Aphids alter host-plant nitrogen isotope fractionation

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Edited* by Peter M. Vitousek, Stanford University, Stanford, CA, and approved May 17, 2011 (received for review May 20, 2010)

Plant sap-feeding insects and blood-feeding parasites are frequently depleted in ^{15}N relative to their diet. Unfortunately, most fluid-feeder/host nitrogen stable-isotope studies simply report stable-isotope signatures, but few attempt to elucidate the mechanism of isotopic trophic depletion. Here we address this deficit by investigating the nitrogen stable-isotope dynamics of a fluid-feeding herbivore-host plant system: the green peach aphid, *Myzus persicae*, feeding on multiple brassicaceous host plants. *M. persicae* was consistently more than 6‰ depleted in ^{15}N relative to their hosts, although aphid colonized plants were 1.5‰ to 2.0‰ enriched in ^{15}N relative to uncolonized control plants. Isotopic depletion of aphids relative to hosts was strongly related to host nitrogen content. We tested whether the concomitant aphid ^{15}N depletion and host ^{15}N enrichment was coupled by isotopic mass balance and determined that aphid ^{15}N depletion and host ^{15}N enrichment are uncoupled processes. We hypothesized that colonized plants would have higher nitrate reductase activity than uncolonized plants because previous studies had demonstrated that high nitrate reductase activity under substrate-limiting conditions can result in increased plant $\delta^{15}N$ values. Consistent with our hypothesis, nitrate reductase activity in colonized plants was twice that of uncolonized plants. This study offers two important insights that are likely applicable to understanding nitrogen dynamics in fluid-feeder/host systems. First, isotopic separation of aphid and host depends on nitrogen availability. Second, aphid colonization alters host nitrogen metabolism and subsequently host nitrogen stable-isotope signature. Notably, this work establishes a metabolic framework for future hypothesis-driven studies focused on aphid manipulation of host nitrogen metabolism.

host–parasite interaction | nitrogen budget | plant–herbivore interaction | trophic enrichment

Nitrogen, required for the synthesis of nucleic and amino acids, is essential for development and growth. Found in the atmosphere, soil, and oceans in inorganic forms (1), animals depend on plants and microorganisms for nitrogen fixation and organic nitrogen supply in the form of proteins and amino acids. Universally, metabolic fractionation during nitrogen fixation, assimilation, respiration, and excretion results in differences in the ratio of nitrogen stable isotopes in different tissues within organisms (2) and at different levels within ecosystems (3). Typically, consumers are enriched in ^{15}N by ~2‰ to 3‰ relative to their diet (2, 4, 5). However, important exceptions exist. Fluid-feeders, a group that includes blood-feeding parasites and hempiteran insects (2), frequently have been found to deviate from these typical patterns such that fluid-feeders are usually reported as showing no enrichment or even depletion in ^{15}N relative to their diet (2, 6–17). Although it is now commonly accepted that fluid-feeders deviate from typical patterns of enrichment, these observations have stimulated considerable speculation (2, 14, 18) but little, if any, research targeted at explaining the mechanistic basis of such atypical patterns of nitrogen enrichment.

Here we elucidate some factors contributing to atypical patterns of nitrogen isotopic enrichment in fluid-feeder/host systems by examining experimentally the interaction of an aphid and its hosts in a metabolic context. Aphids are fluid-feeding plant herbivores that, facilitated by an ancient and obligate symbiosis with the γ-proteobacteria *Buchnera aphidicola*, feed exclusively on phloem sap (19). In aerobic environments, host plants that do not form nitrogen-fixing associations uptake nitrogen mostly in the form of nitrate (NO_3^-) (20) and process it into amino acids through a series of conserved metabolic pathways (Fig. 1). Although the C:N ratio of phloem sap is similar to that of the diets of other insects that show typical patterns of isotopic enrichment (21, 22), the nitrogen profile of phloem sap is unbalanced and deficient in essential amino acids (23) so that aphids are nutritionally dependent on *Buchnera* (19, 24). Movement of nitrogen in this fluid-feeder/host system mostly occurs up the food chain, but some nitrogen, in the form of aphid-derived proteins delivered in watery saliva during aphid feeding, moves in the reverse direction, from fluid-feeder back to host (e.g., refs. 25–27).

What follows is a series of experiments that investigate the nitrogen stable-isotope dynamics of an aphid/host system, that of the green peach aphid, *Myzus persicae*, feeding on multiple brassicaceous hosts. First, in investigating the effect of aphid load on insect and host nitrogen stable-isotope signature, we found aphids to be depleted in ^{15}N relative to hosts and that aphid herbivory can alter the nitrogen stable-isotope signature of host plants. Second, we demonstrate that aphid nitrogen stable-isotope fractionation is dependent on host nitrogen availability. Third, in a labeling experiment we test the hypothesis that aphid feeding alters the nitrogen stable-isotope signature of host plants because aphids are retaining ^{14}N and secreting heavier ^{15}N labeled molecules back into the host. Fourth, because increased nitrate reductase activity has been shown to increase plant $\delta^{15}N$ signature (28), we test the hypothesis that aphid-colonized host plant ^{15}N enrichment results from an increase in host nitrate reductase activity. Finally, we describe the metabolic basis of host-plant enrichment and develop a framework for future hypothesis-driven research.

Results

Aphids Are Depleted in ^{15}N Relative to Hosts. Following a series of initial experiments on cabbage and radish seedlings, where we found that aphids are heavily depleted in ^{15}N relative to their diet (Fig. S1) and host plants colonized by aphids are enriched in ^{15}N relative to control plants (Fig. S2), we designed a single experiment with two aphid genotypes and two aphid loads feeding on cabbage. This cabbage aphid–host interaction experiment combined the separate initial experiments on load effects into a single experiment that aimed to test the null hypothesis of no difference in $\delta^{15}N$ of treatment seedlings with heavy and light aphid loads vs. control seedlings without aphids. Consistent with results from the initial experiments, we find that aphids feeding at both high and low density on cabbage are depleted in ^{15}N greater than 6‰ relative to their hosts (Fig. 2). Furthermore, we find that although aphid genotype had no effect on the $\Delta^{15}N$ (trophic separation: i.e., $\delta^{15}N_{aphid} - \delta^{15}N_{host}$) of aphids and their host plants, aphid density significantly affected $\Delta^{15}N$ such that the $\Delta^{15}N$ of aphids

Author contributions: A.C.C.W. and L.d.S.L.S. designed research; A.C.C.W., L.d.S.L.S., and K.B.H. performed research; A.C.C.W. and L.d.S.L.S. analyzed data; and A.C.C.W. and L.d.S.L.S. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1007065108/-/DCSupplemental.

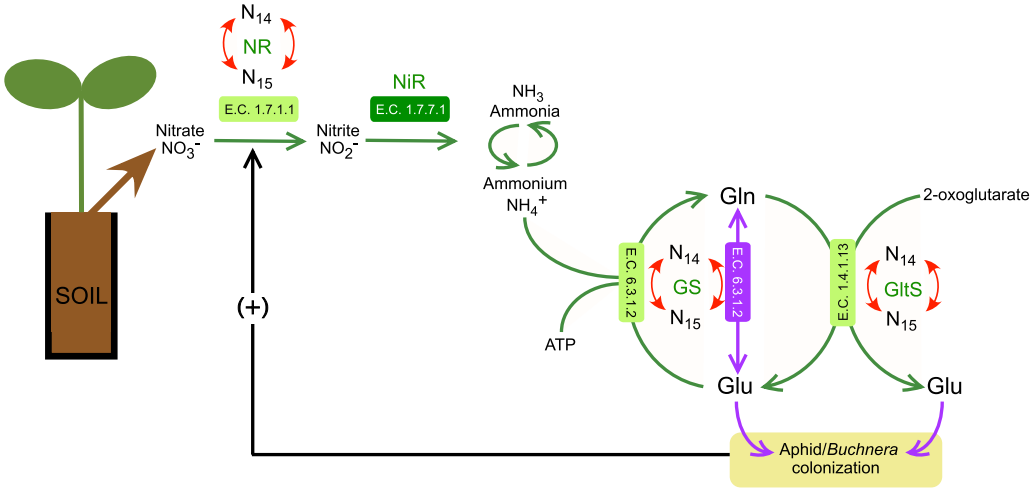

Fig. 1. Schematic of plant nitrogen assimilation showing uptake of nitrate (NO_3^-) from soil where it is then converted by the enzyme nitrate reductase (NR) to nitrite (NO_2^-). The metabolic conversion of nitrite to ammonia (NH_3) is catalyzed by nitrite reductase (NiR). Ammonia then feeds into the GS/GOGAT cycle of amino acid biosynthesis in the form of ammonium (NH_4^+) where the enzyme glutamine synthetase (GS) converts glutamate + ammonium to glutamine. Glutamate synthase then liberates glutamate via input of glutamine + 2-oxoglutarate. Phloem sap containing free amino acids is then ingested by aphids who, in collaboration with their obligate intracellular bacterial symbiont, *B. aphidicola*, upgrade and collaboratively recycle the available mostly nonessential amino acids to meet their essential amino acid needs (for review of aphid/Buchnera amino acid metabolism, see ref. 19). Green arrows and boxes show plant metabolism and purple arrows and boxes illustrate aphid metabolism. Reactions that are known to fractionate against ^{15}N are marked with the circular $^{14}N/^{15}N$ symbol around the abbreviation of the enzyme's name (43). Enzymes with evidence of transcriptional up-regulation in aphid-colonized plants are shown in light green with black text [E.C. 1.7.1.1 (41), E.C. 6.3.1.2 (44), and E.C. 1.4.1.13 (45)]. As indicated by the black arrow linking the aphid/*Buchnera* box back to nitrate reductase, here we report that aphid colonization results in increased host plant nitrate reductase activity (Fig. S3).

feeding at low density was significantly more negative than that of aphids feeding at high density (Fig. 2).

Aphid Herbivory Alters the $\delta^{15}N$ Signature of Host Plants. At high and low density, aphid herbivory significantly increases the ^{15}N content of colonized plants relative to uncolonized host plants (Fig. 3*A*). The effect of aphid herbivory on host nitrogen content is small but significant under conditions of heavy aphid colonization (Fig. 3*B*). These results are consistent with our initial work, where we found that aphid herbivory significantly increases the ^{15}N content of heavily colonized cabbage and radish seedlings relative to uncolonized seedlings (Fig. S2).

Aphid Nitrogen Stable-Isotope Signature Is Dependent on Host Nitrogen Availability. Observations made during the initial series of experiments led us to hypothesize that the $\Delta^{15}N$ of aphids is only negative when host nitrogen content is high. Furthermore, in a series of related experiments with *Arabidopsis* we discovered that the nitrogen content of *Arabidopsis* can be blindly manipulated by fertilizing each plant with a single bead of osmocote slow-release fertilizer. Thus, we designed an experiment to investigate the relationship between host-plant nitrogen content and aphid $\Delta^{15}N$. The results of this experiment demonstrate that the relationship between host-plant nitrogen content and aphid nitrogen stable-isotope signature is negative and linear (Fig. 4). That is, when nitrogen content is low, aphid $\Delta^{15}N$ is positive, but when host percentage of N is > ~4%, aphid $\Delta^{15}N$ is negative (Fig. 4). Importantly, as illustrated by the four colored datapoints in the lower right of Fig. 4, we found that the negative linear relationship described by the *Arabidopsis* data are consistent with results from the cabbage aphid–host interaction experiment (Fig. 2). Furthermore, we find that aphid nitrogen content reflects the nitrogen content (diet quality) of host plants, such that aphids feeding at low density (blue and purple spots) have smaller C:N ratios than aphids feeding at high density (red and orange spots) (the range of C:N values for aphids feeding at low density was 4.7–7.4 and the range of C:N values for aphids at high density was 5.1–9.0).

Changes in the $\delta^{15}N$ Signature of Colonized Plants Does Not Result from Aphid Secretion of ^{15}N-Enriched Protein into Plant Phloem Sap. To test the hypothesis that concomitant aphid ^{15}N depletion/host-plant ^{15}N enrichment results from aphids retaining ^{14}N and secreting heavier ^{15}N-labeled molecules back into hosts, we performed an isotopic enrichment experiment. By raising aphid colonies on cabbage seedlings enriched to twice the natural concentration of ^{15}N, we established enriched aphids with $\delta^{15}N$ values that were 113.9‰ ± SE 32.7‰ greater than those of unenriched aphids. At termination of the enrichment experiment, enriched aphids still possessed $\delta^{15}N$ signatures heavier than their unenriched counterparts: enriched aphids $\delta^{15}N = 7.9‰ \pm SE\ 5.0‰$, unenriched aphids $\delta^{15}N = -3.4‰ \pm SE\ 0.7‰$.

Consistent with the cabbage aphid–host interaction experiment (Fig. 3), control plants with unenriched aphids were significantly enriched in ^{15}N compared with uncolonized control plants (Fig. 5). However, we found no difference between plants colonized by

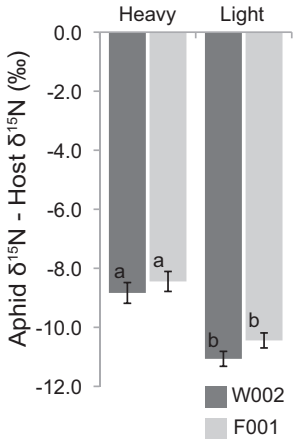

Fig. 2. Two genotypes of *M. persicae* (W002 and F001) feeding on cabbage seedlings are depleted in ^{15}N relative to their host plant irrespective of feeding density (Paired *t* tests: heavy load, $t = -35.6$, $P = 0.0000$; light load, $t = -56.5$, $P = 0.0000$). Aphid load significantly affected the degree of host/aphid isotopic separation but aphid genotype had no effect (ANOVA Model: $F = 26.18$, $P = 0.0000$; aphid load: $F = 49.43$, $P = 0.0000$; aphid genotype: $F = 2.93$, $P = 0.0953$). For each genotype by load treatment $n = 10$. Error bars represent ± SEM.

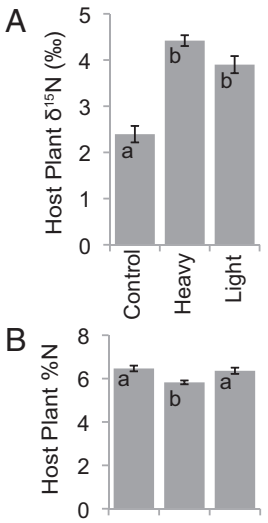

Fig. 3. The effect of aphid feeding on host nitrogen stable-isotope signature and nitrogen content. (*A*) Cabbage seedling $\delta^{15}N$. The $\delta^{15}N$ of cabbage seedlings colonized by aphids is significantly greater than that of uncolonized control plants (one-way ANOVA: $F = 41.65$, $P = 0.000$). (*B*) Cabbage seedling nitrogen content. A one-way analysis of variance with Scheffé multiple comparison test demonstrates that plants heavily colonized with aphids are significantly depleted in nitrogen relative to control plants ($P = 0.002$), but the nitrogen content of host plants with a light load of aphids are indistinguishable from those of control plants without aphids ($P = 0.829$). For each treatment, $n = 20$. Error bars represent ± SEM.

enriched vs. unenriched aphids and no significant difference between plants colonized by enriched aphids and uncolonized control plants (Fig. 5). Although surprising, the failure to find a difference in $\delta^{15}N$ of control plants and plants colonized by enriched aphids was likely driven by the small sample sizes ($n = 4$) and large variance. That said, this experiment powerfully demonstrates that changes in the $\delta^{15}N$ signature of colonized plants does not result from aphid secretion of ^{15}N-enriched protein into plant phloem sap.

Nitrate Reductase Activity Is Elevated in Aphid Parasitized Host Plants. Increased nitrate reductase activity has been shown to increase plant $\delta^{15}N$ signature under substrate-limiting conditions

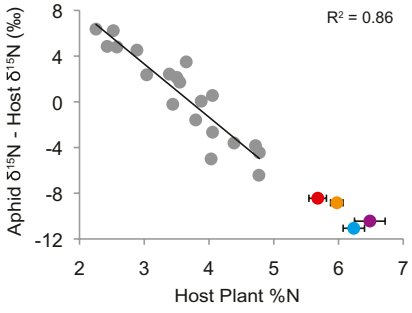

Fig. 4. The relationship between host-plant percentage of nitrogen and isotopic separation of aphids and their host plants is negative and linear (*M. persicae* feeding on *Arabidopsis*: light gray spots; $n = 20$, $y = -4.6524x + 17.266$, $t = -10.35$, $P = 0.0000$). Data from the cabbage aphid/host interaction experiment (Figs. 1 and 2) is plotted in color and shows patterns consistent with the *Arabidopsis* data. Red spot: F001 heavy load; orange spot: W002 heavy load; blue spot: W002 light load; purple spot: F001 light load. $n = 10$ for each colored spot. Error bars represent SEM and for host plant percentage of N plotted on the *y* axis are so small that the colored spots obscure them.

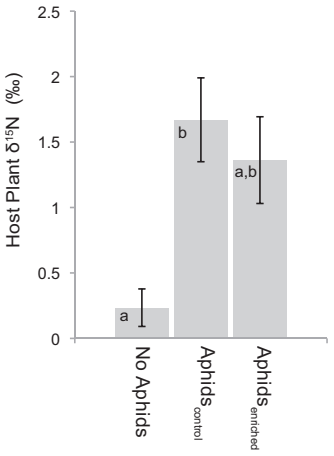

Fig. 5. The increase in $\delta^{15}N$ of host plants parasitized by aphids does not result from aphids excreting ^{15}N-enriched molecules back into host seedlings. There was no difference in the $\delta^{15}N$ of cabbage plants parasitized by unenriched aphids and aphids enriched in ^{15}N ($P = 0.719$). However, plants parasitized by control aphids were significantly enriched in ^{15}N compared with unparasitized control plants ($P = 0.012$). For each treatment, $n = 4$. Error bars represent ± SEM.

(28). Thus, to test the hypothesis that aphid-colonized host-plant ^{15}N enrichment results from an increase in host nitrate reductase activity, we quantified the activity of nitrate reductase in colonized and uncolonized cabbage seedlings. The nitrate reductase activity of plants colonized by aphids was double that of control plants without aphids (Fig. S3) (Wilcoxon rank-sum: $z = -2.570$, $P = 0.0102$).

Discussion

Diet Quality Drives $\Delta^{15}N$ Patterns in Aphid/Host Systems. Aphids feed on plant phloem sap, a diet exceptionally difficult to sample from most hosts in sufficient quantities to robustly measure nitrogen stable-isotope signatures. It is fortunate, therefore, that previous research has demonstrated that the nitrogen stable-isotope signature of plant-leaf tissue provides a reliable phloem sap proxy (8, 15), a result we confirmed for both cabbage and radish seedlings during preliminary work for the present study (*Materials and Methods*).

Previous nitrogen stable-isotope studies in aphids have reported ^{15}N trophic enrichment (6), lack of aphid ^{15}N enrichment (8, 15), and even aphid ^{15}N depletion (29). In the majority of our experiments we found the $\Delta^{15}N$ of *M. persicae* to be significantly negative (Fig. 2 and Fig. S1). However, in working with genetically identical aphids feeding on prereproductive *Arabidopsis* plants, we found aphids to show everything from heavy depletion through to 6‰ ^{15}N enrichment, a surprising result that was mostly explained by host-plant nitrogen content and that was consistent with results from the cabbage aphid–host interaction experiment (Fig. 4). The combined results of the cabbage and *Arabidopsis* aphid–host interaction experiments that are consistent with the weaker relationship previously described by Sagers and Goggin (15), demonstrate that the relationship between host-plant percentage of nitrogen and isotopic separation of aphids and their host is negative and linear. Notably, this result provides insight into apparent inconsistencies in the isotopic separation of aphids and their host plants.

Aphid Herbivory Alters the $\delta^{15}N$ Signature of Host Plants. The unique discovery of this study is our demonstration that aphid herbivory alters the nitrogen stable-isotope signature of host plants (Fig. 3*A*). Although a large body of work focuses on the interaction of aphids and their host plants, particularly in the context of iden-

tification of plant-defensive responses to aphid herbivory (30, 31), surprisingly little is known about the effects of aphid feeding on host-plant nitrogen metabolism. Given that the effect of aphid feeding on host $\delta^{15}N$ is independent of host species [both cabbage and radish heavily colonized by aphids were enriched in ^{15}N (Fig. S2)], we expect this unique phenomenon to be widespread. The uniqueness of our finding comes from the fact that there are no other fluid-feeder studies that quantify the $\delta^{15}N$ signature of colonized and uncolonized host plants; there are, however, two studies involving parasites that examined colonized and uncolonized hosts. The first study reported an increase in the $\delta^{15}N$ of hepatopancreatic tissue in parasitized freshwater snails (*Lymnaea stagnalis*) relative to unparasitized individuals (32). Unfortunately, the hepatopancreatic tissue from parasitized individuals included parasite tissue. Thus, because Doi et al. (32) did not determine the $\delta^{15}N$ signature of the trematode parasite, it is not possible to determine whether the altered $\delta^{15}N$ signature of the hepatopancreas resulted from a change in the $\delta^{15}N$ signature of the host tissue or if the increase was attributable to inclusion of parasite tissue. The second study examined parasitized and unparasitized tissues of four digenean trematode-marine mollusk systems and found that digenean trematode parasitism induced no significant shifts in mollusk $\delta^{15}N$ signatures (33). Therefore, our finding that aphid herbivory alters the $\delta^{15}N$ signatures of host plants is unique in reporting a consumer altering the stable isotope signature of their diet.

What Processes Drive the ^{15}N Enrichment of Aphid-Colonized Host Plants? It is known that aphid feeding relocates nitrogen within host plants (34, 35) and reduces host nitrogen content (30). It is also known that aphids return nitrogen in the form of proteins back to host plants during feeding (26, 27, 36) and that host-plant responses to feeding by some aphids include measureable changes in the host phloem sap amino acid profile (e.g., refs. 37, 38). We investigated two possible mechanisms that could be driving host plant ^{15}N enrichment in response to aphid feeding. First, we investigated the possibility that the isotopic effect of aphid colonization on host plants results from herbivore/host isotopic mass balance. Second, we investigated activity of nitrate reductase in colonized and uncolonized plants because previous studies had demonstrated that high nitrate reductase activity under substrate-limiting conditions can result in increased plant $\delta^{15}N$ (28).

Aphid Depletion and Host-Plant Enrichment Are Uncoupled. Using an isotopic labeling experiment, we tested the hypothesis that the isotopic effect of aphid herbivory on host plants results from herbivore/host isotopic mass balance. No doubt this hypothesis may appear to some to be far-fetched; however, we set out to test it for several compelling reasons. First, as far as we are aware there is only one model of isotopic trophic-step fractionation that accounts for the common observation of fluid-feeders being depleted in ^{15}N relative to their hosts, and this model uses isotopic mass balance equations (39). Second, the biology of aphid feeding results in a two-way flux of nitrogen between the insect and host plant; aphids extract nitrogen in the form of free amino acids and return nitrogen to phloem in the form of aphid proteins delivered via watery saliva (26, 27, 36). Finally, because our ultimate goal is to determine the mechanistic basis of aphid ^{15}N depletion and host ^{15}N enrichment, we need to determine the extent to which these processes are coupled; that is, are we looking at two separate phenomena—aphid depletion and host-plant enrichment—or a single phenomenon? Within this context, the aphid-enrichment experiment clarifies two important aspects of isotopic fractionation: (*i*) that patterns of host-plant ^{15}N enrichment concomitant with aphid ^{15}N depletion do not result from an isotopic mass balance between aphids and their host plants, and (*ii*) that aphid ^{15}N depletion and host ^{15}N enrichment are uncoupled processes. The demonstration that aphid ^{15}N depletion and host ^{15}N enrichment are uncoupled informs future research directions, as we attempt to determine the mechanistic basis of these two phenomena.

Host-Plant Enrichment Is Concomitant with Increases in Nitrate Reductase Activity. Under normal aerobic conditions, nitrate (NO_3^-) is the most abundant form of nitrogen available to plants that do not form associations with nitrogen-fixing microorganisms (20) (Fig. 1). Thus, under most conditions, such plants meet their organic need for nitrogen by assimilation of nitrate. Host-plant assimilation of nitrate can result in isotopic fractionation of nitrogen because nitrate reductase, the enzyme that converts nitrate to nitrite, fractionates against ^{15}N (40) (Fig. 1). Furthermore, under nitrate-limiting conditions, such as occurs when there is an excess of nitrate reductase and a finite supply of nitrate, isotopic discrimination against ^{15}N is reduced, resulting in an increase in plant $\delta^{15}N$ (28). Because nitrogen assimilation in the form of nitrate is known to increase plant $\delta^{15}N$ signatures under nitrate-limiting conditions, we hypothesized that the high $\delta^{15}N$ values of colonized plants result from an increase in nitrate reductase activity. Indeed, we found that the activity of nitrate reductase in aphid colonized plants was double that of control plants (Fig. S3), a result that is consistent with previous gene-expression work in sorghum (41).

It remains to be determined whether our observation of elevated nitrate reductase activity in aphid-colonized plants results from transcriptional or posttranslational processes (42), and it certainly remains to be determined just how the activation of this plant response occurs. However, given that host-plant $\delta^{15}N$ significantly increased in plants colonized at both high- and low-aphid density (Fig. 3*A*) but that only heavily colonized plants showed a small but significant decrease in nitrogen content (Fig. 3*B*), we think that it is unlikely that the elevated nitrate reductase activity of aphid-colonized plants is simply a plant response to aphid reduction of plant nitrogen concentration. We are not aware of any process that might cause aphid herbivory to increase nitrate reductase activity; however, because aphids are known to directly manipulate host physiology by injection of watery saliva during feeding (27, 30, 31), we speculate that the elevated nitrate reductase activity of aphid-colonized plants results from some specific biochemical or molecular signal injected into the host with aphid saliva.

We remain a long way from thoroughly understanding the nitrogen stable-isotope dynamics of fluid-feeder/host systems, yet the present study provides a useful opportunity to highlight the value of studying stable-isotope dynamics and ecology within a firm metabolic framework. In Fig. 1 we presented the conserved metabolic pathway for nitrogen fixation and assimilation in plants not associated with nitrogen-fixing microbes and show the point of connection between host plant and aphid nitrogen metabolism. Furthermore, within this framework we highlight the reactions that are known to fractionate nitrogen, the enzymes that are transcriptionally up-regulated in response to aphid herbivory (reviewed in ref. 43) and illustrate our finding that aphid feeding positively regulates nitrate reductase activity. Taking these data together, it appears that host plants (by an unknown mechanism) increase rates of nitrogen acquisition and assimilation in response to aphid feeding, which under nitrate-limiting conditions results in an increase in host plant $\delta^{15}N$.

Concluding Remarks on the Nitrogen Stable-Isotope Dynamics of Fluid-Feeders and Their Hosts. We set out to investigate the nitrogen stable-isotope dynamics of an aphid herbivore host-plant system with the goal of beginning to elucidate the basis of variability in nitrogen fractionation in fluid-feeders relative to their diet. In this respect we offer three noteworthy insights. First, isotopic separation of aphids and their host plants depends on nitrogen availability; second, aphid herbivory alters the $\delta^{15}N$ signature of host plants; and third, aphid ^{15}N depletion and host-plant ^{15}N enrichment are uncoupled processes. Although we are

still far from a complete understanding of nitrogen isotope fractionation in the aphid-host system, especially with regard to the likely important role of *B. aphidicola*, the obligate bacterial symbiont of aphids in nitrogen isotope fractionation (18), we have made some important strides in explaining variability in aphid-host ^{15}N signatures and suggest that these insights may usefully inform research in other systems that show atypical patterns of stable isotope fractionation.

Materials and Methods

Details on the experimental design and sampling of the cabbage and *Arabidopsis* aphid–host interaction experiments, isotopic enrichment experiment, and nitrate reductase experiment are provided in *SI Materials and Methods*.

Stable-Isotope Analysis. Isotope ratio mass spectrometry was performed on multiple whole aphids and ~3 mg of ground leaf tissue. Samples were encapsulated in crushed tin cups (5 × 8 mm; Elementar America) and loaded into an elemental analyzer (Eurovector) connected to an Isoprime stable isotope mass spectrometer (Elementar). Upon pyrolysis in the elemental analyzer, the nitrogen and carbon dioxide gasses were separated by gas chromatography and directed to the mass spectrometer for analyses of nitrogen and carbon isotope ratios. Isotope ratios are expressed as $\delta^{15}N$ or $\delta^{13}C$ (‰), where

$$\delta^{15}N \text{ or } \delta^{13}C(‰) = \left[\left(R_{sample}/R_{air}\right) - 1\right] * 1000 \quad [1]$$

and R_{sample} and R_{air} are the $^{15}N/^{14}N$ ratios of the samples and atmospheric nitrogen, respectively, in the case of nitrogen and $^{13}C/^{12}C$ of the samples and the vPDB standard, respectively, in the case of carbon. The precision of analysis was ± 0.1‰ for both elements.

In preliminary experiments, we found that the $\delta^{15}N$ values of cabbage and radish leaves did not differ significantly from phloem sap (aphid diet): $F = 0.57$, $P > 0.50$, a result consistent with two earlier aphid/host studies (8, 15). Having established that leaf tissue provides a reliable proxy for phloem sap in measuring nitrogen stable-isotope ratios, and because of the technical challenges of obtaining sufficient phloem sap for stable isotope analysis, here we measure leaf $\delta^{15}N$ and use it as a phloem $\delta^{15}N$ proxy (15).

Data Analysis. Before analysis, dependent variables were tested for normality and homogeneity of variance. All statistical tests were implemented in STATA 10.0. Full details can be found in the *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Don De Angelis, Ted Fleming, Doug Levey, Jennifer Rudgers, and two anonymous reviewers for useful comments; Philip Kushlan, Derek Jones, and Susanna Chan for technical assistance; Philip Kushlan for useful discussions; and Orou Gaoue, Lucero Sevillano, and Sandro de Andrade for statistical advice. This research was supported by University of Miami start-up funds (to A.C.C.W) and National Science Foundation Grant 0420553 (to L.d.S.L.S.).

1. Gruber N, Galloway JN (2008) An Earth-system perspective of the global nitrogen cycle. *Nature* 451:293–296.
2. McCutchan JH, Lewis WM, Kendall C, McGrath CC (2003) Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur. *Oikos* 102:378–390.
3. Hogberg P (1997) Tansley review No 95—N-15 natural abundance in soil-plant systems. *New Phytol* 137:179–203.
4. DeNiro MJ, Epstein S (1981) Influence of diet on the distribution of nitrogen isotopes in animals. *Geochim Cosmochim Acta* 45:341–351.
5. Schoeninger MJ, DeNiro MJ (1984) Nitrogen and carbon isotopic composition of bone-collagen from marine and terrestrial animals. *Geochim Cosmochim Acta* 48:625–639.
6. Scrimgeour CM, Gordon SC, Handley LL, Woodford JAT (1995) Trophic levels and anomalous $\delta^{15}N$ of insects on Raspberry (*Rubus idaeus L.*). *Isotopes Environ Health Stud* 31(1):107–115.
7. Ostrom PH, Colunga-Garcia M, Gage SH (1997) Establishing pathways of energy flow for insect predators using stable isotope ratios: Field and laboratory evidence. *Oecologia* 109:108–113.
8. Yoneyama T, Handley LL, Scrimgeour CM, Fisher DB, Raven JA (1997) Variation of the natural abundances of nitrogen and carbon isotopes in *Triticum aestivum*, with special reference to phloem and xylem exudates. *New Phytol* 137:205–213.
9. Boag B, Neilson R, Robinson D, Scrimgeour CM, Handley LL (1998) Wild rabbit host and some parasites show trophic-level relationships for $\delta^{13}C$ and $\delta^{15}N$: A first report. *Isotopes Environ Health Stud* 34(1–2):81–85.
10. Neilson R, Brown DJF (1999) Feeding on different host plants alters the natural abundances of $\delta^{13}C$ and $\delta^{15}N$ in *Longidoridae* (Nemata). *J Nematol* 31(1):20–26.
11. Pinnegar JK, Campbell N, Polunin NVC (2001) Unusual stable isotope fractionation patterns observed for fish host-parasite trophic relationships. *J Fish Biol* 59:494–503.
12. Deudero S, Pinnegar JK, Polunin NVC (2002) Insights into fish host-parasite trophic relationships revealed by stable isotope analysis. *Dis Aquat Organ* 52(1):77–86.
13. Power M, Klein GM (2004) Fish host-cestode parasite stable isotope enrichment patterns in marine, estuarine and freshwater fishes from Northern Canada. *Isotopes Environ Health Stud* 40:257–266.
14. Spence KO, Rosenheim JA (2005) Isotopic enrichment in herbivorous insects: A comparative field-based study of variation. *Oecologia* 146:89–97.
15. Sagers CL, Goggin FL (2007) Isotopic enrichment in a phloem-feeding insect: Influences of nutrient and water availability. *Oecologia* 151:464–472.
16. Xu J, Zhang M, Xie P (2007) Trophic relationship between the parasitic isopod *Ichthyoxenus japonensis* and the fish *Carassius auratus auratus* as revealed by stable isotopes. *J Freshwat Ecol* 22:333–338.
17. Schumacher E, Platner C (2009) Nutrient dynamics in a tritrophic system of ants, aphids and beans. *J Appl Entomol* 133:33–46.
18. Cook SC, Davidson DW (2006) Nutritional and functional biology of exudate-feeding ants. *Entomol Exp Appl* 118:1–10.
19. Shigenobu S, Wilson ACC (2011) Genomic revelations of a mutualism: The pea aphid and its obligate bacterial symbiont. *Cell Mol Life Sci* 68:1297–1309.
20. Hopkins WG, Hüner NP (2004) *Introduction to Plant Physiology* (John Wiley & Sons, Hoboken, NJ), 3rd Ed, p 560.
21. Dadd RH (1985) Nutrition: Organisms. *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, eds Kerkut GA, Gilbert LI (Pergamon Press, Exeter, Great Britian), Vol 4, pp 313–390.
22. Focken U (2007) Effect of different ratios of wheat to corn flour in the diet on the development and isotopic composition (delta13C, delta15N) of the red flour beetle *Tribolium castaneum*. *Isotopes Environ Health Stud* 43(2):143–154.
23. Douglas AE (1993) The nutritional quality of phloem sap utilized by natural aphid populations. *Ecol Entomol* 18:31–38.
24. Wilson ACC, et al. (2010) Genomic insight into the amino acid relations of the pea aphid, *Acyrthosiphon pisum*, with its symbiotic bacterium *Buchnera aphidicola*. *Insect Mol Biol* 19 (Suppl 2):249–258.
25. Miles PW, Harrewijn P (1991) Discharge by aphids of soluble secretions into dietary sources. *Entomol Exp Appl* 59(2):123–134.
26. Mutti NS, Park Y, Reese JC, Reeck GR (2006) RNAi knockdown of a salivary transcript leading to lethality in the pea aphid, *Acyrthosiphon pisum*. *J Insect Sci* 6:Article No. 38.
27. Will T, Tjallingii WF, Thönnessen A, van Bel AJE (2007) Molecular sabotage of plant defense by aphid saliva. *Proc Natl Acad Sci USA* 104:10536–10541.
28. Mariotti A, Mariotti F, Champigny ML, Amarger N, Moyse A (1982) Nitrogen isotope fractionation associated with nitrate reductase activitly and uptake of NO_3^- by pearl millet. *Plant Physiol* 69:880–884.
29. Oelbermann K, Scheu S (2002) Stable isotope enrichment ($\delta^{15}N$ and $\delta^{13}C$) in a generalist predator (*Pardosa lugubris*, Araneae: Lycosidae): Effects of prey quality. *Oecologia* 130:337–344.
30. de Vos M, Kim JH, Jander G (2007) Biochemistry and molecular biology of *Arabidopsis*-aphid interactions. *Bioessays* 29:871–883.
31. Goggin FL (2007) Plant-aphid interactions: Molecular and ecological perspectives. *Curr Opin Plant Biol* 10:399–408.
32. Doi H, et al. (2008) Parasite-induced changes in nitrogen isotope signatures of host tissues. *J Parasitol* 94:292–295.
33. Dubois SY, et al. (2009) Digenean trematodes-marine mollusc relationships: A stable isotope study. *Dis Aquat Organ* 84:65–77.
34. Girousse C, Moulia B, Silk W, Bonnemain JL (2005) Aphid infestation causes different changes in carbon and nitrogen allocation in alfalfa stems as well as different inhibitions of longitudinal and radial expansion. *Plant Physiol* 137:1474–1484.
35. Frost CJ, Hunter MD (2008) Herbivore-induced shifts in carbon and nitrogen allocation in red oak seedlings. *New Phytol* 178:835–845.
36. Mutti NS, et al. (2008) A protein from the salivary glands of the pea aphid, *Acyrthosiphon pisum*, is essential in feeding on a host plant. *Proc Natl Acad Sci USA* 105:9965–9969.
37. Dorschner KW, Ryan JD, Johnson RC, Eikenbary RD (1987) Modification of host nitrogen levels by the greenbug (Homoptera:Aphididae): Its role in resistance of winter wheat to aphids. *Environ Entomol* 16:1007–1011.
38. Sandström J, Telang A, Moran NA (2000) Nutritional enhancement of host plants by aphids—A comparison of three aphid species on grasses. *J Insect Physiol* 46:33–40.
39. Olive PJW, Pinnegar JK, Polunin NVC, Richards G, Welch R (2003) Isotope trophic-step fractionation: A dynamic equilibrium model. *J Anim Ecol* 72:608–617.
40. Evans RD (2001) Physiological mechanisms influencing plant nitrogen isotope composition. *Trends Plant Sci* 6(3):121–126.
41. Zhu-Salzman K, Salzman RA, Ahn JE, Koiwa H (2004) Transcriptional regulation of sorghum defense determinants against a phloem-feeding aphid. *Plant Physiol* 134:420–431.
42. Kaiser WM, Huber SC (2001) Post-translational regulation of nitrate reductase: Mechanism, physiological relevance and environmental triggers. *J Exp Bot* 52:1981–1989.
43. Tcherkez G, Farquhar GD (2006) Isotopic fractionation by plant nitrate reductase, twenty years later. *Funct Plant Biol* 33:531–537.
44. Voelckel C, Weisser WW, Baldwin IT (2004) An analysis of plant-aphid interactions by different microarray hybridization strategies. *Mol Ecol* 13:3187–3195.
45. Divol F, et al. (2005) Systemic response to aphid infestation by *Myzus persicae* in the phloem of *Apium graveolens*. *Plant Mol Biol* 57:517–540.