major impediment to defining N budgets in many ecosystems. To estimate N additions from BNF, we applied a tree-scale N mass balance approach to a well-characterized chronosequence of woody legume (*Prosopis glandulosa)* encroachment into subtropical grasslands. We defined spatially discrete single *Prosopis* clusters (aged 28–99 years), and for each calculated BNF as the residual of: soil N (0–30 cm), above- and below-ground biomass N, wet and dry atmospheric N deposition, N trace gas and $N_2$ loss, leaching loss, and baseline grassland soil N at time of establishment. Contemporary BNF for upland savanna woodland was estimated at $10.9 \pm 1.8$ kg N $\text{ha}^{-1}$ $\text{y}^{-1}$, equal to a total of $249 \pm 60$ kg N $\text{ha}^{-1}$ over about 130 years of encroachment at the site. Though these BNF values are lower than previous estimates for *P. glandulosa*, this likely reflects lower plant density as well as low water availability at this site. Uncertainty in soil and biomass parameters affected BNF estimates by 6–11%, with additional sensitivity of up to 18% to uncertainty in other scaling parameters. Differential N deposition (higher rates of dry N deposition to *Prosopis* canopies versus open grasslands) did not explain N accrual beneath trees; iterations that represented this scenario reduced estimated BNF estimates by a maximum of 1.5 kg N $\text{ha}^{-1}$ $\text{y}^{-1}$. We conclude that in this relatively well-constrained system, small-scale mass balance provides a reasonable method of estimating BNF and could provide an opportunity to cross-calibrate alternative estimation approaches.

**Key words:** BNF; $\delta^{15}$N; deposition; *Prosopis*; soil.

## INTRODUCTION

Quantifying nitrogen (N) inputs from biological N fixation (BNF), and thus gauging the importance of fixation in the context of ecosystem-scale N cycling, is complicated by difficulty in measuring this process directly. This is especially true for long-lived woody plants in heterogeneous environments

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**Author contributions** FMS and JPS formulated the original idea and developed methodology, FMS conducted field sampling, performed sample processing and statistical analysis, FMS and JPS interpreted data, and FMS and JPS wrote the manuscript.

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(Boddey and others 2000). Common small-scale (that is, plant- or plot-scale) methods for estimating BNF (such as measuring nitrogenase activity in excised nodules or foliar N isotope composition, Shearer and Kohl 1986), are not appropriate for application in many ecosystems. In drylands, for example, nodule recovery from woody plants can be difficult (Hartley and others 2007) and assumptions of the foliar isotope method are frequently violated (Soper and others 2015). Small-scale methods may also fail to capture important spatial and temporal variability in BNF, introducing errors when scaled to long-term fluxes. Larger-scale, longer-term approaches such as watershed mass balance (where BNF is calculated as the residual of N pools and fluxes) overcome some of these challenges but are complicated by the effects of high variance in each of the input and output terms (Likens 2013). To apply this method, it is necessary to quantify many processes acting simultaneously, including losses of N trace gases or $N_2$ to the atmosphere, hydrologic transport of N, and atmospheric deposition of dissolved N or particulates as well as N accumulation in soils and plant biomass. We propose that some of this uncertainty could be reduced by applying mass balance at smaller spatial scales, where flux values and pool sizes may be more accurately characterized.

Encroachment of N-fixing woody plants into grasslands is one such circumstance where rates of BNF are both poorly defined and of significant interest. Woody encroachment is widespread throughout tropical and subtropical grasslands and savannas (Stevens and others 2017) and is often facilitated by N-fixing trees and shrubs in the family Fabaceae (Asner and Martin 2004). In the US, encroachment has already affected or has potential to affect 220–330 million ha of land (Houghton and others 1999; Pacala and others 2001). The potentially large inputs of fixed N that often accompany woody legume encroachment are associated with significant changes in storage, gas flux, biochemical composition and spatial distribution of carbon (C) and N within ecosystems (Stock and others 1995; Archer and others 2001; Martin and others 2003; Boutton and Liao 2010; Barger and others 2011; Creamer and others 2013).

Encroachment of the leguminous tree *Prosopis glandulosa* (honey mesquite) into grasslands of south Texas offers a tractable system in which to apply a mass balance approach to estimate inputs from BNF using a well-characterized space-for-time chronosequence. Encroachment in the region has been ongoing for the last 100–200 years (Archer 1995) and is associated with significant increases in ecosystem N storage (Archer and others 2001). *Prosopis* recruits only into open grassland areas and not beneath existing canopies (Archer and others 1988) and can develop into spatially discrete clusters made up of a single *Prosopis* individual with a diverse woody understory. Allometric equations have been developed for this *Prosopis* system to estimate N storage in above ground plant tissue (Northup and others 2005). N gas loss rates, both for $N_2$ and N trace gases, have been recently estimated for upland grassland and *Prosopis* grove cover (Soper and others 2016a, b). A long-term measurement record for wet N deposition rates exists nearby and modeled dry deposition rates are available (National Atmospheric Deposition Program). Though leaching losses are uncertain, estimates from other systems, long-term soil N balances and sensitivity analyses can be used to constrain this value. Other input and loss pathways, including N fixation by free-living bacteria, uplift of N from deep soil, plant biomass removal by herbivore grazing, and fire, are likely to be insignificant in this system (Boutton and Liao 2010). BNF can therefore be inferred as the difference between inputs, outputs and storage for individual spatially discrete tree clusters. Because the system is not in steady-state, rates of fixed N input over time can be approximated by regressing mass balance-derived fixation estimates for individual tree clusters against cluster age.

Increases in soil N are sometimes observed beneath encroaching woody canopies even in the absence of N-fixing species (Eldridge and others 2011). Though it could theoretically be driven by a decrease in N losses (gas or hydrologic) resulting from encroachment, this ‘island of fertility’ effect has usually been attributed (at least in part) to greater rates of dry N deposition to tree canopies compared to adjoining grasslands or bare soil (Archer and others 2001; Boutton and Liao 2010). This occurs because certain N-containing compounds, especially ammonia, have greater deposition velocities to tall, aerodynamically rough woody canopies than to relatively low statured grass or herbaceous vegetation (Fowler and others 1989; Bobbink and others 2010). However, the relative influence of differential dry deposition versus BNF on soil N accrual during encroachment of *Prosopis* or other woody legumes has not been quantitatively explored.

We studied a well-characterized encroachment chronosequence of developing *Prosopis glandulosa* clusters in a grassland matrix to infer fixed N inputs over the last 100 years. We measured soil N accumulation associated with *Prosopis* clusters of known ages and developed a mass balance using soil and biomass N accrual, N gas and leaching loss and N deposition values to constrain potential BNF rates. We also varied rates of dry N deposition to *Prosopis* canopies to assess the effect of differential deposition on N accumulation around encroaching trees (Archer and others 2001).

## MATERIALS AND METHODS

### Study Site

Sampling was conducted at the Texas A&M AgriLife La Copita Research Area (27°40′N, 98°12′W) in the eastern Rio Grande Plains during 2012, 2013 and 2014. The site consists of savanna parkland with discrete woody patches dominated by one or more *Prosopis glandulosa* (Torr.) var *glandulosa* (honey mesquite, referred to here as *Prosopis*) individuals with a mixed woody understory (including *Zanthoxylum fagara, Celtis pallida, Condalia hookeri* and *Diospyros texana*), embedded in a matrix of $C_4$ grasses, forbs and bare ground. At the time of sampling (2012–2014), forb cover in remnant grasslands was limited or absent compared with historical averages, likely due to low rainfall (Thomas Boutton, personal communication). *Prosopis* is a leguminous tree in the family Fabaceae and generally considered to be capable of substantial rates of BNF (Shearer and others 1983). Though it nodulates readily under both glasshouse and natural conditions (including at this site), its nodules are very difficult to recover under field settings, partly because they occur deep ($>1$ m) in the soil profile (Virginia 1986; Johnson and Mayeux 1990; Zitzer and others 1996). Putatively N-fixing *Acacia farnesiana* occurs at the site but is limited to a few scattered individuals.

The climate at this site is subtropical with typically warm, moist winters and hot, dry summers. Mean annual precipitation is 680 mm, occurring year-round with maxima in May and September. Mean annual temperature is 22.4°C with an average growing season of 289 days (Archer and others 1988). Soils at the site are sandy loams (Typic and Pachic Argiustolls) with little to no topography. The sampling plot measured 260 × 215 m and has been fenced to exclude livestock grazing since 1985. Fire history for the site is not formally known, but documentation (beginning ~1950) does not record any burning for this plot, and heavy grazing prior to that likely kept fire probability low (Bai and others 2013).

### Plant Density and Demography Measurements

Basal diameter was measured for all *Prosopis* individuals within a 200 × 100 m (2 ha) area within the sampling plot. For single-bole individuals, tree ages were calculated from basal diameter according to Stoker (1997), using equations developed specifically for upland single-bole *Prosopis* at this site. For plants with multiple or split boles at the soil surface, these equations were applied to each stem, possibly underestimating the age of some trees within the plot. Approximately 2.5 trees per hectare had basal diameters at least 40 cm (up to 52 cm), exceeding the range for which the size-age equations were developed.

### Soil Sampling and Analysis

All soil samples were taken from discrete *Prosopis* clusters, defined as individual, single-stemmed *Prosopis* trees with a non-fixing woody understory, surrounded completely by remnant grassland and at least 6 m from the dripline of another *Prosopis* individual. All remnant grassland samples were taken at least 2 m from the drip line of the nearest woody plant and at least 4 m from the drip line of a *Prosopis.* To characterize N distribution around trees, soil cores (0–15 and 15–30 cm depth) were taken every 30 cm outward from the bole in an easterly direction to about 1 m past the drip line of eight *Prosopis* individuals ranging in age from 28 to 99 years (7–14 cores each). Surface litter, if present, was brushed aside to sample mineral soil. This age range was selected based on the availability of trees at the site that matched the selection parameters. *Prosopis* at this site displays no directional pattern of understory plant density (Franco-Pizaña and others 1995). Ten cores (0–15 and 15–30 cm depth) were sampled in remnant grassland areas.

All soil samples were passed through a 2 mm screen to remove large organic fragments (minimal or not present) and dried for 3 days at 105°C. Unsieved replicate cores were used to determine bulk density, using a core of 4 cm diameter. No obvious soil compaction occurred in these sandy soils and no rocky material was present in the samples. Soils were homogenized using a ball mill (Retsch, MM-2, Haan, Germany) and analyzed for $\delta^{15}$N and %N using a continuous flow isotope ratio mass spectrometer (Model Delta V Advantage; Thermo-Scientific, Bremen, Germany). All isotope analyses were conducted at the Cornell University Stable Isotope Laboratory (COIL).

## Plant Biomass and N Content Calculations

Above-ground biomass ($AGB_P$) N accrual in *Prosopis* tissue (large stems, small stems, standing dead wood and foliage) was calculated using equations developed by Northup and others (2005) specifically for *Prosopis glandulosa* at this site. *Prosopis* below-ground biomass ($BGB_P$) was calculated as a percentage of $AGB_P$ (35 ± 11%, BGB fraction for *Prosopis caldenia* in semi-arid Argentinian savannas; Risio and others 2014). Proportion of $BGB_P$ composed of coarse versus fine roots was taken as the average of twelve monthly measurements for the top 10 cm of soil in *Prosopis* groves at this site (44 ± 8% coarse, 56 ± 8% fine; Hibbard and others 2001). N content for *Prosopis glandulosa* coarse roots was assumed to be the same as stem wood (0.93 ± 0.28% N; Northup and others 2005) and for fine roots, was assumed to be 1.99% N (value for *Prosopis juliflora*; Jha and Mohapatra 2009). Proportion $BGB_P$ and root size distribution was held constant with tree age.

To determine N content in understory shrubs, the relationship between woody understory area (for understory shrubs with basal diameter ≥4 cm) and *Prosopis* age was derived from Archer and others (1988). This canopy area was then converted to equivalent N mass ($AGB_U$) using N concentration equations from Northup and others (2005). Insufficient data were available to calculate root biomass N for these species.

Grassland-specific above-ground biomass and below-ground biomass ($AGB_G$ and $BGB_G$) values for this site were taken from Boddey and others (2000) and Liu and others (2010) and N concentrations in common grassland species from Hartley and others (2007) and Creamer and others (2013).

## Other N Inputs and Losses

Atmospheric wet N deposition ($NH_4^+$ and $NO_3^-$) rates were obtained from a National Atmospheric Deposition Program (NADP) monitoring station at Beeville, TX (~90 km northeast of the study site) for the years 1984–2014 (www.nadp.sws.uiuc.edu). Modeled dry N deposition rates for the La Copita site for the years 2000–2013 came from the NADP Total Deposition maps (www.nadp.sws.uiuc.edu/committees/tdep/tdepmaps/). Average values for each of these fluxes were calculated and held constant over the last century.

N trace gas flux rates (sum of NO, $NO_Y$, $NH_3$ and $N_2O$) were measured for upland *Prosopis* groves and grasslands at this site from 2012 to 2014 and scaled to annual rates using temperature and rainfall (Soper and others 2016a). Rates are averages for 2000–2010 and were held constant for the last century. Proportional $N_2$ flux rates were taken from Soper and others (2016b). Maximum potential $N_2$ emissions following soil wetting events of 15 mm or greater were applied to annual estimates of post-wetting N fluxes from Soper and others (2016a). $N_2$ production between rainfall events was considered to be negligible.

No areal leaching values (to below 30 cm) are available for this site. Leaching values for both grassland and *Prosopis* areas were initially set at 4 kg N $ha^{-1}$ $y^{-1}$, comparable to values for mixed grass/*Prosopis* cover (below 25 cm) in the Jornada basin (3.8 kg N $ha^{-1}$ $y^{-1}$; Reichmann and others 2013). Though *Prosopis* root biomass at 0–30 cm and below is greater than for grassland (Weltz and Blackburn 1995; Midwood and others 1998), potentially decreasing leaching losses, inorganic N concentrations are greater beneath *Prosopis* and have been observed to correlate with leaching rates (Reichmann and others 2013). Both cover types show similar low or negligible rates of deep hydrologic infiltration under normal conditions (Weltz and Blackburn 1995), but sporadic large rainfall events may lead to episodic leaching. Runoff is likewise low and comparable under both cover types, though may also be impacted by sporadic large rainfall events (Weltz and Blackburn 1995).

## Estimating BNF by Difference

A mass balance framework was developed to estimate BNF inputs for seven individual *Prosopis* tree clusters of known ages, according to the premise

$$\frac{dN}{dt} = I - O \qquad (1)$$

where N is the change to the standing N pool (soil and biomass N) of each cluster (which is initially equal to standing soil and biomass pool under grassland cover). Inputs ($I$) consist of atmospheric N deposition and BNF, and outputs ($O$) consist of hydrologic leaching and N gas flux. Variables are summarized in Table 1, and the model is represented in Figure 1.

Total contemporary soil N beneath individual *Prosopis* trees was calculated from radial soil core samples, adjusted for bulk density, to 30 cm depth. Total soil N was integrated over a circular area, the

**Table 1.** Mass Balance Input Variables

| | Component | Grassland value | *Prosopis* value | Source |
|---|---|---|---|---|
| Fluxes | N trace gas loss | 0.65 ± 0.03 | 0.56 ± 0.02 | Soper and others (2016a) |
| | $N_2$ loss | 0.07 ± 0.00 | 0.10 ± 0.00 | Soper and others (2016b) |
| | Wet N deposition ($NH_4^+ + NO_3^-$) | 2.77 ± 0.81 | 2.77 ± 0.81 | National Atmospheric Deposition Program (Beeville, TX, 1984–2013). |
| | Dry N deposition | 3.88 ± 0.81 | 3.88 ± 0.81 | National Atmospheric Deposition Program (modeled, 2000–2013) |
| | Leaching | 4 ± 1 | 4 ± 1 | Reichmann and others (2013) |
| Standing pools | Above ground biomass N | 8.11 ± 2.82 | Function of tree age (0.1–5.6 kg per tree) | Grassland: Liu and others (2011), Creamer and others (2013). *Prosopis*: Northup and others (2005). Woody understory: Archer (1988), Northup and others (2005) |
| | Below-ground biomass N | 10.08 ± 8.03 | Function of tree age (0.1–2.0 kg per tree) | Grassland: Liu and others (2011), Creamer and others (2013). *Prosopis:* Proportion BGB from Risio and others (2014). Proportion coarse/fine roots from Hibbard and others (2001). Root N content from Jha and Mohapatra (2009), Northup and others (2005) |
| | Bulk soil N (0–30 cm depth) | 1965 ± 358 | Function of tree age (0.1–10.1 kg per tree) | Measured |

*Rate values are in units of kg N ha$^{-1}$ y$^{-1}$ unless indicated. Standing stock values are in units of kg N ha$^{-1}$. Values are mean ± 1 SD. BD basal diameter in cm.*

radius of which was determined by the distance from the tree bole to the point at which N concentration (per unit soil volume) coincided with average grassland levels. This circular area is referred to as the area of influence (AOI) of a given tree. AOI increased linearly with tree age ($\text{AOI} = 0.47 \times \text{age} - 8.86$, $r^2 = 0.65$).

Separate annual N gas loss rates (N trace gases plus $N_2$) were used for grassland and beneath *Prosopis* canopies (gaseous N flux, $GF_G$ and $GF_P$, respectively). Wet and dry deposition (total N deposition, TD) rates were initially held equal for both grassland and *Prosopis* canopies. Leaching rates were initially held equal for both cover types ($L_G$ and $L_P$). Age-specific *Prosopis* biomass N and woody understory biomass N ($AGB_P$, $BGB_P$ and $AGB_U$) were summed to generate a value for total biomass N ($TB_P$).

N concentration in the grassland at the time of *Prosopis* cluster establishment was subtracted from contemporary soil N to calculate accrual of soil N under *Prosopis* canopies ($S_P$). This historical grassland N concentration (the ‘grassland baseline’, $S_{G\ n=0}$) was calculated by taking average contemporary grassland soil N ($S_G$) plus grassland biomass N ($TB_G$), subtracting rates of total N deposition (TD)

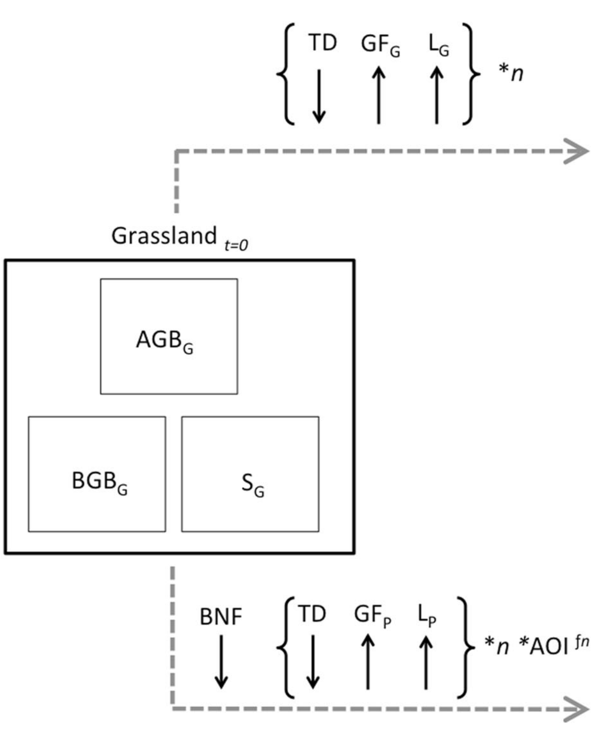

**Figure 1.** Schematic of the N mass balance model for two landscape elements, remnant grassland patches or *Prosopis* clusters, representing N pools (*boxes*) and fluxes (*arrows*). Historical grassland patches (Grassland $_{t=0}$) can either remain as grassland over time ($n$ years, Grassland $_{t=n}$) or develop into tree clusters of age $n$ (*Prosopis* $_{t=n}$). The standing N pool for each landscape element is made up of above ground biomass (AGB), below-ground biomass (BGB), and soil (S) pools. N fluxes into and out of the system consist of wet and dry atmospheric N deposition (TD), N gas emissions (GF), or leaching through the soil profile (L). The total magnitude of these inputs or outputs for each landscape element is determined by time since cluster formation ($n$) and for *Prosopis* also by cluster area (AOI, a function of $n$). The final term, biological N fixation (BNF), is calculated as the difference between total N for Grassland $_{t=0}$ *and Prosopis* $_{t=n}$ for each cluster. The resulting value (total N fixed per tree) is then regressed against tree age ($n$) to estimate the rate of BNF.

and adding gas loss ($\mathrm{GF_G}$) and leaching loss ($L_\mathrm{G}$) for $n$ years, where $n$ = tree age.

$$S_{\mathrm{G}n=0} = S_\mathrm{G} + \mathrm{TB_G} - \mathrm{GF_G} \times n - L_\mathrm{G} \times n - \mathrm{TD} \times n \tag{2}$$

For individual *Prosopis* trees at current age $n$, N inputs derived from fixation (BNF) for the cluster AOI were calculated according to:

$$S_\mathrm{P} + \mathrm{TB_P} = \mathrm{BNF} + S_{\mathrm{G}n=0} + \mathrm{TD} \times \mathrm{AOI}^n + \mathrm{GF_P} \times \mathrm{AOI}^n + L_\mathrm{P} \times \mathrm{AOI}^n \tag{3}$$

where AOI increased linearly as a function of tree age (see above).

The BNF value for each cluster was then regressed against cluster age to derive fixation rate. To generate areal BNF estimates, this per-tree BNF rate was scaled using measured tree age and density for the plot.

## Sensitivity Analysis

Single-parameter sensitivity analysis (Katz 2002) was applied to determine which components (pools, fluxes and scaling parameters) of the mass balance most strongly affected estimates of BNF. Most parameters were initially set to their mean values (Table 1) and then one parameter at a time was varied by ±1 SD. The mass balance was then recalculated and the resulting BNF estimate expressed as a percentage change from the initial (base) scenario. The grassland bulk soil N value (used to define the area of N accumulation around *Prosopis* clusters) was varied only by +1 SD, because soil N sampled radially away from trees usually did not reach as low as the grassland mean −1 SD (Figure 6A) and thus this accumulation value could not be computed. No error estimate was available for total biomass N (*Prosopis* and understory N) and so the proportional error for total biomass was used instead (Northup and others 2005). No error estimate was available for %N value for fine roots (Jha and Mohapatra 2009). For leaching and deposition, varying values had no effect on BNF estimates when the same rate was applied to both cover types. Because of high uncertainty in the leaching parameter, several additional scenarios were tested in which leaching was also increased or decreased with encroachment, by 50–200% of the mean. For the final scaling parameter (applying the BNF-tree age function to measured tree age/density distribution from the field site), the sensitivity analysis tests the effect of including or excluding multiple boles per tree.

## Differential Dry N Deposition Scenarios

Additional sensitivity analyses were conducted to determine whether differential deposition to woody canopies (versus low-stature grasslands) is an important driver of N accumulation in tree clusters that might influence estimates of BNF. It is challenging to develop a simple representation of N deposition to individual *Prosopis* canopies versus grassland, due to the myriad factors that affect deposition to vegetative surfaces (including N species, concentration, boundary layer, canopy structure, stomatal conductance, surface wetness etc.; (Shearer and Kohl 1986; Wesely and Hicks 2000). Three simplified deposition scenarios were considered: even dry N deposition across the landscape, dry deposition weighted by surface area (calculated leaf area for *Prosopis* individuals, with grassland considered to be a plane equal to ground surface area) and dry deposition weighted by surface area *plus* two times greater deposition velocity for *Prosopis* leaf surfaces. Total dry deposition was held constant at 3.88 kg N ha$^{-1}$ y$^{-1}$ and background deposition rates to grassland were reduced to compensate for increased canopy deposition. Changes in percentage canopy cover over time were calculated from current tree age distribution, plus allometric equations developed by Stoker (1997). *Prosopis* leaf area was found by calculating foliar biomass as a function of basal diameter (Houghton and others 1999; Pacala and others 2001; Northup and others 2005), and multiplying by average specific leaf area values for the species (Asner and Martin 2004; Bai and others 2008). As *Prosopis* is winter deciduous, it was assumed that trees had no standing foliage for 3 months per year (Stock and others 1995; Archer and others 2001; Nelson and others 2002; Martin and others 2003; Boutton and Liao 2010; Barger and others 2011; Creamer and others 2013).

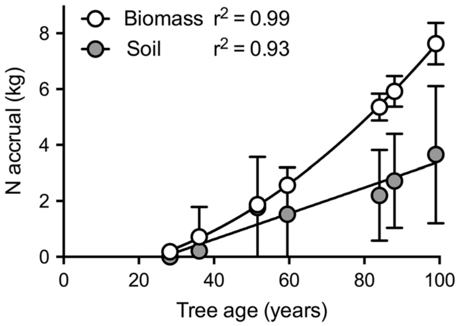

**Figure 2.** N accrual in plant biomass (*Prosopis* and understory) and soil N (new N, above grassland baseline at time of establishment) for *Prosopis* clusters of varying ages. $n = 7$. Values are for individual *Prosopis* trees ± 1 SD.

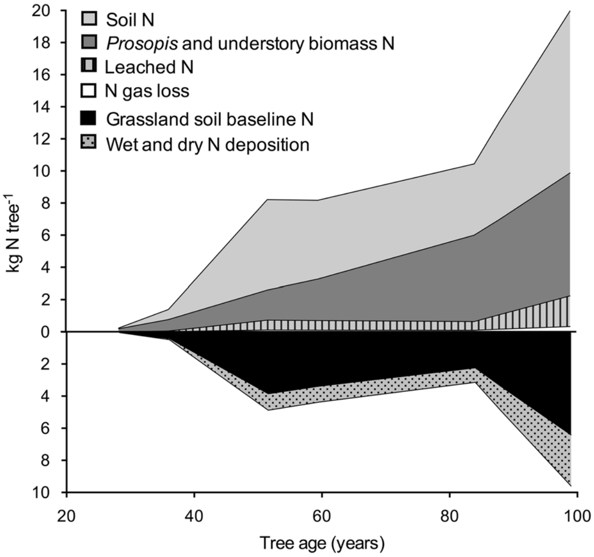

**Figure 3.** Mean N inputs and losses for individual *Prosopis* trees of varying ages (kg N tree$^{-1}$ over tree lifetime). $n = 7$ trees. BNF is calculated as the sum of N in soil and *Prosopis* tissue (above and below-ground, plus aboveground understory tissue), plus gaseous N losses, minus N derived from deposition, minus baseline N concentration in pre-encroachment grassland at the time of establishment (adjusted for grassland atmospheric deposition and gas loss).

## Statistical Analysis

Statistical analyses were performed in R (R Core Team 2014) and GraphPad Prism 6.0 (GraphPad Software Inc, La Jolla, CA, USA). Best-fit regressions for tree age versus other parameters (N accumulation in soil and biomass, mass balance

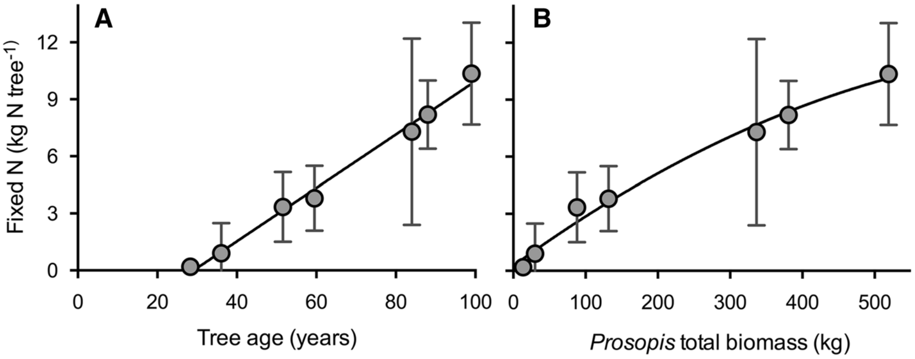

**Figure 4.** Mass balance output for total N derived from BNF (kg) per tree versus **A** tree age (years) or **B** total above- and below-ground biomass per tree (kg). Values are for individual *Prosopis* trees ± 1 SD.

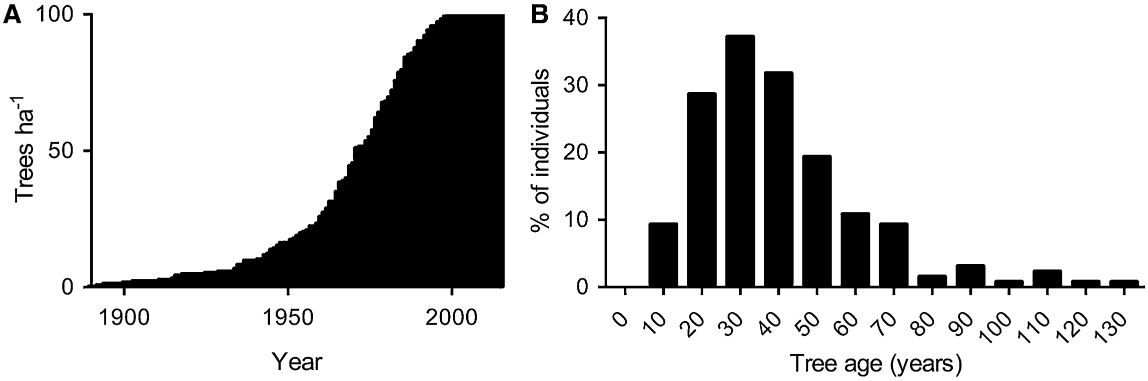

**Figure 5.** **A** Upland *Prosopis glandulosa* density (trees ha$^{-1}$) from 1890–2010 in a 2-ha upland area of the La Copita Research Area and **B** age distribution of trees in 2014. Ages calculated from basal diameter according to Stoker (1997) ($n$ = 201).

BNF estimates, AOI) were selected using Akaike's Information Criteria (AIC). %N values were first subject to Johnson Su transformation to improve normality. ANCOVA was used to test for significant differences in slopes between differential N deposition scenarios.

## RESULTS

### N Mass Balance Along the Prosopis Age Chronosequence

If N deposition additions and N gas and leaching losses remain constant in this ecosystem over time, the balance of the two would lead to a net annual N addition of 1.93 kg N ha$^{-1}$ y$^{-1}$ (grassland values, Table 1). This is equivalent to an increase in grassland N stocks (at 0–30 cm depth) of approximately 5% N over the last 50 years and is consistent with soil N values measured at this site between 1990 and 1995 (Archer 1995). Because of this slight imbalance in inputs and losses, the baseline grassland soil N content (kg N ha$^{-1}$, 0–30 cm) increases through time and is described by the equation $N = -1.93 \times YBP + 1965$, where YBP = years before present (2014).

Accrual of N in soil since *Prosopis* cluster establishment (above the grassland baseline) increases linearly with *Prosopis* age from 28 to 99 years (N (kg) = 0.0462 × age − 1.223, $r^2 = 0.93$, $P < 0.0005$; Figures 2, 3). Combined N accrual in *Prosopis* and understory biomass also increases with tree age, according to N (kg) = 0.0007 × (age)$^2$ + 0.0099 × (age) − 0.6434 ($r^2 = 0.93$, $P < 0.0001$; Figures 2, 3). Data from Archer and others (1988) and Archer (1995), and observations during this study indicate that understory biomass does not begin to accrue in a cluster until the central *Prosopis* is about 30 years old.

When grassland baseline N, gas loss, leaching, wet and dry N deposition estimates (for a given cluster area and age) are subtracted from biomass and soil accrual N values, the remainder is attributed to inputs of fixed N from *Prosopis* (Figures 3, 4). This remainder is positive and increases linearly with tree age according to the equation: N (kg per tree) = 0.1404 × (age) − 4.086 ($r^2 = 0.99$, $P < 0.0001$; Figure 4A), with net N accrual from BNF in

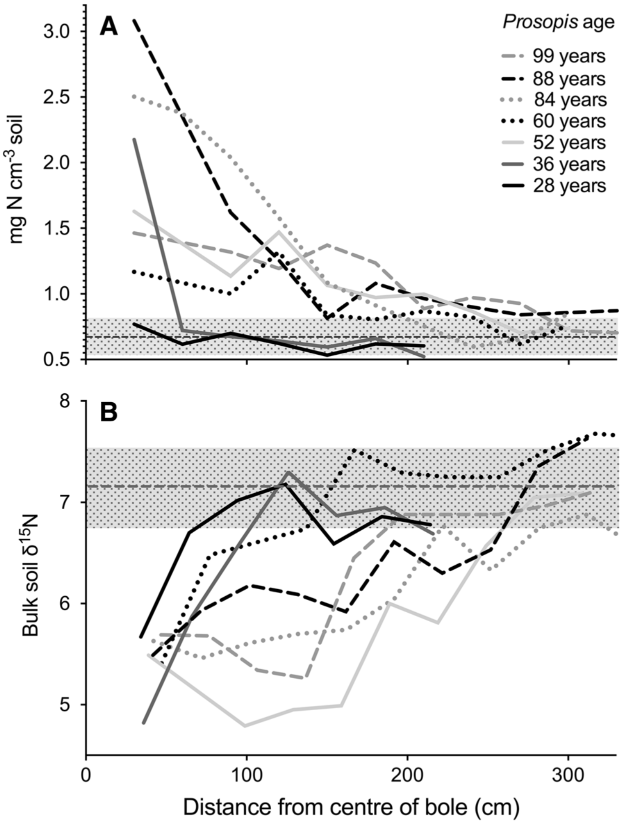

**Figure 6.** **A** Soil N concentration (mg N cm$^3$ soil) and **B** $\delta^{15}$N with distance from bole for *Prosopis glandulosa* trees of varying ages (0–15 cm depth). For both *panels, gray dashed line* and *shaded area* represents average grassland soil N concentration or $\delta^{15}$N, ±1 SD ($n$ = 10).

a cluster beginning at 29 years after *Prosopis* establishment. Expressed as a function of *Prosopis* tree biomass (above- and below-ground), the rate of N accrual from BNF declines as biomass increases ($1.661 \times 10^5 \times (\text{age})^2 + 0.0276 \times (\text{age}) + 0.2641$, $r^2 = 0.99$, Figure 4B).

## Prosopis Density and Demography

Average 2014 upland *Prosopis* density at the site was 101 trees ha$^{-1}$ (Figure 5A), corresponding to canopy area cover of 13.6%. Of these, 19% of trees had more than one stem arising from the base. *Prosopis* stems ranged in age from 13 to 131 years, with a mean age of 45.3 years (Figure 5B).

## Scaling to Landscape

Extrapolating the *Prosopis* BNF–age relationship to the landscape scale (using measured tree density and age; Figure 5) generates a contemporary annual BNF estimate of 10.9 ± 1.8 kg N ha$^{-1}$ y$^{-1}$ for this upland semi-arid, subtropical savanna woodland. Accounting for increasing *Prosopis* density and maturity in the plot over time (Figure 5), this is equal to 249 ± 60 kg ha$^{-1}$ of fixed N inputs from *Prosopis* since encroachment began about 130 years ago. Extrapolating *Prosopis* basal diameter-age equations beyond the 100 years they were originally calibrated for had the effect of increasing total BNF estimates by 2.0% (data not shown).

## Sensitivity Analysis

Thirteen components of the mass balance (11 pool/flux values and two scaling parameters) were varied to determine the sensitivity of estimated areal BNF rates to input values (Table 2). When each pool or flux value was varied by 1 SD, estimates of BNF were most affected by variation in the bulk soil N concentration parameter (11%), followed by below-ground biomass estimates for *Prosopis* (6%; Table 2). Error associated with the tree age-fixed N regression (Figure 4A) affected areal BNF estimates by 9–10%. For about 20% of *Prosopis* trees with multiple stems, counting stems individually likely decreased estimates of tree age while increasing estimates of tree density. If ignoring multiple stems, areal BNF estimates were reduced by 18%, to 8.9 kg N ha$^{-1}$ y$^{-1}$ (Table 2).

Estimates of fixed N inputs are relatively insensitive to rates of dry deposition to *Prosopis* canopies. Assuming dry deposition of 3.88 kg N ha$^{-1}$ y$^{-1}$ (constant over time) and weighting deposition by *Prosopis* leaf area decreases annual BNF by 6.3% (to 10.2 kg N ha$^{-1}$ y$^{-1}$, Table 2), in comparison with even deposition (no significant difference in slope,

**Table 2.** Relative Importance of Each Variable in the Mass Balance, Representing Percent Change in Estimated Areal BNF (kg N $ha^{-1}$ $y^{-1}$) When Each Component was Varied as Indicated

| Component | Varied by | Relative error (%) |
|---|---|---|
| Pools | | |
| Above-ground biomass N | ±1 SD | |
| Grassland | | < 1 |
| *Prosopis* | | −2 to 2 |
| Below-ground biomass N | ±1 SD | |
| Grassland | | < 1 |
| *Prosopis* | | −6 to 6 |
| Bulk soil N | +1 SD[b] | 11 |
| Fluxes | | |
| N Gas loss | ±1 SD | |
| Grassland | | > −1 to < 1 |
| *Prosopis* | | > −1 to < 1 |
| Wet N deposition[a] | | 0 |
| Dry N Deposition | Leaf area-weighted | 6 |
| | Leaf area-weighted + 2 × deposition velocity | 14 |
| Leaching | Increasing with encroachment by | |
| | 2 kg N $ha^{-1}$ $y^{-1}$ | 2 |
| | 4 kg N $ha^{-1}$ $y^{-1}$ | 5 |
| | 8 kg N $ha^{-1}$ $y^{-1}$ | 10 |
| | Decreasing with encroachment by | |
| | 2 kg N $ha^{-1}$ $y^{-1}$ | −2 |
| | 4 kg N $ha^{-1}$ $y^{-1}$ | −5 |
| | 8 kg N $ha^{-1}$ $y^{-1}$ | −9 |
| Scaling parameters | | |
| Tree age-BNF function | $S_{yx}$ | −10 to 9 |
| Tree age distribution and density | Excluding multiple stems per tree | 18 |

*Leaf area weighted = dry deposition weighted by average annual leaf area (where leaf area is proportional to tree age for Prosopis and grassland considered to be 1 $m^2/m^2$).*
*Leaf area weighted + 2 × deposition velocity = dry deposition weighted by surface area plus two times greater deposition velocity to Prosopis leaf surfaces than to grassland.*
*$S_{yx}$ = standard error of the estimate for linear regression.*
*[a] Because the same rate is applied to both grassland and Prosopis cover, changing the input parameter does not affect estimates of BNF.*
*[b] Value for +1 SD only. Soil N around most Prosopis trees did not reach as low as the mean grassland value −1 SD, so this value could not be calculated (Figure 6B).*

$P < 0.127$), Figure 4). Further adjusting this rate by assuming both leaf area-weighted deposition and a doubling of deposition rate to *Prosopis* leaf surfaces reduces BNF by 13.5% (to 9.4 kg N $ha^{-1}$ $y^{-1}$, Table 2; $P < 0.008$).

Varying rates of leaching for grassland and *Prosopis* cover affected estimates of BNF. The base scenario (4 kg N $ha^{-1}$ $y^{-1}$) maintains a fairly steady state of N inputs (deposition) versus losses (trace gases and leaching) in the grasslands pre-encroachment (net accrual 1.9 kg N $ha^{-1}$ $y^{-1}$). If grassland leaching values were significantly higher or lower, this implies significant accrual or depletion of grassland N over the last century and there is no evidence that this has occurred. When leaching was varied during woody encroachment (different rates applied to grassland and *Prosopis* clusters), the effect on BNF estimates increased with the difference between rates. Increasing leaching during encroachment by either 2, 4 or 8 kg N $ha^{-1}$ $y^{-1}$ (for example, by setting the rate of grassland leaching to zero and the rate for *Prosopis* clusters to 2, 4 or 8 kg N $ha^{-1}$ $y^{-1}$) increased BNF estimates by 2.4, 4.7 and 10.0%, respectively (Table 2). Assuming the opposite (leaching rates decrease during encroachment by the same magnitudes) reduced BNF estimates by 2.4, 4.9 and 9.5%, respectively (Table 2). Because gas losses from this ecosystem are both comparatively small and do not differ significantly between *Prosopis* and grassland cover (Table 1, Soper and others 2016a; b), the resulting sensitivity to these parameters is less than 1%.

## N and $\delta^{15}$N Distribution in Soil

There was a positive relationship between *Prosopis* age and distance to which the tree influenced soil N content and $\delta^{15}$N in the top 15 cm of soil (Figure 6A, B), where N concentrations were highest and $\delta^{15}$N values lowest closer to the tree bole. For N content, this relationship was described by $D = 3.726 \times \text{age} - 5.3151$ ($D$ = distance in cm from the center of the *Prosopis* bole, $r^2 = 0.65$, $P > 0.03$). For $\delta^{15}$N, this relationship was described by $D = 2.3164 \times \text{age} + 60.949$ ($r^2 = 0.40$, $P > 0.13$). For $\delta^{15}$N, values increased significantly with distance from the bole for trees older than 28 years ($P < 0.05$). For %N, values decreased significantly moving away from the bole for trees older than 36 years ($P < 0.05$).

## DISCUSSION

Applying a mass balance approach to N inputs and losses in developing woody clusters in a subtropical savanna over about 100 years, we find that nitrogen fixation by *Prosopis glandulosa* represents an input of 0.14 kg N tree$^{-1}$ y$^{-1}$ for trees aged 29 years and older. This is equivalent to 10.9 ± 1.8 kg N ha$^{-1}$ y$^{-1}$ at current *Prosopis* densities. BNF thus represents a large input term and is primarily responsible for the observed increases in ecosystem N storage during *Prosopis* encroachment. The mass balance is relatively insensitive to changing distribution of dry atmospheric N deposition, which has been previously identified as a potential driver of N accumulation in this system (Archer and others 2001; Boutton and Liao 2010). BNF rates are most sensitive to error associated with scaling from a per tree to areal basis, which can affect estimates by up to 18% (2.0 kg N ha$^{-1}$ y$^{-1}$). $\delta^{15}$N patterns in soil, where $\delta^{15}$N is lowest adjacent to *Prosopis* boles and increases moving toward remnant grassland, are also consistent with *Prosopis* trees being a significant point source of isotopically light (~0‰, Shearer and Kohl 1986) fixed N.

### N Accrual and BNF in Time and Space

The balance between N accrual, gas loss and deposition inputs since establishment was negative for all *Prosopis* clusters measured in this study, indicating a missing source of N that is almost certainly *Prosopis* BNF. Total N attributed to BNF increased linearly with tree age from 28 to 99 years. This suggests that after trees begin accruing N, BNF inputs remain constant even as trees increase in size and soil N content increases. However, because the rate of tree biomass accrual increases with time, BNF on a per unit biomass basis decreases as trees mature. Net N accrual in 28-year-old trees, the youngest we measured, was very small (less than 0.2 kg), and regression equations fitted to all trees indicated that 29 years is the approximate threshold at which N begins to accrue. The lack of younger trees identified at the site may be a legacy of recent drought-induced mortality of smaller individuals, or suppression of establishment by increased grass growth after the cessation of grazing at the site approximately 30 years ago.

There is conflicting evidence regarding the relationship between tree age, size and BNF rates. Increasing soil inorganic N concentrations associated with tree development would usually be expected to suppress BNF (Archer and others 1988; Hartwig 1998). However, in this case, previous isotopic measurements suggested that contribution of BNF to tree N nutrition increases with age (Northup and others 2005; Soper and others 2015), and this study found evidence that rates of BNF remain stable over development. Several features of this system may explain why this pattern differs from that seen elsewhere, perhaps as a result of shifting limitations at different growth or establishment phases. Trees in this system establish into a grassland system with generally low availability of resources needed to support BNF (particularly water and soil phosphorus; Hartwig 1998) and initially have a relatively small root system with which to access these resources. Trees grow slowly at this site, and a 28-year-old *Prosopis* still has a basal diameter of only approximately 8 cm (Stoker 1997; Eldridge and others 2011) and height of less than 2 m. Resource limitation early in life may also explain why *Prosopis* does not appear to begin fixing N for some time, although demand for N may be strong. As root systems expand with age, the ability to acquire these resources may increase, along with their general availability. Available soil P, for example, increases dramatically with time since encroachment (Kantola 2012), and soil moisture is greater beneath shaded, well-developed canopies (Archer and others, 2001). Increasing resource availability could thus allows for proportionally increasing rates of BNF with tree age (Boutton and Liao 2010; Soper and others 2015) to support, without necessarily saturating, plant demand for N.

Though encroachment has occurred at this site for the past 100–200 years, this process has not yet gone to completion on many upland portions of the landscape (Archer 1995; Archer and others 2001; Boutton and Liao 2010). We found that more than half of the trees surveyed in an upland plot were 40 years or younger, with a fifth 29 years or younger. As we did not measure net N accrual from BNF until this age, it is likely that BNF rates in this ecosystem will continue to increase as these trees mature. Assuming no recruitment or mortality, maturation of existing trees would be expected to increase BNF rates to about 14.2 ± 0.6 kg N ha$^{-1}$ y$^{-1}$ within the next 15 years.

## Sensitivity to Atmospheric Deposition Rates

N deposition represents a significant input of N to this system, accounting for ~31% of total N accrual observed in *Prosopis* clusters after 100 years. Some authors have suggested that differential dry N deposition during encroachment may explain in part the N soil enrichment frequently observed beneath woody invaders (Fowler and others 1989; Bobbink and others 2010; Boutton and Liao 2010; Eldridge and others 2011). We examined this by varying rates of deposition to *Prosopis* canopies in our calculations. Though other woody biomass is present in the woody clusters we sampled, the central *Prosopis* individuals represented the greatest height and leaf area (Shearer and Kohl 1986; Archer and others 1988; Boddey and others 2000). Although it is challenging to calculate actual deposition rates, scenarios in which we weighted dry deposition by *Prosopis* leaf area or leaf area plus two times deposition velocity reduced total N attributed to BNF by only about 13% (within the uncertainty range of our base estimate) over the last 100 years. This suggests that while uneven deposition could play a role in N accrual beneath woody species, it is not the primary driver of N enrichment in this ecosystem.

We made the assumption that total wet and dry deposition rates in this region remained constant over time and have no reason to believe that this scenario is not broadly true. While the proximity of the site to coastal oil refineries may contribute to N deposition, these refineries have been active since the 1930s, comprising most of the time period considered in this study. Additionally, total deposition rates (averaging 6.5 kg N $ha^{-1}$ $y^{-1}$ for 2000–2013, NADP) for this area are relatively low compared with more developed parts of North America and so likely do not reflect strong temporal increases associated with anthropogenic development seen elsewhere.

## Comparison to Other Fixation Estimates

Estimates of BNF by woody legumes vary widely. Though our values fall on the low end of those estimated for other natural *Prosopis*-dominated systems, this likely reflects lower plant density and environmental conditions (especially low water availability) at this site. Rundel and others (1982) used a mass balance approach to estimate BNF rates of 23–40 kg N $ha^{-1}$ $y^{-1}$ in a non-water-limited stand with 34% *Prosopis glandulosa* cover in the Sonoran Desert. In a water-limited stand in Texas, Johnson and Mayeux (1990) used nodule abundance to estimate rates of 45–150 kg N $ha^{-1}$ $y^{-1}$ at 50% canopy cover. By contrast, we estimated contemporary BNF rates (for ~ 14% *Prosopis* canopy cover) of 10.9 ± 1.8 kg N $ha^{-1}$ $y^{-1}$, increasing to 14.2 ± 0.6 kg N $ha^{-1}$ $y^{-1}$ when all existing individuals reach maturity. Adjusting for canopy cover would bring our estimate into the lower end of the other reported ranges. In a 10-year-old irrigated planting of another sub-species of *P. glandulosa,* Abrams and others (1990) used a simple mass balance to calculate BNF rates of around 100 kg N $ha^{-1}$ $y^{-1}$. Given the strong relationship between water availability and potential symbiotic fixation in *Prosopis* and other legumes (Zitzer and others 1996; Zahran 1999; Archer and others 2001), it is likely that the much higher fixation rates calculated for that system reflect physiological than methodological differences. By contrast, López Villagra and Felker (1997) found much lower rates (2–2.8 kg N $ha^{-1}$ $y^{-1}$) in another planted stand using a foliar $\delta^{15}$N approach. Given that our previous work in *Prosopis* has identified significant issues with application of the foliar $\delta^{15}$N method (Soper and others 2015), we conclude that mass balance provides a more robust approach for estimating BNF rates in *Prosopis*.

What is the significance of N additions during woody legume encroachment to N budgets at a larger scale? Though continental-scale areal estimates of *Prosopis* cover (and of encroachment and N-fixing plant cover more generally) are scarce (Sobota and others 2013), first-order estimates suggest that contributions may be significant. Van Auken (2000) estimates that the genus *Prosopis* (species *glandulosa, velutina, toreyana and justiflora*) is the dominant woody plant across 38 million ha of semi-arid grasslands of southwestern North America. Assuming *Prosopis* canopy cover of 10–20% over this area (Johnson and Mayeux 1990), and BNF rates from this study of 10.9–14.2 kg N $ha^{-1}$ $y^{-1}$, this generates total *Prosopis* N inputs across North America of 0.2–0.6 Tg N $y^{-1}$. Estimates for total non-cultivated BNF in the USA vary widely (0.5–12.2 Tg N $y^{-1}$ Sobota and others 2013), with recent estimates in the range of about 6.4 Tg N $y^{-1}$ (SAB 2011). Thus, *Prosopis* cover and encroachment may account for between 3 and 9% of total natural BNF on a continental scale.

## Robustness of Mass Balance Approach

We considered wet and dry atmospheric deposition, hydrologic leaching, symbiotic fixation and N trace gas/$N_2$ loss as the primary fluxes of N in and out of spatially discrete *Prosopis* clusters, and soil (to 30 cm), *Prosopis* biomass and woody understory biomass as the primary N accrual reservoirs. Further, we assumed that the baseline level of soil N at the time of *Prosopis* establishment was represented by current grassland soil N concentrations, adjusted to account for historical N deposition, leaching and gas loss. How realistic is this approach? Where certain processes and pools were not considered, their inclusion would tend to increase, rather than decrease, BNF estimates or have negligible effects. We did not consider accrual in small understory shrubs (basal diameter $< 4$ cm) or understory root biomass because estimates were not available for these pools. Assuming similar N content and root distribution to *Prosopis,* we estimate that adding this pool would increase total BNF estimates by about 10%. Litter (not present beneath younger trees and limited to ~ 1 cm or less in larger clusters) and lateral movement of *Prosopis* litter outside of clusters was also not accounted for, as the magnitude of this redistribution is unknown. Patches of biological soil crust are present in some remnant grasslands at the site, though not in the areas we sampled. $\delta^{15}$N of the crusts at this site (1.7‰) is depleted with respect to the top 2 cm of soil (6.1‰) suggesting that active BNF does occur (Soper, unpublished). However, given the small area of cover, crust BNF occurring at even the highest reported rates (9 kg ha$^{-1}$ y$^{-1}$ for undisturbed crusts on the Colorado plateau; (Franco-Pizaña and others 1995; Belnap 2002) would be still be relatively insignificant. Redistribution of N between grassland and *Prosopis* rhizospheres is considered unlikely because *Prosopis* roots do not typically extend beyond the reach of the canopy (Watts 1993; Northup and others 2005). Uplift of N from deeper soil layers (beyond 30 cm) by *Prosopis* roots is possible, but isotopic data indicate that it cannot be a major source of surface soil N under *Prosopis* canopies (Bai and others 2013; Risio and others 2014); bulk soil $\delta^{15}$N decreases with encroachment, whereas $\delta^{15}$N at depth is isotopically enriched with respect to surface grassland soil (Hibbard and others 2001; Boutton and Liao 2010). Finally, significant grazing and fire removal of biomass N are unlikely, given that grazing has been restricted for at least 30 years (stocking rates were likely low prior to that due to low rainfall) and site records bear no evidence of fire for at least 60 years and likely longer (Northup and others 2005; Bai and others 2013).

## Mass Balance Sensitivity to Input Values

As many authors have observed, mass balance estimates are only as accurate as the pool and flux estimates used to build them. For fluxes, the larger the temporal range over which they are measured, the less annual variation matters for mass balance calculations because they come closer to representing the true average over time. Deposition records used here cover 10–30 years and thus provide a good integrated measure of variability. Trace gas fluxes are less certain (3 years of sampling scaled using 10 years of climate data), but the small size of the flux (an order of magnitude less than deposition, for example) makes this of minimal concern. Leaching rates for this ecosystem are by far the least certain, but sensitivity analysis (of scenarios in which leaching rates were increased or decreased during encroachment by up to 8 kg ha$^{-1}$ y$^{-1}$) affected estimates of BNF by less than 10%. Uncertainty (± 1 SD) in measurement and/or estimation of soil and biomass pools also affected BNF estimates, but again by less than 11%. Scaling parameters represented the greatest potential source of uncertainty in BNF estimation, with assumptions about applying fixation rates to multistem trees affecting areal rates by up to 18%. This finding reflects a common trend in ecological studies, where heterogeneity and magnification of errors are of significant concern (Li and Wu 2006).

# CONCLUSIONS

We conclude that in this system, where N inputs and outputs can be well constrained and pool sizes can be accurately measured, mass balance methods offer a useful approach to the challenging task of estimating BNF in woody perennials. This is especially true for systems such as *Prosopis,* where N inputs from BNF may represent a significant and ongoing source of N over large areas, and have important implications for the magnitude of ecosystem carbon storage and future vegetative cover (Archer and others 2001).

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