ABSTRACT

RENNIE, TRACY LEE. Investigating host use and migration by the corn earworm (Noctuidae: *Helicoverpa zea)* using stable isotope and morphometric techniques. (Under the direction of Fred Gould).

The commercialization of cotton and corn, genetically engineered to produce a caterpillar-specific insecticidal protein (Bt toxin) has focused renewed attention on the issue of southward migration of *Helicoverpa zea* because it could have a major effect on the rate at which this pest evolves resistance to the Bt toxin. Corn is a major host of *H. zea* and since there is presently limited use of transgenic Bt corn in the Northern corn-belt southern migration from this corn could result in a larger overall refuge for susceptible *H. zea* and that would decrease the rate of resistance evolution

We applied a stable carbon isotope technique to test the hypothesis that *H. zea* migrates North in early summer, feeds on corn, then migrates back South to feed on cotton. We analyzed the relative abundance of the naturally occurring isotope, ^{13}C, incorporated in wing cuticle of a moth as an indicator of the photosynthetic pathway (corn=C_4, cotton=C_3) of the plant that had been fed upon by it when it was a larva. Our carbon isotope analyses of *H. zea,* captured in pheromone traps, in the Brazos River Valley, Texas, demonstrated that at any given date from late summer to early fall in 2000, 40-100% of the moths developed on C_4 host plants and were present as adults in a cotton growing area where there were no known C_4 hosts suitable for development. Our investigation has also shown significant morphometric differences in moths from C_3 and C_4 hosts. Moths that fed upon a C_4 host during the larval stage typically had larger wing length and were heavier than moths that fed upon a C_3 host. Host plant quality during

larval development may affect fluctuating asymmetry in adult characteristics, such as wing length, causing greater asymmetry in moths whose larvae fed on sub-optimal hosts. Apparently, there was no effect of host plant type on the degree of wing length asymmetry measured in the Texas moth samples, however, there was a significant effect of DATE, and an interaction effect of HOST*DATE. The moths from early in the season were more asymmetrical than moths later in the season. We have provided circumstantial evidence that suggests that *H. zea* moths are migrating into cotton growing regions of the Brazos River Valley from northern regions where corn or some other C_4 host is suitable for larval development.

It is expected that continental hydrogen isotope ratios (δD) in surface water at moth natal sites will be significantly correlated with δD values in moth wings. We examined wings of *H. zea* moths that developed as larvae on corn from known origins across the mid- and eastern US to determine the potential use of hydrogen isotope ratios in *H. zea* wing tissue as geographical indicators of *H. zea* natal origins. However, the δD values in wing tissue did not reflect the continental δD gradient in rainfall. Therefore, without further investigation, pheromone trapped moths cannot be used to determine moth natal origins via stable hydrogen isotope analysis.

INVESTIGATING HOST USE AND MIGRATION BY THE CORN EARWORM (NOCTUIDAE: *HELICOVERPA ZEA)* USING STABLE ISOTOPE AND MORPHOMETRIC TECHNIQUES

By

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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Master of Science

ENTOMOLOGY

Raleigh

2003

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Dedicated to Enya

BIOGRAPHY

Tracy Lee Rennie was born 4 February 1975 in Sechelt, a small town on the west coast of Canada. Tracy lived with her parents Mark Andrew and Leina Fraser Rennie and younger brothers, Richard John and Tyrell William, in Gibsons, British Columbia until she was graduated from Elphinstone Secondary School in 1993. During her deciding years of academia at Elphinstone she was most impressed by the teachings of Mr. Vladimir Murawsky, upon which she developed an invested interest in the biological sciences.

She matriculated at the University of Victoria, BC, in 1994 and obtained her Bachelor of Science (with distinction) in May 1999. Tracy maintained part-time employment through the work-study program at UVic during her bachelor's degree. It was through her work experience in Dr. Livingston's plant physiology laboratory that Tracy decided to pursue employment at the National Hydrology Research Center in Saskatoon, Saskatchewan.

She worked for 16 months as a research assistant in the stable isotope facility under the supervision of Dr. Len Wassenaar. During her last 4 months working in the prairies at NHRC, Tracy combined her work with the beginnings of a Master's degree, which was to be completed at North Carolina State University under the direction of Dr. Fred Gould. Fred Gould was a client of NHRC and offered Tracy a graduate position to apply the stable isotope techniques she had been learning at UVic and NHRC on a project involving corn earworm host use and migration. She left Canada January 2001, with a car-load of her belongings and her pet cat, Enya, of 7 years, in pursuit of her MSc in Entomology at NCSU, Raleigh, North Carolina.

ACKNOWLEDGEMENTS

I would like to express my gratitude first to my committee: JR Bradley, Neal Blair and Fred Gould, whom helped keep my goals in perspective and instill the confidence and enthusiasm I needed to earn this degree. It was a team effort, of which they made me the leader. I thank my committee individually for their expertise in the diverse areas of my research. And I pay special thanks Fred Gould for his genuineness in the role of major advisor. I have heard and seen the horror stories of the relationships between graduate students and their advisors and I am pleased to say that I never encountered a conflict with Fred nor did I feel, at anytime, that I could not approach him with an idea, question, or problem.

This research was supported by the USDA Biotechnology Risk Assessment Program and would not have been feasible without the help of so many collaborators collecting corn earworms; thanks to: Juan Lopez, Steve Micinski, John Adamczyk, Richard Sprenkel, Michael Jackson, Brian Nault, Shelby Fleischer, John Wedberg, Gill Hutchison, and Paul Hagerman. Several friends and colleagues contributed their time and expertise to the preparation and processing of samples for isotope analysis: Tom Maddox, Len Wassenaar, Geoff Koehler and Patty Healy, their efforts are greatly appreciated. Dr. Cavell Brownie provided statistical assistance on the data analysis; she is an invaluable resource of expert advice for statistical analysis in the biological sciences. The help and support from the NCSU Entomology personnel, especially Donna Nye, Noreen Smith, Pat Robertson and Gene Dupree, is sincerely acknowledged. I give a warm thanks to my fellow graduate students and the “Method Road” crew for their support and guidance in

accomplishing this goal that I thought at times was out of reach. A warm thanks to my family and friends from home for believing in me. I thank Dr. John Meyer for giving me the opportunity to participate in his “General Entomology, ENT 425” class as a teaching assistant. The experience I gained will undoubtedly become a valuable asset in future teaching positions that I pursue.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Corn earworm, *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae). *Helicoverpa zea* is polyphagous, multivoltine, highly mobile, highly fecund, and has the ability to diapause. These characteristics are at least in part the factors that make *H. zea* such a successful exploiter of agronomic systems (Fitt 1989). It is the larval stage that is responsible for the serious economic losses in several agricultural crops throughout the mid-south and southeastern United States of America. It is more damaging in areas where it successfully overwinters because in northern areas it typically arrives too late to inflict extensive damage. The corn earworm is managed on US cotton in combination with the budworm (*Heliothis virescens*), better known as the Heliothine complex. Total management cost, including losses in yield, of the Heliothine complex for 2001 was about 224 million dollars (USDA 2002). Hardwick's (1965) comprehensive list of *H. zea's* geographic distribution includes a wide area ranging from Saskatoon, Saskatchewan in the north to the Falkland Islands in the south. *H. zea* has several common names that are usually associated with the crop it is exploiting (e.g. the corn earworm (corn), bollworm (cotton), tomato fruitworm (tomatoes)).

The polyphagous nature of *H. zea* seems almost limitless, however, the species prefers corn in its silking stage to any other host, and is more consistently reported as feeding on corn crops in North America than any other (Hardwick 1965). In Texas, *H. zea* is capable of completing 4-5 generations per season utilizing several plant hosts. First generation *H. zea* larvae primarily feed on wild hosts during the early spring. Texas bluebonnets, *Lupinus texensis* Hook, and Texas paintbrush, *Castilleja indivisa* Engelemann are two wild suitable hosts in the early season (Eger et. al. 1982). Cultivated hosts are more

attractive to the second generation of *H. zea*. These hosts include corn, cotton, soybean, tomato, sorghum, vetch, peanut, alfalfa, tobacco, cucumber, eggplant, okra, cabbage, and peach (Graham et. al. 1972, Cole et. al. 1973, EPA 2001). Corn is most attractive when it begins to silk (Isley 1935, Johnson et. al. 1975). It is during the second generation when corn is silking in Texas and populations of *H. zea* can reach very high densities**.** By the third generation, corn has begun to dry and is not attractive so alternate hosts including cotton, sorghum, and late-planted soybeans become more important and may serve as hosts through the fourth generation (Johnson et. al. 1975, Hillhouse and Pitre 1976). During the final generation *H. zea* may move back into wild hosts such as purslane.

Typically, the development period from egg to adult is 35 days, however, the duration of the larval stage varies with temperature and nutritive value of the host plant (Hardwick 1965, Butler 1976). The adults live for about 10 days. *H. zea* eggs are laid singly, usually on leaf hairs, buds, and corn silk. Fecundity ranges from 500 to 3000 eggs per female. The eggs hatch in about three to four days. Upon hatching, neonates wander about the plant until they encounter a suitable feeding site, normally the reproductive structure of the plant. On corn, they feed their way under the husk to the tender kernels to complete development (Hardwick 1965).

Transgenic Technology. Transgenic is a term that is used to describe an organism that has been modified by genetic engineering to contain DNA that has been asexually transferred from one species to another. The development of genetic transformation in crops enabled corn and cotton plants to express genes that encode insecticidal proteins from the bacterium *Bacillus thuringiensis* Berliner, commonly refered to as Bt (Koziel et. al. 1993). The genes of *Bacillus thuringiensis* subspecies *kurstaki* that produce crystal-

like proteins capable of killing Lepidoptera pests are transferred into plants to express the insecticidal δ-endotoxin Cry1A(b) or Cry1A(c). Plants expressing the Cry1Ab protein are more effective on the beet armyworm (*Spodoptera exigua*) and less effective on *H. zea* compared to Cry1Ac (MacIntosh et. al. 1990, Perlak et. al. 1990). Transgenic crops, such as Bt corn and Bt cotton, express the δ-endotoxin in their tissues, which acts as a stomach poison once it is ingested by the caterpillar (Schnepf et. al. 1998). The δ-endotoxin is continuously expressed throughout the plant's growing season. Continuous, long-term exposure to a pesticide is one of the factors increasing potential selection pressure upon a pest.

Insect resistance management (IRM). By definition IRM is the set of practices aimed at reducing the potential or rate at which insect pests become resistant to a pesticide. The recent commercialization of Bt corn and Bt cotton has focused renewed attention on the issue of southward migration because it could have a major effect on the rate at which *H. zea* may evolve resistance to Cry δ-endotoxin. The rate of Bt-resistance evolution depends on several interacting influences, including genetic factors such as initial frequency of resistance alleles in the pest population, genetic mode and stability of resistance, gene flow among different geographical populations, fitness of resistant individuals; ecological factors such as temporal and spatial distribution of the insect pest on different host plants; and management factors such as refugia and pesticide control (Tabashnik and Croft 1982, Gould 1998, Peck et. al. 1999). To slow the evolution of Bt resistance, the US government requires that toxin-free host plants be available to *H. zea* populations to serve as a refuge where caterpillars with toxin susceptibility genes can survive. A successful refuge-strategy has the embedded assumption that the susceptible

moths population include both homozygous (SS) and heterozygous (RS) toxin-susceptible genes and that rare homozygous resistant (RR) moths are homozygous recessive for the toxin resistance allele.

Research indicates that refugia can significantly slow the evolution of resistance if the refuge produces 500 or more susceptible insects for every resistant insect that developed on a transgenic plant (Gould 1998). A high-dose refuge strategy is defined as the expression of at least 25 times the concentration of Bt toxin required to kill more than 95% of susceptible insects to maximize the impact of susceptible immigrants from refugia (Tabashnik and Croft 1982, Gould 1998, SAP 1998). However, the currently deployed high-dose refuge strategy is not effective for slowing the evolution of resistance in *H. zea* because the levels of toxin in commercialized Bt corn and Bt cotton are not great enough to kill more than 95% of the heterozygotes (EPA 1998).

Stone and Sims (1993) performed a point in time study of the geographical susceptibility of *H. zea* to CryIA(c) and found there to be a 16-fold range of susceptibility throughout the US cotton belt. Although the study by Stone and Sims (1993) was specific in time and endotoxin it does demonstrate that *H. zea* has a natural and variable tolerance to the endotoxin CryIA(c), which suggests that *H. zea* has the ability to adapt to Bt crops of different endotoxin varieties. Burd (2001) was the first to find resistance alleles to the Bt toxin (CryIAc) in field populations of *H. zea*. The EPA-SAP (1998) considered *H. zea* to be the most likely Lepidoptera to develop cross-resistance because of the similarities in the varieties of Bt toxin and the continuous exposure of Bt toxin in corn and cotton throughout the growing season. The amount of insect movement affects the rate at which

local populations evolve resistance and the rate at which resistant populations spread through a region (Gould and Tabashnik1998).

H. zea is a highly mobile and polyphagous insect, capable of utilizing both corn and cotton over the 3-5 generations per year, which may lead to an accelerated adaptation to Bt crops if *H. zea* is continuously exposed to the Bt endotoxin. Our investigation to further understand the extent of *H. zea* migration will be a valuable addition to the scientific assessment made by the EPA regarding regulatory action on Bt corn and Bt cotton. *Bacillus thuringiensis* is the most widely used biologically produced pest control agent (Schnepf et. al. 1998) and Bt IRM is of great importance because of the threat that insect resistance poses to the future of Bt plant-protectants and Bt technology as a whole. Strategic plans made by the EPA to slow the resistance of *H. zea* to Bt crops will be more effective once *H. zea* migration is taken into consideration.

In present efforts to delay resistance to Bt crops, growers must chose from at least one of the three structural refuge options: 95:5 external structured unsprayed refuge; 80:20 external sprayed refuge; 95:5 embedded refuge (EPA 2001). These refuge strategies have been developed based on data that does not include moth migration. If there is substantial southward migration of *H. zea* moths late in the summer then the rate of resistance evolution should decrease due to less selective pressure by the recent decrease of transgenic corn and subsequent increase of refuge in the North. Shelton et al. (2000) compared the level of resistance Bt in the diamondback moths (*Plutella xylostella*) from a greenhouse study to a field study and found that the moths became more susceptible in

the field study as a result of immigration of native susceptibles, which diluted the frequency of resistant alleles. Farmers employing insect resistant management strategies for *H. zea* in Texas must rely on non-Bt (nBt) cotton to serve as a refuge for Bt cotton. It is clear from the literature review that there are several potential hosts for *H. zea* during the season. However, the exact utilization of these hosts is not entirely understood and the complexity of *H. zea* movement amongst various possible hosts requires more research so that each identifiable agroecosystem utilized by this pest, will be considered when incorporating refugia into insect resistance management strategies for *H. zea* and Bt crops.

Supporting evidence of moth migration. There are a number of studies indicating that *H. zea* can migrate North in early to midsummer although none of these studies quantify the percentage of moths from the South that are involved in this migration. One unique approach to documenting northward movement has taken advantage of the fact that plants, which depend upon insects for pollination, have evolved pollen that adheres readily to the insects'exterior. Moths feeding on such plants become naturally marked with this pollen. This provides a useful tool via pollen identification because pollen grains are distinctive and can often be identified to genus (Kapp1969). Furthermore, the distribution and flowering periods of most plants are well known, which helps to establish the origin of captured insects (Hendrix et. al. 1987, 1992, Lingren et. al. 1994). Hendrix *et al.* (1987) identified pollen of two Mimosoideae legumes, *Pithecellobium* spp. (ape's earring or Texas ebony) and *Calliandra* spp. (false mesquite), adhered to the eyes and proboscis of *H. zea* captured in the spring in Arkansas. The nearest populations of

these plant genera were located in southern Texas; thus, these moths had migrated ≥1000km. In conjunction with pollen analysis synoptic weather patterns have been used to identify continuous and discontinuous trajectories for passive, downwind, and true-north displacement of several Lepidopteran pests (Lingren et. al.1994, Westbrook et. al. 1997, 1998). Wind trajectory analysis correlated well with the displacement pattern of *H. zea* marked naturally with *Citrus* pollen and artificially with *Lycopodium clavatum* spores to identify a flight range of at least 660 km in Texas (Westbrook et. al. 1998). Showers and Whitford (1989) showed strong evidence of migration by a mechanistic link between synoptic southerly winds and the recapture of marked black cutworm moths, *Agrotis ipsilon* (noctuid), far away from their release site (≈1000km). Pair et. al. (1987) has shown evidence of *H. zea* migration from MX and the LRGV into the Texas high plains and with synchronous events of moth emergence and high trap captures. Radar profiles of airborne insects, and high pheromone trap captures in Texas provide strong circumstantial evidence that populations of moths produced in the high plains complete a reverse migration late in the growing season (Pair et. al. 1987).

One important factor limiting the use of pollen markers in the study of migratory and feeding activities of insects is that an effective pollen marker must be geographically remote from the areas in which the pollen-bearing insects were caught. We do not know of a unique pollen marker that *H. zea* would naturally encounter in the northern US that would enable us to identify moths captured in the south as northern migrants. Stable isotopes exist as natural markers in biological systems that provide historical information about the study organism (Miller et. al. 1988, Kelly and Finch 1998, Wassenaar and

Hobson 1998). Stable isotope analysis is being recognized as a useful application to better understand ecological systems, including animal migration (see reviews: Gannes et. al. 1998, Hobson 1999).

General description of stable isotopes. Isotopes are forms of elements that are identical in their number of protons but differ in number of neutrons, which results in chemically identical elements that differ in mass. The small mass differences between isotopic forms of an element cause the isotopes to function differently in both physical processes and chemical reactions. Naturally-occurring variations in the abundance of the stable isotopes such as $^{2}H/^{1}H$ (δD), $^{13}C/^{12}C$ ($\delta^{13}C$), and $^{15}N/^{14}N$ ($\delta^{15}N$), in the tissues of animals have been used to trace nutritional origin and migration (Spain and Reddell 1996, Chamberlain 1997, Tayasu et. al. 1998, see review Hobson 1999) since the values of $\delta^{13}C$, $\delta^{15}N$, and δD in plants are transferred through the food webs that depend on them (DeNiro and Epstein 1981, Korner et. al. 1991). In this thesis we present stable isotope ratios of *H. zea* wing tissue and corn kernels.

Isotopic ratios are reported in terms of δ, i.e.

$$\delta D = [R(sample) / R(standard) - 1] \times 1000 \qquad \text{(eq 1.1)}$$

where R is the heavy/light isotope ratio. The δ value is expressed in terms of parts per thousand or per mil (‰). Typically, δ values are negative and interpreted as such: a less negative δ value indicates an enrichment of the heavy isotope relative to lighter isotope, a more negative value represents a sample depleted in the heavier isotope.

Since, the magnitude of natural isotope abundance changes is small, of the order of one part in 10^4 of the major isotope, successful measurement of such small differences necessitates very precise analysis. Isotope ratio mass spectrometry (IRMS) was introduced specifically for the analysis of the light gas stable isotopes (Nier 1947). (see Appendix I for description of an IRMS).

Stable carbon isotopes. Studies of carbon isotope ratios have been of value for studying the host use of herbivores because the rate at which ^{13}C and ^{12}C isotopes are fixed by plants differs among photosynthetic pathways. The two major photosynthetic pathways in higher plants are the Calvin cycle and C_4-dicarboxylic acid pathway, abbreviated as the C_3 and C_4 photosynthetic pathways, respectively, because the first product after CO_2 assimilation is either a 3-carbon chain acid, or a 4-carbon chain acid (Hatch and Slack 1970)**.** C_3 plants are typically dicots whereas C_4 plants are most commonly grasses, which are generally monocots (Smith and Epstein 1971). The stable isotopic ratios of higher plant carbon ($\delta^{13}C$) falls into 2 distinct groups due to isotope fractionation that differs by the photosynthetic pathway of the plant (Smith and Epstein 1971, O'Leary 1988, Boutton 1991). Fractionation is the change in isotopic abundance between chemical species due to physical or chemical processes. The isotopic difference between C_3 and C_4 plants was first recognized in 1968 (Bender) and it has been consistently shown that C_4 plants have proportionally greater content of ^{13}C than do C_3 plants (Tieszen and Boutton 1989). The greater fractionation against the heavier isotope (^{13}C) in C_3 plants results in a more negative (^{13}C-depleted) isotopic signature. The long-term reliability of $\delta^{13}C$ values used to differentiate between C_3 and C_4 plants (Farqhuar et. al. 1989), have provided a

dynamic tool for ecologists, anthropologists, paleontologists, and climatologists interested in the C_3-C_4 composition of diets of both ancient and contemporary organisms (Fry et al. 1978, Miller 1991, Tallamy and Pesek 1996, Webb et. al. 1998, del Rosario et. al. 2002). Lepidoptera wings reflect the carbon isotope fractionation that occurs in photosynthesis and can act as a diagnostic tool for determining the photosynthetic pathway of the larval host plant (Gould et. al. 2002). Carbon isotope analysis is a valuable indicator of alternate host plant use by *H. zea* because its two major hosts are corn (C_4) and cotton (C_3).

Stable hydrogen isotopes. The stable isotopic composition of atmospheric water is a function of temperature and depletion of isotopes that occurs as an air mass moves over continental land masses (Craig 1961, Daansgard 1964). Stable hydrogen isotope ratios in precipitation show continent-wide patterns (Daansgard 1964) with a general trend across North America of decreasing delta-D values in precipitation from the SE to higher latitudes in a NW direction. An increase in latitude, altitude, or distance inland will result in a decrease in δD values (depleted in deuterium) of local precipitation; therefore, there is also a depletion of deuterium from the east coast into the mid-west. Hydrogen naturally exists as two stable isotopes, protium ^{1}H and deuterium ^{2}H (or D). Deuterium, the heavier isotope, has a tendency to precipitate out first from a moist air mass, primarily because of differences in the vapor pressures of heavier and lighter water (i.e. DHO versus H_2O and $H_2{}^{18}O$ versus $H_2{}^{16}O$). In areas where ground water comes from local precipitation its isotopic composition is a weighted average of the annual precipitation.

Water taken up by plant roots and transported in the xylem shows no or very little fractionation between stem water and soil water (Smith and Ziegler 1990). Fractionation of water in plants typically occurs at the site of evapotranspiration in the leaves where evaporative enrichment in the heavier isotopes occurs, for example H_2O evaporates more readily than DHO. Plants that utilize groundwater during photosynthesis have been shown to reflect δD signatures in rainfall during the growing season (Yapp and Epstein 1982). The isotopic composition from the groundwater is eventually detected in higher trophic levels (Cormie et. al. 1994) and can therefore be used in geographical studies to reveal historical information on isotopically distinct regions. Preliminary evidence for using δD analysis of butterfly (Lepidoptera) wings to discern the origin of wing development was provided by Wassenaar and Hobson (1998) in a study using stable isotopes as geographical indicators of natal origins of migratory Monarch butterflies (*Danaus plexippus*). These authors demonstrated that δD values of whole wings from wintering Monarchs in Mexico could be used to trace their natal origins throughout their breeding range in the United States. Hobson et. al. (1999b) compared δD values of field-raised Monarchs to the isotopic composition of their host plant (*Asclepias* sp.) at natal sites throughout eastern North America, which suggests that wing tissue of Monarch butterflies is metabolically inert following synthesis. Results from this study showed a high correlation between the isotopic composition of Monarch (whole wing tissue) and milkweed (host plant-leaf tissue), which in turn corresponded closely with the long-term geographic patterns of δD values in rainfall.

Hydrogen exchange with ambient moisture. Hydrogen forms weak bonds with nitrogen and oxygen and is able to exchange with ambient water vapor. These hydrogen atoms are referred to as exchangeable hydrogen (Schimmelmann 1991). Non-exchangeable hydrogen refers to the portion of carbon-bound hydrogen in organic tissue that is not influenced by uncontrolled hydrogen isotope exchange with ambient moisture. The non-exchangeable hydrogen in a wing should represent the hydrogen isotopic composition of water molecules that were involved in insect growth and wing development. Because the exchangeable hydrogen can come from several sources at different times, it is not historically informative. At least a portion of the exchangeable hydrogen in an insect wing is able to exchange with ambient water vapor after the wing has formed. Therefore, a measurement of total δD would only in part reflect the composition of water that was present during larval feeding. It is possible to remove the uninformative exchangeable hydrogen by equilibrating all wings with water that has a known isotopic value. The isotopic composition of the non-exchangeable hydrogen can provide historic information about materials that were originally metabolized in building the tissues. Standardized samples allow one to compare the isotopic differences between the non-exchangeable hydrogen of individual samples.

Isotope summary. There are several advantages of stable isotope analysis for use in studies of Lepidoptera host use and migration. Firstly, there are large differences in the carbon isotopic composition of plants utilizing different photosynthetic pathways. Lepidoptera wing tissue reflects the isotopic signature of the larval plant host. This is a valuable tool for studying host use of butterflies and moths capable of utilizing different

host types. Secondly, with recent advances in technology, hydrogen isotope analysis is less expensive due to reduced labor and preparation materials compared to the offline equilibration method. Also, the new comparative method (Wassenaar and Hobson *in press*) requires only very small quantities, 350 ±10 μg, and sample throughput has increased to 120-160 (hydrogen samples) per day (Wassenaar and Hobson *in press*). Thirdly, several Lepidoptera species are known to migrate distances large enough to cross over continental isotopic gradients, which make them an excellent candidate for this technique (Smith and Sears 1982, Hendrix and Showers 1992, Wassenaar and Hobson 1998).

Weight and wing length. Although adult weight is a reasonable estimate of moth size and quality it can be affected by age, activity, and nectar/water feeding during the adult stage (Hardwick 1965). In contrast, wing length of adult insects apparently does not change after the wings are fully expanded and sclerotized, so any influence on wing size would have occurred during larval development and not during the adult stage. The larval stage provides a more sensitive estimate of the effects of stress because disruption of development typically occurs early in life.

Fluctuating asymmetry (FA). Small, random deviations from symmetry in bilaterally symmetrical individuals are commonly described by frequency distributions of absolute values of right-left (R-L) size differences, described here as fluctuating asymmetry (FA) (Palmer 1994). FA is widely recognized as the inability of the genotype to effectively buffer against environmental perturbations (Van Valen 1962). Inferences made on

developmental stability can be characterized by the pattern of FA. FA has traditionally been used as an indicator of genetic and environmental stress including chemical exposure and may give a general measure of the health of a population (Clarke 1992, Leary and Allendorf 1989). Traits that directly affect insect performance may result in strong correlations between asymmetry and fitness differences (Thornhill 1992).

If the quality and type of plant host affects phenotypic characteristics of adult moths then it may be possible to use morphological variation as a natural marker for determining larval host use, temporally and/or spatially. Also, morphological variation could be used in conjunction with other natural markers such as stable isotope ratios to increase the power of tests used to identify moth natal origin. Morphological variation in a population of *H. zea* may result from the variety of hosts that the insect is capable of exploiting and the nutritional value associated with each host through time and space. Some *H. zea* larvae may survive exposure to Bt toxin or other insecticides, which may cause variation in symmetry, size, or fitness of adults. These examples, if distinguishable within a population, could be potential indicators of the larval environmental quality.
These former examples, if distinguishable within a population, could be potential indicators used to determine factors about the larval environment.

CHAPTER II

STABLE CARBON ISOTOPE AND PHENOTYPIC ANALYSIS OF PHEROMONE TRAPPED, *HELICOVERPA ZEA*, FROM THE BRAZOS RIVER VALLEY, TEXAS

ABSTRACT

During 2000, male adult *Helicoverpa zea* were captured in pheromone traps in the Brazos River Valley, Texas. Stable carbon isotope analysis was performed on moth wings to determine the photosynthetic physiology of the larval host plant. We found that *H. zea* moths can be separated isotopically into 2 distinct groups, C_3 (-21 to -28) and C_4 (-10 to -15)‰, depending on the photosynthetic pathway of the plant that the larva developed on. In our study area, at any given date between February and October 2000, 40-100% of the moths developed on a non-cotton C_4-host. Moths that fed upon a C_4 host during the larval stage typically had larger wing length and were heavier than moths that fed upon a C_3 host. *H. zea* larvae were collected during August and September 2002 from non-Bt corn and Bt corn hybrids, grown at the Tidewater Research Station (TRS) near Plymouth, North Carolina. Analysis of fluctuating asymmetry (FA) in wing dimensions was conducted separately on moths collected in TX and NC to assess larval environmental quality. Our results indicate that there was no effect of host plant type on the degree of wing length asymmetry measured in the TX moth samples, however, there was a significant effect of DATE, and an interaction effect of HOST*DATE. The moths from early in the season were more asymmetrical than moths later in the season. Fluctuating asymmetry of mean wing length was compared between moths collected in NC that originated from Bt and non-Bt corn. Moths from Bt corn showed significantly greater asymmetry than moths from non-Bt corn. We have provided circumstantial evidence that suggests that *H. zea* moths are migrating into cotton growing regions of the Brazos River Valley from northern regions where corn or some other C_4 host is suitable for larval development.

INTRODUCTION

Insect resistance management. The recent commercialization of cotton and corn, genetically engineered to produce a delta-endotoxin from *Bacillus thuringiensis* Berliner (Bt), has focused renewed attention on the issue of southward migration because it could have a major effect on the rate at which *Helicoverpa z*ea Boddie may evolve resistance to this caterpillar-specific insecticidal protein (Bt toxin). To slow the evolution of Bt resistance, the US government requires that toxin-free host plants be available to *H. zea* populations as a refuge where caterpillars with toxin susceptibility genes can survive**.** In present efforts to delay resistance to Bt crops, growers must chose from at least one of the three structural refuge options: 95:5 external structured unsprayed refuge; 80:20 external sprayed refuge; 95:5 embedded refuge (EPA 2001). These refuge options have been developed based on data that does not include alternate host use or moth migration.

Because corn is a major host of *H. zea,* the limited use of transgenic corn in the Northern corn-belt results in a larger refuge that should decrease the rate of resistance evolution if there is substantial southward migration of *H. zea* moths late in the summer.

It is clear from the literature (Graham et. al. 1972, Cole et. al. 1973, Hillhouse and Pitre 1976, Eger et. al. 1982) that there are several possible alternate hosts for *H. zea* during the season. However, we lack quantitative data on utilization of these hosts. The complexity of *H. zea* movement amongst various alternate hosts requires more study so that each identifiable agroecosystem utilized by this pest will be considered when

incorporating refugia into insect resistance management strategies for *H. zea* and Bt crops.

The primary objective of this study was to determine larval host use based on analysis of season long pheromone trap collections of *H. zea* in the Brazos River Valley, a region in Central Texas. Two approaches were pursued in support of the primary objective. First, moth wings were examined by stable isotope analysis to determine the degree of larval feeding on host plants with different photosynthetic pathways. This technique seemed promising because *H. zea* is considered to have two major hosts, cotton and corn, that differ by their utilization of either the C_3 or C_4 photosynthetic pathways. Second, collections were examined for phenotypic differences that could indicate the type of host on which the adult *H. zea* had developed as larvae. This approach was undertaken based on the assumption that larvae, which developed on sub optimal hosts, would differ phenotypically from those that developed on optimal hosts.

Stable isotope analysis. Previous work had shown that wings of moths that developed as larvae on plants with C_3 physiology had ratios of ^{13}C:^{12}C (i.e. $\delta^{13}C$) that differed from those of moths whose larvae had developed on plants with C_4 physiology (Gould et al. 2002). A moth that is identified as having a $\delta^{13}C$ value similar to a C_4 plant can be classified as not having developed on cotton, or any other C_3 host. Similarly, a $\delta^{13}C$ value that is within the $\delta^{13}C$ range for a C_3 plant would indicate that the moth did not develop on corn, or any other C_4 host. Therefore, our reports of C_3 and C_4 moths collected in the Brazos River Valley, Texas, can eliminate plant types as a food source during the larval

stage but cannot conclusively identify different host species utilizing the same photosynthetic pathway. For example, when we report that the moth had a C_4 signature, we can say with certainty that the larva did not feed on a cotton plant or any other C_3 plant, but it could have developed on sorghum, corn, or a C_4 native plant.

Phenotypic analysis. Measurements of moth dry body weight and wing length were used to examine seasonal patterns in moth quality, and affects of larval host on moth quality. Although adult male weight is a reasonable estimate of moth size and quality (Tammaru et. al. 2002) it can be affected by age, activity, and nectar/water feeding during the adult stage. In contrast, wing length as estimated based on vein measurements of adult insects apparently does not change after the wings are fully expanded and sclerotized, so any influence on wing size would have occurred during larval development and not during the adult stage. In bilaterally symmetrical organisms, fluctuating asymmetry (FA) has traditionally been used as an indicator of genetic and environmental stress including chemical exposure and may give a general measure of the health of a population (Clarke 1992, Leary and Allendorf 1989). Host plant quality during larval development may affect FA in adult characteristics, such as wing length, causing greater asymmetry in moths whose larvae fed on sub-optimal hosts. Development on a host plant that has been genetically treated with the Bt toxin will undoubtedly cause developmental stress and it may increase levels of asymmetry.

MATERIALS AND METHODS

Field collection of *Helicoverpa zea* moths in Texas. Adult *Helicoverpa zea* were collected by sex pheromone trappings from 18 February to 2 October, 2000, in Brazos River Valley a region in Central Texas about 350 miles north of the Lower Rio Grande Valley (LRGV) (Figure 2.1). The 6 Harstack® pheromone traps used were each baited with Zealure®, a synthetic sex pheromone that attracts male *H. zea.* However, as a precaution, a randomly selected set of moths was examined for *H. zea* characteristics. Live moths were removed from the traps 3 times per week with the interval between collections ranging from 2-4 nights of moth activity. There were a total of 28 collection dates. The time interval between collection dates was 1 or 2 weeks. Approximately 100 moths were randomly sampled from each of these 28 collection dates and shipped to North Carolina State University in plastic bottles filled with 95% ethanol.

Sampling of Texas moths. For each of the 28 collection dates, 25 moths were randomly selected from each sampling date. Whole moths were placed on paper towel to air dry prior to analysis. The same moths were first used for phenotypic analysis and subsequently for isotopic analysis.

Collection of North Carolina moths: *H. zea* larvae were collected, at 3 separate times during August and September 2002, from non-Bt corn and Bt corn hybrids, grown at the Tidewater Research Station (TRS) near Plymouth, NC. One hundred ears of corn per plot were examined for the presence of a 4^{th} or 5^{th} instar *H. zea.* Ears found to contain a larva in the 4th or 5th instar were collected and placed, by plot, in 68.1 liter buckets containing

2 inches of potting soil and a wooden rack to elevate the ears above the soil. The buckets were stored in a shed. After 7 to 12 days the potting soil was sifted for *H. zea* pupae. If any ears still contained large larvae, they were placed back into the buckets and the soil was resifted within 5 days. Pupae were put in 1 oz. diet cups, weighed, and stored in a rearing room at 29°C; 65 - 70% humidity; 15 hrs light and 9 hrs dark. Male adults were placed in 70% ETOH. Only male *H. zea* were used for phenotypic analysis.

Isotopic analysis of Texas moths. Gould et. al. (2002) showed that storing moths in ethanol prior to analysis had no effect on the carbon isotope ratios of the wings. Each left forewing was prepared for carbon isotopic analysis by cutting it into small pieces and placing the pieces into a 5 x 9 mm tin capsule. The capsule was then tightly folded into a cube that would fit into an autosampler. Scissors and forceps used to handle the wings were cleaned with 90% ethanol between each sample. The wing tissue was converted to CO_2 by micro-Dumas combustion using a Carlo Erba NA 1500 CHN Analyzer coupled to a Finnigan Delta C Mass Spectrometer, at the University of Georgia. The isotope standard reference material was bovine muscle powder (NIST-RM 8414) from the National Institute of Standards and Technology (NIST) with a precision of about ± 0.1‰ for $\delta^{13}C$. All $\delta^{13}C$ values are relative to V-PDB scale.

Determining measurement error for phenotypic analysis. All measurements were performed by T.R. to prevent any inter-observer measurement error. Measurement repeatability was determined for wing length by using a subset (n=21) of the moths collected in North Carolina (n=71) and conducting a blind repeat measurement of wing

length on the same day as the initial measurements. Paired left and right forewings were analyzed for directional asymmetry. All differences were subjected to paired t-tests for significance at the 0.05 probability level.

Phenotypic analysis. Only male *H. zea* moths from Texas and North Carolina were analyzed. After moths were air-dried whole body weight was determined to the nearest milligram using a microbalance (Mettler AE50). Measurements of the left and right forewing of each moth (n=605; e.g. Texas 2-18-00(#5): LW=16.625mm, RW=16.216mm) was conducted with a MTI™ digital video camera, mounted on an Olympus SZx12 scope, interfaced to a personal computer with version 3.0 Image-Pro Plus. Individual wings were laid flat and covered with a glass slide to hold them in place while photographing. All measurements were made to the nearest 10^{-3} mm at 3.5X@0.5X magnification. Wing dimensions measured for the purposes of our study were defined based on the wing venation of a typical noctuid moth described by Borror et. al. (1989). The 3 dimensions we measured are: (1) M_3, measured as a straight line starting at the forewing base and ending at the outer margin of the forewing on the M_3 vein; (2) Cu_2, which is the first branch of the cubitus and was measured by tracing a line from the basal portion of the branch to the outer margin of the wing; (3) Medial, was measured from the main stem of the medial vein at the wing base to its branching point in the middle of the wing (Figure 2.2). Our initial sample size included 700-paired forewings, however only 605 pairs were used for the analysis because some wings were damaged to the point that the described wing dimension(s) was unrecognizable.

Analysis of fluctuating asymmetry. We analyzed our data with two indices of fluctuating asymmetry, FA1 and FA2 as described in Palmer (1994). The FA1 index is the absolute value of the difference in right versus left wing lengths for a single moth, and therefore has the advantage of being simple to explain and understand. FA2 represents the FA1 of a moth divided by the average length of its left and right wing lengths. FA2 is a more accurate index of asymmetry when there is a substantial difference in the average wing lengths of moths from different samples being compared. This is to say, the FA1 index may prove to be misleading if a comparison is made between insects with very large and very small wings. For example a moth wingspan of 100 mm that differs in the left and right wing length by 1 mm is going to be relatively more symmetric than a moth wingspan of 10 mm where the left and right wing differ by 1 mm. The indices, FA1 and FA2, were subjected to a two-way ANOVA using PROC GLM in SAS (1999). Our analysis includes an investigation of the effect of DATE, HOST and an interaction effect of DATE*HOST on the dependent variables of asymmetry: FA1 and FA2.

RESULTS

Isotope results. Figure 2.3 presents the $\delta^{13}C$ values of 696 *H. zea* collected in the Brazos River Valley, Texas. The $\delta^{13}C$ values for the individual moths fall into 2 groups - corresponding to the range in value attributed to C_3 and C_4 plants. Based on previous work (Gould et al. 2002), moths with $\delta^{13}C$ values between -28 and -21 units per mil (‰) are considered to have a C_3 host signature, whereas moths with $\delta^{13}C$ values between -15 and -10 ‰ developed on C_4 host. There are three moths "X" with $\delta^{13}C$ values that lie between the two assigned groupings, $(-21 < X < -15)$‰. Figure 2.4 shows the proportion of C_4 to C_3 adult moths captured in the Brazos River Valley from 18 February to 2 October. The dotted line in figure 2.4 represents the expected decline in *H. zea* population after corn in this area of Texas has reached 100% silking and begins to dry (Johnson *et al*. 1975, Lopez *pers*. *comm*.).

Phenotypic analysis:

Determining the most appropriate measurement of moth size: The left and right forewings of Texas moths were analyzed separately for correlations between dry weight versus specific wing measurements, and between the 3 specific wing measurements themselves (Table 2.1). Table 2.2 shows a correlation matrix of phenotypic characteristics of *H. zea* collected in the Brazos River Valley. The M_3 wing length for the left and right wing had the highest correlation with weight (LW: 0.58; RW: 0.61) compared to the other 2 wing dimensions. Both the Cu_2 and Medial wing lengths were highly correlated with M_3 (LW: 0.86, 0.86; RW: 0.89, 0.89) but were less correlated with each other (LW: 0.60; RW: 0.66). Since the M_3 dimension had the highest correlation

with body weight and was highly correlated with the other two measurements, it was chosen as the best wing characteristic for further analysis (Table 2.2).

Determining effects of Bt on size and symmetry. Repeat measures of 3 wing dimensions of the same set of 21 individual NC moths showed no differences (M_3, P= 0.1073; Cu_2, P=0.0511; Medial, P=0.0482) between measurements (Table 2.7). Bt moths were significantly smaller in all 3 mean wing dimensions than non-Bt moths (M_3, P <0.0001; Cu_2, P <0.0001; Medial, P= 0.0021); however, Bt moths had a mean weight significantly heavier than non-Bt moths (P= 0.0029) (Table 2.8). The moths from nBt and Bt corn in NC were at least 3X heavier than the moths captured in Texas, including moths from both C_3 and C_4 host types (Table 2.10). Fluctuating asymmetry was compared between Bt and non-Bt moths using indices, FA1 and FA2, for the 3 separate mean wing dimensions. Only one comparison was significant, which was the FA2 index used to compare the M_3 wing lengths between Bt and non-Bt moths, which showed greater asymmetry in the Bt moths (Table 2.9).

Effects of host on wing length, weight, and fluctuating asymmetry: Hereafter, the term wing length will be used when referring to the analysis of the M_3 measurements, unless otherwise stated. Wing length and weight are highly variable within collection dates; however, separating the C_3 moths from the C_4 moths reduces some of the variation. Moths that fed upon a C_4 host during the larval stage typically had larger wing length and were heavier than moths that fed upon a C_3 host (Figure 2.5 and 2.6). A two-way ANOVA on wing length and moth weight shows that some of the variation can be

explained by the effect of HOST, and DATE, as well as an interaction effect of HOST*DATE (Table 2.4). Figures 2.7 (a&b) and 2.8 (a&b) illustrate the seasonal variation in standard deviations of the mean lengths and weights for moths from a C_3 and C_4 hosts, respectively.

In Figure 2.9 the ratio of *H. zea* wing length to dry body weight across the cropping season provides a preliminary analysis of suggestive differences between locally produced and immigrant *H. zea* moths. Ratios nearest to 1 indicate moths more uniform in size compared to larger ratios, which indicate moths with a comparatively larger wingspan and lighter body. The smallest ratios between June and July are in synchrony with the emergence of *H. zea* from local corn. Ratios are highest for C_4 moths between March and May and between August and September when *H. zea* immigration is expected from areas where corn silks earlier or later in the season and is unavailable as a suitable host in the study area. An analysis of variance shows that DATE has a significant effect on the ratio of wing length to body weight for C_4 moths (P= 0.0310); however, there is no apparent effect of DATE on the length/weight ratio for C_3 moths (P= 0.7723).

The wing length of the left and right forewings of 605 individual moths were averaged and the overall sample mean and standard deviation was 16.3165 ±0.9325 mm. The mean of FA1 was 0.2821 mm (SD ±0.2778, n=605), indicating that approximately 68% of the moths measured have left and right wings which differ in length by 0.5599 mm or less (i.e. 0.2821 + 0.2778 mm). The mean FA2 (unitless index) is 0.0173 (SD ±0.01692, n=605), indicating that for approximately 68% of the moths measured, the left and right

wings differ from each other by 3.42 % or less of their average length. As stated in the introduction the index FA2 is often a more accurate estimator of the asymmetry in wing vein length, however, it should be noted here that the indices, FA1 and FA2, are not substantially different from each other and this is probably because average difference in *H. zea* wingspan is small (~25mm).

Figures 2.10 and 2.11 show a relationship between date, host and asymmetry; there appears to be no effect of host plant type on the degree of asymmetry, however, there appears to be some relationship between date and asymmetry. The moths from early in the season are more asymmetrical, having a higher FA1 and FA2 index, than moths later in the season. A two-way ANOVA on the indices FA1 and FA2 of asymmetry shows that some of the variation can be explained by the significant effect of DATE, and an interaction effect of HOST*DATE. There was no effect of HOST on asymmetry. The significance of these variables on asymmetry is illustrated in Table 2.5.

DISCUSSION

Helicoverpa zea plant host use:

Stable carbon isotope analysis. There is a definite $\delta^{13}C$ grouping based on the physiology of plant host. We have demonstrated this with 696 individual moths collected at Brazos River valley, Texas (Figure 2.3). Organisms that develop on plants with C_3 physiology typically have a $\delta^{13}C$ range of -22 to -33‰ and plants with C_4 physiology have a $\delta^{13}C$ range of -9 to -16‰ (Boutton et. al. 1991). The most evident grouping from Fig 2.3 is: C_3 (-21 to -28) and C_4 (-10 to -15)‰, which agrees with Boutton et. al. (1991) findings. Gould et. al. (2002) analyzed the carbon isotope signature of moths that developed from field larvae raised in the laboratory on either corn or cotton and found no overlap between cotton-reared and corn-reared moths. Of the 696 individuals that we measured we found a few moths that showed intermediate $\delta^{13}C$ values ($-21 < X < -15$)‰. We speculate that these individuals with intermediate values may have fed, as larvae, for a considerable amount of time on both corn and one of the dicot weeds that are often found in corn fields. Alternatively, they could have fed on another host that had an intermediate isotope value. Insect integument may not be devoid of metabolic activity once it is formed. Miller et. al. (1985) showed that the carbon isotope ratio in cockroach chitin (*Periplaneta Americana* L.) is not fixed and will reflect changes in diet of differing carbon isotope ratios. Tallamy and Pesek (1996) showed that beetle chitin of Chrysomelids is not entirely inert since the adult diet caused a shift in the carbon isotope ratio from the C_4 signature (-9.94 ± 0.10‰) of the larval host to an intermediate value (-18.42 ± 0.07‰) in the adult that fed strictly on a C_3 host (-23.70 ± 0.13‰) during the adult stage. Although the 2 previous studies show isotopic shifts in chitin, non-chitinous

tissues such as Lepidoptera wing cuticle may have little or no metabolic activity to result in a detectable isotopic shift influenced by adult diet. Out of 696 moths we observed intermediate isotope values in 3 individuals, which suggests that it is uncommon for larvae to feed on more than one host.

Alternate host use of *H.zea* moths collected in Texas. Winter survival of *H. zea* has been well studied (Roach and Adkisson 1971, Stadelbacher and Pfrimmer 1972, Rummel et. al. 1986, Pair et. al. 1987) and conditions in Texas report that commencement of diapause peaks within late September through October and the onset of emergence occurs from mid-April to mid-May (Graham and Fife 1972, Rummel et. al. 1986). Our study showed that the greater portion of moths collected from February to early April were identified as having a C_4 isotopic signature whereas a greater percentage of moths collected throughout April into mid-May had a C_3 signature (Figure 2.4). The latter is best explained by the emergence of overwintering pupae whose larvae had fed on Texas cotton prior to diapause. But where are the overwintering C_4 moths coming from if there were little or no suitable C_4 host plants in Texas during the fall? Peak C_4 moth captures during March could be the result of immigrants from southern Texas and Mexico that had developed on early season corn. Weather patterns during March provide transport opportunities for airborne insects to be moved rapidly from northeast MX and southern TX northward into central TX (Harstack et. al. 1986, Muller and Tucker 1986). Since, the tobacco budworm (*Heliothis virescens*) emerges from diapause earlier than *H. zea* it is highly unlikely that the large numbers of *H. zea* caught in pheromone traps were from overwintering moths, in this study area, because *H. virescens* typically begin emerging

early April and peak trap capture of *H. zea* were observed during March (Harstack et. al. 1986). Ratios of wing length to dry body weight for *H. zea* identified as having a C_4 isotope signature may prove to be a valuable measurement for distinguishing immigrant moths from locally produced moths. We expect migratory moths to have larger ratios values compared to moths produced locally because migrants will use more of their fat reserves during flight, which should result in a higher wing length to body weight ratio than moths that are locally produced. We observed higher ratios for C_4 moths between March and May and between August and September when *H. zea* immigration is expected from areas where corn silks earlier or later in the season, respectively, and is unavailable as a suitable host in the study area compared to the most abundant local production of C_4 moths in the study area, which occurs during June and July. We have supporting evidence of immigration from our analysis of variance, which showed a significant DATE effect on the ratio of wing length to body weight for C_4 moths ($P= 0.0310$). Our preliminary analysis of wing length to dry body weight ratios for *H. zea* throughout the cropping season in the Brazos River Valley are far from conclusive; however, future analysis of moths from other years may result in a stronger trend.

A recent update on *H. zea* susceptibility to pyrethroids in the cotton belt showed an increasing tolerance; however, Texas had the lowest percent survival at both 5 and 10 μg/vial dose, 1.9% and 0.4% respectively, n=958 (Martin et. al. 1999). Therefore, it can be expected with the widespread use of highly toxic insecticides, including pyrethroids, on cotton during August that the decline in *H. zea* would minimize the amount of moths entering diapause in Texas. However, *H. zea* immigrating into this area during the fall

would escape the insecticide applications earlier in the summer and could develop on late season cotton, thus, contributing C_3 moths to the following spring population.

Corn in Texas is planted early in the growing season and it is during the second generation of *H. zea* when corn is silking and populations of *H. zea* can reach very high densities. A significant proportion of *H. zea* that develop on early season corn (C_4 host) are expected to emerge during June and early July resulting in a higher percentage of C_4 moths compared to C_3 moths in response to host plant availability and quality. *H. zea* moths were observed in high proportion of C_4 to C_3 host type during June (Fig 2.4), the time that corresponds with the maturation of the Texas corn (NASS 2002). The *H. zea* developing on wild hosts during the spring contribute little to the widespread infestations during the summer (Rummel et. al.1986, Raulston et. al. 1998), suggesting that corn is the major host contributing to the *H. zea* population in June and July. This pattern also agrees with Raulston et. al. (1990) study in the Lower Rio Grand Valley (LRGV), Texas, showing peak adult emergence from fruiting corn in early June.

By the third generation, corn silks have dried and corn is not as attractive as alternate hosts including cotton (C_3) and sorghum (C_4) (Johnson et al. 1975, Hillhouse and Pitre 1976). The production of locally produced C_4 moths is expected to approach zero after July due to the absence of suitable C_4 hosts; however, our results demonstrate a relatively high percentage of C_4 moths (as high as 90%) present during August that slowly decline into October, but fall-off no less than 40%. These results agree with Gould et. al. (2002) study in Louisiana and Texas of data collected over 4 years (1997-2000). This means that

at any point in time of our study, cotton or any other C_3 host could not have produced more than 60% of *H. zea* moths. These results demonstrate the presence of moths between July and October that had fed on C_4 hosts, in regions of Texas consisting almost exclusively of C_3 host plants - there are no other recorded C_4 hosts of *H. zea* in the Brazos River Valley area during this time suitable as *H. zea* hosts (Lopez *pers*. *comm*.). There are at least four hypotheses that may explain the high abundance of C_4 moth later into the season. First hypothesis: larvae are actually developing on drying corn. Drying corn is an unsuitable host for *H. zea* and it is unlikely to support large population sizes for generations late in the season. Second hypothesis: *H. zea* populations in September could be the result of a summer aestivation; however, there are no published reports that *H. zea* is able to aestivate in the study area (Lopez et al. 1995). Third hypothesis: larvae are developing on other C_4 hosts such as sorghum or wild hosts with a C_4 signature. It is an unlikely that a high abundance of *H. zea* came from sorghum or wild hosts late in the season because these are minor hosts in the study area and would contribute relatively little to the late season populations. It is believed that *H. zea* migrants into the northern corn-belt states perish with the onset of winter (Rabb and Kennedy 1979, Fitt 1989). However it is possible that as corn in the northern corn-belt states begins to dry *H. zea* migrate south via wind trajectories to cotton to complete another generation (Fourth hypothesis). We have provided circumstantial evidence that suggests that *H. zea* moths are migrating into cotton growing regions of the Brazos River Valley from northern regions where corn or some other C_4 host is suitable for larval development. Lopez et al. (1995) investigated the temporal pattern of *H. zea* in the Brazos River Valley during 1990 and 1991 and found that it was indicative of moth immigration. Even though their moth

collections were not separated into host type (C_3 and C_4) by way of stable carbon isotope analysis, the influx of moths from late August to mid-October suggests immigration because the dramatic increase cannot be accounted for on the basis of earlier populations.

Measurement error in wing length analysis.

Texas moths. Left wing lengths were measured first followed by the right wing lengths in a more-or-less sequential order by collection date, commencing with February samples and measuring wings through to October samples. Most of the measurements were made within a period of a month. Plant type, C_3 or C_4, was unknown and randomized within each collection date; therefore, the effect of host on fluctuating asymmetry within collection dates is not biased and is presumably unaffected by comparison between dates. However, the effect of date on FA for moths collected in Texas may involve measurement error due to non-randomized analysis.

North Carolina moths. Moths collected in Texas were analyzed phenotypically during the summer of 2001. Weight was measured on a microbalance and therefore was not influenced by the observer; however, wing dimensions were orderly measured by a single observer. First, each wing dimension for the left wings was measured for each collection date followed by right wing dimensions for each collection date. For all 3 wing dimensions of the Texas moths, the left wings were significantly larger than those of the right wings suggesting directional asymmetry (Table 2.2). In a follow up study to test the cause for directional asymmetry observed in the Texas moths, *H. zea* were collected from corn in North Carolina and were randomized by left and right wings and measured within

the same day. The same day analysis differed from the Texas moth analysis, which included daily measurements over a month duration. Results of wing asymmetry in the NC moths were variable across wing dimensions, however, M_3 for both the nBt and Bt moths showed no directional asymmetry (Table 2.2). Directional asymmetry observed in the Texas moths may have been an artifact of measurement error due to ordered sampling of left and then right wings. We also repeated measurements for a random subset of NC moths (n=21) on the same day of the initial analysis. The moths collected in NC had better repeatability of wing dimension measurements than moths collected in TX, which suggests that the NC moths may be a better estimator of directional asymmetry (Table 2.2).

Phenotypic analysis. Phenotypic differences between C_3 and C_4 adult moths, collected in pheromone traps in Texas, typically resulted in heavier C_4 moths complimented with a larger wing length (Fig 2.5 & 2.6). Of particular interest are the late season moths, these results are surprising if in fact the C_4 moths are representing a local population of moths in the Texas area that had developed on senescing corn. Senescing corn becomes dry and is less likely to support larval development, Fletcher (1941) showed that food with high moisture content was more conducive to larval survival; therefore, it is highly unlikely that any *H. zea* that survive on drying corn would be larger than *H. zea* that developed on a C_3 host. The most dominant C_3 host in Texas during the late summer is cotton. Cotton in the Texas area typically supports a 3rd and 4th generation of *H. zea*. Cotton being most preferred, by *H. zea* for oviposition, when corn is unavailable. However, the C_4 moths

could represent a migrant population that had developed on corn during the silking stage in the northern corn-belt states where corn matures late into the growing season.

Pupal weight has been shown to have a positive correlation with adult lepidopteran fitness (Leuck and Perkins 1972, Tammaru et. al. 2002) and has been used as an indicator of *H. zea* fitness for larvae that survive exposure to the Bt toxin in transgenic crops, for example, Storer et. al. (2001) have shown that larvae exposed to the Bt toxin have reduced pupal weight by 33%. There is a significant interaction effect of host type and seasonal date on adult male moth size for both the C_3 and C_4 moths collected in Texas. The smaller sized C_3-moths could be the result of feeding on a sub-optimal host. Or there could be a significant contribution of survivors from Bt cotton that are smaller in wing length on average presumably due to the exposure of the Bt toxin during development (Table 2.8).

Moths whose larvae survive exposure to the Bt toxin are expected to weigh less than moths that developed on nBt corn, which is not the case with the moths collected from Bt corn in NC where the Bt moths weighed significantly more than the nBt moths from the same location (Table 2.8). The mean weight difference between the Bt and nBt moths from NC is 0.012 g (P= .0029), which is significant but not especially alarming. However, in our study both the Bt and nBt moths from NC weighed about 3X more than the TX moths (compared to C_3 and C_4 moths separately or together). The TX moths are identified by stable isotope analysis has having developed on either C_3 and C_4 hosts, C_3 moths are expected to have come from local hosts (based on our study area) and therefore

not subjected to the stress of migration, which means their weight is expected to be more comparable to the NC moths than C_4 moths. However, the NC moths were killed immediately upon emergence which would make them as heavy as possible and C_3 moths caught in pheromone traps will have aged and depleted some of their fat reserves. We did show, from our study in the Brazos River Valley, that C_3 moths typically weighed more than C_4 moths (Figure 2.6). What we don't know is what proportion, if any, of our pheromone captured moths in TX came from a Bt host? It is unlikely that there is a significant contribution in our trap captures of survivors from Bt crops. What we find interesting is the magnitude of 3X the weight for moths collected in NC (directly from nBt and Bt corn as larvae) compared to trap captured moths in TX (Table 2.10). We cannot conclude from this study why the moths from NC corn are substantially heavier than the Texas moths, but we can present the differences between the two collection methods and suggest how they may affect weight. Moths from NC were killed within the day of emergence, whereas, moths captured in pheromone traps in Texas are variable in age. Older moths especially those that have migrated depend on reserves for nutritional support and will have depleted their fat reserves, therefore, weighing less than recently emerged adults. Moths collected in pheromone traps are more likely to suffer wing damage because they tend to lose scales and tatter their wings trying to fly in a confined space. The TX moths were stored in alcohol for over a year, whereas, the NC moths were stored in alcohol less than a month. TX moths analyzed from long-term storage (>1 yr) in alcohol were often missing legs, antennae and a large portion of scales.

Summary of techniques used to determine *H. zea* plant host use. Our investigation into the use of stable carbon isotope analysis to determine which moths are feeding on plants other than cotton has proved to be a reliable technique. Phenotypic analysis of adult characteristics may provide supporting evidence for determining a more specific host type other than just C_3 or C_4 host. Fluctuating asymmetry of moth traits such as wing dimensions or differences in male moth weight could possibly be used as an indicator of host quality, which may further suggest the identity of the host type (i.e. corn opposed to sorghum, Bt opposed to non-Bt host). Our findings show significant phenotypic differences between moths raised on either a C_3 or C_4 host or those raised Bt opposed to non-Bt corn; however, further investigation is needed to determine the circumstances in which phenotypic analysis may be a reliable and useful indicator of larval plant host.

IRM and alternate host use. Changes in cyclicity due to prolonged development have potential to cause asynchronous population peaks with host development. Stinner et. al. (1977) model to demonstrate the effects of intraspecific competition on survival and population cyclicity showed that increased development variability had a large impact on the apparent generation time (the more variable development is the larger the reduction in generation time). The effects on population cyclicity are of particular significance in polyphagous species such as *H. zea* that respond to various hosts and their respective phenological stages. Not only are different hosts and their respective stages capable of affecting *H. zea* development rate, crops sprayed with Bt or transgenically expressing the Bt toxin have been shown to slow larval development (Storer et. al. 2001). *Helicoverpa armigera* (Hübner) is China's equivalent of *H. zea* (Hardwick 1965), a major pest of cotton, corn, and sorghum. Wu et. al. (2002) have been studying *H. armigera* in relation

to Bt crops and have observed longer larval development on Bt cotton than non-Bt cotton. Longer larval development on Bt crops could cause the overall emergence of moths to be later than in non-Bt crops. For instance, if larvae are developing on a sub-optimal host such as Bt-cotton in Texas then moth emergence in Texas may occur late in the summer. Late moth emergence in Texas could prove to be beneficial in regards to insect resistance management because it may coincide with moths that are immigrating into Texas from non-cotton (such as corn), non-Bt hosts in the north (Figure 2.4a). Whereas, *H. zea* moth emergence from non-Bt cotton in Texas may not coincide with the moth emergence from Bt-cotton in Texas defeating the purpose of a local refuge by decreasing the likelihood that mating of susceptibles with potentially resistant *H. zea* will occur. Adequate migration of susceptible genotypes into Bt crops may support the refuge strategy for slowing the evolution of resistance. Seasonal synoptic weather patterns clearly depict wind trajectories that are suitable for statewide moth migration (Westbrook et. al. 1997, 1998) in both the northern and southern directions in TX (Pair et. al. 1997). However, moth behavior has not been taken into account here and cannot be concluded from these trajectories alone. It is unknown whether the moths immigrate into an area to oviposit, mate, die, overwinter, or feed before further migration. Also, their fitness after long distance travel has yet to be determined. Fecundity, competitive behavior, and survival rate are all important characteristics that may be negatively affected by the harsh conditions of long distance migration. Importantly, fecundity is highly variable in *H. zea* and is affected by larval nutrition (Isley 1935). Mortality during the egg and early larval stages are extremely high in *H. zea* (Hardwick 1965). Therefore, migrant moths may be of little significance in regards to inflated pest infestations depending on the suitability of

the larval host. Another important consideration is that migratory moths may be exposed, over multiple generations, to sub-lethal doses of the Bt toxin, increasing the selection pressure for resistance. Desirable *H. zea* immigrants into Bt crop areas should have at least two important characteristics. They should be susceptible to the Bt toxin, and their arrival time should coincide with the emergence time of resistant individuals from local Bt crops. It is advantageous for *H. zea* in the northern corn-belt states to migrate south as corn becomes too dry so they can escape the onset of winter and to exploit periods of resource abundance occurring later in the season.

Questions raised from this study. Do females have a different flight behavior than males? The pheromone traps only captured males thus the inferences made from this study can only project onto the male population of *H. zea*. Perhaps females are more inclined than males to take flight for the chance of being relocated to a suitable host for oviposition. Therefore, not including females in the analysis may lead to bias conclusions regarding alternate host use. Do moth survivors of larvae exposed to Bt toxin, have the same fitness and flight activity as moths that have not been exposed to toxin?

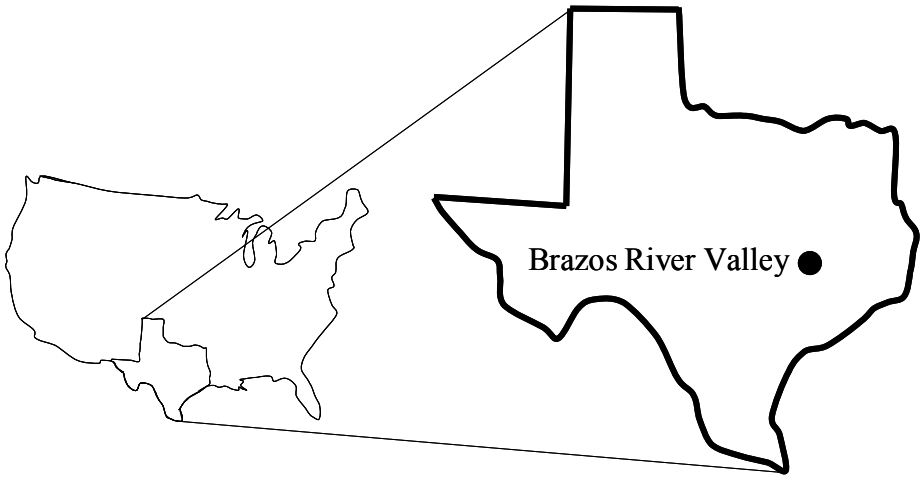


Figure 2.1. Collection site in the Brazos River Valley near College Station in Brazos County, Texas.

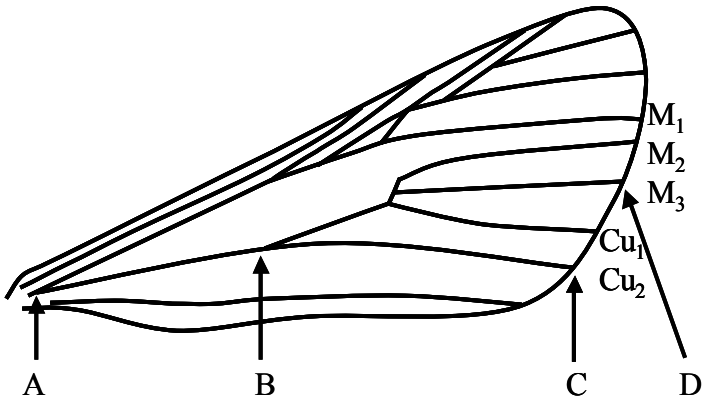


Figure 2.2. Diagram of the right forewing of *H. zea* showing abbreviations for selected longitudinal veins. M, medial; Cu, cubitus. Wing dimensions: M_3 = straight line from point A to D; Cu_2 = straight line from point B to C; Medial = straight line from point B to C.

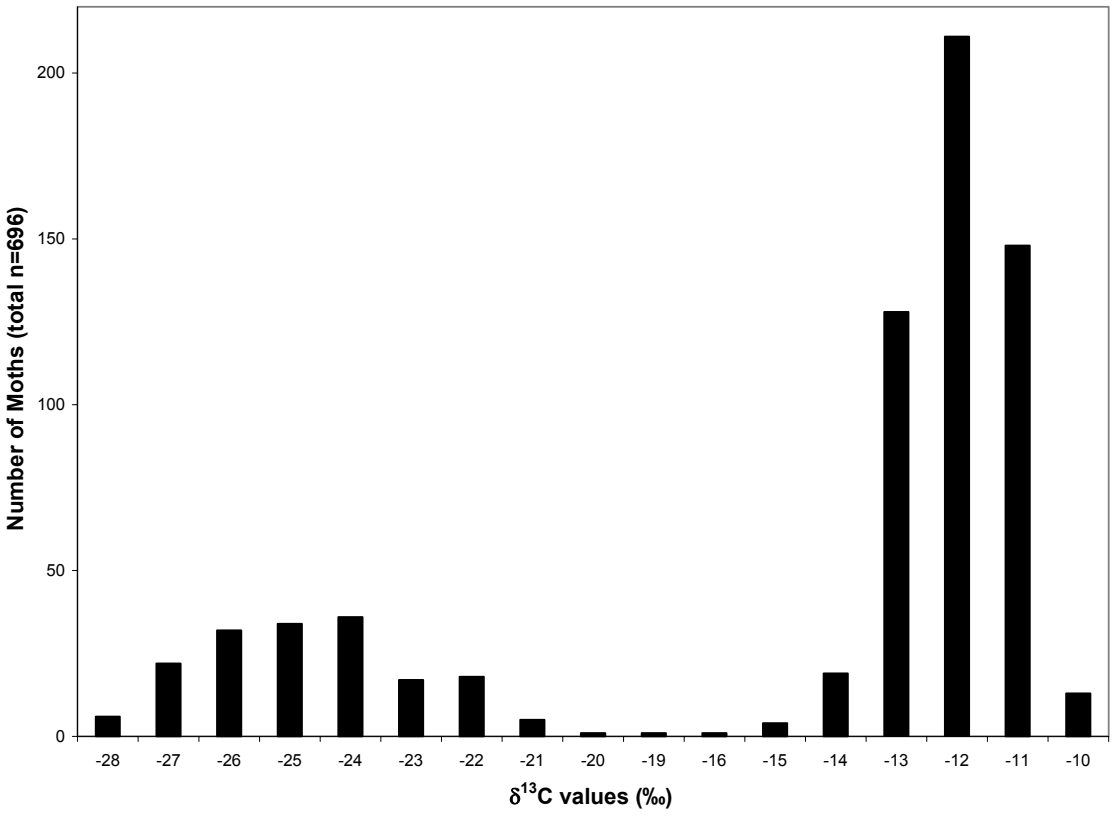


Figure 2.3. $\delta^{13}C$ distribution of pheromone trapped moths from the Brazos River Valley area, Texas from 18 February to 2 October 2000. The $\delta^{13}C$ values for the individual moths fall into 2 groupings. These groupings are recognized as a C_3 host type, which includes moths with $\delta^{13}C$ values between -28 and -21 ‰, and a C_4 host type having $\delta^{13}C$ values between -15 and -10 ‰.

Table 2.1. Wing dimension to dry body weight correlations of left and right forewings of pheromone trapped moths from the Brazos River Valley, Texas. The average seasonal correlation is highest for wing length, M_3, of both the left (0.58) and right (0.61) wing.

	M_3		Cu_2		Medial	
DATE	**LW**	**RW**	**LW**	**RW**	**LW**	**RW**
18-Feb	0.513	0.417	0.637	0.568	0.285	0.188
24-Feb	0.351	0.529	0.302	0.581	0.286	0.31
1-Mar	0.809	0.819	0.765	0.718	0.614	0.753
9-Mar	0.827	0.736	0.681	0.733	0.567	0.478
17-Mar	0.781	0.797	0.755	0.76	0.671	0.656
22-Mar	0.724	0.747	0.607	0.692	0.484	0.589
27-Mar	0.786	0.852	0.427	0.521	0.706	0.769
3-Apr	0.59	0.715	0.515	0.544	0.294	0.623
10-Apr	0.824	0.731	0.695	0.751	0.753	0.606
18-Apr	0.817	0.78	0.789	0.759	0.697	0.517
6-May	0.73	0.773	0.619	0.632	0.714	0.727
11-May	0.699	0.815	0.703	0.795	0.549	0.782
16-May	0.39	0.718	0.58	0.595	0.437	0.571
23-May	0.456	0.366	0.38	0.377	0.494	0.342
1-Jun	0.661	0.649	0.599	0.518	0.541	0.631
7-Jun	0.399	0.536	0.475	0.468	0.242	0.485
13-Jun	0.317	0.387	0.369	0.392	0.199	0.273
26-Jun	0.572	0.541	0.629	0.594	0.301	0.389
29-Jun	0.3	0.279	0.438	0.096	0.172	0.29
5-Jul	0.827	0.874	0.826	0.79	0.645	0.778
21-Jul	0.512	0.497	0.414	0.475	0.419	0.415
26-Jul	0.087	0.225	0.138	-0.139	0.272	0.235
2-Aug	-0.274	-0.257	-0.199	-0.165	-0.276	-0.265
11-Aug	0.857	0.853	0.787	0.849	0.794	0.836
18-Aug	0.674	0.704	0.708	0.641	0.629	0.732
22-Aug	0.878	0.89	0.873	0.886	0.829	0.846
15-Sep	0.799	0.862	0.733	0.721	0.837	0.838
2-Oct	0.326	0.241	0.225	0.117	0.379	0.351
MEAN	0.579714	0.609857	0.5525	0.545321	0.483357	0.526607
STDEV	0.271463	0.26593	0.2387927	0.274034	0.250809	0.255214

Table 2.2. Correlation matrix of wing dimensions and weight for left and right forewings of *H. zea* collected in Texas, 2000.

LW	wt	M_3	Cu_2	Medial		RW	wt	M_3	Cu_2	Medial
wt	1	-	-	-		wt	1	-	-	-
M_3	0.58	1	-	-		M_3	0.61	1	-	-
Cu_2	0.553	0.863	1	-		Cu_2	0.545	0.886	1	-
Medial	0.483	0.858	0.595	1		Medial	0.527	0.892	0.664	1

Table 2.3. Mean left and right wing differences (L-R) for moths collected in Texas and North Carolina. P-values followed by an asterisk are significant.

	Texas moths			nBt moths, NC			Bt moths, NC		
	M_3	Cu_2	Medial	M_3	Cu_2	Medial	M_3	Cu_2	Medial
n	613	606	689	40	41	40	19	24	21
mean	0.158	0.087	0.023	-0.061	-0.116	0.070	0.156	-0.129	0.228
stdev	0.770	0.185	0.549	0.264	0.223	0.338	0.368	0.286	0.396
sterr	0.031	0.008	0.017	0.042	0.035	0.053	0.084	0.058	0.086
p-value	**<0.0001***	**<0.0001***	**<0.0001***	**0.1513**	**0.0016***	**0.1953**	**0.0461**	**0.0376***	**0.0184***

Table 2.4. Type III results of phenotypic analysis of variance for *H. zea* collected in the Brazos River Valley, 2000. P-values followed by an asterisk are significant.

Dependent Variable: M3 wing dimension			
Effect	F value	df	p value
DATE	3.27	1, 27	< 0.0001*
HOST	112.83	1, 1	< 0.0001*
DATE*HOST	2.13	1, 23	0.0016*
Dependent Variable: dry body weight			
Effect	F value	df	p value
DATE	8.87	1, 27	< 0.0001*
HOST	52.03	1, 1	< 0.0001*
DATE*HOST	2.45	1, 23	0.0002*

Table 2.5. Type III results for indices of fluctuating asymmetry, FA1 and FA2, of *H. zea* collected in the Brazos River Valley, 2000. P-values followed by an asterisk are significant.

Dependent Variable: FA1			
Effect	F value	df	p value
DATE	3.72	1, 27	< 0.0001*
HOST	0.3	1, 1	0.5814
DATE*HOST	0.127	1, 21	0.0106*
Dependent Variable: FA2			
Effect	F value	df	p value
DATE	3.68	1, 27	< 0.0001*
HOST	0.02	1, 1	0.9025
DATE*HOST	1.84	1, 21	0.0132*

Table 2.6. Frequency distribution of plant host type for *H. zea* collected in the Brazos River Valley, Texas, 2002.

Julian Day	Host: C3	Host: C4	Total
49	**5**	**20**	25
55	**4**	**21**	25
61	**7**	**17**	24
69	**6**	**19**	25
77	**13**	**12**	25
82	**7**	**17**	24
87	**10**	**15**	25
94	**5**	**19**	24
101	**11**	**14**	25
109	**12**	**13**	25
127	**17**	**8**	25
132	**13**	**12**	25
137	**11**	**14**	25
144	**0**	**25**	25
153	**1**	**24**	25
159	**0**	**25**	25
165	**0**	**25**	25
178	**0**	**25**	25
181	**0**	**25**	25
187	**0**	**25**	25
203	**4**	**21**	25
208	**2**	**23**	25
215	**2**	**23**	25
224	**4**	**21**	25
231	**3**	**22**	25
235	**5**	**20**	25
259	**15**	**10**	25
276	**14**	**11**	25

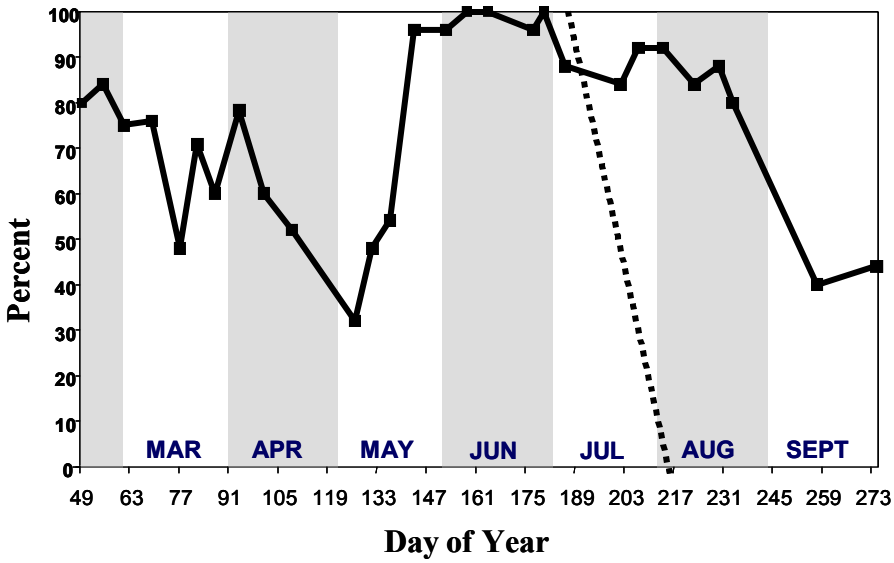


Figure 2.4. This graph illustrates the percentage of moths identified as having a C_4 isotopic signature from pheromone trapped captures in the Brazos River Valley, Texas, from 18 February to 2 October 2000. The dotted line represents the expected decline in *H. zea* population after corn in this area of Texas has reached 100% silking and begins to dry.

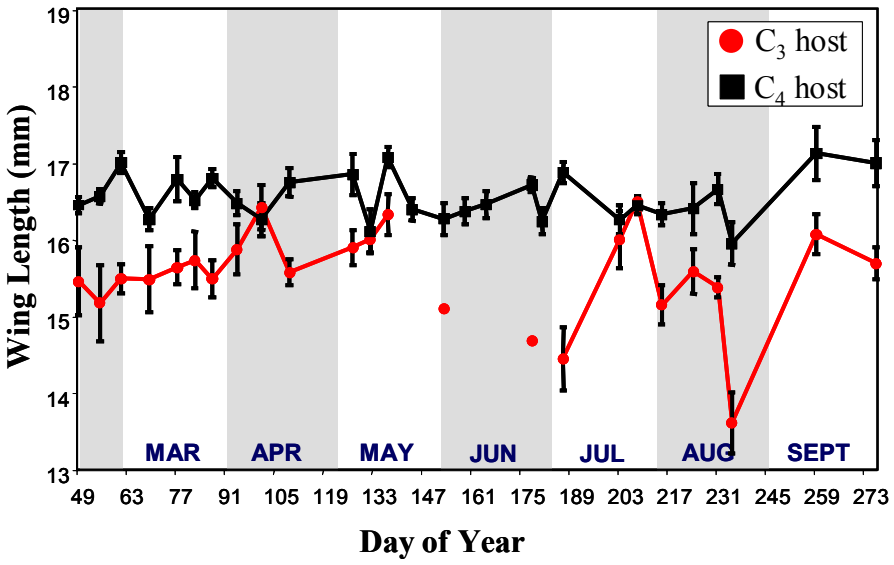


Figure 2.5. ***H. zea*** **moths from each collection date in Texas are separated by host type and plotted as a function of M_3 wing dimension. Moths that fed upon a C4 host during the larval stage have a typically longer M_3 dimension than moths that fed upon a C_3 host. The line interruption indicates collection dates (7 June, 13 June and 29 July) when the sample set contained 100% C_4 moths.**

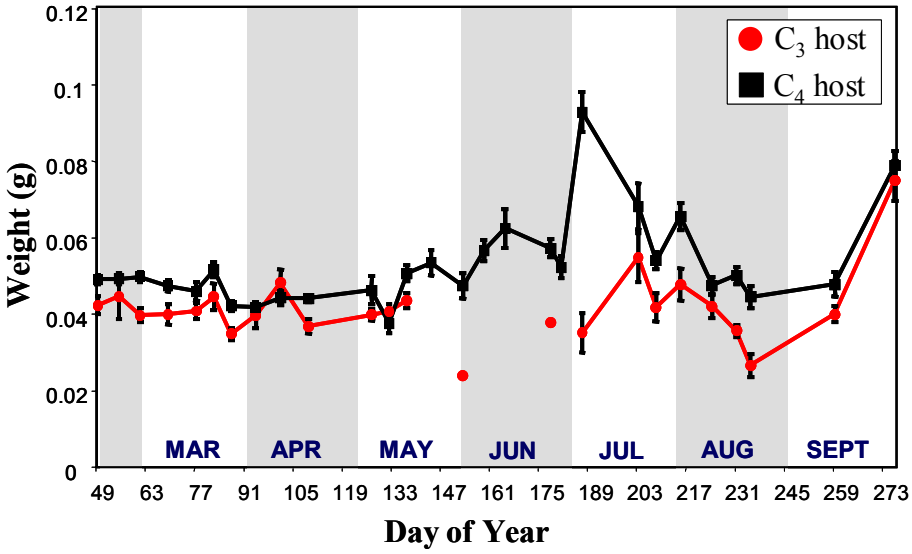


Figure 2.6. *H. zea* moths from each collection date in Texas are separated by host type and plotted as a function of dry body weight. Moths that fed upon a C_4 host during the larval stage are typically heavier in dry body weight than moths that fed upon a C_3 host. The line interruption indicates collection dates (7 June, 13 June and 29 July) when the sample set contained 100% C_4 moths.

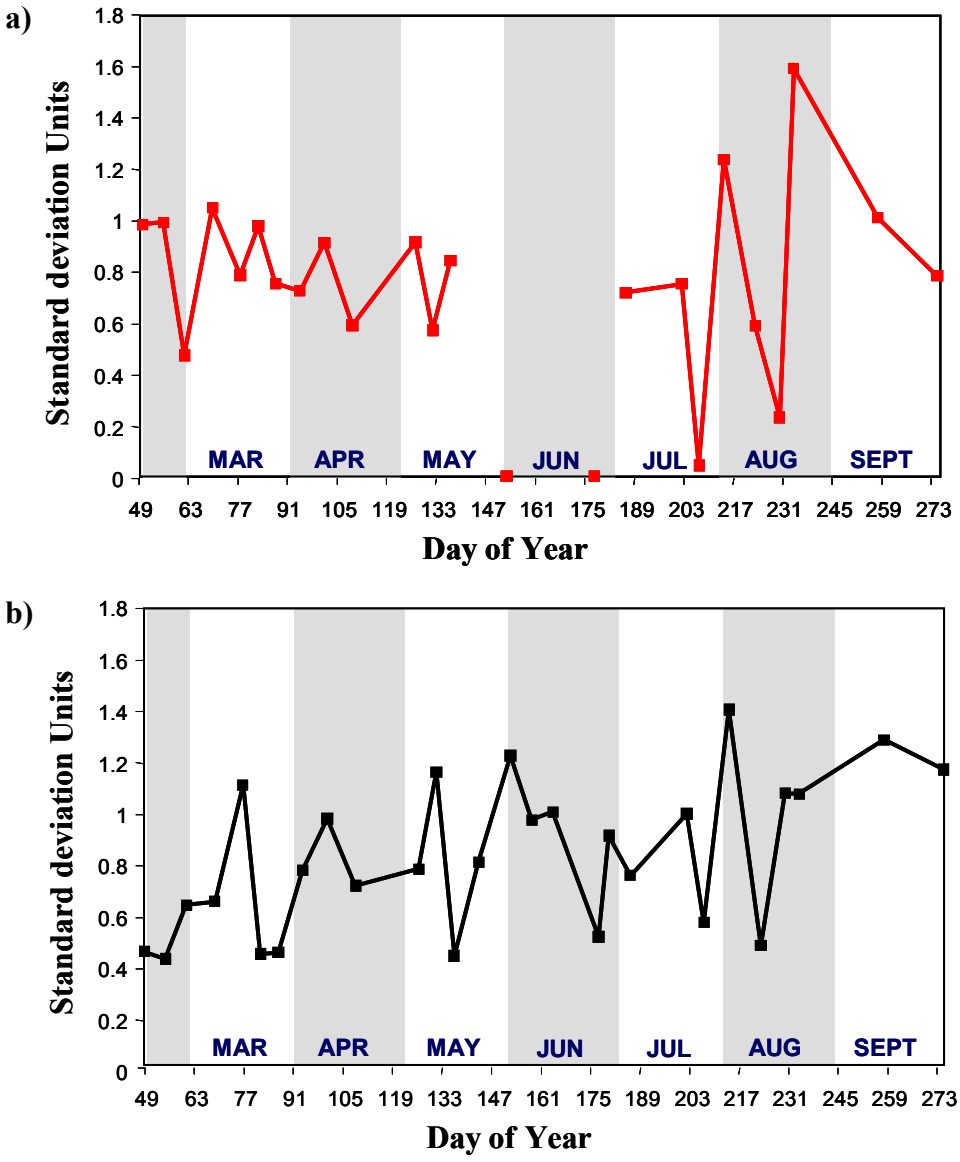


Figure 2.7. *H. zea* moths identified as host type, C_3 or C_4, are separated into two graphs a and b respectively, to illustrate the standard deviation about mean wing length at each collection date. The line interruption indicates collection dates (7 June, 13 June and 29 July) when the sample set contained 100% C_4 moths.

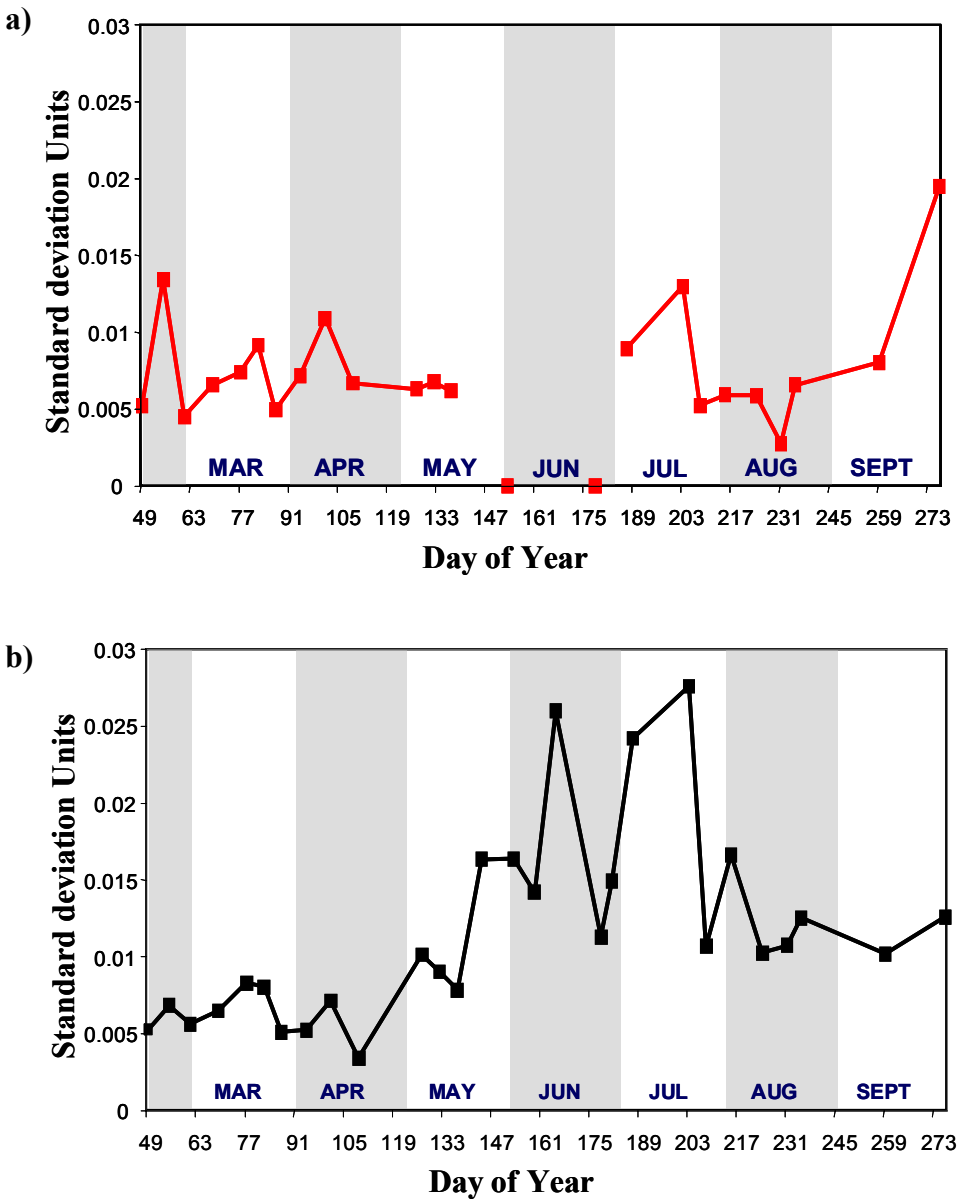


Figure 2.8. ***H. zea*** **moths identified as host type, C_3 or C_4, are separated into two graphs a and b respectively, to illustrate the standard deviation about mean body weight at each collection date. The line interruption indicates collection dates (7 June, 13 June and 29 July) when the sample set contained 100% C_4 moths.**

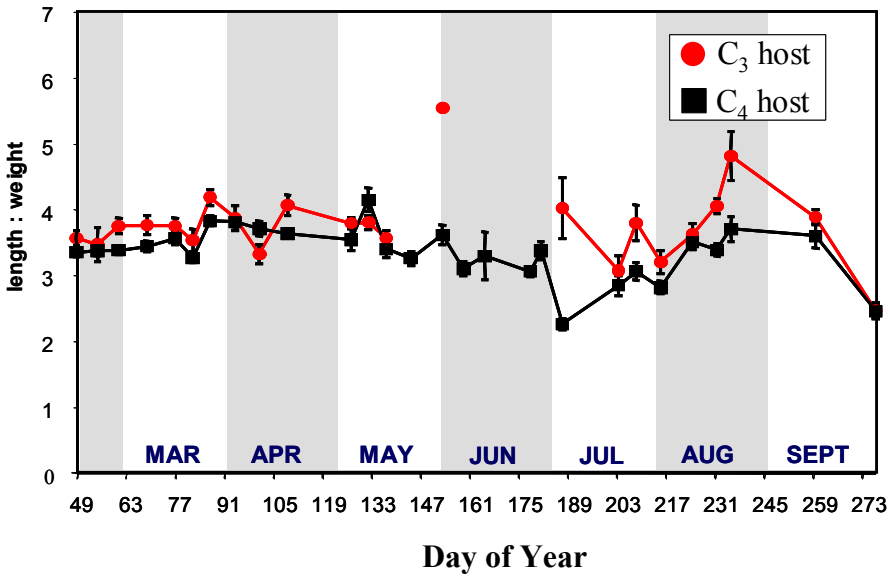


Figure 2.9. Ratios of wing length (M_3 dimension) to dry body weight of *H. zea* from pheromone trap captures in the Brazos River Valley, TX, 2000.

Table 2.7. Mean length (mm) differences between replicates (initial – rep1) of *H. zea* wings. *H. zea* moths collected from both Bt and non-Bt corn in Plymouth, North Carolina, 2002.

n=21	M_3	Cu_2	Medial
mean	-0.112007	0.064314	-0.137913
stdev	0.30447	0.142084	0.300397
sterr	0.066441	0.031005	0.065552
p-value	**0.107372**	**0.051177**	**0.048243**

Table 2.8. Moth weight (g) and wing dimensions (mm) of *H. zea* collected from Plymouth, North Carolina, 2002. Each wing dimension (M_3, Cu_2, Medial) is a mean of an average for left and right forewing lengths of each individual moth. P-values followed by an asterisk are significant.

nBt moths	**wt**	**M_3**	**Cu_2**	**Medial**
mean	0.1703	16.58096	7.595465	7.763073
stdev	0.021736	0.579771	0.32103	0.367688
sterr	0.003205	0.09167	0.049536	0.058137
n	46	40	42	40
Bt moths	**wt**	**M_3**	**Cu_2**	**Medial**
mean	0.182	15.81032	7.165211	7.400369
stdev	0.021755	0.856366	0.465814	0.488688
sterr	0.004351	0.191489	0.097129	0.109274
n	25	20	23	20
p-value	**0.0029***	**<0.0001***	**<0.0001***	**0.0021***

Table 2.9. Fluctuating asymmetry (FA) differences between moths raised on Bt and non-Bt corn at Plymouth, NC, 2002. P-values followed by an asterisk are significant.

wing length	**M_3**	**M_3**	**Cu_2**	**Cu_2**	**Medial**	**Medial**
nBt moths	**FA1 (mm)**	**FA2**	**FA1 (mm)**	**FA2**	**FA1 (mm)**	**FA2**
mean	0.188	0.011	0.177	0.024	0.271	0.035
stdev	0.193	0.012	0.177	0.024	0.209	0.027
sterr	0.031	0.002	0.028	0.004	0.033	0.004
n	40	40	41	41	40	40
Bt moths	**FA1 (mm)**	**FA2**	**FA1 (mm)**	**FA2**	**FA1 (mm)**	**FA2**
mean	0.306	0.019	0.244	0.034	0.316	0.044
stdev	0.248	0.016	0.193	0.027	0.326	0.046
sterr	0.057	0.004	0.039	0.005	0.071	0.010
n	19	19	24	24	21	21
p-value	**0.0505**	**0.0295***	**0.1601**	**0.1073**	**0.5188**	**0.3404**

Table 2.10. Dry body weight (g) of *H. zea* moths collected from corn in North Carolina and from pheromone traps in Texas.

	NC moths		Texas moths		
host	nBt (C4)	Bt (C4)	C4	C3	C4 + C3
mean	0.170	0.182	0.054	0.043	0.051
stdev	0.022	0.022	0.018	0.013	0.017
sterr	0.003	0.004	0.001	0.001	0.001
n	46	25	523	173	696

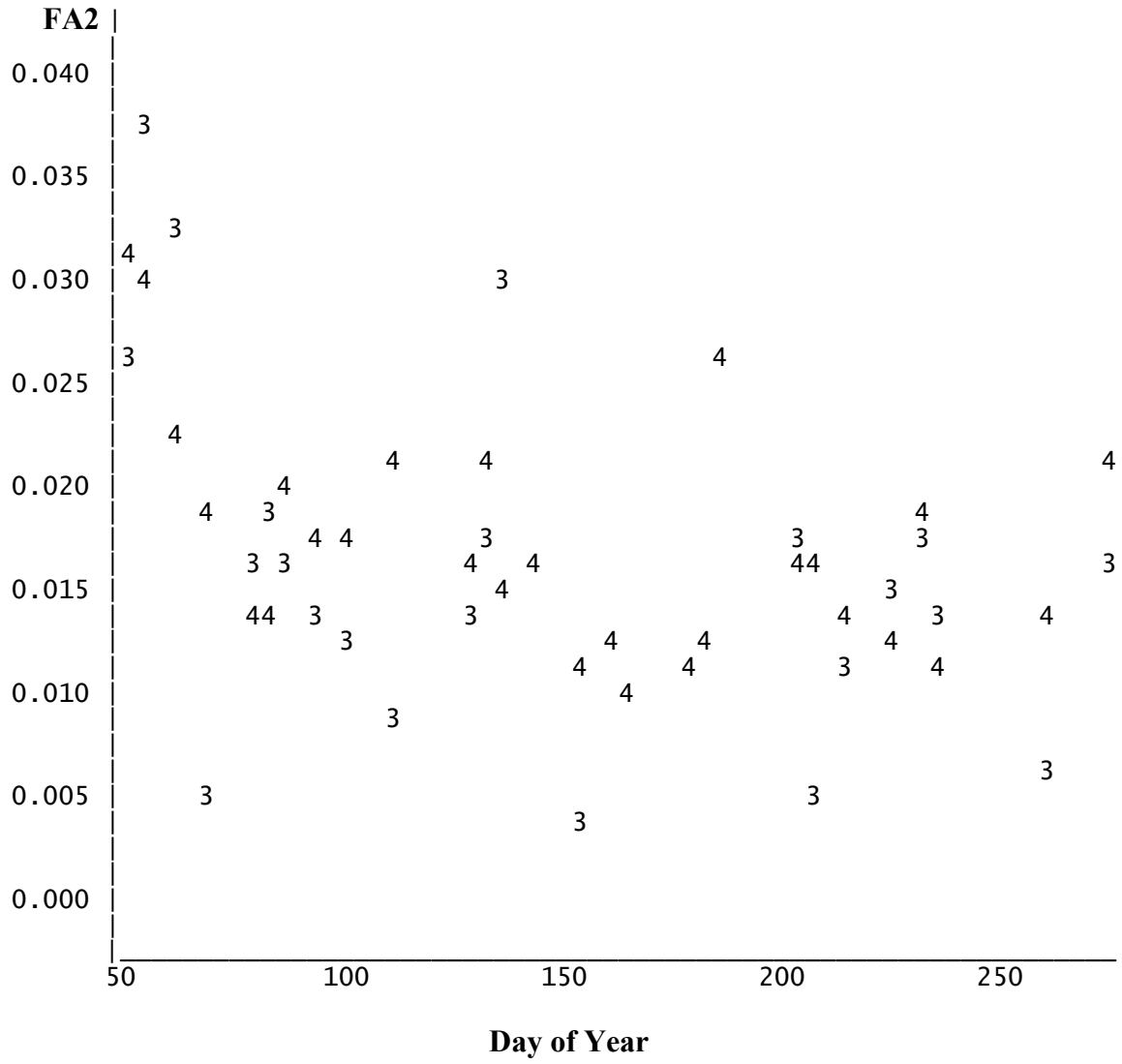


Figure 2.10. Fluctuating asymmetry index, FA1, plotted against date. Symbol represents host type.

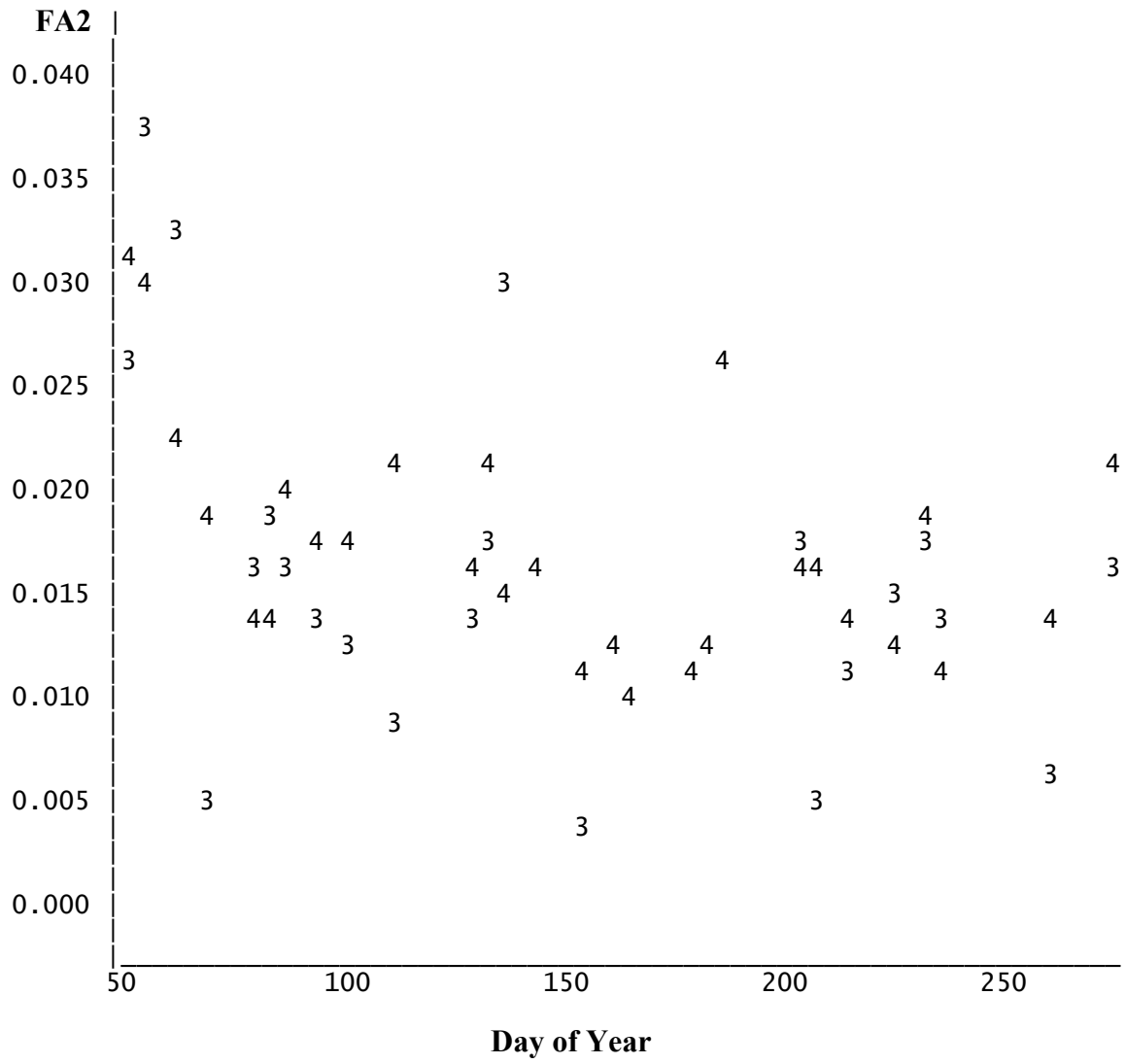


Figure 2.11. Fluctuating asymmetry index, FA2, plotted against date. Symbol represents host type.

CHAPTER III

STABLE HYDROGEN ISOTOPE ANALYSIS OF HAND COLLECTED *HELICOVERPA ZEA* FROM THE MID-EAST AND EASTERN UNITED STATES OF AMERICA

ABSTRACT

During the cropping seasons of 1999 and 2000, *Helicoverpa zea* individuals were hand collected from corn in their final instar, or from the soil beneath corn plants as pupae at 12 sites in the United States. Field collected *H. zea* were reared in the laboratory. Upon emergence from pupation, adult moths were killed and stored in alcohol. Moth wings were subjected to hydrogen isotope analysis to determine geographic δD patterns in *H. zea* wings and whether or not the δD values measured in the wing reflected those of continental rainfall patterns. The δD values in wing tissue did not reflect the continental δD gradient in rainfall. Moths reared from larvae collected in Florida (field-corn) and Virginia (sweet corn) were the most depleted in deuterium, -121 ±12 SD and -123 ±5 SD ‰, respectively, even though they were expected to have the least depletion based on δD values in local surface water. The North Carolina moths had the most enriched δD value -77 ±5 SD ‰. The most North Western state that was sampled was Minnesota and its average δD value was –87 ±6 SD ‰.

INTRODUCTION

Helicoverpa zea moths are considered to be highly mobile (Fitt 1989) but conclusive information about the extent of long distance movement by this moth species is lacking (Pair et. al. 1987, Westbrook et. al. 1998). Developing an understanding of *H. zea* migration became increasingly important as these insects began to evolve resistance to conventional insecticides, because of concern about the spread of this resistance. Now there is a possibility that they will develop resistance to transgenic plants that produce toxins derived from *Bacillus thuringiensis* Berliner (Bt) (Lutrell et. al. 1999). For example, Burd (2001) has recently found resistance alleles to the Bt toxin, CryIA(c), in field populations of *H. zea*, which underscores the need for implementing resistance management strategies. Due to its wide geographical distribution and the diverse agroecosytems in which *H. zea* thrives, each identifiable agroecosystem utilized by this pest, should be considered when incorporating refugia into Bt resistance management strategies for *H. zea*. To slow the evolution of Bt resistance, the US government requires that toxin-free host plants be available to *H. zea* populations as a refuge where caterpillars with toxin susceptibility genes can survive. In chapter 2 of this thesis I demonstrate that *H. zea* larvae are utilizing hosts other than cotton during the late summer and early fall. The stable carbon isotope analysis that I used shows that these moths carry an isotopic signature similar to plant hosts that utilize the C_4 photosynthetic pathway. There are no known C_4 plants in the Brazos River Valley that can support the abundance of C_4 moths that we observed during the late summer and early fall. Therefore, our carbon isotope findings are indicative of *H. zea* migration from areas of suitable C_4 host plants. To further support our unexpected findings of alternate host use

by *H. zea* I attempted to use isotope ratios of hydrogen to trace the natal origin of *H. zea* moths collected in Texas.

Lepidopteran movement patterns can be difficult to trace with conventional ecological methods. However, there have been several relatively successful attempts to determine the geographical range of movement in moth species (Hendrix et. al. 1987, Pair et al. 1987, Showers et. al. 1989, Lingren et. al. 1994; Westbrook et al. 1997 and references within). Recently Westbrook et. al. (1998) combined moth markers and weather data to determine *H. zea* movement pattern and dispersal in Texas. Moths marked naturally with *Citrus* pollen and artificially with *Lycopodium clavatum* spores were used to successfully identify a flight range of at least 660 km and wind trajectory analysis correlated well with the displacement pattern of these marked *H. zea* (Westbrook et. al. 1998). These and other techniques used to analyze moth migration tend to provide circumstantial evidence, or qualitative information on when and where a moth population is migrating but actual quantitative data on the number of migrants at specific locations is needed to develop appropriate resistance management strategies. Previous studies with birds and butterflies have demonstrated that measurements of stable hydrogen isotopes in wing tissue can be useful in providing quantitative information on their movement patterns in animals that are difficult to follow (Hobson and Wassenaar 1997, Wassenaar and Hobson 1998; Hobson 1999). The amount of deuterium in atmospheric water vapor and precipitation decreases moving from the equator to more northern regions. Therefore the ground water in northern areas, and plants that use this water are depleted in deuterium compared to more equatorial latitudes.

Butterflies (Lepidoptera) that feed on plants during the larval stage incorporate the hydrogen isotope signature of the plant into their wing tissue (Hobson et al. 1999b)**.** Thus, it is expected that continental hydrogen isotope patterns in plant hosts at moth natal sites will be significantly correlated with δD values of moth wings (Wassenaar and Hobson 1998). To relate the isotope ratios of wing tissue to groundwater, isotope fractionation during assimilation of organic hydrogen into the body tissue must be considered (see Appendix II for a description of isotope fractionation). Only when migration occurs across isotopically distinct regions can the hydrogen isotope content of wings from pheromone trapped moths be used in tracing moth natal origins.

The objective of this chapter was to investigate geographic δD patterns in *H. zea* wings on a continental scale to determine the potential use of hydrogen isotope ratios in *H. zea* wing tissue as geographical indicators of *H. zea* natal origins. We examined wings of *H. zea* moths that developed as larvae on corn from known origins across the mid- and eastern US to determine if the δD values reflected those of continental rainfall patterns. We also looked at hydrogen isotope ratios in corn kernels over time to determine possible sources of isotope fractionation.

MATERIALS AND METHODS

In this chapter we present hydrogen isotope composition of *H. zea* wing tissue and corn kernels. The isotope value (δD) is expressed in terms of per mil (‰), deviation of the measured sample from an accepted standard, Vienna Standard Mean Ocean Water (VSMOW) with an absolute ratio of D/H equal to 0.00015576, and calibrated to the VSMOW-SLAP (Standard Light Antarctic Precipitation) standard scale (Hagemann et. al. 1970). SMOW and SLAP were measured several times against the reference gas, which produces a calibration line from which a simple linear equation is used to correct the raw δD values. A secondary reference material, (IAEA-CH-7) polyethylene foil (PEF), is included with the sample procedure because it is more chemically related to our sample material than the waters (SMOW and SLAP). The equation derived from SMOW-SLAP is used to make sure that δD values for PEF are –100‰. PEF δD values were used to correct the sample δD values. Typically, δD values are negative and interpreted as such: a less negative δD value indicates an enrichment of the ^{2}H (D) relative to ^{1}H, a more negative value represents a sample depleted in the heavier isotope.

Hydrogen fractionation between plant and insect tissue. Late instar *H. zea* were reared in the laboratory on the corn ear on which it had been feeding in the field. A layer of soil at the bottom of a container provided the substrate necessary for pupation. Once the insect dropped to the soil to pupate the kernels were removed from the corn ears and were oven-dried. Upon emergence from pupation, adult moths were killed and stored in alcohol prior to δD analysis. The isotopic shift between the insect and the kernel was quantified with stable isotope analysis described below.

Temporal study of hydrogen isotopic values:

Temporal study #1, year 2000. Sweet corn planted on 2 separate dates (May 4th and 16th) (Cabrera 2001) at the Central Crops Research in Johnston County, NC, were sampled for kernels from the first planting on July 1st and 8th and from the second planting on July 8th and 19th. Kernel samples were subjected to hydrogen isotope analysis to determine if the isotopic composition of kernels was affected by the kernel stage of maturity. Also larvae were collected from corn in Washington, Mississippi, on 3 different days in July (3rd, 10th, 25th) to investigate any isotopic changes associated with corn age that may be reflected in the moth wings.

Temporal study #2, year 2001. Collections of field-corn were taken from non-Bt corn (seed type: DKC 69-70AF) that was planted 4 May 2001 at the Central Crops Research Station, Johnston County, North Carolina. Irrigation occurred on 3 dates: July 18 and 23 and August 7, in the amount of 1.1, 1.0, and 1.0 inches/acre, respectively. Corn ears were randomly picked from the north facing side of the corn plants. One ear from each of 4 separate plants was collected weekly from July 16 to August 20. In the laboratory, within 2 hours of ear removal in the field, husk and silk were removed and kernels were cut away from the cob. Wet weight was measured, and then kernels were oven-dried for 3 days at 65°C before dry weight was measured. A sample of approximately 10 kernels from each ear for each temporal study was pulverized to a fine homogenized powder in a Wig-L-Bug® grinding mill in preparation for hydrogen isotope analysis.

Geographic Comparison. *H. zea* individuals were collected as larvae from corn plants in the final instar, or from the soil as pupae at 12 sites in the US (Figure 3.3, Table 3.5). The larvae were reared in the laboratory on corn ears obtained from their collection sites. Upon emergence from pupation, adult moths were killed and stored in alcohol prior to hydrogen isotope analysis. Variation between sample batches processed on the mass spectrometer at different times (i.e. more than a month apart) may lead to biases when analyzing δD values. We observed a significant difference between forewings separated into two batches (i.e. left and right wings). The difference we observed in repeat wing measurements were for 8 moths collected in Plymouth, NC, the left wings were processed in June 2000 and as a follow-up we ran the right wings in November 2000 (Table 3.5).

Determination of exchangeable hydrogen. The hydrogen isotopic values (δD) of organic tissue can be divided into 2 components: an exchangeable (δD_{ex}) and non-exchangeable (δD_n) fraction. If p_{ex} represents the percent exchangeable hydrogen, the following mass balance equation can be used (Cormie et. al. 1994, Chamberlain et. al. 1997):

$$\delta D = p_{ex}\delta D_{ex} + (1- p_{ex})\, \delta D_n \qquad \textbf{(Eq. 3.1)}$$

where δD, is the isotopic value of total hydrogen. The non-exchangeable hydrogen in a wing should represent the hydrogen isotopic composition of water molecules that were involved in insect growth and wing development. At least a portion of the exchangeable hydrogen in a wing is able to exchange with ambient water vapor after the wing was formed. Therefore, a measurement of total δD would only in part reflect the composition

of water that was present during larval feeding. It is possible to remove the uninformative exchangeable hydrogen by equilibrating all wings with water that has a known isotopic value. This water is referred to as equilibration water. If δD_w is the δD value of the equilibration water, and Δ is the isotopic fractionation between exchangeable hydrogen of wing tissue and water such that:

$$\delta D_{ex} = \delta D_w + \Delta \qquad \textbf{(Eq. 3.2)}$$

Eq.3.2 becomes:

$$\delta D = p_{ex}(\delta D_w + \Delta) + [(1- p_{ex})\, \delta D_n] \qquad \textbf{(Eq. 3.3)}$$

Rearrangement:

$$\delta D = p_{ex}\delta D_w + [(1- p_{ex})\, \delta D_n + p_{ex}\Delta] \qquad \textbf{(Eq. 3.4)}$$

Where p_{ex} in $p_{ex}\delta D_w$ is the slope (m) and $[(1- p_{ex})\, \delta D_n + p_{ex}\Delta]$ is the slope intercept (b). There is an unknown fractionation between the injected water and the sample material ($p_{ex}\Delta$). Therefore, the isotopic value of hydrogen that has exchanged with the wing tissue is not identical to the δD value of the equilibration water (Schimmelmann 1991). All samples were equilibrated at the same temperature, which renders the fractionation between the injected water and the sample material inconsequential for the purpose of our isotopic comparisons. By replacing exchangeable hydrogen in a set of wing samples with equilibration water treatments that have different δD_w it is possible to determine p_{ex} and thereby assess only the δD of the non-exchangeable hydrogen (Wassenaar and Hobson 2000).

To determine the percent of exchangeable hydrogen in corn kernels we ran experiments similar to those of Wassenaar and Hobson (2000), which involved equilibration of the

samples with waters of a wide range of hydrogen isotope values (-135, +115, and +525 ‰). Water (200µl) was injected into sealed tubes containing corn kernels at a constant temperature of 130 ± 0.1 °C for 2 hours. The slope of the regression line of water δD_w versus final δD primarily represents the proportion of exchangeable hydrogen in the sample tissue. All organic samples, except kernel samples from the "*Temporal study #2, year 2001*" were standardized by steam equilibration of known δD value and corrected for the percent exchangeable hydrogen determined by this experiment and Wassenaar and Hobson (2000).

Hydrogen isotope analysis:

Offline steam equilibration. Dried wings were solvent cleaned 3 times with a 2:1 chloroform:methanol solution to remove lipids and surface oils. It is desirable to remove lipids because they have a wide range of δD values and are typically much more depleted in deuterium than other tissue types (Park and Epstein 1961; DeNiro and Epstein 1977). Weight was recorded for each wing and the entire wing was loaded into 9mm Vycor™ break-seal tubes with 1g of cupric oxide (CuO) and 30mg of copper (Cu) metal for equilibration, whereas; 7.5mg of powdered samples (such as kernels) were loaded in a 9x5mm tin boat before introducing the sample into the break-seal tubes. All samples for hydrogen analysis were equilibrated with steam of known isotopic composition (-135‰) to eliminate uncontrolled, temperature dependent hydrogen isotope exchange with ambient water vapor. Samples were equilibrated by evacuating each break-seal tube for 1 hour at 130°C to remove any absorbed ambient moisture. After 1 hour of evacuation, 200 µl of water with a known isotopic value was injected into each tube for equilibration at

constant temperature (130 ± 0.1ºC) for 2 hours (Wassenaar and Hobson 2000). Reference materials (IAEA-CH-7) polyethylene standard (having no-exchangeable hydrogen) were always included within sample groups (9 samples: 1 reference) to ensure quality control. The internal standards provided a means to test for analytical errors throughout laboratory procedures, which included exposure to equilibration methods, sample combustion, gas separation, reduction to H_2 gas, and analysis on the mass spectrometer.

Water was evacuated after the 2 hr equilibration period and sample tubes were flame sealed under vacuum and combusted overnight (gradual heating and cooling) at 850°C for 2 hours. The gas in the break-seal was released into a vacuum-sealed gas separation line and was immediately cryogenically trapped in a glass loop that was immerged in a mixture of dry ice and liquid nitrogen that had a temperature between –70 and –80 °C. Both H_2O and CO_2 froze at this temperature; however, nitrogen remained in the gas phase and was evacuated before H_2O and CO_2 were cryogenically separated. Water and CO_2 were cryogenically transferred and trapped into two separate 6 mm Pyrex tubes and were flame sealed. Prior to gas separation, between 40 and 60 mg of zinc alloy (Biogeochemical Laboratories, Indiana University) was loaded into each Pyrex tube for water collection. Sealed Pyrex tubes containing the water sample and zinc alloy were kept in the freezer for long-term storage to reduce the chance of leakage. Pyrex tubes containing CO_2 were stored in a cabinet at room temperature. On the day of analysis the water-containing tubes were loaded into a hot reaction plate at 500ºC for 30 min for the reduction of water-H. Stable hydrogen isotope measurements were performed using dual inlet isotope-ratio mass spectrometry (Micromass Optima) at the National Hydrology Research Center (NHRC), Saskatoon, Saskatchewan, Canada.

On-line comparative equilibration. Kernel samples from the "*Temporal study #2, year 2001*" were processed differently from the offline steam equilibration method. The NHRC isotope facilities in Saskatoon have recently upgraded their IRMS to a Continuous Flow -IRMS (CF-IRMS) that enabled them to modify the previous method while still correcting for the effects of exchangeable hydrogen through the use of calibrated in-house working standards, naming the new method - comparative equilibration (Wassenaar and Hobson *in press*). The in-house standards are pre-calibrated with internationally accepted standards (i.e.VSMOW). The in-house working standard that was used in our study is CFS, which is a 2 kg homogenized mixture of chicken feathers from a single geographical location. The sample and standard stand at room temperature for 96 hours in the NHRC stable isotope facilities to equilibrate with laboratory air moisture prior to stable hydrogen isotope analysis.

Kernel samples from 2001 were analyzed during the summer of 2002 with the new "comparative equilibration" method. Pulverized kernel samples (100 ±10 μg) were loaded into 4 x 3.2 mm silver capsules and allowed to air equilibrate on the shelf with ambient lab air moisture at room temperature for 96 hours prior to stable hydrogen isotope analysis. Isotope measurements for the kernels were performed on H_2 derived from high-temperature flash pyrolysis and by CF-IRMS. A GC column was used to resolve the sample H_2 from N_2 and CO. The resolved H_2 sample pulse was then introduced to the IRMS (Micromass Isoprime™ with electrostatic analyzer) via an open split capillary. We ran 2 repeats for each kernel sample from each ear. An overall isotopic average was calculated for the kernel measurements of 4 ears for each collection date.

RESULTS

Determination of exchangeable hydrogen. Hydrogen in corn kernels available for isotopic exchange at a constant temperature of 130 ± 0.1 ºC was determined by the slope of the line on a plot of δD_w versus δD will give the percent of exchangeable hydrogen by using Eq. 3.3, $\delta D = p_{ex}(\delta D_w + \Delta) + [(1 - p_{ex})\ \delta D_n]$ (Figure 3.2). For these experiments we find an equation of $\delta D = 0.1956\ \delta D_w + 6.1882$ ($r^2 = 0.9974$). Thus the percent of hydrogen exchange is approximately 19.6%. This value (0.1956) was used to standardize hydrogen isotope measurements of corn kernels. Our calculated percent of carbon-bound hydrogen isotope exchange in kernels (19.6 %) is essentially the same as 19 ±0.7 % determined for butterfly wing tissue by Wassenaar and Hobson (1998).

Isotopic differences due to time of processing. Table 3.4 shows a mean difference of 5‰ between paired moth wings (Right-Left) processed similarly for stable hydrogen isotope analysis in two separate batches that were run 4 months apart from each other. The isotopic difference between batches is significant (P= 0.007). We did not, however, find a significant difference in left and right wings run in the same batch. The left and right wings for 3 individual moths collected in Greensboro, NC, differed by 1 per mil each.

Hydrogen fractionation between plant and insect tissue. Standardized samples of corn kernels and *H. zea* were compared to determine the fractionation between paired samples of kernels and wings from the moths that had fed on those kernels. The mean δD for a sample of 9 moth wings was -90 ± 7SD‰ and for 9 samples of homogenized corn

kernels was -52 ± 4SD‰, denoting an apparent mean fractionation of -39± 7SD ‰ between *H. zea* wing tissue and corn kernels.

Temporal study of hydrogen isotopic values.

Temporal study #1, year 2000. Kernels collected from sweet corn at the Central Crops Research Station in Clayton showed significant differences in deuterium enrichment (Table 3.1). The kernels obtained in Table 3.1 differ in maturity due to different planting dates and collection dates; otherwise they were subject to similar growing conditions. A comparison between kernel δD values of the same planting dates shows an enrichment of 29‰ in the older kernels for the first planting (early season) and no change in δD values in the kernels from the second planting (Table 3.1). A comparison of kernels collected on the same date (July 8) from the two plantings shows a difference of 17‰.

In a temporal comparison of *H. zea* moths collected from field-corn in Washington County, Mississippi on the 3rd, 10th, and 25th of July 2000 we did not observe a temporal effect on δD values (Table 2).

Temporal study #2, year 2001. Kernels collected weekly for six weeks, between July 16th and August 20th, were processed for isotopic analysis (Table 3.3). Ears collected on July 16th were in an early stage of development, whereas, the ears collected on August 20th had completed development and were hard and pale (dent). The kernels at the end of the growing season were more enriched in deuterium (-43 ±3.3SE) than the kernels at the

very beginning of the season (-66 ±1.9SE). The kernels decreased in moisture content by about 20 percent biweekly (Table 3.3).

Geographic Comparison. Hydrogen isotope ratios for *H. zea* collected as larvae or pupae from known locations are listed in Table 3.5 and plotted on the map of gradient patterns of δD in average rainfall in Figure 3.3. The δD values in wing tissue did not reflect the δD values of groundwater from where the larvae developed according to the isotopic gradient in rainfall. Moths reared from larvae collected in Florida (field-corn) and Virginia (sweet corn) were surprisingly the most depleted in deuterium, -121 ±12 SD and -123 ±5 SD ‰, respectively, even though they were expected to have the least depletion based on δD values in local surface water. The North Carolina moths had the most enriched δD value -77 ±5 SD ‰. The most North Western state that was sampled was Minnesota and its average δD value was –87 ±6 SD ‰.We observed isotopic ranges as great as 48‰ between *H. zea* individual wing samples that were obtained from the same location (Table 3.5).

DISCUSSION

The use of stable hydrogen isotopes for determining the natal origin of highly mobile insects is new, and very few studies have been conducted with this technique. When we began our project, we adopted the approach used in the benchmark study of monarch butterfly movement by Wassenaar and Hobson (1998). Unfortunately, we did not foresee a number of important biological differences between the trophic biology of monarch butterflies and *H. zea* moths. In hindsight, we should have started our study by first studying the temporal and geographic patterns of hydrogen isotopes in the host of our target insect. Based on the Wassenaar and Hobson (1998) study we assumed that if *H. zea* was migrating across isotopically distinct regions, then the δD value of the wings from moths captured in pheromone traps would reflect the δD values of the moths' natal origins and would be significantly different from the isotopic value at the site where they were collected. In our study, the δD values in wing tissue from *H. zea* moths did not reflect the δD values of groundwater, from where they developed and were collected as larvae, according to Hobson and Wassenaar's (1997) configuration of weighted average growing season δD values for precipitation in North America. This is evident in Figure 3.3 where we overlaid the wing δD values, of moths that developed at known locations, on the continental gradient of δD values in average rainfall. We found, in many cases, drastically different δD values from those of rainfall. Instead our results are puzzling, showing no isotopic trend or consistent offset compared to average rainfall. For instance, moths reared from larvae collected in Florida (field corn) and Virginia (sweet corn) were surprisingly the most depleted in deuterium, -121 ±12 SD and -123 ±5 SD ‰, respectively. Whereas, the Carolina moths had the most enriched δD values of -77 ±5

SD ‰. The most NW state that was sampled was Minnesota and its average δD value was –87 ±6 SD ‰. According to the Global Network of Isotopes in Precipitation (GNIP) values in the southeast such as Florida are typically the most enriched in deuterium and yet we are finding our most enriched samples in Carolina, which is a considerable increase in latitude and therefore Carolina raised moths are expected to be depleted in deuterium compared to moths developing in Florida (Figure 3.3). There is enough idiosyncratic variation of δD values for moth wings within the same isoline, where the differences are frequently more than 10‰. Mean differences of 10‰ between collection sites within the same isolines do not offer enough resolution to decipher moth natal origins within the southern states where the gradient differences in rainfall differ only by about 5‰, in other words there is too much overlap (Figure 3.3). These findings were unexpected and have been difficult to interpret. However, we attempt to account for some of the variability based on our results from field tests and examples or explanations of variation within the literature of similar biological systems.

Our data suggest that some of the variation observed in moths collected at the same location may be due to time of processing (Table 3.4). The mean difference among batches was 5‰, which is small compared to the other isotopic variation we have observed. However, it may be useful for future hydrogen isotope analysis to include a standard in each batch that comes from a large, homogenized group of moth wings. Isotopic ranges are listed in Table 3.5 to emphasize the extent of δD variability observed in moths within the same location with the greatest difference being 48‰, although most ranges were less than 20‰. Hobson et al. (1999b) results of mean δD values for wild

reared Monarchs showed a progressive depletion in deuterium with increasing latitude from San Antonio Texas (-82‰) to Springstein, Manitoba (-137‰). The largest isotopic range of δD values we observed in *H. zea* from a single location (48‰) nearly encompasses the range of mean δD values Hobson et al (1999b) observed in Monarchs across the entire eastern US (55‰).

Some of the variability may be explained by the nature of the host plant since these moths were raised on cultivated corn, a major crop in the US, opposed to wild hosts. In some regions of the US corn is irrigated during dry periods. For instance, the cornfields at the Central Crops Research Station in Clayton, NC, are irrigated during dry periods with basin water located at the field station. The isotopic composition of waters from springs and closed inland basins may differ from groundwater because of evaporative enrichment. Variation in sources of fresh water can be as large as 200‰ at a single location (Daansgard 1964). If, in fact, the variability seen in the moth wing δD values stems from the inputs of agriculture, then tracing migrant moths to their natal origins via the hydrogen isotope technique cannot be applied to *H. zea* because cultivated corn in the US is dynamic and cannot be used to develop a reliable mapping system.

To the best of our knowledge, very few studies have paid attention to the temporal hydrogen isotopic composition of the study organism's diet or host plant(s) and their different tissue types and how this may affect the isotopic composition of the study organism temporally. Hydrogen isotope fractionation between captive reared organisms and their diet have been investigated but these studies involve diet of known isotopic

composition and not of diet at different phenological stages (Miller 1985, Hobson and Wassenaar 1997). Typically the analysis for tracing migratory species is to measure the δD in animal tissue and compare it to δD values of previously published continent-wide patterns of average rainfall (Kelly and Finch 1998, Wassenaar and Hobson 1998). We suggest more investigation into the dynamics of the δD values in host plants, in the case of herbivores, or whatever the diet may be of the study organism.

Isolated tissues of an organism may show enrichment or depletion of the heavy isotope depending on specific metabolic pathways. The bulk of *H. zea* larval diet in midseason, when on corn, are kernels. We have looked at the relationship between isotopic composition of *H. zea* adults and the kernels they fed upon as larvae and found that the mean hydrogen isotopic ratio in wing tissue is offset from the kernels by about -39‰. Oddly, in comparison to a laboratory rearing experiment with another species of Lepidoptera, the hydrogen isotopic fractionation between adult Monarchs and their larval host plants (milkweed) was negligible (Hobson et. al. 1999b). They did however observe a significant δD offset between the water and the milkweed plants.

We have also investigated temporal changes in isotopic composition of drying kernels. While our data are far from conclusive, we did find an overall temporal D-enrichment in kernels (Table 3). Dent kernels late in the growing season have less water compared to milk stage kernels earlier in the season, which may mean that there is evaporative enrichment within the kernel resulting in a progressively large proportion of heavy kernel water for synthesis of carbohydrates over time. Our study did not include a controlled

experiment to look at the effects of temporal isotopic changes in kernels on moth wing tissue, however, we do have moths collected from the same corn field in Mississippi over a 3 week period and these data do not show a significant change in isotopic composition (Table 3.2). Our data indicate that this temporal isotopic difference of kernels within a single field of corn can be larger than the difference between isotopic gradients of continental groundwater (Table 3.1 (between July 1st and 8th) & 3.3), but in other cases there may be no change within a field (Table 3.1 (between July 8th and 19th) & 3.2). What we don't know with certainty is whether or not the isotopic differences seen in kernels is temporally integrated during larval development or is the isotopic composition of the kernel during the latter instar(s) selectively incorporated into the wing tissue of the adult moth? If δ values are varying in isotopic composition in moth wing tissue due to temporal δD differences from the larval diet, where the differences are greater than the continental isotopic gradients, then it is possible to make false conclusions of the natal origins of pheromone captured moths. Furthermore, although most *H. zea* larvae feed on kernels, larvae can also consume leaves, husk, and silk. The isotopic variation within these tissues has not been studied in detail. Helliker and Ehleringer's (2000, 2002) investigation into the oxygen isotope ratios of leaf water in C_4 plants (monocots) provided evidence of significant isotopic variation within a single monocotyledon leaf where they showed a progressive evaporative enrichment along parallel veins, a pattern that does not occur in dicots or C_3 grasses. A large and distinct increase in $\delta^{18}O$ was observed in whole grass blades, with some base-to-tip differences exceeding 50‰ (Helliker and Ehleringer 2000). It is well known that $\delta^{18}O$ and δD in meteoric waters are related ($\delta D = \delta^{18}O + 10$)

(Craig 1961) meaning that an enrichment of ^{18}O in leaf water due to evaporative transpiration would subsequently result in an enrichment of D in leaf water.

The aforementioned observations are somewhat contradicting since the examples of fractionation within water basins, corn kernels and monocot leaves typically result in an enrichment of deuterium and ^{18}Oxygen; however, the isotopic offset between the kernels and moths was observed as a substantial depletion in deuterium.

Our research and review of the literature on this subject raises a point of concern relative to those studies that allude to the simplicity at which the hydrogen isotope ratios in animal tissues can be directly related to the growing-season average δD values in precipitation (Chamberlain et. al. 1997, Kelly and Finch 1998, Hobson et. al. 1999) without more emphasis on the isotopic variation in the animal's diet at a single location (Hobson et. al. 1999a). Clearly, continual refinements are needed in our knowledge about how the stable hydrogen isotope ratios in rainfall relate to local food webs involving migratory species.

To identify the primary source of isotopic variability we recommend a detailed study that examines the changes in isotopic composition in the host plant. The study should begin with a controlled experiment in the greenhouse that involves potted corn plants irrigated only with water of a unique isotopic composition. The corn plants should be analyzed to determine the isotopic composition of different plant parts (i.e. leaves, husk, silk, kernel).

And this analysis should be done at different phenological stages. This method could provide the missing link to our observation of unexpected and highly variable isotope results for *H. zea* moth wings raised on corn plants.

Figure 3.1. Conceptual evolution of isotopic ratios due to condensation and preferential rainout of isotopically heavy water during continentward movement of atmospheric water vapor from oceanic source regions.

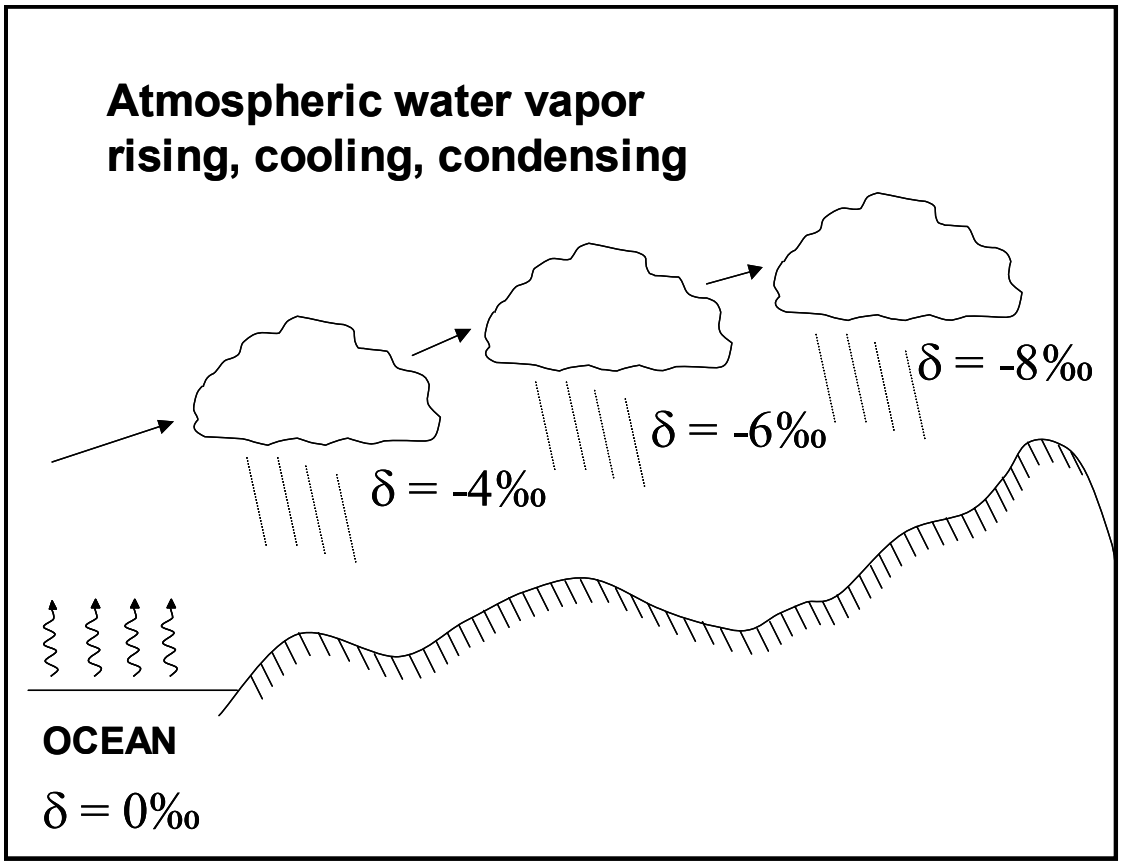


Figure 3.2. Corn kernel equilibrations using a wide isotopic range of vapors for 2 hrs at 135 ± 0.1 °C

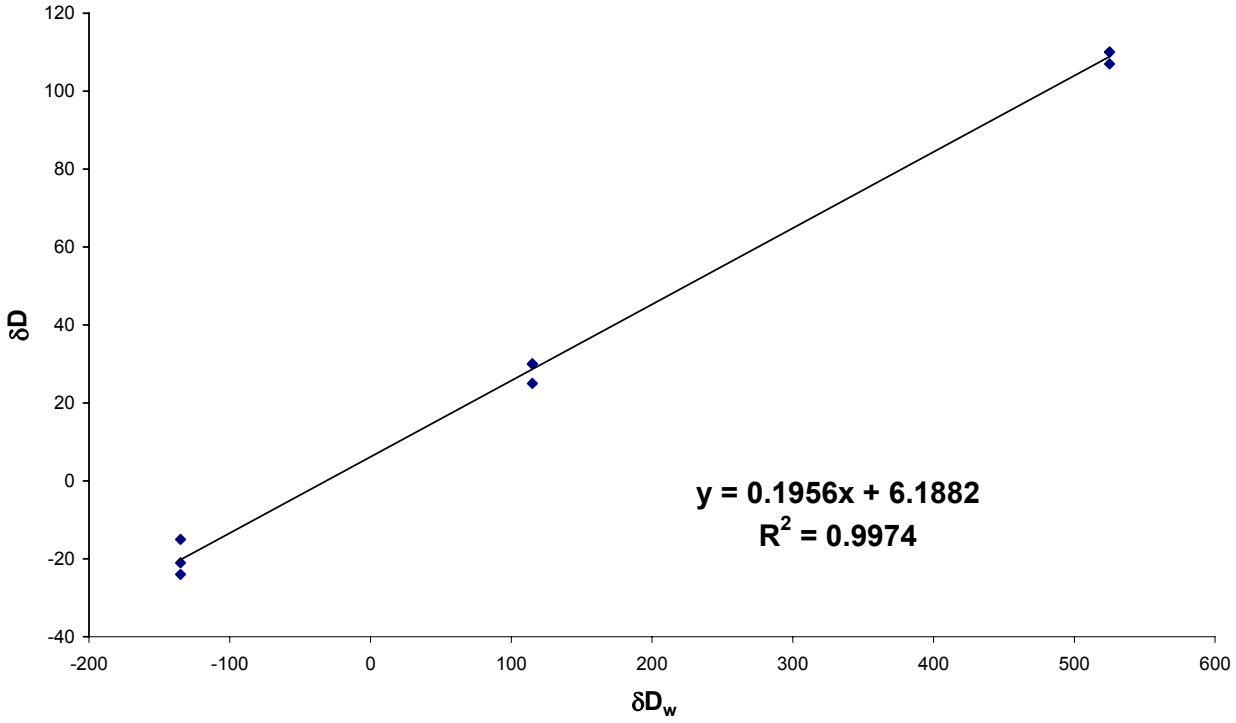
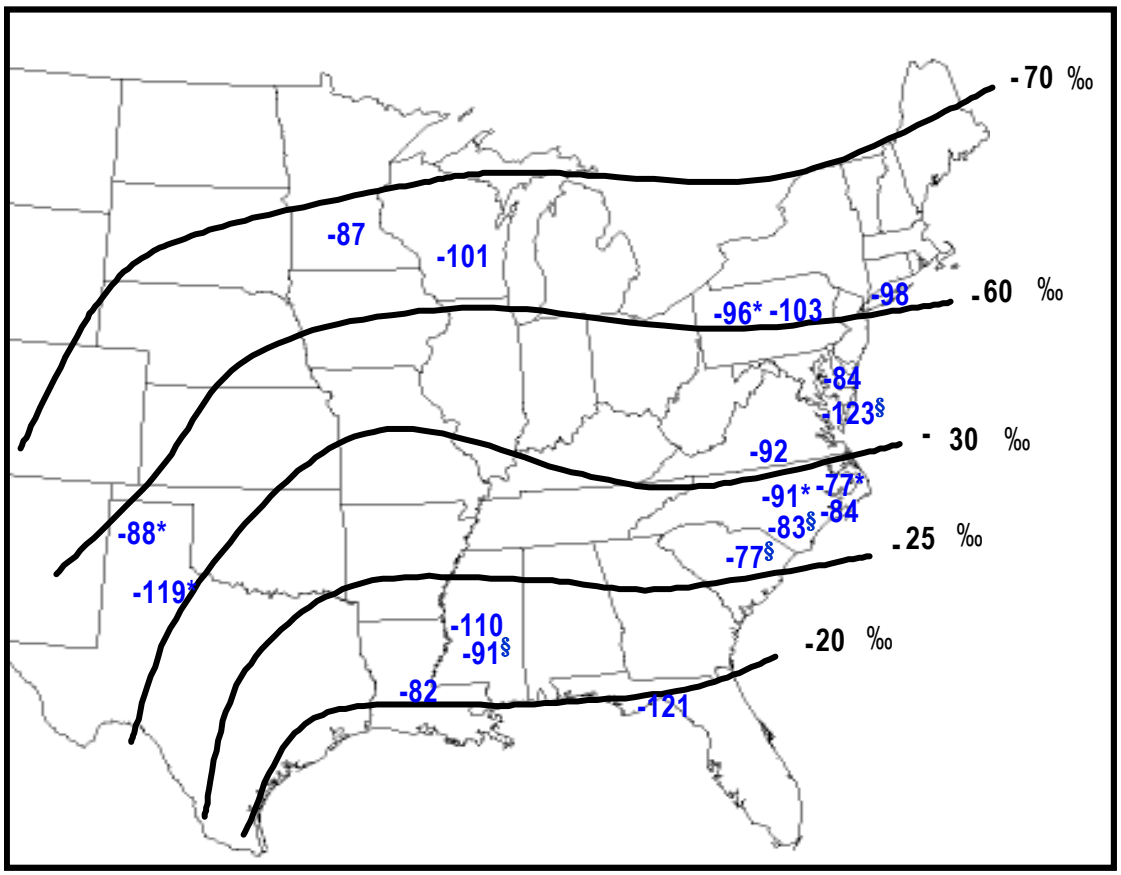


Figure 3.3. Gradient patterns of δD in average rainfall, and distribution of wing tissue δD values collected from corn fields, across North America in 1999* and 2000.



δD contours adapted from Hobson and Wassenaar (1997).

Table 3.1. Temporal comparison of hydrogen isotope ratios (δD) from sweet corn (kernels) that differ by date planted and date collected in Clayton, North Carolina, 2000.

date planted	4-May	4-May	16-May	16-May
date collected	1-Jul	8-Jul	8-Jul	19-Jul
isotope values (‰) ± SD	-64 ± 3	-35 ± 3	-52 ± 3	-52 ± 4
n	3	3	3	9

Table 3.2. Temporal comparison of hydrogen isotope ratios (δD) from *H. zea* (moths) collected from field corn in Washington County, Mississippi during July 2000.

date collected	3-Jul	10-Jul	25-Jul
isotope values (‰) ± SD	-113 ± 5	-114 ± 5	-105 ± 10
n	12	4	7

Table 3.3. Hydrogen isotope ratios (δD) from corn (kernels) collected during the summer of 2001 from different corn plants on the same field at the Central Crops Research Station in Clayton, North Carolina.

date	n	δD	sterr	stdev	% moisture
16-Jul-01	4	-66	1.9	6.4	79
23-Jul-01	4	-41	1.8	6.1	75
30-Jul-01	4	-40	1.1	3.8	58
4-Aug-01	4	-33	1.9	6.7	51
13-Aug-01	4	-36	1.5	5.3	37
20-Aug-01	4	-43	3.3	11.6	34

* the δD value –105‰ was excluded from 7/16 sample set

Table 3.4. Hydrogen isotope ratios (δD) measured in paired *H. zea* forewings. Left wings were processed in batch 1 and right wings were processed in batch 2 (4 month interval). The isotopic difference between batches is significant*.

batch 1	batch 2	(b2-b1)
-85	-84	1
-74	-70	4
-88	-84	4
-83	-78	5
-95	-90	5
-86	-75	11
-83	-71	12
-97	-79	18
mean		5
stdev		3
p-value		**0.00701***

Table 3.5. Geographic distribution of δD values from moth wing tissue.

batch	state	county/city	month	day	year	n	delta D	SD	‰ range	host			
A	MN	X	Sept	1	2000	12	-87	6	19	FC			
A	WI	X	Sept	20	2000	4	-101	5	10	FC			
A	PA	X	Sept	20	1999	4	-96	8	17	FC			
A	PA	X	Sept	21	2000	3	-103	6	13	FC			
A	NY	Long Island	Sept	5	2000	5	-95	3	7	FC			
A	NY	Long Island	Aug	22	2000	3	-101	4	6	FC			
B	VA	*South*	July	X	2000	11	-92	10	25	SC	-197 outlier excl (-101 incl)		
A	VA	Painter	July	26	2000	3	-84	9	19	FC			
B	VA	Painter	Aug	12	2000	12	-123	5	X	SC			
B	SC	Charleston	June	16	2000	12	-77	5	15	SC			
B	NC	Plymouth	Sept	X	1999	7	-77	6	15	FC			
A	NC	Plymouth	Oct	6	2000	8	-84	5	17	FC			
C	NC	Clayton	July	X	2000	10	-91	7	X	SC			
E	NC	Clayton	Aug	X	1999	8	-83	4	13	X			
B	MS	Washington	July	3	2000	12	-113	5	16	FC			
B	MS	Washington	July	10	2000	4	-114	5	11	FC			
B	MS	Washington	July	25	2000	7	-105	10	31	FC	-89 outlier incl		
B	MS	Washington	July	11	2000	11	-91	5	48	SC	-51 outlier excl (-88 incl)		
B	FL	Quincy	June	29	2000	9	-121	12	34	FC			
A	LA	Red River	July	5	2000	3	-85	3	5	FC			
A	LA	Red River	July	10	2000	8	-78	7	25	FC			
D	TX	Lubbock	X	X	1999	10	-119	13	42	FC			
A	TX	Lubbock	Aug	4	1999	3	-113	2	4	FC			
D	TX	Amarillo	X	X	1999	10	-88	7	25	FC			
A	TX	Amarillo	Aug	24	1999	4	-92	11	23	FC			

Batch indicates sampes that were run on the IRMS within a month of eachother; X indicates no data; all values in this table were obtained using the steam equilibration method

NOTE: Tables 3.1 through 3.5 isotope ratios are represented as δD in units per mil (‰).

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APPENDIX I

Description of an isotope-ratio mass spectrometer (IRMS). In general terms a mass spectrometer is an instrument that separates charged particles on the basis of their mass differences. The three essential components of a mass spectrometer include: a source, a magnetic field, and ion collectors. Molecules are ionized by an electron beam source before they enter a magnetic field that accelerates and separates the positively charged ions down a flight tube where they are detected by a series of ion collectors. The mechanism for separating the gas into components of different masses occurs in the magnetic field where the ions are deflected into circular paths whose radii are proportional to the masses of the isotopes. The collector cups are arranged in such a way that they receive the different mass components. Amplifiers attached to the collectors convert the ionic impacts into voltage, which is then converted into a frequency. The valuable measurement is the ratio of the individual signals and not the absolute intensity, which is dependent on the amount of gas that is introduced into the mass spectrometer

APPENDIX II

Isotope fractionation. Isotope fractionation can be described as the process that results in changes in the relative abundance of isotopes of an element. Fractionation is the result of physical-chemical processes such as: the relative rates of reaction, diffusion, or evaporation; temperature; bond-breaking and formation; whether it is a kinetic or thermodynamic isotope effect; whether it is a primary or secondary isotope effect. The magnitude of the isotope effect is proportional to the mass differences between isotopes. Detectable isotope fractionation occurs in elements such as H, C, N, O, S, Li, and B where the relative mass differences between isotopes of the same element are large compared to elements with a higher proton number. The largest variations in isotope ratios are found for hydrogen where the isotope deuterium (^{2}H or D) has double the mass of the isotope protium (^{1}H or H)). The lighter isotope generally diffuses, evaporates and reacts more rapidly than its heavier counterpart; for example, in physiochemical fractionation, the differences in bond strength for isotopes of the same element provide for differences in their reaction rates; the heavy isotope will have a stronger bond and require greater energy to dissociate than the bond of a light isotope (O'Leary et al. 1992). The equilibrium fractionation factor (α) describes how isotopes are partitioned between 2 phases and is defined as the ratio of the numbers of any two isotopes in one chemical compound divided by the corresponding ratio for another chemical compound. For example:

Isotope exchange reaction $\mathbf{H_2{}^{18}O + ½\ C^{16}O_2 <-> H_2{}^{16}O + ½\ C^{18}O_2}$

Equilibrium constant K $$\mathbf{K = \frac{(C^{18}O_2)^{1/2}\,(H_2{}^{16}O)}{(C^{16}O_2)^{1/2}\,(H_2{}^{18}O)}}$$

Rearrangement $K = \frac{(^{18}O/^{16}O)CO_2}{(^{18}O/^{16}O)H_2O} = \alpha$ (alpha)