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The Influence of Endosymbiont Metabolism on the Δ15N Value of the Pea Aphid, Acyrthosiphon pisum

Philip F. Kushlan
pkushlan@gmail.com

UNIVERSITY OF MIAMI

THE INFLUENCE OF ENDOSYMBIONT METABOLISM ON THE $\Delta^{15}N$ VALUE OF THE PEA APHID, *ACYRTHOSIPHON PISUM*

By

Philip F. Kushlan

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Philip F. Kushlan

Approved:

_____
Alexandra C.C. Wilson, Ph.D.
Assistant Professor of Biology

_____
Terri A. Scandura, Ph.D.
Dean of the Graduate School

_____
Leonel Sternberg, Ph.D.
Professor of Biology

_____
Georg Jander, Ph.D.
Associate Scientist
Boyce Thompson Institute,
Ithaca, New York

_____
Marjorie Oleksiak, Ph.D.
Assistant Professor of Marine Biology and Fisheries

KUSHLAN, PHILIP (M.S., Biology)
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The use of stable nitrogen isotope data in ecological and physiological studies is based in the assumption that nitrogen fractionates predictably during metabolism, leading to a broadly conserved pattern whereby consumers are isotopically enriched with respect to their diets. The application of stable isotope data to such studies is limited is by our understanding of the factors in that cause variability in the $\Delta^{15}N$ values of consumers. In particular, parasites and fluid-feeders have been shown to demonstrate isotopic depletion with respect to their food sources. One factor that has been suggested to influence the $\Delta^{15}N$ values seen in fluid-feeding consumers is the presence of endosymbionts and their contribution to nitrogen metabolism. The experiments described in this thesis directly test the hypothesis that the endosymbiotic bacteria *Buchnera aphidicola* is influencing the $\Delta^{15}N$ value of the pea aphid on host alfalfa plants. Here I find that although aphids cured of their bacterial symbionts are less isotopically depleted than untreated aphids, they are still not enriched with respect to their phloem sap diet, indicating that endosymbiont metabolism alone is not responsible for the isotopic depletion observed in pea aphids. Metabolism of nitrogen in the pea aphid-*Buchnera* symbiosis has been well described with decades of physiological studies and with the publication of the pea aphid and *Buchnera* genomes. The two key features of metabolism in the pea aphid-*Buchnera* symbiosis are the recycling of waste ammonia by the aphid and the upgrading of the

nonessential amino acids found in phloem sap to essential amino acids through collaborative metabolism between the pea aphid and *Buchnera*. Consistent with the described role of *Buchnera* in nitrogen metabolism, amino acid analyses of symbiotic and aposymbiotic aphids demonstrates an accumulation of the nonessential amino acids glutamine and glutamate and lower amounts of essential amino acids in the aposymbiotic aphids. I tested the influence of dietary amino acid profile on the $\Delta^{15}N$ value of pea aphids and found that aphids are only isotopically depleted when they feed on diets with unbalanced amino acid compositions and are isotopically enriched when fed on a diet with a balanced profile of amino acids. I used isotopically labeled fructose to determine whether the difference in $\Delta^{15}N$ value of pea aphids on diets of varying amino acid profiles is correlated to the amount of *de novo* amino acid synthesis occurring in the aphid. I found that there was a significantly higher incorporation of the labeled carbon backbone in the protein of pea aphids feeding on the unbalanced diets, supporting the idea that increased *de novo* amino acid synthesis are responsible for the differences in $\Delta^{15}N$ values among aphids feeding on the two diets. The findings of this study highlight the influence of endosymbionts on the $\Delta^{15}N$ values for pea aphids, demonstrate that dietary amino acid composition can influence the $\Delta^{15}N$ value of pea aphids through the demand for metabolic upgrading of amino acids, and provide a model for the study of $\Delta^{15}N$ values in systems where metabolism has been well characterized by experimental and genomic data.

To the thousands of aphids who gave their lives for this project,

May god have mercy on your pea-eating souls.

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PREFACE - PROJECT AIMS, SCOPE, AND OVERVIEW

Project aims

The aims of this project are to test whether the metabolic processing of nitrogen by the endosymbiont *Buchnera aphidicola* is influencing the whole-body $\Delta^{15}N$ value of the pea aphid (*Acyrthosiphon pisum*) and to determine the influence of dietary amino acid content on that $\Delta^{15}N$ value.

Project scope and overview

In this thesis, I first present an introduction on isotopic enrichment in natural systems including the use of stable isotopes in ecological studies. I then present an overview of what is known about the discrimination factors of consumers and possible explanations for the deviation of fluid-feeders from the typical pattern of isotopic enrichment. I conclude the background section with an explanation of why pea aphids and their host plants are an ideal system in which to test the influence of endosymbiont metabolism on the $\Delta^{15}N$ values of a fluid-feeding consumer.

After the introduction chapter, I present the findings of my research in background, experimental design and methods, results, and discussion sections. The data presented address several hypotheses. I first test the hypothesis that *Buchnera* is influencing the $\Delta^{15}N$ value of pea aphids on host plants with a comparison of symbiotic and aposymbiotic pea aphids on alfalfa. Other studies have indicated that the overall nitrogen percentage of an aphid's diet can influence $\Delta^{15}N$ values. I tested the hypothesis that when the amino acid concentration in an aphid's diet is held constant, the amino acid

profile of the diet can influence the aphid's $\Delta^{15}N$ value. Finally, I tested the hypothesis that the change in $\Delta^{15}N$ value seen in pea aphids on diets of varying amino acid profiles correlates to a difference in the amount of *de novo* synthesis of amino acids required by the aphid. Lastly, I discuss these the inferences that can be made from the results of this study about the specific metabolic mechanisms involved in generating a $\Delta^{15}N$ value in the pea aphid-*Buchnera* symbiosis.

In the concluding section I recapitulate the major findings of this study, discuss future directions for this research and discuss the broader implications of these results for studies of nitrogen fractionation and.

CHAPTER I. PROJECT BACKGROUND

PART 1. Fractionation of nitrogen and the use of $\delta^{15}N$ in ecological studies

Mechanisms of fractionation in nitrogen metabolism

Stable isotope analysis has been an important tool in studies of ecology and physiology for decades. New applications for stable isotopes are continually being found as we improve our understanding of the isotopic patterns found in nature and the underlying mechanisms that generate them. The most common isotopes of interest include carbon, nitrogen, oxygen, and sulfur. This study focuses primarily on nitrogen. Nitrogen isotope values are typically expressed as $\delta^{15}N$ signatures, which uses the isotopic value of atmospheric nitrogen as a baseline, is measured in parts per thousand (‰), and is defined by the formula (from DeNiro and Epstein 1981):

$$\delta^{15}N = [(^{15}N/^{14}N)_{sample}/(^{15}N/^{14}N)_{air}) - 1]*1000$$

A common metric of interest to ecologists is the discrimination factor, $\Delta^{15}N$, which is defined as the difference in the $\delta^{15}N$ value between a consumer and its food source and determined by the formula (from Peterson and Fry 1987):

$$\Delta^{15}N = \delta^{15}N_{consumer} - \delta^{15}N_{diet}$$

A central principle underlying ecological studies that are reliant on nitrogen stable isotope values is that the isotopic signature of an organism tends to reflect that of its diet (Ambrose and DeNiro 1986, DeNiro and Epstein 1981) and that consumers are almost always enriched in $\delta^{15}N$ with respect to their food sources, that is, they have a positive $\Delta^{15}N$ value. On the scale of an ecosystem this means that increasing $\delta^{15}N$ values are

observed in higher levels of the food chain. Different studies have put the average value of trophic enrichment in the range of 2.3‰ to 3.4‰ increase per trophic level (Caut et al. 2009, McCutchan et al. 2003, Minagawa and Wada 1984, Post 2002, Robbins et al. 2010, Vander Zanden et al. 1997, Vanderklift and Ponsard 2003). This pattern of trophic enrichment is ubiquitous among ecosystems ranging from open oceans to saltwater marshes, estuarine wetlands, African savannah, and the arctic (Abrantes and Sheaves 2010, Ambrose and DeNiro 1986, Ehleringer et al. 1986, Fry 1988, Hobson and Welch 1992, Minagawa and Wada 1984, Pang and Nriagu 1977). The consistency of this pattern across such a wide range of ecosystem arises from the fact that the underlying mechanism is a physical process that occurs during fundamental metabolic reactions.

In all organisms, nitrogen is incorporated into cells and bodies primarily through amino acids, which are the building blocks of proteins and also serve as precursors for the synthesis of nucleotides, hormones, and neurotransmitters like serotonin (from tryptophan) and dopamine (from tyrosine)(Campbell et al. 2008). When amino acids are degraded or synthesized, their amine group is either released or incorporated in the form of free ammonia (facilitated by the forward and reverse actions of synthase or ligase enzymes) or it is directly transferred to or from another amino acid (facilitated by transamination enzymes). In the course of many of these reactions, the lighter ^{14}N isotope is utilized at a quicker rate than the physically heavier ^{15}N isotope (Macko et al. 1986). The preferential use of one isotopic form of an element by an enzyme or reaction is termed “fractionation” and the fractionation of nitrogen during the metabolism of amino acids is thought to be the driving mechanism behind the observed pattern of ^{15}N trophic enrichment. While it may seem counterintuitive that consumers become isotopically

heavier when it is the lighter nitrogen isotope that is preferentially used to make the amino acids that become the proteins of the organisms, tropic enrichment results because "nitrogen metabolism" includes both assimilation and excretion. The constant turnover of proteins and other amine-containing compounds in the cell is such that the ultimate fate of most of the nitrogen consumed is to be freed as ammonia and excreted in some form. As a result the nitrogen that an organism excretes tends to be isotopically lighter than its food, and the heavier ^{15}N isotope is left behind in the free amino acid pool that eventually becomes incorporated into the structural proteins that make up the body (Gannes et al. 1997). Although it is widely assumed that transamination reactions are primarily responsible for the isotopic fractionation involved in nitrogen metabolism, there is a scarcity of experiments directly linking transaminase activity to isotopic shifts. One study has attempted to use the activity of a single transaminase enzyme to predict trophic shifts however they found that it was not possible unless the exact amount of food consumed and dietary protein content were known (Gaye-Siessegger et al. 2007). This study highlighted the fact that isotopic fractionation is the result of the overall patterns of nitrogen metabolism in a consumer and not just the activity of single enzymes.

The amount of fractionation that occurs during metabolic reactions is termed the "isotopic effect", and the size of the isotopic effect depends on the enzymes involved, the concentration of the substrates and other physiological factors such as temperature or pH (reviewed in Tcherkez 2011). Typically, the isotopic effect is very small (deviating from a neutral effect of 1.0 by about 0.001 to 0.06 in either direction) where reactions favoring ^{15}N have a value less than one and reactions favoring ^{14}N have a value higher than one, and is calculated by the formula (after Mariotti et al. 1981)

$$\propto_{ps} = \left({}^{15}N_p/{}^{14}N_p\right)/\left({}^{15}N_s/{}^{14}N_s\right),$$

where $\propto_{ps}$ is the proportion of the heavier isotope incorporated, and $^{15}N_p/^{14}N_p$ and $^{15}N_s/^{14}N_s$ are the proportions of each isotope in the product and the substrate respectively.

Two types of isotopic effects act on nitrogen during metabolic reactions. The "kinetic effect" is the effect of irreversible reactions where breaking or making a bond using the lighter ^{14}N is favored and the ^{15}N isotope is discriminated against. The "thermodynamic effect" is the effect that is seen in reversible reactions, and this effect is dependent on the concentration of reactants. The kinetic effect is always higher than one (favoring the lighter ^{15}N isotope) but the thermodynamic effect may be either higher or lower than one depending reactant concentrations (Tcherkez 2011). The discrimination factor of consumers is the result of the fractionation that occurs as a result of the combined influence of the kinetic effect and the isotopic effect.

Transamination reactions are one class of metabolic reactions that has been shown to preferentially favor the lighter nitrogen isotope. Transamination reactions are catalyzed by transaminases, which transfer an amine group from one amino acid to an a-keto acid backbone creating a new and different amino acid. Aspartate aminotransferase (EC 2.6.1.1) facilitates a reversible reaction that converts glutamate and oxaloacetate to and from aspartate and 2-oxoglutarate. It has been demonstrated that aspartate aminotransferase has an isotopic effect of 1.0083 when producing aspartate and 1.0017 in the reverse reaction producing glutamate (Macko et al. 1986). Although the reaction catalyzed by aspartate aminotransferase is a reversible reaction, the isotopic effect values determined by Macko and colleagues (1986) were determined almost entirely by measuring the kinetic effect because the substrate concentrations and experimental timing

ensured that the reaction was only occurring in one direction. Subsequent experiments have demonstrated that the isotopic effect in the conversion of glutamate to aspartate is actually less than one, meaning that heavy, ^{15}N isotope is favored (Rishavy and Cleland 2000) and it is possible that the results of Macko *et al* may be due to the temperatures or concentrations used (Tcherkez 2011). While the isotopic effect of aspartate aminotransferase (EC 2.6.1.1) has been relatively well studied, the isotopic effects of most other transaminases have not been experimentally measured. That said, all transaminases operate using a similar mechanism involving a protonated Schiff base that covalently attaches the carbon backbones of an amino and an α-keto acid to facilitate transfer of the amine group. Thus, it is reasoned that the isotopic effect is likely to be similar in all transamination reactions (Tcherkez 2011).

Transaminases are not the only enzymes that have been demonstrated to fractionate nitrogen. Other enzymes that have been experimentally shown to fractionate nitrogen include asparagine synthetase (isotopic effect of 1.022 from Stoker et al. 1996), glutamine synthetase (isotopic effect of 1.016 from Yoneyama et al. 1993), glutamate synthase, also called GOGAT (isotopic effect of 1.022 from Rishavy and Cleland 2000) and glutamate dehydrogenase (isotopic effect of 1.014 from Weiss et al. 1988). Each of the reactions catalyzed by these enzymes are critically important to nitrogen metabolism in the pea aphid-*Buchnera* symbiosis and are discussed in more detail later in this chapter.

Nitrogen isotopes in ecological and physiological studies

The largely consistent pattern of trophic ^{15}N enrichment has resulted in a large collection of studies that utilize nitrogen stable isotope values. $\delta^{15}N$ values are frequently used in cases where feeding patterns and trophic relationships are difficult or impossible to observe directly (uses of nitrogen stable isotopes in ecological studies are reviewed in Crawford et al. 2008, Gannes et al. 1997, Hood-Nowotny and Knols 2007, Peterson and Fry 1987, Thompson et al. 2005, Wang et al. 2004, Wolf et al. 2009). Some studies have attempted to utilize equations to determine trophic structure based solely on the $\delta^{15}N$ values of food web members. For example, Vander Zanden (1997) created such a formula, which was later modified by (Post 2002) to define trophic level as:

$$TL = l + (\delta^{15}N_c - \delta^{15}N_{base})/ \Delta_n,$$

where l is the trophic level of the base food (with primary producers occupying the l level 1), $\delta^{15}N_c$ is the value of the consumer, $\delta^{15}N_{base}$ is the value of the base food, and Δ_n is the estimated change in $\Delta^{15}N$ per trophic level. This final term is included because the amount of change appears to be greater in higher trophic levels than lower ones (Post 2002). This model was tested by investigating the correlation of model estimates of the trophic position of several freshwater fish species against their trophic position determined by nitrogen stable isotope analysis of gut content, and the correlation between model estimates and diet-based estimates was significant and positive (Vander Zanden et al. 1997).

Other studies have attempted to use $\delta^{15}N$ values to more relatively assign trophic levels to consumers (Abrantes and Sheaves 2010, Gallagher and Dick 2010, Kling et al. 1992, Nakazawa et al. 2010, Post 2002, Rau et al. 1992, Vander Zanden et al. 1997)

or to build entire food webs (Canbana and Rasmussen 1994, Fanelli et al. 2009, Fry 1988, Layman et al. 2007). One benefit to using $\delta^{15}N$ values is that there can be temporal variation in the incorporation of nitrogen into different body tissues. Tissues with high levels of activity tend to incorporate nitrogen isotopes faster, and so the composition of liver cells for example, will reflect recent dietary inputs while the composition of bone collagen will reflect dietary inputs over a longer period of time (Dalerum and Angerbjorn 2005). Similarly, tissues that are periodically discarded, such as shells, feathers, and hair, will retain the composition of dietary inputs at the time that they were made (Bowen et al. 2005) and so changes in isotopic composition can also be assessed at different temporal levels.

$\delta^{15}N$ values have also been used in ecological studies in more specific applications. It has been found that $\delta^{15}N$ values in an organism tend to increase during periods of starvation (Adams and Sterner 2000, Boag Brian et al. 2006, Haubert et al. 2005, Webb et al. 1998), and so $\delta^{15}N$ values can be used as a noninvasive indicator of an organism's health in natural settings. The mechanism that causes enrichment with starvation is thought to be the same as the typical mechanisms that cause trophic enrichment, except that rather than consuming and metabolizing a typical diet, the starving organism is breaking down and metabolizing its own proteins. A few studies however, have shown that in some organisms, namely cockroaches and some diverse reptile species, starvation does not lead to enrichment in $\delta^{15}N$ (McCue 2008, McCue and Pollock 2008), and so the physiology of a particular consumer species should always be considered when investigating the question of starvation. In another specific use of $\delta^{15}N$ values, Stapp and Salkeld (2009) used the isotopic signatures of fleas and the blood of

grasshopper mice and prairie dogs to determine that grasshopper mice are likely sharing fleas and facilitating the spread of a plague-causing bacterium among prairie dog families. Another study used $\delta^{15}N$ values to determine how specialized the diets of two ant species were over a diverse geographic range, with the working hypothesis being that ants that feed solely on a host plant would have a lower isotopic signature than ones with more opportunistic foraging behaviors (Trimble 2004). Senn *et al.* (2010) used $\delta^{15}N$ values to assign trophic positions to pelagic tuna and to correlate those positions with lifestyle (coastal or oceanic) and with mercury accumulation levels. All of these studies that use $\delta^{15}N$ values, from the common dietary reconstruction and trophic assignments to the more specific applications, are dependent on the consistency of the pattern of trophic enrichment.

PART 2. Isotopic patterns of fluid-feeders and pea aphids as a study system

Fluid feeders and the role of endosymbionts in nitrogen fractionation

The use of nitrogen isotopes in trophic level assignment, dietary reconstruction, and other related studies is based on the pattern of trophic enrichment, and so the use of $\delta^{15}N$ values in such capacities, and especially in food web reconstruction, is limited by our understanding of some well-documented exceptions to the pattern. Fluid-feeding consumers tend to exhibit $\delta^{15}N$ values that are either depleted or not enriched with respect to their hosts. The first indication of this trend came from a study that found that parasitic intestinal nematodes were enriched in $\delta^{15}N$ with respect to their rabbit hosts

while parasitic cestodes were $\delta^{15}N$ depleted (Boag et al. 1998). Two broader studies that looked at a total of four taxa of fish parasites on 12 different species of fish found endoparasite cestode and nematode species were significantly depleted in $\delta^{15}N$ with respect to their host fishes while the ectoparasite isopod and copepod species were either depleted, enriched, or isotopically indistinguishable from their host (Deudero et al. 2002, Pinnegar et al. 2001). Other studies that focus on plant and insect interactions have found that phloem-feeding insects are either depleted or not enriched with respect to their diet. This has been shown in several aphid species including potato aphids, *Macrosiphum euphorbiae,* on pumpkin plants ($\Delta^{15}N$ = -1.2 ‰ Sagers and Goggin 2007), oat bird cherry aphids, *Rhopalosiphum padi*, on wheat plants ($\Delta^{15}N$ = 0.0 ‰ Yoneyama et al. 1997), raspberry aphids, *Amphorophora idaei*, on raspberry plants ($\Delta^{15}N$ = from + 0.86 to +3.58‰ Scrimgeour et al. 1995), black bean aphid, *Aphis fabae*, on common bean ($\Delta^{15}N$ = -1.0 ‰ Schumacher and Platner 2009), another unknown aphid species on sorghum ($\Delta^{15}N$ = 0.0‰ Ostrom et al. 1997), and green peach aphids, *Myzus persicae* on brassicaceous host plants ($\Delta^{15}N$ = -6.0 ‰ Wilson 2011).

It is of critical importance that the deviation of fluid-feeders from the otherwise ubiquitous pattern of trophic enrichment be accounted for because the entire field of nitrogen-based stable isotope ecology is dependant on our understanding of the mechanisms underlying these patterns. Additionally, many fluid-feeders are highly influential on food web structures, and parasites in particular have a unique role in the connectivity and complexity of food webs. To date there have been many attempts to incorporate parasites into food-web studies and their inclusion has been consistently found to have a dramatic effect on several parameters of food-webs including an increase

in species richness, the proportions of top species, number of trophic levels, and an increase in the percentage of omnivory in the models (Amundsen et al. 2009, Arias-Gonzalez and Morand 2006, Chen et al. 2008, Hatcher et al. 2006, Hernandez and Sukhdeo 2008, Huxham et al. 1995, Marcogliese and Cone 1997, Mouritsen and Poulin 2002, Thompson et al. 2005). The complex nature of the life history of parasites and the obvious implications for connectivity has led to several recent calls for more studies into the incorporation of parasites into food webs (Byers 2009, Lafferty et al. 2006, Poulin 2010), with one paper calling parasites the "ultimate missing links" in food web reconstruction (Lafferty et al. 2008).

While the importance of incorporating parasites into food web studies should not be understated, the bigger question raised by fluid-feeders that have been observed to deviate from the pattern of trophic enrichment is what the mechanisms responsible for this deviation are and whether those mechanisms can give us insight into the more subtle and widely documented variability among all consumers that have been studied (*e.g.* Vanderklift and Ponsard 2003)?

Many reviews and experimental studies have attempted to elucidate the mechanisms of fractionation that influence discrimination factors in ecological studies (*e.g.* Gannes et al. 1997, McCutchan et al. 2003, Vanderklift and Ponsard 2003, Wolf et al. 2009). Some of the key influences of discrimination factors that have been proposed or examined are simple mass-balance effects, the interference of metabolism by nitrogenous substrates (Pinnegar et al. 2001), diet quality (Adams and Sterner 2000, Oelbermann and Scheu 2002, Robbins et al. 2005, Robbins et al. 2010), nitrogen recycling and the influence of endosymbiont metabolism (Cook and Davidson 2006,

Davidson et al. 2003, Feldhaar et al. 2009). A mass-balance effect is essentially an explanation that has to do with the inputs and outputs in a consumer. In this case it would be the most simplistic explanation - the bioaccumulation of ^{15}N can be explained if an organism absorbs the heavier isotope at a higher rate than it excretes it (Martinez del Rio and Wolf 2005). Although this idea makes intuitive sense, it has been directly tested with conflicting results. Sponheimer (2003) found that the $\delta^{15}N$ signature of llama's excreta was not more negative than the signature of their food and a lack of isotopically depleted excreta has also been seen in other organisms that demonstrate trophic enrichment (Tsahar et al. 2007). Power and Klein (2004) tested whether the discrimination values of fluid-feeding cestodes might be related to the parasites feeding on the hosts' diet rather than on the host itself. They compared those cestodes to both the muscle tissue and to the diet of several fish species and found that they were depleted in $\delta^{15}N$ with respect to host muscle in each case but that they were enriched with respect to the fish's diet in two species and depleted in one, suggesting that direct consumption of the hosts food may be responsible for the observed depletion in some cases. While this explanation may be sufficient for the cestode species studied in that particular study it cannot account for the observed depletion of cestode species that feed on muscle away from gut tissue and certainly cannot explain the observed depletion in insect fluid-feeders. Another possibility is that consumers with a similar fluid-feeding life history may simply ingest most of the free amino acids they need from their fluid food source, without the requirement of metabolic conversion (Spence and Rosenheim 2005), however this seems unlikely given that some of those food sources such as phloem sap are deficient in the essential amino acids needed to build proteins and metabolic conversion of nonessential

amino acids to essential amino acids has been experimentally demonstrated (Prosser and Douglas 1992). Mass balance explanations are likely too simplistic to explain the depletion seen in fluid-feeders as they mainly consider the inputs and outputs and don't account for the metabolism that occurs in between.

It has also been suggested that an excess of nitrogenous substrate or product, such as may be expected with fluid-feeding organisms, could form complexes that inhibit transamination and the accompanying fractionation (Velick and Vavra 1962). It is difficult to imagine that this effect could be influential in an organisms like the pea aphid, where high levels of transamination in the synthesis of essential amino acids is required and a reduction in the ability to perform those transaminating reactions would have a debilitating effect on the aphid.

Besides mass balance explanations and the idea that transamination may be inhibited by dietary nitrogen, more specific explanations related to nitrogen recycling, diet quality, and endosymbiont metabolism have also been suggested. I address all three explanations here in turn as they are interrelated and are collectively what I believe are the three driving mechanisms behind the isotopic depletion seen in fluid-feeders. Nitrogen recycling is defined as the incorporation of free ammonia into amino acids (Prosser and Douglas 1992). Metabolized ammonia contains a higher amount of the proportionately lighter ^{14}N isotope and Pinnegar *et al.* (2001) discuss the possibility that parasites may be able to recycle the this ammonia that would otherwise be excreted from a host into the amino acid glutamate by the reverse glutamate-dehydrogenase reaction or that differential metabolism of more isotopically depleted amino acids could cause relative isotopic depletion of the whole-body value (Pinnegar et al. 2001). Although the

influence of nitrogen recycling on discrimination factors has not been experimentally tested, it is related to a large field of studies related to diet quality in that recycling is only required in organisms that feed on a low quality diet.

In examining the role of diet quality in influencing the discrimination factors of consumers, it is important to delineate between a diet's "quality" and the diet's amino acid profile. The term quality is oftentimes used to refer solely to the overall amount of protein in the diet, as measured by ratio of carbon to nitrogen (the C:N ratio). However the amino acid profile of a diet can also be considered a measure of diet quality with respect to how close it is to matching the amino acid requirements of the consumer – a diet with an amino acid profile that is similar to that of the needs of a consumer would be a high quality diet for that organism. There have been several predictions made about $\Delta^{15}N$ values with respect to the amount of nitrogen in diets. It has been predicted that discrimination values should decrease with diets of a balanced amino acid profile and should increase with increased protein quality (Martinez del Rio and Wolf 2005, Olive et al. 2003). The predicted negative correlation between $\Delta^{15}N$ values and the closeness of dietary amino acid composition to a consumer's amino acid requirements has been experimentally tested and has good support. For example, Robbins *et al.* (2005) found that among 21 species of birds and mammals, $\Delta^{15}N$ values decreased with protein quality (as defined by the extent to which the essential amino acid spectrum of the absorbed proteins matches the animal's requirements). Another study looked at the validity of mixed diet models by feeding rats different combination of foods with known amino acid compositions (Robbins et al. 2010). It was found that the $\Delta^{15}N$ values for the mixed diets differed from what would be expected based on the weighted averages of the individual

foods and that diets with complementary amino acid profiles generated lower than expected $\Delta^{15}N$ values while diets with non-complementary amino acid profiles led to higher than expected $\Delta^{15}N$ values. The implication of this experiment was that the biological processes that generate changes in $\delta^{15}N$ signatures are complex and dependent on the overall amino acid composition of an organism's diet. Highlighting the importance of the amino acid composition in determining $\Delta^{15}N$ values is the mixed support for dietary quality (the overall amount of protein in an organisms diet) influencing $\Delta^{15}N$. In two separate studies examining the influence of metabolic rate on isotopic turnover, it was found that with one species of bat the high basal metabolic rate did not translate into a high isotopic turnover rate (Voight and Matt 2004) while in another species of bat there was a much higher than average rate of turnover seen (Miron et al. 2006). As the bats were closely related and their metabolic rates were similar, it was determined that the higher protein quality in the diet was responsible for the higher isotopic fractionation. The influence of protein quality was directly tested by Tsahar *et al.* (2007) and it was found that in birds fed a low protein content diet there was a larger $\Delta^{15}N$ value and that excreta was significantly depleted with respect to the birds' tissues while in birds fed a high protein diet the $\Delta^{15}N$ values were lower and their excreta was not depleted. A positive correlation was found between $\Delta^{15}N$ values and dietary protein content in warblers (Pearson et al. 2003) and in Nile tilapia (Focken 2001) but a negative correlation was found in fruit-eating birds (Tsahar et al. 2007) and no effect of protein quality was found in the rat study of Robbins *et al.* (2010). So it appears that the amino acid profile of a consumers diet may be a more reliable predictor of that organism's $\Delta^{15}N$ value than the bulk protein content.

If amino acid composition and/or the amount of nitrogen a consumer's diet are important influences in determining $\Delta^{15}N$ values, then it stands to reason that the adaptive mechanisms that organisms have evolved to deal with poor quality diets may contribute significantly to the discrimination factor. One such adaptation is the development of intimate relationships with endosymbionts. The possibility that the metabolic recycling of nitrogenous materials by endosymbionts is the contributing mechanism behind the lack of trophic enrichment in fluid-feeders was first proposed by Davidson *et al.* (2003). It was observed that among a wide range of ant species, the ones that had lower $\Delta^{15}N$ values tended to feed on plant exudates and honeydew, and many of those ant species had specialized pouch structures in which to house bacterial symbionts. Davidson *et al.* (2003) hypothesized that if the endosymbiotic bacteria in ants were recycling nitrogen, the ants would have a lower than expected $\delta^{15}N$ signature and would appear more "herbivorous" than they actually are (as herbivores have a lower trophic shift than higher level consumers). Direct tests of the contribution of endosymbiont metabolism to the $\Delta^{15}N$ value of a host species are scarce. One study compared two closely related species of foraminifers and found that the species that harbored dinoflagellate symbionts had a lower $\Delta^{15}N$ value than the species without the symbiont, even though they both had the same food sources and similar life histories (Uhle et al. 1997). This study however was only a comparison and not an experimental test. The question of endosymbiont metabolism influencing the $\Delta^{15}N$ values of an ant species was directly tested by Feldhaar (2009), who compared the discrimination factors in ant pupae that were cured of their bacteria with antibiotics delivered from chemically defined (holidic) diets, generating so-called "aposymbiotic" pupae. Two different holidic diets were used. Both contained all

the trace metals, vitamins, and growth factors needed for normal growth, but one contained ample essential amino acids and one contained no essential amino acids and was instead compensated with higher levels of nonessential amino acids. It was found that there was no difference in the $\Delta^{15}N$ values of symbiotic and aposymbiotic ant pupae that had been fed the diet with abundant essential amino acids, however for reasons that are not explained the researchers did not treat the ant pupae that were fed the diets without essential amino acids with antibiotics. If the endosymbionts are thought to be contributing to the amino acid metabolism because the ants' diet is lacking in essential amino acids, then it might be expected that their contributions and therefore isotopic influences would be minimized on a diet where the need to synthesize essential amino acids was alleviated. A direct test of the influence of endosymbiont metabolism on the discrimination factor of a consumer that is feeding on a natural food source is needed for resolution of the question of the role of endosymbiont metabolism on the discrimination factors of fluid-feeders.

Pea aphids as a study system for the role of endosymbionts in nitrogen fractionation

Pea aphids are an ideal study system in which to examine the role of endosymbiont metabolism on nitrogen fractionation. The symbiosis between aphids and *Buchnera aphidicola* is well described, especially with respect to nitrogen metabolism, and the recent publication of the symbiont genome (Shigenobu et al. 2000) and the pea aphid genome (Consortium 2010) has confirmed much of what was predicted from experimental evidence about the metabolic capabilities of the pea aphid and *Buchnera*. Aphids can be considered plant parasites, they feed exclusively on plant phloem sap and

oftentimes individual aphids will stay on a single host plant for their entire lives. Plant phloem sap has very low levels of essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) and high levels of nonessential amino acids like asparagine and glutamine in the case of alfalfa and fava or serine in the case of pea (Girousse et al. 1991, Sandstrom and Pettersson 1994). The two roles that *Buchnera* has been hypothesized to contribute to the aphid are in nitrogen recycling and nitrogen upgrading. Nitrogen recycling refers to the incorporation of nitrogenous waste, *i.e.* ammonia, back into amino acids while nitrogen upgrading refers to the conversion of nonessential amino acids and metabolic intermediates into essential amino acids (Adams and Sterner 2000).

There is good evidence that nitrogen recycling is done solely by the pea aphid while *Buchnera* is primarily involved in nitrogen upgrading. The primary experiment indicating that nitrogen recycling by *Buchnera* is not occurring showed that similar small amounts of ammonia are found in the honeydew of symbiotic and aposymbiotic aphids (Sasaki et al. 1990) indicating that *Buchnera* doesn't utilize free ammonia. Uniquely among insects with sequenced genomes, the pea aphid has lost several key enzymes related to the urea cycle including ornithine carbamoyltransferase (Enzyme Commission number (EC 2.1.3.3), acetyleornithine deacetylase (EC 3.5.1.16), argininosuccinate synthase (EC 6.3.4.5), and argininosuccinate lyase (EC 4.3.2.1) and so they cannot produce urea or the amino acid arginine. It would be expected that without the ability to produce urea, there would be high levels of ammonia in the honeydew (excreta) of aphids, however only trace amounts are found (Sasaki et al. 1990), indicating that the ammonia is recycled into amino acids by the aphid. Direct tests of *Buchnera*'s role in

nitrogen recycling have been done using aphids that are treated with antibiotics to cure them of their *Buchnera*. Such so-called aposymbiotic aphids do not exhibit malaise, impaired ability to penetrate plant tissues, changes in osmoregulation, or assimilation of dietary amino acids (Prosser et al. 1992, Wilkinson 1998), but they do grow and develop more slowly than symbiotic aphids and they do not produce as many progeny. Several studies have shown that aposymbiotic aphids have higher levels of glutamine in their honeydew than symbiotic aphids (Prosser and Douglas 1992, Sasaki et al. 1993) while symbiotic aphids had higher levels of glutamine as well as arginine. Because of its role in the urea cycle and the fact that it contains four nitrogen atoms, it has been proposed that arginine is a primary "excretory" amino acid for symbiotic aphids and that glutamine, which also has multiple nitrogen atoms, may be a second "excretory" amino acid when *Buchnera* are disrupted. The most likely mechanism for the aphid's incorporation of ammonia back into amino acids is through the reactions of glutamine synthetase (EC 6.3.1.2) and glutamate synthase (EC 1.4.1.13), which can incorporate ammonia into glutamate and 2-oxoglutarate to create glutamine and glutamate respectively. The activity of glutamine synthetase is the same in the tissues of symbiotic and aposymbiotic aphids and it was found that aposymbiotic aphids that have been fed on a diet containing no glutamine still produce glutamine in their honeydew (Sasaki et al. 1993), so the genomic data and experimental data both suggest that nitrogen recycling is done solely by the aphid.

Another test of the respective influence of upgrading and recycling was done by Prosser and Douglas (1992). They fed symbiotic and aposymbiotic aphids on a series of diets that had either increasing overall nitrogen quality (overall amino acid content) or

increasing essential amino acid content. There was no difference between the performance of symbiotic and aposymbiotic aphids on diets of very low amino acid content, but there was a drastic difference in the performance of the two groups on diets of very low essential amino acid content. This suggests that the role of *Buchnera* may be more significant in upgrading nitrogen than in recycling it because if they could recycle ammonia effectively then the symbiotic aphids would perform much better than aposymbiotic aphids on the diet of low protein quality.

The evidence for *Buchnera*'s role in amino acid upgrading is well established. Aposymbiotic aphids have a lower protein content and higher free amino acid content than symbiotic aphids, indicating that there is less overall production of protein occurring in the aposymbiotic aphids (Liadouze et al. 1995, Prosser and Douglas 1992) and when essential amino acids are injected directly into the haemocoel of aposymbiotic aphids, protein synthesis is increased, indicating that essential amino acids are limiting protein production in aphids without *Buchnera*-mediated reactions (Wilkinson and Ishikawa 1999). The effect was the same when phenylalanine alone was injected (Wilkinson and Ishikawa 2000), possibly indicating that phenylalanine is the single most limiting essential amino acid for protein synthesis in aposymbiotic aphids. This is consistent with the genomic data as *Buchnera* codes for almost the entire phenylalanine synthesis pathway while the pea aphid only facilitates one of the necessary reactions (Shigenobu et al. 2000, Wilson A.C.C. et al. 2010). Labeled glutamine was found to be incorporated into protein ten fold higher in symbiotic than aposymbiotic aphids (Sasaki and Ishikawa 1993) and labeled aspartate, glutamate, and glutamine have been shown to undergo substantial catabolism in symbiotic aphids (Febvay et al. 1995), presumably as nitrogen

donors for the synthesis of essential amino acids. When fed an unbalanced diet based on the amino acid composition of alfalfa sap, aposymbiotic aphids were found to have high levels of nonessential amino acids asparagine, aspartate, and glutamine and low levels of essential amino acids like isoleucine, tyrosine, phenylalanine, and threonine in their free amino acid pools (amino acids that are not a part of proteins) (Liadouze et al. 1995, Wilkinson and Douglas 1996). It is thought that the symbiotic aphids are able to maintain their amino acid profiles on balanced and unbalanced diets while aposymbiotic aphids are unable to fully utilize the nonessential amino acids to produce their own essential amino acids because they lack the necessary reactions that would otherwise be facilitated by *Buchnera*.

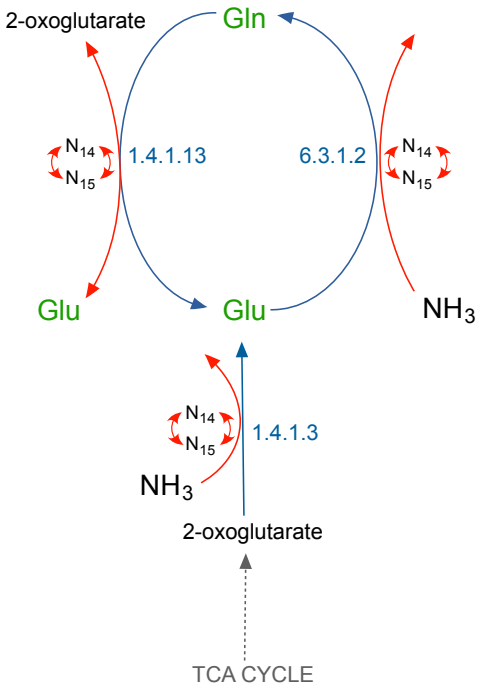
The interface of the shared nitrogen metabolism in the pea aphid-*Buchnera* symbiosis is in the aphids' bacteriocytes and the symbiosomal membranes that surround the individual *Buchnera* cells. There is evidence that glutamine and not glutamate can pass from the aphid into the bacteriocyte and that glutamate and not glutamine can pass from the bacteriocyte into the *Buchnera* cells (Sasaki and Ishikawa 1995). This may be the key to the recycling of ammonia for the aphid, because glutamate is the direct nitrogen donor in the synthesis of nearly all the essential amino acids. Glutamate donates an amino group to synthesize six out of nine essential amino acids: histidine, isoleucine, leucine, lysine, phenylalanine, and valine. Methionine gets its amino group from the nonessential amino acid serine and serine either gets its amino group from glutamate if it's being made from the products of glycolysis or else it gets it from free ammonia if it's being made from pyruvate. Similarly, threonine either gets its amino group from aspartate or through glycine, which itself can either get its amino group from serine (and ultimately

from one of the two ways mentioned above) or else from alanine (in which case it ultimately comes from glutamate). So even the three essential amino acids that don't directly get their ammonia groups from glutamate are likely to get them indirectly from glutamate. Both glutamine synthetase (EC 6.3.1.2), which incorporates ammonia into glutamate to make glutamine, and glutamate synthetase (EC 1.4.1.13), which turns glutamine into two molecules of glutamate are upregulated in bacteriocytes compared to whole-body aphid tissues (Hansen and Moran 2011). If glutamine is needed to transport amino groups into the bacteriocyte and glutamate is needed to transport amino groups into *Buchnera* cells, then it's possible that free ammonia would be incorporated into these amino acids rather than excreted so that the synthesis of essential amino acids will never be limited by the availability of amino donating groups. Another consideration in the limiting of amino acid synthesis and metabolism in the pea aphid/*Buchnera* symbiosis is the cofactor pyridoxal phosphate, which is required in the activity of all transamination reactions. *Buchnera* has lost the genes necessary to synthesize this cofactor and are completely reliant on the pea aphid for its production, giving another potential source of regulation in the symbiosis.

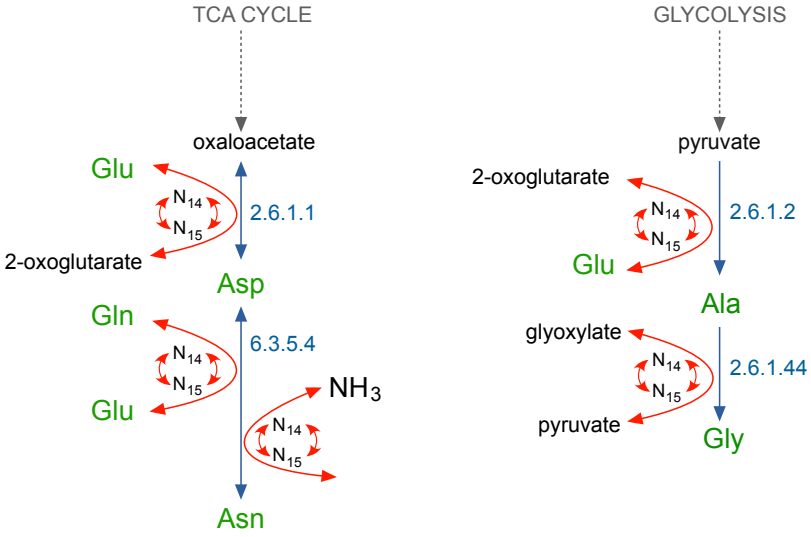
The unique value of studying isotopic discrimination factors in the pea aphid-*Buchnera* symbiosis is that the amino acid profiles of their food sources and their own tissues are known and our knowledge of the metabolic reactions facilitated by each member of the symbiosis allows us to formulate specific hypotheses about how amino acids are converted from the amino acid composition found in phloem sap to that of the aphid. Knowledge of the precise metabolic reactions that are occurring in a system will allow me to interpret experiments into the influence of endosymbiont metabolism and dietary

composition on the pea aphid's $\Delta^{15}N$ value in the context of a well-understood metabolic framework.

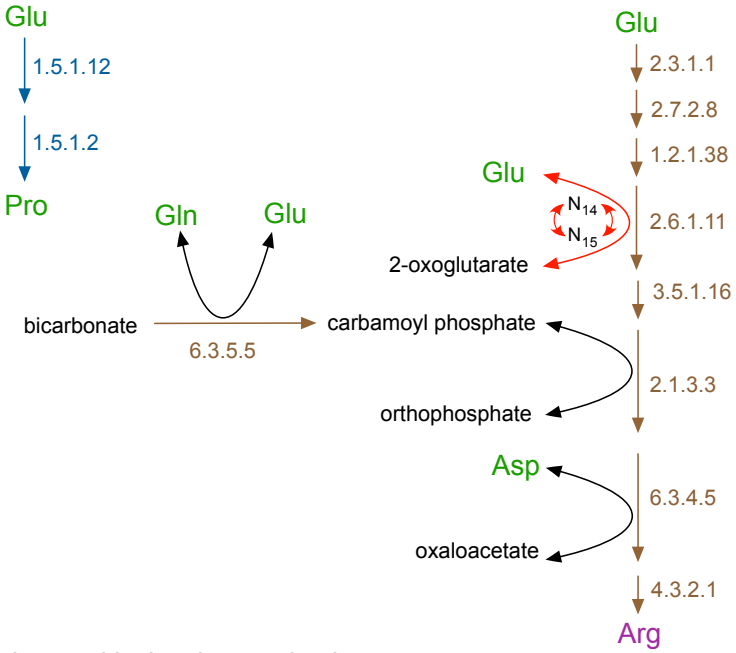
(A) Glutamate and glutamine metabolism



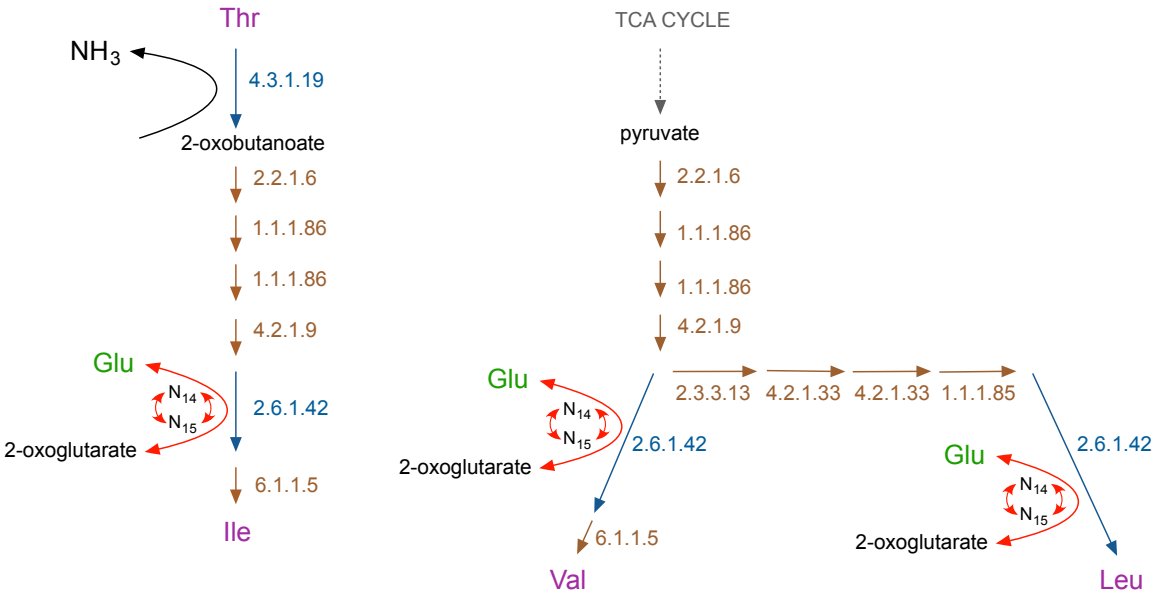
(B) Aspartate, asparagine, and alanine metabolism



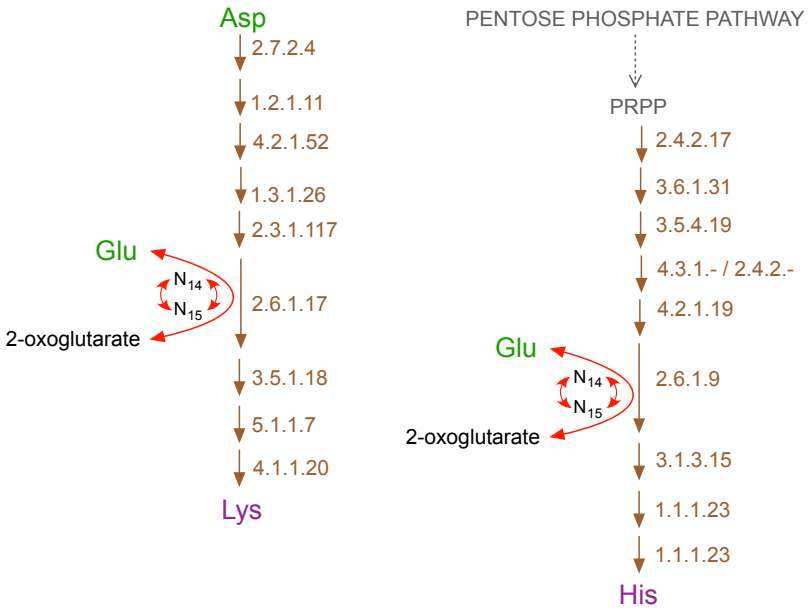
(C) Proline and arginine synthesis



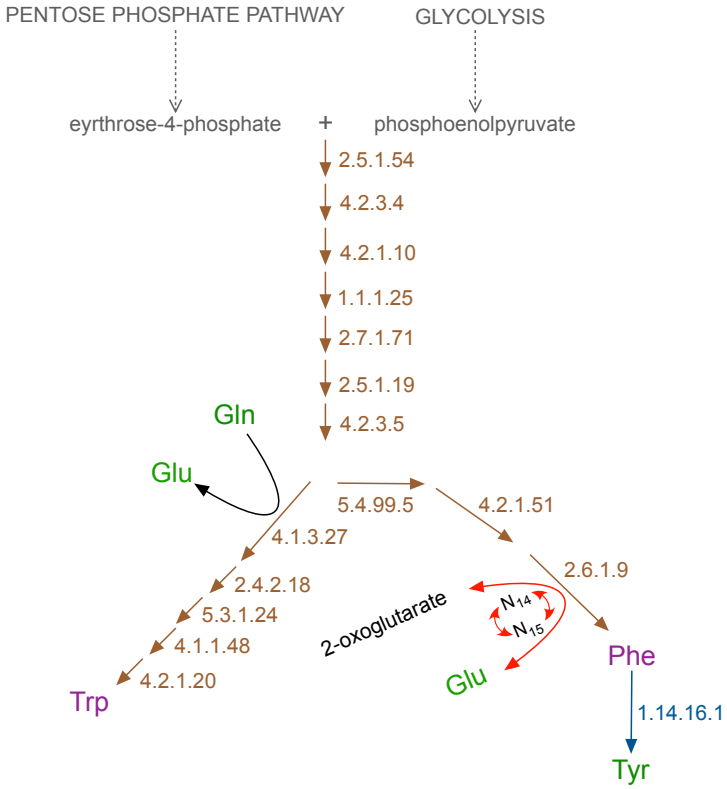
(D) Valine, leucine, and isoleucine synthesis



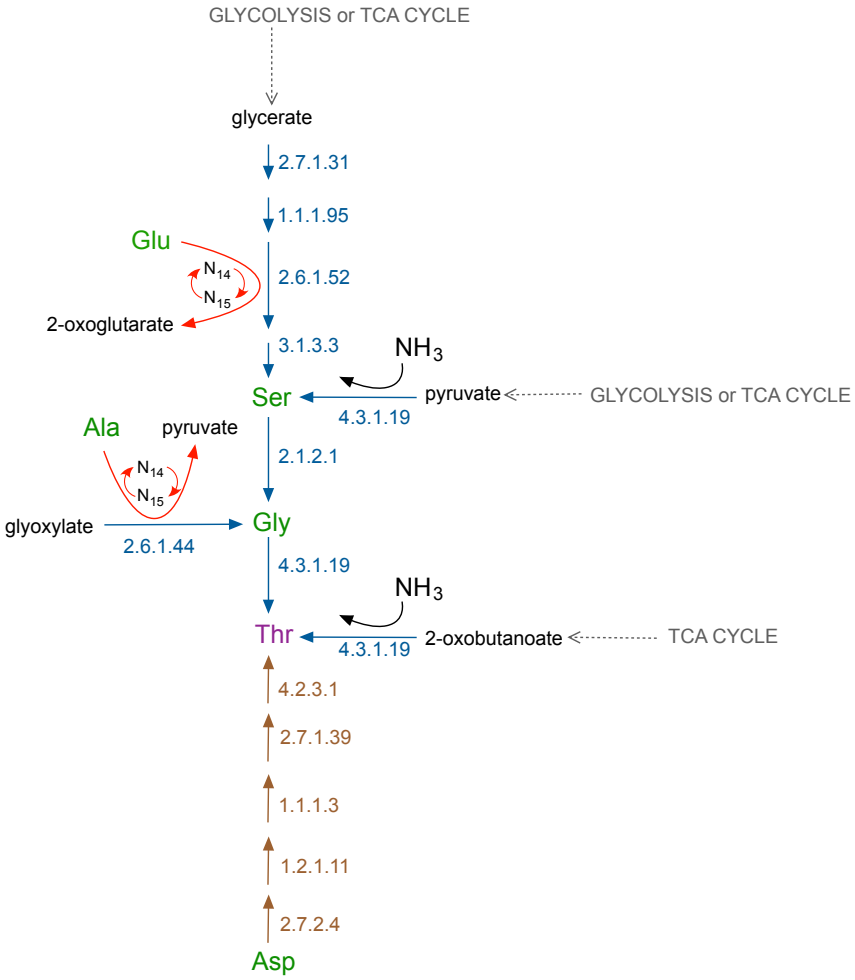
(E) Lysine and histidine synthesis



(F) Tryptophan, tyrosine, and phenylalanine synthesis



(G) Glycine, serine, and threonine synthesis



(H) Cysteine and methionine synthesis

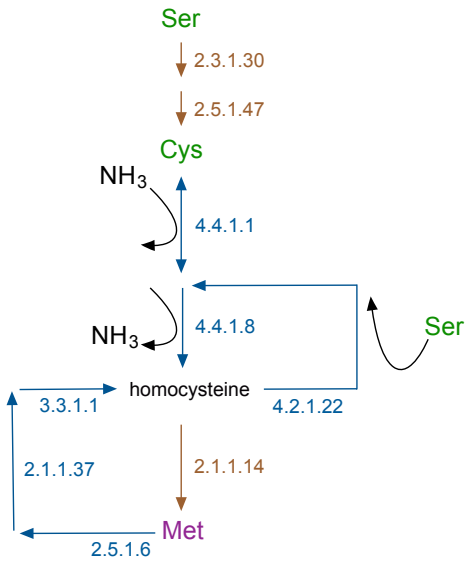


Figure 1. Fractionating reactions are involved in the synthesis of amino acids in the pea aphid/*Buchnera* symbiosis.

Depiction of the metabolic pathways for the synthesis of all 20 amino acids in the pea aphid/*Buchnera* symbiosis. Reactions are indicated by colored arrows with the corresponding Enzyme Commission (E.C.) numbers for the enzymes that facilitate those reactions given to the right or below their respective arrows. Blue and brown arrows and E.C. numbers represent enzymes and reactions coded for and facilitated by the pea aphid and *Buchnera* respectively. Swooping arrows indicate the removal or addition of nitrogen into the pathway and the source or end product for that nitrogen. Red swooping arrows with a nitrogen cycling symbol indicate reactions where there is either experimental evidence that the fractionation of nitrogen occurs in that reaction or else experimental evidence for fractionation in a reaction that transfers nitrogen by the same mechanism. Black swooping arrows indicate that there is no evidence that nitrogen is fractionated in that reaction or in other reactions with the same mechanism of nitrogen transfer. Arrows do not represent the directionality or reversibility of reactions but rather the dominant direction of amino acid metabolism in the aphid. (A) Direct evidence for fractionation of nitrogen exists for glutamine synthetase (EC 6.3.1.2 Yoneyama et al. 1993) and glutamate dehydrogenase (EC 1.4.1.3 Weiss et al. 1988) and indirect evidence for fractionation exists for glutamate synthase (EC 1.4.1.13) as it functions as an amidotransferase and evidence for fractionation exists from similarly acting carbamyl-phosphate synthase (EC 6.3.5.5) and asparagine synthetase (EC 6.3.5.4) (Rishavy and Cleland 2000, Stoker et al. 1996). (B) Direct evidence for fractionation exists for aspartate aminotransferase (EC 2.6.1.1 Macko et al. 1986) and for asparagine synthetase (EC 6.3.5.3 Rishavy and Cleland 2000) and indirect evidence exists for alanine aminotransferase (2.6.1.2) and alanine-glyoxylate transaminase (EC 2.6.1.44) as they have a mechanistically similar function to aspartate transaminase, with the transfer of nitrogen being facilitated by a Schiff-base formation and the use of pyridoxl phosphate as an cofactor. (C) Indirect evidence exists for acetyleornithine transaminase (EC 2.6.1.11) as it has a mechanistically similar function to aspartate transaminase (repeat refs for these indirect evidences). (D) Indirect evidence exists for branched chain amino acid transaminase (EC 2.6.1.42) as it has a mechanistically similar function to aspartate transaminase. (E) Indirect evidence exists for succinyldiaminopimelate transaminase (EC 2.6.1.17) and histidinol phosphate transaminase (EC 2.6.1.9) as they have a mechanistically similar function to aspartate transaminase. (F) Indirect evidence exists for histidinol phosphate transaminase (EC 2.6.1.9) as it has a mechanistically similar function to aspartate transaminase. (G) Indirect evidence exists for alanine glyoxylate transaminase (EC 2.6.1.44) and phosphoserine transaminase as they have a mechanistically similar function to aspartate transaminase. (H) There are no reactions with evidence of fractionation in this pathway. Figures were generated from pathway information available on the KEGG database (http://www.genome.jp/kegg/).

CHAPTER 2: STUDIES OF THE EFFECTS OF ENDOSYMBIONT METABOLISM AND DIETARY NITROGEN ON THE $\Delta^{15}N$ VALUE OF THE PEA APHID

PART 1. Background

Stable isotope analysis has been an important tool for the study of animal ecology and physiology for decades and new applications for this tool are continually being found as we improve our understanding of the isotopic patterns found in nature and the mechanisms that generate them. A central principle underlying nitrogen-based stable isotope studies is that the isotopic signature of an organism tends to reflect that of its diet (DeNiro and Epstein 1981) and that consumers are almost always enriched in $\delta^{15}N$ with respect to their food sources, that is, they have a positive discrimination factor ($\Delta^{15}N$) (Macko et al. 1986). This pattern of isotopic enrichment is generated by several families of enzymes that have been shown to fractionate nitrogen (through the preferential use of one or the other isotopic forms of nitrogen) during their reactions, most notably the transamination reactions (Macko et al. 1986). This pattern of isotopic enrichment through trophic levels has led to a wide range of applications for nitrogen isotopes in ecological and physiological studies (reviewed in Wang et al. 2004, Thompson et al. 2005, Hood-Nowotny and Knols 2007, Crawford et al. 2008). Despite their widespread application, our ability to reliably use nitrogen stable isotopes in ecological and physiological studies is limited by our understanding of the variability in the discrimination factors seen in natural systems, and as such, biological variability in $\Delta^{15}N$ values has resulted in much interest in determining the mechanisms that influence discrimination factors such as mass balance effects, interference of metabolism by nitrogenous substrates, starvation, diet

quality, nitrogen recycling, and the influence of endosymbiont metabolism (reviewed in Gannes et al. 1997, McCutchan et al. 2003, Vanderklift and Ponsard 2003, Wolf et al. 2009).

One group of organisms that have been shown to deviate from the pattern of isotopic trophic enrichment are fluid feeders such as parasitic cestodes and nematodes (Boag B. et al. 1998, Deudero et al. 2002, Pinnegar et al. 2001) and phloem feeders including several aphid species. A lack of enrichment or depletion has been shown in potato aphids, *Macrosiphum euphorbiae,* on pumpkin plants ($\Delta^{15}N$ = -1.2 ‰ Sagers and Goggin 2007), oat bird cherry aphids, *Rhopalosiphum padi*, on wheat plants ($\Delta^{15}N$ = 0.0 ‰ Yoneyama et al. 1997), raspberry aphids, *Amphorophora idaei*, on raspberry plants ($\Delta^{15}N$ = from + 0.86 to +3.58‰ Scrimgeour et al. 1995), black bean aphid, *Aphis fabae*, on common bean ($\Delta^{15}N$ = -1.0 ‰ Schumacher and Platner 2009), another unknown aphid species on sorghum ($\Delta^{15}N$ = 0.0‰ Ostrom et al. 1997), and green peach aphids, *Myzus persicae* on brassicaceous host plants ($\Delta^{15}N$ = -6.0 ‰ Wilson 2011).

This study focuses on the influence of endosymbiont metabolism on the variable $\Delta^{15}N$ values seen in fluid-feeding consumers. Davidson *et al.* (2003) first suggested that the metabolism of nitrogen by endosymbionts might influence $\Delta^{15}N$ values in a variety of ant species. This idea was directly tested by Feldhaar (2009), who compared the $\Delta^{15}N$ values of untreated ant pupae to those of pupae that had been treated with antibiotics to cure them of their endosymbionts. They found that there was no difference in the $\Delta^{15}N$ values, however their results may be an artifact of the experimental conditions chosen, as explained later in the discussion. To date this is the only experiment to test the contribution of endosymbiont metabolism on a consumer's $\Delta^{15}N$ value aside from one

other study that compared two closely related species of foraminifers and found that the species that harbored dinoflagellate symbionts had a lower $\Delta^{15}N$ value than the species without the symbiont, even though they both had the same food sources and similar life histories (Uhle et al. 1997). There have been several studies addressing the influence of dietary nitrogen percentage and amino acid composition on $\Delta^{15}N$ values, and two studies have demonstrated that the nitrogen percentage of a host plant influences the $\Delta^{15}N$ value of the aphids species (Sagers and Goggin 2007, Wilson A.C.C. et al. 2011). Since it is known that *Buchnera*'s primary role is to compensate for a phloem sap diet that has a low nitrogen content and unbalanced amino acid profile, dietary composition will also be a focus of this study.

Pea aphids are an ideal study system in which to examine the role of endosymbiont metabolism on nitrogen fractionation. The symbiosis between aphids and *Buchnera aphidicola* is well described, especially with respect to nitrogen metabolism, and the recent publications of the symbiont genome (Shigenobu et al. 2000) and the pea aphid genome (Consortium 2010) has confirmed and expanded on much of what was predicted from experimental evidence about the metabolic capabilities of the pea aphid and *Buchnera*. This study will test three hypotheses. First, that metabolism of nitrogen by *Buchnera* is influencing the $\Delta^{15}N$ value of the aphid. Second, that the amino acid profile of the aphids' diet is a driving influence on the discrimination factor of the aphid, and three, that an influential mechanism determining the $\Delta^{15}N$ value of the aphid is the amount of *de novo* amino acid synthesis and the associated transamination reactions that are required of the aphid based on the amino acid profile of their diet.

PART 2. Experimental Design and Methods

Determining the $\Delta^{15}N$ value of pea aphids on alfalfa plants

There are currently no published reports of nitrogen isotopic signatures for pea aphids and their host plants. To determine whether pea aphids exhibit similar discrimination factors to other aphid species that have been studied I selected four genotypes of pea aphid (5A, 7A, A2A, and LSR1) and determined their $\Delta^{15}N$ values on host alfalfa plants. Multiple genotypes were used to control for potential genotypic effects and those four genotypes were selected because they were all originally collected on host alfalfa plants in the field and because they all accepted alfalfa (*Medicago sativa*) as a host plant after being kept long term in isofemale cultures on fava plants (*Vicia faba*, var. Windsor). Cultures of each genotype were allowed to develop through two generations on alfalfa plants so that the aphids could acclimatize to the new host plant and any maternal effect on the embryos from feeding on fava plants would be eliminated. After two generations, adult aphids from the alfalfa cultures were allowed to deposit nymphs onto caged, 7-day old alfalfa plants (n=5 adults per plant per genotype) for 24 h at which time the adults were removed and excess nymphs were removed such that ten nymphs remained on each plant. The aphid nymphs were allowed to grow on the alfalfa plants for 14 more days, at which time they were sampled. The conditions in the incubator were kept at 21° C with a 16:8 hour light to dark (L:D) photoperiod at all times.

To sample the aphids, 3 individuals from each plant were placed in a pre-weighed tin analysis cup (5 x 8 mm, Elementar America, New Jersey), which was pinched shut

and put in a 60° C oven to dry for 48 hours. To sample alfalfa, the entire plant was extracted from the soil by the roots and washed with distilled water and a soft bristled brush to remove the soil, exuviae, and honeydew. The leaves from each plant were then cut off at the petiole with a scalpel and stored in envelopes to dry in a 60° C oven for 48 hours. After 48 h, a second weight measurement was taken to get the dry weight of the aphid samples and the alfalfa leaves were ground up with a porcelain mortar and pestle in liquid nitrogen and weighed into tin cups. Around 0.30 mg of dry aphid tissue and 2.25 mg of dry plant tissue were used for each sample. The tin cups were loaded into an elemental analyzer (Eurovector, Milan, Italy) connected to an Isoprime stable isotope mass spectrometer (Elementar, hanau, Germany) and isotopic signatures of the samples were determined. These analyses were performed at the University of Miami's Stable Isotope Core Facility with $\delta^{15}N$ being determined using the formula, $\delta^{15}N\ [(^{15}N/^{14}N)_{sample}/(^{15}N/^{14}N)_{air}) - 1]*1000$ and with the precision of the analyses being ± 0.1 ‰.

Comparing the $\Delta^{15}N$ values of symbiotic and aposymbiotic pea aphids on alfalfa plants

To test the influence of symbiont metabolism on the $\Delta^{15}N$ signature of the pea aphids, antibiotics were used to disrupt the endosymbiont *Buchnera aphidicola* and create so-called "aposymbiotic" aphids. To administer the antibiotic, rifampicin (Sigma, cat. #R3501) an artificial diet (diet A0 from Febvay et al. 1988) with modified sucrose concentrations as in Febvay (1999) was used. To make the diet, each of the ingredients (given in Table S#) were weighed out and dissolved into 100 mL of distilled water. The diet was then adjusted to a pH of 7.55 with KOH and sterilized with a vacuum filter (0.22

mm), and aliquots of 15 ml were stored in sterile Falcon© tubes at -80° C. When the diets were ready to be used, an appropriate number of aliquots were defrosted and 0.05 mg/mL of rifampicin was added to half of the diet to be used. To make the artificial diet cages, small Parafilm© squares were sterilized by soaking for 5 minutes in 10% bleach and 5 minutes in 10% ethanol with thorough rinsing with distilled water after each soak. A single square was then stretched tight across a 2 cm tall, 3 cm diameter Plexiglas cylinder and 300 mL of the diet was aliquoted on top with a micropipette and sterile tips. A second piece of Parafilm© was then used to sandwich the diet in between the sheets such that the diet was sealed in a sachet atop the cage.

Due to the scale of this experiment, the four genotypes were tested in four separate runs. Adult aphids were taken from alfalfa cultures and transferred to artificial diet cages that contained the untreated or rifampicin-laced diet. Five adults were transferred to each diet replicate and 20 untreated and 20 rifampicin-laced diet cages were used. The aphids were allowed to deposit their larvae for 24 hours at which point the adults were removed and the larvae were allowed to feed on the diets for 48 hours. These 2-day old larvae were then transferred to individual caged, 7-day old alfalfa plants, with only aphids from a single diet being transferred to a single plant to maintain replication. 10 larvae were transferred to each of 20 plants, with 10 plants being infested with symbiotic nymphs and 10 being infested with aposymbiotic nymphs. These nymphs were left on the plants to develop to maturity for 14 days, at which time they were sampled as described above.

Verification of Buchnera *reduction in aposymbiotic aphids*

In order to determine that the antibiotic-treated aphids did in fact have their *Buchnera* disrupted, molecular quantification using RT-PCR was performed and performance data was collected (because it has been shown that aposymbiotic pea aphids have a longer developmental time, lower growth rate, and lower fecundity than symbiotic ones (Douglas 1992, Sasaki et al. 1991). To perform the molecular quantification, one aphid per replicate was collected (n =10 symbiotic aphids and 10 aposymbiotic aphids for each genotype) and DNA extractions were performed with a DNeasy DNA isolation kit (Qiagen, cat. #69506). The DNA samples were quantified with a Nanodrop spectrophotometer, visualized with gel electrophoresis, and stored at -20° C until RT-PCR was performed. The aphids were collected at day 10 to ensure that all replicates would be at least fourth instars and that the *Buchnera* hadn't yet begun to decline in the symbiotic aphids, as has been previously been shown to occur in older aphids (Whitehead and Douglas 1993). *gluD* was selected as an aphid housekeeping gene, with primers forward – 5'-TGTCTATGTTGTCGAGGCAC and reverse 5'– TTACGACTGCGTATGGATCG and *cysG* was selected as a single-copy *Buchnera* gene with primers forward 5'-AGGTGGTGATCCCTTTATTTTC and reverse 5'– GAGTATTTGCGATGTGTCAGTG (from Wilson Alex C.C. et al. 2006). PCR conditions are given in Table S3.

To verify that the *Buchnera* populations were indeed reduced in the antibiotic-treated aphids, we first determined an average Ct value for *gluD* and *cysG* for each aphid that was tested and we used the $2^{\wedge-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) to perform a

relative comparison of gene expression in symbiotic and aposymbiotic pea aphids using the formula below, where $2^{-\Delta\Delta CT}$ gives the fold change expression from our control group (symbiotic aphids) to the experimental group (aposymbiotic aphids):

$\Delta\Delta CT = (CT_{cysG} - CT_{gluD})_{aposymbiotic} - (CT_{cysG} - CT_{gluD})_{symbiotic}$

To collect the performance data, 20 extra alfalfa plants (so-called "performance plants"), were planted along with the 20 experimental plants during each run of the experiment. The number of adult aphids on each replicate was counted every day starting at day seven, and the average time to adulthood was calculated for each replicate after 14 days on the plant. On the first day that an adult appeared in a replicate that aphid was weighed and transferred to a clean performance plant. In cases where multiple adults were present in a replicate on the first day they appeared, all the adults were weighed and an average was taken but only one aphid was transferred to the performance plant. These performance aphids were allowed to develop on their plants for 14 days after they were transferred, and the number of offspring was counted on the 14th day of adulthood.

Comparing the $\Delta^{15}N$ values of pea aphids on complete, alfalfa-based, and Asn-only diets

To test the influence of amino acid composition on the $\Delta^{15}N$ value of the pea aphid, three artificial diets with the similar amino acid concentrations (between 252.5 and 260 mmol/L) and the same sucrose concentrations (584 mmol/L, 20% w/v) but differing amino acid profiles were used (Fig. S2). In addition to the complete diet described above, a diet based on the amino acid profile of alfalfa phloem sap designed by Febvay (1999) and a diet with asparagine as the sole amino acid were made. The diets and cages were

made as described above and adult aphids of genotype 5A from alfalfa cultures were placed on the diets for 24 h. Five adults were used per cage and n=10 for each diet. After 24 h, the adults and all but 10 nymphs were removed from each cage and the nymphs were allowed to feed for 10 days, with the diets being changed every three days to prevent contamination of the diet with mold or bacteria. After 10 days, the aphids were sampled, dried, and analyzed with isotope ratio mass spectrometry.

Estimating the amount of amino acid synthesis from ingested carbon sources in symbiotic and aposymbiotic pea aphids on complete, alfalfa-based, and Asn-only diets

To get a measure of how much of the pea aphid's amino acid requirement was being met by *de novo* synthesis using carbon backbones originating from sugar sources, an experiment was performed in which symbiotic and aposymbiotic aphids were raised on three artificial diets of differing amino acid composition that contained isotopically labeled fructose. The diets were the complete, alfalfa-based, and asparagine-only diets described above and they were made without sugar being added and without the pH being adjusted before the aliquots were stored at -80° C. When the aliquots were ready to be used, the sucrose was added at the same concentrations as before, but with 300 mg/mL of D-[UL-$^{13}C_6$] fructose (Omicron Biochemicals, cat. #FRU-011) added and a corresponding amount of sucrose removed so as to maintain the sugar molarity of the diet. The diets were then adjusted to a pH of 7.55 with KOH and filter sterilized a second time before use. Thirty cage replicates were used for each of the three diets and half of the diets had 0.05 mg/mL of rifampicin added to them initially. 5 adult aphids were allowed to deposit nymphs on each diet for 24 h and then they were removed along with extra nymphs such that each replicate had exactly 10 nymphs. At this time the 10 aphids

were collectively weighed and an average was calculated for each replicate. Half of the diets contained 0.05 mg/mL of rifampicin initially and 48 h after the adults were removed the cages had the diets changed out and replaced with fresh diets that did not contain rifampicin. The aphids were allowed to develop on these diets for 10 days, with the diets being changed every two days and then an average weight was again taken for the aphids in each replicate and the replicates with at least 5 aphids remaining were subjected to a chloroform methanol precipitation protein extraction to remove the carbon-rich chitin (Wessel and Flugge 1984). These samples along with 10 mL samples of each diet were dried for 48 h at 60° C and analyzed with isotope ratio mass spectrometry.

In order to interpret the $\delta^{13}C$ signatures from an experiment utilizing isotopically labeled artificial diets in a biologically meaningful way, we developed a parameter that we termed the transamination value "T". This parameter does not directly measure the level of enzyme activity or the exact amount of transamination occurring in the aphid, rather it gives a comparative metric of the amount of *de novo* synthesis occurring in the aphids of amino acids from carbon backbones originating from sugar sources. We started by adjusting the $\delta^{13}C$ values so that we're only considering the signatures of the newly synthesized amino acids using the formula:

$$\delta^{13}C_{final} = P_{new}(\delta^{13}C_{new}) + P_{initial}(\delta^{13}C_{initial})$$

where $\delta^{13}C_{final}$, $\delta^{13}C_{new}$, and $\delta^{13}C_{initial}$ are the isotopic signatures of the final aphid protein, the newly synthesized protein, and the original protein in the aphids at the beginning of the experiment respectively and P_{new} and $P_{initial}$ are the proportions by weight of newly synthesized protein and the initial protein respectively. The final $\delta^{13}C$ signatures of aphid samples and initial $\delta^{13}C$ signatures were known and the proportions of new and initial

aphid protein was estimated from the initial and final bulk weights of the aphids. Plugging those into the equation then gave us our unknown term P_{new}.

Next, we calculated the isotopic contribution of the amino acids as well as the contribution of the other carbon-containing ingredients in each diet using the following formula:

$$\delta^{13}C_{total} = X(\delta^{13}C_{AAs}) + (1 - X)(\delta^{13}C_{other})$$

where $\delta^{13}C_{total}$, $\delta^{13}C_{AAs}$, and $\delta^{13}C_{other}$ represent the isotopic signatures of the total diet, the amino acids, and the other diet components respectively and X represents the proportion by weight of carbon from the amino acids in the diet. We had previously determined the $\delta^{13}C$ signatures of each of the amino acids in the diets, and because we knew the weight of each amino acid in the diets and the weight of carbon in each amino acid, we could estimate the carbon signature of the amino acid component of the diets. Then, because we had determined the total signature of the diets, we could solve for the unknown quantity which was the signature of the other carbon-containing ingredients in the diets.

Finally, knowing the $\delta^{13}C$ signature of the newly synthesized protein, we were able to estimate a transamination parameter "T" according to the formula:

$$\delta^{13}C_{new} = T(\delta^{13}C_{other}) + (1 - T)(\delta^{13}C_{AAs})$$

where T represents our transamination value, the proportion of the $\delta^{13}C$ signature that can be accounted for by *de novo* synthesis from a carbon backbone that did not originate from amino acids.

Statistical Analyses

Prior to analysis dependent variables were tested for normality and homogeneity of variance. In cases where normality and homogeneity of variance were met, I proceeded with an appropriate parametric test. In cases where these assumptions were not met, I applied the appropriate nonparametric test. In experiments that used multiple aphid genotypes, I first tested for a genotypic effect. In all cases, no genotypic effect was found and therefore the data was pooled across genotypes before use. All tests were performed with STATA version 10.0.

PART 3. Results

Pea aphids demonstrate a lack of isotopic enrichment with respect to host plants

The $\delta^{15}N$ signatures for four genotypes of pea aphid and their host alfalfa plants show that pea aphids demonstrate a similar lack of isotopic enrichment with respect to host plants similar to other aphid species that have been studied (Fig. 2.). There was no significant genotypic effect on the $\delta^{15}N$ signature of aphids or for the alfalfa plants (ANOVA, $F = 0.77$, $p > 0.53$). The combined alfalfa $\delta^{15}N$ values were significantly higher than the combined aphid $\delta^{15}N$ values ($N = 20$, Wilcoxon matched-pairs signed rank, $z = -2.023$, $p < 0.05$).

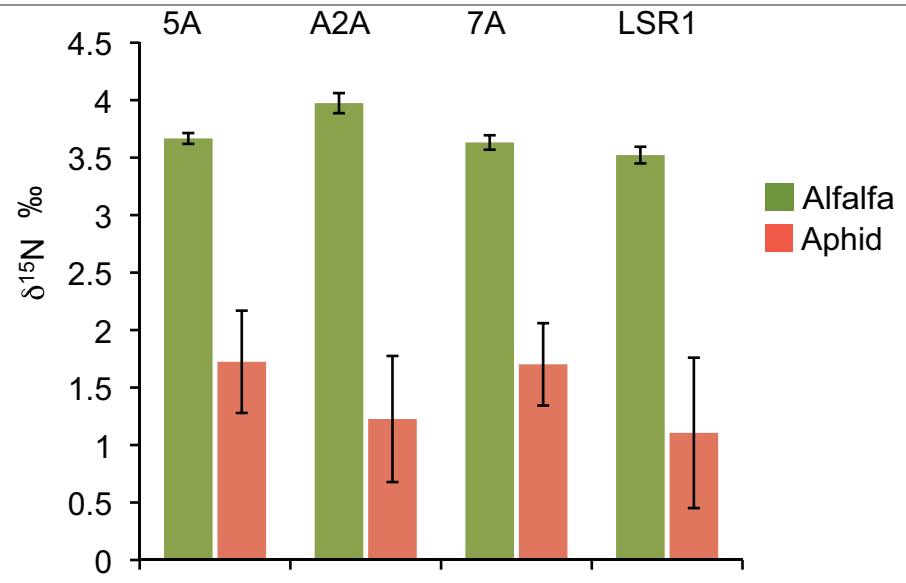


Figure 2. Pea aphids are depleted in $\delta^{15}N$ with respect to host alfalfa plants.

$\delta^{15}N$ signatures for alfalfa plants and four genotypes of pea aphid. Aphid and alfalfa $\delta^{15}N$ values are paired and N=5 for each genotype. Error bars represent ± S.E.M.

Aposymbiotic aphids are less isotopically depleted than symbiotic aphids.

In order to test the influence of endosymbiotic metabolism on the $\Delta^{15}N$ values of pea aphids on host plants, the same four genotypes (5A, A2A, 7A, and LSR1) were used in separate trials of an experiment comparing the $\Delta^{15}N$ values of symbiotic and aposymbiotic pea aphids on alfalfa plants (Fig. 3). For each genotype used, there was a significant difference between the $\Delta^{15}N$ values of the symbiotic aphids and the aposymbiotic aphids on alfalfa plants (Mann-Whitney U $p < 0.05$). There was no genotypic effect on $\Delta^{15}N$ values for either the symbiotic or aposymbiotic treatments (ANOVA, $F = 0.63$ for symbiotic treatment, $F = 2.27$ for aposymbiotic treatment, $p > 0.05$ in each case). For the four combined genotypes, the aposymbiotic aphids had

significantly larger $\Delta^{15}N$ values than the symbiotic aphids (Wilcoxon rank sum, z = 5.727, p <0.0001).

The performance data are given in table S4 and the reduction of *Buchnera* was confirmed for a single aphid on each replicate of this experiment. The $2^{-\Delta\Delta CT}$ values for the comparison of the *Buchnera* housekeeping gene to the aphid housekeeping gene in symbiotic to aposymbiotic aphids were 0.0025 for 7A, 0.0025 for A2A, 0.0029 for 5A, and 0.0046 for LSR1, demonstrating that for each experiment the expression of *Buchnera* gene was reduced by between -217 and -400 fold in aposymbiotic aphids compared to symbiotic aphids.

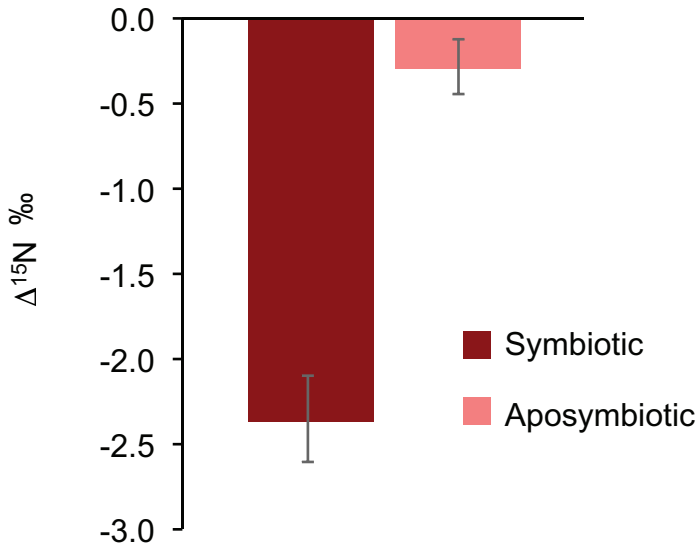


Figure 3. Aposymbiotic aphids are less depleted in $\delta^{15}N$ with respect to alfalfa plants than symbiotic aphids.
The discrimination factors for symbiotic and aposymbiotic pea aphids on host alfalfa plants. This experiment includes four aphid genotypes with paired alfalfa data (N= 40 for aposymbiotic and 39 for symbiotic treatments). Error bars represent ± S.E.M. propagated for the S.E.M.s of aphid and alfalfa $\delta^{15}N$ values.

Differences between $\Delta^{15}N$ values in symbiotic and aposymbiotic aphids may be explained by their respective abilities to synthesize amino acids

An amino acid analysis of symbiotic and aposymbiotic pea aphids from the genotype LSR1 trial of this experiment showed differences in the amino acid profiles of the whole-body aphid samples. There is more glutamate/glutamine in the aposymbiotic aphid than in the symbiotic aphid (29.89% and 12.27% respectively) and there was a general pattern of symbiotic aphids having higher percentages of essential amino acids than aposymbiotic aphids (Ile, Leu, Lys, Phe, Thr, and Val).

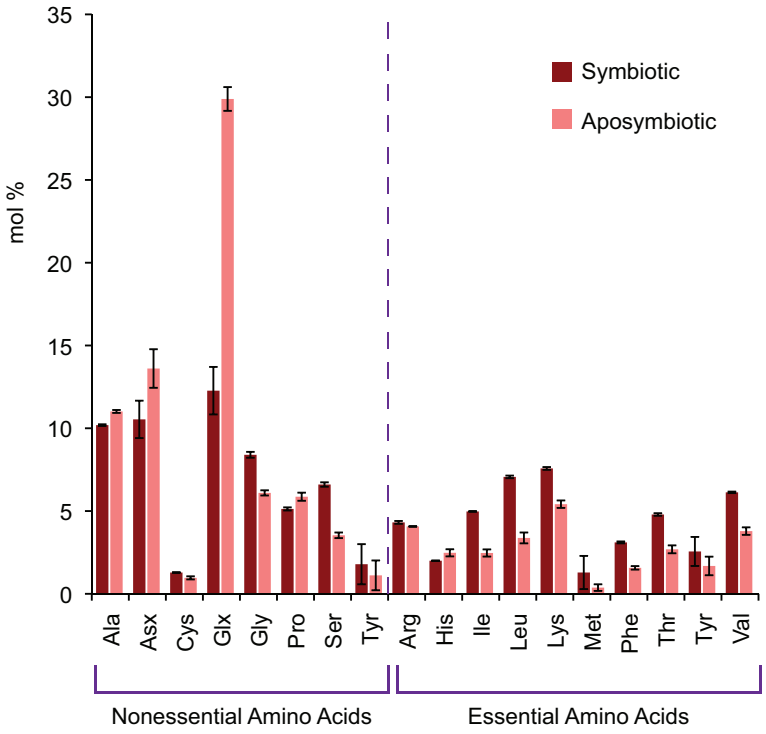


Figure 4. Amino acid profiles of symbiotic and aposymbiotic pea aphids are different.
Amino acid profiles of whole-body tissues of symbiotic and aposymbiotic pea aphids fed on host alfalfa plants. Amino acids are separated into nonessential and essential and are delineated by a purple dotted line. Error bars represent ± S.E.M. for two runs (HC hydrolysis and performic acid oxidation) of the same aphid sample.

Diet composition affects the $\Delta^{15}N$ values of pea aphids.

I tested the hypothesis that the $\Delta^{15}N$ values of pea aphids were influenced by the amino acid composition of their diets. On three artificial diets of varying amino acid composition, the $\Delta^{15}N$ values for aphids on complete diet are significantly higher than the values for aphids on alfalfa-based and asparagine-only diet (Fig. 4) (ANOVA, $F = 213.51$, $p < 0.0001$, Scheffe's method $p < 0.001$ for comparisons of complete diet and alfalfa-based diet and complete diet and asparagine-only diet). There was no significant difference between the $\Delta^{15}N$ values of pea aphids on alfalfa-based diet and on asparagine-only diet.

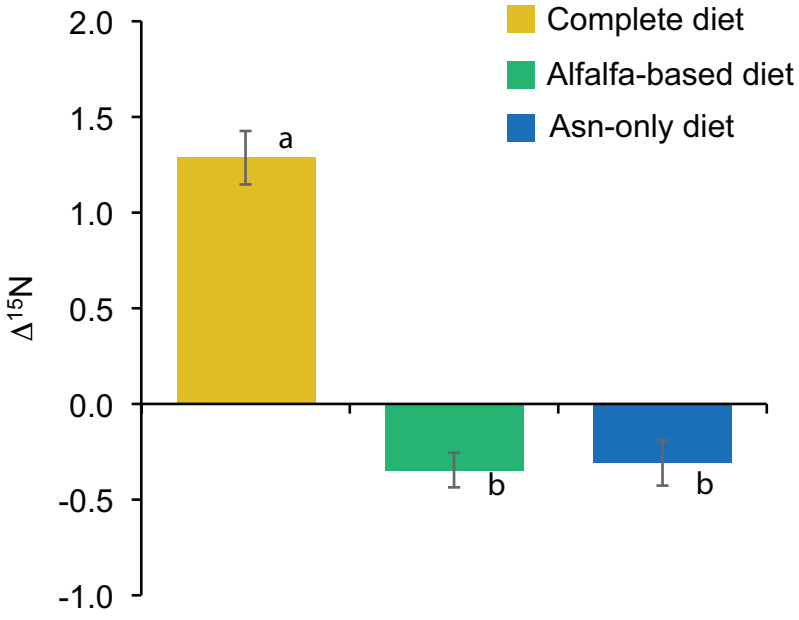


Figure 5. Diet amino acid composition influences the $\Delta^{15}N$ values of pea aphids.
Discrimination factor $\Delta^{15}N$ for pea aphids on complete, alfalfa-based, and asparagine-only artificial diets (see Fig. S1 for amino acid profiles of each diet). Letters "a" and "b" represent significantly different values. N = 10 for each diet and error bars represent ± S.E.M.

The level of de novo *amino acid synthesis is higher in aphids feeding on diets with more unbalanced amino acid compositions.*

I used ^{13}C-labeled fructose in each of the three diets used in the preceding experiment to test the hypothesis that the Δ^{15}N values of pea aphids are influenced by the amount of *de novo* amino acid synthesis required by their diets. There was a significant difference between the "T" values of symbiotic aphids on each of the three artificial diets (Krustal-Wallice, $p < 0.001$), with the progressively higher "T" values on the diets with more unbalanced amino acid profiles. There was also a significant difference between the "T" values of aposymbiotic aphids (Krustal Wallice, $p < 0.05$) (Fig. 5), with progressively lower "T" values on the diets with more unbalanced amino acid profiles. In comparisons between symbiotic and aposymbiotic pea aphids, there was a significant difference in the "T" values for each of the three diets used, with symbiotic aphids having a higher "T" value than aposymbiotic aphids on the complete diet and with aposymbiotic aphids having the higher "T" value on the two unbalanced diets (Two-sample T-test, $p < 0.01$ for each diet).

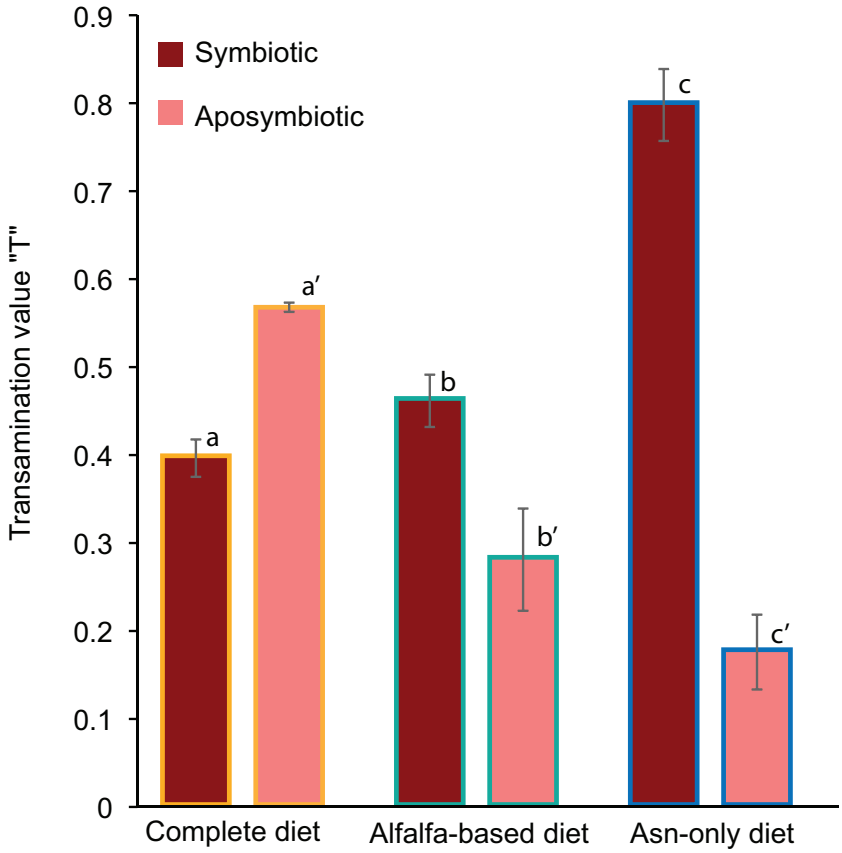


Figure 6. Diet amino acid composition affects transamination values in symbiotic and aposymbiotic pea aphids.

Transamination value "T" for symbiotic and aposymbiotic aphids on three diets (color coded orange for complete, green for alfalfa-based, and blue for asparagine-only). Letters "a", "b", and "c" represent significant differences between diets for symbiotic and aposymbiotic treatments. N = 8, 9, and 8 for complete, alfalfa-based, and asparagine-only diets respectively. Error bars represent ± S.E.M.

PART 4. Discussion

Pea aphids are depleted with respect to host plants but Buchnera aphidicola *is only partly responsible for the depletion.*

The isotopic depletion seen in the pea aphid with respect to host alfalfa plants is consistent with previous studies of aphids (see values in introduction), with an average $\Delta^{15}N$ value of -2.26‰ (Fig. 1). Aposymbiotic aphids are less isotopically depleted on host alfalfa plants than symbiotic pea aphids (with average $\Delta^{15}N$ values of -0.28 and -2.35‰ respectively), but importantly they are still not enriched with respect to their food source, indicating that symbiont metabolism is not entirely responsible for isotopic depletion in this system (Fig. 2). The amino acid analysis indicates that amino acid metabolism in symbiotic and aposymbiotic aphids is different, and this difference is highlighted by the accumulation of glutamate/glutamine as well as the lower amounts of essential amino acid present in the aposymbiotic aphids.

The amino acid profile of the diet is the primary determinant of the pea aphid's $\Delta^{15}N$ values.

Because the aposymbiotic aphids are still isotopically depleted with respect to their host plants, there must be another mechanism behind the depletion seen in aphids. Artificial diets of varying amino acid composition confirm that the unbalanced profile of the aphid's phloem sap diet is primarily responsible for the observed depletion (Fig. 4). All three diets have the same concentration of sucrose and similar concentrations of amino acids, so it can be concluded that the difference in the isotopic shift is related to the amino acid profile, and not the overall nitrogen content of the diet.

The amino acid profile of the aphid's diet determines the amount of de novo *synthesis and transamination that is required.*

On the diet with a complete and balanced assortment of amino acids, there was a lower amount of transamination and not only was the isotopic depletion no longer seen, but the aphids were actually enriched in $\delta^{15}N$ with respect to their host plants. Importantly, the higher levels of *de novo* synthesis seen in the asparagine-only diet with respect to the alfalfa-based diet do not correlate with a change in the aphids' $\Delta^{15}N$ values on those diets. This is a clear indication that the upgrading of nonessential amino acids to essential amino acids by *Buchnera* is not the only influence on the aphid's $\Delta^{15}N$ value and suggests that the recycling of ammonia by the aphid for the synthesis of those amino acids may also be contributing to the isotopic depletion observed. Although more conversion of asparagine to glutamate is occurring due to the higher levels of asparagine in the diet, there is only so much glutamate that is needed for the synthesis of the other amino acids and so the isotopic effect no longer changes once abundant glutamate is present (Fig. 6).

Conclusions and future directions.

This study demonstrates that endosymbiont metabolism can influence a consumer's discrimination value, that the amino acid profile of the diet is a primary determinant for a consumer's discrimination value, and that the amount of *de novo* amino acid synthesis and the associated transamination reactions are the mechanism behind the influence of the dietary amino acid profile. With the amino acid profiles of the symbiotic and aposymbiotic aphids as well as the published amino acid profile of alfalfa phloem sap and the published genomes of the pea aphid and *Buchnera*, we can piece together a

complete story of how nitrogen metabolism in the pea aphid-*Buchnera* symbiosis results in a depleted discrimination factor. The dominant amino acid in alfalfa phloem sap is asparagine (up to 70 molar %)(Girousse et al. 1991, Sandstrom and Pettersson 1994), and so the general pattern of amino acid metabolism in pea aphids must involve turning the abundant asparagine from the phloem sap to glutamate, which is then made available to *Buchnera* as a nitrogen donor in the synthesis of all the essential amino acids. There is evidence that glutamine and not glutamate can pass from the aphid into the bacteriocyte and that glutamate and not glutamine can pass from the bacteriocyte into the *Buchnera* cells (Sasaki and Ishikawa 1995). So to get glutamate to the *Buchnera* cells from the asparagine in the phloem sap, the asparagine must be converted to aspartate to glutamate and to glutamine before it can be transported across the symbiosomal membrane. The glutamine must then be converted to glutamate in the symbiosomal space before it can be transported across the bacterial membrane to the *Buchnera* cells to be used as a nitrogen donor for the synthesis of essential amino acids. The key enzymes involved in this pathway are the reverse reaction of asparagine synthetase, aspartate transaminase, and then glutamine synthetase, all of which have been shown to fractionate nitrogen (Tcherkez 2011). In aposymbiotic aphids, there are still fractionating steps involved in the conversion of asparagine to glutamate, but there is a far lower amount of transamination occurring in the synthesis of other amino acids, and this is reflected in the data shown in Fig. 5.

The isotopic effects of amino acid metabolism in this system can be visualized in Fig. 6. Asparagine synthetase, aspartate transaminase, and glutamine synthetase are all fractionating reactions, as are the transaminating steps in the production of each essential

amino acid (described in the figure legend of Fig. 6). In each case, the kinetic effect (where the lighter isotope is favored for purely physical reasons) should not be mitigated by the thermodynamic effect (where the concentrations of reactants and products can alter the overall fractionation) because in each of these reactions it would be expected that there would be an excess of the reactants or precursor amino acids such that they would not be limiting.

The influence of dietary amino acid composition may explain the results of Feldhaar, Gebaur, and Bluthgen (2009), who found that there was no difference in the discrimination factors between symbiotic ants *Camponotus floridanus* and aposymbiotic ants that had been cured of their endosymbiont *Blochmannia* when fed on artificial diet. The diet they chose provided all the amino acids, vitamins, cofactors, and growth factors that the ant needs to grow, and so whatever contribution that the endosymbiont was making to their metabolism is likely minimized to the point where any isotopic effect would no longer be seen. Additionally, although two previous studies into the effect of dietary nitrogen on the $\Delta^{15}N$ values of aphids have shown a correlation between $\Delta^{15}N$ values and the nitrogen percentage of the host plant (Sagers and Goggin 2007; Wilson *et al.* 2011), I did not find a similar correlation in this experiment (R^2 value of the best fit line is only 0.04) even though ranges of plant nitrogen percentages and $\Delta^{15}N$ values were similar among all three experiments. Further experiments into the relative contributions of dietary nitrogen percentage and dietary amino acid composition to a consumer's $\Delta^{15}N$ value are needed to resolve this question.

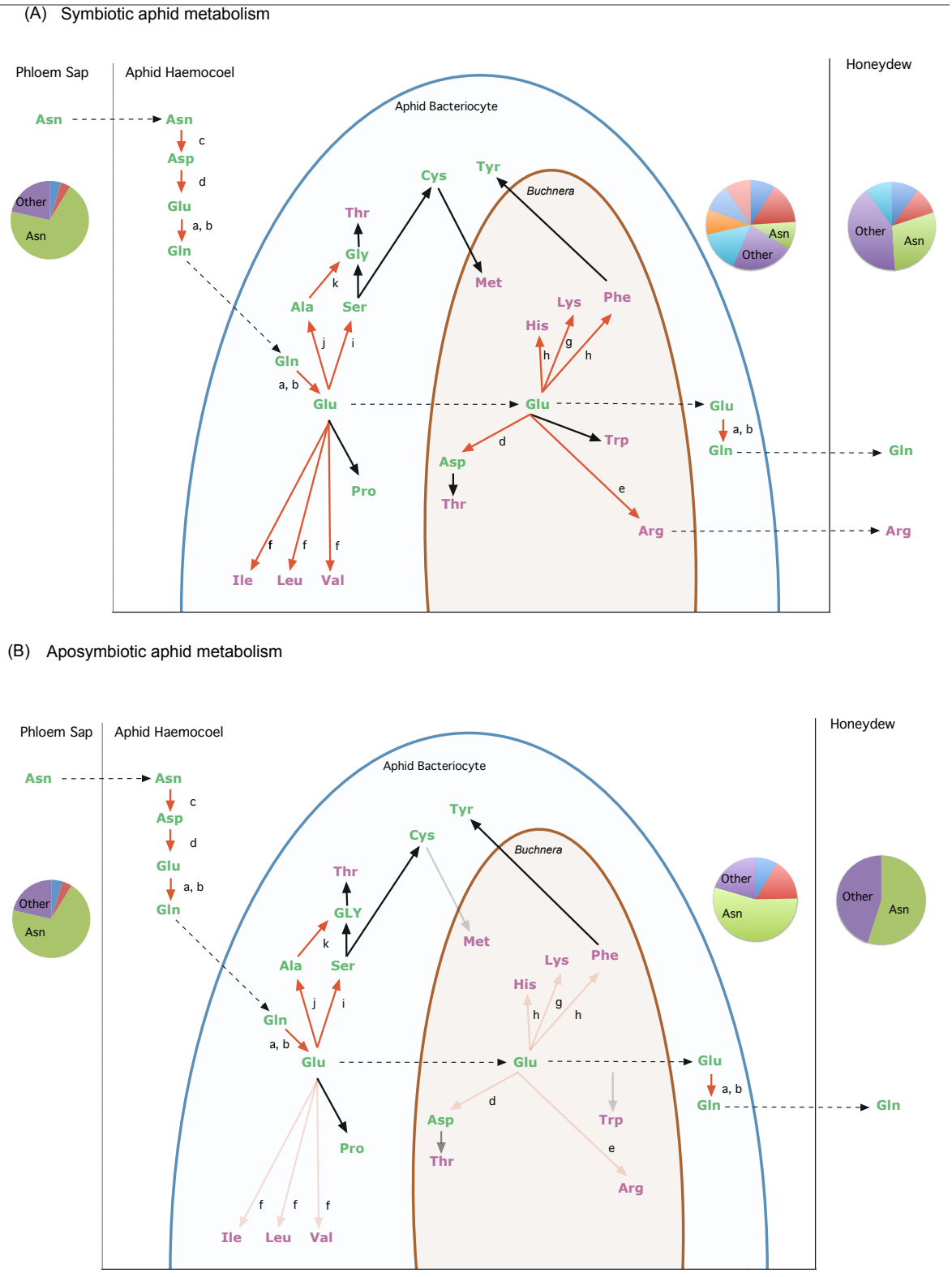


Figure 7. Visualization of metabolic fractionation in the pea aphid

Depiction of the isotopic fractionation of nitrogen during amino acid biosynthesis in the pea aphid/*Buchnera* APS symbiosis. This figure is not intended to show the full details of amino acid metabolism (previously outlined in Wilson *et al.* 2010 and Shigenobu and Wilson 2011) but rather the overall pattern of amino acid synthesis and the pathways where fractionating reactions are thought to occur in this system. Panels A and B show amino acid synthesis in symbiotic and aposymbiotic aphids and green and purple text represents the nonessential and essential amino acids, respectively. Histograms indicate the molar percentage of individual amino acids in the phloem, aphid haemoceol, and honeydew (from Girousse *et al*. 1991, Liadouze *et al.* 1995, and Sasaki *et al.* 1990 respectively). Only amino acids with a molar percentage greater than 10% of the total are shown, the rest are grouped into a category labeled "other". Dashed arrows represent transport across a biological membrane, while transport of most amino acids is yet unknown, there is evidence that glutamine and not glutamate is transported across the symbiosomal (aphid bacteriocyte) membrane and glutamate and not glutamine is transported across the bacterial membrane (Sasaki and Ishikawa 1995). Solid red arrows indicate pathways involving one or more fractionating reaction and black arrows represent pathways without such reactions. The location of the arrows in the bacteriocyte or *Buchnera* cell indicates the location where the fractionating reaction takes place and not where the entirety of the metabolic pathway is occurring. Most reactions in this system have not been experimentally tested as to their effect on nitrogen fractionation and are indicated as fractionating based on the similarity in function to other enzymes that have been experimentally tested. Among the enzymes with experimental evidence supporting fractionation are **(a)** glutamine synthetase (Yoneyama *et al.* 1993) **(b)** glutamate synthetase (Rishavy *et al*. 2000) **(c)** asparagine synthetase (Rishavy *et al.* 2000), and **(d)** aspartate transaminase (Macko *et al.* 1986). Other transaminating reactions are likely to cause fractionation because of their similar mechanism to aspartate transaminase (Tcherkez 2011) such as Schiff base formation and the use of glutamate as an amino-group donor include **(e)** acetyleornithine transaminase, **(f)** branched-chain aminotransferase, **(g)** succinyldiaminopimelate transaminase **(h)** histidinol-phosphate transaminase, and **(i)** phosphoserine transaminase, and **(j)** alanine transaminase. **(k)** Alanine-glyoxylate transaminase is distinct from the other transaminases coded for by the pea aphid/*Buchnera* genomes in that it uses alanine and not glutamate as a nitrogen donor, however as the underlying mechanism still involves the transfer of an amino group from one amino acid to another keto-acid, it is very likely that this reaction also results in fractionation.

The two major contributions of this study to stable isotope ecology are the recognition of the roles that endosymbionts may play in generating the discrimination factors of their hosts and the model that is provided with by use of amino acid analyses and genomic data in this study. It is important that future ecological or physiological

studies take into account the role of endosymbionts and the particular metabolism occurring in organisms before generalizations about their life history or trophic interactions can be made. This study also provides a framework in which future studies of variation in discrimination factors can be based. While the aphid may not be an ideal study system for examining other influences on discrimination factors, researchers should look to other organisms with fully sequenced genomes to address these questions. By knowing the amino acid composition of the aphid and its food source and by knowing the specific metabolic reactions and particularly the fractionating reactions in our system, I as able to make specific and testable hypotheses about the underlying mechanisms causing observed $\Delta^{15}N$ values, and this approach should be utilized whenever possible.

CHAPTER III. CONCLUDING THOUGHTS

This study demonstrates that endosymbiont metabolism can influence a consumer's discrimination value, that the amino acid profile of the diet is a determinant for a consumer's discrimination value even when the overall concentration of amino acids is held constant, and that the amount of *de novo* amino acid synthesis and the associated transamination reactions is correlated to the observed influence of the dietary amino acid profile on the aphid's $\Delta^{15}N$ value. The study will contribute to the field of stable isotope ecology in two ways. First, it has highlighted that endosymbionts can in fact influence the discrimination factor of consumers and may be partly responsible for the variability of discrimination factors seen in natural systems. Secondly, this study provides a framework upon which future studies into the variability of discrimination factors should ideally be based - in systems where the amino acid composition of the consumer and it's diet as well as the metabolic capabilities from genomic data are known.

It is clear that endosymbiont metabolism does influence the discrimination factor of pea aphids on alfalfa plants, but also that the metabolism of endosymbionts is not the driving mechanism behind the observed isotopic depletion in aphids. Using the terminology of previous studies into the metabolism of aphids, the nitrogen upgrading facilitated by the metabolic collaboration between the pea aphid and *Buchnera* and the nitrogen recycling facilitated solely by the pea aphid are the key mechanisms responsible for the overall isotopic signature of the pea aphid. Although it is tempting to assume that the discrimination factor seen in symbiotic aphids on alfalfa is the result of both upgrading and recycling while the discrimination factor in aposymbiotic aphids is the

result of solely recycling, the truth is that the two processes are interrelated and must be considered in relation to each other. The recycling of waste ammonia by the pea aphid is necessary to provide the glutamate that is the nitrogen donor for all the essential amino acids synthesized by *Buchnera*. When the aphid is cured of it's *Buchnera* the synthesis of essential amino acids is no longer occurring, but the aphid is still recycling ammonia into glutamine via the GOGAT cycle because it does not have the ability to synthesize urea or even the nitrogen-rich amino acid arginine without *Buchnera*. Further studies into resolving the respective influence of nitrogen recycling and nitrogen upgrading on the $\Delta^{15}N$ value of pea aphids are needed and must involve the correlation of specific metabolic pathways to changes in $\Delta^{15}N$ values.

The pea aphid is an ideal study subject for the influence of endosymbiont metabolism and for the influence of amino acid composition, but it's specialized life history makes it unsuitable for studies into other influences such as starvation or mixed food sources, and so it is important that other systems with detailed genomic and compositional information be identified. Additionally the nascent field of compound specific stable isotope analysis will be an invaluable tool as we will be able to measure the contribution of particular amino acids to the overall signature of a consumer. At the present time in the pea aphid-*Buchnera* system, the contributions of specific amino acids could only be estimated by comparing the isotopic signatures of aphids that have been fed diets with a single amino acid omitted, however such an experiment would reflect the influence of the synthesis of that amino acid under an altered metabolic framework as opposed to the natural metabolism that would be occurring on the aphid's normal diet.

Future studies into the variables that affect discrimination factors will be crucial to the expanded use of stable isotopes in nitrogen-based ecological studies. Better insight into the mechanisms that cause variability in discrimination factors will allow us to make more accurate interpretations of isotopic data taken from the field and more accurate predictions about what effects different diets and environmental conditions should have on a particular consumer's isotopic signature. Particularly with respect to fluid-feeders, the ability to incorporate certain parasite species into isotope-based food webs will be very important as these parasites have large influences on connectivity and the flow of materials through food webs.

LITERATURE CITED

Abrantes K, Sheaves M. 2010. Use of a d13C-d15N relationship to determine animal trophic positions in a tropical Australian estuarine wetland. Australian Ecology 35: 96 - 103.

Adams TS, Sterner RW. 2000. The effect of dietary nitrogen content on trophic level 15-N enrichment. Limnology and Oceanography 45: 601 - 607.

Ambrose SH, DeNiro MJ. 1986. The isotopic ecology of East African mammals. Oecologia 69: 395 - 406.

Amundsen P-A, Lafferty KD, Knudsen R, Primicerio R, Klemetsen A, Kuris AM. 2009. Food web topology and parasites in the pelagic zone of a subarctic lake. Journal of Animal Ecology 78: 563 - 572.

Arias-Gonzalez JE, Morand S. 2006. Trophic functioning with parasites: a new insight for ecosystem analysis. Marine Ecology Progress Series 320: 43 - 53.

Boag B, Neilson R, SCrimgeour CM. 2006. The effect of starvation on the planarian Arthurdendyus triangulatus (Tricladida: Terricola) as measured by stable isotopes. Biology and Fertility of Soils 43: 267 - 270.

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 in Environmental and Health Studies 34: 81 - 85.

Bowen G, J., Wassenaar LI, Hobson KA. 2005. Global application of stable hydrogen and oxygen isotopes to wildlife forensics. Oecologia 143: 337 - 348.

Byers JE. 2009. Including parasites in food webs. Trends in Parasitology 25: 55 - 57.

Campbell NA, Reece JB, Urry LA, Cain M, L., Wasserman SA, Minorsky PV, Jackson RB. 2008. Biology. New York: Pearson Benjamin Cummings.

Canbana G, Rasmussen JB. 1994. Modelling food chain structure and contaminant bioaccumulation using stable nitrogen isotopes. Nature 372: 255 - 257.

Caut S, Angulo E, Courchamp F. 2009. Variation in discrimination factors (d15N and d13C): the effect of diet isotopic values and applications for diet reconstruction. Journal of Applied Ecology 46: 443 - 453.

Chen HW, Liu WC, Davis AJ, Jordan F, Hwang MJ, Shao KT. 2008. Network position of hosts in food webs and their parasite diversity. Oikos 117: 1847 - 1855.

Consortium IAG. 2010. Genome sequence of the pea aphid *Acyrthosiphon pisum*. PLoS Biology 8: e1000313.

Cook SC, Davidson DW. 2006. Nutritional and functional biology of exudate - feeding ants. Entomologia Experimentalis et Applicata 118: 1 - 10.

Crawford K, McDonald RA, Bearhop S. 2008. Applications of stable isotope techniques to the ecology of mammals. Mammal Review 38: 87 - 107.

Dalerum F, Angerbjorn A. 2005. Resolving temporal variation in vertebrate diets using naturally occuring stable isotopes. Oecologia 144: 647 - 658.

Davidson DW, Cook SC, Snelling RR, Chua TH. 2003. Explaining the abundance of ants in lowland tropical rainforest canopies. Science 300: 969 - 972.

DeNiro MJ, Epstein S. 1981. Influence of diet on the distribution of nitrogen isotopes in animals. Geochimica et Cosmochimica Acta 45: 341 - 351.

Deudero S, Pinnegar JK, Polunin NVC. 2002. Insights into fish host-parasite trophic relationships revealed by stable isotope analysis. Diseases of Aquatic Organisms 52: 77 - 86.

Douglas AE. 1992. Requirement of pea aphids (*Acyrthosiphon pisum*) for their symbiotic bacteria. Entomologia Experimentalis et Applicata 65: 195 - 198.

Ehleringer JR, Rundel PW, Nagy KA. 1986. Stable isotopes in physiological ecology and food web research. TREE 1: 42 - 45.

Fanelli E, Cartes JE, Rumolo P, Sprovieri M. 2009. Food-web structure and trophodynamics of mesopelagic-suprabenthic bathyal macrofauna of the Algerian Basin based on stable isotopes of carbon and nitrogen. Deep-Sea Research I 565: 1504 - 1520.

Febvay G, Delobel B, Rahbe Y. 1988. Influence of the amino acid balance on the improvement of an artificial diet for a biotype of *Acyrthosiphon pisum* (Homoptera: Aphididae). Canadian Journal of Zoology 66: 2449 - 2452.

Febvay G, Liadouze I, Guillaud J, Bonnot G. 1995. Analysis of energetic amino acid metabolism in *Acyrthosiphon pisum*: a multidimensional approach to amino acid metabolism in aphids. Archives of Insect Biochemistry and Physiology 29: 45 - 69.

Febvay G, Rahbe Y, Rynkiewicz M, Guillaud J, Bonnot G. 1999. Fate of dietary sucrose and neosynthesis of amino acids in the pea aphid, *Acyrthosiphon pisum*, reared on different diets. Journal of Experimental Biology 202: 2639 - 2652.

Feldhaar H, Gebaur G, Bluthgen N. 2009. Stable isotopes: past and future in exposing secrets of ant nutrition (hymenoptera Formicidae). Myrmecological News 13: 3 - 13.

Focken U. 2001. Stable isotopes in animal ecology: The effect of ration size on the trophic shift of C and N isotopes between feed and carcass. Isotopes in Environmental and Health Studies 37: 199 - 211.

Fry B. 1988. Food web structure on Georges Bank from stable C, N, and S isotopic compositions. Limnology and Oceanography 33: 1181 - 1190.

Gallagher CP, Dick TA. 2010. Trophic structure of a landlocked Arctic char *Salvelinus alpinus* population from southern Baffin Island, Canada. Ecology of Freshwater Fish 19: 39 - 50.

Gannes LZ, O'Brien DM, Martinez del Rio C. 1997. Stable isotopes in animal ecology: assumptions, caveats, and a call for more laboratory experiments. Ecology 78: 1271 - 1276.

Gaye-Siessegger J, Focken U, Abel H, Becker K. 2007. Dietary back-calculation using stable isotopes: can activities of enzymes involved in amino acid metabolism be used to improve estimates of trophic shifts in fish? Isotopes in Environmental and Health Studies 42: 129 - 141.

Girousse C, Bonnemain JL, Delrot S, Bournoville R. 1991. Sugar and amino acid composition of phloem sap of *Medicago sativa:* a comparative study of two collecing methods. Plant Physiology and Biochemistry 29: 41 - 48.

Hansen A, Moran N. 2011. Aphid genome expression reveals host-symbiont cooperation in the production of amino acids. Proceedings of the National Academy of Sciences: 1013465108.

Hatcher MJ, Dick JTA, Dunn AM. 2006. How parasites affect interactions between competitors and predators. Ecology Letters 9: 1253 - 1271.

Haubert D, Langel R, Scheu S, Ruess L. 2005. Effects of food quality, starvation and life stage on stable isotope fractionation in Collembola. Pedobiologia 49: 229 - 237.

Hernandez AD, Sukhdeo MVK. 2008. Parasites alter the topology of a stream food web across seasons. Oecologia 156: 613 - 624.

Hobson KA, Welch HE. 1992. Determination of trophic relationships within a high Arctic marine food web using d13C and d15N analysis. Marine Ecology Progress Series 84: 9 - 18.

Hood-Nowotny R, Knols BG. 2007. Stable isotope methods in biological and ecological studies of arthropods. Entomologia Experimentalis et Applicata 124: 3 - 16.

Huxham M, Raffaelli D, Pike A. 1995. Parasites and food web patterns. Journal of Animal Ecology 64: 168 - 176.

Kling GW, Fry BW, O'Brien J. 1992. Stable isotopes and planktonic trophic structure in arctic lakes. Ecology 73: 561 - 566.

Lafferty KD, Dobson AP, Kuris AM. 2006. Parasites dominate food web links. Proceedings of the National Academy of Sciences 103: 11211 - 11216.

Lafferty KD, et al. 2008. Parasites in food webs: the ultimate missing links. Ecology Letters 11: 533 - 546.

Layman CA, Arrington AD, Montana CG, Post DM. 2007. Can stable isotope ratios provide for community-wide measures of trophic structure? Ecology 88: 42 - 48.

Liadouze I, Febvay G, Guillaud J, Bonnot G. 1995. Effect of diet on the free amino acid pools of symbiotic and aposymbiotic pea aphids, *Acyrthosiphon pisum*. Journal of Insect Physiology 41: 33 - 40.

Livak, KJ, and Schmittgen, TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-DDCT} method. Methods 25: 402 - 408.

Macko SA, Fogel Estep ML, Engel MH, Hare PE. 1986. Kinetic fractionation of stable nitrogen isotopes during amino acid transamination. Geochimica et Cosmochimica Acta 50: 2143 - 2146.

Marcogliese DJ, Cone DK. 1997. Parasite communities as indicators of ecosystem stress. Parassitologia 39: 227 - 232.

Mariotti A, Germon JC, Hubert P, Kaiser P, Letolle R, Tardieux A, Tardieux P. 1981. Experimental determination of nitrogen kinetic isotope fractionation: some principles; illustration for the denitrification and nitrification processes. Plant and Soil 62: 413 - 430.

Martinez del Rio C, Wolf BO. 2005. Mass-balance models for animal isotopic ecology. Enfield and Plymoth: Science Publishers, Inc.

McCue MD. 2008. Endogenous and environmental factors influence the dietary fractionation of 13C and 15N in hissing cockroaches Gromphadorhina portentosa. Physiological and Biochemical Zoology 81: 14 - 24.

McCue MD, Pollock ED. 2008. Stable isotopes may provide evidence for starvation in reptiles. Rapid Communications in Mass Spectrometry 22: 2307 - 2314.

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.

Minagawa M, Wada E. 1984. Stepwise enrichment of 15-N along food chains: further evidence and the relation between delta 15-N and animal age. Geochimica et Cosmochimica Acta 48: 1135 - 1140.

Miron LL, GHerrera LG, Ramirez N, Hobson KA. 2006. Effect of diet quality on carbon and nitrogen turnover and isotopic discrimination in blood of a New World nectarivorous bat. Journal of Experimental Biology 209: 541 - 548.

Mouritsen KN, Poulin R. 2002. Parasitism, climate oscillations and the structur5e of natural communities. Oikos 97: 462 - 468.

Nakazawa T, Sakai Y, SHsieh C-h, Koitabashi T, Tayasu I, Yamamura N, Okuda N. 2010. Is the relationship between body size and trophic niche position time-invariant in a predatory fish? First stable isotope evidence. PLoS Biology 5: e9120, 9121 - 9125.

Oelbermann K, Scheu S. 2002. Stable isotope enrichment (d15N and d13C) in a generalist predator (*Pardosa lugubris,* Araneae: Lycosidae): effects of prey quality. Oecologia 130: 337 - 344.

Olive PJW, Pinnegar JK, Polunin NVC, Richards G. 2003. Isotope trophic-step fractionation: a dynamic equilibrium model. Journal of Animal Ecology 72: 608 - 617.

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.

Pang P, C., Nriagu J, O. 1977. Isotopipc variations of the nitrogen in Lake Superior. Geochimica et Cosmochimica Acta 41: 811 - 814.

Pearson SF, Levey DJ, Greenberg CH, Martinez del Rio C. 2003. Effects of elemental composition on the incorporation of dietary nitrogen and carbon isotopic signatures in an omnivorous songbird. Oecologia 135: 516 - 523.

Peterson BJ, Fry B. 1987. Stable isotopes in ecosystem studies. Annual Review of Ecology and Systematics 18: 293 - 320.

Pinnegar JK, Campbell N, Polunin NVC. 2001. Unusual stable isotope fractionation patterns observed for fish host-parasite trophic relationships. Journal of Fish Biology 59: 494 - 503.

Post DM. 2002. Using stable isotopes to estimate trophic position: models, methods, and assumptions. Ecology 83: 730 - 718.

Poulin R. 2010. Network analysis shining light on parasite ecology and diversity. Trends in Parasitology 26: 492 - 498.

Power M, Klein GM. 2004. Fish host-cestode parasite stable isotope enrichment patterns in marine, estuarine and freshwater fishes from northern Canada. Isotopes in Environmental and Health Studies 40: 257 - 266.

Prosser WA, Douglas AE. 1992. A test of the hypotheses that nitrogen is upgraded and recycled in an aphid (*Acyrthosiphon pisum*) symbiosis. Journal of Insect Physiology 38: 93 - 99.

Prosser WA, Simpson SJ, Douglas AE. 1992. How an aphid (*Acyrthosiphon pisum*) symbiosis responds to variation in dietary nitrogen. Journal of Insect Physiology 38: 301 - 307.

Rau GH, Ainley DG, Bengtson JL, Torres JJ, Hopkins TL. 1992. 14N/14N and 13C/12C in Weddell Sea birds, seals, and fish: implications for diet and trophic structure. Marine Ecology Progress Series 84: 1 - 8.

Rishavy MA, Cleland WW. 2000. 13C and 15N kinetic isotope effects on the reaction of aspartate aminotransferase and they tyrosine-225 to penylalanine mutant. Biochemistry 39: 7546 - 7551.

Robbins CT, Felicetti LA, Sponheimer M. 2005. The effect of dietary protein quality on nitrogen isotope discrimination in mammals and birds. Oecologia 144: 534 - 540.

Robbins CT, Felicetti LA, Florin ST. 2010. The impact of protein quality on stable nitrogen isotope ratio disscrimination and assimilated diet estimation. Oecologia 162: 571 - 579.

Sagers CL, Goggin FL. 2007. Isotopic enrichment in a phloem-feeding insect: influences of nutrient and water availability. Oecologia 151: 464 - 472.

Sandstrom J, Pettersson J. 1994. Amino acid composition of phloem sap and the relation to intraspecific variation in pea aphid (*Acyrthosiphon pisum*) performance. Journal of Insect Physiology 40: 947 - 955.

Sasaki T, Ishikawa H. 1993. Nitrogen recycling in the endosymbiotic system of the pea aphid, *Acyrthosiphon pisum*. Zoological Science 10: 779 - 785.

Sasaki, T, and Ishikawa, H. 1995. Production of essential amino acids from glutamate by mycetocyte symbionts of the pea aphid, *Acyrthosiphon pisum*. Journal of Insect Physiology 41: 41-46.

Sasaki T, Hayashi H, Ishikawa H. 1991. Growth and reproduction of the symbiotic and aposymbiotic pea aphids, *Acyrthosiphon pisum* maintained on artificial diets. Insect Physiology 37: 749 - 756.

Sasaki T, Fukuchi N, Ishikawa H. 1993. Amino acid flow through aphid and its symbiont: studies with 15N-labeled glutamine. Zoological Science 10: 787 - 791.

Sasaki T, Akoki T, Hayashi H, Ishikawa H. 1990. Amino acid composition of the honeydew of symbiotic and aposymbiotic pea aphids *Acyrthosiphon pisum*. Journal of Insect Physiology 36: 35 - 40.

Schumacher E, Platner C. 2009. Nutrient dynamics in a tritrophic system of ants, aphids and beans. Journal of Applied Entomology 133: 33 - 46.

Scrimgeour CM, Gordon SC, Handley LL, Woodford JAT. 1995. Trophic levels and anomalous d15N of insects on raspberry (*Rubus idaeus L.*). Isotopes in Environmental and Health Studies 31: 107 - 115.

Senn DB, Chesney EJ, Blum JD, Bank MS, Maage A, Shine JP. 2010. Stable isotope (N, C, Hg) study of methylmercury sources and trophic transfer in the northern Gulf of Mexico. Environmental Science Technology 44: 1630 - 1637.

Shigenobu S, Watanabe H, Hattori M, Sakaki Y, Ishikawa H. 2000. Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. Nature 407: 81 - 86.

Spence KO, Rosenheim JA. 2005. Isotopic enrichment in herbivorous insects: a comparative field-based study of variation. Oecologia 146: 89 - 97.

Sponheimer M, Robinson TG, Roeder BL, Passey BH, Ayliffe LK, Cerling TE, Dearing MD, Ehleringer JR. 2003. An experimental study of nitrogen flux in llamas: is 14N preferentially excreted? Journal of Archaeological Science 0: 1649 - 1655.

Stapp P, Salkeld DJ. 2009. Inferring host-parasite relationships using stable isotopes: implications for disease transmission and host specificity. Ecology 90: 3268 - 3273.

Stoker PW, O'Leary MH, Boehlein SK, Shcuster SM, Richards NGJ. 1996. Probing the mechanism of nitrogen transfer in Escherichia coli asparagine synthetase by using heavy atom isotope effects. Biochemistry 35: 3024 - 3030.

Tcherkez G. 2011. Natural 15N/14N isotope composition in C3 leaves: are enzymati isotope effects informative for predicting the 15N-abundance in key metabolites? Functional Plant Biology 38: 1-12.

Thompson RM, Mouritsen KN, Poulin R. 2005. Importance of parasites and their life cycle characteristics in determining the structure of a large marine food web. Journal of Animal Ecology 74: 77 - 85.

Trimble, ST, Sagers, SL. 2004. Differential host use in two highly specialized ant-plant associations: evidence from stable isotopes. Oecologia 74 - 82.

Tsahar E, Wolf N, Izhaki I, Arad Z, Martinez del Rio C. 2007. Dietary protein influences the rate of 15N incorporation in blood cells and plasma of Yellow-vented bulbuls (Pucnonous xanthopygos). Journal of Experimental Biology 211: 459 - 485.

Uhle ME, Macko SA, Spero HJ, Engel MH, Lea DW. 1997. Sources of carbon and nitrogen in modern planktonic foraminifera: the role of algal symbionts as determined by bulk and compound specific stable isotope analysis. Organic Geochemistry 27: 103 - 113.

Vander Zanden JM, Cabana G, Rasmussen JB. 1997. Comparing trophic position of freshwater fish calculated using stable nitrogen isotope ratios (delta N-15) and literature dietary data. Canadian Journal of Fisheries and Aquatic Sciences 54: 1142 - 1158.

Vanderklift MA, Ponsard S. 2003. Sources of variation in consumer-diet delta 15-N enrichment: a meta analysis. Oecologia 136: 169 - 182.

Velick SF, Vavra J. 1962. A kinetic and equilibrium analysis of the glutamic oxaloacetate transaminase mechanism. The Journal of Biological Chemistry 237: 2109 - 2122.

Voight CC, Matt F. 2004. Nitrogen stress causes unpredictable enrichments of 15-N in two nectar-feeding bat species. Journal of Experimental Biology 207: 1741 - 1748.

Wang J, Lin G, Huang J, Han X. 2004. Applications of stble isotopes to study plant-animal relationships in terrestrial ecosystems. Chinese Science Bulletin 49: 2339 - 2347.

Webb SC, Hedges REM, Simpson SJ. 1998. Diet quality influences the delta13C and delta15N of locusts and their biochemical components. The Journal of Experimental Biology 201: 2903 - 2911.

Weiss PM, CChen CY, Cleland WW, Cook PF. 1988. Use of primary deuterium and nitrogen-15 isotope effects to deduce the relative rates of steps in the mechanisms of alanine and glutamate dehydrogenases. Biochemistry 27: 4814-4822.

Wessel D, Flugge UI. 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Analytical Biochemistry 138: 141 - 143.

Whitehead LF, Douglas AE. 1993. Populations of the symbiotic bacteria in the parthenogenetic pea aphid (*Acyrthosiphon pisum*) symbiosis. Proceedings of the National Academy of Sciences 254: 29 - 32.

Wilkinson TL. 1998. The elimination of intracellular microorganisms from insects: an analysis of antibiotic-treatment in the pea aphid (*Acyrthosiphon pisum*). Comparative Biochemistry and Physiology 119: 871 - 881.

Wilkinson TL, Douglas AE. 1996. The impact of aposymbiosis on amino acid metabolism of pea aphids. Entomologia Experimentalis et Applicata 80: 279 - 282.

Wilkinson TL, Ishikawa H. 1999. The assimilation and allocation of nutrients by symbiotic and aposymbiotic pea aphids, *Acyrthosiphon pisum*. Entomologia Experimentalis et Applicata 91: 195 - 201.

Wilkinson TL, Ishikawa H. 2000. injection of essential amino acids substitutes for bacterial supply in aposymbiotic pea aphids (*Acyrthosiphon pisum*). Entomologia Experimentalis et Applicata 94: 58 - 91.

Wilson ACC, Sternberg LdSL, Hurley KB. 2011. Aphids alter host-plant nitrogen isotope fractionation. Proceedings of the National Academy of Sciences: 1007065108.

Wilson ACC, Dunbar HE, Davis GK, Hunter WB, Stern DL, Moran NA. 2006. A dual-genome microarray for the pea aphid, *Acyrthosiphon pisum*, and its obligate bacterial symbiont, *Buchnera aphidicola*. BMC Genomics 7: 50 - 60.

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 Molecular Biology 19: 249 - 258.

Wolf N, Carleton SA, Martinez del Rio C. 2009. Ten years of experimental animal isotopic ecology. Functional Ecology 23: 17 - 26.

Yoneyama T, Kamachi K, Yamaya T, Mae T. 1993. Fractionation of nitrogen isotopes by glutamine synthetase isolated from spinach leaves. Plant and Cell Physiology 34: 489 - 491.

Yoneyama T, Handley LL, Scrimgeour CM, Fisher DB, Raven JA. 1997. Variations of the natural abundances of nitrogen and carbon isotopes in *Triticum aestivum*, with special reference to phloe and xylem exudates. New Phytologist 137: 205 - 213.

APPENDIX I. GLOSSSARY

* **Aposymbiotic aphids** – aphids that have been cured of their endosymbiotic bacteria with antibiotic treatment or heat shock (Wilkinson and Douglas 1996).

* **Discrimination factor ($\Delta^{15}N$)** – the difference between the $d^{15}N$ signature of a consumer and the $\delta^{15}N$ value of its diet (Peterson and Fry 1987).

* **Kinetic isotopic effect** – the unequal rate of chemical reaction between two isotopes of the same element, always larger than 1 because making or breaking a bond with ^{15}N is disfavored compared to those with ^{14}N (Tcherkez 2011).

* **Isotopic fractionation** – the preferential accumulation of one isotope over the other by physical or chemical processes(Peterson and Fry 1987).

* **Nitrogen recycling** – the incorporation of waste ammonia back into amino acids (Prosser and Douglas 1992).

* **Nitrogen upgrading** – the synthesis of essential amino acids from nonessential amino acids (Prosser and Douglas 1992).

* **Thermodynamic isotopic effect** – the portion of isotopic fractionation influenced by the concentration of products and reactants, can be larger or smaller than one depending on the conditions of the reaction (Tcherkez 2011).

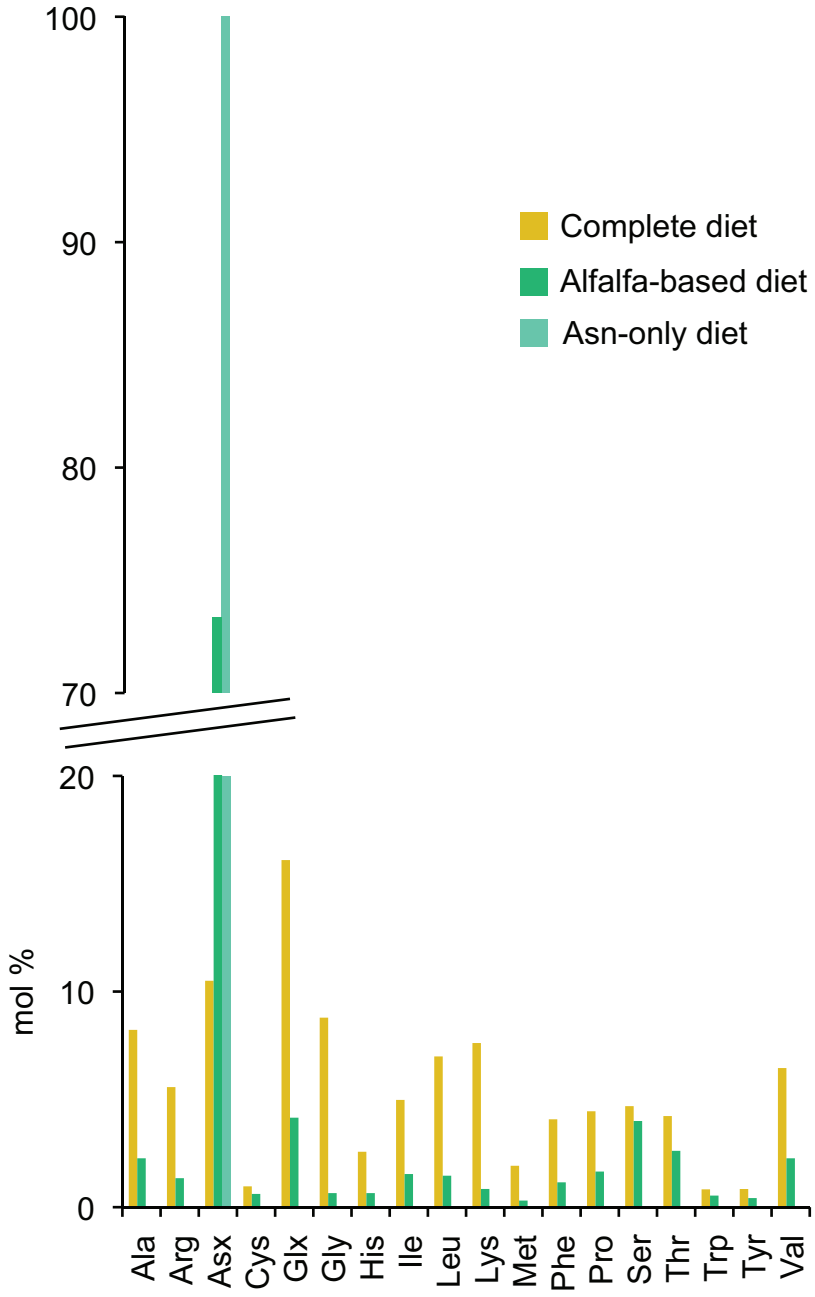


Figure S1. Amino acid profiles of artificial diets.
The amino acid profiles of the complete, alfalfa-based, and asparagine-only diets. Complete and alfalfa-based diets are derived from (Febvay et al. 1988)and (Febvay et al. 1999) respectively. The asparagine-only diet was designed specifically for this experiment.

Complete diet

L-amino acids	**mM**	**g/100 mL**
Alanine	20.06	0.1787
beta-Alanine	0.7	0.0062
Arginine	14.06	0.2449
Asparagine, H_20	19.88	0.2311
Aspartic acid	6.63	0.0882
Cysteine	2.44	0.0622
Glutamic acid	10.15	0.1493
Glutamine	30.49	0.4456
Glycine	22.19	0.1666
Histidine, HCl, H_20	6.49	0.1360
Isoleucine (allofree)	12.56	0.1648
Leucine	17.65	0.2315
Lysine, mono HCl	19.22	0.3511
Methionine	4.85	0.0724
Ornithine, mono HCl	0.56	0.0094
Phenylalanine	10.29	0.1700
Proline	11.23	0.1293
Serine	11.83	0.1243
Threonine (allofree)	10.67	0.1271
Tyrptophan	2.09	0.0427
Tyrosine	2.13	0.0386
Valine	16.29	0.1908
*Sucrose	584	19.9903

Vitamins	**mg/100mL**
p-aminobenzoic acid	10
L-ascorbic acid	100
Biotin	0.1
D-calcium pantothenate	5
Choline chloride	50
Folic acid	1
i-Inositol	42
Nicotinamide	10
Pyridoxin HCl	2.5
Riboflavin	0.5
Thiamine di-HCl	2.5

Other	**mg/100mL**
$CuSO_{4,}$ $5H_2O$	0.3004
FeCl3, $6H_2O$	2.6704
MnCl2, 4H2O	0.4133
NaCl	2.54
ZnCl2	0.83
Calcium citrate	11.4363
Cholesteryl benzoate	2.5
$MgSO_4$, $7H_2O$	242
KH2PO4	250

Alfalfa-based diet

L-amino acids	**mM**	**g/100 mL**
Alanine	5.4	0.0481
beta-Alanine	0.5	0.0045
Arginine	3.5	0.0610
Asparagine, H_20	179	2.3649
Aspartic acid	11.9	0.1584
Cysteine	1.6	0.0281
Glutamic acid	4.9	0.0721
Glutamine	5.9	0.0862
Glycine	1.7	0.0128
Histidine, HCl, H_20	1.7	0.0356
Isoleucine (allofree)	4	0.0525
Leucine	3.8	0.0498
Lysine, mono HCl	2.2	0.0402
Methionine	0.8	0.0119
Ornithine, mono HCl	0.4	0.0067
Phenylalanine	3	0.0496
Proline	4.3	0.0495
Serine	10.4	0.1093
Threonine (allofree)	6.8	0.0810
Tyrptophan	1.4	0.0286
Tyrosine	1.1	0.0199
Valine	5.9	0.0691
*Sucrose	584	19.9903

Vitamins	**mg/100mL**
p-aminobenzoic acid	10
L-ascorbic acid	100
Biotin	0.1
D-calcium pantothenate	5
Choline chloride	50
Folic acid	1
i-Inositol	42
Nicotinamide	10
Pyridoxin HCl	2.5
Riboflavin	0.5
Thiamine di-HCl	2.5

Other	**mg/100mL**
$CuSO_{4,}$ $5H_2O$	0.3004
FeCl3, $6H_2O$	2.6704
MnCl2, 4H2O	0.4133
NaCl	2.54
ZnCl2	0.83
Calcium citrate	11.4363
Cholesteryl benzoate	2.5
$MgSO_4$, $7H_2O$	242
KH2PO4	250

Asparagine-only diet

L-amino acids	mM	g/100 mL
Alanine	0	0
beta-Alanine	0	0
Arginine	0	0
Asparagine, H_20	260.2	3.44
Aspartic acid	0	0
Cysteine	0	0
Glutamic acid	0	0
Glutamine	0	0
Glycine	0	0
Histidine, HCl, H_20	0	0
Isoleucine (allofree)	0	0
Leucine	0	0
Lysine, mono HCl	0	0
Methionine	0	0
Ornithine, mono HCl	0	0
Phenylalanine	0	0
Proline	0	0
Serine	0	0
Threonine (allofree)	0	0
Tyrptophan	0	0
Tyrosine	0	0
Valine	0	0
*Sucrose	584	19.9903

Vitamins	mg/100mL
p-aminobenzoic acid	10
L-ascorbic acid	100
Biotin	0.1
D-calcium pantothena	5
Choline chloride	50
Folic acid	1
i-Inositol	42
Nicotinamide	10
Pyridoxin HCl	2.5
Riboflavin	0.5
Thiamine di-HCl	2.5

Other	mg/100mL
$CuSO_4$, $5H_2O$	0.3004
FeCl3, $6H_2O$	2.6704
MnCl2, 4H2O	0.4133
NaCl	2.54
ZnCl2	0.83
Calcium citrate	11.4363
Cholesteryl benzoate	2.5
$MgSO_4$, $7H_2O$	242
KH2PO4	250

Figure S2. Artificial diet formulae
Formulae for artificial diets based on Febvay *et al.* 1999 and Febvay *et al.* 1995

PCR program

Temperature	Time	
95	5:00	
95	0:15	40x
50	0:15	
60	0:20	
95	0:15	
60	0:15	

PCR conditions

Reagent	Concentration
Eppendorf Real MasterMix (cat. #954160203)	1x
Forward primer	0.2 μM
Reverse primer	0.2 μM
DNA template	about 2.5 ng/μL

Figure S3. PCR conditions and program for qPCR verification of *Buchnera* reduction in aposymbiotic pea aphids.
Analyses were performed in four separate runs corresponding the four genotypic

	Average age to adulthood		Average weight at adulthood		Average number of progeny	
	Symbiotic	Aposymbiotic	Symbiotic	Aposymbiotic	Symbiotic	Aposymbiotic
7A	12.23	15.48	1.458	0.702	16.0	0
A2A	11.78	15.54	1.404	0.461	18.3	0.1
5A	12.92	15.46	1.458	0.587	15.1	0
LSR1	11.68	13.88	1.202	0.659	13.9	0
average	12.15	15.09	1.381	0.602	15.8	0.03
standard error	0.28	0.40	0.06	0.05	0.93	0.03

Figure S4. Performance data for symbiotic and aposymbiotic pea aphids.
Average age to adulthood, average weight at adulthood, and average number of progeny for symbiotic and aposymbiotic pea aphids of each of the four genotypes tested. N = 10 for each genotype and the averages are given above.