Large variation in nitrogen isotopic composition of a fertilized legume

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A B S T R A C T

Plant nitrogen isotopic compositions are highly variable and are influenced by a diversity of environmental and anthropogenic factors, including the application of animal-derived fertilizers. Legumes that acquire most of their nitrogen from atmospheric N_2 (rather than mineralized soil nitrogen) tend to have relatively low $\delta^{15}N$ values (consistently around 0‰), and it has been presumed that their $\delta^{15}N$ values are largely or wholly unaffected by fertilization. This study presents nitrogen isotopic data from leguminous (garden bean, *Phaseolus vulgaris*) and non-leguminous (summer squash, *Cucurbita pepo*) plants subjected to seabird guano fertilization while growing under controlled conditions. Both bean and squash tissue $\delta^{15}N$ values were substantially increased by seabird guano fertilization: +16.3 to +19.2‰ for bean and +19.6 to +24.5‰ for squash. The results of this study demonstrate that the enrichment in plant ^{15}N resulting from seabird guano fertilization occurs consistently in non-maize species. Moreover, it demonstrates that under conditions of high soil nitrogen availability, leguminous plants may obtain a substantial portion of their nitrogen through the uptake of inorganic soil nitrogen (ammonium and nitrate), rather than atmospheric N_2. In general, where the $\delta^{15}N$ values of fertilizers differ substantially from that of endogenous soil nitrogen and mineralized nitrogen derived from the fertilizer is readily available, a significant manuring effect can be expected in leguminous plants.

1. Introduction

Plant nitrogen isotopic compositions are influenced by a variety of environmental factors at global, continental, regional, and local scales. These include temperature, water availability, mycorrhizal associations, and relative openness of the nitrogen cycle (Craine et al., 2009; Handley et al., 1999; Szpak et al., 2013). Plant $\delta^{15}N$ values are strongly influenced by the application of fertilizers derived from animal waste (Bogaard et al., 2007; Choi et al., 2002; Fraser et al., 2011; Szpak et al., 2012a). Additionally, irrespective of anthropogenic influence, plants that have symbiotic associations with N_2-fixing bacteria (rhizobia) such as beans, peas, lentils, chickpeas, and peanuts (legumes, family Fabaceae) consistently have $\delta^{15}N$ values around 0‰ (Fig. 1). Legume $\delta^{15}N$ values are typically lower than non N_2-fixing plants (Yoneyama et al., 1986) because they derive a portion of their nitrogen from atmospheric N_2, which has a nitrogen isotopic composition of 0‰ (Mariotti, 1983). There is little fractionation of ^{15}N during N_2-fixation (Kohl and Shearer, 1980), resulting in plant tissues with nitrogen isotopic compositions consistently around 0‰.

Legumes have been significant components of prehistoric agricultural systems throughout much of the world, including: Europe (Bogaard, 2004), the near east (Abbo et al., 2009), Africa (D'Andrea et al., 1999), South Asia (Fuller and Harvey, 2006), East Asia (Crawford et al., 2005), North America (Hart et al., 2002), Mesoamerica (Staller and Carrasco, 2010), and South America (Pearsall, 2008). Because of their high protein content relative to other plants, legumes can represent a substantial portion of protein in human diets (Graham and Vance, 2003). The proteinaceous component of the diet is that which is most commonly assessed in isotopic paleodietary studies because the tissue most frequently analyzed (bone collagen) is derived principally from dietary protein (Jim et al., 2004). It is therefore especially crucial that the range and variation in carbon and nitrogen isotopic compositions of leguminous plants be understood. Regional surveys of cultivated plants provide one means towards this end (Szpak et al., 2013; Warinner et al., 2013), but because the nitrogen isotopic compositions of any plants collected from modern contexts are strongly influenced by local agricultural practices (which are unlikely to be analogous with those practiced in antiquity), these data must be interpreted cautiously in paleodietary analyses. Studies of controlled settings

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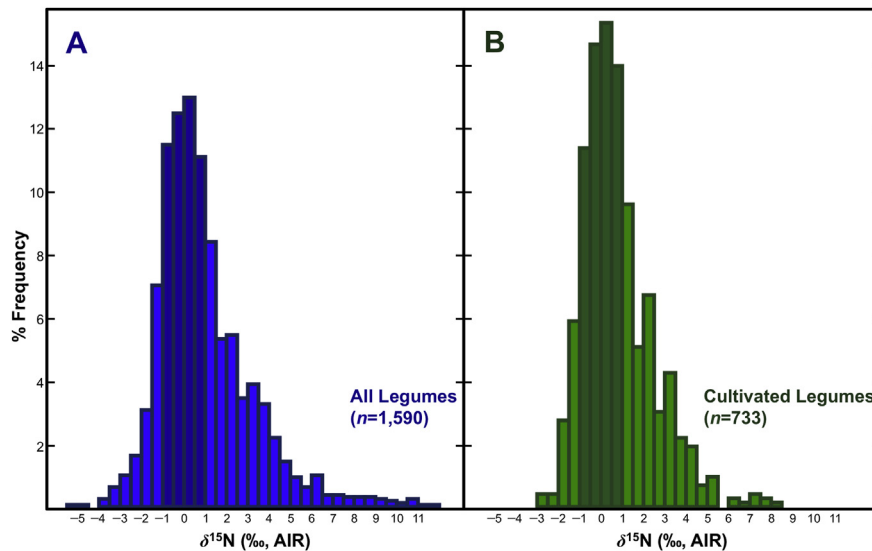
Fig. 1. Histograms representing nitrogen isotopic compositions of leguminous plants. Data were taken from published literature (see Appendix) and are for above-ground plant tissues only. (A) Measurements from both cultivated (e.g. bean, pea, chickpea, groundnut) and wild (e.g. clover, *Acacia* spp., *Inga* spp.) species. (B) Measurements from cultivated, edible legumes only. The darker shaded bars represent $\delta^{15}N$ values between -1 and $+1‰$, which represent 47.8% of the surveyed measurements for all legumes and 55.1% of the cultivated legumes.

are necessary to fully understand the complexities of plant nitrogen isotopic compositions.

It has been suggested that leguminous (N_2-fixing) plants are minimally affected by manuring relative to non-legumes (Bogaard et al., 2013; Fraser et al., 2011). On the basis of nitrogen isotopic data from a number of long-term experimental fields, Fraser et al. (2011) concluded that legume $\delta^{15}N$ values are not affected, or minimally affected, by manure application unless the application is at a very high rate over the long term. Aside from the rate and duration of application, an important consideration in terms of the effects of manuring on plant $\delta^{15}N$ values is the *type* of manure used. While in the vast majority of cases, nitrogen isotopic compositions of manured plants tend to be higher than unfertilized plants, the magnitude of this difference is highly variable, both between and within different fertilizer types.

The purpose of the present study was to examine the effects of seabird guano on the nitrogen isotopic composition of a nitrogen-fixing plant (common garden bean, *Phaseolus vulgaris*) in a controlled setting. Seabird guano is a fertilizer that was of tremendous agricultural importance in the nineteenth century and was mined extensively from nearshore islands off the western coast of South America from the 1840s through to the 1870s (Cushman, 2013). This fertilizer may also have been used in by various groups in the Andes prior to the arrival of Europeans (Garcilaso de la Vega, 1966; Julien, 1985; Kubler, 1948; Netherly, 1977; Nordt et al., 2004). In addition to having implications for dietary reconstruction in the Andean region of South America (see Szpak et al., 2012a), the high nitrogen isotopic composition of seabird guano ($\delta^{15}N > +20‰$) makes assessment of the relative importance of fertilizer- and atmospheric-nitrogen to plant growth much less ambiguous than other fertilizers (e.g. cattle or sheep manure).

2. Materials and methods

2.1. Experimental design

Common beans (*Phaseolus vulgaris* L., garden bean 'provider', The Cook's Garden®, Lot 3, 2010) and summer squash (*Cucurbita pepo*, early summer crookneck, Burpee®, Lot 6, 2010) were grown in a walk-in growth chamber at the Biotron Institute for Experimental Climate Change Research at the University of Western Ontario. Growth chamber conditions were: 25/18 °C (day/night temperature), 13 h photoperiod (185 W fluorescent bulbs), 60% relative humidity (Szpak et al., 2012b). Both beans and squash (four replicates each) were grown in 2 L free-draining (perforated at the base) plastic containers; substrate was Pro-mix® for containers (75–85% sphagnum moss, 15–25% perlite and limestone).

Seedlings were sprouted in the 2 L containers in the absence of any fertilizers. Peruvian seabird guano (Guano Company International) was applied to the surface of the soil at a rate of 5 g/ container 5 days after seedling emergence (four replicates for beans and squash). The nitrogen isotopic composition of the guano was previously determined to be $+26.7 \pm 0.6‰$ (Szpak et al., 2012b). Four replicate controls (no fertilizer applied) of both beans and squash were grown simultaneously under the same conditions. Leaf and fruit samples were harvested 65 d after planting.

2.2. Sample preparation

Leaf and fruit samples were cleaned of visible foreign matter and frozen (-25 °C) immediately after sampling. Leaf samples consisted of entire leaves excluding petioles; for squash three leaves were sampled and for beans all leaves on each plant were sampled. Fruit samples consisted of entire fruits for squash. For bean fruit samples, all beans within the largest pod at the time of harvest were homogenized; the pod was not included in the sample. Additionally, for one control and one fertilized plant, single beans from the second largest pod at the time of harvest were processed individually to assess any potential within-fruit variation in nitrogen isotopic composition. Prior to isotopic analysis, samples were dried at 90 °C for at least 48 h (leaves) or 96 h (fruit) and then ground to a homogenous fine powder using a mechanical shaker (Crescent Wig-L-Bug). This material was subsequently dried at 90 °C for at least 48 h.

Two commercial samples of Peruvian seabird guano were also analyzed for comparative purposes: Original Sea Bird Guano (Guano Company International, reported N–P–K 13–12–2) and Peruvian Seabird Guano (Sunleaves Garden Products, reported N–P–K 12–11–2). One ~50 g sample was taken from each of five

454 g bags of each commercial guano. These samples were ground, homogenized, and dried at 90 °C for 72 h.

2.3. Stable isotope analysis

Nitrogen isotopic ($\delta^{15}N$) and elemental (% N) compositions were determined using a Thermo Finnigan Delta V Plus isotope ratio mass spectrometer coupled to a Costech elemental analyzer at the Laboratory for Stable Isotope Science (LSIS) at The University of Western Ontario (London, ON, Canada). Following sample combustion, CO_2 was removed with a Carbo-Sorb trap.

Sample $\delta^{15}N$ values were calibrated to AIR with USGS40 (accepted value $\delta^{15}N = -4.5 \pm 0.1‰$) and USGS41 (accepted value $\delta^{15}N = +47.6 \pm 0.2‰$). In addition to USGS40 and USGS41, internal (keratin, average $\delta^{15}N = +6.4‰$) and international (IAEA-N-1, accepted $\delta^{15}N = +0.4 \pm 0.2‰$; IAEA-N-2, accepted $\delta^{15}N = +20.3 \pm 0.2‰$) standard reference materials were analyzed to monitor analytical precision and accuracy. For keratin mean $\delta^{15}N$ within runs was +6.2 to +6.5‰ and mean $\delta^{15}N$ among all runs was $+6.4 \pm 0.1‰$ ($n = 59$). For IAEA-N-1 mean $\delta^{15}N$ within runs was +0.4 to +0.6‰ and mean $\delta^{15}N$ among runs was $+0.5 \pm 0.1‰$ ($n = 12$). For IAEA-N-2 mean $\delta^{15}N$ within runs was +20.2 to +20.5‰ and mean $\delta^{15}N$ among runs was $+20.3 \pm 0.2‰$ ($n = 27$). For plant samples, reproducibility was $\pm0.08‰$ for $\delta^{15}N$ and $\pm0.04\%$ for %N (mean differences between duplicate pairs for 13 replicates).

Nitrogen isotopic and elemental compositions were compared between treatment and control groups with an unpaired t-test using R for Mac OS X (R Development Core Team, 2007).

3. Results

3.1. Seabird guano $\delta^{15}N$

Nitrogen isotopic and elemental compositions for the two commercial guano samples are presented in Table 1 along with similar data for the guano used in this experiment (Szpak et al.,

Table 1
Nitrogen isotopic and elemental compositions for Peruvian seabird guano samples. Values in boldface are means ± one standard deviation for each variety of guano.

Guano	$\delta^{15}N$ (‰, AIR)	% N
Peruvian Seabird Guano (Guano International Company)[a,b]	+26.1	11.5
	+27.3	11.3
	+26.6	11.0
	+26.1	11.2
	+27.4	11.0
	+26.7 ± 0.6	**11.2 ± 0.2**
Locally collected Peruvian seabird guano[b]	+38.1	8.6
	+37.6	8.8
	+37.8	8.5
	+37.8	8.7
	+39.1	6.7
	+38.1 ± 0.6	**8.2 ± 0.9**
Original Peruvian seabird guano (Guano Company International)	+27.4	7.5
	+26.0	10.5
	+30.5	7.2
	+27.8	7.0
	+31.8	8.0
	+29.0 ± 2.6	**8.2 ± 1.6**
Peruvian seabird guano (Sunleaves Garden Products)	+22.8	12.3
	+21.9	13.4
	+23.4	11.8
	+23.2	12.4
	+25.5	9.5
	+23.5 ± 1.5	**11.8 ± 1.7**

[a] Guano used in this experiment.
[b] Five replicates of a single homogenized sampled rather than samples from five discrete bags.

2012b) and seabird guano purchased at a local market in Trujillo, Peru (Szpak et al., 2012a). Mean $\delta^{15}N$ values for Peruvian seabird guano ranged from $+23.5 \pm 1.5‰$ to $+38.1 \pm 0.6‰$. These are consistently the highest $\delta^{15}N$ values recorded for any fertilizer of organic or inorganic origin (Bateman and Kelly, 2007). The large sample-to-sample differences may be the product of environmental variation among different guano islands. Processes such as nitrogen mineralization and ammonia volatilization will vary according to temperature and water availability (Cassman and Munns, 1980; Ernst and Massey, 1960), and different islands may produce guano with distinct nitrogen isotopic compositions. Additionally, variable species of guano birds (guanay cormorant, Peruvian booby, Peruvian brown pelican) on different islands may further drive geographic variation in guano $\delta^{15}N$ values because of dietary or metabolic differences among species. Such observations are consistent with the significant differences in fertilizer quality (presumably nitrogen content) observed during the extensive mining of Peruvian seabird guano in the middle of the nineteenth century (Cushman, 2013).

3.2. Plant ^{15}N enrichment with fertilization

Nitrogen isotopic and elemental compositions for the control and fertilized beans and squash are presented in Table 2. Results are

Table 2
Nitrogen isotopic and elemental compositions for control and fertilized beans and squash. Values in boldface are means ± one standard deviation for each tissue by treatment.

Species	Tissue	Treatment	$\delta^{15}N$ (‰, AIR)	% N
Squash	Leaf	Control	+3.7	1.4
			+5.4	1.8
			+5.1	1.4
			+2.3	1.1
			+4.1 ± 1.4*	**1.4 ± 0.3****
		Guano	+20.0	3.4
			+25.4	3.9
			+18.8	6.0
			+30.9	3.2
			+23.8 ± 5.5*	**4.1 ± 1.3****
	Fruit	Control	+0.1	2.7
			+0.3	2.7
			+0.3	2.5
			+0.3	1.9
			+0.2 ± 0.1*	**2.5 ± 0.4****
		Guano	+23.1	3.1
			+23.2	3.3
			+25.5	3.4
			+27.0	3.5
			+24.7 ± 1.9*	**3.3 ± 0.2****
Bean	Leaf	Control	+0.2	4.5
			+0.7	3.9
			+0.4	4.1
			+0.3	5.3
			+0.4 ± 0.2*	**4.4 ± 0.6***
		Guano	+19.1	4.5
			+20.2	3.9
			+25.0	4.1
			+14.2	5.3
			+19.6 ± 4.4*	**4.4 ± 0.6***
	Fruit	Control	+0.8	5.4
			−0.4	4.5
			−0.8	4.5
			+0.3	4.2
			0.0 ± 0.7*	**4.6 ± 0.5***
		Guano	+16.4	6.2
			+18.1	5.7
			+16.5	6.1
			+13.9	9.1
			+16.2 ± 1.7*	**6.8 ± 1.6***

Mean values significantly differ (unpaired t-test) between guano-fertilized and control treatments at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

summarized in Fig. 2A for $\delta^{15}N$ and Fig. 2B for %N. Control bean leaves and fruits had $\delta^{15}N$ values between -1 and $+1‰$, as expected for a nitrogen-fixing legume (Fig. 1). For both leaf and fruit tissues the guano-fertilized plants were characterized by significantly higher $\delta^{15}N$ values and %N than the control plants (Table 2). The difference in $\delta^{15}N$ between the control and guano-fertilized plants was extremely large: $+19.2‰$ (bean leaf), $+16.3‰$ (bean fruit), $+19.6‰$ (squash leaf), and $+24.5‰$ (squash fruit). These increases in $\delta^{15}N$ are much larger than typically reported in studies examining fertilizers derived from terrestrial mammals (Bogaard et al., 2007; Choi et al., 2002; Fraser et al., 2011; Lim et al., 2007; Rapisarda et al., 2010; Šturm et al., 2011). However, the results are comparable with studies of maize fertilized with seabird guano, where differences between fertilized and control plant leaves and fruits ranged from $+14.9‰$ to $+30.4‰$ depending on the tissue sampled and the amount of fertilizer applied (Szpak et al., 2012a, 2012b).

3.3. Within-fruit variation in $\delta^{15}N$

Nitrogen isotopic compositions for three individual beans sampled within single pods for the control and fertilized beans are presented in Table 3. For comparative purposes, nitrogen isotopic compositions from three individual maize kernels sampled from single cobs from control and guano-fertilized plants grown in the same growth chamber, under the same conditions (but different pot sizes and guano application rates) are also presented in Table 3. There was little variation between individual beans or maize kernels, with $\leq 1‰$ variation between the three measurements. In each case, the $\delta^{15}N$ of any one of the three individual beans or kernels measured was within $1‰$ of the $\delta^{15}N$ value derived from a homogenized sample from the same plant. That there is little variation for the guano-fertilized beans and maize is significant because
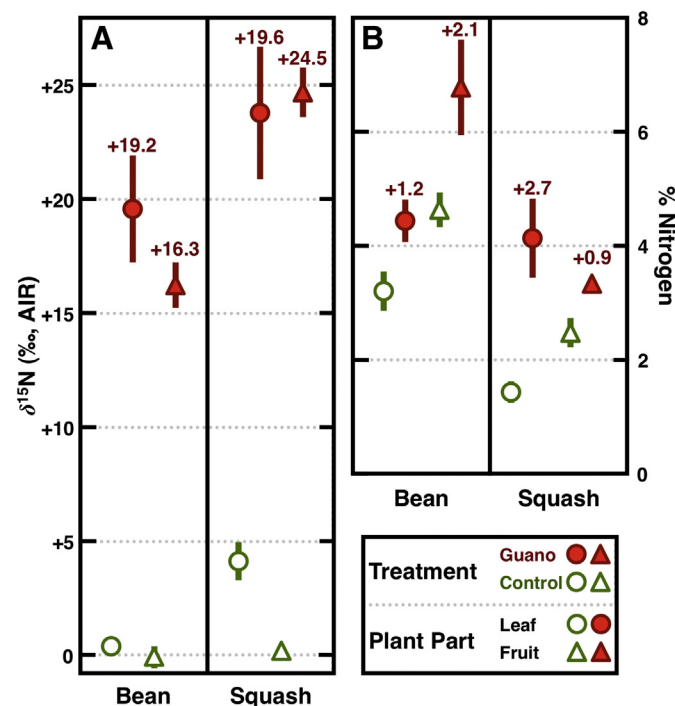
Table 3
Nitrogen isotopic composition of individual beans and maize kernels taken from the same pod or cob.

Sample	$\delta^{15}N$ (‰, AIR)				
	1	2	3	Δ[a]	Homogenized[b]
Bean (Control)	+0.7	+0.1	+0.0	0.7	+0.2
Bean (Guano)	+16.5	+16.4	+16.4	0.1	+16.4
Maize (Control)[c]	+2.0	+1.7	+1.8	0.3	+2.0
Maize (Guano)[c]	+25.0	+25.2	+25.1	0.2	+25.2
Maize (Guano)[c]	+34.6	+33.6	+33.6	1.0	+33.6

[a] Difference between maximum and minimum $\delta^{15}N$ values.
[b] Homogenized $\delta^{15}N$ for the same plant and tissue from: all beans within the largest pod (for beans) or all kernels (for maize) homogenized prior to analysis.
[c] Maize $\delta^{15}N$ values associated with experiments described in Szpak et al. (2012a) performed under the same conditions with different fertilizer application rates.

there is consistently more variation between fertilized plants relative to control plants within treatments (Szpak et al., 2012a, 2012b). This larger variation is most likely a product of rapidly changing $\delta^{15}N$ for the mineralized soil nitrogen as processes associated with large fractionations such as ammonia volatilization and nitrification (Mariotti et al., 1981; Mizutani et al., 1985; Robinson, 2001) occur following fertilizer application. Even under these conditions, there is little variation observed within a single bean pod or maize cob, which suggests that $\delta^{15}N$ values obtained from individual seeds in archaeobotanical contexts should closely approximate those of all seeds within the plant from which it originated.

4. Discussion

4.1. Dietary implications of guano fertilization of C_3 plants

Seabird guano has been previously demonstrated to cause extremely large increases in maize tissue $\delta^{15}N$ values in growth chamber (Szpak et al., 2012b) and field (Szpak et al., 2012a) studies. The results of this study demonstrate comparable effects in beans and squash. While additional data from other cultigens would be useful, it is unlikely that any species grown with seabird guano fertilization would not be characterized by the extremely high $\delta^{15}N$ values observed thus far for maize, beans, and squash.

The implications of guano fertilization for C_3 plants (such as beans and squash) are quite different than for C_4 plants (maize). Because of the high $\delta^{13}C$ value of C_4 plants (c. $-12‰$), the utilization of guano-derived nitrogen has the effect of isotopically imitating a high-trophic level marine resource (high $\delta^{13}C$, high $\delta^{15}N$) when bulk collagen $\delta^{13}C$ and $\delta^{15}N$ values are considered (Szpak et al., 2012a). Conversely, no potential food sources exist in terrestrial or marine environments with $\delta^{13}C$ values of $-26‰$ (typical of most C_3 plants) and $\delta^{15}N$ values of $>+20‰$. Whereas the consumption of guano-fertilized maize and high trophic level marine resources cannot be easily distinguished on the basis of bulk bone collagen $\delta^{13}C$ and $\delta^{15}N$ values (Szpak et al., 2012a), the consumption of appreciable quantities of C_3 crops fertilized with seabird guano would produce a relatively unique consumer isotopic signature with low $\delta^{13}C$ and very high $\delta^{15}N$ values. This phenomenon is illustrated in Fig. 3 using a simplified mixing polygon for the Andean region of Peru. The area of the mixing polygon defines the possible range of consumer isotopic compositions that can exist for a given range of sources (foods) in bivariate space (Phillips and Gregg, 2003). While additional sources certainly exist, it is only those with extreme δ-values that define the area of the minimum area-mixing polygon; any additional source isotopic compositions plotting inside the polygon will not alter its area or geometry. In Fig. 3B, the fertilization of only C_4 plants with seabird guano does

Fig. 2. Nitrogen isotopic (A) and elemental (B) compositions for control (open symbols) and guano-fertilized (filled symbol) bean and squash leaves (circles) and fruits (triangles). Values are means $\pm$ one standard deviation. Numbers above fertilized symbols indicate difference in $\delta^{15}N$ (A) or %N (B) between guano treatment and control.
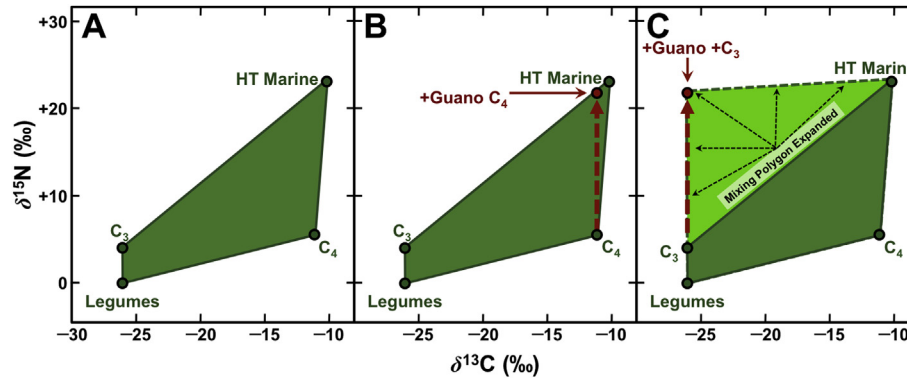
Fig. 3. Simplified mixing polygons for a generalized set of dietary resources under three different circumstances: (A) no guano fertilization, (B) guano fertilization of C_4 plants, (C) guano fertilization of C_3 plants. HT Marine = high trophic level marine consumer.

not alter the size or shape of the polygon (provided unfertilized C_4 plants are also a feasible source). Accordingly, the possible distribution of consumer $\delta^{13}C$ and $\delta^{15}N$ values should not vary markedly between the scenarios presented in Fig. 3A and B. Conversely, the fertilization of C_3 plants with seabird guano drastically alters the area of the mixing polygon, nearly doubling its size (Fig. 3C). This situation creates the possibility of consumers with low $\delta^{13}C$ and high $\delta^{15}N$ values, and would alter the distribution of consumer $\delta^{13}C$ and $\delta^{15}N$ values in bivariate space if seabird guano was used to fertilize C_3 plants. The fertilization of both C_3 and C_4 plants with seabird guano would produce the same isotopic pattern as in Fig. 3C. In summary, diets composed of large portions of C_3 plants fertilized with seabird guano are not likely to be confused with other subsistence strategies on the basis of bulk collagen carbon and nitrogen isotopic data because of the expansion of the mixing polygon depicted in Fig. 3. Consumption of mixed C_3 and C_4 plants fertilized with seabird guano would also produce a distinctive distribution of consumer $\delta^{13}C$ and $\delta^{15}N$ values, lacking the positive linear correlation often observed for humans consuming variable portions of terrestrial and marine foods (Richards and Hedges, 1999).

4.2. Influence of fertilizers on N_2 fixation by legumes

Leguminous plants form symbiotic relationships with soil bacteria (*Rhizobium*) that infect roots, forming characteristic nodules. These bacteria have the capacity to reduce atmospheric N_2 to ammonium (NH_4^+) via the nitrogenase enzyme (Howard and Rees, 1996). This ammonium is exported from the bacteria into the plant nodule cytosol where it is assimilated into organic nitrogen (Mylona et al., 1995). The growth of the nodules containing the bacteria and the reduction of N_2 to NH_4^+ is metabolically supported by photosynthates (sucrose in most species) provided by the host plant (Rawsthorne et al., 1980). Effectively, the nodule acts as a carbon sink and nitrogen source for the plant (Mylona et al., 1995). There is variation among different legume species according to local environmental conditions, but the plant may allocate greater than 30% of net photosynthate production to support nodule growth and N_2 reduction by rhizobia (Schubert, 1986). Thus, the acquisition of fixed nitrogen by legumes represents a considerable metabolic investment. Legumes do not lack the capacity to take up inorganic nitrogen from the soil, and typically the degree to which they rely on nitrogen acquired through symbiotic N_2-fixation will vary with the availability of mineralized nitrogen (ammonium and nitrate) in the local environment (Ledgard and Steele, 1992).

Because leguminous plants contribute substantial amounts of protein to human diets worldwide (Tharanathan and Mahadevamma, 2003), the rate at which they assimilate N_2 from the atmosphere under field conditions has been investigated extensively. Table 4 presents estimates of the relative percentage of nitrogen derived from the atmosphere relative to the soil or fertilizer for a number of cultivated legumes. Important to note, both within and among species, is the wide variation observed for the amount of nitrogen acquired via fixation. This variation is caused by a number of environmental factors (water availability, temperature, competition with other plants for soil nitrogen). The most important factor, however, is the availability of inorganic nitrogen in the soil (Liu et al., 2011).

Under conditions of high nitrate availability (as occurs with the application of nitrogenous fertilizers), nodule formation and N_2-fixation in legumes decreases (Ledgard et al., 1996; Müller and Pereira, 1995; Nesheim et al., 1990). This is true both for applications of inorganic (chemical) and organic fertilizers. For example, Menneer et al. (2003) observed a decline in the percentage of nitrogen obtained from the atmosphere from 84% to 22% (for clover) following cow urine application; this suppression of nitrogen fixation gradually ceased after nearly one year. Vinther (1998) found the application of cattle dung reduced N_2 fixed by clover, but only by 10% and only within 10 cm of dung pats. Other studies have similarly observed significant reductions in legume nitrogen fixation with the application of urine (Saunders, 1982; Vinther, 1998) or slurry (mixture of urine, feces, and general barnyard waste) fertilizers (Nesheim et al., 1990; Rasmussen et al., 2012), and much smaller reductions with dung application (Jørgensen and Jensen, 1997).

The results of the present study clearly demonstrate a suppression of nitrogen fixation in the common bean (*Phaseolus vulgaris*) when seabird guano is used as a fertilizer. The difference between the mean $\delta^{15}N$ values of guano-fertilized and control leaves is +19.2‰ for bean and +19.6‰ for squash (Fig. 2). This similar difference suggests that the fertilized beans obtained a large portion, or possibly all, of their nitrogen from seabird guano during the formation of the leaves – a similar logic (^{15}N natural abundance method) is used extensively in assessments of nitrogen fixation rates in plants (Shearer and Kohl, 1986). At the very least, the beans and squash obtained similar amounts of nitrogen from the guano during leaf formation. That there is a not a similar difference in $\delta^{15}N$ between the fertilized and control fruits (+16.3‰ for bean, +24.3‰ for squash) may be driven by variable demands for nitrogen during fruit formation by these two species. Specifically, beans may have acquired a larger portion of nitrogen from N_2-fixation towards the end of the growth period (during pod filling) when the vast majority of absorbed nitrogen is allocated to the fruit (Dubois and Burris, 1986). During fruit development in the common bean,

Table 4
Estimations of the percentage of nitrogen obtained from the atmosphere (% Ndfa) for select leguminous cultigens. %Ndfa values are reported as means or ranges.

Location	%Ndfa[a]	Reference
Lentil (*Lens culinaris*)		
Canada (Alberta)	67	(Rennie and Dubetz, 1986)
Canada (Western)	23	(Bremer et al., 1988)
Canada (Western)	25	(Bremer et al., 1988)
Nepal	78	(Maskey et al., 2001)
New Zealand	17–24	(Haynes et al., 1993)
Pakistan	50–87	(Shah et al., 2003)
Syria	55–68	(Kurdali et al., 1997)
Fababean (*Vicia faba*)		
Austria	72–79	(Zapata et al., 1987)
Canada (Alberta)	85	(Rennie and Dubetz, 1986)
Canada (Western)	58	(Bremer et al., 1988)
Canada (Western)	57	(Bremer et al., 1988)
Nepal	85	(Maskey et al., 2001)
New Zealand	4–34	(Haynes et al., 1993)
Portugal	59–82	(Carranca et al., 1999)
Pea (*Pisum sativum*)		
Australia (Western)	65–81	(Armstrong et al., 1994)
Canada (Alberta)	79	(Rennie and Dubetz, 1986)
Canada (Saskatchewan)	13–97	(Androsoff et al., 1995)
Canada (Western)	33	(Bremer et al., 1988)
Canada (Western)	41	(Bremer et al., 1988)
Denmark	65–92	(Hauggaard-Nielsen et al., 2010)
Denmark	68–75	(Hauggaard-Nielsen et al., 2009)
France	46–52	(Hauggaard-Nielsen et al., 2009)
Germany	81–86	(Hauggaard-Nielsen et al., 2009)
Italy	63–76	(Hauggaard-Nielsen et al., 2009)
New Zealand	16–22	(Haynes et al., 1993)
Portugal	8–82	(Carranca et al., 1999)
United Kingdom	72–83	(Hauggaard-Nielsen et al., 2009)
Chickpea (*Cicer arietinum*)		
Canada (Western)	82	(Rennie and Dubetz, 1986)
Nepal	79	(Maskey et al., 2001)
Portugal	44–83	(Carranca et al., 1999)
Syria	60	(Kurdali, 1996)
USA (California)	51–93	(Lee et al., 2011)
Cowpea (*Vigna unguiculata*)		
Ghana	9–90	(Belane and Dakora, 2009)
Ghana	56–96	(Belane and Dakora, 2010)
South Africa	49–81	(Belane et al., 2011)
South Africa	44–56	(Makoi et al., 2009)
Zambia	58–60	(Nyemba and Dakora, 2010)
Groundnut (*Vigna subterranea*)		
Austria	55–75	(Kumaga et al., 1994)
Nepal	57	(Maskey et al., 2001)
South Africa	33–98	(Mohale et al., 2013)
Zambia	27–70	(Nyemba and Dakora, 2010)
Common bean (*Phaseolus vulgaris*)		
Austria	27–67	(Hardarson et al., 1993)
Brazil	12–53	(Hardarson et al., 1993)
Canada (Western)	32–50	(Rennie and Kemp, 1983)
Chile	27–60	(Hardarson et al., 1993)
Colombia	32–47	(Hardarson et al., 1993)
Guatemala	22–73	(Hardarson et al., 1993)
Japan	37–47	(Kimura et al., 2004)
Mexico	19–26	(Vásquez-Arroyo et al., 1998)
Mexico	0–58	(Hardarson et al., 1993)
Peru	13–56	(Hardarson et al., 1993)
USA (Wisconsin)	5–32	(Pereira et al., 1989)
Zambia	3–55	(Nyemba and Dakora, 2010)

[a] Estimate of the % nitrogen derived from the atmosphere.

nitrogen obtained from fixation is allocated *exclusively* to the fruit (Dubois and Burris, 1986) and the rate of fixation is highest at this stage (Kumarasinghe et al., 1992). Because of the high nitrogen content of legumes (Mattson, 1980), it is possible that less guano-derived nitrogen was available during fruit development for the bean relative to the squash. Accordingly, there may have been a higher demand for nitrogen obtained through N_2-fixation during pod-filling, even for guano-fertilized plants. Regardless, a large portion of the nitrogen in the bean fruit was still derived from the guano. This outcome illustrates the potential for legume $\delta^{15}N$ values to be significantly impacted by animal fertilizers, although these effects will be strongest where mineralized nitrogen derived from the fertilizer is readily available to plants and the nitrogen isotopic composition of the fertilizer differs significantly from that of the soil and atmospheric nitrogen. These conditions are most likely to be met with seabird guano, poultry manure, and slurry fertilizers, but are relatively unlikely in the case of solid manure derived from terrestrial herbivores (e.g. cattle, sheep) as is evidenced by the data presented by Fraser et al. (2011).

5. Conclusions

The large enrichment in plant tissue ^{15}N caused by seabird guano occurs in both squash and beans, confirming previous results obtained for maize (Szpak et al., 2012a, 2012b). The consumption of substantial amounts of C_3 plants fertilized with seabird guano would produce a unique consumer isotopic signature (low $\delta^{13}C$, very high $\delta^{15}N$) that would be difficult to confuse with other subsistence strategies. Our data show that the nitrogen isotopic compositions of legumes can be greatly impacted by animal-derived fertilizers under certain circumstances.

The following general expectations are consistent with the results of our study, as well as those of Fraser et al. (2011), who found little to no effect of cattle manure fertilization on legume $\delta^{15}N$ values. First, where animal fertilizers contain principally organic nitrogen and have a relatively low nitrogen isotopic composition, legume $\delta^{15}N$ values should be minimally impacted. Second, legume $\delta^{15}N$ values will be increasingly impacted as: (a) the $\delta^{15}N$ value of the fertilizer increases and therefore becomes more distinct from the $\delta^{15}N$ value of endogenous soil nitrate and ammonium, and atmospheric N_2, (b) the amount of mineralized nitrogen available from the fertilizer increases due to suppression of nodulation and N_2 fixation in legumes, and/or (c) the amount and frequency of fertilizer application increase, again, increasing the supply of mineralized nitrogen derived from the fertilizer, rather than the soil.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jas.2014.02.007.

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