

Limitations of stable carbon isotope analysis for determining natal host origins of tobacco budworm, *Heliothis virescens*

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Abstract

Differences in the stable carbon isotope ratios of plants utilizing the C3 vs. C4 photosynthetic pathway have been used to broadly identify the natal host origins of herbivorous insects. This study explored whether adequate variation exists between the carbon isotope ratios of different C3 plants in the host range of *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) to enable accurate identification of natal host-plant species. Isotope ratio mass spectrometry (IRMS) analysis of $^{13}\text{C}/^{12}\text{C}$ ratios of moths reared on four crop plant species [*Gossypium hirsutum* (L.), *Nicotiana tabacum* L., *Glycine max* (L.) Merrill, and *Arachis hypogaea* L.] and two common weed species [*Geranium carolinianum* L. and *Linaria canadensis* (L.) Chaz.] revealed a range of $\delta^{13}\text{C}$ values within that expected for plants utilizing the C3 photosynthetic pathway. Analysis of vegetative and reproductive tissues from the plants utilized in the study resulted in statistically different $\delta^{13}\text{C}$ values for some plant species; nevertheless, the range of $\delta^{13}\text{C}$ values observed for many plant species overlapped. Significant differences in mean $\delta^{13}\text{C}$ values were detected between groups of moths reared on different host-plant species, but there was no significant correlation between the $\delta^{13}\text{C}$ values of moths vs. the $\delta^{13}\text{C}$ value of plant tissue on which they were reared. Feral tobacco budworm moths collected over 3 years were found to have carbon isotope ratios consistent with those having fed on C3 plants, confirming little utilization of C4 plant species by the insect. Results demonstrate that within the range of C3 host plants tested, carbon isotope signatures are not sufficiently unique to enable a reliable determination of natal origin of feral tobacco budworm with current IRMS technology.

Introduction

Quantifying the seasonal utilization of host-plant species by the tobacco budworm, *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae), is currently an important area of investigation in cotton growing regions of the USA. Naturally occurring stable isotopes of carbon have been used as biological markers to determine movement patterns of migratory animals (Hobson, 1999) and to resolve the natal host origins of phytophagous insects (Tallamy & Pesek, 1996; Gould et al., 2002).

Two stable carbon isotopes exist in the environment, and they often occur in predictable ranges in particular tissue types (Peterson & Fry, 1987; Hobson, 1999). The overwhelming majority of carbon (ca. 98.98%) exists as

^{12}C while only a small proportion (ca. 1.11%) exists as ^{13}C . Variation in the ratio of ^{13}C to ^{12}C can be measured via isotope ratio mass spectrometry (IRMS) and is reported as $\delta^{13}\text{C}$ or parts per thousand deviation from a recognized standard (Dawson & Brooks, 2001; Pate, 2001). For example, plants utilizing the C3 and C4 photosynthetic pathways have distinct non-overlapping ranges of stable carbon isotope ratios (Smith & Epstein, 1971). Carbon isotopes present in a plant are acquired by a herbivore when it feeds upon the plant's tissues. Using carbon isotope ratios to determine the proportion of an individual food source consumed by a single organism that utilizes multiple food sources can be problematic (Phillips & Gregg, 2003); however, individual tobacco budworm larvae typically develop on a single host plant. Because carbon isotope ratios remain relatively unchanged as carbon moves from plant to consumer, the carbon isotope composition of the herbivore

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may be used to link it to its host plant (Post, 2002). The ability to resolve the natal host origin of an organism using carbon signatures is dependent on the existence of different carbon isotope ratios in the host-plant species. This technology has been utilized to broadly identify the natal host origin (C3 vs. C4 host plants) of rootworms (Chrysomelidae) (Tallamy & Pesek, 1996), field-collected corn earworm, *Helicoverpa zea* (Gould et al., 2002), and European corn borer, *Ostrinia nubilalis* (Bontemps et al., 2004); for a review, see Hood-Nowotny & Knols (2007).

While the existence of substantial differences in the stable carbon isotope ratios of C3 vs. C4 plants have been well-established, plant species within a single photosynthetic pathway differ to a much more limited extent, and it is unknown whether the insects that feed on them can be distinguished on the basis of carbon isotope composition. Even if host-plant species can be discerned by the isotopic composition of their tissue, this does not guarantee that the same isotope ratios will be present in herbivores (Gannes et al., 1997). The range of reported $\delta^{13}\text{C}$ values for plants utilizing the C3 photosynthetic pathway is about 15 parts per thousand (-20‰ to -35‰) (Bender, 1971; Cerling et al., 1997), and it is possible that consistent differences in carbon isotope ratios exist between some C3 plant species. Water-use efficiency is an important factor determining the amount of ^{13}C that will be present in plant tissues. Plants with greater water use efficiency are more depleted in ^{13}C (Farquhar & Richards, 1984), and variation in water-use efficiency between plant species in the host range of the tobacco budworm could provide a mechanism leading to discriminating differences in carbon isotope ratios.

Previous investigations of host utilization by tobacco budworm employed costly larval or pupal sampling, and could only roughly estimate adult production from a given host (Neunzig, 1969; Schneider, 2003; Abney et al., 2007). Alternatively, the use of stable carbon isotopes as biological markers could provide relatively low-cost, high-resolution data regarding the host-use patterns of this economically important insect. Before such data can be generated, the ability to differentiate between host-plant species and insects reared on them on the basis of isotopic variation must be validated. We initiated this study to evaluate the stable carbon isotope composition of six important C3 host-plant species and tobacco budworm moths reared on them. Because of the relatively narrow range of carbon isotope ratios in C3 plants, we did not expect to be able to differentiate between all of the plant species tested. Nevertheless, for this technique to prove useful for scientists developing insecticide-resistance management strategies, it would only need to be able to reliably differentiate one of the major hosts or group of hosts from all the others. We

determined whether any of the plants tested could be reliably separated from the other species based on their carbon isotope ratios. We also examined the relationship between carbon isotope ratios in host plants and in tobacco budworm to determine if the natal host origin of moths could be resolved. The results of these explorations will provide a valuable reference for agricultural entomologists seeking to utilize carbon isotopes in ecological studies.

Materials and methods

Insects

Heliothis virescens used in all experiments reported here were obtained from a laboratory colony (strain YDK) maintained at North Carolina State University (NCSU), Raleigh, NC, USA.

Controlled rearing study: greenhouse experiment

A preliminary greenhouse study was conducted to determine if differences in carbon isotope ratios between tobacco budworm adults reared on cotton and those reared on tobacco were sufficient to warrant further investigation. Cotton variety DP 50 and flue-cured tobacco variety K-326 were planted into commercial media (Metromix 200®; The Scotts Company, Marysville, OH, USA) in 15-cm diameter sterilized clay pots in a greenhouse at NCSU on 14 February 2001. Plants were hand watered daily until introduction of tobacco budworm larvae. Three neonate tobacco budworms were placed on the upper one-third of each of 10 cotton and tobacco plants on 17 May. Immediately following infestation, pots containing the plants were transferred to water-filled moats constructed on greenhouse benches. Moats minimized the chance of disturbing larvae during overhead watering and prevented larval movement between plants. The larvae were allowed to feed uninterrupted until just prior to pupation, at which time final instars were removed from plants and transported to the laboratory to complete development on excised tissue from the respective host plant. A total of seven and 11 moths were reared on cotton and tobacco, respectively. Adults were frozen shortly after eclosion and stored at $-5\text{ }^{\circ}\text{C}$ until preparation for analysis of carbon isotope composition. All tissue samples were analyzed at the Stable Isotope Mass Spectrometry Facility in the Department of Soil Science located at NC State University. Insect samples were prepared for analysis by removing the right forewing of each moth and placing it in a tin combustion capsule. Wing tissue has been successfully utilized in previous studies to resolve the natal host origins of phytophagous insects (Tallamy & Pesek, 1996; Gould et al., 2002). Different tissue types can fractionate and sequester ^{12}C and ^{13}C atoms in slightly different ratios; by

limiting our investigation to the carbon signature of the forewing, we expected our results to be directly comparable from individual to individual.

Post et al. (2007) report that variation in lipid content can significantly bias the results of stable carbon isotope analyses. They further conclude that lipids should be accounted for in analysis of terrestrial animal tissue when the lipid content is high (>10%). A C:N ratio of around 4 in terrestrial animals indicates low-lipid content and suggests that lipids should not significantly affect the results of stable carbon isotope analyses (Post et al., 2007). The mean C:N ratio of forewings from *H. virescens* reared in the greenhouse for this study was 4.13 (SD = 0.21). Because we estimated lipid content in wing tissue to be very low, and because there was very little variation in lipid content in the population tested, no measures were taken to account for lipid content in this study.

The samples were analyzed using a CE Elantech NA 2500 elemental analyzer (Lakewood, NJ, USA) coupled to a Thermo Finnigan DELTA plus mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) via a Conflo II open split interface (Thermo Fisher Scientific Inc.) at the stable isotope facility at NCSU. Student's t-test was used to compare sample means.

Host-plant tissue collection

Tests were conducted to determine whether several common plant hosts of the tobacco budworm in North Carolina could be distinguished on the basis of their carbon isotope composition. Cotton (*Gossypium hirsutum*), soybean (*Glycine max*), peanut (*Arachis hypogea*), tobacco (*Nicotiana tabacum*), old field toadflax (*Linaria canadensis*), and Carolina geranium (*Geranium carolinianum*) were collected at various developmental stages and multiple locations in North Carolina. Cotton was obtained from Wilson and Johnston Counties on 21 July and from Martin County on 29 July and 20 August. Soybean and peanut samples were acquired from Johnston County on 2 and 17 September and 2 and 23 September, respectively. Tobacco was collected on 24 June and 29 July from Martin County and on 1 and 29 July from Johnston County. Old field toadflax was collected from sites in Edgecombe and Lenoir Counties on 30 April and from Johnston County on 1 May. Carolina geranium was obtained from Edgecombe and Lenoir Counties on 30 April. Although old field toadflax and Carolina geranium may be temporally isolated from cotton, soybean, and peanut, these two species do occur simultaneously with tobacco early in the growing season. The period in which tobacco, cotton, soybean, and peanut serve as suitable hosts for tobacco budworm development overlaps significantly in North Carolina (Abney et al., 2007). The temporal variation in the collection of plant

tissue in this study enabled analysis of carbon signatures in different phenological stages of host plants; this does not imply that the phenological stages of these hosts suitable for tobacco budworm development are temporally isolated. On each sample date at each location, tissue was collected from 10 individual plants of a single species and combined in a resealable plastic storage bag. Tissue samples were transported to NCSU and stored at 0 °C until processed for analysis. Plant samples were dried in a laboratory oven at 43 °C for 8 days and then ground to a fine powder. Approximately 1.4 mg of homogenized ground plant tissue was placed in a tin combustion capsule for each plant sample examined. Plant tissue was analyzed using the same technique described earlier for moth wings. The data analysis for plant tissue composition was generated using SAS general linear model (GLM) procedure (SAS Institute Inc., 2000–2004).

Controlled rearing study: field experiment

Within a single plant species, carbon isotope composition may be influenced by a number of factors including photosynthetic rate, moisture availability, and the plant structure tested. For isotope analysis to provide useful insight into natal host origin, the ratio of stable carbon isotopes conferred to tobacco budworms feeding on a single host-plant species must remain consistent over a wide geographic region within a referenced period of time. A field study was initiated to test the hypothesis that tobacco budworms reared on a particular host species would have similar stable carbon isotope composition regardless of location of origin or plant structure fed upon. Tobacco budworm larvae were reared on four species of crop hosts (tobacco, cotton, soybean, and peanut) and two weed host species (old field toad flax and Carolina geranium) in 2003.

Tobacco budworm larvae were reared in the laboratory on field-collected old field toad flax and Carolina geranium from Johnston and Wake Counties, NC, respectively. Plants were cut at ground level, placed in distilled water, and transported to the laboratory where the foliage was rinsed in tap water and allowed to air dry. Plants were then placed singly into no. 50 water pics (Aquapic®; Syndicates Sales Inc., Kokomo, IN, USA) containing distilled water. Neonate tobacco budworms were placed individually on plants using a size 0 camel hair paint brush. The plants were secured via water pics in a polystyrene foam base and placed in a 20 × 20 × 40 cm Plexiglas® (Altuglas International, Philadelphia, PA, USA) box with two 400 cm² screen-covered openings for ventilation. Larvae were transferred to fresh plant material as needed, and distilled water was added to water pics as necessary. Prepupae were collected from the plants and transferred to 60 × 15 mm plastic Petri

dishes for pupation. Following adult eclosion, wings from individual insects were prepared as previously described for greenhouse-reared insects and subjected to IRMS analysis ($n = 8$ per host species).

Tobacco, cotton, soybean, and peanuts were planted in small test plots at the Central Crops Research Station in Johnston County, NC, and cotton and tobacco were planted in small plots on a private research farm in Martin County, NC. Sleeve-type field cages were constructed from polypropylene floating row cover (Gardens Alive®, Lawrenceburg, IN, USA) and placed over individual plants of each host species; neonates (strain YDK) were placed directly on plant tissue within the cages using a size 0 camel hair paint brush. Tobacco budworms were reared on tobacco in early and late July and on cotton in mid-July and mid-August; the insects were reared on soybean and peanut in late August. Cages were sealed at the bottom by securing the polypropylene material around a stalk or branch with plastic-coated twist wire. The tops of cages were folded over twice and held fast with two no. 3 gem clips. Cages were monitored twice each week, and larvae were transported to the laboratory just prior to pupation. Pupae were held in individually labeled 60×15 mm plastic Petri dishes until adult eclosion at which time moths were frozen and then prepared for isotope analysis as previously described (cotton reared: $n = 37$, tobacco reared: $n = 40$, peanut reared: $n = 9$, and soybean reared: $n = 6$). The carbon isotope composition data for adult wings were analyzed using SAS GLM and MIXED procedures (SAS Institute Inc., 2000–2004).

The relationship between carbon isotope ratios in host plants and in tobacco budworm adults was also studied. Pearson's correlation coefficient was generated from SAS correlation analyses (PROC CORR; SAS Institute Inc., 2000–2004).

Feral insect collection

Tobacco budworm adults were evaluated to determine whether the ranges of carbon isotope ratios found in feral populations were consistent with the host plants tested and/or moths from controlled rearing studies. Male tobacco budworm moths were collected in Harstack-type pheromone traps in North Carolina each summer from 2001 to 2003 in the central coastal plain counties of Greene, Pitt, Edgecombe, and Wilson. Moths were removed from the traps twice weekly and transported to NCSU where they were stored at 0°C . A random subsample of 10 moths per year for each year of the study was collected and prepared for analysis of stable carbon isotope composition. Feral moths from all 3 years were combined and included as 'unknown' in statistical analyses of moths from known hosts.

Results

Controlled rearing study: greenhouse experiment

The $\delta^{13}\text{C}$ values of tobacco budworm moths reared on tobacco in the greenhouse differed and did not overlap with the $\delta^{13}\text{C}$ values of moths reared on cotton. The $\delta^{13}\text{C}$ value of moths reared on tobacco (mean \pm SE $\delta^{13}\text{C} = -29.89\text{‰} \pm 0.18$) was significantly lower ($t = 2.47$, d.f. = 6, and $P < 0.05$) than value for moths reared on cotton (mean \pm SE $\delta^{13}\text{C} = -26.21\text{‰} \pm 0.14$).

Host-plant tissue analysis

The $\delta^{13}\text{C}$ values of the six host-plant species tested were consistent with those expected for plants utilizing the C3 photosynthetic pathway (Griffith, 1991). Significant differences in $\delta^{13}\text{C}$ values were observed for tissue from different plant species ($F = 6.33$, $P = 0.0042$); however, there was considerable overlap in the range of $\delta^{13}\text{C}$ values between species (Table 1).

Table 1 Mean and range of $\delta^{13}\text{C}$ values for four crop and two weed hosts of *Heliothis virescens* collected at multiple locations in North Carolina and dates in 2003

| Plant tested | Mean $\delta^{13}\text{C}$ (SEM) ¹ | $\delta^{13}\text{C}$ range (‰) |
|-------------------|-----------------------------------------------|---------------------------------|
| Toadflax | -29.30 (0.06)a | -29.5 to -29.1 |
| Carolina geranium | -28.65 (0.45)a | -29.1 to -28.2 |
| Cotton | -28.35 (0.45)ab | -29.1 to -27.1 |
| Peanut | -27.35 (0.35)bc | -27.7 to -26.9 |
| Soybean | -27.20 (0.90)bc | -28.1 to -26.3 |
| Tobacco | -26.92 (0.15)c | -27.4 to -26.5 |

¹Means within a column followed by the same letter are not significantly different ($P > 0.05$).

Controlled rearing study: field experiment

IRMS analysis of wings from laboratory- and field-reared tobacco budworm revealed $\delta^{13}\text{C}$ values that were within the range expected for insects feeding on C3 plants. Insects reared on old field toadflax, Carolina geranium, or peanut were significantly more depleted in ^{13}C than were insects reared on cotton, soybean, or tobacco ($F = 19.27$, $P = 0.0001$). There were no other significant differences between insects reared on different known hosts. The data collected from insects reared in the field on cotton and tobacco did not corroborate the findings of greenhouse experiments. While insects reared in the field on cotton had a $\delta^{13}\text{C}$ value (mean \pm SE $\delta^{13}\text{C} = -26.55\text{‰} \pm 0.17$) similar to those reared in the greenhouse, *H. virescens* reared on tobacco in the field were consistently more enriched in ^{13}C (mean \pm SE $\delta^{13}\text{C} = -26.16\text{‰} \pm 0.13$) than those reared on tobacco in the greenhouse. Additionally,

Table 2 Mean and range of $\delta^{13}\text{C}$ values for wings of *Heliothis virescens* adults reared on four crop hosts in the field and two weed hosts in the laboratory in 2003

| Host plant | Mean $\delta^{13}\text{C}$ (SEM) ¹ | $\delta^{13}\text{C}$ range (‰) |
|-------------------|-----------------------------------------------|---------------------------------|
| Toadflax | -28.07 (0.32)c | -28.65 to -27.60 |
| Carolina geranium | -28.59 (0.32)c | -29.26 to -28.19 |
| Cotton | -26.55 (0.15)b | -28.88 to -25.16 |
| Peanut | -27.85 (0.30)c | -28.96 to -25.76 |
| Soybean | -26.47 (0.37)ab | -28.09 to -25.12 |
| Tobacco | -26.16 (0.14)ab | -27.46 to -24.35 |
| Unknown | -25.76 (0.31)a | -29.8 to -22.20 |

¹Means within a column followed by the same letter are not significantly different ($P > 0.05$).

the range of mean $\delta^{13}\text{C}$ values obtained from moths reared on several of the host plants including cotton and tobacco was shown to overlap (Table 2). Host plant*tissue type and host plant*tissue type*location interactions were also significant ($F = 25.87$, $P = 0.001$ and $F = 8.17$, $P = 0.001$, respectively). (The $\delta^{13}\text{C}$ values of moths reared on vegetative and reproductive cotton and tobacco at two locations are given in Table 3). There was no significant correlation between the mean $\delta^{13}\text{C}$ value of moths reared on specific host plants vs. the mean $\delta^{13}\text{C}$ value of host-plant tissue (Pearson's correlation coefficient = 0.64, d.f. = 4, and $\alpha = 0.05$).

Feral insect analysis

The $\delta^{13}\text{C}$ values observed for feral *H. virescens* adults (Table 2) were similar to those seen in the controlled rearing experiment conducted in the field and were consistent with larval development on C3 hosts. No significant variation in carbon isotope composition was observed between years ($F = 2.88$, $P = 0.07$). The mean $\delta^{13}\text{C}$ value for feral moths combined from all 3 years was significantly different than moths reared on toadflax, Carolina geranium, cotton, and peanut. There was no difference between the mean $\delta^{13}\text{C}$ values of feral moths and moths reared on tobacco and soybean (Table 2).

Discussion

Preliminary greenhouse studies indicated that IRMS might provide a useful tool for elucidating the natal host origin of tobacco budworm. However, further investigation revealed that environmental variation is apparently too great to enable the use of stable carbon isotope methods as a means for distinguishing moths originating from larvae that fed on different C3 host-plant species. Analysis of tissue from a set of plant species collected in the field as well as analysis of tobacco budworm moths reared on those plants resulted in a wide range of $\delta^{13}\text{C}$ values for tissue from individual plants within a species and individual insects reared on the same host-plant species. Additionally, a range of overlapping $\delta^{13}\text{C}$ values was seen for plant tissue from different species as well as insects reared on different species. Significant host plant*tissue type and host plant*location*tissue type interactions further demonstrate the lack of homogeneity of carbon isotope ratios in insects reared on vegetative or reproductive structures of the same host species at different locations. To accurately discriminate between populations, the difference between the two population means must be at least twice as large as the standard deviation for the combined population. Although significant variation was observed between mean $\delta^{13}\text{C}$ values for different plant species and insects reared on different host species, the differences between population means were not sufficiently large in either case to permit reliable determination of plant species or host-plant species.

Variation observed in the amount of ^{13}C present in a specific tissue type among plants of a single species and the resulting variation in the herbivore is likely due in part to differences in moisture availability as the plants grow. Plants growing in conditions of adequate moisture are expected to have higher photosynthetic rates, greater transpiration, and greater leaf conductance than plants of the same species growing under moisture stress (Farquhar et al., 1989). This in turn leads to greater abundance of ^{13}C in plants grown under water-deficit conditions compared

Table 3 $\delta^{13}\text{C}$ values for plant tissue type and mean $\delta^{13}\text{C}$ values for *Heliothis virescens* adults reared on cotton reproductive tissue and tobacco vegetative and reproductive tissue at two locations in North Carolina in 2003

| Host plant | Location | Tissue type | Plant tissue $\delta^{13}\text{C}$ | Mean wing $\delta^{13}\text{C}$ (SD) |
|------------|-----------------|--------------|------------------------------------|--------------------------------------|
| Cotton | Johnston County | Reproductive | -28.9 | -25.9 (0.63) |
| Cotton | Martin County | Reproductive | -29.6 | -27.1 (0.96) |
| Tobacco | Johnston County | Vegetative | -26.5 | -26.7 (0.28) |
| Tobacco | Johnston County | Reproductive | -26.8 | -25.4 (0.47) |
| Tobacco | Martin County | Vegetative | -26.9 | -26.8 (0.37) |
| Tobacco | Martin County | Reproductive | -27.4 | -25.8 (1.01) |

to those grown with ample moisture (Martin & Thorstenson, 1988; Farquhar et al., 1989). Plant species that have higher water-use efficiencies may be expected to have different ratios of stable carbon isotopes than their less-efficient relatives (Smith & Epstein, 1971). This phenomenon could provide a mechanism for separating plant species utilizing the C3 photosynthetic pathway on the basis of their carbon isotope composition if differences in water-use efficiencies between species are sufficiently great. However, in these studies, we were unable to differentiate between plants species using IRMS as the plant-to-plant variation within a species at different locations proved to be quite large. The very narrow, non-overlapping range of $\delta^{13}\text{C}$ values seen in insects reared in the greenhouse on both cotton and tobacco could be the result of the continuous excess water provided to the host plants.

The range of $\delta^{13}\text{C}$ values of feral moths collected in 2001, 2002, and 2003 was consistent with feeding on hosts utilizing the C3 photosynthetic pathway. The $\delta^{13}\text{C}$ value of several individual moths collected in 2001 and 2002 was higher than any of the $\delta^{13}\text{C}$ values observed for plant tissue or insects from the controlled rearing experiments in 2003. This result could indicate utilization of a host plant(s) by tobacco budworm populations in these years that was/were not included in our studies. Given that there were no insect samples tested in 2003 with similarly elevated ^{13}C levels, it is likely that environmental variation between years is responsible for this observed variation in carbon isotope composition.

Although statistically significant differences in ^{13}C were detected between some of the C3 host plants tested, carbon isotope signatures were not sufficiently unique to enable a reliable determination of the natal origin of feral tobacco budworm with current IRMS technology. Stable isotopes can be powerful tools when used as biological markers in ecological studies, but careful evaluation and validation of techniques must be conducted to ensure reliable conclusions.

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