2014 *SOUTHEASTERN NATURALIST* 13(4):663–672

A Molecular Analysis of Herbivory in Adults of the Invasive Bean Plataspid, *Megacopta cribraria*

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Abstract - *Megacopta cribraria* (Bean Plataspid or Kudzu Bug) is an invasive phytophagous stink bug that was first detected in North America in 2009 and has subsequently spread across the southeastern US. It is thought to feed primarily on *Pueraria montana* (Kudzu), *Glycine max* (Soybean), and a few other legumes. We investigated the diet of adult Bean Plataspids by analyzing DNA found in their guts. We extracted DNA from adult Bean Plataspid viscera and PCR-amplified and sequenced an exon of the chloroplast trnL gene. Our results indicate that Bean Plataspid adults not only feed on Kudzu and other legumes, but also on a variety of angiosperms and some conifers. We discuss possible implications of the invasive plataspid's broad diet, and propose that it might also imply that Bean Plataspid is an even more threatening pest than was previously thought.

Introduction

Although invasion of exotic species into extant communities has been commonplace historically, the recent increase in the rate of their spread associated with human activity has become a cause for concern (Lodge 1993, Roman and Darling 2007). Proposed characteristics of successful invasive species include having *r*-selected traits, high dispersal rates, single-parent reproduction, vegetative reproduction, high genetic variability, phenotypic plasticity, eurytopy, a large native range, polyphagy, and human commensalism (Lodge 1993). However, the role of some of these traits in successful invasion, including breadth of diet, is unclear. In a study comparing either established vs. failed species or invasive vs. noninvasive species, Kolar and Lodge (2001) found that breadth of diet was not a significant characteristic in determining species' success. However, in a later risk assessment for alien fishes in North America, they found that breadth of diet, when used in a classification and regression tree along with other characteristics (i.e., minimum temperature threshold and two measures of relative growth) predicted failed and successful fishes with 94% accuracy (Kolar and Lodge 2002). Also, there is evidence for a broader diet in highly invasive *Apis mellifera scutellata* Lepeletier (Africanized Honey Bee) when compared to *Apis mellifera* L. (European Honey Bee) (Villanueva and Roubik 2004), and Roman and Darling (2007) concluded that phenotypic plasticity and generalism, which if broadly defined include breadth of diet, are key characteristics of successful invasive species.

In addition to characteristics of the invading species, successful invasion may also be dependent upon propagule pressure, a function of the number of propagules

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Manuscript Editor: Richard Brown

per introduction event and the number of introduction events (Roman and Darling 2007); the biological composition of the invaded communities (Lodge 1993); and the presence of a suitable niche in the invaded territory (Zhu et al. 2012).

Megacopta cribraria (Fabricius) (Bean Plataspid or Kudzu Bug) was first detected in the US in northern Georgia in 2009 and since has spread rapidly throughout the Southeast, extending into northeastern Louisiana, central Florida, and southern Delaware as of the spring of 2014 (Center for Invasive Species and Ecosystem Health 2014). Heteropterans like Bean Plasaspid use piercing mouthparts to feed on plants by sucking their pre-digested tissue and sap (Schaeffer and Panizzi 2000). This invasive plataspid feeds on *Pueraria montana* var. *lobata* (Willd.) (Kudzu) and *Glycine max* (L.) (Soybean), and Bean Plataspid has been observed on various legumes and other angiosperms (Eger et al. 2010, Zhang et al. 2012). This species can cause a biomass reduction of nearly 25% in Kudzu (Zhang et al. 2012) and 20% in Soybeans (Roberts et al. 2013). However, to date, its diet has only been determined observationally.

In North America, Bean Plataspid appears to have come from Japan, because a single Japanese mitochondrial haplotype was found across the insect's range in Georgia during its early spread (Jenkins and Eaton 2011). More thorough mitochondrial haplotype analysis indicated that it belongs to a clade that includes *Megacopta punctatissima* (Montandon) (Japanese Common Plataspid Stinkbug) from the island of Kyushu in Japan; *M. punctatissima* and *M. cribraria* may represent local populations within the same species rather than distinct species (Hosokawa et al. 2014). Therefore, it appears that invasive Bean Plataspid in the southeastern US arrived as one or a few propagules. Consequently, propagule pressure may not be a major factor in the establishment of this pest in North America.

Knowledge of Bean Plataspid's diet could prove important in understanding the role of breadth of diet in the insect's rapid spread and might also prove beneficial in developing pest-control protocols. Therefore, in this study, we sought to confirm the broad diet of this invader using molecular analysis of gut content. We used modifications of the methodologies used to determine the gut contents of Elateridae (wireworms) (Staudacher et al. 2011) and Caelifera (grasshoppers) (Ibanez et al. 2013) in order to investigate Bean Plataspid's diet.

Methods

Collection of specimens

We collected approximately ten adult Bean Plataspids from each of seven sites in Alabama and Tennessee (Fig. 1). Additionally, we collected three individual bugs from the east wall of Propst Hall, Samford University, Birmingham AL (site S, Fig. 1). We processed bugs from site S immediately but preserved other specimens in 95% ethanol until DNA extraction.

DNA extraction

In a preliminary experiment to test the feasibility of this assay, we vortexed the individual adults from site S in 600 µL Nuclei Lysis Solution (NLS) from Wizard Genomic DNA Isolation kit (Promega, Madison, WI), then placed each adult in 600

µL NLS and homogenized the sample using a handheld Micro-Pellet Homogenizer (Wilmad LabGlass, Vineland, NJ). For each bug, we extracted DNA from each of these two samples following the Wizard Genomic DNA Isolation kit protocol so that two samples for each S adult were prepared: the one vortexed in NLS represented external DNA and the homogenized sample represented DNA from the entire bug.

Similarly, we vortexed the ten bugs from site C (Fig. 1, Table 1) in 600 µL NLS and extracted DNA from this bug-exterior sample as described above. After vortexing these ten bugs, we dissected out their viscera in Grace's Medium (Life Technologies, Grand Island, NY), combined the visceral masses, and homogenized them in 300 µL NLS. We then added an additional 300 µL NLS and extracted DNA following the Wizard protocol.

For the remaining pooled 10-bug homogenates (that is, all except S and C specimens), we extracted DNA only from the visceral masses.

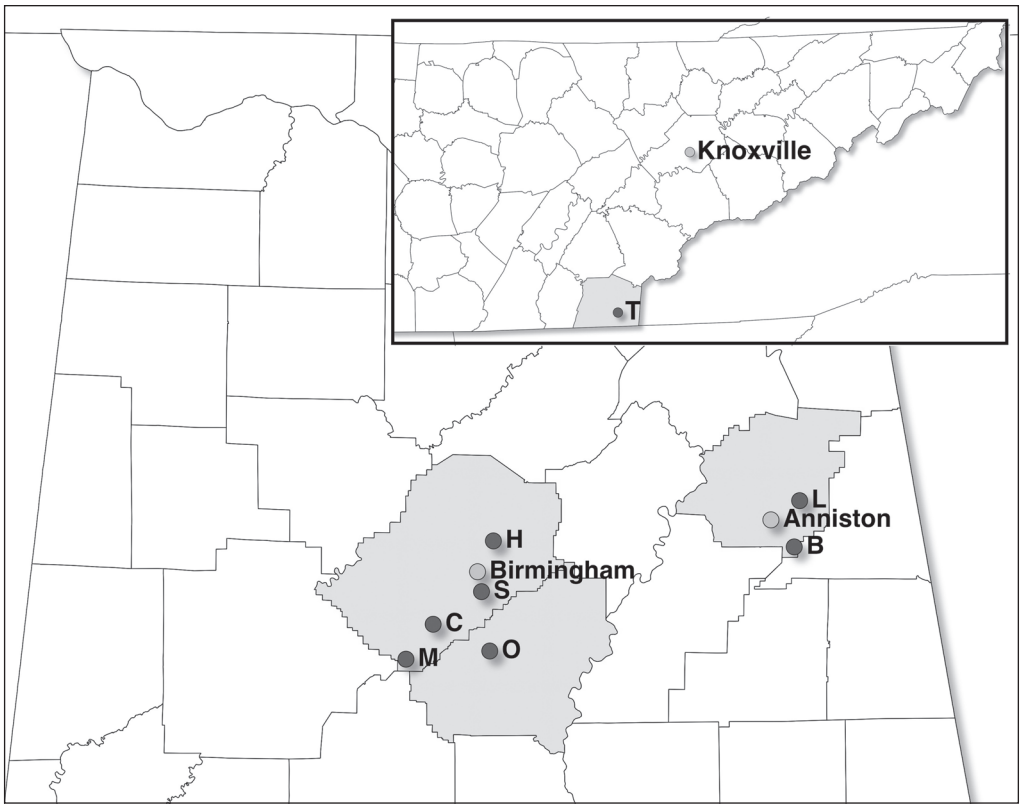

Figure 1. *Megacopta cribraria* collection sites. Collection sites of adult *M. cribraria* are indicated by letters. The large map is northern Alabama and the insert is southeastern Tennessee. Latitude, longitude, county, and state of sites: B = 33.58477°, -85.75534°, Cleburne County, AL; C = 33.37591°, -86.94879°, Jefferson County, AL; H = 33.60550°, -86.75000°, Jefferson County, AL; L = 33.71201°, -85.73590°, Cleburne County, AL; M = 33.27956°, -87.03826°, Jefferson County, AL; T = 35.06699°, -84.46177°, Polk County, TN. For sites H and L, two nearby sites were sampled, designated H1, H2, L1, and L2 in Table 1.

PCR-amplification and sequencing of plant chloroplast DNA

For the individual adults from site S, we PCR-amplified a 153-bp fragment of the ribulose-bisphosphate carboxylase (rbcL) chloroplast-DNA (ctDNA) gene using the rbcL19 (5'-AGA TTC CGC AGC CAC TGC AGC CCC TGC TTC-3') and rbcLZ1 (5'-ATG TCA CCA CAA ACA GAG ACT AAA GCA AGT-3') primers (Poinar et al. 1998). For all samples, including those from site S, we amplified a

Table 1. Plants with closest match to trnL-c/d amplified DNA from viscera of adult Bean Plataspids. Collections were made from two nearby locations at sites H and L, designated as H1, H2, L1, and L2. GenBank-database accession numbers are listed for each clone. Plants listed are BLAST matches with the closest published sequence. Also shown are the sequence lengths, percent pair-wise matches, and percent query coverage for each top BLAST match. Each BLAST match named here represents multiple hits. S2, S4, and S5 represent DNA from individual adults from site S (Fig. 1). WSC = White Sweet Clover, Sorghum = *Sorghum bicolor* (L.) Moench, Black Walnut = *Juglans nigra* L., and Loblolly Pine = *Pinus taeda* L.

Site	Collected from	# of clones	Accession #	Best match	Length	% pairwise	% query coverage
C	Unknown						
		7	KJ015980–KJ015986	Pine	518–519	99.4–100.0	100.0
		2	KJ015987–KJ015988	Red Oak	519	99.0	100.0
		8	KJ015989–KJ015996	Peanut	602–603	99.3–100.0	100.0
M	Kudzu						
		6	KF616444–KF616449	Kudzu	534	99.4–99.9	99.1
T	WSC						
		13	KF616409–KF616421	WSC	275–276	99.3–100.0	100.0
		1	KF616422	Tomato	540	99.8	100.0
		3	KF616423–KF616425	Kudzu	534	99.2–99.4	99.1
H1	Kudzu						
		8	KF616450–KF616457	Kudzu	532–534	99.1–100.0	99.1
H2	Unknown monocot						
		5	KF616426–KF616430	Sorghum	483	99.6–100.0	100.0
		1	KF616431	WSC	275	100.0	100.0
		1	KF616432	Lettuce	475	99.8	100.0
		1	KF616433	Kudzu	534	99.1	99.1
L1	Unknown						
		8	KF616434–KF616441	Lespedeza	555	99.3–99.8	99.2
		2	KF616442–KF616443	Kudzu	534	99.4–99.8	99.1
L2	Unknown						
		8	KF616400–KF616407	Kudzu	534	99.4–100.0	99.1
		1	KF616408	Sweet Gum	532	99.8	100.0
B	Kudzu						
		9	KF616458–KF616466	Kudzu	534	99.4–100.0	99.1
		1	KF616467	Red Oak	519	100.0	100.0
O	Unknown						
		6	KF616391–KF616396	Lespedeza	555	99.1–99.6	98.2
		2	KF616397–KF616398	Sweet Gum	532	99.8	100.0
		1	KF616399	Black Walnut	560	98.6	100.0

trnL (UAA) exon using the trnL-c (5' CGA AAT CGG TAG ACG CTA CG 3') and trnL-d (5' GGG GAT AGA GGG ACT TGA AC 3') primers (Taberlet et al. 1991), which amplify a fragment that is generally over 600 bp in length but which may be as small as approximately 300 bp long in Bean Plataspid (Table 1). PCR-cycler steps were 5 min at 94 °C, 33 x (1 min at 94 °C, 1 min at 57 C°, 1 min at 72 C°), 10 min at 72 °C. We used GoTaq HotStart Green or Colorless master mix (Promega) for amplification and a Miniopticon RL-TM PCR System (Bio-Rad; Hercules, CA). We purified trnL amplicons using a 2% EGel Size-Select Gel (Life Technologies) and cloned fragments using the TOPO-TA Cloning Kit with the pCR 2.1 Vector and One-Shot Chemical Transformation (Life Technologies). M13-F and M13-R colony sequencing was performed by GeneWiz (South Plainfield, NJ).

DNA sequence analysis

We constructed consensus-trnL sequences from the M13-F- and M13-R-generated sequences for most clones, and searched for similar sequences using Geneious Pro software version 6 or 7 (created by Biomatters, Auckland, NZ, and available at http://www.geneious.com) which searches multiple sequence-databanks. For those sequences where a complete M13-F/R consensus was not determined, we used the single M13-F- or M13-R-generated trnL sequence to find the closest matches. We used Geneious Pro software for all analysis and storage of sequence data and submitted sequences to GenBank.

Results

In the preliminary experiment to determine if DNA analysis could detect ingested plant material and if this DNA could be used to determine the diet of adult Bean Plataspid, although PCR-amplification of extracted external plant DNA showed very weak rbcL-bands, DNA from the whole-bug homogenates showed strong bands of the predicted rbcL-fragment size (Fig. 2). However, sequencing of these short fragments did not yield sequences long enough to determine plant type.

Table 1, continued.

Site	Collected from	# of clones	Accession #	Best match	Length	% pairwise	% query coverage
S2	Building wall						
		8	KF616468–KF616475	Loblolly Pine	522	99.4–99.8	100.0
		1	KF616476	Kudzu	534	100.0	99.1
S4	Building wall						
		8	KF616477–KF616484	Black Medic	387	99.2–100.0	100.0
		1	KF616485	Lettuce	476	98.9	100.0
		3	KF616486–KF616488	Loblolly Pine	522	99.6–99.8	100.0
		1	KF616489	Red Oak	476	99.6	100.0
S5	Building wall						
		3	KF616490–KF616492	Loblolly Pine	522	99.4–99.6	100.0
		3	KF616493–KF616495	Walnut	560	98.8	100.0
		2	KF616496–KF616497	Red Oak	519	99.8	100.0

Consequently, we used trnL PCR amplification in subsequent analyses. In adults collected from site C (Fig. 1), plant DNA of the appropriate size (300–600 bp) was not detected after trnL-amplification using the bug-exterior extract (Fig. 3). Also,

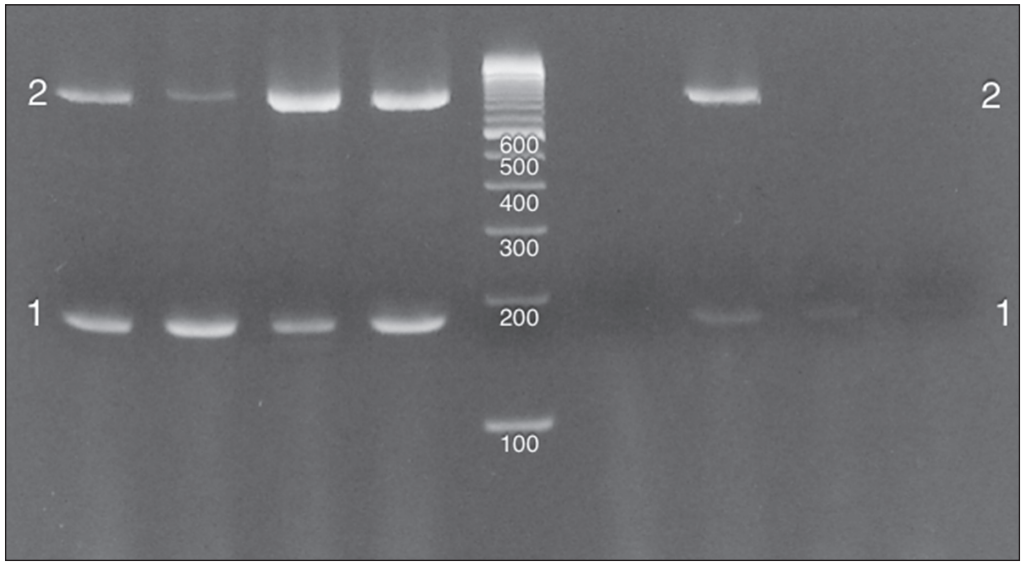

Figure 2. Agarose gel electrophoresis of amplified rbcL ctDNA from Bean Plataspid surface and from the whole bug. Marker DNA in the center lane was TrackIt (Life Technologies) with the fragment lengths in bp indicated. Whole-bug DNA bands are in the first four lanes. External DNA bands are in the last four lanes. Visible bands at 1 are approximately of the expected size. The identity of the larger bands at 2 was not determined.

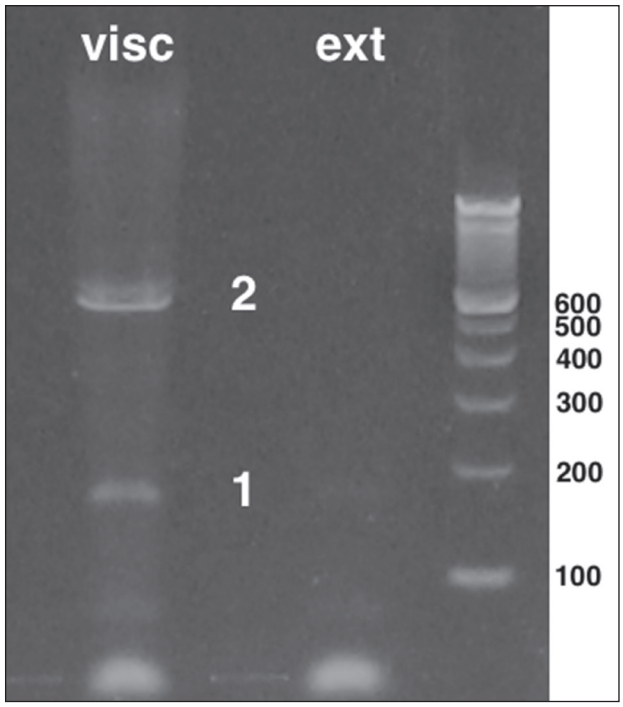

Figure 3. Agarose gel electrophoresis of amplified trnL ctDNA from Bean Plataspid's surface and from bug viscera. ext lane = DNA from exterior of Bean Platasids, visc lane = DNA from Bean Plataspid viscera.

gel-purification and sequencing of the small amplicon band that did appear (ext lane, band 1 in Fig. 3) yielded no plant sequences. These two results demonstrate the lack of detectable plant DNA on the exterior surface of these bugs. Although sequencing of the small amplicon from the viscera of these same bugs also yielded no plant DNA (visc lane, band 1 in Fig. 3), BLAST searches of DNA from the two large viscera bands (visc lane, bands at 1 in Fig. 3) showed the presence of *Pinus* sp. (pine), *Quercus rubra* L. (Red Oak), and (*Arachis hypogaea* L. (Peanut) trnL DNA (site C samples; Table 1), demonstrating the feasibility of this protocol to describe Bean Plataspid diets.

PCR-amplification of trnL fragments, sequencing, and BLAST searches of cloned trnL sequences from all sites demonstrated the presence of DNA from various vascular plants (Table 1), many of which were found in the immediate vicinity of the collection site. A variety of monocot, dicot, and gymnosperm taxa were identified in the analyses: the legumes Kudzu, *Medicago lupulina* L. (Black Medic), *Melilotus alba* Medik. (White Sweet Clover), *Lespedeza* sp. (lespedeza), and Peanut, the non-leguminous dicots Red Oak, *Juglans* sp. (walnut), *Liquidambar styraciflua* L. (Sweet Gum), *Solanum lycopersicum* L. (Tomato), and *Lactuca sativa* L. (Lettuce), the monocot *Sorghum bicolor* (L.) (Sorghum), and at least one species of pine (Table 1). We detected more than one plant species in the three individual analyses carried out on each bug (site S). Although Kudzu is abundant approximately 10 m from the S-collection site, only one of 29 cloned trnL fragments from the three adult Bean Plataspids matched Kudzu. Also, all adults from site C (Fig. 1) were collected from Kudzu, but we found no Kudzu in their viscera, only pine, Red Oak, and Peanut (Table 1). However, we also collected bugs from sites M and B from Kudzu and these samples showed only or predominantly Kudzu in their viscera (Table 1).

Discussion

In this report, we have demonstrated the effectiveness of using DNA isolation, trnL PCR-amplification, cloning, and sequencing to identify the plant diet of adult Bean Plataspids. To date, this invasive plataspid's diet has been assumed to be primarily Kudzu, Soybean, and a few other legumes. We have confirmed observational evidence suggesting that adults feed on a broader range of plants. Because very little plant DNA was amplified from site S external DNA samples using the rbcL primers (Fig. 2) and because no plant DNA was amplified from site C external DNA samples using the trnL primers (those we used for plant variety determination), the amplified plant DNA identified in our study appears to have come from ingested plant material. During collection, we observed Bean Plataspid eggs and nymphs only on Kudzu and expected to find this invasive legume to be the adults' primary, if not sole, diet. However, our evidence indicates that herbivory by Bean Plataspid adults may not be as specialized as heretofore assumed. Contrary to the conclusion that Bean Plataspid adults frequently observed on various plants simply represent resting bugs (Melacon 2013), it appears that they may instead represent non-discriminant herbivory during migration. However, another possible explanation for

the observed diversity of gut plant-material could be foraging of surface particles as is known to occur when plataspid nymphs acquire symbiotic bacteria (Fukatsu and Hosokawa 2002). It is possible that pollen or other plant material, which may not be a primary nutrition source, is ingested in this manner.

Bean Plataspid is reported to prefer Kudzu and Soybean as its reproductive hosts (Zhang et al. 2012), but our results indicate a broader dietary host range for adults. A diverse diet for Bean Plataspid adults could be a contributing factor in the rapid spread of this bug in the southeastern US. Although it seems clear that other dispersal factors such as hitchhiking are of major importance (Halbert and Eger 2010), it is possible that dietary diversity could be a characteristic of importance in the spread of Bean Plataspid as it feeds during migration to the preferred reproductive host.

Surprisingly, in our study, we found very little Kudzu in the guts of Bean Plataspids collected from site S, which was approximately 10 m from Kudzu (Table 1). Even more surprising was the gut content of bugs from site B (Table 1) that were collected on Kudzu, but for which our analysis revealed no Kudzu in the clones analyzed. This finding may indicate that the choice of other plants is not simply due to the lack of Kudzu. There are several possible explanations for the lack of Kudzu in the guts of bugs from sites S and B. The first is PCR-primer bias; that is, our primers might more readily anneal with non-Kudzu template DNA. However, we wish to point out that six of the 12 total trnL-generated amplicons (represented by the first 11 sites plus the 3 adults from site S in Table 1) showed the presence of Kudzu even when other plant DNA was detected in the viscera. The second possible explanation for the lack of Kudzu in the gut samples is that our sampling at each site was limited. For each site, a limited number of clones were established, ranging from six to 29 (Table 1). Our results suggest that Kudzu is rare but not necessarily absent from sampled bugs from these two sites. The third possible explanation for the lack of Kudzu DNA in some of our samples is that the bugs we collected might have represented adults that had been visiting other sites and had only recently returned to, or migrated to this particular Kudzu site. If so, migrating adults might seem to have a broader diet. Any tendency toward a broader or non-Kudzu dietary preference in migratory adults could prove to be important in the rate of spread of Bean Pataspid.

In our next project, we will study dietary preferences of all Bean Pataspid stages, including migrating adults. We will also use more complete ctDNA-sequencing methodology (multiple loci, massively parallel sequencing) to conduct more thorough gut-content assays to increase our knowledge of the plant species eaten by each insect. Our goal will be to further define the diet of this invasive stink bug in order to elucidate the role of breadth of diet in the spread of Bean Plataspid.

Our data suggests that the diet of Bean Plataspid adults is not limited to a few hosts but may consist of various available plants, which also suggests that the potential ecological impact of this invasive species might be even greater than originally imagined.

Acknowledgments

This research was made possible by the Department of Biological and Environmental Sciences at Samford University and NSF REU Award Number: 1327466 (Interdisciplinary research in a diverse Appalachian ridge and valley ecosystem). We also wish to thank M. Fincher and K. Bakkegard for Bean Plataspid specimens; M. Fincher, A.A. Fodor, and G. Gentry for reviewing this manuscript; and E. Hayes, M.E. Butts, and Samford BIOL 334 students for their technical assistance.

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