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Original Article

Investigating patterns of symbiotic nitrogen fixation during vegetation change from grassland to woodland using fine scale $\delta^{15}N$ measurements

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ABSTRACT

Biological nitrogen fixation (BNF) in woody plants is often investigated using foliar measurements of $\delta^{15}N$ and is of particular interest in ecosystems experiencing increases in BNF due to woody plant encroachment. We sampled $\delta^{15}N$ along the entire N uptake pathway including soil solution, xylem sap and foliage to (1) test assumptions inherent to the use of foliar $\delta^{15}N$ as a proxy for BNF; (2) determine whether seasonal divergences occur between $\delta^{15}N_{xylem\ sap}$ and $\delta^{15}N_{soil\ inorganic\ N}$ that could be used to infer variation in BNF; and (3) assess patterns of $\delta^{15}N$ with tree age as indicators of shifting BNF or N cycling. Measurements of woody N-fixing *Prosopis glandulosa* and paired reference non-fixing *Zanthoxylum fagara* at three seasonal time points showed that $\delta^{15}N_{soil\ inorganic\ N}$ varied temporally and spatially between species. Fractionation between xylem and foliar $\delta^{15}N$ was consistently opposite in direction between species and varied on average by 2.4‰. Accounting for these sources of variation caused percent nitrogen derived from fixation values for *Prosopis* to vary by up to ~70%. Soil–xylem $\delta^{15}N$ separation varied temporally and increased with *Prosopis* age, suggesting seasonal variation in N cycling and BNF and potential long-term increases in BNF not apparent through foliar sampling alone.

Key-words: *Prosopis glandulosa*; *Zanthoxylum fagara*; biological nitrogen fixation; season; soil inorganic N; xylem sap.

INTRODUCTION

Biological nitrogen fixation (BNF) in natural ecosystems remains one of the most poorly estimated terms in the global N cycle, primarily because methodological challenges limit accurate quantification (Galloway *et al*. 2004; Cleveland *et al*. 2010). This is especially true for symbiotic N fixation in woody perennials, the dominant source of fixed N in many ecosystems (Boddey *et al*. 2000). This difficulty limits our ability to investigate controls over fixation rates and their sensitivity to environmental variation (Vitousek *et al*. 2002, 2013; Sprent 2005), which in turn limits extrapolation of BNF rates to large scales.

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N stable isotope ratios have long been recognized as a powerful tool for tracing BNF, because N generated through BNF tends to differ in the ratio of heavy (^{15}N) to light (^{14}N) isotopes ($\delta^{15}N$) compared with other sources (Shearer & Kohl 1986; Boddey *et al*. 2000). One common application in natural systems is the ^{15}N natural abundance method, which compares $\delta^{15}N$ of plant tissue (in practice, usually foliage) of target fixing species and non-fixing reference plants in a common environment (Shearer & Kohl 1986; Boddey *et al*. 2000). Because the non-fixing plant has only one potential N source (soil-derived) while the fixer has two (atmospheric N_2 and soil-derived N), the difference in $\delta^{15}N_{foliage}$ is assumed to be directly proportional to the proportion of N obtained from fixation (Shearer & Kohl 1986; Robinson 2001). This method has proved useful for application in relatively simple cropping or agroforestry systems, but presents several challenges that limit its application in natural environments (Boddey *et al*. 2000). Firstly, the method necessarily assumes that the soil N source is isotopically consistent spatially and through time (Shearer & Kohl 1986; Handley & Scrimgeour 1997). However, localized differences in N cycling, turnover rates between rhizospheres and the accumulation of isotopically light N from legume litter inputs are likely to alter the isotopic composition of soil solution N both spatially and temporally in a heterogeneous landscape (Shearer & Kohl 1989; Boddey *et al*. 2000; Yoneyama *et al*. 2000; Boutton & Liao 2010; Bai *et al*. 2013). Secondly, it may be difficult or even impossible to locate multiple suitable reference species in many environments, either because ecologically similar woody species do not co-occur or because fixing and non-fixing species overlap in their $\delta^{15}N$ values (Högberg 1997; Boddey *et al*. 2000). Thirdly, it is necessary to assume that isotopic fractionation during plant uptake, assimilation and turnover is either small or comparable between unrelated species. This is despite evidence that differential mycorrhizal colonization affects fractionation during N uptake (Handley & Raven 1992; Evans 2001) and that the site of nitrate assimilation (roots or shoots) is variable between species and affects tissue $\delta^{15}N$ values (Evans 2001). Finally, the temporal resolution of the method is limited; $\delta^{15}N_{foliage}$ represents an integrated isotopic signature of N laid down during leaf formation and subsequently altered by poorly quantified turnover and resorption during leaf lifetime, on scales of months

to years (Shearer & Kohl 1986; Gebauer & Schulze 1991; Evans 2001). Leaf lifetime often cannot be readily controlled for between species during sampling, especially in the tropics where many woody species exist as evergreens (Eamus & Prior 2001). This resolution precludes the testing of hypotheses about abiotic or phenological controls over N fixation that might produce variation at sub-annual scales.

We hypothesized that more explicit sampling of $\delta^{15}N$ of N pools along the pathway from the soil solution to leaves may offer a mechanism to test assumptions of the foliar $\delta^{15}N$ method, overcome certain limitations and provide novel information to address hypotheses about N fixation in natural systems. Specifically, we measured the $\delta^{15}N$ of the plant-accessible soil N pool, plant xylem sap and foliage at sub-annual scales. Accurate isotopic measurement of low concentrations of inorganic N ($\delta^{15}N_{soil\ inorganic\ N}$) in soil extracts is now feasible (Stephan & Kavanagh 2009; Lachouani *et al*. 2010; Bell 2012). Xylem sap ($\delta^{15}N_{xylem\ sap}$), a mixture of recently acquired fixed and soil-derived N (and likely some resorbed N) (Yoneyama 1995; Yoneyama *et al*. 2000; Canton *et al*. 2005; Millard & Grelet 2010), precedes fractionation steps involved in leaf incorporation and turnover. Divergence between soil and xylem $\delta^{15}N$ values ($\Delta^{15}N_{soil\ inorganic\ N\text{-}xylem}$) could be compared between individuals, or in a single individual across time, as a potential measure of relative fixed N contribution, removing direct comparisons with reference plants. As both soil inorganic and xylem N pools have turnover times on the order of minutes/hours to perhaps days (Hayashi *et al*. 1997; Stark & Hart 1997; Booth *et al*. 2005), they have the potential to provide much finer temporal resolution than foliage. $\delta^{15}N_{xylem\ sap}$ values from woody plants are scarce in the literature, but evidence from N-fixing and non-fixing trees measured along an Australian continental rainfall gradient shows significant differences in the $\delta^{15}N_{xylem\ sap}$ between fixers and non-fixers and suggests that $\delta^{15}N_{xylem\ sap}$ may provide useful isotopic information not available from foliar sampling (Soper *et al*. unpublished data).

Encroachment of the N-fixing tree legume *Prosopis glandulosa* (honey mesquite) into grasslands of the Rio Grande Plains offers a useful setting in which to test this extended sampling approach. *Prosopis* is widespread throughout the southern United States and is the dominant N-fixer over more than 38 million hectares (Van Auken 2000). However, its root nodules are difficult to recover in the field and the acetylene reduction method has not been applied successfully (Johnson & Mayeux 1990; Felker 2009), making isotopic approaches particularly appealing. At the site of this study, *Prosopis* seedlings recruit into remnant grasslands of known soil composition and, over time, develop a woody understory forming 'islands' embedded within a relatively homogenous soil matrix. These single *Prosopis* clusters can be accurately aged (Archer *et al*. 1988; Flinn *et al*. 1994; Stoker 1997), and the result is a chronosequence of time since symbiotic BNF introduction. Over this encroachment chronosequence, well-characterized shifts in soil parameters such as increases in inorganic N availability and available phosphorus have been observed (Virginia & Jarrell 1983; Hibbard *et al*. 2001; Boutton & Liao 2010; Kantola 2012). These parameters are known to suppress (in the case of inorganic N) or increase (P) nodule formation and N fixation in other woody perennials (Hartwig 1998; Mortier *et al*. 2011; Augusto *et al*. 2013), and variation in this system could be used to test hypotheses about long-term controls over fixation during succession. Finally, *Prosopis* fixation rates may vary on seasonal time scales in response to pronounced changes in temperature and rainfall (Archer *et al*. 2001), supported by observations of seasonal variation in leaf N isotope composition (Bai *et al*. 2009) and root nodule morphology and abundance (Johnson & Mayeux 1990; Zitzer *et al*. 1996).

In this study, we applied expanded isotopic sampling to investigate N fixation dynamics in *P. glandulosa* in a subtropical, semi-arid woodland. *Zanthoxylum fagara* (lime prickly ash), an associated woody non-fixer, was investigated as a control for variation that may arise from processes other than fixation. The goals were (1) to test the assumptions of the foliar $\delta^{15}N$ method, specifically that $\delta^{15}N_{soil\ inorganic\ N}$, soil–plant fractionation events and plant internal N fractionation are comparable between plant species and over time; (2) to investigate changes in *Prosopis* $\delta^{15}N_{xylem\ sap}$ sub-annually in relation to fluctuations in $\delta^{15}N_{soil\ inorganic\ N}$, and determine whether these differences could be used to infer sub-annual variation in N fixation; and (3) to use a woody encroachment chronosequence to determine whether measures of $\delta^{15}N_{soil\ inorganic\ N}$ and $\delta^{15}N_{xylem}$ show patterns with tree age suggestive of shifting N fixation or N cycling.

MATERIALS AND METHODS

Study site

Sampling was conducted at the Texas A&M AgriLife La Copita Research Area (27°40′N, 98°12′W), 65 km west of Corpus Christi, Texas, USA, on the Rio Grande Plains during 2011–2013. The site consists of savanna parkland with discrete woody patches dominated by one or more *P. glandulosa* (Torr.) var. *glandulosa* (honey mesquite, referred to here as *Prosopis*) individuals, usually embedded in a matrix of C_4 grasses and bare ground. *Prosopis* is a leguminous tree in the family Fabaceae, and generally considered to be capable of substantial rates of BNF (Shearer *et al*. 1983). Although it nodulates readily under both glasshouse and natural conditions (including at this site), its nodules are difficult to recover under field settings, partly because they occur deep (>1 m) in the soil profile (Virginia *et al*. 1984; Johnson & Mayeux 1990; Zitzer *et al*. 1996). *Prosopis* is winter deciduous and individuals in this study replaced their leaves completely between January and March of both 2012 and 2013. Foliar samplings taken between replacement events are referred to here as 'leaf cohorts'. *Z. fagara* (L.) (lime prickly ash, referred to hereafter as *Zanthoxylum*) is in the family Rutaceae and is the most abundant non-fixing woody plant at the site. It is hypothesized that *Zanthoxylum* individuals recruit only in the understory of a *Prosopis* nurse plant, but they may persist long after the *Prosopis* dies and many large free-standing individuals exist at this site with no evidence of

the original nurse plant present. *Zanthoxylum* is evergreen, with leaf turnover occurring throughout the growing season (March–October) and has an average leaf lifetime of 116 days (Nelson *et al*. 2002).

The climate 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, and minima in July/August. During the study period (August 2011–August 2012), the site was drier than average, experiencing 329 mm annual rainfall. Mean annual temperature is 22.4 °C with an average growing season of 289 days. Air temperature and precipitation data were sampled every 10 min by a weather monitoring system inside the sampling plot (NexSens Technology, Fairborn, OH, USA). Soils at the site are sandy loams (Typic and Pachic Argiustolls) with little to no topography. The sampling plot measured 200×100 m and has been fenced to exclude livestock grazing since 1985.

Plant tissue sampling

Nineteen *Prosopis* individuals and 10 *Zanthoxylum* individuals were sampled for xylem and foliage in January, May and August of 2012, with a smaller subset of individuals ($n = 12$ and $n = 6$ for *Prosopis* and *Zanthoxylum*, respectively) sampled in August 2011. Additional foliage was sampled from the same trees in February, May and August of 2013. *Prosopis* tree age ranged from 25 to 136 years, calculated by basal diameter calibrated for upland clusters at this site (Flinn *et al*. 1994; Stoker 1997). Sampled individuals typically had a diverse woody understory and were selected to have no other potentially N-fixing woody plants within 10 m of the bole. *Zanthoxylum* individuals were located at least 6 m from the drip line of the nearest potentially N-fixing woody plant. *Prosopis* is characteristically the first species to establish in the former C_4 grasslands at the site (Bai *et al*. 2012), so that tree age corresponds to the number of years the soil immediately surrounding each tree has been experiencing symbiotically-fixed N inputs.

Foliage was sampled at the four cardinal locations from each tree, although some or all individuals of *Prosopis* had no attached foliage at the January and February sampling points. In August 2013, additional foliar samples were taken from six individuals of other non-fixing woody species *Condalia hookeri* (M. C. Johnst.), *Diospyros texana* (Scheele), *Celtis pallida* (Torr.) and an N-fixing tree *Acacia farnesiana* (L.) Willd. located in the same plot. Individuals were located at least 6 m from the drip line of the nearest potentially N-fixing woody plant. Samples were dried for 3 d at 60° C, ground and analyzed for $\delta^{15}N$ using a continuous flow isotope ratio mass spectrometer (IRMS) (Model Delta V Advantage; ThermoScientific, Bremen, Germany). All isotope analyses were conducted at the Cornell University Stable Isotope Laboratory (COIL).

Branch xylem sap was collected as in Pate *et al*. (1994), with care taken to fully remove phloem tissue before extraction. Collection occurred between 0400 and 0900 h to control for potential diel variation in N metabolism (Peuke *et al*. 2013). Sap was stored on ice and frozen prior to analysis. Sap from three to six branches was bulked into a single sample per tree and 25–420 μL of xylem sap was freeze-dried and analyzed for $\delta^{15}N$. A subsample of xylem sap from each time point was assayed for ATP using a Sigma ATP Bioluminescent Assay Kit (Sigma Aldrich, St Louis, MO, USA) to check for phloem sap contamination (Rennenberg *et al*. 1996).

Soil sampling and analysis

At the same time points as xylem samples, two soil cores were taken beneath each tree, within 50 cm of the bole, and divided into two depth increments (0–15 and 15–30 cm). The majority of fine roots, responsible for nutrient uptake from soil (Eissenstat 1992), occur in the top 30 cm of soil for *Prosopis* (Ansley *et al*. 2014), and for woody elements overall greatest total root biomass occurs with the top 15 cm (Midwood *et al*. 1998). Soil was passed through a 2 mm screen to remove large organic fragments and was then extracted within 4 h of collection by shaking for 1 h in 1:4 (w/v) 1 M KCl (Fisher Scientific, Pittsburgh, PA, USA). The supernatant was filtered through Whatman 41 cellulose filter paper (GE Healthcare Life Sciences, Piscataway, NJ, USA) and frozen prior to analysis. Subsamples of cores were weighed before and after drying for 3 d at 105 °C to determine percent moisture. NH_4^+ and NO_3^- concentrations in KCl extracts were analyzed colorimetrically using a Lachat flow injection analyzer (Hach Co., Loveland, CO, USA) at the Cary Institute of Ecosystem Studies Rachel L. Carson Analytical Facility (Millbrook, NY, USA).

To determine $\delta^{15}N$ of NO_3^- and NH_4^+, KCl extracts from two soil cores per tree (0–15 cm) were bulked and a subsample of individuals (10 *Prosopis*, 7 *Zanthoxylum*) were analyzed for each time point. $\delta^{15}N$ of NO_3^- was measured using the microbial denitrifier method at the Facility for Isotope Ratio Mass Spectrometry at University of California, Riverside, controlling for trace NO_3^- contamination in the KCl reagent (Bell 2012). A microdiffusion method was used to determine $\delta^{15}N$ of NH_4^+, as per Stephan & Kavanagh (2009), using a diffusion volume of 40 mL in 90 mL polypropylene containers. Samples were diffused for 7 d at 25° C, and filters were analyzed for $\delta^{15}N$ at COIL. A minimum recovery cut-off of >99% of sample N was applied for all samples described here. Filter papers used during extraction were identified as a source of NH_4^+ contamination and controlled for as per Stephan & Kavanagh (2009), using a minimum of five replicate controls matched to each individual batch of filter paper used during extraction. $\delta^{15}N$-NO_3^- and $\delta^{15}N$-NH_4^+ values were weighted by relative concentration in the soil KCl extracts in order to generate a single value for $\delta^{15}N_{\text{soil inorganic N}}$.

Calculation of %Ndfa values

As a theoretical exercise, percent N derived from fixation (%Ndfa) was calculated for *Prosopis* using the $\delta^{15}N$ foliar natural abundance technique (Shearer & Kohl 1986). This value was then adjusted to account for observed variation

in soil solution $\delta^{15}N$ and $\Delta^{15}N_{xylem\text{-}foliage}$ (both assumed to be constant in the foliar method) between species for two sampling dates. This exercise was intended to indicate the broad sensitivity of %Ndfa to assumptions of the method (rather than absolute values), as application of the technique did not conform to suggested conditions including use of multiple non-fixing reference species and a separation between species of >5‰ (Shearer & Kohl 1986; Högberg 1997).

Shearer *et al.* (1983)'s values for *P. glandulosa* leaves grown hydroponically without added N (–1.3 ± 0.5‰, *B*) were used in calculations and were in agreement with values generated using *Prosopis* seed and rhizobial inoculum from the study site (–1.0 ± 0.2‰; Soper, unpublished data). To account for the lower baseline soil solution $\delta^{15}N$ in *Zanthoxylum*, the divergence between species $\delta^{15}N_{soil\ inorganic\ N}$ (averaged over the January–August 2012 sampling period) was subtracted from $\delta^{15}N_{foliage}$. To account for xylem–foliar fractionation, a Δ value of 1.07‰ (taken from field observations for *Prosopis* in this study) was applied to *B* and species- and sampling point-specific $\Delta^{15}N_{xylem\text{-}foliage}$ was subtracted from $\delta^{15}N_{foliage}$. When both corrections were applied together, sampling date-specific $\delta^{15}N_{soil\ inorganic\ N}$ values were used in order to keep temporal scales of N uptake and integration comparable between source and sink. Error propagation was performed as per Shearer *et al.* (1983).

Statistical analysis

Statistical analyses were performed in R (R Core Team 2012) and JMP Pro v10.0.0 (SAS Institute, Cary, NC, USA). Significance was set at $\alpha = 0.05$, unless otherwise indicated.

Pooled *t*-tests were used to determine significant differences between the two species for $\delta^{15}N_{foliage}$, $\delta^{15}N_{soil\ inorganic\ N}$, $\Delta^{15}N_{soil\ inorganic\ N\text{-}xylem}$ and $\Delta^{15}N_{xylem\text{-}foliage}$ at each time point, after testing for homogeneity of variance and normality. Paired *t*-tests were used to determine significance of differences ($\Delta^{15}N$) between N pools.

Simple linear regressions were used to determine relationships between $\delta^{15}N_{xylem\ sap}$ between years for individual plants; $\delta^{15}N_{foliage}$ between years for individual plants, plant age and soil inorganic N concentrations, $\delta^{15}N_{xylem\ sap}$, $\delta^{15}N_{foliage}$, $\delta^{15}N_{soil\ inorganic\ N}$ and $\Delta^{15}N_{soil\ inorganic\ N\text{-}xylem}$ (*Prosopis* only); and soil inorganic N concentrations and $\delta^{15}N_{soil\ inorganic\ N}$ and $\delta^{15}N_{xylem\ sap}$. Distribution of residuals was examined to confirm appropriateness of fit. Mixed-effect models were used to analyzed date effects and generate *P*-values, with tree identity incorporated as a random effect to account for repeated sampling of individuals. Tukey's honest significant difference (HSD) post-hoc test was used to determine significance of differences between individual time points. For $\delta_{15}N_{xylem\ sap}$ and $\delta^{15}N_{foliage}$, a mixed-effect model was created using sampling date and tree age as fixed effects and tree identity as a random effect to generate restricted maximum likelihood variance components estimates. One-way analysis of variance (ANOVA) was used to compare $\delta^{15}N_{foliage}$ between multiple species.

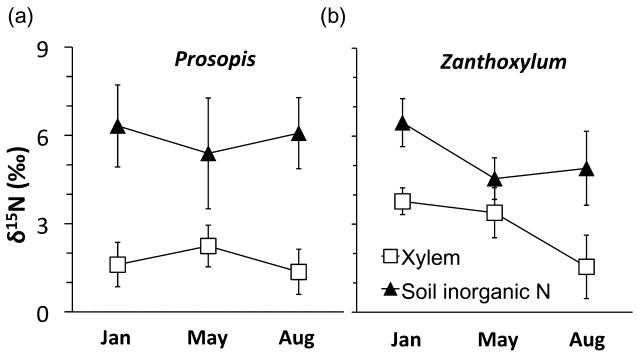

Figure 1. $\delta^{15}N_{xylem}$ and $\delta^{15}N_{soil\ inorganic\ N}$ for (a) N-fixing *Prosopis glandulosa* and (b) non-fixing *Zanthoxylum fagara* at three time points in 2012. $\delta^{15}N_{soil\ inorganic\ N}$ is a concentration-weighted value for $^{15}N\text{-}NO_3^-$ and $^{15}N\text{-}NH_4^+$ in 1 M KCl soil extracts from the rooting zone (0–15 cm). Soils were sampled from below each tree (<50 cm from trunk) within 3 h of xylem sampling. Extracts from two soil cores per tree were bulked for analysis. Values represent mean ± 1 SD.

RESULTS

Temporal and interspecies variability in $\delta^{15}N$ and changes between N pools

In both *Prosopis* and *Zanthoxylum*, $\delta^{15}N_{soil\ inorganic\ N}$ was enriched with respect to $\delta^{15}N_{xylem}$ (Fig. 1; Table 1). In *Zanthoxylum*, both $\delta^{15}N_{soil\ inorganic\ N}$ and $\delta^{15}N_{xylem}$ showed variation over time of up to 1.92 and 2.23‰, respectively

Table 1. Average fractionation ($\Delta^{15}N$) between N pools for *Prosopis glandulosa* and *Zanthoxylum fagara* at three sample dates in 2012

	Prosopis		*Zanthoxylum*	
Month	$\Delta^{15}N_{soil\ inorganic\ N\text{-}xylem}$ (‰)	$\Delta^{15}N_{xylem\text{-}foliage}$ (‰)	$\Delta^{15}N_{soil\ inorganic\ N\text{-}xylem}$ (‰)	$\Delta^{15}N_{xylem\text{-}foliage}$ (‰)
January	4.42^{a} ***	1.23^{a} ***	2.67^{ab} ***	-0.65^{a} *
May	2.91^{a} ***	1.05^{a} ***	1.16^{b} NS	-0.91^{a} *
August	4.37^{a} ***	0.51^{b} ***	3.35^{a} **	-2.64^{b} ***

Lowercase letters indicate significant differences between time points determined by mixed-effect modelling followed by Tukey's honest significant difference post-hoc test.
P-values indicate fractionation values significantly different from zero (paired *t*-test). ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.
NS, not significant.

Table 2. Results for paired *t*-tests testing differences in $\delta^{15}N$ of N pools and fractionation ($\Delta^{15}N$) between pools for *Prosopis glandulosa* and *Zanthoxylum fagara* at three sample dates in 2012

	Between-species comparisons				
Month	$\Delta^{15}N_{soil\ inorganic\ N\text{-}xylem}$ (‰)	$\Delta^{15}N_{xylem\text{-}foliage}$ (‰)	$\delta^{15}N_{soil}$ (‰)	$\delta^{15}N_{xylem}$ (‰)	$\delta^{15}N_{foliage}$ (‰)
January	**0.027**	**<0.001**	0.811	**<0.001**	**<0.001**
May	**0.045**	**<0.001**	0.282	**<0.001**	**<0.001**
August	0.291	**<0.001**	0.074	0.315	**<0.001**

Numbers in bold indicate significant differences ($P < 0.05$).

($P < 0.0099$ and $P < 0.0001$; Fig. 1). Separation between the two pools ($\Delta^{15}N_{soil\ inorganic\ N\text{-}xylem}$) was also variable through time ($P < 0.04$), ranging from 1.16‰ in May up to 3.35‰ in August 2012 (Fig. 1; Table 1). In *Prosopis*, $\delta^{15}N_{xylem}$ varied between time points by up to 0.7‰ ($P < 0.0001$) but $\delta^{15}N_{soil\ inorganic\ N}$ did not (Fig. 1; Table 1). $\Delta^{15}N_{soil\ inorganic\ N\text{-}xylem}$ was greater for *Prosopis* than for *Zanthoxylum* at two of the three time points, from 2.91 to 4.42‰ ($P < 0.08$; Fig. 1; Tables 1 and 2). In August 2012, the difference between isotopic composition of soil solution inorganic N ($\delta^{15}N_{soil\ inorganic\ N}$) between the species reached 1.17‰ ($P < 0.07$, pooled *t*-test; Fig. 1), but was otherwise relatively spatially constant.

Within species, both *Zanthoxylum* and *Prosopis* showed significant separation between $\delta^{15}N_{xylem}$ and $\delta^{15}N_{foliage}$ ($\Delta^{15}N_{xylem\text{-}foliage}$) at all time points ($P < 0.05$; Table 1). However, the direction of fractionation between these pools was consistently opposite between the two species ($P < 0.001$; Fig. 2; Tables 1 and 2). Averaged across a year, $\Delta^{15}N_{xylem\text{-}foliage}$ was 1.07 ± 0.12‰ (± 1 SE) for *Prosopis*, and -1.35 ± 0.25‰ for *Zanthoxylum*, leading to a difference in fractionation factors of 2.42 ± 0.28‰ between species (Fig. 2).

%Ndfa values for *Prosopis* were sensitive to variations in $\Delta^{15}N_{xylem\text{-}foliage}$ between species and over time. When foliage-derived %Ndfa values were adjusted for species variation in $\Delta^{15}N_{xylem\text{-}foliage}$, average values dropped from 73 to 35% and 61 to 5% for January and August, respectively (Table 3). Accounting for average species differences in $\delta^{15}N_{soil\ inorganic\ N}$ had a smaller effect, reducing foliage-derived %Ndfa by only 2–4%. When the soil correction was applied in a time point-specific manner along with the xylem correction, an effect of sampling date was also apparent.

Variability in $\delta^{15}N$ between *Prosopis* individuals

Prosopis individuals were consistently ranked in their relative xylem and foliar $\delta^{15}N$ values across a 12 month period, that is, the values of individual trees relative to each other remained constant despite temporal variation over the course of the year (Fig. 3). $\delta^{15}N_{xylem}$ was similar between August of 2011 and 2012, with linear regression producing a slope close to 1 ($\delta^{15}N_{xylem\ Aug\ 2012} = 1.0837 \times \delta^{15}N_{xylem\ Aug\ 2011} +$

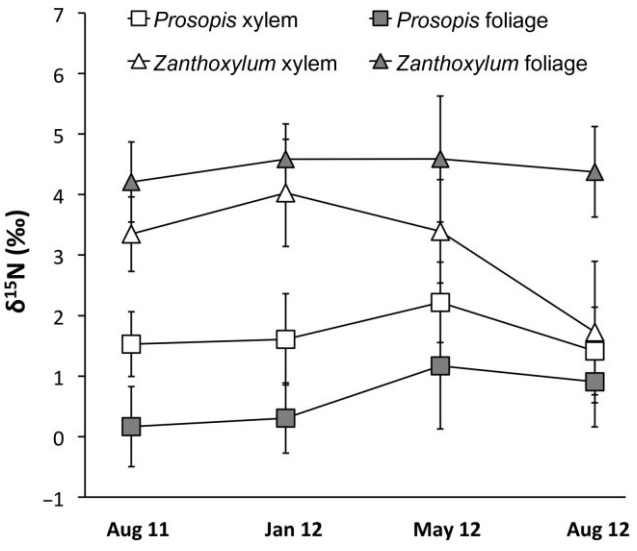

Figure 2. $\delta^{15}N_{xylem\ sap}$ and $\delta^{15}N_{foliage}$ for f N-fixing *Prosopis glandulosa* and non-fixing *Zanthoxylum fagara* at four time points in 2011 and 2012. Values represent mean ± 1 SD. Statistical relationships are presented in Table 1.

Table 3. Sensitivity of percent nitrogen derived from fixation (%Ndfa) values for *Prosopis glandulosa* to variation in $\delta^{15}N_{soil\ inorganic\ N}$ and xylem–foliar fractionation ($\Delta^{15}N_{xylem\text{-}foliage}$) between species and sampling date calculated using *Zanthoxylum fagara* as a non-fixing reference. Values are for tissues sampled in January and August 2012, ± 1 SE. Note that these values are intended to provide a general illustration of the effects of accounting for assumptions, rather than absolute values, as application of %Ndfa technique deviates from suggested conditions (Shearer & Kohl 1986; Högberg 1997).

	%Ndfa	
Calculation method	January 2012	August 2012
Foliage (conventional)	73 ± 7	61 ± 6
With soil correction[a]	75 ± 7	65 ± 6
With xylem–foliar fractionation correction[b]	35 ± 6	5 ± 14
With soil and xylem–foliar fractionation correction[b]	34 ± 8	27 ± 11

[a]Using average soil solution $\delta^{15}N$ (January–August 2012).
[b]Using sampling point-specific soil solution $\delta^{15}N$.

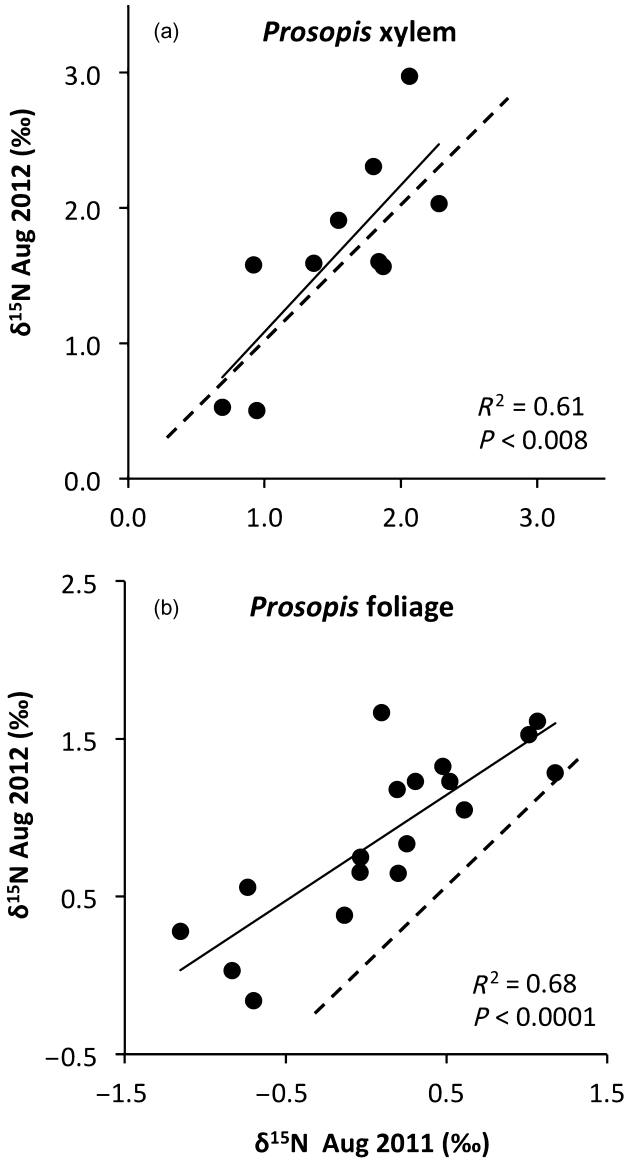

Figure 3. Linear relationships between $\delta^{15}N_{xylem\ sap}$ (a) and $\delta^{15}N_{foliage}$ (b) of individual *Prosopis glandulosa* trees sampled in August 2011 and August 2012. Dashed line represents 1:1 $\delta^{15}N$ relationship. Foliage was replaced completely between the two sampling points. *P*- and R^2 values derived from linear regression.

0.0016, $R^2 = 0.61$, $P < 0.008$; Fig. 3a). In a mixed-effect model, sample date was a significant fixed effect in the model ($P < 0.0001$), although tree age was not ($P > 0.05$), and 64.2% of the residual variation was explained by individual tree identity.

$\delta^{15}N_{foliage}$ values were also correlated across years at the individual plant level, despite being offset between years by ~0.8‰ (linear regression, $^{15}N_{foliage\ Aug\ 2012} = 0.6715 \times \delta^{15}N_{foliage\ Aug\ 2011} + 0.8082$, $R^2 = 0.68$, $P < 0.0001$; Fig. 3b). Again, sample date was a significant fixed effect in the mixed-effect model ($P < 0.0001$) and tree age was not ($P > 0.05$), and 84.1% of the residual variation in $\delta^{15}N_{foliage}$ was explained by individual tree identity.

This same consistency in $\delta^{15}N_{xylem}$ and $\delta^{15}N_{foliage}$ at the individual level over time was not observed in *Zanthoxylum*, and after accounting for date as a fixed effect in a mixed-effect model individual tree identity accounted for 16% or less of residual variation.

Effect of *Prosopis* age on plant and soil inorganic $\delta^{15}N$ and soil N concentration

Prosopis tree age was not a predictor of $\delta^{15}N_{foliage}$ (Fig. 4a) or $\delta^{15}N_{xylem}$ (Fig. 4b; $P > 0.05$). However, $\delta^{15}N_{soil\ inorganic\ N}$ in the rooting zone of *Prosopis* increased with tree age ($R^2 = 0.23$, $P < 0.06$) by 2.05‰ from 20 to 100 years (predicted, $\delta^{15}N_{soil\ inorganic\ N} = 0.0257 \times Age + 4.497$; Fig. 4c). As a result of this increasing soil baseline, the $\Delta^{15}N_{soil\ inorganic\ N\text{-}xylem}$ value increased comparably over the same age range (2.10‰ predicted, $R^2 = 0.12$, $\delta^{15}N_{soil\ inorganic\ N} = 0.0263 \times Age + 2.477$ $P < 0.07$; Fig. 4d).

Soil inorganic N concentrations ($NH_4^+ + NO_3^-$, 0–15 cm depth) increased linearly with *Prosopis* age (linear regression, $R^2 = 0.62$, $P < 0.0001$; Fig. 5a). Increasing soil inorganic N also correlated positively with $\delta^{15}N_{soil\ inorganic\ N}$ concentrations in soils under *Prosopis* (linear regression, $R^2 = 0.26$, $P < 0.02$; Fig. 5b), although not in soils under *Zanthoxylum* (data not shown).

Effect of soil N concentration on xylem $\delta^{15}N$

In *Zanthoxylum*, there was a strong negative relationship between $\delta^{15}N_{xylem}$ and soil NH_4^+, NO_3^- and total inorganic N concentrations when all time points were considered together (linear regression: $R^2 = 0.48$, $P < 0.0001$; $R^2 = 0.33$, $P < 0.005$; $R^2 = 0.47$, $P < 0.0002$, respectively) (Fig. 6a). However, $\delta^{15}N_{xylem}$ was not correlated with $\delta^{15}N_{soil\ inorganic\ N}$. No relationship was observed among any of these parameters in *Prosopis* ($P > 0.05$; Fig. 6b).

Inter-annual and between-species variation in foliar $\delta^{15}N$

Over the course of 2 years, *Prosopis* showed strong temporal variation in $\delta^{15}N_{foliage}$, whereas non-fixing *Zanthoxylum* did not (Fig. 7; *Zanthoxylum*: $P > 0.05$: *Prosopis*: $P < 0.0001$). When individual *Prosopis* leaf cohorts were considered, the average $\delta^{15}N_{foliage}$ increased between cohorts 1 (2011) and 2 (2012) by 0.73‰ [$P < 0.0001$, 95% confidence interval (CI) = 0.58–0.89‰] and between cohorts 2 and 3 (2013) by 2.12‰ ($P < 0.0001$, 95% CI = 1.94–2.30‰). During February–April of this study, when new leaf cohorts were produced, total precipitation varied among years (148, 137 and 70 mm in 2011, 2012 and 2013, respectively) and was lower than the average rainfall for this interval over the period 1979–2009 (186 mm; National Climatic Data Centre; http://www.ncdc.noaa.gov).

When six species at the site were assessed for $\delta^{15}N_{foliage}$ in August of 2013, *Prosopis* was not different from three of the four non-fixing species with the exception of *D. texana*, which

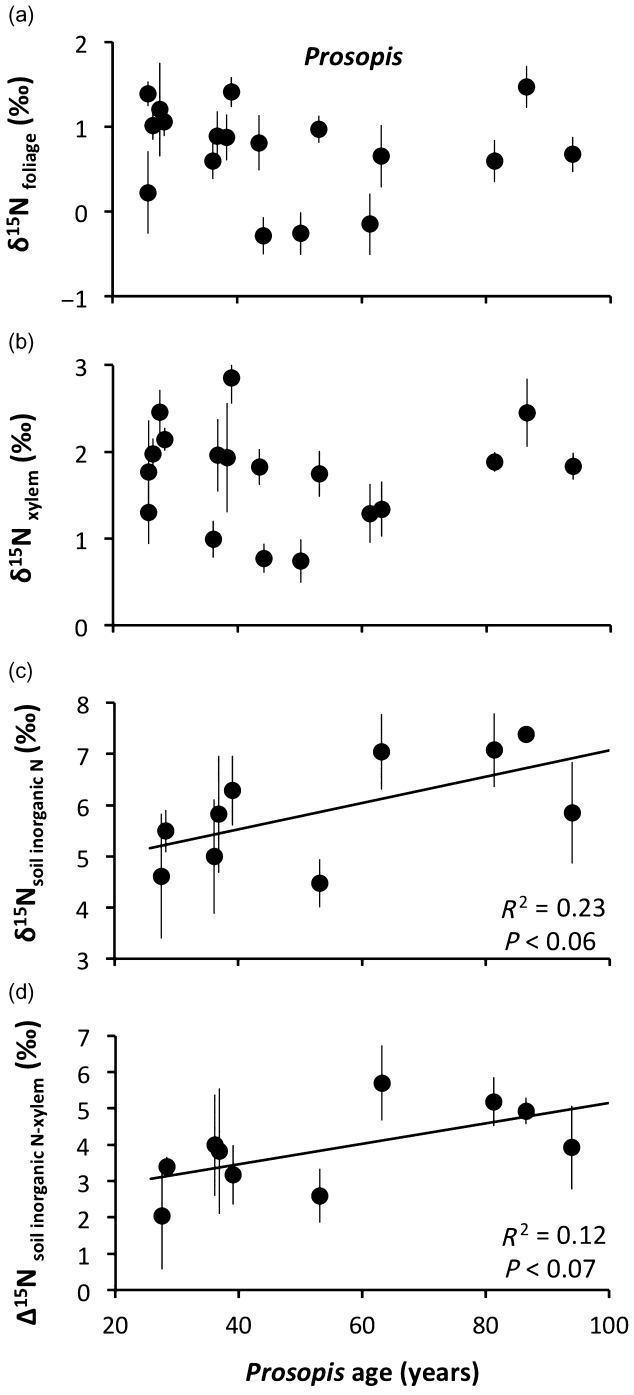

Figure 4. (a) $\delta^{15}N_{foliage}$ for *Prosopis glandulosa* trees ranging from 20 to 100 years in age. (b) $\delta^{15}N_{xylem}$ sap for *Prosopis* trees ranging from 20 to 100 years in age. (c) Linear relationship between $\delta^{15}N_{soil\ inorganic\ N}$ from soils at 0–15 cm depth in the rooting zone of *Prosopis* and tree age. (d) Linear relationship between $\Delta^{15}N$ (difference between $\delta^{15}N_{soil\ inorganic\ N}$ and $\delta^{15}N_{xylem\ sap}$) for *Prosopis* individuals and tree age. All values represent the mean of three time points in 2012 ± 1 SE. *P*-values derived from linear regression.

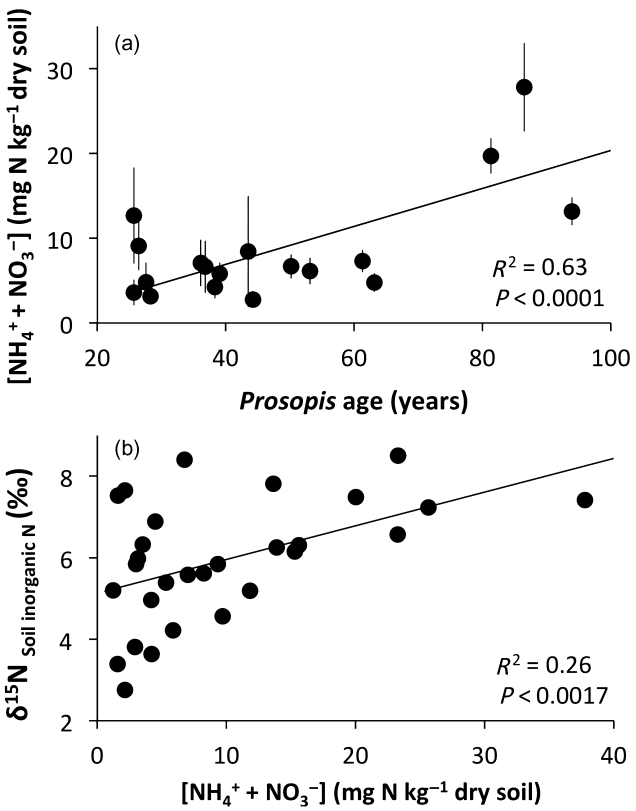

Figure 5. (a) Linear relationship between total inorganic N concentration ($NH_4^+ + NO_3^-$) in 1 M KCl soil extracts (0–15 cm) and age of *Prosopis glandulosa* under which soil was sampled. Values represent mean of three time points in 2012 ± 1 SE. (b) Linear relationship between $\delta^{15}N_{soil\ inorganic\ N}$ and total inorganic N concentration ($NH_4^+ + NO_3^-$) in each sample. *P*- and R^2 values derived from linear regression.

was depleted with respect to *Prosopis* (Fig. 8). Additionally, putatively N-fixing *A. farnesiana* $\delta^{15}N_{foliage}$ was enriched in compared with all species except *Zanthoxylum*. At the previous six time points considered in 2011 and 2012, *Prosopis* $\delta^{15}N_{foliage}$ was significantly depleted with respect to *Zanthoxylum* (Fig. 7).

DISCUSSION

Explicit isotopic sampling of plant and soil $\delta^{15}N$ pools along the N uptake pathway suggests that applying the foliar $\delta^{15}N$ natural abundance method to estimate BNF would be challenging in this subtropical *Prosopis* savanna woodland. The requirement that the $\delta^{15}N$ of non-BNF-derived N sources be constant among species (Shearer & Kohl 1986) was not met, as the concentration-weighted $\delta^{15}N_{soil\ inorganic\ N}$ was not always the same in N-fixing *Prosopis* and non-fixing *Zanthoxylum* (Fig. 1). This parameter also showed significant seasonal variation in *Zanthoxylum* (up to 1.9‰ over 3 months), suggesting that the soil inorganic $\delta^{15}N$ source is neither temporally nor spatially constant. Additionally, fractionation during N assimilation and transport were not comparable between the N-fixing and reference species.

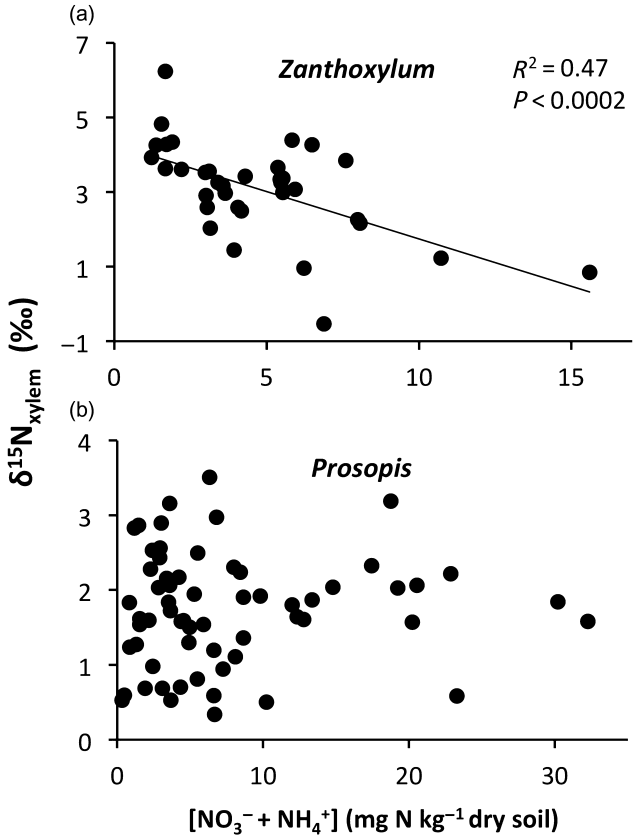

Figure 6. (a) Linear relationship between total inorganic N concentration ($NH_4^+ + NO_3^-$, mg N kg^{-1} dry soil, 1 M KCl soil extracts, 0–15 cm) and $\delta^{15}N_{xylem}$ in *Zanthoxylum fagara* and (b) no significant linear relationship between the two values in *Prosopis glandulosa*. *P*- and R^2 values derived from linear regression.

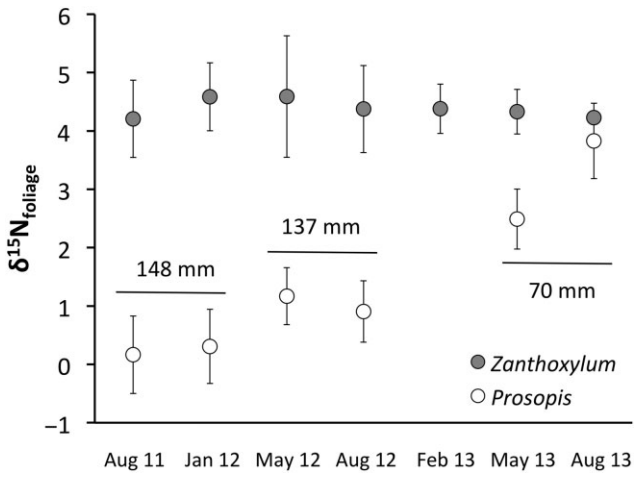

Figure 7. Seasonal variation in $\delta^{15}N_{foliage}$ for N-fixing *Prosopis glandulosa* and non-fixing *Zanthoxylum fagara* from August 2011 to August 2013. Horizontal lines indicate different leaf cohorts in *Prosopis*, accompanied by total precipitation (mm) at the site during the 3 month growing period (February to April, inclusive) when each leaf cohort was laid down. Missing value occurs where *Prosopis* trees had no foliage at time of sampling. Values represent mean ± 1 SD.

Zanthoxylum and *Prosopis* differed consistently in the magnitude and direction of the separation between xylem and foliar $\delta^{15}N$ ($\Delta^{15}N$), leading to an average $\Delta^{15}N$ difference between species of 2.4‰. Applying this fractionation factor to percent N derived from fixation calculations for *Prosopis* was sufficient to reduce assumed fixation by 50% or more. These observations demonstrate that applying the natural abundance foliar $\delta^{15}N$ method would be untenable in this and similar ecosystems.

Within-plant fractionation has the potential to occlude or even mislead the interpretation of foliar $\delta^{15}N$ as a proxy for BNF. Foliage is usually observed to be ^{15}N depleted with respect to xylem sap (Pate *et al.* 1993; Schmidt & Stewart 2003; Peuke *et al.* 2013), as we saw in *Prosopis* (Fig. 2). Our observation of enriched foliar tissue in *Zanthoxylum* is rare, with only one observation found in the literature (Yoneyama *et al.* 2000). Foliar isotope ratios are influenced by several processes, many of which are likely to differ between taxa (e.g. Britto & Kronzucker 2013), including preferences for N forms with different isotopic ratios (Yoneyama 1996), distribution of nitrate reduction between roots and shoots (Andrews 1986; Comstock 2001; Werner & Schmidt 2002; Tcherkez & Hodges 2008), relative leaf turnover/export of N compounds with differing $\delta^{15}N$ signatures (Tcherkez 2011; Gauthier *et al.* 2012), and leaf age and lifetime (Kolb & Evans 2002; Peuke *et al.* 2013). Regardless of the mechanisms of fractionation, the opposing patterns observed in *Prosopis* and *Zanthoxylum* create a separation between foliar isotopic values that significantly overemphasizes the apparent contribution of BNF (Table 3).

We observed variable relationships between soil source $\delta^{15}N$ and xylem $\delta^{15}N$ in the two species. In *Zanthoxylum*, but not *Prosopis*, the N isotopic composition of xylem was

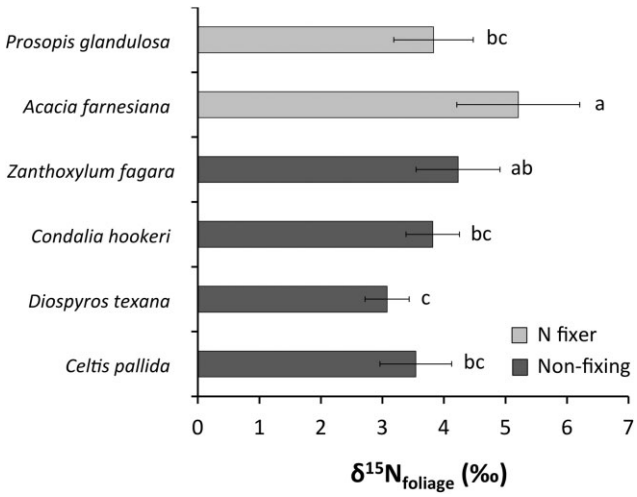

Figure 8. $\delta^{15}N_{foliage}$ for four co-occurring non-fixing (dark grey) and two N-fixing (light grey) woody plants, sampled in August 2013. Values represent means ± 1 SD. Letters indicate significant differences between species (Tukey's post-hoc test).

strongly negatively related to the concentration of soil inorganic N (both ammonium and nitrate), but not to its isotopic composition, that is, $\delta^{15}N_{xylem}$ became more depleted at higher soil inorganic N concentrations (Fig. 6). To our knowledge, this relationship has not previously been investigated in natural ecosystems under ambient soil N concentrations, and is counter to general predictions of concentration/fractionation relationships for nitrate (Evans 2001). The $\delta^{15}N$ of xylem sap reflects the relative content of nitrate and organic N compounds and integrates a complex set of fluxes (Handley & Scrimgeour 1997; Peuke *et al*. 2013). Nitrate taken up from soil may be partly reduced in root tissue. During reduction, this nitrate is fractionated by nitrate reductase (Robinson *et al*. 1998) producing ^{15}N-depleted organic N compounds that are either incorporated directly into root tissue or exported to the xylem. The remaining, unreduced, ^{15}N-enriched nitrate pool is exported to the xylem and reduced in the shoot (Robinson *et al*. 1998). Ammonium, both soil derived and as the product of N fixation, is immediately incorporated into organic compounds prior to xylem transport (Evans 2001) and does not show the same shift in isotopic distribution within the plant (Werner & Schmidt 2002).

We propose two potential mechanisms that may account for the inverse relationship between soil N concentration and xylem $\delta^{15}N$ relationship that we observed in *Zanthoxylum*. The relative degree of nitrate reduction in roots versus shoots may have decreased with external nitrate concentration, resulting in a smaller proportion of the increased nitrate pool being reduced in roots and reducing enrichment of the remaining nitrate pool exported in the xylem. This is however the opposite of what Kolb and Evans (2003) observed in barley (*Hordeum vulgare*) grown with increasing nitrate concentrations, where the root-to-shoot ratio of nitrate reductase activity increased and exported nitrate was more enriched. Alternatively, shifts in N source preference with concentration have been also observed in woody species (Kronzucker *et al*. 1997). Here, *Zanthoxylum* may have increased uptake of ammonium relative to nitrate as external ammonium concentrations increased, leading to a decreased proportion of enriched nitrate in the xylem sap. However, the ratio of the two compounds did not change with concentration.

Substantial increases in foliar $\delta^{15}N$ occurred across three *Prosopis* leaf cohorts over 2 years (>2.8‰), but no comparable variation was seen in non-fixing *Zanthoxylum* (Fig. 7), raising an important point for general interpretation of foliar $\delta^{15}N$ data. As a result of this temporal variation, foliar $\delta^{15}N$ did not differ between *Prosopis* (or N-fixing *A. farnesiana*) and non-fixing plants at the single time point when we sampled multiple species (Fig. 8), although it likely would have 2 years previously. Few studies that rely on the foliar $\delta^{15}N$ method consider sampling time as an important consideration, or conduct multiple staggered samplings, despite the fact that sample date has been recognized as an important determinant of the relative isotopic rankings of different species for some time (e.g. Handley & Scrimgeour 1997). Given our observation of variable relationships between soil solution, xylem and foliar $\delta^{15}N$, it would be invalid to directly compare isotopic values between species to interpret this shift as directly proportional to decreasing BNF.

The increase in $\delta^{15}N_{foliage}$ observed through time across all *Prosopis* trees studied (Fig. 7) suggests external environmental change is potentially affecting the isotopic composition of soil solution N; internal plant N assimilation and partitioning; rates of BNF; or all three. Precipitation was notably variable during this time, declining over 50% between 2012 and 2013 in the February–April period when *Prosopis* leaves were flushed and coincident with an increase in $\delta^{15}N_{foliage}$ of 2.1‰. Low water availability has been linked to decreased nodule abundance and acetylene reduction rates (a proxy for nitrogenase activity) in *Prosopis* and other woody legumes (Zitzer *et al*. 1996; Zahran 1999) and could account for our observation here. However, both drought and rates of BNF have been shown to alter internal ^{15}N distribution in herbaceous plants (Handley *et al*. 1999; Wanek & Arndt 2002), making it difficult to attribute the increasing foliar isotope values solely to decreasing BNF.

We observed seasonal variation in *Prosopis* xylem $\delta^{15}N$ that was largely independent of shifts in the isotopic composition of the soil inorganic N pool (Fig. 1; Table 1). Controls on N fixation, especially the effect of abiotic conditions (in the context of global climate change for example), are of significant interest to the scientific community (Vitousek *et al*. 2013) and existing seasonal variation may provide an opportunity to investigate these controls on manageable time scales in natural environments. However, it is difficult to attribute our observations to only changes in BNF because of similar observed shifts in the xylem–soil relationship of non-fixing *Zanthoxylum*. Most likely, these shifts integrate changes in both fractionation events during uptake and BNF effects over time. Previous experiments with soybean have shown that a nodulating strain had lower xylem $\delta^{15}N$ values than the non-nodulating strain grown under the same field conditions, indicating that the isotopic signature of fixation is likely detectable in N transported from the roots (Yoneyama *et al*. 2000). Both that study and Schmidt & Stewart (2003) looking at Australian *Acacias* observed variation in xylem sap $\delta^{15}N$ that was apparently influenced by abiotic conditions. Although it may prove challenging to parse out the effects of abiotic (temperature, water availability) and biotic (plant phenology) influences, the present study does demonstrate that measurable isotopic variation occurs and could be used to investigate the effect of these processes on N cycling in a natural ecosystem. Potential also exists to compare xylem data with that derived from other methods, for example, nodule abundance and acetylene reduction activity, to begin to address questions about *in situ* N fixation.

Individual *Prosopis* trees showed a high degree of consistency in tissue $\delta^{15}N$ relative to each other over time (Fig. 3) and non-fixing *Zanthoxylum* did not, potentially indicating sustained differences in BNF rates between *Prosopis* individuals. This consistency was not explained by age or any soil N variable tested. Instead, individual *Prosopis* tree identity accounted for 60–80% of the observed variation after accounting for sampling date. Although heterogeneous availability of other soil nutrients necessary for BNF or degree of

mycorrhizal colonization (Hobbie & Högberg 2012) could account for this observation, its restriction to *Prosopis* suggests that genotypic factors affecting N fixation rates (e.g. genotype-mediated plant–rhizobial interactions; Mytton *et al*. 1977) may be a probable explanation.

Prosopis encroachment into grassland and subsequent growth greatly increases soil inorganic N concentrations (McCulley *et al*. 2004; Fig. 5), a factor that might be expected to suppress rates of BNF as trees age (Hartwig 1998). In this study, *Prosopis* $\delta^{15}N_{foliage}$ was constant among trees ranging in age from 20 to 100 years (Fig. 4), suggesting constant rates of BNF. However, over this same chronosequence, soil solution inorganic $\delta^{15}N$ increased linearly by >2‰, leading to an increased separation ($\Delta^{15}N$) between tissue and soil isotopic values with time since establishment. This suggests increasing rates of BNF by *Prosopis* over time. In order for $\delta^{15}N_{xylem}$ to remain stable while $\delta^{15}N_{soil\ inorganic\ N}$ falls (assuming that fractionation of soil N upon uptake does not change with plant age), there must be a corresponding increasing output of isotopically depleted N, assumed to come from BNF. This implies either (1) that there is increased survival of trees that have a greater BNF capacity (e.g. as a result of plant–*Rhizobium* genotype interactions; Mytton *et al*. 1977), leading to an age distribution in which older trees are likely to be inherently higher N fixers; or (2) that fixation capacity increases with plant age. The latter could result from increasing access to resources (such as phosphorus or soil moisture) that might limit fixation (Hartwig 1998; Augusto *et al*. 2013). At this site, available P in the rooting zone increases significantly with *Prosopis* age (Kantola 2012), and larger root systems increase access to soil moisture, shown to correlate with nodule abundance (Zitzer *et al*. 1996).

Alternatively, increasing fractionation of soil solution N upon uptake with plant age could explain our observation, but seems mechanistically less likely. Increasing $\delta^{15}N_{soil\ inorganic\ N}$ values with plant age also correlated strongly with an increase in the concentration of inorganic N in the rooting zone (Fig. 5). Laboratory experiments have demonstrated that isotopic fractionation of N upon uptake increases depending on external N availability in certain herbaceous species (Evans 2001; Yoneyama *et al*. 2001; Kolb & Evans 2003), although the concentrations used are typically very high. In natural systems, it is assumed that N availability is too low relative to plant N demand for such a fractionation to be realized (Evans 2001; Hobbie & Högberg 2012). Even if uptake discrimination did occur at field concentrations, the contribution of fractionation during this step would need to be great enough to counter the increase in the $\delta^{15}N$ of the total soil inorganic N pool that we observed with increasing concentration.

In conclusion, the explicit sampling of $\delta^{15}N$ in xylem, foliar and soil pools can provide useful information on seasonal and long-term variation describing N fixation, fractionation, N use physiology in woody perennials and ecosystem-scale N cycling. For example, explicit sampling of the isotopic composition of plant available soil N sources, and comparison with simultaneously sampled xylem sap, can be used to infer patterns of increasing contribution of BNF to plant N nutrition over time not apparent via tissue sampling alone. Measuring and subsequently being able to control for the composition of the soil pool identified other sources of variability in plant isotopic composition, such as individual tree identity. In addition, seasonal variation identified here as well as long-term potentially climate-driven shifts in foliar isotopic values introduce variation that significantly affects interpretation of potential BNF, emphasizing the importance of repeated sampling over time. Finally, applying this method to test the assumptions of the foliar ^{15}N technique demonstrates violation of assumptions that preclude its use in many systems, further highlighting the need for alternative approaches to investigating BNF in natural ecosystems.

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AUTHOR CONTRIBUTIONS

F.M.S., J.P.S. and T.W.B. formulated the original idea and developed methodology. F.M.S., T.W.B. and J.P.S. conducted field sampling. F.M.S. analyzed samples and performed statistical analysis. F.M.S. and J.P.S. interpreted data and wrote the manuscript, and T.W.B. provided editorial advice.

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