Using Stable Isotope Analysis to Examine Fall Armyworm (Lepidoptera: Noctuidae) Host Strains in a Cotton Habitat

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ABSTRACT *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), or fall armyworm, is an important agricultural pest of several crops in the Western Hemisphere, including cotton (*Gossypium* L.). Two morphologically identical host strains of fall armyworm exist that differ in plant host use and habitat distribution. The corn-strain is a primary pest of corn, *Zea mays* L., whereas the rice-strain is the majority population infesting rice (*Oryza* spp.) and turfgrass (*Cynodon* spp.). With the increased use of *Bacillus thuringiensis* (Bt) toxin-expressing cotton varieties and the necessity of ensuring adequate refuge areas to prevent the spread of Bt toxin resistance, it is crucial to identify the alternative plant hosts available for the fall armyworm population infesting cotton. Stable isotope analysis combined with the molecular analysis of strain-specific markers was used to investigate whether one or both strains routinely develop on cotton grown in the Mississippi delta. We found that the majority of fall armyworm adults present during the early cotton growing season arose from C_4 plants (e.g., corn and sorghum, *Sorghum vulgare* Pers.) and that the only strain likely to be developing on cotton (a C_3 plant) in substantial numbers was the corn-strain. The population distribution patterns observed were consistent with corn providing an important refuge for the fall armyworm strain infesting cotton and suggested that late season populations in the Mississippi delta may be migrants from more northern corn areas.

KEY WORDS fall armyworm, *Spodoptera frugiperda*, cotton, stable isotope, host strains

Spodoptera frugiperda (J.E. Smith) (Lepidoptera: Noctuidae), or fall armyworm, is responsible for substantial economic damage to forage and sweet corn, *Zea mays* L., sorghum, *Sorghum vulgare* Pers., and several turfgrass varieties (e.g., *Cynodon* spp.), and it can be a major pest in cotton (*Gossypium* L.) (Sparks 1979, Pashley 1988). The highly polyphagous behavior of fall armyworm is in part due to the presence of two strains that differ in host preference (Pashley 1986). The corn-strain is generally associated with corn and sorghum, whereas the rice-strain is preferentially found in turfgrass. The two strains are morphologically identical, so they can only be distinguished by molecular methods (Pashley 1986; Lu et al. 1992, 1994; Pashley and Ke 1992; Lu and Adang 1996; McMichael and Prowell 1999; Prowell et al. 2004).

Identifying the host plant preferences for each strain is important for understanding basic questions in population dynamics and strain divergence as well as for the development of effective methods for pest control in specific crops. An example of the latter is the widespread and growing use of *Bacillus thuringiensis* (Bt) toxin-expressing plants, particularly in cotton. To reduce the likelihood of generating Bt-resistance among insect herbivores, the U.S. Environmental Protection Agency proposed that nontoxic host plants be made available to the targeted pests to serve as a "refuge" to maintain the proportion of susceptible insects (United States Environmental Protection Agency 2001). With polyphagous pests such as fall armyworm, it is possible that alternative hosts in the Bt-cotton growing areas could serve as natural refuges, obviating the need to set acreage aside for that purpose. Identifying the fall armyworm strain associated with cotton is a necessary first step to determining whether such alternative hosts are present in sufficient numbers. Because the two host strains also differ in their susceptibility to commonly used insecticides and to Bt transgenic plants, knowing the strain identity of the infesting population could influence the type of control measures recommended (Pashley et al. 1987, Veenstra et al. 1995, Adamczyk et al. 1997).

The identity of the strain of fall armyworm most likely to infest cotton remains largely uncertain, particularly in the United States. Preliminary studies comparing protein polymorphisms showed that larvae collected from cotton in Ecuador were more similar to

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the corn-strain than the rice-strain (Pashley 1986), and genetic examination of Brazilian fall armyworm populations showed substantial gene flow between larvae isolated from maize and cotton fields (Martinelli et al. 2006). Although both studies imply that the corn-strain infests both corn and cotton, it is not clear whether this observation applies to U.S. populations or whether the rice-strain might also be a significant pest.

The use of larvae to identify the fall armyworm strain associated with a particular plant is potentially problematic for plant hosts that are secondary or sporadic targets of infestations. These collections tend to be biased for fall armyworm populations present during unusual "outbreak" seasons when infestation levels are high, and so may not be representative of the typical behaviors of the two strains. Furthermore, these surveys may not be predictive of adult numbers if there are substantial strain differences in fitness during later larval and pupal stages (Fitt 1989). It is the capacity of plant hosts to support adult insect development that is most relevant to assessing the contribution of different plant types to the overall fall armyworm population and their effectiveness as Bt refuges.

A useful method for estimating plant host use was developed for the study of *Helicoverpa zea* (Boddie), or bollworm, which like fall armyworm is a polyphagous pest of corn and cotton (Gould et al. 2002). The stable carbon isotope ratio ($^{13}C/^{12}C$) in adult wings, commonly designated as $\delta^{13}C$, was used to determine whether the specimen arose from a C_3 (i.e., cotton) or C_4 (i.e., maize) plant host. Studies in Lepidoptera have found that although adult behavior can influence the isotope ratio, the primary determinant is the larval diet (Ponsard et al. 2004). Gould et al. (2002) was able to show that corn can serve as a refuge for *H. zea* infesting Louisiana Bt cotton and used the observed seasonal changes in the proportions of C_3 and C_4 moths to infer patterns of plant host use and migratory behavior.

We adapted this strategy to examine changes in $\delta^{13}C$ values in the adult fall armyworm population in cotton-dominated areas of Washington Co., MS, which lies within the Mississippi river delta region. Fall armyworm does not survive the winters in this area, so the cotton infestation arises from migrant populations originating from more southern overwintering sites. The primary objective was to determine whether the strains differed in their usage of cotton and, if so, to identify which contributed most to infestations. The implications of these data on our understanding of fall armyworm population dynamics and the development of pest management strategies are discussed.

Materials and Methods

Trap Collection and Sites. Pheromone trapping of adult males was performed in cotton growing areas in Washington Co. to estimate changes in fall armyworm populations in 2004 and 2005. Traps were placed adjacent to large cotton fields, and within 3 miles of corn, sorghum, and soybean [*Glycine max* (L.) Merr.] acreages. The trapping method has been described previously (Meagher and Gallo-Meagher 2003). Standard plastic Unitraps were baited with a commercially available fall armyworm pheromone (Scenturion lures, Suterra, Bend, OR), and they contained insecticide strips (Hercon Environmental Co., Emigsville, PA). Collections from traps were made at various intervals, ranging from 7 to 14 d. Moths were placed dry in plastic bags or in vials containing 95% ethanol and stored at 4°C.

Information on Crop Progress and Conditions. Data for fall armyworm infestation levels in cotton as a proportion of acreage infested was obtained from the Mississippi State University Extension Service (Williams 2006). Information about crop progress and conditions in Mississippi during 2004 and 2005 was obtained from the National Agricultural Statistics Service (USDA 2006).

Samples from Known Hosts. Both bollworms and fall armyworms (corn-strain colony) were reared throughout their larval stages on known hosts to demonstrate the nonoverlapping ranges of $\delta^{13}C$ values that correspond to larval development on C_3 or C_4 plant hosts. Bollworms were reared on corn and cotton, whereas fall armyworms were reared on corn, cotton, soybean, and broadleaf signal grass [*Brachiaria platyphylla* (Grisebach) Nash.].

Sample Preparation and Stable Isotope Analysis. For a given moth, a forewing was removed using dissection scissors and placed on tissue paper to evaporate remaining ethanol. Approximately 0.4–0.6 mg of wing material was removed and placed into 5- by 9-mm tin capsules. The corresponding body was placed in a plastic 1.5-ml vial containing fresh 95% ethanol for DNA analysis. Both wings and carcass were labeled in a manner that allowed future matching.

Stable isotope analysis was conducted at two locations. Initial studies were performed at the USDA–ARS, Southern Insect Management Research Unit (Stoneville, MS) by using an elemental combustion system (ECS4010 CHNSO, Costech Instruments, Milan, Italy) coupled to a mass spectrometer (Finnigan Delta^{Plus} Advantage, Thermo Electron Corporation, Waltham, MA) using a Conflo III Interface to measure stable carbon isotope ratio ($^{13}C/^{12}C$ or $\delta^{13}C$). The isotope standard reference material used in these analyses was acetanilide. Subsequent samples were analyzed at the Stable Isotope/Soil Biology Laboratory of the University of Georgia, Institute of Ecology, Athens, GA. Here, samples were analyzed using a Carlo Erba NA 1500 CHN Analyzer coupled to a Finnigan-MAT Delta C mass spectrometer by using a Conflo II Interface. The isotope standard reference material was bovine muscle powder.

DNA Preparation. The bodies remaining after the stable isotope analysis were individually homogenized in 4 ml of phosphate-buffered saline (PBS; 20 mM sodium phosphate and 150 mM NaCl, pH 8.0) in a 15-ml test tube by using a tissue homogenizer (PRO Scientific Inc., Oxford, CT). Cells and tissue were pelleted by centrifugation at 6000 $\times$ *g* for 5 min at room temperature. The pellet was resuspended in 800

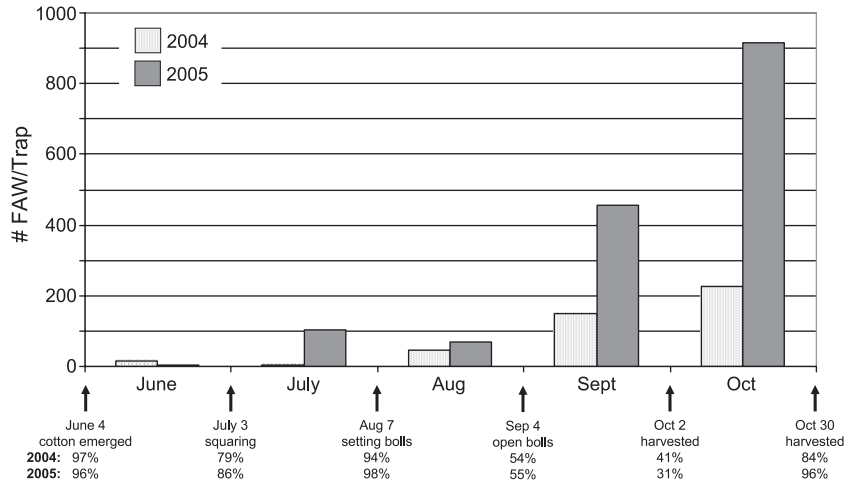

Fig. 1. Pheromone trap captures of fall armyworms from cotton growing areas in the Mississippi delta region from June to October in 2004 and 2005. The progression of the local cotton crop is indicated by arrows and was obtained from the USDA Weekly Weather Crop Reports for the appropriate dates in 2004 and 2005 (USDA 2006).

μl of cell lysis buffer (0.2 M sucrose, 0.1 M Tris-HCl, pH 8.0, 0.05 M EDTA, and 0.5% sodium dodecyl sulfate), transferred to a 1.5- or 2.0-ml microcentrifuge tube, and incubated at 55°C for 5 min. Proteins were precipitated by the addition of 100 μl of 8 M potassium acetate. The supernatant was transferred to a Zymo-Spin III column (Zymo Research, Orange, CA) and processed according to manufacturer's instructions. The DNA preparation was increased to a final volume of 40 μl with distilled water. Each polymerase chain reaction (PCR) reaction required 1 μl of the DNA preparation.

PCR Analysis. PCR amplification of the mitochondrial *COI* gene was performed in a 30-μl reaction mix containing 3 μl of 10X reaction buffer, 1 μl of 10 mM dNTP, 0.5 μl of 20 μM primer mix, 1 μl of DNA template (between 0.05 and 0.5 μg), and 0.5 μl of *Taq*DNA polymerase (New England Biolabs, Beverly, MA). The thermocycling program was 94°C (1 min), followed by 33 cycles of 92°C (45 s), 56°C (45 s), 72°C (1 min), and a final segment of 72°C for 3 min. Primers were synthesized by Integrated DNA Technologies (Coralville, IA). Amplification of the *COI* region used the primer pair *COI-58 F* (5′-GGAATTTGAGCAG GAATAGTAGG-3′) and *COI-1058R* (5′-ACACCT GTTAATCCTCCTACAG-3′) to produce a 1.0-kb fragment. The fragment contains two MspI sites, one specific to the corn-strain and the other to the rice-strain. Digestion of the PCR product with MspI results in two different restriction patterns that can be identified by agarose gel electrophoresis. The MspI restriction enzyme was diluted in 1X reaction buffer to a concentration of 1 U/μl, and 5 μl was added to 8 μl of each PCR reaction. Digestions were at 37°C for 1–2 h. Two microliters of 6X gel loading buffer was added to each sample, which was run on a 1.8% PCR grade agarose (Fisher, Hampton, NH) horizontal gel.

Statistical Analysis. Analysis of variance (ANOVA) (PROC GLM, SAS Institute, 2003) was used to compare the proportion of moths analyzed in each category among sampling dates.

Results

Crop Description and Pheromone Trap Captures. The Mississippi cotton crop followed similar progression patterns during 2004 and 2005 (USDA 2006). More than 95% of the cotton had emerged by 5 June, with ≈80% progressing to the squaring stage by 3 July and over half in open bolls by the beginning of September (Fig. 1). The cotton harvest began in mid-September, and it was 84% (2004) and 96% (2005) complete by 30 October.

Pheromone trap capture numbers during the cotton growing season (June–October) were about threefold higher in 2005 (1,548 per trap) than 2004 (440 per trap), consistent with reports of higher cotton infestation rates based on crop inspections in the Mississippi delta region in 2005 (Williams 2006). Substantial numbers of trap captures did not occur until August in 2004 and July in 2005, with substantial increases in both years occurring in September and continuing into October (Fig. 1).

Distribution of Fall Armyworm Arising from C_3 and C_4 Plants. As a control for the stable isotope methodology, $\delta^{13}C$ analysis was performed for fall armyworm and bollworm specimens raised on the C_3 plants cotton and soybean or the C_4 hosts corn and signalgrass. Bollworms had previously been shown to display nonoverlapping peaks when raised on C_3 and C_4 plants (Gould et al. 2002). We found that cotton-raised bollworm and fall armyworm gave $\delta^{13}C$ values ranging from -28 to -23, compared with -13 to -8 for those raised on corn (Fig. 2). One outlier (out of 10 samples) was observed with the corn-raised fall armyworm (-23), most likely a mislabeled specimen. Similar results were obtained with fall armyworm raised on soybean or

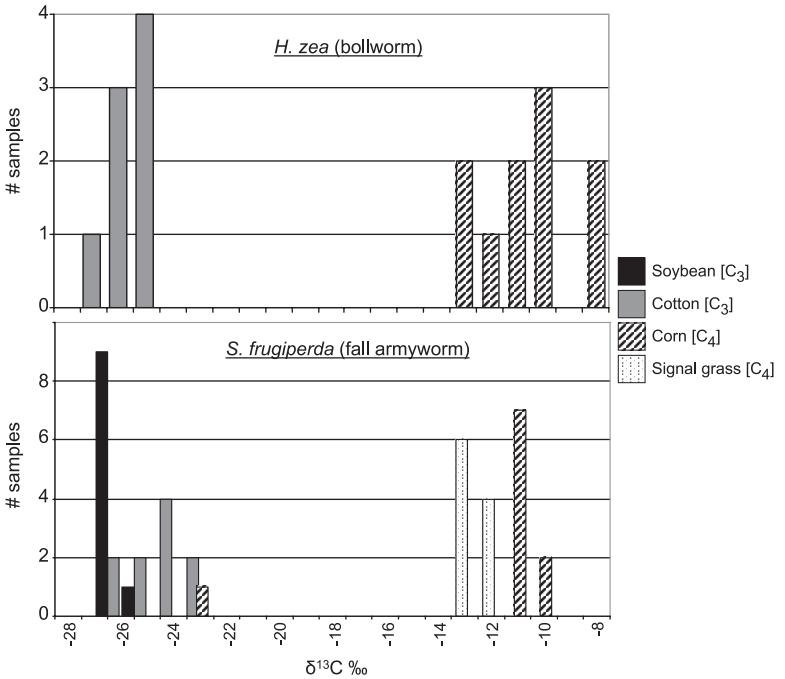

Fig. 2. Distributions of $\delta^{13}C$ values from laboratory bollworms and fall armyworm lines grown on C_3 or C_4 plants. Bollworms were raised on cotton or corn until pupation. Wings were dissected from newly eclosed adults and tested for $\delta^{13}C$ values. Fall armyworms were raised on cotton, corn, soybean, and signalgrass and treated similarly.

signalgrass, with values ranging from −27 to −26 and −13 to −12, respectively. From these analyses we defined values lower than −20 as indicating development on C_3 plants, between −20 and −15 as intermediates from unspecified plant hosts, and values greater than −15 as indicating a C_4 plant origin.

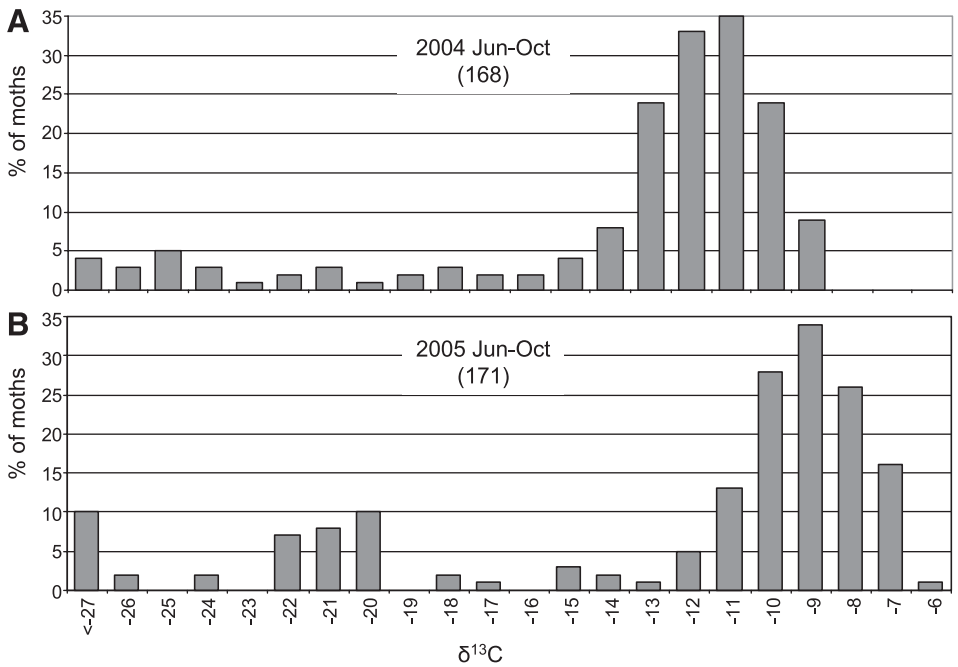

Fig. 3. Distribution of $\delta^{13}C$ values from wings of field-collected fall armyworm in 2004 and 2005. On the y-axis is plotted the percentage of samples at the given $\delta^{13}C$ value. Numbers in parentheses indicate number of samples analyzed. (A) Data from June to October in 2004. (B) Data from June to October in 2005.

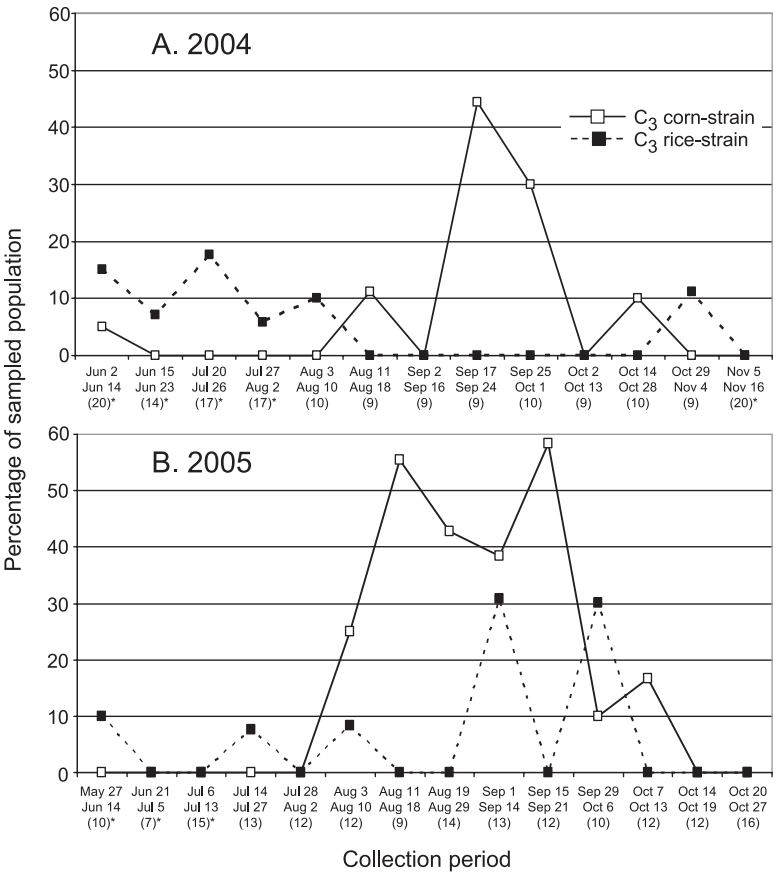

Fig. 4. Proportion of C_3 fall armyworm of each strain from field collections described as a percentage of the total samples tested. The first and last dates of each collection period are labeled on the x-axis. Each data point represents corn-strain (open squares) or rice-strain (solid squares) with a C_3 signature as a percentage of the total (C_3 and C_4) specimens sampled (in parentheses) for each collection period. (A) Data from 2 June to 16 November 2004. (B) Data from 27 May to 27 October 2005. Asterisks indicate data pooled from more than one collection.

Similar criteria were used previously to study bollworm (Gould et al. 2002).

The fall armyworms collected by pheromone trapping in 2004 and 2005 were tested for their $\delta^{13}C$ values. In 2004, 79% (133/168) were categorized as having developed on C_4 plants (Fig. 3A). The remaining specimens were evenly distributed between −27 to −15, with no obvious C_3 peak. This large variance in the C_3 range and the presence of values intermediate between C_3 and C_4 could be due to several reasons, including a range of C_3 plant hosts with variable $\delta^{13}C$ values, individual larvae feeding on both C_3 and C_4 plants, and adult feeding significantly altering the isotopic ratio in the wings. However, in the latter case, studies in other Lepidoptera showed that although the adult diet had minor effects, the wing $\delta^{13}C$ values primarily reflected the larval host plant (Ponsard et al. 2004).

In 2005, there was roughly the same proportion of specimens in the C_4 range (81%; 138/171), but the C_3 subgroup showed a greater tendency to cluster (Fig. 3B). C_3 $\delta^{13}C$ values ranged from −58 to −21, but 61% (20/33) fell in the −22 to −20 range. The majority (28/33) of the C_3 population was captured in August and September.

Distribution of Fall Armyworm Strains. In 2004, only 13% (21/168) of the sampled population from 2 June to 16 November were unambiguously derived from a C_3 plant host. The C_3 proportion rose substantially above the seasonal mean only during the two collections that occurred in the period spanning 17 September to 1 October, when levels of 44% (4/9) and 30% (3/10), respectively, were observed (Fig. 4A). All the C_3 samples tested from these collections (7/7) were of the corn-strain. In comparison, the C_3 samples from the preceding period, 2 June to 16 September, were primarily of the rice strain (9/11), whereas only two C_3 specimens were collected from 2 October to 16 November. Despite the low overall C_3 numbers, the proportion (mean) of C_3 corn-strain observed from 17 September to 1 October (37 ± 7%) was significantly higher ($F = 35.9$; df = 2, 10; $P < 0.0001$) than the preceding 2 June to 16 September (2 ± 2%) or the subsequent 2 October to 16 November (3 ± 3%) periods.

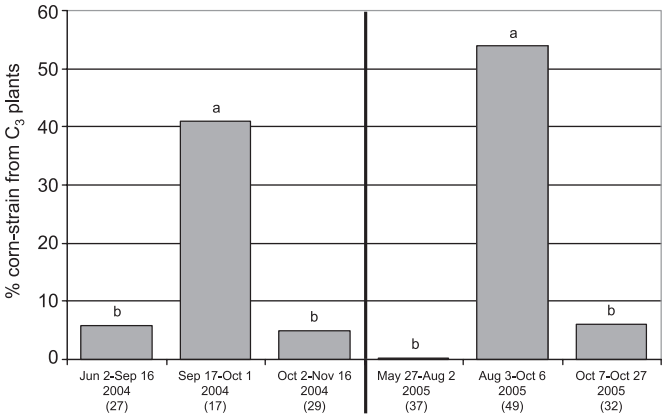

Fig. 5. Average percentage of moths arising from C_3 host plants in field-collected corn-strain samples collected from selected periods during the 2004 and 2005 growing seasons. To obtain the percentage, the number of C_3 corn-strain was divided by the total number of corn-strain samples tested for each collection listed in Fig. 4. Each column represents the average percentage for the collections during the given time period (numbers in parentheses indicate total number of samples). The collection periods with the highest proportion of C_3 corn-strain samples were identified for 2004 (17 September–1 October) and 2005 (3 August–6 October). Columns with different letters differ significantly ($F = 11.8$; df = 2, 10; $P = 0.0023$ for 2004 and $F = 24.8$; df = 2, 11; $P < 0.0001$ for 2005).

An overall higher C_3 proportion was observed in 2005, comprising 25% (42/171) of the specimens tested from June to October. The distribution pattern was similar to 2004 except that the mid-season increase in C_3 proportions began a month earlier in August (Fig. 4B). From 3 August to 6 October, 51% (38/74) of the tested samples were from C_3 plant hosts, compared with 4 and 5% from the previous 27 May–2 August and subsequent 7 October–27 October periods, respectively. This increase was primarily due to changes in the corn-strain subgroup, as these made up 77% (27/35) of the C_3 specimens tested during the peak period. As in 2004, the proportion (mean) of the C_3 corn-strain class during the 3 August–6 October period (38 ± 8%) was significantly higher ($F = 13.2$; df = 2, 11; $P = 0.0012$) than observed for the earlier 27 May to 2 August (0%) and subsequent 7–27 October (6% ± 6) periods. In comparison, significant differences were not observed for changes in the C_3 rice-strain proportions for these periods in either 2004 or 2005.

Discussion

By analyzing pheromone trap collections for strain-specific markers and isotopic carbon levels, we were able to derive a quantitative description of fall armyworm population dynamics in a cotton-dominated habitat. Our prediction was that changes in the fall armyworm subpopulation arising from cotton, which exhibited a C_3 signature, would correspond to seasonal variations in the availability of the plant host. Fall armyworm can feed on squares, blooms, and bolls and has an ≈37-d developmental cycle on cotton (Pitre and Hogg 1983). Assuming that infestation began in mid-June when approximately half the crop was squaring, we estimated that the first adults arising from cotton should have been present by August, with numbers increasing into September. Harvesting began in mid-September in both years, interrupting further larval development by eliminating the plant host. Pupae that formed before harvesting could continue to develop in the ground, emerging after an ≈11-d pupal period previously observed for cotton-derived fall armyworm (Pitre and Hogg 1983). As these adults moved on to new food sources, we anticipated a continued decline of the cotton-derived population as harvesting continued through October.

The corn-strain best displayed the expected pattern for a cotton pest. After being largely absent during the early cotton growing season, the C_3 corn-strain population increased rapidly in 17 September to 1 October 2004 and 3 August to 6 October 2005 periods (Fig. 4). During these collections, the proportion of corn-strain developing from C_3 plants were at its highest levels, reaching averages of 41 ± 3% in 2004 and 54 ± 8% in 2005 (Fig. 5). These compare with the preceding and subsequent periods when on average >90% of the corn-strain captured in both years displayed the C_4 isotope signature. These results are all consistent with the corn-strain being the primary fall armyworm subpopulation infesting and developing on cotton. We cannot preclude the possibility that nearby soybean plantings might also contribute to the local C_3 population, but this seems unlikely to be significant given previous studies indicating high (94%) larval mortality on this host (Pitre and Hogg 1983).

Fall armyworm is not known to diapause so the annual infestations in the Mississippi delta cotton fields are presumed to originate from migrants that overwinter in southern Texas or Florida (Luginbill 1928, Pair et al. 1986). In our studies, the early specimens of both strains collected by pheromone trapping arose primarily from a C_4 plant host, representing >70% of June–July trap captures in 2004 and >90% in 2005. Corn is likely to be a primary C_4 source for these

early-season moths, because it is a preferred host of the corn-strain and large plantings were present within a few miles of the test site. The rice-strain was found in substantial numbers during this period, particularly in 2004 (data not shown). Although the rice-strain was initially identified infesting rice in Puerto Rico (Pashley et al. 1985), fall armyworm is not a major pest of rice crops in North America. The preferred plant hosts are pasture and turf grasses, and substantial infestations in corn and has also occasionally been observed (Pashley 1989, Nagoshi and Meagher 2004). Whether this early season C_4 rice-strain population originates from the same source as the corn-strain is not known.

Migrants may also be a primary contributor to late season adult populations in the Mississippi delta. Gould et al. (2002) discussed the possibility of a north-to-south migration to explain the presence of C_4 bollworms in areas and during periods when appropriate C_4 plant hosts were absent. A similar pattern arose with fall armyworm after the cotton was harvested in October. Trap captures continued to increase substantially and the specimens captured in October in both years were >90% of C_4 origin, indicating that these were not derivatives from the recently harvested cotton crop (Figs. 1 and 4). The source of these moths (of both strains) is unknown, because there was no obvious candidate C_4 hosts in the vicinity of the trapping site. One possibility is that these come from more northern and later developing corn and sorghum fields. There have been earlier suggestions for a return north-to-south migration of fall armyworm to overwintering areas, based largely on pheromone trap collection patterns and the observation of atmospheric conditions that should be conducive to such long-range movements (Pair et al. 1986, Mitchell et al. 1991).

These results represent, to our knowledge, the first investigation of North American fall armyworm populations for strain preference in cotton. Even though adult males of both strains were present in the area, only the corn-strain displayed evidence of development on cotton in substantial numbers. This strongly suggests that corn, the primary plant host for the corn-strain, can potentially serve as a refuge for the fall armyworm population most likely to infest Bt-cotton. The experiments demonstrate that a strategy combining traditional larval and adult collection methods with carbon isotope analysis can provide a useful and detailed description of strain-specific population dynamics and plant host usage in a given habitat and season. We think this level of detail will be necessary to understand and predict the seasonal movements of fall armyworm and to identify the habitats that are the sources of infesting populations.

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