2005 Beltwide Cotton Conferences, New Orleans, Louisiana - January 4 - 7, 2005

ASSESSING THE UTILITY OF STABLE CARBON ISOTOPES FOR DETERMINING NATAL HOST ORIGINS OF TOBACCO BUDWORM, HELIOTHIS VIRESCENS, IN A HOST SPECIES RICH AGRO-ECOSYSTEM

Mark R. Abney, Clyde E. Sorenson, Fred Gould and Julius R. Bradley Jr.
NC State University
Raleigh, NC

Abstract

This study was conducted to investigate whether stable carbon isotope ($^{13}C/^{12}C$) ratios in the wings of tobacco budworm, *Heliothis virescens*, might serve as a diagnostic tool for elucidating the natal host origin of this economically important pest. Isotope ratio mass spectrometry (IRMS) analysis of $^{13}C/^{12}C$ ratios of moths reared in the field on four crop-plant species and in the laboratory on two common weed species revealed a range of $\delta^{13}C$ values within that expected for plants utilizing the C3 photosynthetic pathway. Despite early greenhouse experiments showing significant differences in $\delta^{13}C$ values between budworms reared on cotton and tobacco, no significant differences in mean $\delta^{13}C$ values were detected between moths reared on any of the host plant species in the field. Analysis of vegetative and reproductive tissues from the plants utilized in the study likewise resulted in $\delta^{13}C$ values that could not be separated statistically on the basis of plant species. Feral tobacco budworm moths collected over three years were found to have carbon isotope ratios consistent with those having fed on C3 plants. The average $\delta^{13}C$ value of feral insects collected within a single year appeared to be closely associated with the total accumulated rainfall for the months May through August. Results demonstrate that within the range of C3 host plants tested, no unique carbon isotope signature exists that would enable a reliable determination of natal origin of feral TBW with current IRMS technology.

Introduction

The looming specter of Bt resistant tobacco budworm populations is an ongoing concern for producers and IPM practitioners throughout the U.S. Cotton Belt. This potentially serious pest of both cotton and tobacco has a long history of developing resistance to conventional insecticides, and while not yet reported in the field, resistance to Bt toxins has been shown in laboratory selected budworm strains (Gould et al. 1992). Because of the economic and environmental significance of transgenic Bt cotton, the US EPA mandates the use of specific resistance management strategies in an effort to prolong the life of this important technology. The high dose/refuge strategy currently employed for Bt resistance management of Heliothines in cotton is based on a number of assumptions including the level of adult insect production from non-Bt refuge areas. The development of an effective insecticide resistance management strategy requires extensive knowledge of the biology of the insect and the environment in which it exists, and quantifying the seasonal utilization of host plant species by the tobacco budworm is currently an important area of investigation. Sufficient budworm production in existing weedy or non-selected alternate crop hosts could eliminate the need for the planting of costly non-Bt cotton refuges currently required by EPA's heliothine resistance management strategy.

An oligophagous insect, the tobacco budworm's pattern of host utilization varies with the appearance and phenology of suitable weedy and cultivated plant species within a growing season and from one geographic region to the next. In the Midsouth, weedy plant species serve as hosts for the first generation of budworms, while subsequent generations are more likely to be associated with cotton, the insect's predominate crop host in the region. Similarly, first generation budworms in the Southeast develop almost exclusively on weeds; however, a greater variety and abundance of host plants occurring throughout the growing season in this region reduces the importance of cotton for later generations (Abney et al. 2004). Investigations in North Carolina in the 1960's found old field toadflax, Carolina geranium, and tobacco to be significant resources for early season tobacco budworm development (Neunzig 1969). Later budworm generations in North Carolina can be found on tobacco, cotton, and peanut, and to a lesser extent soybean. Additionally, a number of weedy plant species, most notably members of the genus *Rhexia*, may be locally abundant through the summer months and serve as an important source of budworm production. The spatial and temporal diversity of the tobacco budworm's host suite in North Carolina cotton production regions may reduce the impact and therefore the necessity of non-Bt cotton refuges in the state.

Isotopes are atoms of the same element with an equal number of protons and electrons but varying in their number of neutrons. Naturally occurring stable isotopes can be used as biological markers as they often exist in predictable ranges in particular tissue types. Two stable isotopes of carbon exist in the environment. The overwhelming majority of carbon (98.98%) exists as ^{12}C while only a small proportion (1.11%) exists as ^{13}C. Variation in the ratio of ^{13}C to ^{12}C can be measured via IRMS and is reported as "δC" or parts per thousand deviation from a recognized standard (Dawson and Brooks 2001, Pate 2001). For example, plants utilizing the C3 and C4 photosynthetic pathways have distinct non-overlapping ranges of stable carbon isotope ratios. Because isotopes present in a plant are acquired by an herbivore when it feeds upon the plant's tissues, the carbon isotope composition of the herbivore can be used to link it to its host plant. Isotope ratio mass spectrometry (IRMS) is used to measure the relative abundance of specific isotopes in a sample of given material, and this technology has recently been utilized to broadly identify the natal host origin (C3 vs. C4 host plants) of field collected corn earworm, *Helicoverpa zea*, adults (Gould et al. 2002). While differences in the stable carbon isotope ratios of C3 vs. C4 plants have been well established, it is not known whether plant species within a single photosynthetic pathway can be distinguished on the basis of carbon isotope composition. This research project was designed to determine the utility of isotope ratio mass spectrometry (IRMS) for identifying the natal host origins of field collected tobacco budworm moths.

Materials and Methods

Insects.

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

Controlled Rearing Study: Greenhouse Experiment.

A preliminary greenhouse study was conducted to determine if differences in carbon isotope ratios between budworm adults reared on cotton and 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®) in 6-inch diameter sterilized clay pots in a greenhouse at NC State University on 14 February 2001. Plants were hand watered daily until introduction of tobacco budworm larvae. Three neonate tobacco budworm larvae were placed on the upper 1/3 of each of 10 cotton and tobacco plants on 17 May. Immediately following infestation, plants were transferred to water filled moats constructed on greenhouse benches. Moats provided continuous water thereby reducing the chance of disturbing larvae with overhead watering and also prevented larval movement from plant to plant on a single bench. The budworms were allowed to feed uninterrupted until just prior to pupation at which time final instar larvae were removed from plants and transported to the lab to complete development. A total of 7 and 11 adult budworms were reared from cotton and tobacco respectively. Adults were frozen shortly after eclosion and stored at -5°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 by removing the right forewing of each tobacco budworm to be analyzed and placing it in a tin combustion capsule. The samples were analyzed using a CE Elantech NA 2500 elemental analyzer coupled to a Therma Finnigan DELTA plus mass spectrometer via a Conflo II open split interface.

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 tobaccum*), old field toadflax (*Linarria 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, NC 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. 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 NC State University and stored at -5° C until processed for analysis. Plant samples were dried in a laboratory oven at 110°F for 8 days and then ground to a fine powder. Approximately 1.4 mg of homogenized ground plant tissue were placed in a tin combustion capsule for each plant sample examined. Plant tissue was analyzed using the same technique described earlier for moth wings.

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 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 and two weed host species in 2003.

Budworm larvae were reared in the lab on field collected old field toad flax and Carolina geranium from Johnston and Wake Co, NC respectively. Plants were cut at ground level, placed in distilled water, and transported to the lab where they were rinsed in tap water, allowed to air dry, and then placed singly into #50 water pics (Aquapic®, Syndicates Sales Inc., Kokomo, IN) containing distilled water. Neonate TBW larvae (strain YDK) 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 20x20x40cm Plexiglas® box with two 400cm^2 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 x 15mm 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.

Tobacco, cotton, soybean, and peanuts were planted in small test plots at the Central Crops Research Station in Clayton, 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) and placed over individual plants of each host species; neonate budworm larvae (strain YDK) were placed directly on plant tissue within the cages using a size 0 camel hair paint brush. Budworms were reared on tobacco in early and late July and on cotton in mid July and mid 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 #3 gem clips. Cages were monitored twice each week, and larvae were transported to the lab just prior to pupation. Pupae were held in individually labeled 60 x 15mm plastic petri dishes until adult eclosion at which time moths were frozen and then prepared for isotope analysis as previously described.

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 NC State University where they were stored at -5°C. A random sub-sample of ten moths per year for each year of the study was collected and prepared for analysis of stable carbon isotope composition.

Results

Controlled Rearing Study: Greenhouse Experiment.
The $\delta^{13}C$ values of tobacco budworm moths reared on tobacco in the greenhouse differed from and did not overlap the $\delta^{13}C$ values of moths reared on cotton. The mean $\delta^{13}C$ value of moths reared on tobacco (-29.2114‰) was significantly lower than the mean value for moths reared on cotton (-26.2114‰) at $\alpha = 0.05$.

Host Plant Tissue Analysis.
The $\delta^{13}C$ values of the six host plant species tested were consistent with those expected for plants utilizing the C3 photosynthetic pathway; however, there was considerable overlap in the range of values obtained for each species (Table 1). The $\delta^{13}C$ values of a single tissue type from the same plant species varied by collection date for peanut, by location for Carolina geranium, and by both collection date and location for cotton and tobacco. Variation was seen in the $\delta^{13}C$ values recorded for different tissue types (vegetative vs. reproductive tissue) of the same species for

cotton, tobacco, and soybean; tissue types were not evaluated separately for peanut, Carolina geranium, or old field toadflax.

Table 1. Range of δ 13C values for four crop and two weed hosts of tobacco budworm collected at multiple locations and dates.

Plant Tested	Date of Collection	Collection Location	Structures Tested	δ 13C range (‰)
Tobacco	6/24/2003	Martin Co., NC	Leaves	-27.4 to -26.5
	7/1/2003	Johnston Co., NC	Leaves	
	7/21/2003	Johnston Co., NC	Flowers	
	7/29/2003	Martin Co., NC	Leaves	
	7/29/2003	Martin Co., NC	Flowers	
Cotton	7/21/2003	Wilson Co., NC	Leaves	-29.1 to -27.1
	7/21/2003	Johnston Co., NC	Leaves	
	7/21/2003	Johnston Co., NC	Large Squares	
	7/29/2003	Martin Co., NC	Leaves	
Toadflax	4/30/2003	Edgecombe Co., NC	Whole plant	-29.5 to -29.1
	4/30/2003	Lenoir Co., NC	Whole plant	
	5/1/2003	Johnston Co., NC	Whole plant	
C. Geranium	4/30/2003	Edgecombe Co., NC	Whole plant	-29.1 to -28.2
	4/30/2003	Lenoir Co., NC	Whole plant	
Peanut	9/2/2003	Johnston Co., NC	Leaves	-27.7 to -26.9
	9/17/2003	Johnston Co., NC	Leaves	
Soybean	9/2/2003	Johnston Co., NC	Leaves/Stems	-28.1 to -26.3
	9/23/2003	Johnston Co., NC	Pods	

Controlled Rearing Study: Field Experiment.

IRMS analysis of wings from laboratory and field reared tobacco budworm revealed $\delta^{13}C$ values that were within the range expected for insects feeding on C3 plants. The data collected from insects reared in the field on cotton and tobacco did not corroborate the findings of earlier greenhouse experiments; budworms reared on tobacco in the field were consistently less depleted in ^{13}C than those reared on tobacco in the greenhouse. Additionally, the range of mean $\delta^{13}C$ values obtained from budworms reared on tobacco and cotton at different locations and dates were shown to overlap (-26.81‰ to -25.36‰ and -27.70‰ to -25.64‰ respectively). The mean $\delta^{13}C$ values of budworms reared on Carolina geranium and old field toadflax were also very similar (-28.59‰ and -28.07‰ respectively). Peanut and soybean reared insects (mean $\delta^{13}C$ = -28.00‰ and -26.47‰ respectively) likewise could not be separated from those reared on other hosts based on these data.

Feral Insect Analysis.

The $\delta^{13}C$ values observed for feral budworm adults (Table 2) were similar to those seen for budworms in the controlled rearing experiment conducted in the field and were consistent with larval development on C3 hosts. Significant variation in carbon isotope composition was observed between years. Differences in $\delta^{13}C$ values from year to year appear to be correlated with rainfall amounts during the growing season. Budworm moths collected during 2003, the year with greatest in-season precipitation, were the most depleted in ^{13}C ($\delta^{13}C$= -26.80‰). Moths collected during the droughty summer of 2002 contained the greatest proportion of ^{13}C ($\delta^{13}C$= -24.95‰), while budworms collected in 2001 were found to have intermediate levels of ^{13}C ($\delta^{13}C$= -25.54‰).

Table 2. Mean $\delta^{13}C$ values observed for tobacco budworm moths collected in pheromone traps in Eastern North Carolina in 2001, 2002, and 2003.

Year	n	Mean $\delta^{13}C$ (‰)
2001	10	-25.54
2002	10	-24.95
2003	10	-26.80

Discussion

Though preliminary greenhouse studies indicated IRMS might provide a useful tool for elucidating the natal host origin of tobacco budworm, further investigation revealed that environmental variation is apparently too great to enable separation of C3 host plants on the basis of stable carbon isotope ratios. Analysis of plant tissue collected in the field as well as analysis of budworms reared on those plants resulted in a wide range of $\delta^{13}C$ values for both plants and insects that could not be separated on the basis of 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. The stomata of a plant growing in conditions of adequate moisture are expected to remain open more than those of a plant growing under moisture stress. This in turn leads to a greater depletion of ^{13}C in plants with ample moisture compared to those under stress. By nature, plant species that have higher water use efficiencies may be expected to have different ratios of stable carbon isotopes than their less efficient relatives. 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 specific species at different locations and times proved to be quite large. The very narrow, non overlapping range of $\delta^{13}C$ values seen in insects reared in the greenhouse on both cotton and tobacco may be explained by the continuous excess water provided to the host plants.

The analysis of feral male tobacco budworm moths collected in pheromone traps over three years provided further evidence that stable carbon isotope composition is dependant upon the moisture available to the host plant. The mean $\delta^{13}C$ value of moths varied by year, and the level of depletion of ^{13}C was directly related to the amount of rainfall recorded during the growing season in each year. Though variation was observed between years, the range of $\delta^{13}C$ values of moths collected in all years was consistent with feeding on hosts utilizing the C3 photosynthetic pathway. The $\delta^{13}C$ value of several individual moths collected in 2001 and 2002 was higher than any of the $\delta^{13}C$ 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 more likely that results in 2001 and 2002 are due to variation in carbon isotope composition caused by differences in moisture availability.

We can conclude from our data, that within the range of C3 host plants tested, no unique carbon isotope signature exists that would enable a reliable determination of the natal origin of feral tobacco budworm with current IRMS technology. Future studies will investigate other techniques for determining host origin including the search for secondary plant metabolites unique to a specific host species that might serve as biological markers within the insect.

Literature Cited

Abney, M.R., C.E. Sorenson, and J.R. Bradley Jr. 2004. Alternate crop hosts as resistance management refuges for tobacco budworm in North Carolina. Proc. Beltwide Cotton Conf. 1413-1416.

Dawson, T.E., and P.D. Brooks. 2001. Fundamentals of stable isotope chemistry and measurement. pp. 1-18. *In* M. Unkovich, J. Pate, A. Mcneill, and D. J. Gibbs [eds.], Stable isotope techniques in the study of biological processes and functioning of ecosystems. Kluwer Academic Publishers, Boston, Mass.

Gould F, N. Blair, M. Reid, T.L. Rennie, J. Lopez , S. Micinski. 2002. *Bacillus thuringiensis*-toxin resistance management: Stable isotope assessment of alternate host use by *Helicoverpa zea*. Proceedings of the National Academy of Sciences, Vol 99: 16581-16586.

Gould, F, A. Martinez-Ramirez, A. Anderson, J. Ferre, F.J. Silva and W.J. Moar. 1992**.** Broad-spectrum resistance to *Bacillus thuringiensis* toxins in *Heliothis virescens*. Proceedings of the National Academy of Sciences, Vol 89: 7986-7990.

Neunzig, H.H. 1969. The biology of the tobacco budworm and the corn earworm in North Carolina. North Carolina Agricultural Experiment Station. Tech. Bu. No. 196. 76 pp.

Pate, J.S. 2001. Carbon isotope discrimination and plant water-use efficiency: case scenarios for C_3 plants. pp 19-36. *In* M. Unkovich, J. Pate, A. Mcneill, and D. J. Gibbs [eds.], Stable isotope techniques in the study of biological processes and functioning of ecosystems. Kluwer Academic Publishers, Boston, Mass.