Using a Stable Isotope to Label Seeds and Seedlings of an Invasive Shrub, *Lonicera maackii*

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Seed dispersal is a crucial process in most plant invasions, but is notoriously difficult to study. One technique to identify the maternal source of dispersed seeds and newly established seedlings is labeling with a stable isotope. We tested whether foliar application of ^{15}N-labeled urea would result in sufficient ^{15}N enrichment to discriminate among seeds and seedlings grown from those seeds of the invasive shrub *Lonicera maackii* (Amur honeysuckle). We subjected mature *L. maackii* to all combinations of three concentrations of ^{15}N-labeled urea (0.025 g L^{-1} [0.003 oz gal^{-1}], 0.20 g L^{-1}, and a 0 g L^{-1} control) and three temporal treatments (one application in August, one application in September, and five applications spaced every three weeks from June through August). Seeds were collected September to November; some of these were analyzed for $\%^{15}$N and others allowed to germinate and grow into seedlings under two treatments (in potting mix in greenhouse and in woodlot soil outdoors). Seedlings were harvested midway through the next growing season. We found that seeds from plants subjected to the three different concentrations had significantly different $\%^{15}$N levels, and there was a significant interaction between concentration and temporal treatment: the highest seed $\%^{15}$N levels were from plants sprayed five times with ^{15}N-labeled urea, and the second highest from plants sprayed once in September. Similar patterns in $\%^{15}$N levels were found in seedlings, except that those from the 0.025 g L^{-1} spray treatment were only distinguishable from controls for seedlings grown outdoors in woodlot soil. These findings demonstrate that a single foliar application of ^{15}N in early September is sufficient to label both seeds and seedlings of this invasive shrub, enabling one to identify the source of field-collected seeds or seedlings. This provides a tool for studying patterns and processes in seed dispersal of Amur honeysuckle and potentially other invasive plants.

Nomenclature: Amur honeysuckle, *Lonicera maackii* (Rupr.) Herder.

Key words: Amur honeysuckle, foliar spray, ^{15}N, seed dispersal, urea.

Dispersal and establishment are integral parts of species invasion (Allendorf and Lundquist 2003; Richardson et al. 2000) and largely influence the pattern of invasive spread. Depending on the mode of dispersal, invasive spread may occur along an ''invasion front'', concentrically outward from smaller satellite foci, or a combination of these (Dietz 2002; Moody and Mack 1988). Effective management of invasive species depends on an understanding of how new sites become colonized (Edwards and Leung 2009; Moody and Mack 1988); therefore, an understanding of their dispersal dynamics and recruitment patterns is critical. However, it is difficult to link population patterns to dispersal patterns due the difficulty in determining the source of the new recruits; this is especially true when rare, but greatly important, long-distance dispersal events are involved (Nathan 2006; Nathan and Muller-Landau 2000; Wang and Smith 2002).

A variety of different techniques have been employed to try to link plant recruits to their parent source (Carlo et al. 2009; Wang and Smith 2002), but an emerging method described as having great potential is stable isotope labeling. Stable isotopes have been used for numerous ecological and environmental applications (Michener and Lajtha 2007), but Carlo et al. (2009) were the first to apply them to seed dispersal using foliar application. Carlo et al. (2009) used foliar application of ^{15}N-labeled urea; ^{15}N enters through the epidermis and becomes incorporated into plant tissues including seeds (Schmidt and Scrimgeour 2001). As seeds are a sink for nitrogen translocated from the foliage (Rentsch et al. 2007), they would acquire a greater ^{15}N to ^{14}N ratio than found naturally. Carlo et al. (2009) found

DOI: 10.1614/IPSM-D-12-00053.1

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Management Implications

Understanding the patterns and processes of seed dispersal can inform strategies for managing and eradicating invasive plants in landscapes. For example, deciding where to invest resources to detect and treat an invasive species depends on the relative importance of long-distance dispersal vs. diffusion (expansion of existing populations). For species where diffusion is important, resources should be focused on treating the advancing front of existing populations, whereas for species where long-distance dispersal is important, patrolling outside the invaded area for newly established individuals and patches would be more effective.

Seed dispersal, however, is notoriously difficult to study, in part because it is challenging to determine the source (mother plant) of dispersed seeds and new seedlings. One potential method for matching seeds/seedlings with sources is stable isotope labeling, where one or more mature plants is treated with a stable isotope that is then incorporated in the tissue of the seed.

This study shows that a stable isotope of nitrogen, ^{15}N, can be used to label both the seeds and seedlings of Amur honeysuckle, one of the most problematic invasive shrubs in the Eastern and Midwestern United States. A single foliar application of ^{15}N-labeled urea in late summer was sufficient to label seeds that matured that fall, so that they could be distinguished, via laboratory analysis of the N isotope ratio, from seeds of unsprayed plants. Furthermore, seedlings harvested midway through the next growing season retained sufficient ^{15}N that those from sprayed mother plants could be distinguished from those from unsprayed mothers.

These findings demonstrate that it is possible to use stable isotope labeling to determine the relative importance of different seed sources in an invasion, for example distinguishing how many new seedlings are from seeds of an isolated new population/horticultural planting vs. from a more distant but larger invasive population.

that seeds of ^{15}N-treated adults of two herbaceous plants, *Solanum americanum* Mill. and *Capsicum annuum* L., reliably had elevated ^{15}N levels; the ^{15}N signal was retained in growing *S. americanum* seedlings for a month after germination, and seeds from adults treated with different dosages could be differentiated.

The objective of this study was to determine whether the seeds and seedlings of an invasive shrub, *Lonicera maackii* (Rupr.) Herder (Caprifoliaceae; Amur honeysuckle), can be labeled with ^{15}N. This multi-stemmed, deciduous shrub native to eastern Asia was first introduced to the United States in 1898 (Luken and Thieret 1995). Since its introduction, *L. maackii* has spread throughout much of the Eastern and Midwestern U.S. and now is recorded in 26 states and Ontario, Canada; over much of its introduced range, it is listed as an invasive plant or a noxious weed (USDA 2012).

In this study we tested (1) if ^{15}N-urea solution applied to *L. maackii* foliage in summer results in the signal being detectable in seeds and seedlings, (2) the best time to apply the solution, (3) whether one treatment is sufficient or multiple treatments are required, (4) which of two solution concentrations is more effective, and (5) if the signal is retained in the labeled seedlings over the course of a growing season. Carlo et al. (2009) found that the strength of the ^{15}N signal in *S. americanum* seedlings was negatively correlated with seedling dry mass as plant nitrogen became diluted with naturally occurring ^{14}N. To be used in seedling recruitment studies, it is necessary for the signal to be detectable in the seedlings for some time after germination to identify the source of successful recruits that survive the early months.

Materials and Methods

Study Species. *Lonicera maackii* was often employed to improve habitat for birds and used as an ornamental shrub in horticultural plantings because of the attractive flowers and high fruit production (Luken and Thieret 1996). First documented as naturalized in Ohio by Braun (1961), *L. maackii* has since become the most common shrub in southwest Ohio (Luken and Thieret 1995) and often dominates old fields and the shrub layer of forests (Hutchinson and Vankat 1997).

Lonicera maackii negatively impacts local ecosystems by decreasing the fecundity of both annual and perennial plants (Gould and Gorchov 2000; Miller and Gorchov 2004) and reducing tree seedling survival and growth (Gorchov and Trisel 2003; Hartman and McCarthy 2004, 2008). Recent evidence suggests that *L. maackii* also has potential allelopathic chemicals, such as phenolic metabolites (Cipollini *et al.* 2008), that reduce seed germination and plant growth (Dorning and Cipollini 2006). In exposed sites, such as forest edges, *L. maackii* shrubs maximize their size through branch production (Luken et al. 1995), thus providing a competitive edge over other plants. *Lonicera maackii* seedlings, however, grow successfully in the shade of larger shrubs, forming dense clumps (Castellano and Boyce 2007). While associated with lower nesting success of songbirds (Schmidt and Whelan 1999), *L. maackii* is dispersed by several species of edge-inhabiting birds (Bartuszevige and Gorchov 2006; Ingold and Craycraft 1983), as well as white-tailed deer (*Odocoileus virginianus*) (Castellano and Gorchov 2013).

Site Selection. This study was conducted in an old field recently invaded by *L. maackii* at the Ecology Research Center (ERC) of Miami University in Oxford, Ohio. The site has a southwest aspect with vegetation comprised of grasses, *L. maackii*, *L. tatarica* L., *Juniperus virginiana* L., *Robinia pseudoacacia* L., *Rubus* sp., etc (S. M. Castellano, unpublished data). The soil on the site consists of Russell-Miamian silt loams, 2 to 6% slopes, moderately eroded and Hennepin-Miamian silt loams, 18 to 25% slopes, moderately eroded (USDA-NRCS 2008).

Sample Selection. In May, 2009, a total of 54 reproductively mature (flowering) *L. maackii* shrubs, ranging in

https://doi.org/10.1614/IPSM-D-12-00053.1 Published online by Cambridge University Press

height from 1.20 m to 2.75 m (4 to 9 feet), were selected for isotopic labeling. To ensure that each shrub could be treated with minimal potential of cross-shrub contamination, we used only shrubs that were not touching or overhanging other test shrubs.

^{15}N Treatment. Solutions of ^{15}N-enriched urea (98+% purity; Cambridge Isotope lab) were prepared in distilled water in the following concentrations: 0.025 g L^{-1}, 0.20 g L^{-1}, and a 0 g L^{-1} control. Coco-Wet® (Spray-n-Grow, Rockport TX) wetting agent, a nonreactive mixture of modified cocodiethanolamide (90%), was added to each concentration (including control), in accordance with manufacturer's recommendations, to ensure the adherence of the urea solution to shrub foliage and to enhance nutrient delivery into the leaf. Shrubs were randomly assigned to nine treatment combinations: the three concentrations crossed with the following three "temporal treatments": five applications three weeks apart beginning June 5 and ending August 27, 2009, one application on August 6, 2009, and one application on September 4, 2009. These intervals were chosen to investigate if a single application is sufficient for labeling and what treatment date was most effective. Six replicate shrubs were assigned to each of the nine treatments. All foliage and twigs were sprayed using a hand pump sprayer at a rate of approximately 750 ml (0.8 quarts) per shrub. Steps were taken to minimize airborne drift such as holding other shrubs away from treated shrubs, spraying only during windless conditions, and maintaining a downward spray pattern. Treatment was only done on days without precipitation.

Fruit and Seed Handling. Mature fruit was collected from each shrub from September to November 2009. Seeds were hand removed from fruit pulp, then air-dried and stored at room temperature (approximately 23 C [73 F]). From each treatment some seeds were selected for germination and seedling growth and other seeds were prepared for ^{15}N analysis. Seeds from 11 shrubs were analyzed in a preliminary study (Castellano and Gorchov 2010), and one shrub did not produce fruit. For analysis of seeds of the other 42 shrubs, we selected two seeds from each shrub, representing all combinations of the three concentrations and three temporal treatments. Each seed was packed into a separate foil cup and these were arranged in a 96-well plate for analysis.

Seedlings. Seeds from all treatment groups were planted in soil to determine if the ^{15}N signal can be detected in young seedlings after a growing season. Two growing methods were used: (1) seeds sown in Metro-Mix 360 potting soil grown under greenhouse conditions and (2) seeds sown in soil collected from a typical woodlot in Darke County Ohio and grown in an outdoor garden. No N fertilizer was applied to either soil. Greenhouse pots were watered with distilled water and garden pots were watered with rainwater and, occasionally, tap water. Dried seeds were sown in March 2010 and allowed to grow throughout the summer. In most cases, 20 seeds from each adult shrub were planted in a pot for each of the two methods. In cases where fewer than 20 seeds were collected from a shrub, they were split between the two growing methods.

On July 16, 2011, for growing method one, and August 13, 2011, for growing method two, one seedling from each pot was selected for destructive sampling. For some pots no surviving seedlings reached adequate size for nitrogen analyses, so a total of 50 seedlings (4 to 6 per treatment combination) from method 1 and 34 seedlings (2 for each of the September spray treatments and 3 to 6 for each of the other combinations) from method 2 were sampled and analyzed. Each seedling was dried to a constant mass in a drying oven, then its leaves were finely ground, homogenized, and approximately 4 to 10 mg was packed into a foil cup. Cups were arranged in a 96-well plate for analysis.

^{15}N Analysis. All samples were sent to University of California at Davis Stable Isotope Facility for analysis using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer for determination of $\%^{15}$N and calculation of the isotopic ratio, δ^{15}N. Data ($\%^{15}$N values) were analyzed using a sequential 2-way ANOVA with ^{15}N concentration and temporal treatment as the two fixed factors, with separate variances for each concentration, using the nlme package (Pinheiro et al. 2012) in the R language (v. 2.15.0, R Core Development Team). As each data set was unbalanced, each ANOVA tested for the effect of ^{15}N concentration without controlling for temporal treatment, the effect of temporal treatment after controlling for concentration, and the interaction after controlling for both main effects.

Results and Discussion

Seeds. $\%^{15}$N values of seeds differed significantly based on the concentration of ^{15}N applied ($P < 0.0001$), with seeds from shrubs sprayed with the lower concentration of ^{15}N-urea (0.025 g L^{-1}) having $\%^{15}$N, and therefore δ^{15}N, values intermediate between those of controls and those sprayed with the higher concentration (0.20 g L^{-1}) (Figure 1). Temporal treatment did not significantly affect seed $\%^{15}$N, but there was a significant interaction between concentration and temporal treatment ($P < 0.0001$); for those plants receiving ^{15}N-urea, spraying five times throughout the summer resulted in the highest $\%^{15}$N and spraying only in September resulted in the second highest (Table 1). The same pattern was evident for another batch of seeds from these shrubs that was analyzed earlier (Castellano and Gorchov 2010).

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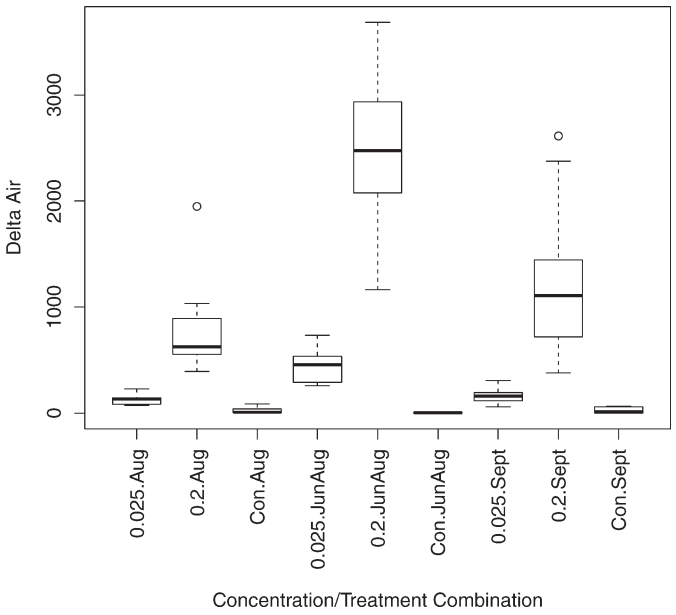

Figure 1. Box plots of $\delta^{15}N$ values of seeds collected from shrubs that were subjected to three different concentrations of ^{15}N-enriched urea [0.025 g L^{-1}, 0.20 g L^{-1}, and control (0 g L^{-1})] using three different temporal treatments (Aug = August 6, 2009, Sept = September 4, 2009, JunAug = 5 applications three weeks apart beginning June 5, 2009). The dark line is the median, the boxes define the upper and lower quartiles (75th and 25th percentiles); the whiskers show the standard deviations, and the open circles are outliers.

Seedlings. The $\%^{15}N$ values of both seedlings grown in the outdoor garden in woodlot soils and seedlings grown in greenhouse conditions were significantly affected by concentration and by the interaction between concentration and temporal treatment, but there was no direct effect of temporal treatment (Tables 2 and 3). Seedlings from seeds of shrubs that had been sprayed with the higher concentration of ^{15}N-enriched urea consistently showed the highest $\%^{15}N$ values, but those from shrubs sprayed with the lower concentration were only distinguishable from untreated controls in the woodlot soils (Figures 2 and 3). For both sets of seedlings, the highest $\%^{15}N$ values were from parents sprayed five times with ^{15}N-urea and the second highest from those sprayed once in September, the same pattern found for seeds.

The differentiation in seedling $\%^{15}N$ values among the three spray concentrations was lower for greenhouse-grown seedlings than it was for seedlings grown in woodlot soil (Figure 3 vs. Figure 2). We attribute this to the greater dilution of ^{15}N with N taken up by the seedlings in the greenhouse, based on the larger size, and much lower $\%^{15}N$ values, of the greenhouse-grown seedlings. This pattern is consistent with the pattern of decreased $\delta^{15}N$ signal with greater seedling size demonstrated for *S. americanum* by Carlo et al. (2009).

These results show that foliar application of ^{15}N-enriched urea on an invasive shrub is effective in labeling seeds and seedlings with $\%^{15}N$, and hence $\delta^{15}N$, signals higher than controls. Thus it is possible to distinguish the source (treated vs. nontreated parent) of both field-collected seeds (e.g. from seed traps) and seedlings.

For *L. maackii* at least, we recommend a single application in early September, as this involves much less labor and ^{15}N-enriched urea than multiple applications. In early September, *L. maackii* fruits are still immature, with seeds not yet filled; fruit maturation and seed dispersal do not occur until late fall (Bartuszevige et al. 2006). However, it is likely that spraying during this species' flowering period (late April to late May in our area) would also be effective, as application of ^{15}N-urea to petals of several species effectively enriched the $\delta^{15}N$ of seeds (Carlo and Norris 2012).

Our findings indicate it is feasible to use two different concentrations of ^{15}N-enriched urea, applied in early

Table 1. Analysis of variance of $\%^{15}N$ (atom percent ^{15}N) of two seeds per shrub, with shrubs assigned to one of three ^{15}N concentrations crossed with three temporal treatments.

	numDF	denDF	F-value	P-value
(Intercept)	1	42	19867.5	< .0001
conc	2	33	120.9	< .0001
treat	2	33	2.8	0.074
conc × treat	4	33	19.8	< .0001

Table 2. Analysis of variance of leaf $\%^{15}N$ from seedlings grown from labeled seed in Darke County, OH soil in an outdoor garden. Parent shrubs were assigned to one of three ^{15}N concentrations crossed with three temporal treatments.

	numDF	denDF	F-value	P-value
(Intercept)	1	25	1296932.4	< .0001
conc	2	25	55.0	< .0001
treat	2	25	0.8	0.4601
conc × treat	4	25	11.2	< .0001

Table 3. Analysis of variance of leaf $\%^{15}N$ from seedlings grown from labeled seed in Metro-Mix under greenhouse conditions. Parent shrubs were assigned to one of three ^{15}N concentrations crossed with three temporal treatments.

	numDF	denDF	F-value	P-value
(Intercept)	1	41	1100421.4	< .0001
conc	2	41	18.5	< .0001
treat	2	41	0.5	0.6186
conc × treat	4	41	7.3	0.0002

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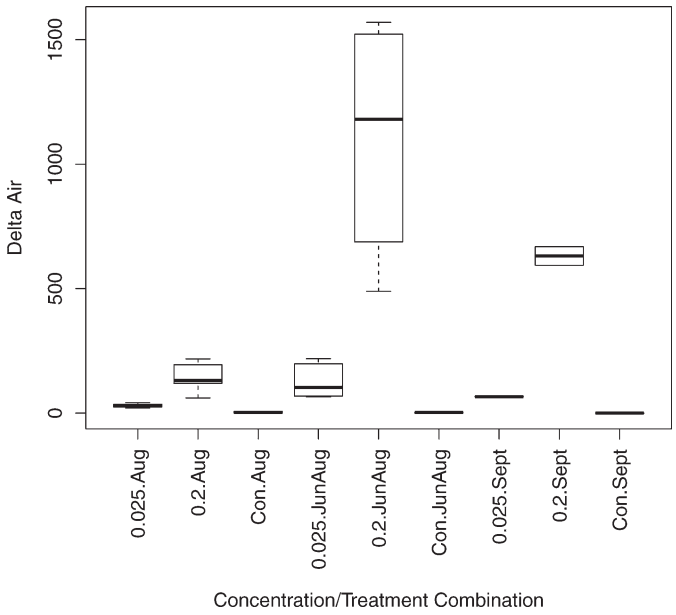

Figure 2. Box plots of $\delta^{15}N$ values of seedlings grown outdoors in woodlot soil from seeds of shrubs that were subjected to three concentrations of ^{15}N-labeled urea using three temporal treatments, as in Figure 1.

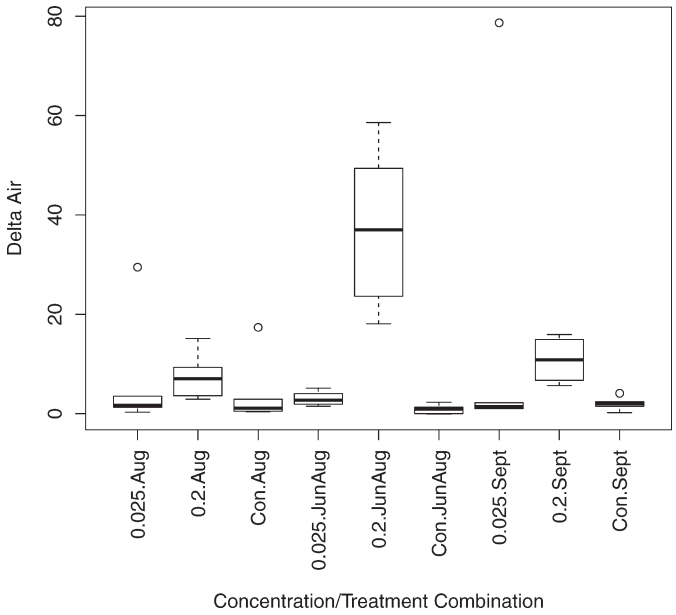

Figure 3. Box plots of $\delta^{15}N$ values of seedlings grown in the greenhouse in potting soil from seeds of shrubs that were subjected to three concentrations of ^{15}N-labeled urea using three temporal treatments, as in Figure 1.

September, to differentially label seeds and woodlot-grown seedlings from different sources, as Carlo et al. (2009) demonstrated for three concentrations in *S. americanum* and *C. annuum*.

This study shows it is feasible to use stable isotope labeling to address invasion biology questions. For example, the extent to which new populations (nascent foci, sensu Moody and Mack [1988]) grow via local recruitment vs. via seed dispersal from larger populations could be investigated by foliar application of ^{15}N-enriched urea to adult plants in the new populations, and analysis of the next year's seedling cohort. Stable isotope ratios can also be used to estimate the proportion of a 'batch' of dispersed seeds (e.g. in a seed trap or fecal clump) that originated from a labeled source plant or patch, as Morales et al. (2012) demonstrated in their study of mistletoe dispersal, by grinding seeds together and analyzing the isotopic signal with a Bayesian mixing model.

Acknowledgments

This research was supported by Sigma Xi and the Miami University Department of Botany's Academic Challenge Program. We thank Ann Rypstra and Rodney Kolb for facilitating this research at the Miami University Ecology Research Center, Hank Stevens for assistance on statistical analyses, and two anonymous reviewers for constructive comments.

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Received June 29, 2012, and approved November 6, 2012.